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Genetic variants of the *LIN28B* gene predict severe radiation pneumonitis in patients with non-small cell lung cancer treated with definitive radiation therapy

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Abstract

Background—LIN28 is an RNA-binding protein that not only plays key roles in multiple cellular developmental processes and tumorigenesis, but also is involved in tissue inflammatory response. However, no published study has investigated associations between genetic variants in *LIN28* and radiation-induced pneumonitis (RP) in patients with non-small cell lung cancer (NSCLC) treated with definitive radiation therapy.

Methods—We genotyped eight potentially functional single nucleotide polymorphisms (SNPs) of *LIN28A* (rs11247946 T>C, rs3811464 C>T, rs11581746 T>C, and rs12728900 G>A), and *LIN28B* (rs314280 A>G, rs12194974 G>A, rs17065417 A>C and rs314276 C>A) in 362 patients with NSCLC, who received definitive radio (chemo) therapy. The associations between RP risk and genotypes were assessed by hazards ratio (HR) in Cox proportional hazards regression analysis with time to event considered with and without adjustment for potential confounders.

Results—Multivariate analyses found that patients carrying *LIN28B* rs314280 AG and AA/AG or rs314276 AC and AA/AC genotypes had a higher risk of grade 3 RP (for rs314280 AG and AA/AG versus GG, adjusted HR= 2.97 and 2.23, 95% CI, 1.32–6.72 and 1.01–4.94, *P* = 0.009 and 0.048, respectively; for rs314276 AC and AA/AC versus CC, adjusted HR = 2.30 and 2.00, 95% CI, 1.24–4.28 and 1.11–3.62, and *P* = 0.008 and 0.022, respectively). Further stratified analyses showed a more profound risk in the subgroups of the age <65 years subjects, males, stage III/IV, ever smokers and MLD ≥ 19.0 Gy.

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Conflict of interest statement

None declared.

Conclusion—Genetic variants of *LIN28B*, but not *LIN28A*, may be biomarkers for susceptibility to severe RP in NSCLC patients. Large, prospective studies are needed to confirm our findings.

Keywords

LIN28; polymorphisms; inflammation; Hazards ratio; Non-small cell lung cancer; radiation pneumonitis

1. Introduction

Lung cancer is the leading cause of cancer death in both men and women in the United States, with an estimated 228,190 new cancer cases and 159,480 deaths in 2013 [1]. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases [2]. Radiotherapy, alone or in combination with concurrent or sequential chemotherapy, is the standard treatment approach for patients with inoperable or unresectable NSCLC, and such a definitive therapy improves local control and overall survival. However, radiation pneumonitis (RP), which can occur after radiation therapy as a result of inflammation of normal lung tissues injured by radiation, has been identified as one of the most common dose-limiting complications of thoracic radiation; in these NSCLC patients, approximately 10–20% have experienced severe RP (grade 3), and almost half of the patients who developed severe RP died of this radiation complication [3]. Previous studies have demonstrated an association between RP risk and multiple therapeutic and patient-related factors, such as Karnofsky performance status (KPS), dosimetric parameters, smoking status and plasma inflammatory cytokine levels [4]. Recently, some genetic variants, such as single nucleotide polymorphisms (SNPs) of several genes, are also shown to be associated with an increased risk of severe RP in patients with NSCLC [5, 6], suggesting that genetic factors may play an important role in RP development.

Studies have indicated that microRNAs (miRNAs), a class of approximately 19–23 nucleotide long RNA molecules in the genome, play critical roles in posttranscriptional gene regulation [7, 8]. miRNAs can negatively regulate target gene transcription through hybridization to incomplete, complementary sequences in the 3' untranslated region (UTR) of their target messenger RNAs (mRNAs). This process results in either the degradation of target mRNAs or repression of their translation. Studies have found that LIN28 (including LIN28A and LIN28B) is an important regulator of miRNAs and mRNAs [9, 10]. For example, LIN28 not only binds to the non-coding miRNAs but also binds directly to thousands of mRNAs. Therefore, LIN28 functions to regulate not only the translation of mRNAs important for cell growth and metabolism, but also the biogenesis of miRNAs, particularly the *let-7* family miRNAs [11, 12]. More recently, some studies have reported that *let-7* overexpression significantly reduced IL-6 expression, which is a major mediator of the tissue inflammatory response [13, 14]. Although the inflammation mechanism of Lin28 is still largely unknown, it is believed that LIN28B plays a key role in regulation of inflammation by regulating IL-6 expression. Indeed, it has been identified that the nuclear factor- κ B (NF- κ B) regulates the expression of a wide range of genes involved in inflammation and that NF- κ B activates LIN28B but inhibits *let-7* miRNA expression, resulting in higher levels of IL-6 by NF- κ B activation [13–15].

Functional experiments have demonstrated that LIN28 regulates radiosensitivity of human lung cancer cells [16]. Several studies, including genome-wide association studies (GWAS), also have reported that genetic variants of the *LIN28* gene were associated with type 2 diabetes mellitus and some cancer susceptibility [17–21]. But no published study has evaluated the associations between genetic variants of the *LIN28* gene and risk of RP in cancer patients who had received radiotherapy. Considering the LIN28/let7 might play important roles in the mechanism of inflammation induced by irradiation, we hypothesized that SNPs of the *LIN28* gene are associated with risk of RP in patents with NSCLC and thus are biomarkers for predicting RP risk of NSCLC patients undergoing definitive radiation therapy. To test this hypothesis, we evaluated associations between *LIN28* genotypes and severe RP in a well-defined cohort of NSCLC patients with detailed information about the definitive radiotherapy they have received.

2. Materials and Methods

2.1. Study populations

The characteristic details of subjects used in the present study have been previously reported [5, 6]. Briefly, the study population included 393 patients who had DNA samples available and follow-up data from a dataset of NSCLC patients treated with definitive radiation at a single institution between March 1998 and June 2009. Among these 393 patients, except for 31 who developed recurrent diseases or underwent surgical resection before radiotherapy, the remaining 362 patients had both radiation dosimetric data and documented information on RP with the complete follow-up data. We interviewed each of the 362 eligible patients to obtain data on tobacco smoking. Those who had smoked <100 cigarettes in their lifetime were considered “never smokers”, and all others were considered “ever smokers”. Techniques for treatment planning and delivery changed considerably during the study period. For example, the 3-dimensional (3D) CT-based simulation and 3D conformal radiation therapy (3D-CRT) was used before July 2004, and the 4-dimensional CT-based simulation with respiratory motion management and intensity modulation radiation therapy (IMRT) or proton beam radiation (PBT) was used thereafter [22]. The characteristics of patients, tumors, and treatment are described in Table 1, which are consistent with our previous reports [6]. Severe RP (grade 3) was defined according to the National Cancer Institute’s Common Terminology Criteria for Adverse Events, version 3.0. Symptomatic RP that interfered with daily activities or required the administration of oxygen was scored as grade 3. Briefly, RP had been diagnosed by reviewing all available radiographic images, including thoracic x-ray, CT, or positron emission tomography (PET)/CT scans; severity was established and confirmed by reviewing dictated clinical notes. During radiation therapy, patients were seen at least weekly; thereafter, patients were followed up at one to three months after therapy and then every three months. Follow-up evaluations included an interval history and physical examination and chest CT or PET/CT, pulmonary functional tests, and routine blood tests. The time to RP development was calculated from the start of radiation therapy; patients not experiencing either endpoint were censored at the date of the last follow-up or death [5]. The present study was approved by The University of Texas M.D. Anderson Cancer Center institutional review board, and the Health Insurance Portability and Accountability Act (HIPAA) regulations were strictly followed.

2.2. Selection of SNP and Genotyping

The *LIN28A* and *LIN28B* genes have been mapped to chromosomes, 1p36.11 and 6q21, respectively. *LIN28A* encodes an approximately 25-kDa protein, whereas *LIN28B* has a similar molecular structure and biologic functions as *LIN28A* [18, 21, 23]. We searched the National Institute of Environmental Health Sciences Genome Program's SNPs database (<http://www.ncbi.nlm.nih.gov/projects/SNP>) and related literature to identify all potentially functional SNPs of *LIN28* that met at least two of the following four criteria: (1) a minor allele frequency ≥ 0.05 in Caucasians, (2) affecting transcription factor binding site (TFBS) activity in the putative promoter or intron regions (FuncPred, <http://snpinfo.niehs.nih.gov/snpfunc.htm>), (3) previously reported associations with diseases [18–21], and (4) not in high linkage disequilibrium (LD), i.e., $LD \leq 0.8$. With assistance of the online searching tools of HapMap database (<http://hapmap.ncbi.nlm.nih.gov>), we selected and genotyped eight potentially functional SNPs of the *LIN28A* (rs11247946 T>C, rs3811464 C>T, rs11581746 T>C, and rs12728900 G>A), and *LIN28B* (rs314280 A>G, rs12194974 G>A, rs17065417 A>C and rs314276 C>A) genes using the TaqMan assay with the Sequence Detection Software on an ABI-Prism 7900 instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

We extracted genomic DNA from the buffy coat fraction of the blood samples by using a blood DNA mini kit (Qiagen, Inc.) according to the manufacturer's instructions. The DNA purity and concentrations were determined by spectrophotometer measurement of absorbance at 260 and 280 nm. Primers and probes were supplied by Applied Biosystems. Each plate included four negative controls (no DNA), duplicated positive controls, and eight repeat samples. Amplification was done under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95 °C for 15 s and 60°C for 1 min. For all genotypes, the assay success rate was >99%, and the repeated sample's results were 100% concordant.

2.3. Real-time RT-PCR for expression levels of LIN28B mRNA

The expression levels of *LIN28B* mRNA were examined by quantitative RT-PCR using the total RNA that was isolated from peripheral blood mononuclear cells (PBMCs) of 105 cancer-free controls with the TRIzol reagent (Invitrogen™, Carlsbad, CA). *LIN28B* mRNA expression levels were detected by using TaqMan gene expression assays with the master mix reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time RT-PCR was performed using the ABI-Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate and the expression level of *LIN28* mRNA was calculated relative to that of *18S*.

2.4. Statistical Analysis

Patients were grouped by their genotypes. All statistical analyses were performed with SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC), unless stated otherwise. Cox proportional hazards regression analysis was performed to evaluate effect of genotypes on RP risk by calculating the hazard ratios (HRs) and 95% confidence intervals (CIs). Kaplan-Meier analysis was performed to assess the effect of different genotypes on cumulative probability of RP. In addition, a multivariate Cox regression analysis was performed to adjust for other covariates. Likelihood ratio tests were also performed for each multivariate

Cox regression model to assess the goodness of fit. A 2-sided test with a P value ≤ 0.05 was considered statistically significant. We also used the false-positive report probability (FPRP) to test for false positive associations [24]. For all significant genetic effects observed in our study, we calculated FPRP with prior probabilities of 0.0001, 0.001, 0.01, 0.1, and 0.25. The HR was set close to the observed value in our study, and a probability <0.2 was considered a noteworthy. All tests were two sided and $P < 0.05$ was considered significant.

3. Results

3.1. Patient Characteristics and Risk of severe RP (grade 3)

As shown in Table 1, there were 200 men and 162 women, with a median age of 65 years (range, 35 to 88 years) in the pooled group. Of these patients, 297 (82.0%) were Caucasians, 85% had stage III/IV diseases according to the 6th edition of the AJCC stage grouping criteria, and 90.9% were treated with a combination of chemotherapy and radiotherapy. The median radiation dose received by patients was 66 Gy (range, 50.0–87.5 Gy) with a median mean lung dose (MLD) of 19.0 Gy (range, 2.7–30.6 Gy). The median follow-up time for assessment of RP after radiation therapy was 20.6 months (range, 1.0 to 157.6 months). The overall incidence of severe RP was 15.5%, and the median occurrence time for severe RP (grade 3) was 3.6 months (95% CI, 2.1–10.1 months). We evaluated the association between RP and clinic-pathologic characteristics to identify any confounding factors, including age, sex, ethnicity, KPS, disease stage, tumor histology, smoking status, use of chemotherapy, radiation dose and MLD. In the univariate analysis, we found that only MLD was significantly associated with severe RP (grade 3) (MLD ≥ 19.0 Gy vs. MLD <19.0 Gy, crude HR = 3.12, 95% CI 1.73–5.64, $P < 0.001$) (Table 1 and Fig. 1a). No other clinic-pathologic characteristics were found to be associated with severe RP risk in this study population.

3.2. Association between severe RP (grade 3) and LIN28 SNPs

Table 2 shows the results of univariate and multivariate analyses of the associations between *LIN28* genotypes and severe RP (grade 3) using the Cox proportional hazards regression analysis. We found that the *LIN28B* rs314280 A>G and rs314276 C>A SNPs were significantly associated with hazards of severe RP (grade 3). Compared with the rs314280 GG genotype, the variant AG and AA/AG genotypes were associated with more than 2-fold increased hazards of severe RP (AG and AA/AG versus GG; crude HR = 2.87 and 2.42; 95% CI, 1.27–6.45 and 1.10–5.34; $P = 0.011$ and 0.029, respectively) (Fig. 1b). Similar results were observed in multivariate analyses with adjustment for disease stage, chemotherapy history, smoke status and MLD (Table 2) that were considered potential confounding factors of RP [4, 5]. In addition, The *LIN28B* rs314276A variant allele (i.e., AC and AA/AC) was also associated with significantly increased hazards of grade 3 RP in both univariate and multivariate analyses. Specifically, compared with the rs314276 CC genotype, the variant AC and AA/AC genotypes were associated with a statistically significantly increased hazards of severe RP (AC and AA/AC versus CC; crude HR = 2.14 and 2.05; 95% CI, 1.15–3.96 and 1.14–3.71; $P = 0.016$ and 0.017, respectively; adjusted HR = 2.30 and 2.00; 95% CI, 1.24–4.28 and 1.11–3.62; $P = 0.008$ and 0.022, respectively) (Table 2 and Fig. 1c). In contrast, no associations were observed between the genotypes of

other SNPs of *LIN28B* (rs12194974 G>A and rs17065417 A>C) and *LIN28A* (rs11247946 T>C, rs3811464 C>T, rs11581746 T>C, and rs12728900 G>A) and hazards of severe RP (Table 2).

The LD analysis showed that all SNPs were in low LD using our genotyping data (i.e. *LIN28B* polymorphisms, $r^2 = 0.14$ for rs314280 G>A and rs12194974 G>A; $r^2 = 0.10$ for rs314280 G>A and rs17065417 A>C; $r^2 = 0.35$ for rs314280 G>A and rs314276 C>A; $r^2 = 0.01$ for rs12194974 G>A and rs17065417 A>C; $r^2 = 0.05$ for rs12194974 G>A and rs314276 C>A; $r^2 = 0.04$ for rs17065417 A>C and rs314276 C>A; Fig. 2a), the LD results were consistent with these calculated by using the CEPH (Utah residents with ancestry from northern and western Europe) population of HapMap data (Rel 28 Phase II+ III) (Fig. 2b). These suggest that each SNP may have an independent effect. Furthermore, we examined the combined effects of rs314280 and rs314276 genotypes. As shown in Table 2, when we used “0–1” risk genotypes as the reference, we found that carriers with risk genotypes on both SNPs (AA-AA, AA-AC, AG-AA or AG-AC) had significantly increased hazards of RP (adjusted HR=2.55, 95% CI=1.50–4.35; $P = 0.0006$).

To further characterize biological significance of the *LIN28B* rs314280 and rs314276, we conducted correlation analysis between genotypes of rs314280 and rs314276 and mRNA expression levels of *LIN28B* in PBMCs from 105 cancer-free controls. As we expected from the literature, we found that there were no detectable expressions of *LIN28B* in these PBMCs (data not shown).

3.3. Stratified analyses

We then performed stratified analyses to evaluate the effects of variant genotypes on the hazards of RP by age, sex, race, KPS, stage, tumor histology, smoking status, and chemotherapy status, and MLD and radiation dose (Table 3). The results showed that the increased hazards associated with the variant A allele of rs314276 and risk genotypes (AA/AG and AA/AC) of the combined rs314280 and rs314276 were more evident in subjects who were <65 years old (adjusted HR = 2.74 and 3.79, 95% CI = 1.11–6.73 and 1.76–8.13, $P = 0.029$ and 0.0006, respectively), males (adjusted HR = 2.41 and 3.65, 95% CI = 1.12–5.22 and 1.79–7.44, $P = 0.025$ and 0.0004, respectively), or having stage III and IV (adjusted HR = 3.11, 95% CI = 1.74–5.59, $P = 0.0001$ for combined) or squamous cell carcinomas (adjusted HR = 3.24 and 3.54, 95% CI = 1.05–10.05 and 1.39–9.01, $P = 0.041$ and 0.008, respectively), ever smokers (adjusted HR = 1.99 and 2.68, 95% CI = 1.08–3.68 and 1.54–4.65, $P = 0.028$ and 0.0005, respectively), and having radio-chemotherapy (adjusted HR = 1.92 and 2.61, 95% CI = 1.04–3.54 and 1.50–4.52, $P = 0.037$ and 0.0006, respectively), MLD ≥ 19.0 Gy (adjusted HR = 3.76, 95% CI = 2.00–7.07, $P < 0.0001$ for combined), and radiation dose ≥ 66 (adjusted HR = 2.89 and 5.20, 95% CI = 1.08–7.70 and 2.13–12.67, $P = 0.034$ and 0.0003, respectively). For rs314280, the increased hazards associated with the variant A allele of rs314280 was more evident only for stage III and IV (adjusted HR = 3.11, 95% CI = 1.74–5.59, $P = 0.017$), MLD ≥ 19.0 Gy (adjusted HR = 3.76, 95% CI = 2.00–7.07, $P = 0.009$), and radiation dose ≥ 66 (adjusted HR = 3.19, 95% CI = 1.01–10.03, $P = 0.047$) (Table 3).

Because most of the significant findings were in the subgroup analyses, we calculated the FPRP values for all the observed significant associations. As shown in (Table 4), when the assumption of a prior probability was 0.25, the association with the combined *LIN28B* (AA/AG and AA/AC) genotypes was still noteworthy for all subjects (FPRP = 0.009), age <65 years (FPRP = 0.036), males (FPRP = 0.022), stage III and IV (FPRP = 0.006), ever smokers (FPRP = 0.009), radio-chemotherapy (FPRP = 0.011) as well as for MLD >19.0Gy (FPRP = 0.005); the association with *LIN28B* (rs314276 AA/AC) genotypes was only noteworthy for all subjects (FPRP = 0.007). When the assumption of prior probability was 0.1, the association with the combined *LIN28B* (AA/AG and AA/AC) genotypes was still noteworthy for all subjects (FPRP = 0.028), stage III and IV (FPRP = 0.019), ever smokers (FPRP = 0.027), radio-chemotherapy (FPRP = 0.031) as well as for MLD >19.0 Gy (FPRP = 0.014).

4. Discussion

In the present study, we evaluated the associations between genetic variants of *LIN28A* and *LIN28B* genes and risk of severe RP in patients with NSCLC treated with definitive radiotherapy. We found that *LIN28B* rs314280 variant A genotypes were associated with a significantly increased risk of grade 3 RP, compared with the GG genotype. Furthermore, we found that the AA/AC genotypes of *LIN28B* rs314276 were also associated with a significantly increased risk of grade 3 RP, compared with its common CC genotype. Stratified analyses showed that the increased risk was more evident in subgroups of subjects of <65 years, males, having squamous cell carcinoma, ever smokers, and having radio-chemotherapy. Although multiple testing had been performed in the present study, the results of FPRP indicated that the combined effect of risk genotypes was less likely to be false positive. Therefore, our data indicated that *LIN28B* (rs314280 and rs314276) genetic variants may independently and jointly have an effect on the risk of severe RP in NSCLC patients treated with radiotherapy, suggesting that these variants may be useful biomarkers for RP susceptibility in lung cancer patients. Our data also indicated that patients at highest risk of severe RP were those who were carrying risk genotypes on both SNPs (AA/AG and AA/AC) and received MLD >19.0 Gy. These findings suggest that the MLD should be controlled as low as possible for patients with the rs314280 AA/AG or rs314280 AA/AG genotypes to avoid high risk of severe RP. Because MLD is known before the radiation has begun, genotyping *LIN28B* at rs314280 and rs314276 could be used as a predictive biomarker for prescribing highly personalized radiation (chemo) therapy once our results are validated in clinical trials.

Experimental and clinical as well as epidemiological studies have suggested a strong association between inflammation and radiation pneumonitis [25–27]. Inflammation is a physiological response to cellular and tissue damage, including radiation-induced damage. Appropriate response to this damage is tightly regulated through a balance between pro-inflammatory and anti-inflammatory cytokines and signaling molecules [28, 29]. Inflammatory cytokines are the key to modulating and ameliorating the inflammation-related effects following lung irradiation as the observed RP. Indeed, lung irradiation induces the expression of pro-inflammatory cytokines; for example, interleukin (IL-6) levels were significantly higher during and after thoracic irradiation therapy in lung cancer patients

who developed RP [30, 31], whereas *let-7* miRNA overexpression significantly reduced IL-6 expression. Recently, some published studies have indicated that LIN28 links inflammation to cell transformation [13, 14], LIN28B functions as a post-transcriptional regulator of *let-7* miRNA biogenesis by inhibiting the microprocessor-mediated cleavage of pri-*let-7* miRNAs [12], and NF- κ B activates LIN28B and inhibits *let-7* miRNA expression, leading to overexpression of IL-6 by NF- κ B activation [13–15]. It is well known that LIN28B is a homologue of LIN28A [20], and both LIN28A and LIN28B also play an important role during multiple cellular development processes and tumorigenesis in humans. For example, overexpression of LIN28B has been shown to be more frequent in human cancers, such as colon cancer, hepatocellular carcinomas, and NSCLC [10, 23] as well as to be able to stimulate tumor growth [32]. More recently, one study has found that LIN28 regulates radiosensitivity of human lung cancer cells [16], in which, by using LIN28-siRNA, LIN28 was found to be specifically inhibited, leading to upregulation of the *let-7a* expression but downregulation of the K-Ras protein expression and resulting in the radiosensitization of lung cancer cells. Taken together, it is confirmed that LIN28 functions to regulate not only the biogenesis of miRNAs, particularly the *let-7* family miRNAs, but also the translation of mRNAs important for cell growth and metabolism [9]. Our data further implied that *LIN28B* may be involved in inflammatory response. However, the exact inflammation mechanism of LIN28, especially in the RP development, is still unclear.

Several GWAS studies have identified some genetic variants of *LIN28B* (i.e., rs314276, rs314277 and rs314280) that were associated with age at menarche and age at natural menopause as well as the timing of puberty [18, 19, 33]. In a GWAS of 15,297 Icelandic women subjects, Sulem *et al.* reported that the *LIN28* rs314280 T allele was associated with earlier age at menarche [18]. In another GWAS of 21,087 European women population, it was shown that *LIN28B* rs314276 C allele was associated with the timing of puberty [19]. To date, however, no published study has investigated the association between genetic variants of *LIN28* and radiation treatment-related toxicities. More recently, only one study investigated the associations between genetic variants of the *LIN28* gene and risk of type 2 diabetes mellitus (T2DM) [17]. In a hospital-based association study of 588 T2DM patients and 588 age and sex matched controls, Zhang *et al.* demonstrated that rs3811463 polymorphism was associated with increased risk of T2DM in a Chinese population [17].

In the present study, we found that among NSCLC patients, severe RP was more likely to occur in those carrying *LIN28B* rs314276 AA/AC and rs314280 AA/AG variant genotypes than in those carrying the *LIN28B* rs314276 CC and rs314280 GG genotypes, respectively. These two SNPs are potentially functional, because they are located either in intron 2 (rs314276) or at the 5' end (rs314280) of the *LIN28B* gene [18] that are predicted to affect the binding activity at the transcription factor binding sites (TFBS); thereby, we speculated that these *LIN28B* variants may result in altered gene expression or regulation of signaling function, which may be the mechanisms underlying the increased radiosensitivity of the irradiated tissues or RP susceptibility, as we observed in the current study. Therefore, we further tested for correlation between these two SNPs and expression levels of *LIN28B* mRNA in peripheral blood mononuclear cells (PBMCs) of 105 cancer-free controls. However, by using RT-PCR, as reported in a previous study [18], we did not detect

expression of *LIN28B* in these cells, which is consistent with the reports that *LIN28B* expression was undetectable or detected at very low levels in most normal adult tissue [9, 11, 34]; furthermore, we did not have lung cancer tumor tissues for the detection of *LIN28B* expression. Therefore, we were not able to correlate *LIN28B* expression with the RP-associated genetic variants in the target tissues in the present study. However, our genotype results are consistent with the function of LIN28 that is involved in the inflammation mechanism.

To the best of our knowledge, the present study provided for the first time the evidence that potentially functional SNPs of the *LIN28B* gene may predict risk of severe RP in lung cancer patients who have received definitive radiotherapy. However, the present study has several limitations. Firstly, the exact molecular mechanisms by which the *LIN28B* SNPs influence the risk of RP were not fully understood, as they were beyond the scope of the current study. Secondly, we used the candidate SNP/gene approach that allowed us to focus on potentially common, functional SNPs in the *LIN28* gene, but this approach did not include all representative or tagging SNPs in the entire gene. Some important SNPs but with a low frequency might have been missed or the observed associations may have been due to genetic linkages with other untyped SNPs. Therefore, additional investigations of the tagged or functional SNPs are warranted and will be the subject of future studies.

In summary, the present study demonstrated that the selected potentially functional SNPs of *LIN28B* (rs314280 and rs314276) may independently and jointly affect the development of severe RP in NSCLC patients treated with radiotherapy. Further large, prospective studies are essential to confirm our findings. Once validated, *LIN28B* SNPs may be useful biomarkers for susceptibility to RP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RP	Radiation-induced pneumonitis
NSCLC	Non-small cell lung cancer
SNPs	Single nucleotide polymorphisms
MLD	mean lung dose
HR	Hazards ratio

95% CIs   95% Confidence intervals

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Figure 1.

The risk of radiation pneumonitis by selected clinical factors and polymorphisms of the *LIN28B* gene is illustrated according to (a) mean lung dose (MLD). Gy indicates grays; (b) *LIN28B* rs314280 AG+AA versus GG; and (c) *LIN28B* rs314276 AC+AA versus CC genotypes.

Figure 2.

Positions of the four potentially functional single-nucleotide polymorphisms (SNPs) in *LIN28B* and pairwise linkage disequilibrium (LD) between them. The lower panel shows the LD plot of the *LIN28B* region, using the genotyping data from the present study (a) and CEPH (Utah residents with ancestry from northern and western Europe) population from the international HapMap project (Rel 28 Phase II + III) (b). Each number in grid represents the pairwise D' and r² between SNPs. All polymorphisms were in low LD with each other in both populations.

Table 1

Characteristics(n = 362) of NSCLC patients received definitive radiation therapy

Parameter	No.(%)	HR	95%CI	P*
Sex				
Male	200(55.0)	1.00		
Female	162(45.0)	0.85	0.50–1.44	0.536
Age(years)				
<65	181(50.0)	1.00		
65	181(50.0)	0.96	0.57–1.63	0.886
Race				
White	297(82.0)	1.00		
Black	65(18.0)	0.99	0.50–1.97	0.984
KPS				
<80	74(20.4)	1.00		
80	288(79.6)	0.62	0.34–1.12	0.111
Stage groups				
I, II	54(14.9)	1.00		
III, IV	307(84.8)	0.82	0.42–1.63	0.580
Missing	1(0.3)			
Histology				
Adenocarcinoma	137(37.8)	1.00		
Squamous cell	127(35.1)	0.99	0.53–1.85	0.981
Other	98(27.1)	1.08	0.56–2.06	0.825
Smoking status				
Never	32(9.1)	1.00		
Ever	330(90.9)	1.18	0.43–3.27	0.746
Chemotherapy				
No	33(8.8)	1.00		
Yes	329(90.9)	1.30	0.47–3.59	0.616
Mean lung dose(Gy)				
<19.0	181(50.0)	1.00		
19.0	181(50.0)	3.12	1.73–5.64	<0.001
Radiation dose(Gy)				
<66	173(47.8)	1.00		
66	189(52.2)	1.00	0.59–1.69	0.999

* P values were calculated by Cox proportional model using univariate analysis.

NSCLC: non-small cell lung cancer.

Univariate and multivariate analyses of associations between *LIN28* genotypes and severe RP(grade 3) in NSCLC patients received definitive radiation therapy

Table 2

Genotypes	Patient No.	Event No.	Crude		<i>P^a</i>	Adjusted		<i>P^b</i>
			HR	95% CI		HR	95% CI	
<i>LIN28A</i> (rs11247946)								
TT	166	24	1.00			1.00		
CT	147	23	1.10	0.62–1.94	0.751	1.08	0.61–1.92	0.784
CC	49	9	1.37	0.64–2.94	0.424	1.28	0.59–2.77	0.526
CC/CT	196	32	1.16	0.68–1.97	0.580	1.33	0.67–1.93	0.644
<i>LIN28A</i> (rs3811464)								
CC	92	17	1.00			1.00		
CT	177	24	0.66	0.36–1.23	0.194	0.66	0.36–1.24	0.199
TT	93	15	0.84	0.42–1.68	0.618	0.92	0.45–1.85	0.805
TT/CT	270	39	0.72	0.41–1.27	0.259	0.74	0.42–1.32	0.305
<i>LIN28A</i> (rs11581746)								
TT	255	41	1.00			1.00		
CT	97	14	0.89	0.48–1.63	0.703	0.86	0.47–1.58	0.625
CC	10	1	0.61	0.08–4.45	0.627	0.62	0.08–4.49	0.632
CC/CT	107	15	0.86	0.48–1.56	0.624	0.84	0.46–1.52	0.558
<i>LIN28A</i> (rs12728900)								
GG	195	31	1.00			1.00		
AG	133	22	1.05	0.61–1.81	0.861	1.04	0.60–1.80	0.896
AA	34	3	0.59	0.18–1.92	0.378	0.60	0.18–1.98	0.406
AA/AG	167	25	0.96	0.57–1.63	0.877	0.96	0.56–1.62	0.865
<i>LIN28B</i> (rs314280)								
GG	95	7	1.00			1.00		
AG	168	35	2.87	1.27–6.45	0.011	2.97	1.32–6.72	0.009
AA	99	14	1.74	0.70–4.32	0.231	1.36	0.54–3.38	0.512
AA/AG	267	49	2.42	1.10–5.34	0.029	2.23	1.01–4.94	0.048
<i>LIN28B</i> (rs12194974)								

Genotypes	Patient No.	Event No.	Crude		<i>P^a</i>	Adjusted		<i>P^b</i>
			HR	95% CI		HR	95% CI	
GG	280	42	1.00			1.00		
AG	76	13	1.20	0.64–2.23	0.567	1.37	0.73–2.57	0.329
AA	6	1	1.10	0.15–7.96	0.929	2.16	0.29–16.26	0.455
AA/AG	82	14	1.19	0.65–2.18	0.571	1.40	0.76–2.59	0.281
LIN28B(rs17065417)								
AA	290	47	1.00			1.00		
AC	68	9	0.74	0.36–1.51	0.410	0.77	0.38–1.57	0.470
CC	4	0	N/A					
CC/AC	72	9	0.73	0.36–1.48	0.377	0.75	0.37–1.54	0.435
LIN28B(rs314276)								
CC	154	15	1.00			1.00		
AC	155	31	2.14	1.15–3.96	0.016	2.30	1.24–4.28	0.008
AA	53	10	1.83	0.82–4.07	0.139	1.42	0.63–3.19	0.395
AA/AC	208	41	2.05	1.14–3.71	0.017	2.00	1.11–3.62	0.022
Combined effect of risk genotypes in rs314280 and rs314276								
0–1 ^c	211	29						
2 ^c	95	27	2.07	1.22–3.49	0.007	2.55	1.50–4.35	0.0006

RP = radiation pneumonitis; NSCLC = non-small cell lung cancer; CI = confidence interval; HR = hazard ratio; N/A, not applicable.

^a *P* values were calculated by Cox proportional model using univariate analysis.

^b *P* values were calculated with adjustment for smoking status, disease stage, chemotherapy history, and mean lung dose.

^c Total number of unfavorable genotypes

Table 3

Stratification analysis for associations between *LIN28B* variant genotypes and severe RP(grade 3) in NSCLC patients received definitive radiation therapy

Variables	rs314280 (patient/event)		rs314276 (patient/event)		Adjusted HR ^a (95%CI)	Combined effect of risk genotypes ^b		Adjusted HR ^a (95%CI)
	GG	AA/AG	CC	AA/AC		0-1	2	
Age, yr								
<65(median)	43/5	109/24	73/6	79/23	2.20(0.83-5.82)	2.74(1.11-6.73)	109/11	3.79(1.76-8.13)
65(median)	42/9	112/18	66/9	88/18	1.16(0.51-2.62)	1.49(0.67-3.34)	102/18	1.46(0.64-3.33)
Gender								
Males	45/6	120/27	83/9	82/24	2.31(0.94-5.71)	2.41(1.12-5.22)	119/15	3.65(1.79-7.44)
Females	40/8	101/15	56/6	85/17	1.11(0.47-2.66)	1.65(0.65-4.20)	92/14	1.68(0.70-3.99)
Race								
White	51/10	220/36	111/13	140/33	1.35(0.66-2.76)	1.79(0.94-3.41)	160/23	2.21(1.23-3.98)
Black	34/4	21/6	28/2	27/8	3.68(0.93-14.61)	3.13(0.63-15.69)	51/6	6.41(1.76-23.34)
KPS								
<80	72/11	175/30	108/11	139/30	1.68(0.83-3.39)	1.78(0.89-3.56)	173/22	2.48(1.33-4.63)
80	13/3	46/12	31/4	28/11	1.37(0.37-5.17)	2.90(0.91-9.28)	38/7	2.42(0.84-6.95)
Stage group								
I, II	10/6	34/4	20/2	24/8	0.37(0.10-1.36)	3.64(0.74-17.89)	28/8	0.84(0.16-4.45)
III, IV	75/8	186/38	119/13	142/33	2.54(1.18-5.49)	1.80(0.94-3.42)	183/21	3.11(1.74-5.59)
Histology								
Adenocarcinoma	32/5	84/16	52/5	64/16	1.34(0.49-3.70)	2.32(0.84-6.42)	76/10	2.11(0.89-5.01)
Squamous cell	30/6	78/13	52/4	56/15	1.57(0.57-4.34)	3.24(1.05-10.05)	78/10	3.54(1.39-9.01)
Other	23/3	59/13	35/6	47/10	4.28(1.11-16.57)	0.70(0.24-2.03)	57/9	2.30(0.83-6.38)
Smoking status								
Never	7/2	21/2	10/1	18/3	0.55(0.07-4.69)	3.45(0.21-57.42)	17/3	1.62(0.14-19.30)
Ever	78/12	200/40	129/14	149/38	1.82(0.94-3.50)	1.99(1.08-3.68)	194/26	2.68(1.54-4.65)
Chemotherapy								
No	6/2	22/2	15/1	13/3	3.00(0.20-44.40)	1.79(0.14-22.47)	19/3	7.40(0.27-204.10)
Yes	79/12	198/40	123/14	154/38	1.78(0.93-3.42)	1.92(1.04-3.54)	191/26	2.61(1.50-4.52)
Mean lung dose								

Variables	rs314280 (patient/event)		Adjusted HR ^a (95%CI)		rs314276 (patient/event)		Adjusted HR ^a (95%CI)		Combined effect of risk genotypes ^b		Adjusted HR ^a (95%CI)
	GG	AA/AG			CC	AA/AC			0-1	2	
<19.0 Gy	31/6	135/9	0.43(0.15–1.23)		75/4	91/11	2.15(0.68–6.82)		102/10	64/5	0.87(0.30–2.57)
19.0 Gy	54/8	86/33	2.82(1.29–6.19)		64/11	76/30	1.91(0.96–3.82)		109/19	31/22	3.76(2.00–7.07)
Radiation dose											
<66	42/4	105/22	3.19(1.01–10.03)		67/5	80/21	2.89(1.08–7.70)		102/9	45/17	5.20(2.13–12.67)
66	43/10	116/20	1.19(0.54–2.62)		72/10	87/20	1.47(0.69–3.15)		109/20	50/10	1.49(0.68–3.26)

^a Adjusted for smoking status, disease stage, chemotherapy history, and mean lung dose.

^b The number represents the numbers of unfavorable genotypes for each SNP.

NSCLC: non-small cell lung cancer.

Table 4

False-positive report probability (FPRP) values for associations between severe RP (grade >3) and frequencies of rs314280, rs314276 and combined genotypes in NSCLC patients received definitive radiation therapy

Genotype	Positive HR 95%CI ^a	P ^b	Statistical power ^c	Prior probability			
				0.25	0.1	0.01	0.0001
rs314280: G>A	GG vs. AA/AG						
All subjects	2.23 (1.01–4.94)	0.048	0.394	0.268	0.523	0.924	0.992
Stage III,IV	2.54 (1.18–5.49)	0.018	0.272	0.164	0.371	0.866	0.985
Histology Other	4.28 (1.11–16.57)	0.035	0.135	0.439	0.701	0.963	0.996
MLD 19.0	2.82 (1.29–6.19)	0.009	0.196	0.130	0.309	0.831	0.980
Radiation dose <66	3.19 (1.01–10.03)	0.047	0.212	0.400	0.667	0.957	0.996
rs314276: C>A	CC vs. AA/AC						
All subjects	2.00 (1.11–3.62)	0.022	0.500	0.007	0.284	0.814	0.978
Age < 65	2.74 (1.11–6.73)	0.028	0.246	0.254	0.505	0.918	0.991
Males	2.41 (1.12–5.22)	0.026	0.318	0.195	0.421	0.889	0.988
Histology Squamous cell	3.24 (1.05–10.05)	0.042	0.202	0.383	0.651	0.954	0.995
Ever Smokers	1.99 (1.08–3.68)	0.028	0.506	0.143	0.334	0.847	0.982
Chemotherapy Yes	1.92 (1.04–3.54)	0.037	0.552	0.166	0.374	0.868	0.985
Radiation dose <66	2.89 (1.08–7.70)	0.034	0.231	0.305	0.569	0.935	0.993
Combined	0–1 vs. 2						
All subjects	2.55 (1.50–4.35)	<0.001	0.186	0.009	0.028	0.239	0.760
Age < 65	3.79 (1.76–8.13)	<0.001	0.050	0.036	0.100	0.550	0.925
Males	3.65 (1.79–7.44)	<0.001	0.049	0.022	0.063	0.426	0.882
White	2.21 (1.23–3.98)	0.008	0.370	0.063	0.167	0.668	0.957
Black	6.41 (1.76–23.34)	0.005	0.039	0.273	0.530	0.925	0.992
KPS <80	2.48 (1.33–4.63)	0.004	0.250	0.050	0.136	0.633	0.946
Stage III,IV	3.11 (1.74–5.59)	<0.001	0.070	0.006	0.019	0.174	0.680
Histology Squamous cell	3.54 (1.39–9.01)	0.008	0.115	0.172	0.384	0.873	0.986
Ever Smokers	2.68 (1.54–4.65)	<0.001	0.149	0.009	0.027	0.232	0.753
Chemotherapy Yes	2.61 (1.50–4.52)	<0.001	0.171	0.011	0.031	0.263	0.783
MLD 19.0	3.76 (2.00–7.07)	<0.001	0.025	0.005	0.014	0.135	0.611

Genotype	Positive HR 95%CI ^a	<i>P</i> ^b	Statistical power ^c	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
Radiation dose <66	2.89 (1.08–7.70)	0.034	0.231	0.305	0.569	0.935	0.993	0.999

^aThe adjusted HR.

^bThe omnibus chi-square test of the genotype frequency distributions.

^cCalculated using study subjects to detect a HR of 2.00 with the common genotype used as the reference.

NSCLC: non-small cell lung cancer.