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Potentially Functional variants of *ATG16L2* predict radiation pneumonitis and outcomes in patients with non-small cell lung cancer after definitive radiotherapy

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Abstract

Introduction: Autophagy not only plays an important role in the progression of cancer but also is involved in tissue inflammatory response. However, few published studies have investigated associations between functional genetic variants of autophagy-related genes and radiation pneumonitis (RP) as well as clinical outcomes in patients with non-small cell lung cancer (NSCLC) after definitive radiotherapy.

Methods: We genotyped nine potentially functional single nucleotide polymorphisms (SNPs) in four autophagy-related genes (*ATG2B, ATG10, ATG12* and *ATG16L2*) in 393 NSCLC patients of a North American population and assessed their association with RP, local recurrence-free survival (LRFS), progression-free survival (PFS) and overall survival (OS) in multivariate Cox proportional hazards regression analyses. These patients had NSCLC that was treated by definitive radiotherapy.

Results: We found that the *ATG16L2* rs10898880 CC variant homozygotes had a better LRFS, PFS and OS [adjusted hazards ratio (adjHR) = 0.59, 0.64 and 0.64; 95% confidence interval (95% CI = 0.45-0.79, 0.54-0.96, 0.48-0.84, and 0.48-0.86); and $P = 0.0004$, 0.002, and 0.003, respectively], but a greater risk of developing severe RP, than patients with CC/CT genotypes (adjHR = 1.80, 96% CI = 1.04-3.12, $P = 0.037$). Further functional analyses suggested that the *ATG16L2* rs10898880 C variant allele modulated gene expression of *ATG16L2*.

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Conflict of interest statement

None declared.

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Conclusion: This is the first report that functional *ATG16L2* C variant homozygous genotype may be a predictor of RP, LRFS, PFS, and OS in NSCLC patients after definitive radiotherapy. Additional larger, prospective studies are needed to confirm these findings.

Keywords

Non-small cell lung cancer; Autophagy pathway; Radiation pneumonitis; Local recurrence-free survival; Progression- free survival; Overall survival; Single nucleotide polymorphisms; Cox regression

Introduction

Lung cancer ranks the top for cancer-related mortality, with over a million deaths each year worldwide.[1] In the United States, there will be approximately 222,500 new cases and 155,870 deaths as estimated in 2017.[2] Non-small-cell lung cancer (NSCLC) is the most common histological type, accounting for approximately 90% of all lung cancer patients.[3] Radiotherapy, alone or chemoradiotherapy is still the standard treatment for unresectable locally advanced NSCLC, and such a definitive therapy improves local control and overall survival. Unfortunately, the median survival remains less than a year, and the 2-year survival rate is still 15–20%.[4] Moreover, radiation pneumonitis (RP), which can occur after radiotherapy 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. Among the treated patients, approximately 10-20% have experienced severe RP (grade ≥3), and almost half of these patients who developed severe RP died of this radiation complication.[5] Therefore, the discovery of suitable biomarkers is needed to predict RP and outcomes of NSCLC after definitive radiotherapy. Increasing evidence suggests that molecular and genetic factors may play an important role in RP development and clinical outcomes of NSCLC, which are accessible molecular markers that may provide therapeutic benefits by predicting clinical outcomes of the patients with NSCLC on an individual basis.[6-8]

Recently, accumulating evidence has indicated that autophagy plays an important role in various stages of cancer development and progression, including NSCLC.[9,10] Autophagy is a catabolic process that degrades intracellular components through the lysosomal machinery,[11] which usually functions at a low level but is upregulated in response to nutrient starvation, stress, or DNA damage. The role of autophagy in cancer development and progression is complex, because it can act as either tumor suppressor by degradation of damaged proteins and organelles or as a growth promoter by a mechanism of cell survival depending on many factors like tissue type or tumor stage.[12, 13] Generally, autophagy plays an important anticarcinogenic role at an early stage of carcinogenesis by clearing damaged mitochondria and aberrant protein aggregates that produce ROS. Autophagy-related genes (ATG) play an key role in autophagy, which control autophagic formation, and the down-regulation of ATG genes directly or indirectly accelerates cancer development and progression.[14]

The ATG family in yeast contains 35 members, of which 16 are currently known in humans [i.e. *ATG2A, ATG2B, ATG3, ATG4A, ATG4B, ATG4C, ATG4D, ATG5, ATG6 (BECN1), ATG7, ATG9A, ATG9B, ATG10, ATG12, ATG14, ATG16L1* and *ATG16L2*].[15, 16] Studies

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have demonstrated that frame-shift mutations in ATG genes would lead to premature cessation of amino acid synthesis of the affected proteins and may inactivate their autophagy function.[17] For example, a mutation in *ATG2B* leads to the loss of 1039 of the total 2078 amino acids, while a mutation in *ATG5* leads to the loss of 41 of the total 275 amino acids.[17] Furthermore, deficient *ATG5* in neural cells leads to progressive neurodegeneration, accompanied by the accumulation of cytoplasmic inclusion bodies.[18] Although the exact roles of ATG10, ATG12 and ATG16 are not yet clear, an increased expression of ATG10 was reported to be significantly associated with lymph node metastasis and lymphovascular invasion in colorectal cancer,[19] and overexpression of ATG12 and ATG16 were demonstrated to inhibit autophagosome formation.[20] More recently, ATG12 silencing was shown to significantly reduce breast cancer cell growth in nude mice, which suggests that ATG12 may be an oncogenic protein.[21]

Recent clinical studies suggested that cancer radiotherapy induced autophagy directly or indirectly through DNA damage.[22] There are two primary opposing functions by radiation-induced autophagy: cytoprotective and cytotoxic. While radiation-induced autophagy often serves as a protective function in cell culture-based studies, it is still unclear to what an extent autophagy may be induced by radiation in human cancer cells, although the function of autophagy in response to radiation is inconsistent.[23] Some studies reported that autophagy promoted the anticancer effects of radiotherapy; others showed that upregulation of autophagy were associated with tumor resistance in radiation therapy and that blockade of autophagy contributed to the radiosensitization.[24] More recent reports suggested that inhibition of autophagy led to an increased expression of IL1B and IL6 and that autophagy played an important role in tissue inflammatory response.[25, 26] However, the role of genetic variants of *ATG* genes in RP and outcomes of patients with NSCLC after radiotherapy is largely unknown.

To date, only two studies reported that mRNA expression and genetic variants of ATG genes contributed to survival and brain metastasis in patients with NSCLC in Chinese populations,[8, 27] but none of the published studies have investigated functional genetic variants of ATG genes in association with RP and outcomes of NSCLC patients in an North American population. Potentially functional genetic variants of autophagy genes may alter the host autophagic capacity and thus influence efficiency of therapies.[28] Inspired by these findings, the present study was conducted to test the hypothesis that potentially functional genetic variants in ATG genes are prognostic and predictive for radiation-induced pneumonitis and clinical outcomes in NSCLC patients after definitive radiotherapy. In the present study, we evaluated the effects of nine functional variants in important ATG genes (e.g., *ATGB2, ATG10, ATG12*, and *ATG16L2)* on clinical outcomes among 393 NSCLC patients in a North American population.

Materials and Methods

Study populations

Characteristic details of the study population used in the present study have been described previously.[7] Briefly, the subjects included 474 patients with primary NSCLC who had been treated with definitive radiation and had available DNA samples as well as clinical follow-up

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data at a single institution between March 1998 and June 2009. Patients with an inoperable stage I to III disease and patients with an oligometastatic stage IV disease (with solitary metastases to the bone or brain) were included. The final group analyzed for single nucleotide polymorphisms (SNPs) and clinical outcomes consisted of 393 patients, all of whom received definitive radiotherapy in the initial treatment, and some of whom also received chemotherapy, either concurrent with or subsequent to the radiotherapy. Radiation toxicity events were scored according to the Common Terminology Criteria for Adverse Events v3.0, and details on methods for evaluating local recurrence-free survival (LRFS), progression-free survival (PFS), overall survival (OS) and RP were described elsewhere.[7] Briefly, we used computed tomography with or without positron emission tomography to evaluate RP at each follow-up visit. The time to RP development was calculated from the start of radiation therapy; patients not experiencing either end-point were censored at the date of the last follow-up or death. We interviewed each of the 393 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, but the 4-dimensional CT-based simulation with respiratory motion management and intensity modulation radiation therapy (IMRT) or proton beam radiation (PBT) was used thereafter. The University of Texas M.D. Anderson Cancer Center institutional review board approved the present study, and the Health Insurance Portability and Accountability Act (HIPAA) regulations were strictly followed.

Selection of SNPs and genotyping assays

The selection of candidate genes *ATGB2*,[17, 29] *ATG10*,[8, 30, 31] *ATG12*[21, 32, 33] and *ATG16L2*[34-36] was based on previously published studies, which show a positive association with risk of diseases including cancers. Using the public HapMap SNP database (phase II + III, August10, on NCBI B36 assembly, dbSNP b126) and the HaploView 4.2 software, common SNPs [a minor allele frequency (MAF) ≥ 0.05] in *ATG2B, ATG10, ATG12*, and *ATG16L2* were screened for in their gene regions (within these genes or ± 2-kb flanking regions) in CEU populations. After prediction by using the SNPinfo Web Server (http://snpinfo.niehs.nih.gov/) and RegulomeDB (http://regulomedb.org), candidate SNPs met at least two of the following three criteria: (1) MAF ≥ 0.05 in Caucasians, (2) SNPs were reported in previous association studies or with potential function, e.g., either causing amino acid change or affecting transcription factor binding site (TFBS) activity in the putative promoter, intron or 3’ UTR regions (FuncPred, http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), and (3) not in high linkage disequilibrium (LD), i.e. LD ⩾ 0.8. As a result, we selected nine functional SNPs in *ATG2B, ATG10, ATG12*, and *ATG16L2* genes were selected [*ATG2B* rs17784271 A>G (3’UTR) and rs4900321 A>T (3’UTR); *ATG10* rs10514231 C>T (intron 2), rs6884232 A>G (3’UTR), and rs4703533 C>G (the promoter region); *ATG12* rs26538 C>T (the promoter region) and rs1058600 C>T (3’UTR); *ATG16L2* rs1126205 G>T (the promoter region) and rs10898880 A>C (the promoter region)] (Supplemental Table S1).

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We extracted genomic DNA from the buffy-coat fraction of the whole blood samples by using a blood DNA mini kit (Qiagen, Inc.) by the process according to the manufacturer's instructions. The DNA purity and concentration were determined by spectrophotometer measurement of absorbance at 260 and 280 nm. The genotyping was performed using the TaqMan methodology in 384-well plates and read with the Sequence Detection Software on an ABI-Prism 7900 instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). 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 and 60°C for 1 min for 40 cycles. All SNPs assay success rate was >99% and the repeated sample's results were 100% concordant.

Bioinformatic analysis

Differential expression analysis was performed to evaluate the mRNA expression of *ATG16L2* in lung squamous carcinoma (LUSC), and lung adenocarcinoma (LUAD) and paired adjacent normal tissues using data generated by The Cancer Genome Atlas (TCGA).[37] The ENCODE (https://genome.ucsc.edu/) was used to identify the regulatory potential of the region adjoining the SNP. We assessed the associations between SNPs and mRNA expression levels of *ATG16L2* by expression quantitative trait loci (eQTL) analysis using the data of 338 whole blood cells of individuals of European descendants from GTEx (https://www.gtexportal.org/home/).

Statistical analysis

We estimated the associations of the genotypes with RP and clinical outcomes in the patients by using a Cox proportional hazards regression model, with the time to event as described previously.[7] The Kaplan-Meier method was used to visualize RP, LRFS, PFS and OS by genotype. RP intervals were calculated from the date that treatment began to the time of incidence of severe RP (grade ≥3); LRFS intervals were calculated from the date that treatment began to the time of tumor recurrence or death (LRFS) or the time of progression or death (PFS); and the patients were censored at the time of the final follow-up.[38] The OS interval was calculated from the date of diagnosis until death or the date of last contact. Multivariate Cox proportional hazards regression models were performed to calculate hazards ratios (HRs) and 95% confidence intervals (CIs) of each genotype to estimate its effect on RP, LRFS, PFS, and OS with adjustment for confounding factors. Such factors were identified with stepwise Cox regression models, which were used to estimate independent predictors of NSCLC prognosis, with a significance level of 0.050 for entering and 0.051 for removing the respective explanatory variables. Further stratified analysis was applied to assess stratified groups. All statistical tests were two-sided, with a *P* value of 0.05 considered significant, and all analyses were performed using SAS software version 9.2 (SAS Institute, Cary, NC). We also calculated the false-positive report probability (FPRP) to detect the false-positive association findings.[39] For all the significant results, we calculated FPRP with prior probabilities of 0.0001, 0.001, 0.01, 0.1, and 0.25. The HR was set close to 0.67 (protection) or 1.50 (risk), and a prior probability value of < 0.2 was considered noteworthy.

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Results

Characteristics of the study population

Demographic and clinical characteristics of the 393 patients treated with definitive radiotherapy, with or without concurrent or subsequent chemotherapy are presented in Table 1. There were more men (216, 55.0%) than women (177, 45.0%), with a median age of 65 years ranging between 35 and 88 years; 83.5% were self-reported non-Hispanic white; 85.5% had a stage III or IV disease (309 stage III and 27 stage IV);[40] 91.1% (358 patients) were treated with a combination of radiotherapy and chemotherapy. The median total radiation dose received by the patients was 66 Gy (range, 50-88 Gy), with a median mean lung dose (MLD) of 19 Gy (range, 2.7-30.6 Gy), a median 35 fractions (range, 15-58 fractions) a median GTV of 108.1 cm^3, and a median follow-up time of 23 months (range, 1.0-157.1 months). The overall median RP, LRFS, PFS, and OS times for all the 393 patients were 14.8, 15.2, 10.7, and 23.5 months, respectively. The median occurrence time for severe RP (grade ≥3) was 3.6 months (range, 1.9-3.6 months) after radiation therapy. To identify potentially confounding factors, we evaluated potential associations of severe RP, LRFS, PFS, and OS with clinical and treatment-related characteristics in univariate Cox models for age, sex, ethnicity, KPS, disease stage, tumor histology, smoking status, use of chemotherapy, MLD, ORT, GVT and radiation dose, respectively. In the univariate analysis, we found that the MLD was significantly associated with severe RP, LRFS, PFS, and OS (MLD ≥19.0 Gy vs. MLD <19.0 Gy, crude HR = 3.12, 1.40, 1.35, and 1.45; 95% CI = 1.73-5.64, 1.11-1.76, 1.08-1.69, and 1.15-1.83; P = 0.0002, 0.005, 0.009 and 0.002 for RP, LRFS, PFS, and OS, respectively) (Table 1).

Associations of SNPs in ATG2B, ATG10, ATG12 and ATG16L2 with LRFS, PFS, OS and risk of RP, respectively

Table 2 and Supplemental Table S2 show genotype distributions of the nine SNPs, the results from the multivariate Cox regression analyses of the associations of the nine SNPs with severe RP, LRFS and PFS as well as OS in the NSCLC patients. As shown in Table 2, in the multivariate Cox regression analyses, we found that the *ATG16L2* rs10898880 variant CC homozygotes had a better LRFS, PFS and OS (adjHR = 0.54, 0.57 and 0.59; 95% CI = 0.39-0.76, 0.41-0.79 and 0.41-0.83; P = 0.0005, 0.0009 and 0.003, P_{trend} test = 0.0004, 0.0007 and 0.002, respectively), compared with patients with the AA genotype; and these effects in a recessive genetic model also remained statistically significant for a better LRFS, PFS and OS (adjHR = 0.59, 0.64 and 0.64; 95% CI = 0.45-0.79, 0.48-0.84 and 0.48-0.86; P = 0.0004, 0.002 and 0.003, respectively) after adjustment for potential confounders, including age, sex, histological grade, MLD, disease stage, KPS score, and GTV (Table 2 and Fig. 1a-c). In contrast, we found that patients with the variant *ATG16L2* rs10898880 CC genotype had a greater risk of severe RP (adjHR = 1.80, 95% CI = 1.04-3.12, P = 0.037), compared with patients with AA/AC genotypes (a recessive genetic model), after adjustment for potential confounders, including MLD, disease stage, smoking status, and chemotherapy (Table 2 and Fig. 1d). Our data also showed that patients with the *ATG10* rs4703533 GG variant genotype also had a better LRFS and PFS (adjHR = 0.62 and 0.63; both 95% CI = 0.41-0.95; P = 0.029 and 0.027 for LRFS and PFS, respectively), compared with patients with the CC genotype; and the effect in a recessive genetic model also remained statistically

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significant for a better LRFS, PFS (adjHR = 0.59 and 0.60; 95% CI = 0.39-0.88 and 0.40-0.89; both $P = 0.011$, respectively). While the variant *ATG10* rs4703533 CG or GG genotype was associated with a greater risk of severe RP (adjHR = 2.04 and 2.49; 95% CI = 1.11-3.73 and 1.14-5.45; $P = 0.021$ and 0.023, respectively; P_{trend} test = 0.008), and GG/CG genotypes were associated with a significantly increased hazard of severe RP (adjHR = 2.14, 95% CI = 1.21-3.80, $P = 0.009$), compared with the CC genotype (Table 2). For the other five SNPs, we did not find any significant association with RP, LRFS, PFS and OS under either additive or recessive genetic models (Supplemental Table S2), except the *ATG2B* rs17784271 AG genotype was associated with a poorer LRFS and PFS (adjHR = 1.40 and 1.38; 95% CI = 1.09-1.81 and 1.07-1.77; $P = 0.009$ and 0.012, respectively), compared with the AA genotype in multivariate analysis; and the *ATG12* rs1058600 TT genotype was associated with a poorer LRFS, PFS and OS (adjHR = 1.51, 1.43, and 1.50; 95% CI = 1.08-2.10, 1.03-2.01, and 1.07-2.10; $P = 0.016$, 0.035, and 0.019, respectively), compared with CC/CT genotypes (Table 2).

Stratified analyses by selected variables

We then performed stratified analyses to evaluate the effects of variant genotypes of *ATG16L2* rs10898880 and *ATG10 rs4703533* on the hazards of LRFS, PFS, and OS by age, sex, KPS, disease stage, tumor histology, MLD and GTV under recessive models by using multivariate Cox models for the main effects. As shown in Table 3, the *ATG16L2* rs10898880CC variant homozygotes had a better survival for LRFS, PFS and OS in males (adjHR = 0.52, 0.54 and 0.53; 95% CI = 0.35-0.77, 0.37-0.79 and 0.35-0.79; $P = 0.001$, 0.002 and 0.002, respectively), KPS ≥80 (adjHR = 0.59, 0.64 and 0.63; 95% CI = 0.43-0.82, 0.47-0.88 and 0.45-0.88; $P = 0.002$, 0.006 and 0.006, respectively), stage III/IV (adjHR = 0.59, 0.64 and 0.65; 95% CI = 0.44-0.80, 0.48-0.85 and 0.48-0.88; $P = 0.0006$, 0.002 and 0.005, respectively), compared with patients with AA/AC genotypes, while the CC variant genotype was more prominently associated with an increased risk of severe RP in those of age <65 year (adjHR = 2.52, 95% CI = 1.18-5.37, $P = 0.016$) and stage III/IV (adjHR = 1.90; 95% CI = 1.04-3.48; $P = 0.038$) (Table 3). The *ATG10* rs4703533GG variant genotype was more prominently associated with a better survival for LRFS, PFS and OS in females (adjHR = 0.39, 0.42 and 0.48; 95% CI = 0.20-0.77, 0.22-0.80 and 0.24-0.96; $P = 0.007$, 0.009 and 0.037, respectively), those at age ≥65 (adjHR = 0.47, 0.48 and 0.54; 95% CI = 0.27-0.83, 0.28-0.82 and 0.31-0.95; $P = 0.009$, 0.007 and 0.032, respectively), squamous carcinoma (adjHR = 0.40, 0.38 and 0.44; 95% CI = 0.19-0.85, 0.18-0.81 and 0.20-0.93; $P = 0.018$, 0.012 and 0.032, respectively), and GTV <108 cm^3 (adjHR = 0.37, 0.40 and 0.45; 95% CI = 0.20-0.69, 0.23-0.73 and 0.24-0.84; $P = 0.002$, 0.002 and 0.012, respectively), compared with CC/CG genotypes (Supplemental Table S3). While the ATG12 rs1058600TT variant genotype was more prominently associated with a poorer survival for LRFS, PFS and OS in males (adjHR = 1.59 1.68, and 1.71; 95% CI = 1.02-2.48 1.08-2.62, and 1.09-2.67; $P = 0.040$, 0.022 and 0.019, respectively), those at age ≥65 (adjHR = 1.69, 1.67 and 1.80; 95% CI = 1.08-2.64, 1.07-2.62 and 1.15-2.83; $P = 0.022$, 0.024 and 0.011, respectively), and KPS ≥80 (adjHR = 1.69, 1.70 and 1.66, 95% CI = 1.17-2.44, 1.17-2.46 and 1.14-2.42; $P = 0.005$, 0.005 and 0.008, respectively), compared with CC/CT genotypes (Supplemental Table S4).

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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 associations of severe RP and clinical outcomes with the *ATG16L2* rs10898880CC genotype (a recessive model) were still noteworthy for all subjects (FPRP = 0.144, 0.001 0.004 and 0.010 for RP, LRFS, PFS and OS, respectively), those at age <65 years (FPRP = 0.154, 0.010, 0.015 and 0.099 for RP, LRFS, PFS and OS, respectively), and stage III and IV (FPRP = 0.166, 0.002, 0.006 and 0.016 for RP, LRFS, PFS and OS, respectively). When the assumption of a prior probability was 0.1, the associations of clinical outcomes with the *ATG16L2* rs10898880CC genotype (a recessive model) were still noteworthy for all subjects (FPRP = 0.004, 0.012 and 0.028 for LRFS, PFS and OS, respectively), those at age <65 years (FPRP = 0.031 and 0.042 for LRFS and PFS, respectively), and stage III and IV (FPRP = 0.007, 0.019 and 0.048 for LRFS, PFS and OS, respectively).

For the *ATG10* rs4703533, when the assumption of a prior probability was 0.25, the associations of clinical outcomes with the *ATG10* rs4703533CC genotypes (a recessive model) were still noteworthy for all subjects (FPRP = 0.035, 0.039 and 0.154 for LRFS, PFS and OS, respectively), those at age ≥65 years (FPRP = 0.194, 0.063, 0.047 and 0.139 for RP, LRFS, PFS and OS, respectively), and GVT <108 cm^3 (FPRP = 0.030, 0.035 and 0.090 for LRFS, PFS and OS, respectively), (**Supplemental Table S5**). For the *ATG12* rs1058600, when the assumption of a prior probability was 0.25, the associations of clinical outcomes with the *ATG12* rs1058600TT genotypes (a recessive model) were still noteworthy for all subjects and subgroups (**Supplemental Table S6**).

Differential mRNA expression of ATG16L2 and functional analysis of ATG16L2 rs10898880

We also evaluated differential mRNA expressions levels of *ATG16L2* in tumor tissues from 50 lung squamous carcinomas (LUSC), and 57 lung adenocarcinomas (LUAD) and paired adjacent normal tissues by using the data generated by The Cancer Genome Atlas (TCGA). As shown in Fig. 2a and 2b, the expression levels of *ATG16L2* were significantly lower in LUSC and LUAD, compared with that in the adjacent normal tissues ($P = 1.14\times10^{-6}$ and 3.36×10^{-5}, respectively). To identify the putative functional role of *ATG16L2* rs10898880, functional annotations from the Encyclopedia of DNA Elements (ENCODE) data indicate that rs10898880 is situated at a locus with transcription factor binding, DNase hypersensitivity, and histone modification patterns that characterize as the promoters in several cell types, which present strong signals of active enhancer and promoter functions (Fig. 2c). Moreover, the rs10898880 is within the *ATG16L2* promoter region and is predicted to be located at transcription factor binding sites (TFBS) by the SNPinfo online tool. To provide biologically plausible support for the observed association and prediction, we evaluated the correlation between the rs10898880 SNP and *ATG16L2* mRNA expression levels by genotype, using mRNA expression data of the blood cells from 338 European descendants by eQTL analysis using data from GTEx. The results showed that the rs10898880 variant C allele was associated with increased mRNA expression levels of *ATG16L2* ($P = 1.4\times10^{-10}$) (Fig. 2d). These data strongly suggest that the *ATG16L2* rs10898880 C variant allele may modulate gene expression levels of *ATG16L2*.

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Discussion

In the present study, we have investigated the associations of nine potentially functional SNPs in *ATG2B, ATG10, ATG12* and *ATG16L2* with server RP, LRFS, PFS and OS in 393 NSCLC patients after definitive radiotherapy, with or without chemotherapy. We found that the *ATG16L2* rs10898880 CC variant genotype and *ATG10* rs4703533 GG variant genotype contributed to a significantly better outcome but a greater risk of RP of patients with NSCLC; stratified analyses showed that the *ATG16 L2* rs10898880 variant CC genotype was more evident in subgroups of age <65 years and stage III/IV. Although multiple tests had been performed in the present study, the results of FPRP indicated that the associations of potentially functional C variant of *ATG16L2* rs10898880 with severe RP, LRFS, PFS and OS were less likely to be false positive. We also provided biological evidence that the mRNA expression levels of *ATG16L2* were lower in lung cancer tissues than that in lung normal tissues and that the *ATG16L2* rs10898880 CC variant genotype was associated with higher mRNA expression levels of *ATG16L2* than AA and AC genotypes in the whole blood cells. These data imply that *ATG16L2* rs10898880 C variant may play a role in the severe RP and clinical outcomes of patients with NSCLC by modulating the mRNA expression levels of *ATG16L2*. Therefore, our results suggest that the *ATG16L2* rs10898880 functional C variant may be a useful biomarker for predicting severe RP and clinical outcomes of patients with NSCLC after definitive radiotherapy, once these results have been validated by additional investigations.

Although dysfunctional study of the ATG genes has addressed the roles of autophagy in both cell death and survival,[41] few studies, to date, have explored the role of genetic variants of ATGs in severe RP and clinical outcomes of lung cancer.[8] We selected *ATG2B* because it is a newly discovered gene that participates in the autophagy pathways.[42] As an important player in autophagy, ATG2B is essential for autophagosome formation and necessary for closure of isolation membranes of autophagosomes.[43] The mutations in *ATG2B* have been shown to lead to the loss 1039 of the total 2078 amino acids, and loss-of-function mutations in *ATG2B* have been identified in gastric and colorectal cancers,[17, 44] which was overexpressed in primary hematopoietic cells from patients compared with donor cells.[44] However, the role of genetic variants of *ATG2B* in cancer susceptibility is largely unknown.

In a cohort of 192 non-muscle invasive bladder cancer (NMIBC) patients treated with Bacillus Calmette-Guerin (BCG), Buffen et al. demonstrated that the *ATG2B* rs3759601 SNP was correlated with progression and recurrence of bladder cancer after BCG intravesical instillation therapy.[29] In contrast, in another study of 238 patients with paget disease of bone (PDB) and 264 sex-matched controls, Usategui-Martin et al. found that genotype frequencies did not significantly differ between PDB patients and healthy subjects.[45] In the present study, we observed that *ATG2B* rs17784271 variant G allele was associated with a poor LRFS and PFS, but we did not find an association of the variant genotypes with risk of severe RP and OS in patients with NSCLC, suggesting that the *ATGB2* rs17784271 variant G allele may play a role in the LRF and PFS of NSCLC after definitive radiotherapy.

ATG10 has been mapped to chromosome 5q14, which codes for an autophagic E2-like enzyme essential for the autophagosome formation.[46] A previous study reported that

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increased ATG10 expression was strongly associated with tumor invasion and metastasis, and, as an oncogenic gene, ATG10 may be a potential prognostic maker for colorectal cancer.[31] In one study of 1064 breast cancer cases and 1073 cancer-free controls in a Chinese population, the authors found that genetic variants in *ATG10* rs10514231 were associated with risk of breast cancer.[30] More recently, in another survival study of 1001 Chinese NSCLC patients, Xie et al. observed that variant alleles of rs10514231 and rs1864182 were associated with a poor overall survival, and these variants were associated with the increased methylation levels of cg17942617.[8] The authors also observed that the elevated mRNA expression of *ATG10* predicted a shorter survival.[8] In the present study, we did not observe a significant association between genetic variant in re10514231 and RP as well as clinical outcomes of patients with NSCLC. However, we found that the *ATG10* rs4703533 GG variant genotype contributed to a significantly increased risk of severe RP and a longer LRFS and PFS of patients with NSCLC. These findings need to be validated by other studies with large samples and in-depth functional experiments, and ethnic background should be carefully considered in further studies.

ATG12 plays a critical role in autophagy.[15, 16] Recent studies have indicated that *ATG12* transcript is commonly upregulated in trastuzumab-unresponsive HER2-overexpressing breast cancer cells and that *ATG12* silencing significantly reduced ATG12-shRNA/JIMT1 (breast cancer cell line) tumor growth induced by subcutaneous injection of trastuzumab in nude mice.[21] However, the role of *ATG12* variants in risk and clinical outcomes of cancer is largely unknown. One previously published study assessed the association between the *ATG12* rs26538 polymorphism and risk of breast cancer in 1064 cases and 1073 controls in a Chinese population, but no significant association with cancer risk was found.[30] Other studies found that there was no significant association with survival and brain metastasis of NSCLC in Chinese patients.[8, 27] In the present study, we genotyped two functional SNPs of this gene, and, similarly, we also did not find significant association between *ATG12* rs26538 SNP and RP as well as clinical outcome of Caucasian patients with NSCLC. However, our analysis on the *ATG12* rs1058600 SNP showed that the variant T allele was associated with a better LRFS, PFS and OS, suggesting that the *ATG12* rs1058600 variant T allele may play a role in clinical outcomes of NSCLC after definitive radiotherapy. The functional rs1058600 T variant is located in the 3'UTR of the *ATG12* primary transcript and in the predicted miRNA-binding site (*miR-181* and *miR-494*) (http://snpinfo.niehs.nih.gov/snpfunc.htm). It has been reported that the *miRNA-181* functions as a tumor suppressor or a tumor promotor by influencing expression of the target genes at the posttranscriptional level,[47] and *miR-494* expression was correlated with lung cancer progression in mice.[48] These may partly explain the underlying biological and molecular mechanisms for the observed associations, which need to be further investigated.

It is known that autophagy-related protein 16-like (ATG16L, including ATG16L1 and ATG16L2) is an important regulator of autophagy and plays key roles in the autophagy pathway and tumorigenesis.[49] However, the exact molecular mechanism of ATG16L2 is still unclear. Previously, one study reported that the isoform ATG16L1 was essential for autophagy, while ATG16L2 did not seem to be important.[50] Later, another study of cell responses to cisplatin treatment used the ATG16L2 antibody to monitor expression of autophagy and cell death related genes,[51] which showed that ATG16L2 levels were reduced

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in response to cisplatin, a possible biomarker or target for tumor cell resistance to the platinum-based drugs.[51] Recently, *Atg16L2* mutants were found to have a more severe defect than *Atg16L1* mutants in C. *elegans*.[52] *A* more recent study reported that *ATG16L2* was a ubiquitously expressed homologue of *ATG16L1*, the ATG16L2 interacts with ATG5 and codes for a protein related to autophagy and formation of autophagosome.[53] In one study of 54 patients with multiple sclerosis (MS) and 55 healthy controls in a Chinese population, Yin et al. found that *ATG16L2* might play an important role in autophagy, specifically in T-cells, and served as a potential biomarker to predict clinical relapse of MS.[35] In another study of a GWA scan of 1,174 systemic lupus erythematosus (SLE) cases and 4,248 controls in a Korean population, the authors observed that *ATG16L2* was involved in the association with SLE.[53] Similarly, in a Chinese population study of 363 Crohn's disease cases and 486 controls, the authors observed that mRNA expression levels of *ATG16L2* were lower in T cells of patients than that in controls and that an increasing mRNA expression level of *ATG16L2* in patients with the *ATG16L2* rs11235604 CC genotype were associated with a decreased risk of Crohn's disease.[36] However, the exact role of *ATG16L2* in lung cancer development and progression remains unclear and needs to be further investigated. Based on the important role of *ATG16L2* in the autophagy pathway, we observed associations of two potentially functional SNPs in *ATG16L2* with severe RP and outcomes in 393 NSCLC patients after definitive radiotherapy. Lower mRNA expression levels of *ATG16L2* in the lung cancer tissues and higher mRNA expression levels of *ATG16L2* correlated with rs10898880 CC variant genotype in blood cells were observed, while the rs10898880 variant CC genotype was associated with a better LRFS, PFS and OS, suggesting that the functional genetic variant of *ATG16L2* rs10898880 may play a role in clinical outcomes of patients with NSCLC by modulating the mRNA expression levels of *ATG16L2*. According to the ENCODE project data from UCSC, rs10898880 was associated with considerable levels of histone modification of H3K27 acetylation (H3K27Ac), mono-methylation of lysine 4 (H3K4Me1), and tri-methylation of lysine 4 of the H3 (H3K4Me3) histone protein enrichment, which may be associated with promoter function and histone modifications may influence gene expression.[54] More interesting, our results also showed that among NSCLC patients, severe RP was more likely to occur in those carrying rs10898880 CC genotype than in those carrying the rs10898880 AA/AC genotypes. This finding is consistent with previous reports showing that overexpression of ATG16 inhibited autophagosome formation and resulted in tissue inflammatory response.[20, 25, 26] However, this hypothesis needs to be future tested in future studies.

To the best of our knowledge, the present study is the first focusing only on associations of the potentially functional SNPs of *ATGB2, ATG10, ATG12* and *ATG16L2* with severe RP, LRFS, PFS and OS in NSCLC patients after definitive radiotherapy. However, there were some limitations in the present study. First, we were unable to explore the exact mechanisms by which the ATG SNPs influence severe RP and outcomes of NSCLC patients, because we did not have the access to the target tissues. Second, we used the reported common, functional SNPs, which may not include all representative SNPs in the entire gene. Some other rare functional SNPs, which could influence survival, may have been missed and need to be investigated in future larger studies. Thirdly, we did not find a suitable and accessible patient population for the validation of our results. Finally, additional larger validation

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studies with multiethnic groups are needed to confirm our results, because our prognosis-predicting model was based on a North American patient population.

In conclusion, we found that potentially functional genetic variant of *ATG16L2* predicted risk of RP and better LRFS, PFS and OS in patients with NSCLC treated with definitive radiotherapy with or without chemotherapy. Whether the SNPs have an impact on the prognosis of NSCLC directly through alteration of autophagy or by other, possibly indirectly, mechanisms should be further investigated in the future. Once validated, patients who at high risk of developing RP should receive a closer follow-up and should be considered for adjuvant therapy in the consideration of individualized treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

NSCLC	Non-small cell lung cancer
ATG	autophagy related gene
SNP	single nucleotide polymorphism
RP	radiation pneumonitis
LRFS	local recurrence-free survival
PFS	progression- free survival
OS	overall survival
HR	hazards ratio
CI	confidence interval

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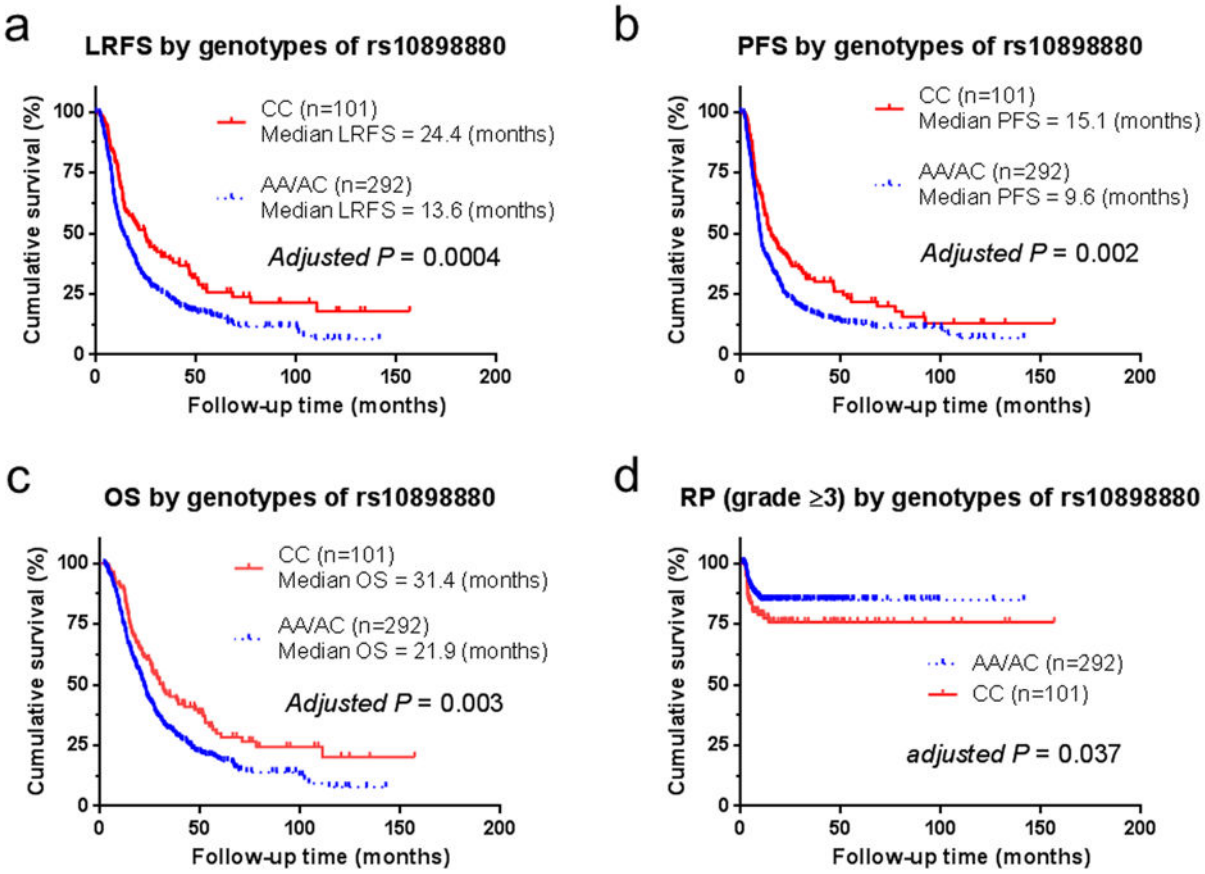

Figure 1.

Kaplan-Meier (KM) analysis for severe RP and clinical outcomes according to *ATG16L2* rs10898880 CC versus AA/AC genotypes (in a recessive model). The CC variant genotype was associated with (**a**) local recurrence-free survival (LRFS; $P = 0.0004$), (**b**) progression-free survival (PFS; $P = 0.002$), (**c**) overall survival (OS; $P = 0.003$), and (**d**) severe radiation pneumonitis (grade ≥3) (RP; $P = 0.037$).

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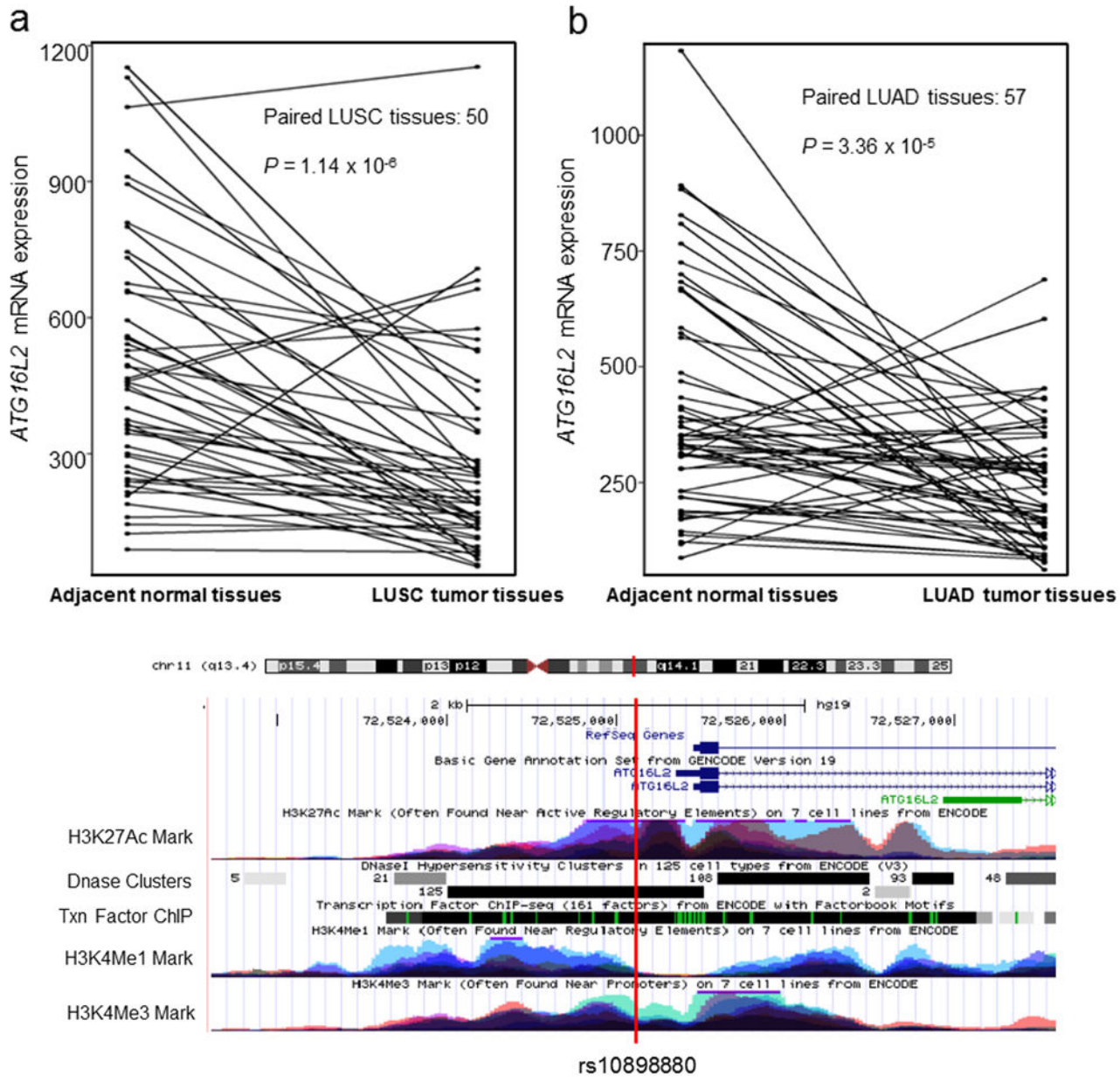

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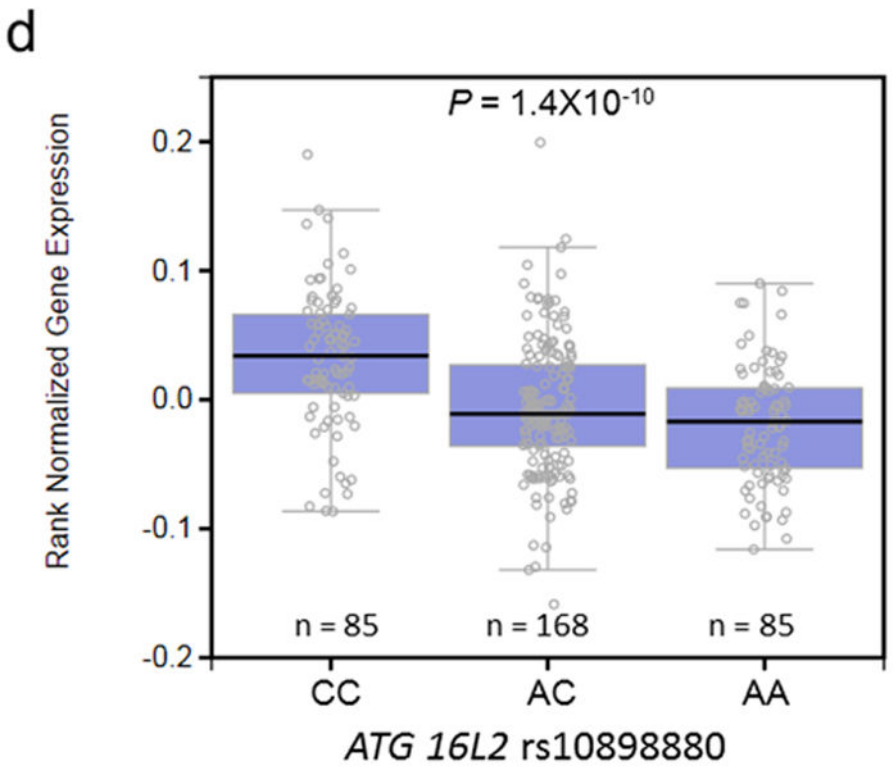

Figure 2.

Differential mRNA expression of *ATG16L2* in lung tissues and functional analysis of *ATG16L2* rs10898880. (**a** and **b**). Differential mRNA expression analysis by using the data generated by The Cancer Genome Atlas (TCGA) showed a lower expression level of *ATG16L2* in lung squamous carcinoma (LUSC) and lung adenocarcinoma (LUAD) than that in paired adjacent lung normal tissues ($P = 1.14\times10^{-6}$ and 3.36×10^{-5}, respectively). (**c**). Expanded view of the ENCODE data for the *ATG16L2* rs10898880. The H3K27Ac, H3K4Me1, and H3K4Me3 tracks showed the genome-wide levels of enrichment of acetylation of lysine 27, the mono-methylation of lysine 4, and tri-methylation of lysine 4 of the H3 histone protein, as determined by the ChIP-seq assays. These levels are thought to be associated with the promoter and enhancer regions. DNase clusters track showed Dnase hypersensitivity areas. Tnx factor track showed regions of transcription factor binding of DNA, as assayed by ChIP-seq experiments. (**d**). Correlation of *ATG16L2*-related mRNA expression with genotypes of rs10898880 in 261 lung adenocarcinoma (LUAD), 198 lung squamous cell carcinomas (LUSC) tumor tissues from The Cancer Genome Atlas (TCGA) database ($P = 0.857$ and 0.974, respectively), and 338 whole blood cells ($P = 1.4\times10^{-10}$) by eQTL analysis using data from GTEx (http://www.gtexportal.org/home/).

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Table 1.

Patient demographics of 393 patients with NSCLC and their associations with hazard of severe RP, and clinical outcomes after definitive radiotherapy

Parameter	RP (radiation pneumonitis, grade ≥3)				LRFS (local recurrence-free survival)				PFS (progression- free survival)				OS (overall survival)			
	0 / 1	HR	95% CI	P^a	0 / 1	HR	95% CI	P^a	0 / 1	HR	95% CI	P^a	0 / 1	HR	95% CI	P^a
Sex																
Male	181 / 35	1.00			36 / 180	1.00			32 / 184	1.00			41 / 175	1.00		
Female	154 / 23	0.76	0.45-1.28	0.300	33 / 144	0.89	0.72-1.11	0.309	24 / 153	0.98	0.79-1.21	0.845	42 / 135	0.88	0.70-1.10	0.263
Age (years)																
<65	172 / 30	1.00			40 / 162	1.00			31 / 171	1.00			49 / 153	1.00		
≥65	163 / 28	1.03	0.61-1.72	0.922	29 / 162	1.15	0.93-1.43	0.206	25 / 166	0.98	0.79-1.21	0.815	34 / 157	1.18	0.94-1.47	0.147
Race																
White	280/ 48	1.00			61 / 267	1.00			49 / 279	1.00			74 / 254	1.00		
Black	55 / 10	1.05	0.53-2.08	0.879	8 / 57	1.13	0.85-1.51	0.399	7 / 58	1.13	0.85-1.50	0.395	9 / 56	1.13	0.85-1.51	0.407
KPS																
≥80	275 / 42	1.00			59 / 258	1.00			47 / 270	1.00			73 / 244	1.00		
<80	60 / 16	**1.80**	**1.01-3.20**	**0.046**	10 / 66	1.28	0.97-1.67	0.079	9 / 67	1.13	0.86-1.48	0.379	10 / 66	**1.36**	**1.03-1.79**	**0.028**
Stage groups[b]																
I,II	45 / 10	1.00			15 / 40	1.00			15 / 40	1.00			15 / 40	1.00		
III, IV	288 / 48	0.78	0.40-1.55	0.480	54 / 282	**1.51**	**1.08-2.10**	**0.015**	41 / 295	**1.80**	**1.29-2.51**	**0.0005**	68 / 268	**1.42**	**1.02-1.98**	**0.039**
Histology																
Squamous	118 / 20	1.00			20/ 118	1.00			17 / 121	1.00			22 / 116	1.00		
Nonsquamous[c]	217 / 38	1.04	0.61-1.79	0.887	49 / 206	0.80	0.64-1.00	0.051	39 / 216	0.93	0.75-1.16	0.529	61 / 194	**0.79**	**0.63-1.00**	**0.046**
Smoking status																
Ever	305 / 54	1.00			64 / 295	1.00			54 / 305	1.00			74 / 285	1.00		
Never	30 / 4	0.85	0.31-2.36	0.760	5 / 29	1.10	0.75-1.62	0.614	2 / 32	1.26	0.88-1.82	0.210	9 / 25	0.95	0.63-1.44	0.821
Chemotherapy																
No	30 / 4	1.00			6 / 28	1.00			6 / 28	1.00			6 / 28	1.00		
Yes	304 / 54	1.27	0.46-3.50	0.648	62 / 296	0.95	0.64-1.39	0.776	49 / 309	1.03	0.70-1.51	0.895	76 / 282	0.94	0.64-1.39	0.759
MLD (Gy)[b,d]																
<19.0	166 / 15	1.00			41 / 140	1.00			34 / 147	1.00			49 / 132	1.00		

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Parameter	RP (radiation pneumonitis, grade ≥3)				LRFS (local recurrence-free survival)				PFS (progression- free survival)				OS (overall survival)			
	0 / 1	**HR**	**95% CI**	***P*[a]**	**0 / 1**	**HR**	**95% CI**	***P*[a]**	**0 / 1**	**HR**	**95% CI**	***P*[a]**	**0 / 1**	**HR**	**95% CI**	***P*[a]**
≥19.0	140 / 41	**3.12**	**1.73-5.64**	**0.0002**	26 / 155	**1.40**	**1.11-1.76**	**0.005**	20 / 161	**1.35**	**1.08-1.69**	**0.009**	32 / 149	**1.45**	**1.15-1.83**	**0.002**
ORT																
IMRT	149 / 18	1.00			36 / 131	1.00			29 / 138	1.00			47 / 120	1.00		
3DCRT	139 / 38	2.09	1.20-3.67	0.010	22 / 155	1.02	0.81-1.29	0.880	18 / 159	1.06	0.84-1.34	0.606	24 / 153	1.17	0.92-1.48	0.215
Other	47 / 2	0.34	0.08-1.47	0.150	11 / 38	0.99	0.69-1.42	0.943	9 / 40	0.97	0.68-1.34	0.857	12 / 37	1.02	0.70-1.47	0.923
GTV [b,e]																
<108 cm^3	151 / 22	1.00			43 / 130	1.00			35 / 138	1.00			53 / 120	1.00		
≥108 cm^3	142 / 32	1.68	0.98-2.89	0.062	22 / 152	**1.66**	**1.31-2.10**	**<0.0001**	18 / 156	**1.70**	**1.35-2.14**	**<0.0001**	25 / 149	**1.85**	**1.45-2.36**	**<0.0001**
Radiation Dose[f]																
<66 Gy	159 / 26	1.00			27 / 158	1.00			24 / 161	1.00			34 / 151	1.00		
≥66 Gy	176 / 32	1.02	0.61-1.7	0.951	42 / 166	0.81	0.65-1.01	0.059	32 / 176	0.84	0.68-1.04	0.110	49 /159	0.83	0.67-1.04	0.111

Abbreviations: NSCLC = non-small cell lung cancer; RP = radiation pneumonitis; LRFS = local recurrence free survival; PFS = progression- free survival; OS = overall survival; 0 = the absence of events; 1 = the presence of events; HR = hazards ratio; CI = confidence interval; KPS = Karnofsky performance status score; MLD = mean lung dose; IMRT = intensity-modulated radiation therapy; 3DCRT = 3-dimensional conformal radiotherapy; GTV = gross tumor volume.

[a] *P* values were calculated by univariate Cox proportional hazards models.

[b] Some missing data.

[c] Included large cell, undifferentiated, and mixed-cell carcinomas

[d] Median 19 Gy

[e] Median 108.1 cm^3

[f] Median 66 Gy

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Table 2.

Associations among genotypes of ATG genes, severe RP, and clinical outcomes in 393 patients with NSCLC after definitive radiotherapy

Genotypes	RP (radiation pneumonitis, grade ≥3)				LRFS (local recurrence-free survival)				PFS (progression- free survival)				OS (overall survival)			
	0 / 1	HR	95% CI	P^a	0 / 1	HR	95% CI	P^b	0 / 1	HR	95% CI	P^b	0 / 1	HR	95% CI	P^b
ATG2B (rs17784271)																
AA	150 / 32	1.00			41 / 141	1.00			34 / 148	1.00			43 / 139	1.00		
AG	148 / 23	0.67	0.39-1.16	0.157	21 / 150	**1.40**	**1.09-1.81**	**0.009**	16 / 155	**1.38**	**1.07-1.77**	**0.012**	30 / 141	1.28	0.99-1.66	0.058
GG	37 / 3	0.45	0.14-1.49	0.192	7 / 30	1.04	0.67-1.61	0.863	6 / 34	1.14	0.74-1.74	0.565	10 / 30	0.90	0.57-1.43	0.657
P_{trend} test				0.078				0.148				0.082				0.513
GG/AG	185 / 26	0.64	0.37-1.08	0.094	28 / 180	**1.32**	**1.04-1.69**	**0.024**	22 / 189	**1.33**	**1.05-1.69**	**0.019**	40 / 171	1.21	0.94-1.54	0.142
AA/AG	298 / 55	1.00			62 / 191	1.00			50 /303	1.00			73 / 280	1.00		
GG	37 3	0.55	0.17-1.75	0.310	7 / 30	0.89	0.59-1.35	0.577	6 / 34	0.98	0.65-1.48	0.922	10 / 30	0.80	0.51-1.25	0.325
ATG10 (rs4703533)																
CC	154 / 18	1.00			31 / 141	1.00			25 / 147	1.00			34 / 138	1.00		
CG	145 / 30	**2.04**	**1.11-3.73**	**0.021**	26 / 149	1.12	0.87-1.45	0.378	20 / 155	1.10	0.86-1.42	0.435	37 / 138	1.04	0.80-1.35	0.795
GG	36 / 10	**2.49**	**1.14-5.45**	**0.023**	12 / 34	**0.62**	**0.41-0.95**	**0.029**	11 / 35	**0.63**	**0.41-0.95**	**0.027**	12 / 34	0.68	0.44-1.04	0.077
P_{trend} test				**0.008**				0.165				0.151				0.199
GG/CG	181 / 40	**2.14**	**1.21-3.80**	**0.009**	38 / 183	0.98	0.77-1.25	0.883	31 / 190	0.97	0.77-1.24	0.829	49 / 172	0.94	0.74-1.21	0.646
CC/CG	299 / 48	1.00			57 / 290	1.00			45 / 302	1.00			71 / 276	1.00		
GG	36 / 10	1.70	0.85-3.37	0.131	12 / 34	**0.59**	**0.39-0.88**	**0.011**	11 / 35	**0.60**	**0.40-0.89**	**0.011**	12 / 34	0.67	0.44-1.01	0.054
ATG12 (rs1058600)																
CC	177 / 27	1.00			38 / 166	1.00			32 / 172	1.00			48 / 156	1.00		
CT	118 / 25	1.47	0.84-2.58	0.175	29 / 114	0.85	0.65-1.11	0.239	22 / 121	0.88	0.68-1.15	0.353	32 / 111	0.90	0.68-1.18	0.439
TT	40 / 6	0.79	0.33-1.93	0.610	2 / 44	1.41	0.99-2.00	0.054	2 / 44	1.36	0.96-1.94	0.085	3 / 43	1.43	1.00-2.05	0.050
P_{trend} test				0.900				0.369				0.361				0.227
TT/CT	158 / 31	1.26	0.74-2.13	0.398	31 / 158	0.98	0.77-1.24	0.834	24 / 165	0.99	0.78-1.25	0.925	35 / 154	1.02	0.79-1.31	0.885
CC/CT	295 / 52	1.00			67 /280	1.00			54 / 293	1.00			80 / 267	1.00		
TT	40 / 6	0.67	0.29-1.57	0.358	2 / 44	**1.51**	**1.08-2.10**	**0.016**	2 / 44	**1.43**	**1.03-2.01**	**0.035**	3 / 43	**1.50**	**1.07-2.10**	**0.019**
ATG16L2(rs10898880)																
AA	83 / 19	1.00			9 / 93	1.00			6 / 96	1.00			13 / 89	1.00		
AC	172 / 18	**0.47**	**0.24-0.91**	**0.024**	33 / 157	0.87	0.65-1.16	0.347	29 / 161	0.84	0.63-1.12	0.231	40 / 150	0.87	0.65-1.17	0.347

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Genotypes	RP (radiation pneumonitis, grade ≥3)				LRFS (local recurrence-free survival)				PFS (progression- free survival)				OS (overall survival)			
	0 / 1	**HR**	**95% CI**	P^a	**0 / 1**	**HR**	**95% CI**	P^b	**0 / 1**	**HR**	**95% CI**	P^b	**0 / 1**	**HR**	**95% CI**	P^b
CC	80 / 21	1.14	0.60-2.18	0.693	27 / 74	**0.54**	**0.39-0.76**	**0.0005**	21 / 80	**0.57**	**0.41-0.79**	**0.0009**	30 / 71	**0.59**	**0.41-0.83**	**0.003**
P_{trend} test				0.645				**0.0004**				**0.0007**				**0.002**
CC/AC	252 / 39	0.68	0.38-1.19	0.177	60 / 231	**0.73**	**0.56-0.96**	**0.024**	50 / 241	**0.73**	**0.56-0.95**	**0.019**	70 / 221	**0.75**	**0.57-1.00**	**0.046**
AA/AC	255 / 37	**1.00**			42 / 250	1.00			35 / 257	1.00			53 / 239	1.00		
CC	80 / 21	**1.80**	**1.04-3.12**	**0.037**	27 /74	**0.59**	**0.45-0.79**	**0.0004**	21 / 80	**0.64**	**0.48-0.84**	**0.002**	30 / 71	**0.64**	**0.48-0.86**	**0.003**

Abbreviations: NSCLC = non-small cell lung cancer; RP = radiation pneumonitis; LRFS = local recurrence-free survival; PFS = progression- free survival; OS = overall survival; 0 = the absence of events; 1 = the presence of events; HR = hazards ratio; CI = confidence interval

[a] *P* values were calculated by Cox proportional model with adjustment for smoking status, mean lung dose, disease stage, and chemotherapy history.

[b] *P* values were calculated by Cox proportional model with adjustment for age, sex, mean lung dose, disease stage, KPS score, history, and gross tumor volume.

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Table 3.

Stratified analysis of *ATG16L2* rs10898880 associated with RP, LRFS, RFS, and OS under recessive models by selected patient positive characteristics

Variables	RP (radiation pneumonitis)				LRFS (local recurrence-free survival)				PFS (progression- free survival)				OS (overall survival)			
	CC/CT (0/1)	TT (0/1)	Adjusted HR (95% CI)	P^a	CC/CT (0/1)	TT (0/1)	Adjusted HR (95% CI)	P^b	CC/CT (0/1)	TT (0/1)	Adjusted HR (95% CI)	P^b	CC/CT (0/1)	TT (0/1)	Adjusted HR (95% CI)	P^b
Overall	255/37	80/21	1.80 (1.04-3.12)	0.037	42/250	27/7	0.59 (0.45-0.79)	0.000	35/257	21/8	0.64 (0.48-0.84)	0.002	53/239	30/71	0.64 (0.48-0.86)	0.003
Sex																
Male	139/23	42/12	1.52 (0.73-3.16)	0.259	21/141	15/3	**0.52 (0.35-0.77)**	**0.001**	19/143	13/4	**0.54 (0.37-0.79)**	**0.002**	25/137	16/38	**0.53 (0.35-0.79)**	**0.002**
Female	116/14	38/9	2.31 (0.96-5.58)	0.062	21/109	12/3	0.71 (0.47-1.08)	0.109	16/114	8/39	0.78 (0.52-1.17)	0.228	28/102	14/33	0.81 (0.53-1.25)	0.341
Age (years)																
<65	134/19	38/11	**2.52 (1.18-5.37)**	**0.016**	26/127	14/3	**0.51 (0.33-0.78)**	**0.002**	20/133	11/3	**0.55 (0.37-0.82)**	**0.003**	34/119	15/34	**0.63 (0.41-0.96)**	**0.033**
≥65	121/18	42/10	1.41 (0.63-3.18)	0.403	16/123	13/3	**0.62 (0.41-0.92)**	**0.019**	15/124	10/4	**0.66 (0.45-0.99)**	**0.043**	19/120	15/37	**0.59 (0.39-0.90)**	**0.014**
KPS																
≥80	209/28	66/14	1.52 (0.78-2.95)	0.216	37/200	22/5	**0.59 (0.43-0.82)**	**0.002**	30/207	17/6	**0.64 (0.47-0.88)**	**0.006**	48/189	25/55	**0.63 (0.45-0.88)**	**0.006**
<80	46/9	14/7	2.39 (0.83-6.89)	0.106	5/50	5/16	0.57 (0.30-1.06)	0.077	5/50	4/17	**0.52 (0.28-0.98)**	**0.043**	5/50	5/16	0.62 (0.33-1.16)	0.134
Stage groups[c]																
I,II	37/7	8/3	1.74 (0.44-6.97)	0.433	11/33	4/7	1.31 (0.41-4.22)	0.651	11/33	4/7	1.14 (0.36-3.63)	0.824	11/33	4/7	1.17 (0.36-3.74)	0.796
III, IV	217/30	71/18	**1.90 (1.04-3.48)**	**0.038**	31/216	23/6	**0.59 (0.44-0.80)**	**0.000**	24/223	17/7	**0.64 (0.48-0.85)**	**0.002**	42/205	26/63	**0.65 (0.48-0.88)**	**0.005**
Histology																
Squamous	86/13	32/7	1.39 (0.52-3.73)	0.516	10/89	10/2	**0.52 (0.33-0.83)**	**0.006**	9/90	8/31	**0.55 (0.35-0.86)**	**0.009**	12/87	10/29	**0.56 (0.35-0.90)**	**0.017**
Nonsquamous[d]	169/24	48/14	**2.01 (1.02-3.97)**	**0.044**	32/161	17/4	**0.69 (0.48-1.00)**	**0.047**	26/167	13/4	0.73 (0.51-1.03)	0.073	41/152	20/42	0.74 (0.51-1.08)	0.117
MLD (Gy)[c]																
<19.0	123/10	43/5	1.75 (0.57-5.39)	0.326	25/108	16/3	**0.56 (0.37-0.86)**	**0.008**	20/113	14/3	**0.54 (0.36-0.82)**	**0.004**	32/101	17/31	**0.64 (0.42-0.99)**	**0.045**
≥19.0	109/26	31/15	1.84 (0.96-3.51)	0.065	16/119	10/3	**0.67 (0.46-0.99)**	**0.044**	14/121	6/40	0.77 (0.53-1.12)	0.173	20/115	12/34	0.67 (0.45-1.00)	0.052
GTV[c,e]																
<108 cm^3	111/12	40/10	2.14 (0.91-5.06)	0.082	28/95	15/3	**0.57 (0.37-0.88)**	**0.009**	23/100	12/3	**0.63 (0.43-0.95)**	**0.026**	36/87	17/33	**0.58 (0.38-0.90)**	**0.015**
≥108 cm^3	109/23	33/9	1.86 (0.83-4.18)	0.130	12/120	10/3	**0.61 (0.41-0.92)**	**0.019**	10/22	8/34	**0.62 (0.41-0.92)**	**0.017**	15/117	10/32	0.68 (0.45-1.02)	0.062

Abbreviations: NSCLC = non-small lung cancer; RP = radiation pneumonitis; LRFS = local recurrence-free survival; PFS = progression- free survival; OS = overall survival; 0 = the absence of events; 1 = the presence of events; HR = hazards ratio; CI = confidence interval; KPS = Karnofsky performance status score; MLD = mean lung dose; GTV = gross tumor volume.

[a] *P* values were calculated by Cox proportional model with adjustment for smoking status, mean lung dose, disease stage, and chemotherapy history.

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[b] *P* values were calculated by Cox proportional model with adjustment for age, sex, mean lung dose, disease stage, KPS score, history, and gross tumor volume.

[c] Some missing data

[d] Large cell, undifferentiated, and mixed-cell carcinomas

[e] Median 108.1 cm^3

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Table 4.

False-positive report probability (FPRP) values for associations between frequencies of *ATG16L2* rs10898880 genotypes (recessive model) and clinical outcomes in 393 NSCLC patients who received definitive radiotherapy

Genotype	Positive HR 95%CI[a]	P[b]	Statistical power[c]	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
RP	AA/AC vs. CC							
All subjects	1.80 (1.04-3.12)	0.036	0.646	**0.144**	0.335	0.847	0.982	0.998
Age <65	2.52 (1.18-5.37)	0.017	0.275	**0.154**	0.353	0.857	0.984	0.998
Stage III,IV	1.90 (1.04-3.48)	0.038	0.566	**0.166**	0.374	0.868	0.985	0.998
LRFS								
All subjects	0.59 (0.45-0.79)	0.0004	0.867	**0.001**	**0.004**	**0.043**	0.313	0.820
Males	0.52 (0.35-0.77)	0.001	0.587	**0.006**	**0.017**	**0.158**	0.654	0.950
Age <65	0.51 (0.33-0.78)	0.002	0.536	**0.010**	**0.031**	0.259	0.779	0.972
Age ≥65	0.62 (0.41-0.92)	0.018	0.857	**0.058**	**0.156**	0.670	0.953	0.995
KPS ≥80	0.59 (0.43-0.82)	0.002	0.838	**0.006**	**0.018**	**0.166**	0.667	0.953
Stage III,IV	0.59 (0.44-0.80)	0.0007	0.857	**0.002**	**0.007**	**0.073**	0.443	0.889
Squamous cell	0.52 (0.33-0.83)	0.006	0.565	**0.031**	**0.089**	0.518	0.915	0.991
Non-squamous cell	0.69 (0.48-1.00)	0.050	0.956	**0.136**	0.320	0.838	0.981	0.998
MLD <19.0	0.56 (0.37-0.86)	0.008	0.698	**0.034**	**0.094**	0.534	0.920	0.991
MLD ≥19.0	0.67 (0.46-0.99)	0.044	0.929	**0.125**	0.301	0.825	0.979	0.998
GTV <108 cm^3	0.57 (0.37-0.88)	0.011	0.723	**0.044**	**0.122**	0.605	0.939	0.994
GTV ≥108 cm^3	0.61 (0.41-0.92)	0.018	0.829	**0.062**	**0.166**	0.687	0.957	0.996
PFS								
All subjects	0.64 (0.48-0.84)	0.001	0.962	**0.004**	**0.012**	**0.118**	0.574	0.931
Males	0.54 (0.37-0.79)	0.002	0.654	**0.007**	**0.020**	**0.185**	0.696	0.958
Age <65	0.55 (0.37-0.82)	0.003	0.680	**0.015**	**0.042**	0.328	0.831	0.980
Age ≥65	0.66 (0.45-0.99)	0.045	0.910	**0.128**	0.306	0.829	0.980	0.998
KPS ≥80	0.64 (0.47-0.88)	0.006	0.936	**0.019**	**0.055**	0.389	0.865	0.985
KPS <80	0.52 (0.28-0.98)	0.043	0.548	**0.191**	0.414	0.886	0.987	0.999
Stage III,IV	0.64 (0.48-0.85)	0.002	0.956	**0.006**	**0.019**	**0.175**	0.682	0.955
Histology Squamous cell	0.55 (0.35-0.86)	0.009	0.662	**0.038**	**0.106**	0.567	0.930	0.992
MLD <19.0	0.54 (0.36-0.82)	0.004	0.641	**0.018**	**0.051**	0.372	0.857	0.984
GTV <108 cm^3	0.63 (0.43-0.95)	0.027	0.865	**0.087**	0.222	0.759	0.969	0.997
GTV ≥108 cm^3	0.62 (0.41-0.92)	0.018	0.857	**0.058**	**0.156**	0.670	0.953	0.995
OS								
All subjects	0.64 (0.48-0.86)	0.003	0.949	**0.010**	**0.028**	0.243	0.764	0.970
Males	0.53 (0.35-0.79)	0.018	0.613	**0.009**	**0.026**	0.228	0.748	0.968
Age <65	0.63 (0.41-0.96)	0.032	0.859	**0.099**	0.249	0.784	0.973	0.997
Age ≥65	0.59 (0.39-0.90)	0.014	0.779	**0.052**	**0.142**	0.645	0.948	0.995
KPS ≥80	0.63 (0.45-0.88)	0.007	0.912	**0.022**	**0.062**	0.422	0.881	0.987
Stage III,IV	0.65 (0.48-0.88)	0.005	0.955	**0.016**	**0.048**	0.355	0.848	0.982

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Genotype	Positive HR 95%CI[a]	P[b]	Statistical power[c]	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
Histology Squamous cell	0.56 (0.35-0.90)	0.017	0.680	**0.068**	**0.180**	0.707	0.961	0.996
MLD <19.0	0.64 (0.42-0.99)	0.045	0.866	**0.135**	0.318	0.837	0.981	0.998
GTV <108 cm^3	0.58 (0.38-0.90)	0.015	0.746	**0.057**	**0.154**	0.667	0.953	0.995

Abbreviations: NSCLC = non-small lung cancer; RP = radiation pneumonitis; LRFS = local recurrence-free survival; PFS = progression- free survival; OS = overall survival; HR = hazards ratio; CI = confidence interval; KPS = Karnofsky performance status score; MLD = mean lung dose; GTV = gross tumor volume.

[a]The adjusted HR.

[b]The omnibus chi-square test of the genotype frequency distributions.

[c]Calculated using study subjects to detect an OR of 2.00 with the common genotype used as the reference in FPRP level 0.2.