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A Single Nucleotide Polymorphism in the *MTHFR* Gene is Associated with Risk of Radiation Pneumonitis in Lung Cancer Patients Treated with Thoracic Radiation Therapy

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Abstract

Background—To study the association between functional single nucleotide polymorphisms (SNPs) in candidate genes from oxidative stress pathways, and risk of radiation pneumonitis (RP) in patients treated with thoracic radiation therapy (RT) for locally advanced lung cancer (LC).

Methods—We reviewed 136 patients treated with RT for LC between 2001 and 2007, and had prior genotyping of functional SNPs in oxidative stress genes including *superoxide dismutase 2* (*SOD2*; rs4880) and *methylenetetrahydrofolate reductase* (*MTHFR*; rs1801131, rs1801133). RP events were retrospectively scored using the Common Terminology Criteria for Adverse Events, version 4.0. Cox proportional hazard regression was performed to identify clinical variables and genotypes associated with risk of grade 2 and grade 3 RP on univariate and multivariate analysis. P-values were corrected for multiple hypothesis testing.

Results—With a median follow-up of 21.4 months, the incidence of grade 2 RP was 29% and grade 3 RP was 14%. On multivariate analysis, after adjusting for clinical factors such as concurrent chemotherapy, and consolidation docetaxel, and lung dosimetric parameters such as V20 and mean lung dose, *MTHFR* genotype (rs1801131; AA versus AC/CC) was significantly associated with risk of grade 2 RP (Hazard ratio [HR]: 0.37; 95% confidence interval [CI]: 0.18-0.76; p=0.006, corrected p=0.018) and grade 3 RP (HR: 0.21; 95% CI: 0.06-0.70; p=0.01; corrected p=0.03). *SOD2* genotype was not associated with RP.

Conclusions—Our study showed an association between *MTHFR* genotype and risk of clinically significant RP. Further study of *MTHFR*-related pathways may provide insight into the mechanisms behind RP.

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Keywords

Radiation pneumonitis; single nucleotide polymorphisms; lung cancer; oxidative stress; MTHFR

Introduction

Radiation pneumonitis (RP) remains a significant barrier to radiation dose escalation to achieve adequate local control in the treatment of locally advanced lung cancer (LC). While technological advances in the delivery of thoracic radiation therapy (RT) including intensity-modulated radiation therapy (IMRT) has allowed gradual dose escalation with potential reduction in toxicity¹, rates of clinically-evident locoregional recurrence remain relatively high at 20-50% in randomized trials of definitive radiation with concurrent chemotherapy in patients with non-small cell lung cancer (NSCLC)²⁻⁴. Further dose escalation may be limited by the increasing risk of normal tissue toxicity, including RP.

In contemporary studies, the risk of symptomatic (grade 2-5) RP ranges from 10-30%⁵⁻⁹, and may be associated with radiation dose, concurrent chemotherapy regimens, use of consolidation docetaxel^{4, 10}, and other clinical factors. Radiation dose to specific lung volumes have been correlated with risk of pneumonitis including volume of lungs receiving > 20 Gray (Gy; V20), > 5 Gy (V5), and mean lung dose (MLD)^{5, 8, 9}. While these studies have helped establish a series of clinically useful dosimetric cutoffs, there may be a continuum of risk of normal tissue toxicity, which may be determined by the interaction between the physical distribution of radiation dose in the lungs and underlying biological factors in the patient.

The pathogenesis of radiation-induced lung injury is not completely understood, but may be due to a combination of direct radiation cytotoxicity to normal lung tissue and secondary inflammatory changes and fibrotic remodeling¹¹. Thus, genetic variation in key genes in DNA repair, inflammation and oxidative stress pathways may ameliorate or exacerbate the effects of a given radiation dose to the lungs. Prior retrospective candidate gene studies in patients treated with RT for LC have shown associations between risk of RP and single nucleotide polymorphisms (SNPs) in the *ataxia-telangiectasia mutated (ATM)*¹², *XRCC1*, *APEX1*¹³, and *p53*¹⁴ genes, key components in cellular signaling and repair response to ionizing radiation, and *transforming growth factor-beta (TGFB1)*, a cytokine involved in fibrotic remodeling¹⁵. Another important class of genes that may play a role in the pathogenesis of RP is oxidative stress pathways genes including *superoxide dismutase 2*, which is involved in free radical scavenging, and *methylenetetrahydrofolate reductase*, which is a key regulator of folate, homocysteine, thiol, methylation, and thymidine metabolism.

In this study, we build upon the existing literature by screening for an association between risk of RP and functional SNPs in candidate genes from oxidative stress pathways in a retrospective cohort of patients treated with thoracic RT for LC.

Methods

Patients

As part of an institutional review board-approved prospective molecular epidemiology protocol, peripheral blood was collected from patients diagnosed with either non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) who were seen at the Massachusetts General Hospital from 1992 to present. The peripheral blood was collected as part of successive candidate gene epidemiological studies to examine associations between

functional SNPs and LC risk and outcomes¹⁶⁻¹⁸. Patient and demographic information were collected at the time of recruitment, and informed consent was obtained to collect follow-up data. More than 85% of eligible patients were recruited in this cohort.

Out of approximately 3982 patients who consented to the protocol between 1992 and 2009, 2334 had prior genotyping of functional SNPs in two candidate genes involved in oxidative stress pathways as listed below. We limited our study to patients treated after 2001, when our department and the hospital adopted widespread utilization of an electronic medical record system that allowed more accurate retrospective collection of clinical data. Out of this group, we retrospectively identified 243 patients who were treated at MGH between 2001 and 2007 with thoracic RT with a minimum dose of 40 Gy. Of these 243 patients, we excluded non-white patients (n=8), patients who were not treated with 3-dimensional conformal RT (3D-CRT) or IMRT and did not have radiation dosimetric data for the lungs (n=57), patients with stage I disease (n=18), patients who were lost to follow-up (<1 month of follow-up) after treatment (n=7), and patients with insufficient peripheral blood for genotyping (n=10). The remaining 136 patients constituted our sample set for this RP and candidate gene SNP association study.

Genotyping

Blood samples were collected from all study participants at the time of recruitment. Germline DNA was isolated from the peripheral blood of each patient using Autopure blood purification kits (Qiagen Sciences, Inc., Germantown, MD, USA). Genotyping was performed using the 5'-nuclease assay (TaqMan) assay and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) on known functional SNPs for candidate genes in oxidative stress pathways including: 1) *superoxide dismutase 2 (SOD2)*; 5482C>T, Val16Ala, rs4880); 2) *methylenetetrahydrofolate reductase (MTHFR)*; 1298A>C, Glu429Ala, rs1801131; and 677C>T, Ala222Val, rs1801133). Genotyping was performed by laboratory personnel blinded to patient status, and a random 5% of the samples were repeated to validate genotyping procedures. Hardy-Weinberg equilibrium was tested using the χ^2 test. Due to the small sample sizes and small number of patients with the homozygous variant genotypes, genotypes were primarily analyzed using the dominant effects model (*SOD2*, rs4880: CC vs. CT/TT; *MTHFR*, rs1801131: AA vs. AC/CC; *MTHFR*, rs1801133: CC vs. CT/TT).

Clinical and Radiation Dosimetric Covariates

Clinical covariates that may be associated with RP were retrospectively collected including, patient age, ECOG performance status (PS), smoking status, pulmonary function test data, AJCC TNM stage (6th edition), tumor size, RT technique, radiation dose, use of concurrent or consolidation chemotherapy, use of consolidation docetaxel, and use of surgery. Smoking status was categorized as: 1) never smokers; < 100 cigarettes in their lifetime; 2) former smokers; quit >1 year prior to diagnosis, and 3) current smokers; smoking at the time of diagnosis or quit < 1 year prior. Lung dosimetric variables including MLD, V5, and V20 were collected from the treatment plans. The lung volume was defined as the total volume of the two lungs minus the volume of the gross tumor volume.

Endpoints

RP events were identified retrospectively and graded using the Common Terminology Criteria for Adverse Events, (CTCAE version 4.0) by two of the investigators (B.M.A. and R.H.M). Both investigators were blinded from the genotyping results and independently reviewed all of the follow-up notes for each patient, and reviewed pertinent radiological imaging to determine a diagnosis and grade of RP. The RP scores from the two investigators were compared using the kappa statistic to estimate the concordance of grading, and any

discordant results were reconciled. The RP grades were analyzed as dichotomized variables at clinically relevant cutoffs: grade 2 RP (use of steroids) versus grade 0-1 RP and grade 3 RP (oxygen requirement) versus grade 0-2 RP. RP was analyzed as a time dependent variable, and was calculated from the initiation of radiation therapy to the time when patients had a RP event or were censored at the time of last follow-up or death.

Statistical Analysis

The cumulative incidence of RP was estimated using the Kaplan-Meier Method. Cox proportional hazard models were performed to identify clinical variables and genotypes associated with risk of RP on univariate and multivariate analysis. For multivariate analysis, variables with a p-value < 0.10 were included in initial models as potential confounders. Stepwise selection was performed to identify variables associated with grade 2 or grade 3 RP. Clinically important confounders such as lung dosimetric parameters were re-introduced into the final model. Since 3 different SNPs were examined, we corrected for multiple hypothesis testing using the method of Benjamini and Hochberg in both univariate and multivariate analyses¹⁹. All statistical testing was done with a two-sided p-value < 0.05 level. All analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Results

Patient, Treatment and Radiation Dosimetric Characteristics

The median age of the patients was 67 (range, 37-85), 51% were female, 90% were current or former smokers, and 95% had ECOG PS of 0-1 (Table 1). In the 118 patients with pulmonary function testing, the median FEV1 was 1.98 L (range, 0.66-4.31 L). The majority of patients were treated with definitive RT without surgery (78%), and the remainder received RT preoperatively (11%) or postoperatively (11%). In total, 96% of patients received chemotherapy, including as induction prior to RT (8%), concurrently with RT (89%) and for consolidation after RT (57%). Of note, 12% of patients received consolidation docetaxel. All patients were treated with IMRT (75%) or 3D-CRT (25%). The median prescribed dose was 63 Gy (range, 41.4-72.7). Dosimetric parameters for the lungs included a median V20 of 34% (9.4-70%), V5 of 51% (14-93%) and MLD of 18.1 Gy (6.1-32.4 Gy).

MTHFR and SOD2 Genotyping

The distribution of the *MTHFR* (rs1801131 and rs1801133) and *SOD2* genotypes are shown in Table 2. All *MTHFR* and *SOD2* polymorphisms examined were in Hardy-Weinberg equilibrium ($p > 0.05$, χ^2 goodness of fit).

Radiation Pneumonitis

With a median follow-up of 21.4 months (range, 1.6-109.5 months) after initiation of RT, the crude incidence of grade 2 RP was 29% (40/136) with an 1-year Kaplan-Meier estimate of 30.5%, and the crude incidence of grade 3 RP was 14% (19/136) with an 1-year Kaplan-Meier estimate of 13.7%. There was excellent concordance between the two independent measures of RP grade using CTCAE version 4.0 with a kappa statistic of 0.81 (95% confidence interval [CI]: 0.72-0.90) when examining the scores on an ordinal scale from 0 to 5. When examining the RP grading as a dichotomous variable of grade 2 and grade 3, the kappa statistics were 0.96 (95% CI: 0.92-1.0) and 0.94 (95% CI: 0.85-1.0), respectively. In total, two cases were discordant between the two independent measures of RP as a dichotomous variable for both grade 2 and grade 3.

Clinical and Dosimetric Variables Associated with RP

On univariate analysis (Table 3), clinical variables associated with grade 2 RP included gender, performance status, MLD, and use of surgery. On multivariate analysis, gender, ECOG PS, surgery, concurrent chemotherapy, and consolidation docetaxel were associated with grade 2 RP (Table 4). Important dosimetric predictors of RP including MLD, V20 and V5 were re-introduced into the final model. The continuous variables such as MLD, V20 and V5 were analyzed both continuously and as categorical variables (e.g. dichotomized at the median); since the models were not significantly different with either approach, the dichotomized variable analysis models are presented.

Univariate and Multivariate Analysis of SNP Association with RP

The *MTHFR* 1298A>C SNP (rs1801131) was associated with risk of grade 2 RP on univariate analysis with a hazard ratio (HR) of 0.51 (95% CI: 0.27-0.97; $p=0.04$; corrected $p=0.12$) for the *MTHFR* 1298 AC/CC versus AA genotype (Table 3). Kaplan-Meier estimates of the incidence of grade 2 RP at 12 months from the initiation of RT were 39.3% for patients with the *MTHFR* 1298 AA genotype versus 22.3% for patients with the *MTHFR* 1298 AC/CC genotypes (Figure 1A). Analysis of the *MTHFR* 1298 genotypes using a co-dominant model (AA vs. AC vs. CC) showed a significant decrease in risk of RP between 1298 AA versus AC genotype, and a non-significant decrease in risk of 1298 AA versus CC genotype on both univariate and multivariate analysis (Table 4). Genotype of *SOD2* (rs4880), and the other SNP from the *MTHFR* gene (rs1801133; 677C>T) were not significantly associated with risk of grade 2 RP on univariate analysis (Table 3).

After adjusting for potential clinical and dosimetric confounders, there remained an association between grade 2 RP and *MTHFR* 1298A>C genotype (rs1801131; 1298AA vs. AC/CC) with an adjusted hazard ratio (AHR) of 0.37 (95% CI: 0.18-0.76; $p=0.006$; corrected $p=0.018$; Table 4). Neither the *SOD2* nor the other *MTHFR* (rs1801133) SNP were associated with risk of grade 2 RP on multivariate analysis (Table 4).

We also examined the relationship between the *MTHFR* 1298C>A SNP and risk of grade 3 RP (Table 5). Kaplan-Meier estimates of the incidence of grade 3 RP at 12 months from the initiation of RT were 22.0% for patients with the *MTHFR* 1298AA genotype versus 5.7% for patients with the *MTHFR* 1298AC/CC genotypes (Figure 1B). *MTHFR* 1298 AC/CC versus AA genotype were significantly associated with decreased risk of grade 3 RP on univariate analysis (HR: 0.30; 95% CI: 0.11-0.83, $p=0.02$; corrected $p=0.06$), and multivariate analysis (AHR: 0.21; 95% CI: 0.06-0.70; $p=0.01$; corrected $p=0.03$).

Discussion

In this cohort of patients treated with thoracic RT for lung cancer, we explored the association between RP and SNPs from candidate genes in oxidative stress pathways. We identified an association between risk of RP and the *MTHFR* 1298A>C genotype (rs1801131). Patients with AC/CC genotypes had a lower risk of both grade 2 and of grade 3 RP compared to patients with the wild-type AA genotype. This association was seen after adjusting for clinical and dosimetric variables associated with risk of RP, and correcting for multiple testing. Patients with an *MTHFR* 1298AC/CC genotype had an unadjusted incidence of grade 2 RP of only 17%, compared to 50% in patients with an AA genotype, raising the hypothesis that AC/CC genotype may be protective. Furthermore, the adjusted hazard ratios of 0.37 (95% CI: 0.18-0.76; $p=0.006$; corrected $p=0.018$) and 0.21 (95% CI: 0.06-0.70; $p=0.01$; corrected $p=0.03$), for grade 2 RP and grade 3 RP, respectively, may suggest a strong underlying biological effect. While there was not a strong association between decreased risk of both 1298AC and 1298CC genotype independently

versus 1298AA nor a variant allele dose effect (i.e. a decrease in risk of RP with each additional C allele), the small sample size in the 1298CC genotype subtype (n=13) likely makes this an underpowered analysis and such a dose effect cannot be excluded. Thus, further work in confirming this hypothesis-generating finding in a larger cohort of patients will be of interest.

The *MTHFR* 1298A>C SNP (rs1801131) results in a glutamate to alanine substitution at codon 439 in exon 7, which is located in the COOH-terminal regulatory domain of the gene, and results in a 30-40% reduction of enzymatic function in the homozygous variant genotype²⁰. The *MTHFR* enzyme plays a central role in the intersection between a number of important metabolic pathways including folate metabolism, thymidine synthesis, homocysteine processing, and synthesis of both sulfhydryl- and methyl-donating species. *MTHFR* is a pivotal enzyme in cell metabolism by catalyzing the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate; the latter is used in methionine synthesis (a key precursor in DNA methylation), while the former is a key building block in thymidine synthesis that is catalyzed by the thymidylate synthase enzyme, and thus may play an important role in DNA repair. Of interest, Batra et al. demonstrated that after total body irradiation in mice, *MTHFR* activity levels decreased while thymidylate synthase activity increased suggesting that the regulation of these enzymes in diverting folate metabolism toward thymidine base synthesis may play an important role in the cellular response to ionizing radiation²¹. Our study identifies an increased risk of RP with the AA genotype, which is associated with higher *MTHFR* enzymatic function and may potentially lower both levels of thiol and thymidine synthesis. Thus, further work to study whether risk of RP is associated with genes involved in these related pathways of thiol, methyl, and pyrimidine synthesis may be of interest.

Strengths of this study include the relative genetic homogeneity of the population, which was achieved by including only white patients to reduce the influence of variation in genotype distribution by race. Secondly, by including only patients with locally advanced lung cancer, the majority received chemotherapy and were treated with similar radiation techniques. Furthermore, by including only patients that received treatment with modern 3D-CRT and IMRT techniques, we were able to control for known dosimetric predictors of RP such as MLD, V5 and V20. Of note, these previously established lung dosimetric parameters were not significantly associated with RP on multivariate analysis in our dataset. The lack of association between MLD, V5, and V20 and RP may be due to the heterogeneity of treatment techniques including IMRT and 3D-conformal RT, use of surgery and use of chemotherapy. Additionally, some of the patients in our series were treated during a time period after publications regarding V5, V20 and MLD had been published,⁵⁻⁸ and may have led to adjustment of clinical practice to meet these new clinical constraints, which would confound the predictive ability of these parameters. However, since the main goal of this study was to identify SNPs associated with RP, controlling for clinical and dosimetric variables as potential confounders of the primary analysis was important, but the independent predictive ability of these variables are less important.

The findings of this study must be interpreted in the context of its retrospective design. First, retrospective assessment of radiation-related toxicity is often difficult due to incomplete follow-up and reliance on physician documentation of these events, which may be biased and/or incompletely documented. To address these challenges, we utilized the CTCAE RP grading system dichotomized at clinically relevant endpoints, which allowed us to assess for RP based on the use of medical interventions (e.g. steroids) for grade 2 RP or new oxygen requirement for grade 3 RP. Furthermore, we used two independent retrospective reviews of the patients' records to generate the RP grades, and had a high concordance rate in the two scores, which suggests consistency in how these events were documented in the medical

records at our institution and subsequently interpreted retrospectively. A second limitation was that the study had a relatively modest number of patients and events, and thus validation in a larger data set will be required. Third, there was heterogeneity in clinical factors that could impact both the risk and/or the retrospective scoring of RP. For example, the use of surgery could certainly impact both the risk of RP and post-operative declines in pulmonary function may impact the ability to reliably score RP. However, we controlled for surgery and other clinical factors in our multivariate model, and we had stringent criteria for scoring RP with highly concordant independent measures of the endpoint as discussed above. Fourth, the rates of RP in this series are fairly high, particularly since the majority of these patients were treated with IMRT which may reflect further heterogeneity such as differences in techniques during the early adoption of IMRT, the use of concurrent chemotherapy, and the use of consolidation docetaxel in some patients, but the high event rate does increase the power of this data set to detect associations with the genotypes studied. Additionally, while the *MTHFR* 1298A>C (rs1801131) was associated with the risk of RP, a second *MTHFR* SNP (rs1801133; 677C>T) was not associated with RP in the test and validation cohorts. This inconsistency may be due to insufficient power in this study to detect an association with the other SNP, different function of these two *MTHFR* SNPs or greater biological effect of the 1298A>C versus the 677C>T SNP. Furthermore, there are numerous other SNPs in the *SOD2* and *MTHFR* gene that were not analyzed in this study, and verification of the results of this study will include further study of other polymorphisms in these genes. Finally, as is the case for most candidate gene studies, the finding of an association between RP and the *MTHFR* SNP (rs1801131) may represent a role of the *MTHFR* gene in mediating the response of lung tissue to radiation injury or alternatively the SNP may simply be a marker for another gene that is in linkage disequilibrium with the SNP. Thus, to further validate these findings, the functional relationship between the *MTHFR* pathway and risk of RP must be determined, such as by assessment of downstream products of the pathway including homocysteine and thymidine. Nevertheless, this study is the first to our knowledge to identify a potential association between a functional SNP in the *MTHFR* gene and risk of radiation-related toxicity.

The results of our study build upon prior published RP and candidate gene association studies. Prior retrospective studies from MD Anderson and Beijing have identified an association between risk of RP and the pro-fibrotic cytokine, *TGF-β*, and DNA damage response and repair genes: *ATM*, *p53*, *APEX1*, and *XRCC1*¹²⁻¹⁵. Of note, the Beijing studies were conducted in a Chinese population of 253, while the MD Anderson study was conducted in a population of 164 patients of several different races. While these studies highlighted the potential importance of circulating cytokines and DNA repair in the risk of RP, we identified a novel association between RP and the *MTHFR* gene, which may provide new insights into the biological mechanisms of radiation injury. However, we did not observe an association between RP and a SNP in the important oxidative stress gene *SOD2* (rs4880). In all the studies to date, the effect estimate of the genotypes found to have an association with grade 2 RP has been relatively large, which is likely a reflection of the relatively small sample sizes in all these studies. All of these cohorts are likely underpowered to detect SNPs with relatively minor biological effects in modulating the risk of RP. For instance, in a post-hoc power calculation for our study, with the sample size of 136 patients and a null hypothesis of a rate of RP of 29%, our study is underpowered to detect an association between RP and a SNP that would confer a less than 11% change in the rate of RP at the 80% level with an alpha level of 0.05. Furthermore, differences in the patient cohorts in each of these institutional studies including genetic variations due to race may make cross-validation and/or pooling of data sets difficult to interpret. The challenge facing the field of radiation oncology will be to improve the prospective gathering of radiation-induced toxicity events, in conjunction with collection of tissue and genetic material.

Conclusions

Our study showed an association between risk of clinically significant RP after radiation therapy for lung cancer and *MTHFR* genotype (1298AA vs. AC/CC; rs1801131). Further validation work including association studies with other enzymes involved in folate metabolism may identify new biomarkers for risk of RP.

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Condensed abstract

This is a candidate gene study examining the association between single nucleotide polymorphisms in oxidative stress genes and radiation pneumonitis in a retrospective cohort of patients treated with thoracic radiation therapy for lung cancer. There was an association between *methylenetetrahydrofolate reductase (MTHFR)* genotype and risk of clinically significant radiation pneumonitis.

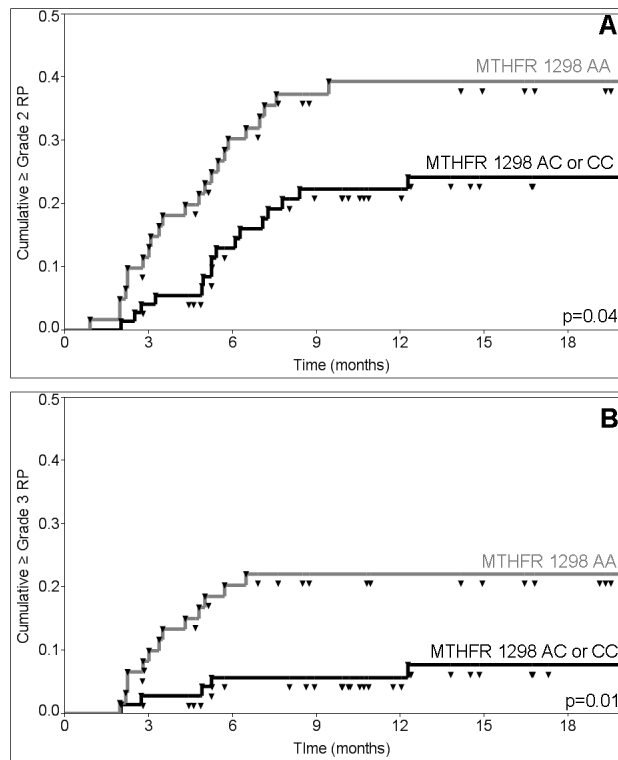


Figure 1. Kaplan-Meier Plots of Cumulative (A) Grade 2 and (B) Grade 3 Radiation Pneumonitis in Patients with *MTHFR* 1298 AA (gray line) versus 1298 AC/CC Genotype (black line).

Table 1

Patient, tumor and treatment characteristics.

	All Patients (n=136)	Grade 0-1 RP (n=96)	Grade 2 RP (n=40)	Grade 3 RP (n=19)
Patient Characteristics				
Median Age (range)	66.7 (37.2-85.2)	66.8 (37.2-85.3)	61.4 (48.0-83.7)	63.9 (51.2-83.4)
Male	67 (49.3%)	41 (42.7%)	26 (65.0%)	15 (79.0%)
Female	69 (50.7%)	55 (57.3%)	14 (35.0%)	4 (21.0%)
ECOG PS				
0	62 (45.6%)	45 (46.9%)	17 (42.5%)	7 (36.8%)
1	67 (49.3%)	49 (51.0%)	18 (45.0%)	9 (47.4%)
2	7 (5.1%)	2 (2.1%)	5 (12.5%)	3 (15.8%)
Smoking Status				
Current	48 (35.3%)	37 (38.5%)	11 (27.5%)	6 (31.6%)
Former	74 (54.4%)	48 (50.0%)	26 (65.0%)	12 (63.2%)
Never	14 (10.3%)	11 (11.5%)	3 (7.5%)	1 (5.3%)
Median Pack-years (range)	49 (0-200)	49 (0-200)	40 (0-150)	55 (0-120)
Median FEV1 in Liters (range)	1.98 (0.66-4.31; n=115)	1.99 (0.74-4.31; n=81)	.98 (0.66-3.44; n=34)	.98 (0.92-3.44; n=18)
Tumor Characteristics				
Histology:				
Squamous cell carcinoma	29 (21.3%)	19 (19.8%)	10 (25.0%)	5 (26.3%)
Adenocarcinoma	64 (47.1%)	49 (51.0%)	15 (37.5%)	5 (26.3%)
Poor differentiated carcinoma [†]	28 (20.6%)	17 (17.7%)	11 (27.5%)	7 (36.8%)
Small cell lung cancer	15 (11.0%)	11 (11.5%)	4 (10.0%)	2 (10.5%)
T-stage				
0	6 (4.4%)	4 (4.2%)	2 (5.0%)	2 (10.5%)
1	31 (22.8%)	26 (27.1%)	5 (12.5%)	1 (5.3%)
2	47 (34.6%)	30 (31.2%)	17 (42.5%)	8 (42.1%)
3	24 (17.6%)	15 (15.6%)	9 (22.5%)	4 (21.0%)
4	28 (20.6%)	21 (21.9%)	7 (17.5%)	4 (21.0%)
N-stage				
0	10 (7.4%)	10 (10.4%)	0 (0.0%)	0 (0.0%)
1	11 (8.1%)	10 (10.4%)	1 (2.5%)	0 (0.0%)
2	80 (58.8%)	53 (55.2%)	27 (67.5%)	14 (73.7%)
3	35 (25.7%)	23 (24.0%)	12 (30.0%)	5 (26.3%)
Stage				
IIB	9 (6.6%)	9 (9.4%)	0 (0.0%)	0 (0.0%)

	All Patients (n=136)	Grade 0-1 RP (n=96)	Grade 2 RP (n=40)	Grade 3 RP (n=19)
IIIA	63 (46.3%)	40 (41.7%)	23 (57.5%)	11 (57.9%)
IIIB	52 (38.2%)	38 (39.6%)	14 (35.0%)	7 (36.8%)
IV	12 (8.8%)	9 (9.4%)	3 (7.5%)	1 (5.3%)
Treatment Characteristics				
Median Radiation Dose (range)	63 Gy (41.4-72.7)	63 Gy (45-72)	66.6 Gy (41.4-72.7)	66.6 Gy (41.4-72.7)
Radiation Technique:				
3D Conformal	34 (25%)	26 (27.1%)	8 (20.0%)	2 (10.5%)
IMRT	102 (75%)	70 (72.9%)	32 (80.0%)	17 (89.5%)
Simulation Technique:				
3D-CT	72 (52.9%)	53 (55.2%)	19 (47.5%)	8 (42.1%)
4D CT	64 (47.1%)	43 (44.8%)	21 (52.5%)	11 (57.9%)
Radiation Sequencing:				
Pre-operative	15 (11.0%)	13 (13.5%)	2 (5.0%)	0 (0.0%)
Radiation alone	106 (78.0%)	69 (71.9%)	37 (92.5%)	19 (100%)
Post-operative	15 (11.0%)	14 (14.6%)	1 (2.5%)	0 (0.0%)
Surgery				
Surgery Type				
Wedge	4 (13.3%)	4 (14.8%)	0	-
Lobectomy	25 (83.3%)	22 (81.5%)	3 (100%)	-
Pneumonectomy	1 (3.3%)	1 (3.7%)	0	-
Any Chemotherapy				
Induction Chemotherapy	11 (8.1%)	10 (10.4%)	1 (2.5%)	1 (5.3%)
Concurrent Chemotherapy	121 (89.0%)	82 (85.4%)	39 (97.5%)	18 (94.7%)
Consolidation Chemotherapy	78 (57.4%)	54 (56.3%)	24 (60.0%)	5 (26.3%)
Consolidation Docetaxel	16 (11.8%)	9 (8.3%)	8 (20.0%)	3 (15.8%)
Bilateral Lung Dose-Volume Histogram				
Median V5 (range)	61.7% (21.7-97.2%)	59.4% (21.7-95.0%)	65.3% (38.0 - 97.2%)	63.8% (42.0-97.2%)
Median V20 (range)	34.0% (9.4-70.0%)	32.4% (9.6-68.7%)	36.3% (21.9-70.0%)	36.4% (22.0-68.0%)
Median MLD (range)	18.1 Gy (6.1-32.4 Gy)	17.6 Gy (6.1-29.3 Gy)	19.8 Gy (11.0-32.4 Gy)	20.5 (13.3-32.4 Gy)

[‡]Includes carcinoma not otherwise specified (NOS) and non-small cell lung cancer NOS.

Abbreviations: CT=computed tomography; FEV1=force expiratory volume in 1 second; IMRT=intensity modulated radiation therapy; MLD=mean lung dose; PS=performance status; V5=volume receiving greater than 5 Gy; V10=volume receiving greater than 10 Gy; V20=volume receiving greater than 20 Gy; RP=radiation pneumonitis.

Table 2

Genotype Frequency

Single Nucleotide Polymorphisms	All Patients (n=136)	Grade 0-1 RP (n=96)	Grade 2 RP (n=40)	Grade 3 RP (n=19)
<i>MTHFR</i> (rs1801131) *				
AA	61 (45.5%)	38 (40.0%)	23 (59.0%)	13 (72.2%)
AC	60 (44.8%)	48 (50.5%)	12 (30.8%)	3 (11.1%)
CC	13 (9.7%)	9 (9.5%)	4 (10.3%)	2 (16.7%)
<i>AC/CC</i>	73 (54.5%)	57 (60.0%)	16 (41.0%)	5 (27.8%)
<i>MTHFR</i> (rs1801133) *				
CC	51 (38.1%)	40 (42.1%)	11 (28.2%)	4 (22.2%)
CT	59 (44.0%)	40 (42.1%)	19 (48.7%)	8 (44.4%)
TT	24 (17.9%)	15 (15.8%)	9 (23.1%)	6 (33.3%)
<i>CT/TT</i>	83 (61.9%)	55 (57.9%)	28 (71.8%)	14 (77.7%)
<i>SOD2</i> (rs4880)				
TT	32 (23.5%)	21 (21.9%)	11 (27.5%)	6 (31.6%)
TC	76 (55.9%)	51 (53.1%)	25 (62.5%)	9 (47.4%)
CC	28 (20.6%)	4 (25.0%)	4 (10%)	4 (21.0%)
<i>TC/CC</i>	104 (76.5%)	72 (78.1%)	29 (72.5%)	13 (68.4%)

* Data available on 134 patients

Abbreviations: RP=radiation pneumonitis.

Table 3
Univariate Cox Regression of Clinical and Dosimetric Factors for Risk of Grade 2 RP

Patient Characteristics	Total Patients (n=136)	Patients with Grade 2 RP (n=40)	Percent of Patients with Grade 2 RP	HR	95% CI	p-value
Patient Characteristics						
Age<66	64	17	26.6%	1.0*	-	-
Age ≥66	72	23	31.9%	1.25	0.67-2.33	0.49
Male	67	26	38.8%	2.22	1.16-4.26	0.017
Female	69	14	20.3%	1.0*	-	-
ECOG PS						
0	62	17	27.4%	1.0*	-	-
1	67	18	26.9%	1.01	0.52-1.96	0.98
2	7	5	71.4%	3.63	1.34-9.87	0.01
Smoking Status						
Current	48	11	22.9%	1.0*	-	-
Former	74	26	35.1%	1.62	0.80-3.29	0.18
Never	14	3	21.4%	0.92	0.26-3.31	0.90
Pack-year<49	68	20	29.4%	1.0*	-	-
Pack-years ≥49	68	20	29.4%	0.98	0.53-1.82	0.95
FEV1<1.98 L	78	55	70.5%	1.0*	-	-
FEV1 ≥1.98 L	58	41	70.7%	0.99	0.53-1.85	0.97
Tumor Characteristics						
Histology:						
SCC	29	10	34.5%	0.96	0.32-2.90	0.95
AdenoCA	64	15	23.5%	1.0*	-	-

	Total Patients (n=136)	Patients with Grade 2 RP (n=40)	Percent of Patients with Grade 2 RP	HR	95% CI	p-value
Carcinoma NOS	28	11	39.3%	1.53	0.69-3.40	0.30
SCLC	15	4	26.7%	1.68	0.77-3.65	0.19
T-stage						
0-1	37	7	18.9%	0.62	0.31-1.24	0.17
2	47	17	36.2%	1.0*	-	-
3	24	9	37.5%	1.19	0.60-2.38	0.62
4	28	7	25.0%	0.84	0.39-1.84	0.67
N-stage						
0-1	21	1	4.8%	0.33	0.14-0.79	0.01
2	80	27	33.8%	1.0*	-	-
3	35	12	34.3%	0.99	0.53-1.84	0.98
Stage						
IIB	9	0	0%	0.0	0.0	0.99
IIIA	63	23	36.5%	1.0*	-	-
IIIB	52	14	26.9%	0.75	0.38-1.44	0.37
IV	12	3	25.0%	0.70	0.21-2.33	0.56
Treatment Characteristics						
Radiation Dose						
<60 Gy	35	6	24.0%	0.70	0.25-1.92	0.49
60 Gy and >66 Gy	40	10	25.0%	1.0*	-	-
66 Gy and >70 Gy	28	12	42.8%	1.86	0.80-4.31	0.15
70 Gy	33	12	36.4%	1.63	0.70-3.78	0.25
Radiation Technique:						
3D Conformal	34	8	23.5%	0.73	0.34-1.59	0.43
IMRT	102	32	31.4%	1.0*	-	-

	Total Patients (n=136)	Patients with Grade 2 RP (n=40)	Percent of Patients with Grade 2 RP	HR	95% CI	p-value
Simulation Technique:						
3D-CT	72	19	26.4%	1.0*	-	-
4D CT	64	21	32.8%	1.28	0.69-2.39	0.43
Radiation Sequencing:						
Pre-operative	15	2	13.3%	0.30	0.07-1.24	0.10
Radiation alone	106	37	34.9%	1.0*	-	-
Post-operative	15	1	6.7%	0.17	0.02-1.25	0.08
Surgery	30	3	10.0%	0.23	0.07-0.74	0.014
Any Chemotherapy	130	39	30.0%	1.66	0.23-12.08	0.62
Concurrent Chemotherapy	121	39	32.2%	5.27	0.72-38.35	0.10
Consolidation Chemotherapy	78	24	30.8%	1.0	0.84-1.18	0.96
Consolidation Docetaxel	16	8	50.0%	2.11	0.97-4.58	0.06
Bilateral Lung Dose-Volume Histogram						
V5<60%	62	13	21.0%	1.0*	-	-
V5 60%	74	27	36.4%	1.90	0.98-3.69	0.057
V20<30%	52	9	17.3%	0.45	0.20-1.04	0.061
V20 30% and <40%	43	15	34.9%	1.0*	-	-
V20 40%	41	16	39.0%	1.15	0.57-2.33	0.70
MLD<18 Gy	66	14	21.2%	1.0*	-	-
MLD 18 Gy	70	26	37.1%	1.95	1.02-3.74	0.044

* Reference group for Cox regression

Abbreviations: CI=confidence interval; CT=computed tomography; FEV₁=force expiratory volume in 1 second; HR=hazard ratio; IMRT=intensity modulated radiation therapy; MLD=mean lung dose; PS=performance status; RP=radiation pneumonitis; V5=volume receiving greater than 5 Gy; V10=volume receiving greater than 10 Gy; V20=volume receiving greater than 20 Gy.

Table 4

Cox Regression Analysis of Risk of Grade 2 RP

	Total Patients (n=136)	Number of Patients with Grade 2 RP (n=40)	Percent of Patients with Grade 2 RP	Univariate Analysis			Multivariate Analysis		
				HR	95% CI	p-value	AHR	95% CI	p-value
Patient Characteristics									
Male	67	26	38.8%	2.22	1.16-4.26	0.017	2.69	1.32-5.49	0.006
Female	69	14	20.3%	1.0*	-	-	1.0*	-	-
ECOG PS									
0	62	17	27.4%	1.0*	-	-	1.0*	-	-
1	67	18	26.9%	1.01	0.52-1.96	0.98	1.03	0.51-2.08	0.92
2	7	5	71.4%	3.63	1.34-9.87	0.01	2.85	0.90-9.00	0.075
Treatment Characteristics									
Surgery	30	3	10.0%	0.23	0.07-0.74	0.014	0.24	0.06-0.91	0.036
Concurrent Chemotherapy	121	39	32.2%	5.27	0.72-38.35	0.10	1.27	1.01-1.59	0.04
Consolidation Docetaxel	16	8	50.0%	2.11	0.97-4.58	0.06	4.42	1.79-10.92	0.001
Bilateral Lung Dose Volume Histogram									
V5<60%	62	13	21.0%	1.0*	-	-	1.0*	-	-
V5 60%	74	27	36.4%	1.90	0.98-3.69	0.057	1.16	0.44-3.10	0.76
V20<30%	52	9	17.3%	0.45	0.20-1.04	0.061	0.77	0.23-2.54	0.66
V20 30% and <40	43	15	34.9%	1.0*	-	-	1.0*	-	-
V20 40%	41	16	39.0%	1.15	0.57-2.33	0.70	1.08	0.50-2.34	0.84

	Total Patients (n=136)	Number of Patients with Grade 2 RP (n=40)	Percent of Patients with Grade 2 RP	Univariate Analysis			Multivariate Analysis		
				HR	95% CI	p-value	AHR	95% CI	p-value
MLD<18 Gy	66	14	21.2%	1.0*	-	-	1.0*	-	-
MLD 18 Gy	70	26	37.1%	1.95	1.02-3.74	0.044	1.19	0.42-3.32	0.75
SNP †									
<i>MTHFR</i> (rs1801131) □									
AA	61	23	37.7%	1.0*	-	-	1.0*	-	-
AC	60	12	20.0%	0.47	0.24-0.95	0.36	0.30	0.14-0.67	00.003
CC	13	4	30.8%	0.68	0.24-1.98	0.48	0.70	0.24-2.07	0.52
<i>AC/CC</i>	73	16	21.9%	0.51	0.27-0.97	0.041	0.37	0.18-0.76	0.006
<i>MTHFR</i> (rs1801133) □									
CC	51	11	21.6%	1.0*	-	-	1.0*	-	-
CT	59	19	32.2%	1.56	0.74-3.28	0.24	1.24	0.57-2.73	0.59
TT	24	9	37.5%	2.05	0.85-4.94	0.11	2.29	0.91-5.76	0.078
<i>CT/TT</i>	83	28	33.7%	1.69	0.84-3.39	0.14	1.48	0.71-3.08	0.29
<i>SOD2</i> (rs4880)									
TT	32	11	10.5%	1.0*	-	-	1.0*	-	-
CT	76	25	32.9%	0.93	0.46-1.88	0.83	1.24	0.60-2.59	0.56
CC	28	4	14.3%	0.40	0.13-1.24	0.12	0.57	0.17-1.85	0.35
<i>CT/CC</i>	104	29	27.9%	0.78	0.39-1.57	0.49	0.84	0.52-2.21	0.85

* Reference group for Cox regression

† Each SNP was independently entered into a multivariate model that adjusted for gender, ECOG PS, use of surgery, use of concurrent chemotherapy, use of consolidation docetaxel, MLD, V5 and V20.

□ Data available on 134 patients

Abbreviations: AHR=adjusted hazard ratio; CI=confidence interval; HR=hazard ratio; MLD=mean lung dose; PS=performance status; RT=radiation therapy; V5=volume receiving greater than 5 Gy; V20=volume receiving greater than 20 Gy; RP=radiation pneumonitis.

Table 5
Univariate and Multivariate Cox Regression analysis of Risk of Grade 3 RP for *MTHFR*

Patient Characteristics	Total Patients (n=136)	Number of Patients with Grade 3RP (n=19)	Percent of Patients with Grade 3 RP	Univariate Analysis			Multivariate Analysis		
				HR	95% CI	p-value	AHR	95% CI	p-value
Patient Characteristics									
Male	67	15	22.4%	4.20	1.40-12.68	0.011	3.90	1.23-12.3	0.02
Female	69	4	5.8%	1.0*	-	-	1.0*	-	-
ECOG PS									
0	62	7	11.3%	1.0*	-	-	1.0*	-	-
1	67	9	13.4%	1.20	0.44-3.24	0.71	1.01	0.35-2.94	0.99
2	7	3	42.9%	5.24	1.35-20.32	0.017	3.99	0.73-21.8	0.11
Treatment Characteristics									
Concurrent Chemotherapy	121	18	14.9%	2.23	0.30-16.68	0.44	1.42	1.08-1.88	0.01
Consolidation Docetaxel	16	3	18.8%	1.39	0.40-4.76	0.60	2.77	0.71-10.9	0.14
Surgery	30	0	0%	0.0	-	0.99	-	-	-
Bilateral Lung DVH									
V5<60%	62	5	8.1%	1.0*	-	-	1.0*	-	-
V5 60%	74	14	18.9%	2.47	0.89-6.87	0.082	1.86	0.35-9.93	0.47
V20<30%	52	4	7.7%	0.38	0.12-1.28	0.12	0.95	0.16-5.64	0.97
V20 30% and <40%	43	8	18.6%	1.0*	-	-	1.0*	-	-
V20 40%	41	7	17.1%	0.91	0.33-2.40	0.85	0.58	0.19-1.74	0.33
MLD<18 Gy	66	6	9.1%	1.0*	-	-	1.0*	-	-
MLD 18 Gy	70	13	18.6%	2.17	0.82-5.71	0.12	1.89	0.35-10.2	0.90

SNP	Total Patients (n=136)	Number of Patients with Grade 3 RP (n=19)	Percent of Patients with Grade 3 RP	Univariate Analysis			Multivariate Analysis			
				HR	95% CI	p-value	AHR	95% CI	p-value	
<i>MTHFR</i> rs1801131 [‡]										
AA	61	13	21.3%	1.0*	-	-	1.0*	-	-	-
AC/CC	73	5	6.8%	0.30	0.11-0.83	0.02	0.21	0.06-0.70	0.01	

* Reference group for Cox regression

[‡]Data available on 134 patients

Abbreviations: AHR=adjusted hazard ratio; CI=confidence interval; HR=hazard ratio; MLD=mean lung dose; PS=performance status; RT=radiation therapy; V5=volume receiving greater than 5 Gy; V20=volume receiving greater than 20 Gy; RP=radiation pneumonitis.