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Original Article

Genetic variants of *BIRC3* and *NRG1* in the NLRP3 inflammasome pathway are associated with non-small cell lung cancer survival

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Abstract: The nod-like receptor protein 3 (NLRP3) is one of the most characterized inflammasomes, and its genetic variation and functional dysregulation are involved in pathogenesis of several cancers. To systematically evaluate the role of NLRP3 in predicting outcomes of patients with non-small cell lung cancer (NSCLC), we performed a two-phase analysis for associations between genetic variants in NLRP3 inflammasome pathway genes and NSCLC survival by using a published genome-wide association study (GWAS) dataset from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. We used multivariate Cox proportional hazards regression analysis with Bayesian false discovery probability (≤0.80) for multiple testing correction to evaluate associations between 20,730 single-nucleotide polymorphisms (SNPs) in 176 genes and overall survival of 1,185 NSCLC patients from the PLCO trial. We further validated the identified significant SNPs in another GWAS dataset with survival data from 984 NSCLC patients of the Harvard Lung Cancer Susceptibility (HLCS) study. The results showed that two independent SNPs in two different genes (i.e., *BIRC3* rs11225211 and *NRG1* rs4733124) were significantly associated with the NSCLC overall survival, with a combined hazards ratio (HR) of 0.83 [95% confidence interval (CI) = 0.74-0.93 and $P = 0.0009$] and 1.18 (95% CI = 1.06-1.31) and $P = 0.002$], respectively. However, further expression quantitative trait loci (eQTL) analysis showed no evidence for correlations between the two SNPs and mRNA expression levels of corresponding genes. These results indicated that genetic variants in the NLRP3 imflammasome pathway gene-sets might be predictors of NSCLC survival, but the molecular mechanisms underlying the observed associations warrant further investigations.

Keywords: Non-small cell lung cancer, NLRP3 inflammasome, genome-wide association study, single-nucleotide polymorphism, overall survival

Introduction

Non-small cell lung cancer (NSCLC) is the most common lung tumor, and the leading cause of cancer-related death worldwide [1]. NSCLC accounts for about 85% of lung cancer including several subtypes, such as adenocarcinoma, squamous cell carcinoma and large cell carcinoma [2]. Although advances have been made in early diagnosis and treatment of NSCLC, the prognosis of NSCLC has been still unfavorable with a dismal 5-year overall survival (OS) rate less than 20% [3], partly due to individual variability in response to available cancer therapies [4]. Therefore, it is important to better understand molecular mechanisms that contribute to individual variability in response to treatments for NSCLC.

It has been reported that inflammation plays a crucial role in carcinogenesis and tumorigenesis, such as cancer cell initiation [5] and promotion [6] as well as tumor progression [7], angiogenesis [8] and invasion [9]. Studies have revealed that the acute inflammation protects the body against infectious pathogens [10]. However, chronic inflammation, which is associated with DNA damage, tissue impairment, and genetic and epigenetic changes, may lead to cancer initiation and progression [11]. Inflammasome, an intracellular multi-protein complex, may form in response to various stimuli [12]. Yet, the exact role of inflammasome in heterogeneous tumorigenesis and thus prognosis of patients with NSCLC remains unclear.

The nod-like receptor protein 3 (NLRP3) is one component of the most characterized inflammasomes, which belongs to the NLR protein family of 22 members, and respond to a wide range of infections and endogenous ligands, such as pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) [13]. One study showed that dysregulated function of the NLRP3 inflammasome pathway was associated with the pathogenesis of several inflammatory diseases [14], while others showed that genetic variants in the NLRP3 inflammasome pathway genes were associated with the development of malignancies, such as chronic myeloid leukemia [15] and melanoma [16]; however, the roles of these genetic variants in initiation and promotion of cancer are still unclear, which may provide insight into therapeutic targets and prognostic significance in NSCLC.

Genome-wide association studies (GWASs) have been proven to be a powerful approach to the disease etiology by genetic analysis of complex diseases and traits [17] and have reported dozens of single-nucleotide polymorphisms (SNPs) to be associated with lung cancer risk. SNPs are characterized by a single nucleotide change in the sequences of a gene, and SNPs in the exons could change amino acid sequence and thus affect functions of the proteins. Those SNPs located in the introns, especially around the 3' untranslated regions (3'UTR), promoter elements and splicing sites, are thought to influence gene expression levels [18]. However, it is still unknown whether SNPs in the NLRP3 inflammasome genes play any roles in cancer growth and progression. In the present study, we explored the association between genetic variants in the NLRP3 inflammasome gene-set and survival of NSCLC patients using the publically available NSCLC GWAS datasets.

Materials and methods

Study populations

As shown in the study flowchart (**Figure 1**), we used a two-phase study design of discovery and replication to uncover any SNPs in the NLRP3 inflammasome gene-set that possibly have an effect on survival of NSCLC patients. The discovery phase included 1,185 NSCLC patients obtained from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, after the application and access approval from National Cancer Institute (NCI). The PLCO trial is a National Cancer Institute (NCI)-funded multicenter randomized screening trial of cancer from ten medical centers in the United States between 1993 and 2011. The screening trial enrolled 77,500 men and 77,500 women aged 55-74. All individuals were randomized to either the intervention arm with screening or the control arm with standard care [19].

The PLCO trial also collected blood specimens from the first screening visit and gathered extensive information about each individual's health status, including demographic information, smoking history and family history of cancer [20]. All participants were followed up for at least 13 years after the enrollment. Genomic DNA extracted from the blood samples was genotyped in a genome-wide scan with Illumina HumanHap240Sv1.0, Human-Hap300v1.1 and HumanHap550v3.0 (dbGaP accession: phs000093.v2.p2 and phs000336.v1.p1) [21, 22]. In 1,187 Caucasian NSCLC patients from the PLCO trial, two with missing follow-up information were excluded, leading to 1,185 eligible NSCLC patients, who had the complete clinicopathological variables and genotype data for analysis. Tumor staging was determined according to the fifth edition American Joint Committee on Cancer staging system. The institutional review boards of each participating institution approved the PLCO trial and the use of biospecimen for further research, and all the subjects signed a written informed consent

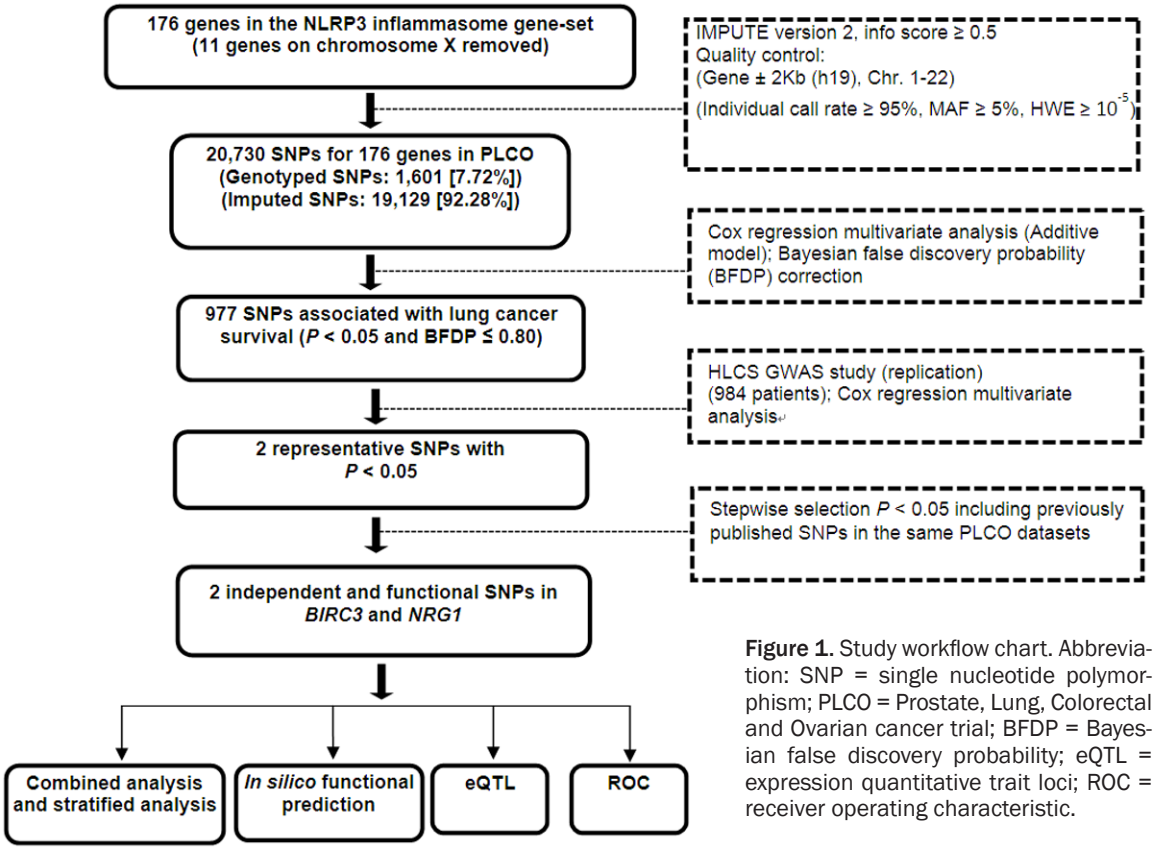


Figure 1. Study workflow chart. Abbreviation: SNP = single nucleotide polymorphism; PLCO = Prostate, Lung, Colorectal and Ovarian cancer trial; BFDP = Bayesian false discovery probability; eQTL = expression quantitative trait loci; ROC = receiver operating characteristic.

permitting the use of their samples and data for future research represented here.

The replication phase used another NSCLC GWAS dataset from the Harvard Lung Cancer Susceptibility (HLCS) Study with 984 histology-confirmed Caucasian NSCLC patients. The histological classification of the tumors was determined by two staff pulmonary pathologists at the Massachusetts General Hospital. The time of blood collection was within 1-4 weeks of the diagnosis for each patient. DNA was extracted from blood samples by using the Auto Pure Large Sample Nucleic Acid Purification System (QIAGEN Company, Venlo, Limburg, Netherlands). Genotyped data was obtained through genome-wise scan with Illumina Humanhap-610-Quad arrays, and imputation was performed by using MaCH based on the 1000 Genomes Project. The comparison of the characteristics between the PLCO and Harvard study is presented in Table S1.

Gene and SNP selection

We selected genes involved in the NLRP3 inflammasome pathway from the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp) and Genecards (https://www.genecards.org/), by the keyword "NLRP3" and "Inflammasome". After removing the duplicated genes and deleting genes in the X chromosome, 176 genes remained as candidate genes for further analysis (Table S2). We first performed imputation for the 176 genes with 500-kb flanking regions by using IMPUTE2 and the 1000 Genomes Project data (phase 3). After imputation, we extracted all the SNPs in these genes and within their ±2 kb flanking regions according to the following criteria: a minor allele frequency (MAF) ≥ 0.05, a genotyping rate ≥ 95%, and Hardy-Weinberg equilibrium P value ≥ 1×10^{-5}. As a result, 1,601 genotyped SNPs and 19,129 imputed SNPs were extracted from the PLCO GWAS dataset.

Statistical analysis

The follow-up time was from the diagnosis of lung cancer to the last follow-up or time of death in both PLCO and HLCS datasets. The follow-up time was defined from lung cancer diagnosis to the last follow-up or time of death. OS was the primary endpoint, and disease-spe-

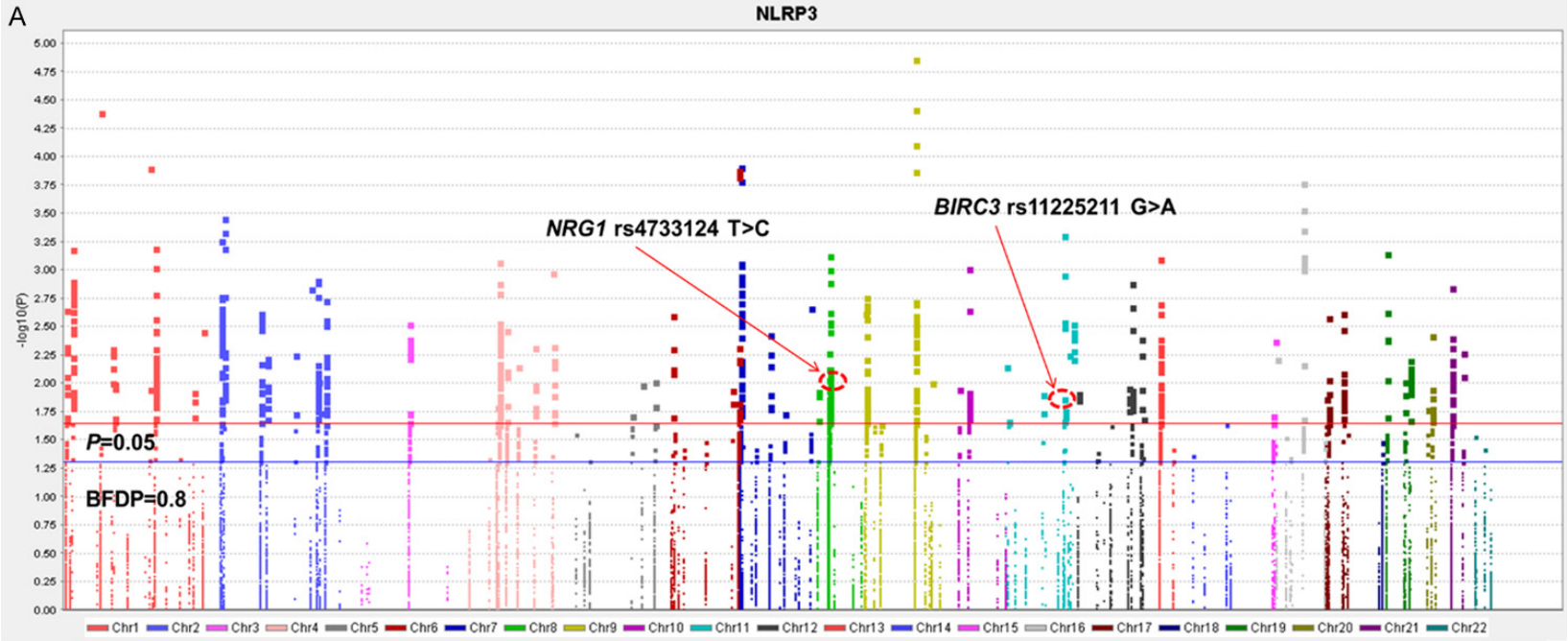
cific survival (DSS) of lung cancer was also available. In the single-locus analysis, we used multivariate Cox proportional hazards regression analysis to evaluate associations between each of 20,730 SNPs and OS/DSS (in an additive genetic model) with adjustment for age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery and the top four principal components of the PLCO dataset using the GenABEL package of R software [23]. Since over 90% of SNPs were imputed with a high level of linkage disequilibrium (LD), we used the Bayesian false discovery probability (BFDP) with a cutoff value of 0.8 for multiple testing correction [24] after the initial evaluation with false discover rate (FDR). We assigned a prior probability of 0.10 to detect an upper bound of HR of 3.0 for an association with variant genotypes or minor alleles of the SNPs with $P < 0.05$. Then, we performed the replication using the HLCS GWAS dataset for those SNPs that satisfied the following conditions: 1) passed the threshold of P value < 0.05 and BFDP ≤ 0.80, 2) potentially functional predicted by HaploReg [25], SNPinfo [26] and RegulomeDB [27], and 3) tagging SNPs based on their LD. To identify independent SNPs, we included the replicated SNPs in a multivariate stepwise Cox model with adjustment for demographical and clinical variables, previous published SNPs as well as the top four principal components of the genotyping data in the PLCO dataset. A meta-analysis was also performed to combine both discovery and replication datasets. A fixed-effects model was applied, If the Cochran's Q-test P value > 0.100 and the heterogeneity statistic (I^2) < 50%; otherwise, a random-effects model was employed. Kaplan-Meier curve was used to depict associations between survival and the genotypes of the SNPs, and the combination of risk genotypes was used to estimate cumulative effects of the identified SNPs.

We further performed an expression quantitative trait loci (eQTL) analysis to assess correlations between genotypes of SNPs and mRNA expression levels by using linear regression analysis with the R (version 3.5.0) software. mRNA expression data of the genes were obtained from lymphoblastoid cell lines derived from the 373 European descendants included in the 1,000 Genomes Project [28]. We also examined the differences in mRNA expression levels between paired tumor tissues and adjacent normal tissues by the paired t test by using the data from the Cancer Genome Atlas (TCGA) database (dbGaP Study Accession: phs000178.v9.p8) [29]. Next, we assessed the association between mRNA expression and survival through Kaplan-Meier (KM) analysis in a pooled dataset (n = 1,928) (http://kmplot.com/analysis/ index.php?p=service&cancer=lung). Finally, we performed the receiver operating characteristic (ROC) curve and time-dependent ROC analysis to illustrate prediction accuracy of the models integrating both clinical and genetic variables on NSCLC survival with the "timeROC" package in R (version 3.5.0) [30]. Unless specified, all statistical analyses were performed with SAS software (version 9.4; SAS Institute, Cary, NC, USA).

Results

Associations between SNPs in the NLRP3 inflammasome gene-set and NSCLC OS in both PLCO and HLCS datasets

The study flowchart is shown in **Figure 1**, and basic characteristics of the 1,185 NSCLC patients have been described previously [31]. In the PLCO discovery with a single-locus analysis in an additive genetic model, the multivariate Cox regression analysis with adjustment for age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery and first four principal components (Table S3) identified 977 SNPs that were significantly associated with NSCLC OS after multiple testing correction by BFDP ≤ 0.8 after failure in the FDR test. The results are summarized in a Manhattan plot (**Figure 2A**). In the replication of the top and potentially functional SNPs by the HLCS dataset, two SNPs in two different genes (i.e., *BIRC3* rs11225211 and *NRG1* rs4733124) were replicated as shown in **Table 1**, in which the multiple testing corrections by both FDR and BFDP are presented, and the association results are also shown in another Manhattan plot (**Figure 2B**). Further combined-analysis of these SNPs in the two datasets showed a much improved OS associated with the *BIRC3* rs11225211 A allele ($P_{adjusted}$ = 0.0009) and a poorer OS associated with the *NRG1* rs4733124 C allele ($P_{adjusted}$ = 0.002), without heterogeneity between the two datasets (**Table 1**).


A
NLRP3
NRG1 rs4733124 T>C
BIRC3 rs11225211 G>A
P=0.05
BFDP=0.8
-log10(P)
Chr1 Chr2 Chr3 Chr4 Chr5 Chr6 Chr7 Chr8 Chr9 Chr10 Chr11 Chr12 Chr13 Chr14 Chr15 Chr16 Chr17 Chr18 Chr19 Chr20 Chr21 Chr22

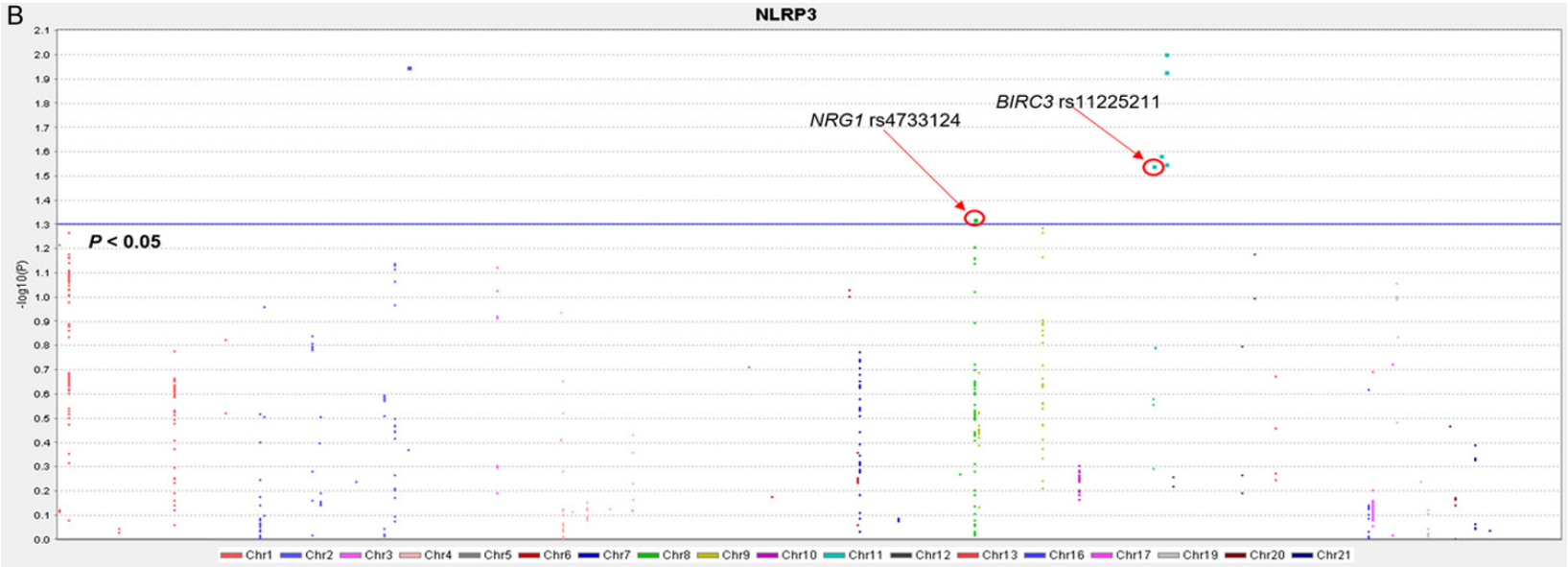

B
NLRP3
NRG1 rs4733124
BIRC3 rs11225211
P < 0.05
-log10(P)
Chr1 Chr2 Chr3 Chr4 Chr5 Chr6 Chr7 Chr8 Chr9 Chr10 Chr11 Chr12 Chr13 Chr16 Chr17 Chr19 Chr20 Chr21

Figure 2. Manhattan plot of SNPs in the NLRP3 inflammasome pathway gene-set. The statistical values across the autosomes for associations between 27,030 SNPs and overall survival are plotted as -log10 *P* values. A. Manhattan plot of 27,030 SNPs in the PLCO trial, the blue horizontal line indicates *P* = 0.05 and the red line indicates BFDP = 0.8. B. Manhattan plot of validated SNPs in the HLCS GWAS study, the blue horizontal line indicates *P* = 0.05.

Identification of independent SNPs associated with OS of NSCLC in the PLCO dataset

To identify potential functional SNPs associated with NSCLC survival, we used three bioinformatics tools (i.e., SNPinfo, RegulomeDB and HaploReg). In the RegulomeDB prediction, *NRG1* rs4733124 has a score of 4, while *BIRC3* rs11225211 has no data available (Table S4). Both the validated SNPs appear to be located in the intron regions with some enhancer histone marks; besides, rs4733124 is involved in two altered motifs (i.e., GZF1/Pax-4), while the rs11225211 is involved in four altered motifs based on the HaploReg prediction. However, there is no obvious evidence for functional relevance of both SNPs based on the SNPinfo prediction.

Next, the two replicated SNPs of *BIRC3* rs11225211 and *NRG1* rs4733124 were included in a multivariate Cox model with adjustment for demographic and clinical covariables and other 15 previously published SNPs as well as the first four principal components available in the PLCO dataset. As a result, both SNPs remained to be independently associated with NSCLC OS (**Table 2**). A regional association plot was made to show the location and neighboring SNPs of each SNP (Figure S1). In the PLCO dataset, patients with the *BIRC3* rs11225211 A allele had a favorable OS and DSS (P_{trend} = 0.014 and 0.035, respectively, **Table 3**), while the *NRG1* rs4733124 C allele had an unfavorable OS and DSS (P_{trend} = 0.009 and 0.048, respectively, **Table 3**). Compared with the reference genotype in a dominant genetic model, *BIRC3* rs11225211 GA+AA genotypes were associated with a favorable OS and DSS (HR = 0.80, 95% CI = 0.69-0.94 and *P* = 0.006 and 0.82, 0.70-0.96 and 0.016, respectively, **Table 3**); *NRG1* rs4733124 TC+CC genotypes were associated with an unfavorable OS (1.21, 1.04-1.41 and 0.014, **Table 3**) but a borderline unfavorable DSS (1.14, 0.97-1.34 and 0.114, **Table 3**).

Combined and stratified analysis of the two independent and functional SNPs in the PLCO dataset

To provide a better estimation of individual hazards of survival, we combined the risk genotypes (i.e., rs11225211 GG and rs4733124 TC+CC) into a genetic risk score (GRS) that was used to divide all NSCLC patients into three groups: zero, one and two risk genotypes. As shown in **Table 3**, in the multivariate analysis, an increased genetic risk score was associated with a poorer survival [HR = 1.21 (1.01-1.46) and *P* = 0.040 for GRS = 1, 1.53 (1.22-1.90) and 0.0002 for GRS = 2, and $P_{trend\ test}$ = 0.0002 for OS; and 1.15 (0.95-1.40), 0.143 for GRS = 1, 1.41 (1.11-1.77) and 0.004 for GRS = 2, and 0.004 for DSS]. To dichotomize the death risk, we devided all the patients into two groups of GRS = 0 and GRS = 1-2. Compared with the group of GRS = 1-2 (the majority), the group with GRS = 0 had a better survival (HR = 0.78, 95% CI = 0.65-0.93, *P* = 0.006 for OS and HR = 0.83, 95% CI = 0.68-0.99, *P* = 0.042 for DSS). These results were further depicted in Kaplan-Meier survival curves (**Figure 3A-D**).

To assess the ability of risk genotypes to predict the survival of NSCLC, we compared the AUC of the model including clinical variables with that including both clinical variables and risk genotypes. The addition of risk genotypes to the prediction model of five-year OS borderline increased the AUC from 88.18% to 88.56% (*P* = 0.052, Figure S2A); similarly, the addition of risk genotypes to the prediction model of five-year DSS also borderline increased the AUC from 88.02% to 88.37% (*P* = 0.078, Figure S2B). Finally, the time-dependent AUC curve was provided to quantitate the ability of risk genotypes to predict NSCLC survival through the entire follow-up period (Figure S2C, S2D).

We further performed stratified analysis to evaluate whether the effect of combined risk genotypes on NSCLC OS and DSS was modified by age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy and surgery. The results showed that the chemotherapy has an interaction with the 1-2 risk genotypes to have a modification effect on OS and DSS (HR = 1.01, 95% CI = 0.80-1.29 and *P* = 0.911 and 1.05, 0.82-1.33 and 0.719, respectively; Table S5), and the surgery also has significant interactions with the 1-2 risk genotypes to have a modification effect on OS and DSS (HR = 0.49, 95% CI = 0.34-0.72 and *P* = 0.0002

Table 1. Combined analysis of the two validated SNPs in the PLCO trial and HLCS study datasets

SNP	Allele[a]	Gene	FDR	BFDP	PLCO (n = 1,185)			HLCS (n = 984)			Combined analysis			
					EAF	HR (95% CI)[b]	*P*[b]	EAF	HR (95% CI)[c]	*P*[c]	HR (95% CI)[d]	*P*[d]	P_{het}[e]	I^2
rs11225211	G>A	*BIRC3*	0.33	0.75	0.17	0.84 (0.73-0.96)	0.014	0.16	0.81 (0.68-0.98)	0.029	0.83 (0.74-0.93)	0.0009	0.780	0
rs4733124	T>C	*NRG1*	0.30	0.74	0.16	1.2 (1.05-1.38)	0.009	0.18	1.15 (1.00-1.33)	0.048	1.18 (1.06-1.31)	0.002	0.714	0

[a]Effect/reference allele; [b]Adjusted for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2, PC3 and PC4; [c]Adjusted for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2 and PC3; [d]additive model; [e]*P* value for heterogeneity by Cochrane's Q test. Abbreviation: PLCO = Prostate, Lung, Colorectal and Ovarian cancer trial; HLCS = Harvard Lung Cancer Susceptibility study; SNPs = Single nucleotide polymorphisms; FDR = False discovery rate; BFDP = Baysian false discovery probability; EAF = Effect allele frequency; HR = Hazards ratio; CI = Confidence interval.

Table 2. Predictors of OS obtained from the final Cox proportional hazards regression analysis in the PLCO dataset

Variables	Category	Frequency	HR (95% CI)[a]	*P*[a]	HR (95% CI)[b]	*P*[b]
Age	Continuous	1185	1.03 (1.02-1.05)	< 0.0001	1.04 (1.02-1.05)	< 0.0001
Sex	Male	698	1.00		1.00	
	Female	487	0.79 (0.68-0.92)	0.002	0.78 (0.67-0.91)	0.002
Smoking status	Never	115	1.00		1.00	
	Current	423	1.61 (1.20-2.15)	0.001	1.83 (1.35-2.46)	< 0.0001
	Former	647	1.59 (1.21-2.09)	0.0009	1.81 (1.36-2.41)	< 0.0001
Histology	AD	577	1.00		1.00	
	SC	285	1.16 (0.96-1.40)	0.120	1.21 (1.00-1.47)	0.049
	Others	323	1.27 (1.08-1.51)	0.005	1.32 (1.10-1.57)	0.002
Stage	I-IIIA	655	1.00		1.00	
	IIIB-IV	528	2.84 (2.34-3.45)	< 0.0001	3.06 (2.51-3.73)	< 0.0001
Chemotherapy	No	639	1.00		1.00	
	Yes	538	0.57 (0.48-0.68)	< 0.0001	0.57 (0.48-0.69)	< 0.0001
Radiotherapy	No	762	1.00		1.00	
	Yes	415	0.96 (0.81-1.13)	0.586	0.96 (0.81-1.13)	0.620
Surgery	No	637	1.00		1.00	
	Yes	540	0.21 (0.16-0.27)	< 0.0001	0.20 (0.15-0.26)	< 0.0001
BIRC3 rs11225211 G>A	GG/GA/AA	814/333/37	0.84 (0.73-0.96)	0.012	0.83 (0.72-0.96)	0.011
NRG1 rs4733124 T>C	CC/CT/TT	841/314/29	1.21 (1.05-1.39)	0.007	1.22 (1.06-1.40)	0.006

[a]The final analysis included age, sex, smoking status, tumor stage, tumor histology, chemotherapy, radiotherapy, surgery, top four principal components and two new validated SNPs (*BIRC3* rs11225211 and *NRG1* rs4733124 in an additive model); [b]Fifteen published SNPs were used for post-stepwise adjustment. Five SNPs were reported in previous publication (PMID: 27557513); One SNP was reported in the previous publication (PMID: 29978465); Two SNPs were reported in the previous publication (PMID: 30259978); Two SNPs were reported in the previous publication (PMID: 26757251); Three SNPs were reported in the previous publication (PMID: 30650190); Two SNPs were reported in the previous publication (PMID: 30989732); Abbreviations: OS = Overall survival; PLCO = Prostate, Lung, Colorectal and Ovarian cancer trial; HR = Hazards ratio; CI = Confidence interval.

and 0.55, 0.36-0.83 and 0.004, respectively; Table S5).

In silico functional validation

According to experimental data from the ENCODE project (Figure S3A, S3B), no potential function of *BIRC3* rs11225211 is predicted, but the *NRG1* rs4733124 is predicted to be located in a DNase I hypersensitive site, which may has potential functions on chromatin rearrangement or expression regulation.

To further explore the potential function of both SNPs, we performed the eQTL analysis for the correlation between genotypes of the SNPs and mRNA expression levels in the 1000 Genomes Project and GTEx datasets. As a result, we found that the *BIRC3* rs11225211A allele was not correlated with mRNA expression in the 1000 Genomes Project or GTEx datasets (Figures S4 and S5A), so was for the *NRG1* rs4733124 C allele (Figure S5B). Finally, to further identify the roles of the genes in the progression of NSCLC, we assessed the mRNA expression levels of these two genes in 111 paired NSCLC tumor and adjacent normal tissue samples obtained from the TCGA database. We found that the expression levels of *BIRC3* were higher in the adjacent normal tissues than in tumor tissues ($P = 0.0003$, Figure S6A), and the higher expression levels were associated with a poorer NSCLC OS (Figure S7A). Interestingly, the expression levels of *NRG1* were also higher in the adjacent normal tissues than in tumor tissues ($P < 0.001$, Figure S6D), but the higher expression levels were not associated with a better NSCLC OS (Figure S7B).

Mutation analyses

Finally, to evaluate the possible role of the SNPs in regulating the gene expression in the target tissues, we accessed the mutation status of *BIRC3* and *NRG1* in lung tumor tissues by using the public database of the cBioPortal for Cancer Genomics. As shown in Figure S8A, S8B, *BIRC3* had a low somatic mutation rate in

Table 3. Associations of the two validated SNPs in the NLRP3 inflammasome pathway gene-set with OS of NSCLC in the PLCO dataset

Genotype	OS Multivariate analysis[a]				DSS Multivariate analysis[a]			
	All	Death (%)	HR (95% CI)	*P*	All	Death (%)	HR (95% CI)	*P*
BIRC3 rs11225211 G>A	n = 1174				n = 1174			
GG	806	553 (68.6)	1.00		806	496 (61.5)	1.00	
GA	331	212 (64.1)	0.80 (0.68-0.94)	0.005	331	191 (57.7)	0.81 (0.68-0.96)	0.013
AA	37	23 (62.2)	0.89 (0.59-1.36)	0.602	37	21 (56.8)	0.94 (0.60-1.46)	0.775
Trend				0.014				0.035
GA+AA	368	235 (63.9)	0.80 (0.69-0.94)	0.007	368	212 (57.6)	0.82 (0.70-0.96)	0.016
NRG1 rs4733124 T>C	n = 1174				n = 1174			
TT	835	541 (64.8)	1.00		835	493 (59.0)	1.00	
TC	310	230 (74.2)	1.19 (1.02-1.40)	0.029	310	198 (63.9)	1.11 (0.94-1.31)	0.238
CC	29	18 (62.1)	1.49 (0.93-2.40)	0.097	29	18 (62.1)	1.68 (1.05-2.71)	0.032
Trend				0.009				0.048
TC+CC	339	248 (73.2)	1.21 (1.04-1.41)	0.014	339	216 (63.7)	1.14 (0.97-1.34)	0.114
NUG[b]	n = 1173				n = 1173			
0	264	159 (60.2)	1.00		264	148 (56.1)	1.00	
1	674	458 (68.0)	1.21 (1.01-1.46)	0.040	674	409 (60.7)	1.15 (0.95-1.40)	0.143
2	235	171 (72.8)	1.53 (1.22-1.90)	0.0002	235	151 (64.3)	1.41 (1.11-1.77)	0.004
Trend				0.0002				0.004
1-2	909	629 (69.0)	1.00		909	560 (61.6)	1.00	
0	264	159 (60.2)	0.78 (0.65-0.93)	0.006	264	148 (56.1)	0.83 (0.68-0.99)	0.042

[a]Adjusted for age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery, and top four principal components: with one subject with missing data for *BIRC3* rs11225211 G>A genotype, one for *NRG1* rs4733124 T>C genotype and 10 subjects with missing data for phenotype; [b]NUG = Number of unfavorable genotypes, unfavorable genotypes were *BIRC3* rs11225211 GG and *NRG1* rs4733124 TC+CC. Abbreviation: SNPs = Single nucleotide polymorphisms; OS = Overall survival; NSCLC = Non-small cell lung cancer; PLCO = Prostate, Lung, Colorectal and Ovarian cancer trial; HR = Hazards ratio; CI = Confidence interval.

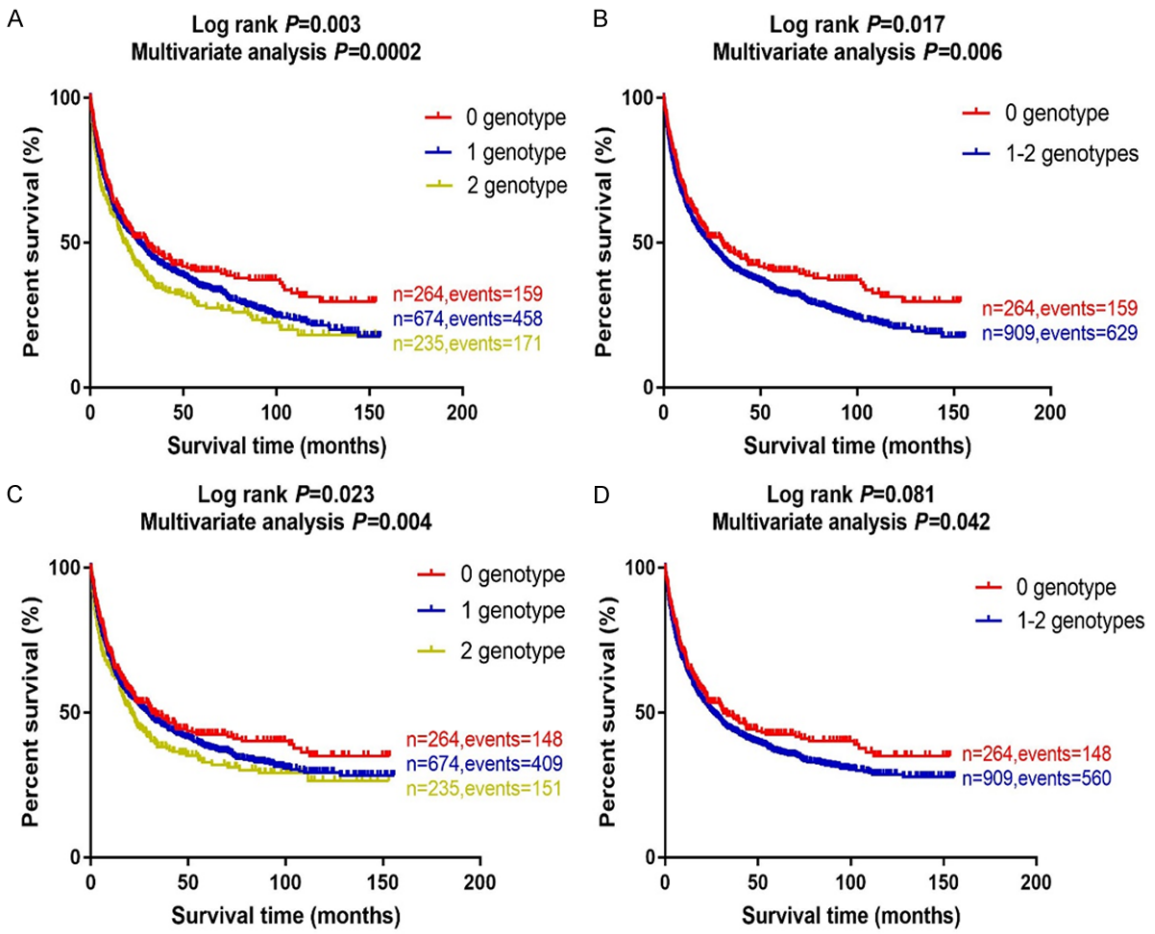


Figure 3. Kaplan-Meier (KM) survival curves for NSCLC patients by combined risk genotypes in the PLCO trial. (A) by 0, 1 and 2 risk genotypes and (B) by 0 and 1-2 risk genotypes for overall survival; (C) by 0, 1 and 2 risk genotypes and (D) by 0 and 1-2 risk genotypes for disease specifical survival in the PLCO trial. Abbreviations: NSCLC, non-small cell lung cancer; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screening trial.

LUSC (0.83%, 4/484) and LUAD (0.76%, 5/660) in the Pan-Lung Cancer Study [29]. In contrast, *NRG1* had a relatively higher somatic mutation rate in LUAD (3.18%, 21/660) and LUSC (1.03%, 5/484) in the Pan-Lung Cancer Study [29]. As shown in the Figure S8C, *BIRC3* and *NRG1* have a close interaction with the frequently altered neighbor genes. Given the relatively higher mutation rate in the *NRG1* gene, the mutation may play a role in the altered functions and expression in the target tissues in addition to the SNPs identified in the present study.

Discussion

In the present study, we found that two novel genetic variants (i.e., *BIRC3* rs11225211 G>A and *NRG1* rs4733124 T>C) in the NLRP3 inflammsome pathway gene-set were significantly associated with NSCLC survival in both PLCO trial and HLCS datasets.

Although the role of inflammasome in the development and progression of lung cancer remains unclear, a series of studies used colitis-induced colon cancer as an animal model to investigate the role of inflammasome in the development and progression of cancers. One study found that the inflammasome components could protect against colitis-associated colon cancer [32]. Other studies suggested that the cancer-related inflammation may promote tumor growth and metastasis. For example, one study reported that the NLRP3 promoted inflammation to induce skin cancer but was dispensable for the asbestos-induced mesothelioma [33]; other studies demonstrated that the activation of NLRP3 inflammasome promoted metastasis of breast cancer to liver and lung tissues [34]

and that the NLRP3 inflammasome could activate the secretion of IL-18 and IL-1β, the principle components of the inflammatory response, in the lung adenocarcinoma cell line A549 [35]. These studies suggest that the NLRP3 inflammasome is involved in carcinogenesis of various organs.

BIRC3 is a family member of inhibitors of apoptosis proteins (IAPs) that may inhibit apoptosis by directly inhibiting the caspase cascade [36-38]. Several studies have reported that overexpression of *BIRC3* is associated with chemoresistance in several malignancies. For example, one study found that BIRC3-positive patients with colon cancer had a shorter disease-free survival after 5-Fu-based chemotherapy [39]. Another study found that the upregulation of BIRC3 was associated with a shorter OS in colorectal cancer [40]. Furthermore, BIRC3 could promote hepatocellular carcinoma epithelial-mesenchymal transition (EMT), cell migration, and metastasis [41]. However, in the present study, we found that the mRNA expression of *BIRC3* was higher in normal lung tissues than in tumor tissues and that the higher expression of *BIRC3* was associated with a poorer OS of NSCLC. Based on the mutation data from the cBioPortal for Cancer Genomics, we speculate that the mutation of *BIRC3* may have some role in regulating its gene expression and thus survival, but the molecular mechanisms underlying the association between the *BIRC3* rs11225211 G>A SNP and outcomes of NSCLC patients need to be further investigated.

Neuregulin 1 (NRG1) is a known ligand for the HER3 (ERBB3) receptor that, when activated by the NRG1 binding, forms a heterodimer with other HER family receptors and regulates the downstream signaling, leading to multiple biology functions, including growth, proliferation, decreased apoptosis, cellular migration and angiogenesis [42]. Oncogenic gene fusions involving *NRG1* have emerged in lung adenocarcinomas and have been proposed to represent a chemo-resistant signaling axis, and such fusion genes are exclusively detected in lung adenocarcinomas of the invasive mucinous subtype in those who never smoked [43]. This subtype tumor usually presents as a multifocal and/or unresectable disease, with no effective treatment [44]. Considering many drugs that target *ERBB2 (HER2)* and *ERBB3 (HER3),* the detecting and targeting of *NRG1* fusions in invasive mucinous lung adenocarcinomas may represent a therapeutic opportunity. To date, few studies have investigated the roles of genetic variants of *NRG1* in the prognosis of cancers, and only one study reported that the M111T, M139I and R438H variants of *NRG1* were associated with reduction of the NRG1 protein levels in patients with Hirschsprung disease [45]. In the present study, we found that the mRNA expression of *NRG1* was higher in normal lung tissues than in tumor tissues in the TCGA dataset, but the expression levels did not have an effect on survival of NSCLC patients. Furthermore, the rs4733124 C allele does not seem to have an effect on the expression of *NRG1* in lung cancer, either; therefore, the mechanism by which *NRG1* rs4733124 T>C may affect survival of NSCLC patients needs to be further investigated.

Although we observed some associations between genetic variants in two NLRP3 inflammasome genes and NSCLS OS, supported by some functional evidence, the exact molecular mechanisms are unclear. Because the eQTL analysis did not find a correlation between the survival-associated genotypes and mRNA expression levels in the unaffected cells, further biochemical studies and functional experiments are required to corroborate the present findings. Although the sample size of PLCO was relatively large, the number of patients in subgroups was still relatively small, which might have reduced the statistical power to detect a weak effect of those SNPs under investigation in one particular subpopulation.

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Atlas (TCGA) database (dbGaP Study Accession: phs000178.v9.p8). A list of contributing investigators and funding agencies for those studies can be found in the end of Supplemental tables. Qingyi Wei was supported by the V Foundation for Cancer Research (D2017-19) and also partly supported by the Duke Cancer Institute as part of the P30 Cancer Center Support Grant (Grant ID: NIH/NCI CA014236). The Harvard Lung Cancer Susceptibility Study was supported by NIH grants 5U01CA209414, CA092824, CA074386 and CA090578 to David C. Christiani.

Disclosure of conflict of interest

None.

Abbreviations

NSCLC, Non-small cell lung cancer; SNPs, single nucleotide polymorphisms; NLRP3, Nod-like receptor protein 3; GWAS, Genome-Wide Association Study; PLCO, the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; HLCS, Harvard Lung Cancer Susceptibility; OS, overall survival; DSS, disease special survival; LD, linkage disequilibrium; BFDP, Bayesian false discovery probability; eQTL, expression quantitative trait loci; TCGA, the Cancer Genome Atlas; ROC, receiver operating characteristic; HR, hazards ratio; CI, confidence interval; AUC, receiver operating characteristic curve.

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Table S1. Comparison of the characteristics between the PLCO trial and the HLCS study

Characteristics	PLCO		HLCS		P^a
	Frequency	Deaths (%)	Frequency	Deaths (%)	
Total	1185	798 (67.3)	984	665 (67.5)	
Median overall survival (months)	23.8		39.9		
Age					
≤ 71	636	400 (62.9)	654	428 (65.4)	
> 71	549	398 (72.5)	330	237 (71.8)	< 0.0001
Sex					
Male	698	507 (72.6)	507	379 (74.7)	
Female	487	291 (59.8)	477	286 (59.9)	0.0006
Smoking status					
Never	115	63 (54.8)	92	52 (56.5)	
Current	423	272 (64.3)	390	266 (68.2)	
Former	647	463 (71.6)	502	347 (69.1)	0.166
Histology					
Adenocarcinoma	577	348 (60.3)	597	378 (63.3)	
Squamous cell carcinoma	285	192 (67.4)	216	156 (72.2)	
Others	323	258 (79.9)	171	131 (76.6)	< 0.0001
Stage					
I-IIIA	655	315 (48.1)	606	352 (58.0)	
IIIB-IV	528	482 (91.3)	377	313 (83.0)	0.003
Missing	2		–		

[a]Chi-square test for comparisons of the characteristics between the PLCO trail and the Harvard study. Abbreviations: PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; HLCS, Harvard lung cancer susceptibility study.

Table S2. List of 176 selected genes in the NLRP3 Inflammasome gene-set from MSigDB and Genecards

Dataset	Name of pathway[a]	Selected genes	Number of genes
KEGG	NOD-like receptor signaling pathway	*BIRC2, BIRC3, CARD18, CARD6, CARD8, CARD9, CASP1, CASP5, CASP8, CCL11, CCL13, CCL2, CCL5, CCL7, CCL8, CHUK, CXCL1, CXCL2, ERBB2IP, HSP90AA1, HSP90AB1, HSP90B1, IKBKB, IKBKG, IL18, IL1B, IL6, IL8, MAP3K7, MAPK1, MAPK10, MAPK11, MAPK12, MAPK13, MAPK14, MAPK3, MAPK8, MAPK9, MEFV, NAIP, NFKB1, NFKBIA, NFKBIB, NLRC4, NLRP1, NLRP3, NOD1, NOD2, PSTPIP1, PYCARD, PYDC1, RELA, RIPK2, SUGT1, TAB1, TAB2, TAB3, TNF, TNFAIP3, TRAF6, TRIP6, XIAP*	62
REACTOME	Inflammasomes	*AIM2, APP, BCL2, BCL2L1, CASP1, HSP90AB1, LOC644816, MEFV, NLRC4, NLRP1, NLRP3, P2RX7, PANX1, PSTPIP1, PYCARD, TXN, TXNIP*	17
REACTOME	The NLRP3 inflammasome	*APP, CASP1, HSP90AB1, LOC644816, MEFV, NLRP3, P2RX7, PANX1, PSTPIP1, PYCARD, TXN, TXNIP*	12
GO	Regultation of NLRP3 inflammasome complex assembly	*ATAT1, CD36, EIF2AK2, GBP5, MEFV, NLRP2P, PYDC1, PYDC2, SIRT2, TLR4, TLR6*	11
Genecards (https://www.genecards.org/)	NLRP3_Inflammasome_Lung cancer	*TP53, EGFR, NLRP3, IL1B, TNF, IL6, CASP8, TGFB1, IFNG, IL10, TLR4, BRCA1, CCL2, NLRP1, PRKN, TP73, TLR2, BIRC3, MEFV, IL1RN, CASP1, SERPINA1, CXCL8, PDCD1, BCL2L1, CASP3, BCL2, TLR9, CCL3, NFKB1, CD36, MAPK1, JUN, FGF2, MAPK8, CASP9, MTOR, P2RX7, IL18, CTLA4, BCL10, RELA, CREB1, HSP90AA1, TLR5, NLRC4, NOD2, HMOX1, AGT, CTSB, CFLAR, FLT3, IL1A, ITGB1, CCL5, CLEC7A, CD274, FN1, NAIP, BIRC2, CARD8, CD209, STAT1, BTK, APAF1, HLA-G, CASR, PTPN22, SYK, MALT1, PSTPIP1, IFNA1, IL17A, C3, IL12B, HMGB1, APOA1, S100B, CASP7, CXCL1, SNCA, PIK3CG, LGALS3, NRG1, C5, ATF6, PML, IL1R1, IFIH1, S100A8, MYD88, S100A9, CD40, LEP, NPPA, TLR6, OLR1, IFNB1, DHX9, CAMP, TLR3, SAA1, JAK1, MAPK14, CCL4, NOD1, HSP90AB1, IL6ST, IRF3, CARD11, CYBB, MIF, IRF7, TREX1, TRIM21, RAC1, CARD9, DDX58, UBC, TLR7, H2AFX, UMOD, IRAK3, XDH, LY96, IL18BP, AGER, TNFAIP3, CD14, IL23A, HSPA1A, IL33, ORMDL3, CYBA, NCF1, IRAK4, IRAK1, LBP, IL27, MYO1C, IL37, ATP6V0A2*	142
Gene removed[a]		*PRKN, BTK, CYBB, TLR7, IRAK1, LOC644816, IKBKG, IL8, TAB3, XIAP, NLRP2P*	11
Total genes			176

[a]Gene in X chromosome was removed; Keyword for MSigDB and Genecards: NLRP3; Inflammasome. Organism: Homo sapiens. Abbreviation: MSigDB = Molecular signatures database.

Table S3. Associations of the first 10 principal components and OS of NSCLC in the PLCO trial

PC*	Parameter Estimate	Standard Error	Chi-Square	*P*
PC1	4.821	1.353	12.697	< 0.001
PC2	-0.681	1.228	0.308	0.579
PC3	-3.054	0.949	10.351	0.001
PC4	-2.837	1.246	5.184	0.023
PC5	-0.910	1.232	0.546	0.460
PC6	1.355	1.252	1.172	0.279
PC7	-0.236	1.218	0.038	0.846
PC8	-1.684	1.322	1.622	0.203
PC9	-1.886	1.267	2.216	0.137
PC10	0.347	1.240	0.078	0.180

*The top four PCs were used for the adjustment for potential population stratification in the multivariate analysis. Abbreviations: OS, overall survival; NSCLC, non-small cell lung cancer; PLCO, the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; PC, principal component.

Table S4. Functional prediction analyses of the two validated SNPs

SNP	Gene	Chr.	Allele	MAF	Regulome DB Score	HaploReg v4.1					
						Promoter histone marks	Enhancer histone marks	DNase	Motifs changed	Selected eQTL hits	dbSNP function annotation
rs4733124	*NRG1*	8	T/C	0.19	4	--	BRST, SKIN	BRST[a], SKIN[b], BRST[c]	GZF1, Pax-4	--	intronic
rs11225211	*BIRC3*	11	G/A	0.16	--	--	BLD	--	4 altered motifs	2 hits	intronic

[a]Breast variant Human Mammary Epithelial Cells; [b]Foreskin Fibroblast Primary Cells; [c]Breast Myoepithelial Primary Cells. Abbreviation: SNP = Single nucleotide polymorphisms; MAF = Minor allele frequency.

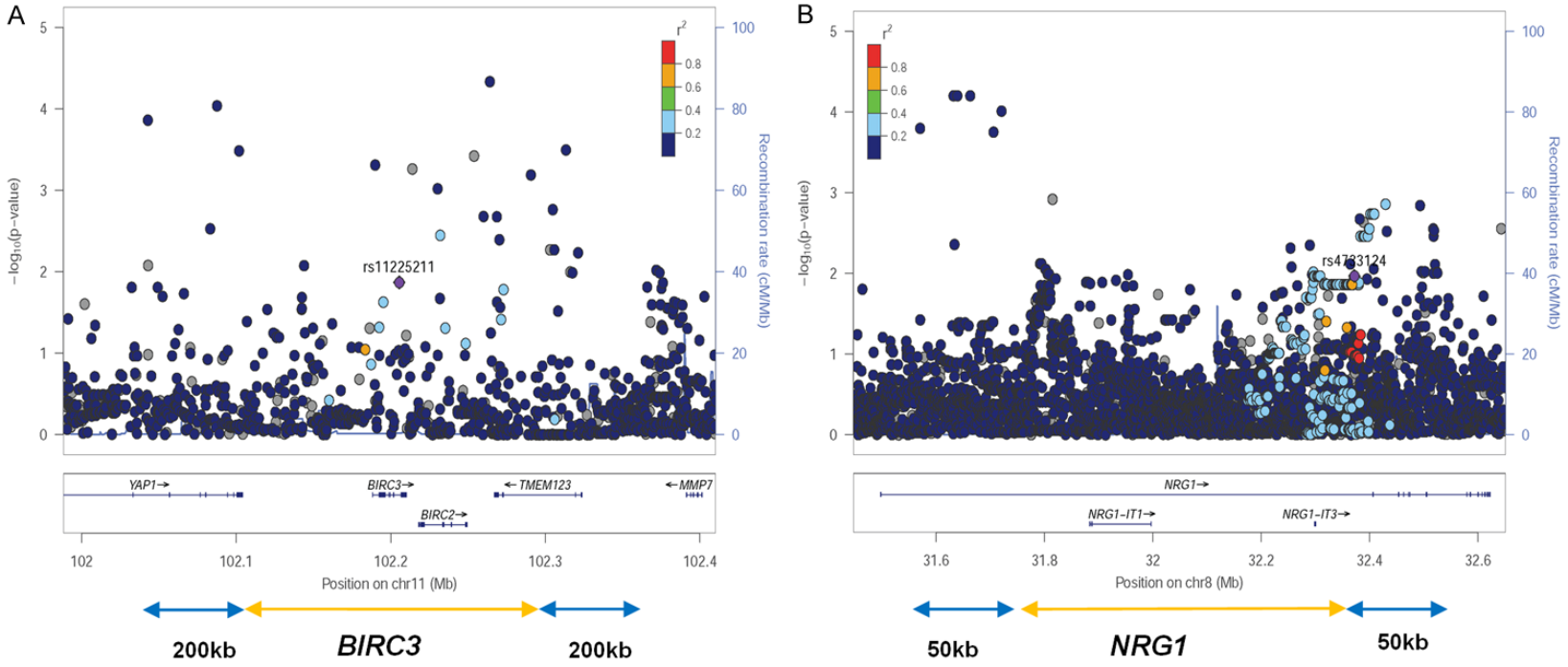

Figure S1. Regional association plots. The left-hand Y-axis shows the -log10 transformation of *P*-value of individual SNPs, which is plotted against the chromosomal base-pair position with an expansion of 500 KB in the flanks of the gene region. The right-hand Y-axis shows the recombination rate estimated for European populations from HapMap Data Rel 22/phase II. A. *XDH* rs141674738; B. *NRG1* rs4733124.

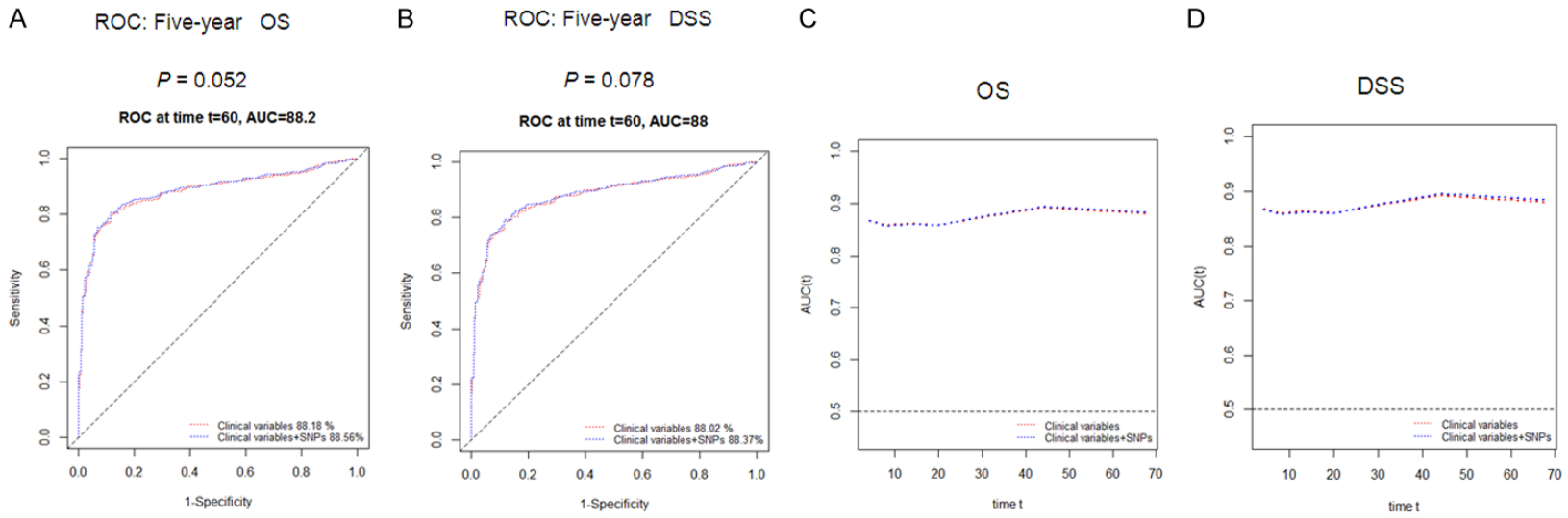

Figure S2. Receiver operating characteristic (ROC) curve and time-dependent area under the ROC curve (AUC) estimation for prediction of OS/DSS using the PLCO dataset. A. Five-year NSCLC overall survival rate, red line indicates clinical variables 88.18%; blue line indicates clinical variables + unfavorable genotypes 88.56%; B. Five-year NSCLC disease-specific survival rate, red line indicates clinical variables 88.02%; blue line indicates clinical variables + unfavorable genotypes 88.37%; C. Time-dependent AUC estimation (OS), based on age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery, principal components and the protective genotypes of the two SNPs. time t = time (months); D. Time-dependent AUC estimation (DSS), based on age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery, principal components and the protective genotypes of the two SNPs. time t = time (months).

Table S5. Stratified multivariate analyses for association between risk genotypes and OS/DSS in NSCLC patients in the PLCO trial

Characteristics	No. of 1-2 risk genotypes		No. of 0 risk genotype		OS Multivariate analysis[a]		P_{inter} [b]	DSS Multivariate analysis[a]		P_{inter} [b]
	All	Death (%)	All	Death (%)	HR (95% CI)	*P*		HR (95% CI)	*P*	
Age (years)										
≤ 71	503	334 (66.4)	131	65 (49.6)	0.80 (0.61-1.05)	0.108		0.92 (0.70-1.21)	0.551	
> 71	406	295 (72.7)	133	94 (70.7)	0.79 (0.62-1.00)	0.051	0.088	0.77 (0.60-1.00)	0.048	0.924
Sex										
Male	533	399 (74.9)	160	105 (65.6)	0.80 (0.64-0.99)	0.044		0.86 (0.68-1.08)	0.201	
Female	376	230 (61.2)	104	54 (51.9)	0.71 (0.53-0.97)	0.030	0.787	0.73 (0.53-1.00)	0.047	0.468
Smoking status										
Never	81	43 (53.1)	33	19 (57.6)	1.04 (0.56-1.95)	0.903		1.01 (0.54-1.89)	0.989	
Current	328	215 (65.6)	87	50 (57.5)	0.72 (0.53-1.00)	0.046		0.80 (0.58-1.12)	0.198	
Former	500	371 (74.2)	144	90 (62.50)	0.81 (0.64-1.02)	0.075	0.558	0.85 (0.66-1.08)	0.178	0.613
Histology										
Adenocarcinoma	429	271 (63.2)	145	75 (51.7)	0.62 (0.48-0.81)	0.0004		0.67 (0.51-0.88)	0.004	
Squamous	220	147 (66.8)	63	43 (68.3)	1.08 (0.76-1.54)	0.681		1.29 (0.89-1.87)	0.177	
Others	260	211 (81.2)	56	41 (73.2)	0.85 (0.60-1.20)	0.342	0.032	0.80 (0.55-1.16)	0.237	0.432
Tumor stage										
I-IIIA	504	260 (51.6)	149	54 (36.2)	0.68 (0.51-0.92)	0.012		0.75 (0.54-1.04)	0.089	
IIIB-IV	405	369 (91.1)	115	105 (91.3)	0.84 (0.67-1.05)	0.119	0.097	0.85 (0.68-1.07)	0.162	0.387
Chemotherapy										
No	490	300 (61.2)	147	66 (44.9)	0.61 (0.46-0.81)	0.0005		0.64 (0.47-0.86)	0.003	
Yes	419	329 (78.5)	117	93 (79.5)	1.01 (0.80-1.29)	0.911	0.007	1.05 (0.82-1.33)	0.719	0.010
Radiotherapy										
No	582	363 (62.4)	178	86 (48.3)	0.75 (0.59-0.95)	0.019		0.80 (0.62-1.04)	0.096	
Yes	327	266 (81.4)	86	73 (84.9)	0.88 (0.67-1.15)	0.334	0.436	0.90 (0.69-1.19)	0.457	0.564
Surgery										
No	497	439 (88.3)	137	125 (91.2)	0.95 (0.77-1.17)	0.614		0.97 (0.78-1.20)	0.763	
Yes	412	190 (46.1)	127	34 (26.8)	0.49 (0.34-0.72)	0.0002	0.002	0.55 (0.36-0.83)	0.004	0.012

[a]Adjusted for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2, PC3 and PC4; One observations missing of *NRG1* rs4733124, One observations missing of *BIRC3* rs11225211; Two observations missing of tumor stage and eight observations missing of chemotherapy/radiotherapy/surgery in PLCO dataset. [b]P_{inter}: *P*-value for interaction analysis between characteristic and number of protective genotypes; Abbreviation: OS = Overall survival; NSCLC = Non-small cell lung cancer; HR = Hazards ratio; 95% CI = 95% Confidence interval.

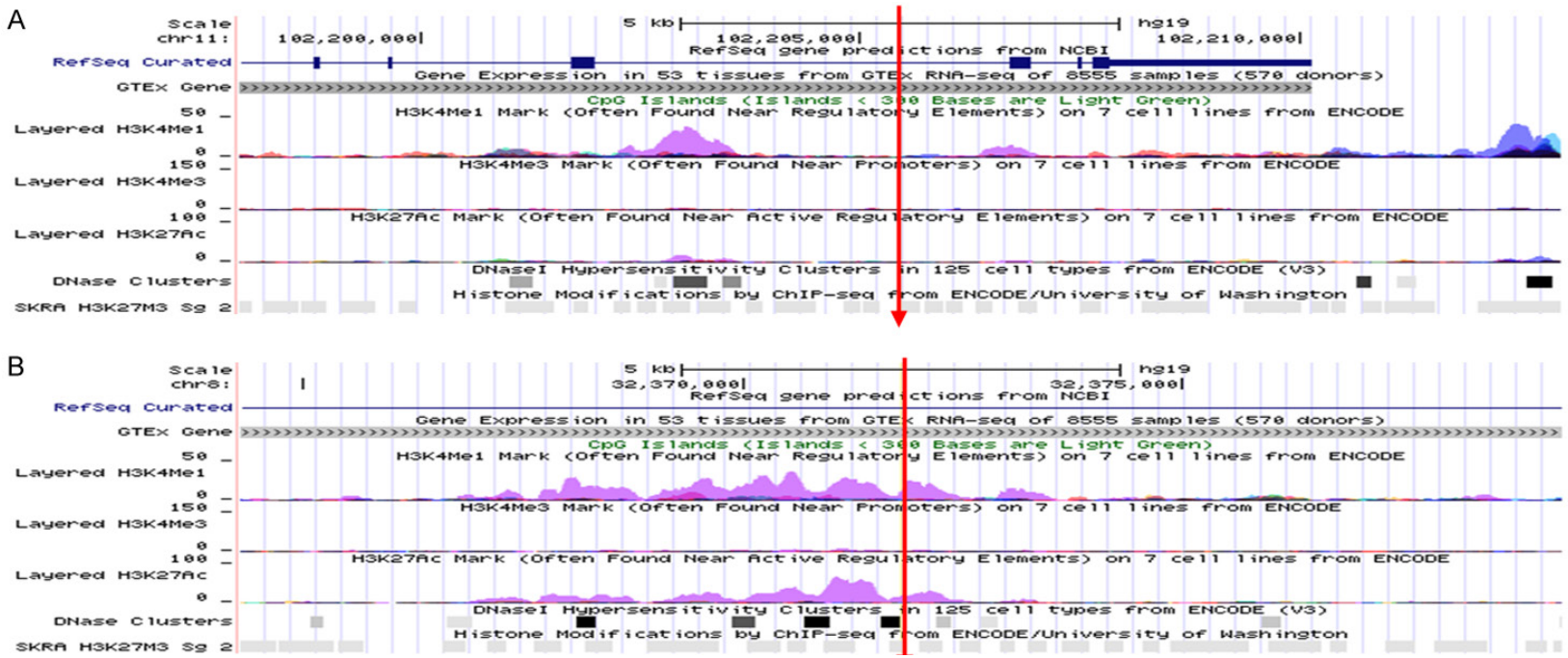

Figure S3. Functional prediction of SNPs in the ENCODE project. (A) Location and functional prediction of SNPs rs11225211, (B) Location and functional prediction of SNPs rs4733124.

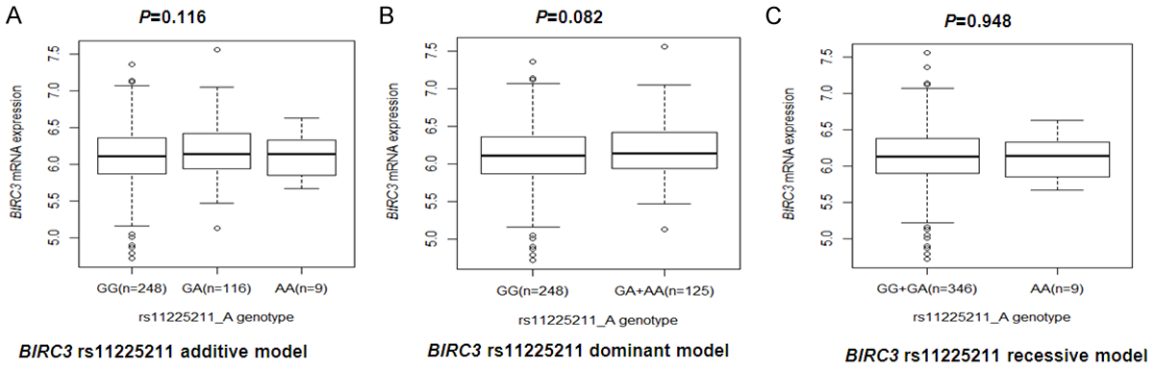

Figure S4. eQTL analysis of *BIRC3* rs11225211 genotypes and corresponding gene mRNA expression in 1000 Genomes project. All the data were from the 1000 Genome Project dataset. (A) rs11225211 additive model (P = 0.116); (B) rs11225211 dominant model (P = 0.082); and (C) rs11225211 recessive model (P = 0.948). Abbreviations: eQTL, expression quantitative trait loci.

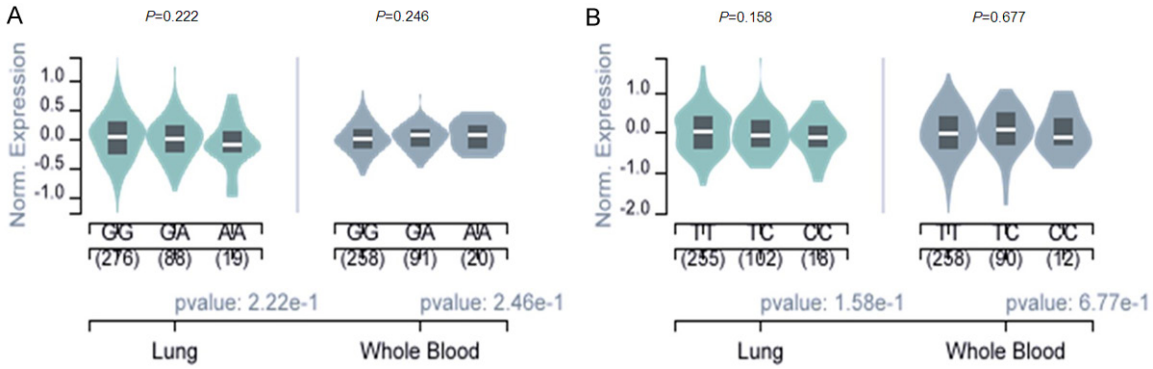

Figure S5. eQTL analysis of *BIRC3* rs11225211 and *NRG1* rs4733124 genotypes and corresponding gene mRNA expression in GTEx. All the data were from the GTEx dataset. A. rs11225211 in the lung tissues (P = 0.222) and whole blood (P = 0.246); B. rs4733124 in the lung tissues (P = 0.158) and whole blood (P = 0.677). Abbreviations: eQTL, expression quantitative trait loci.

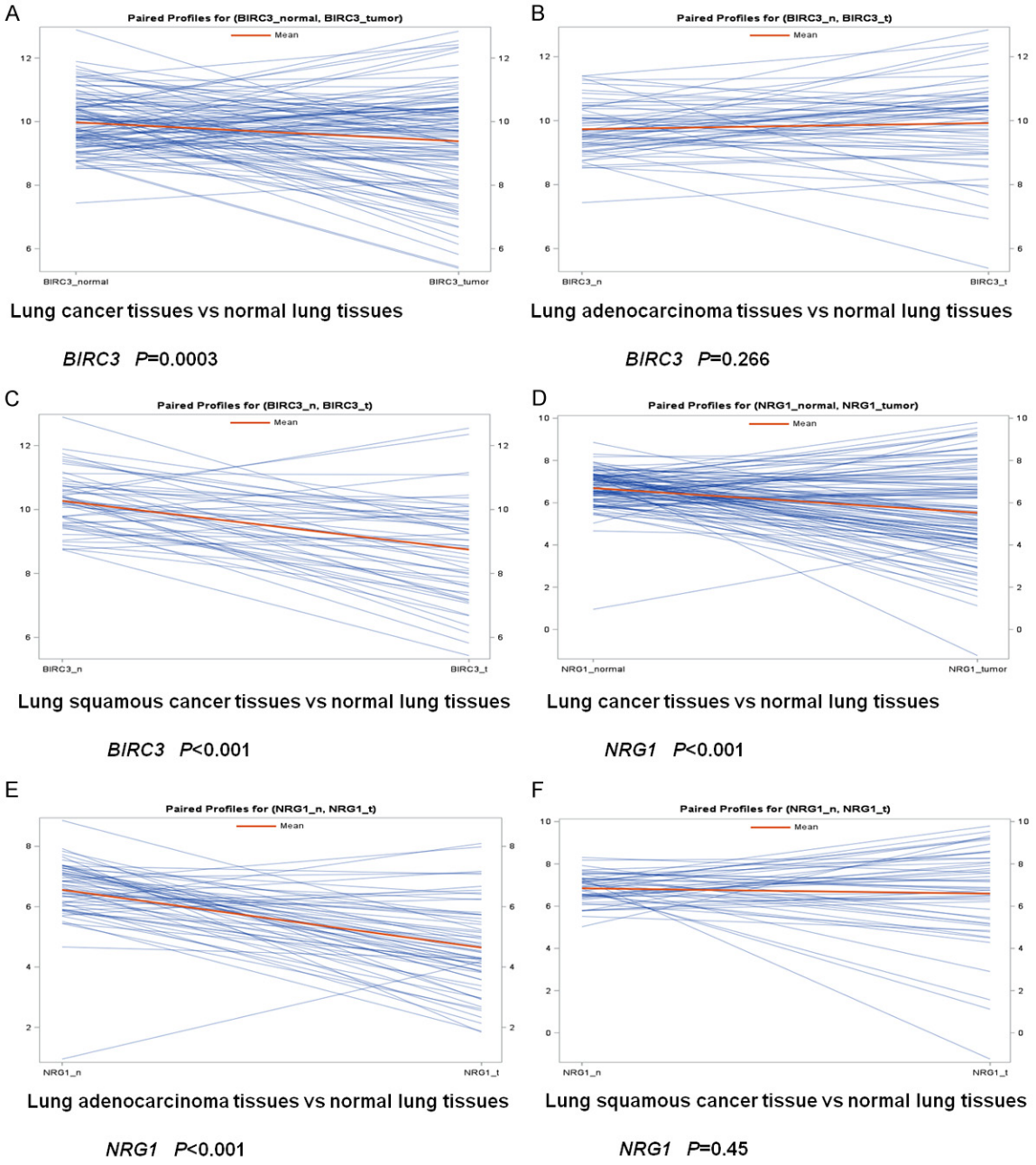

Figure S6. Comparison of mRNA expression levels of *BIRC3* and *NRG1* between lung cancer tissue and normal lung tissues in the TCGA dataset. A. The *BIRC3* mRNA expression levels in the lung cancer tissues were significantly lower than that in the normal lung tissues ($P < 0.001$); B. The *BIRC3* mRNA expression levels in the lung adenocarcinoma tissues were significantly lower than that in the normal lung tissues ($P < 0.001$); C. The *BIRC3* mRNA expression levels in the lung squamous tissues were significantly lower than that in the normal lung tissues ($P < 0.001$); D. The *NRG1* mRNA expression levels in the lung cancer tissues were significantly lower than that in the normal lung tissues ($P < 0.001$); E. The *NRG1* mRNA expression levels in the lung adenocarcinoma tissues were significantly lower than that in the normal lung tissues ($P < 0.001$); F. The *NRG1* mRNA expression levels in the lung squamous tissues were significantly lower than that in the normal lung tissues ($P = 0.45$); BIRC3_t = lung cancer tissues; BIRC3_n = adjacent normal lung tissues; NRG1_t = lung cancer tissues; NRG1_n = adjacent normal lung tissues.

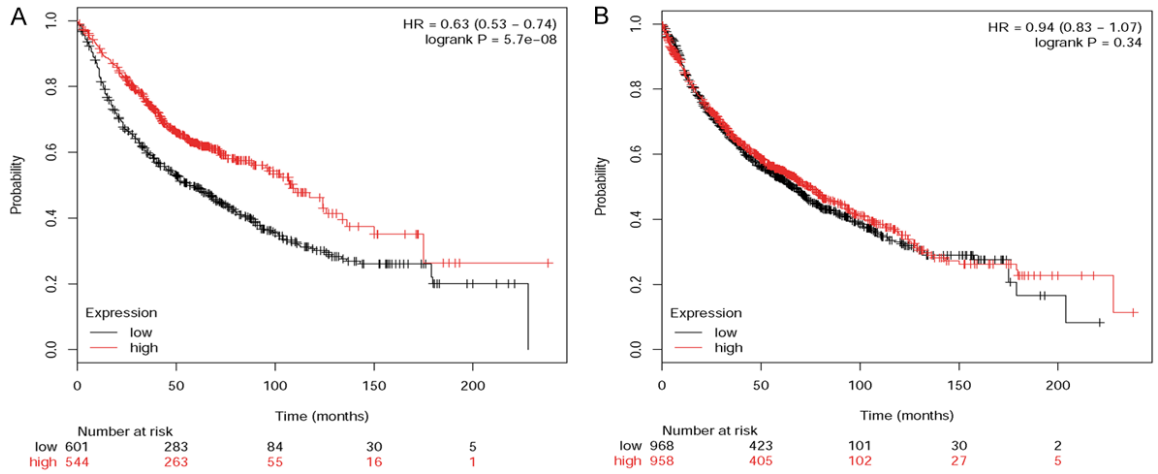

Figure S7. Kaplan-Meier analysis for patients with NSCLC by the two genes. Based on online survival analysis software (www.kmplot.com/analysis). A. High *BIRC3* expression were associated with poorer survival of NSCLC; B. *NRG1* expression were not associated with overall survival of NSCLC significantly.

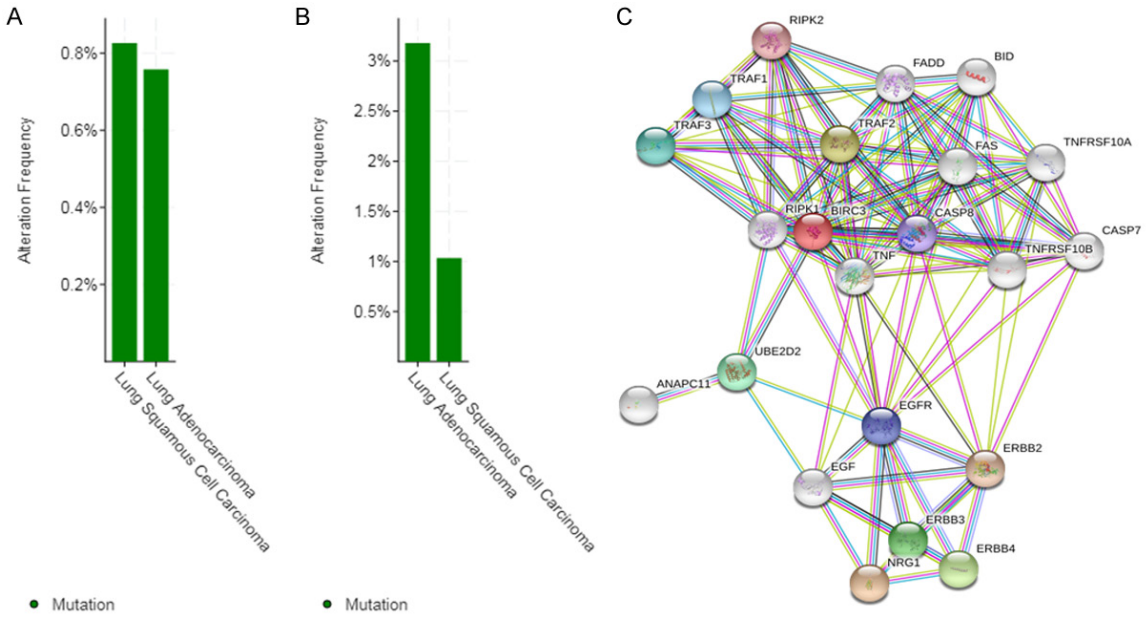

Figure S8. Mutation analysis of *BIRC3* and *NRG1* gene in non-small cell lung tumor tissues by using public available data in the database of the cBioportal for Cancer Genomics (http://www.cbioportal.org) and the network including the most frequently altered neighbor genes (https://string-db.org/cgi/network.pl?taskId = 36YKIt07ing0). A. *BIRC3* had low mutation frequency in LUSC and LUAD; B. *NRG1* had a relatively higher mutation frequency in LUSC and LUAD; C. *BIRC3* and *NRG1* were closely associated with altered neighbor genes.