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Novel genetic variants in *KIF16B* and *NEDD4L* in the endosome-related genes are associated with nonsmall cell lung cancer survival

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The endosome is a membrane-bound organ inside most eukaryotic cells, playing an important role in adaptive immunity by delivering endocytosed antigens to both MHC class I and II pathways. Here, by analyzing genotyping data from two published genome-wide association studies (GWASs), we evaluated associations between genetic variants in the endosome-related gene-set and survival of patients with nonsmall cell lung cancer (NSCLC). The discovery included 44,112 (3,478 genotyped and 40,634 imputed) single-nucleotide polymorphisms (SNPs) in 220 genes in a singlelocus analysis for their associations with survival of 1,185 NSCLC patients from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. After validation of the 821 survival-associated significant SNPs in additional 984 NSCLC patients from the Harvard Lung Cancer Susceptibility Study, 14 SNPs remained significant. The final multivariate stepwise Cox proportional hazards regression modeling of the PLCO dataset identified three potentially functional and independent SNPs (i.e., *KIF16B* rs1555195 C>T, *NEDD4L* rs11660748 A>G and rs73440898 A>G) with an adjusted hazards ratio (HR) of 0.86 (95% confidence interval [CI] = 0.79–0.94, $p = 0.0007$), 1.31 (1.16–1.47, $p = 6.0 \times 10^{-5}$) and 1.27 (1.12–1.44, $p = 0.0001$) for overall survival (OS), respectively. Combined analysis of the adverse genotypes of these three SNPs revealed a trend in the genotype-survival association ($p_{trend} < 0.0001$ for OS and $p_{trend} < 0.0001$ for disease-specific survival). Furthermore, the survival-associated *KIF16B* rs1555195T allele was significantly associated with decreased mRNA expression levels of *KIF16B* in both lung tissues and blood cells. Therefore, genetic variants of the endosome-related genes may be biomarker for NSCLC survival, possibly through modulating the expression of corresponding genes.

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Additional Supporting Information may be found in the online version of this article.

Key words: nonsmall cell lung cancer, endosome pathway, genome-wide association study, single-nucleotide polymorphism, survival

Abbreviations: APC: antigen-presenting cell; AUC: area under the receiver operating characteristic curve; BFDP: Bayesian false discovery probability; CI: confidence interval; DSS: disease-specific survival; EAF: effect allele frequency; eQTL: expression quantitative trait loci; FDR: false discovery rate; GWAS: Genome-Wide Association Study; HLCS: Harvard Lung Cancer Susceptibility; HR: hazards ratio; KIF16B: kinesin family member 16B; LD: linkage disequilibrium; NEDD4L: neural precursor cell expressed developmentally downregulated gene 4-like; NSCLC: nonsmall cell lung cancer; OS: overall survival; PLCO: the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ROC: receiver operating characteristic; SNPs: single nucleotide polymorphisms; TCGA: the Cancer Genome Atlas

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What's new?

Immunotherapy has become a key component of non-small cell lung cancer (NSCLC) treatment. However, not all patients benefit from immunotherapy, and there is increasing need to predict immunotherapy response in order to improve treatment efficacy and patient outcomes. In the present investigation of genes involved in endosome-related pathways, which are suspected of serving important immune-guided anti-tumor functions, three variants, located in the genes *KIF16B* and *NEDD4L*, were associated with NSCLC survival. The survival-associated variant in *KIF16B* was specifically associated with reduced *KIF16B* mRNA expression levels in lung tissue and blood cells, identifying it as a potentially useful biomarker for NSCLC survival.

Introduction

Lung cancer is one of the most common malignancies, with the highest cancer-related mortality worldwide.[1] In the United States, it is estimated that there will be approximately 228,150 new cases and 142,670 deaths from lung cancer in 2019.[2] The most common histological type of lung cancer is nonsmall cell lung cancer (NSCLC), accounting for approximately 85% of all lung cancer patients.[3] Although targeted therapy and immunotherapy have made remarkably improved outcomes in patients with NSCLC and facilitated the development of personalized cancer treatment,[4] the prognosis of NSCLC patients remain heterogeneous, suggesting that genetic factors may play an important role in treatment response and efficacy. Moreover, genetic factors, such as single nucleotide polymorphisms (SNPs), have been shown to have an effect on prognosis of lung cancer patients.[5–7] Therefore, identifying the roles of these genetic factors in development and progression of lung cancer may lead to better personalized management and treatment of NSCLC patients.

To date, few novel and functional SNPs have been identified to be associated with prognosis of lung cancer patients in genome-wide association studies (GWASs). This is because a hypothesis-free GWAS has always focused on the top or most-significant SNPs/genes with a stringent p value after correction of multiple tests for numerous SNPs, and most of the identified top SNPs lack of functional annotations. Recently, the biological pathway-based approach, as a hypothesis-driven method in the post GWAS era, has been applied to the reanalysis of published GWAS datasets to test the cumulative effect of potentially functional SNPs across multiple genes in the same biological pathway. As a result, much fewer SNPs in candidate genes of a significant biological pathway were included in the analysis to avoid the nuisance of multiple tests, which improves the study power to detect statistically significant and biologically important associations for additional functional analysis.

The endosome is a membrane-bound compartment inside eukaryotic cells and plays an important role in the endocytosis of exogenous antigens.[8] Classical antigen-presentation studies have showed that major histocompatibility complex (MHC) class I molecules present peptides derived from proteins synthesized within the cell, whereas MHC class II molecules present exogenous proteins from outside of the cell and in the microenvironment. Emerging evidence indicates that dendritic cells have a specialized capacity of processing exogenous antigens into the MHC class I pathway. This function, known as a cross-presentation, helps dendritic cells to activate the antitumor activity of cytotoxic T lymphocyte (CTL)[9]; thus, the endocytosed antigens from the outside are delivered to both MHC class I and MHC class II pathways through the functioning endosome.

In recent years, the role of the immune system in cancer development and progression has been recognized widely.[10–12] Immunotherapy is now established as the "fourth pillar" of cancer treatment alongside surgery, radiation, and chemotherapy.[13] Immunotherapy alone in patients with a high level of PD-L1 expression or in combination with chemotherapy is now the standard first-line therapy for patients with metastatic NSCLC.[14–16] For patients with stage-III NSCLC treated with chemotherapy and radiation, additional immunotherapy is the current standard of care. However, many patients do not benefit from immunotherapy, and there is an urgent need to identify tumor- and patient-related predictive biomarkers of immunotherapy. Such observations may be due to the killing effect of the immune system in tumor cells being highly dependent on the activation of CTL and CD4+ helper T cells (Th cells). CTL and Th cells are activated by the complex of internalized tumor antigens bonded to MHC class I and MHC class II protein molecules located on the surface of cancer cells and dendritic cells, respectively.[17] Therefore, we hypothesize that genetic variants of the genes involved in the endosome-related pathway in the process of antitumor immune response are associated with NSCLC survival. We tested this hypothesis by using genotyping data of two independently published NSCLC GWAS datasets.

Materials and Methods

Study populations

We used a GWAS dataset from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial as the discovery, which is a randomized control study conducted by the National Cancer Institute (NCI).[16] The PLCO trial included 77,500 men and 77,500 women aged 55–74 years, who were enrolled between the year of 1993 and 2011 from 10 medical centers in the United States; the participants were randomized to either the intervention arm that received a trial screening or the control arm that received standard care instead.[18] All participants provided their blood samples and personal information including smoking status, histologic diagnosis, tumor stage, treatment method and family history at enrollment and

were followed up for at least 13 years after the enrollment.[19] After excluding two individuals who had no follow-up information, a total of 1,185 NSCLC patients were eligible for survival analysis. Genomic DNA extracted from the whole blood samples of the participants were genotyped with Illumina HumanHap240Sv1.0 and HumanHap550v3.0 (dbGaP accession: phs000093.v2.p2 and phs000336.v1.p1).[20,21] The institutional review board of each participating institution had approved the PLCO trial and use of its data, and all the participants had provided a written informed consent permitting the research represented here.

We used another GWAS dataset of 984 histology-confirmed Caucasian NSCLC patients from the Harvard Lung Cancer Susceptibility (HLCS) Study which began in 1992 as the validation. In the HLCS study, the whole blood samples and personal information were collected after diagnosis, and DNA from the blood samples were extracted with Auto Pure Large Sample nucleic acid purification system (QIAGEN Company, Venlo, Limburg, Netherlands) and genotyped by using the Illumina Humanhap610-Quad array. The genotyped data were used for imputation with the Mach3 software based on the sequencing data from the 1000 Genomes Project.[22]

The use of these two GWAS datasets was approved by both the Internal Review Board of Duke University School of Medicine (#Pro00054575) and the dbGAP database administration (#6404). The comparison of the characteristics between the PLCO trial (n = 1,185) and the HLCS study (n = 984) is presented in Supporting Information Table S1.

Gene and SNP selection

The genes involved in the endosome-related pathway were selected by the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp) with the keyword "endosome." After the removal of 44 duplicated genes and six genes in the X chromosome, 220 genes remained as candidate genes for further analysis (Supporting Information Table S2). These genes were used for imputation with IMPUTE2 and the 1000 Genomes Project data (phase 3), in which SNPs within their $\pm$2 kb flanking regions (SNPs located in the 2-kb upstream and downstream of a gene were considered having potential effects on gene transcription) were extracted with the following criteria: an imputation info score ≥0.8 (Supporting Information Fig. S1), a genotyping rate ≥95%, a minor allelic frequency (MAF) ≥ 5%, and Hardy–Weinberg equilibrium (HWE) $\geq 1 \times 10^{-5}$. As a result, 3,478 SNPs were genotyped in the PLCO GWAS dataset (dbGaP accession: phs000093.v2.p2 and phs000336.v1.p1) and additional 40,634 SNPs were imputed.

Statistical analyses

We used multivariate Cox proportional hazards regression analysis to assess the association between each of the selected SNPs and NSCLC survival (in an additive genetic model) in the PLCO dataset, with adjustment for age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery and the first four principal components by using the GenABEL package of R software.[23] We used the Bayesian false discovery probability (BFDP) method with a cut-off value of 0.80 for multiple testing correction to lower the probability of potentially false positive results.[24] We assigned a prior probability of 0.05 to detect an upper bound of a 95% CI for HR of 3.0 for an association with variant genotypes or minor alleles of the SNPs with $p < 0.05$. After that, we validated these chosen SNPs by using the HLCS GWAS dataset. Next, we performed an inverse variance weighted meta-analysis to combine the results of both discovery and validation datasets. In the analysis, Cochran's Q-test and the heterogeneity statistic (I^2) were performed to assess the interstudy heterogeneity. If no heterogeneity was observed between the two datasets ($p_{het} > 0.10$ and $I^2 < 50\%$), a fixed-effects model was implemented. Otherwise, a random-effects model was applied. Furthermore, a multivariate stepwise Cox regression model including the first four principal components of the PLCO dataset, available demographic and clinical variables was performed to identify novel and independent SNP. After that these potential independent SNPs were adjusted for previously published SNPs.

Then, we used the combined genotypes to evaluate the cumulative effects of the identified SNPs and the Kaplan–Meier curve to estimate the 10-year survival probability associated with the genotypes. We also assessed possible interactions with a Cochron's Q-test between subgroups in the stratified analysis, and $p < 0.05$ was considered statistically significant. We then performed the receiver operating characteristic (ROC) curve and time-dependent area under the curve (AUC) with timeROC package of R software (version 3.5.0) to illustrate the prediction accuracy of the model integrating the effects of clinical and genetic variables on NSCLC survival.[25] To evaluate the correlations between SNPs and the corresponding mRNA expression levels, we performed the expression quantitative trait loci (eQTL) analyses with linear regression models using the R software. The mRNA expression data of genes were obtained from two sources: 373 European individuals included in the 1,000 Genomes Project and 369 whole blood samples and 383 normal lung tissue included in the genotype-tissue expression (GTEx) project.[26,27] Then, bioinformatics functional prediction for the identified SNPs was performed with SNPinfo,[28] RegulomeDB[29] (http://www.regulomedb.org) and HaploReg[30] (http://archive.broadinstitute.org/mammals/haploreg/haploreg.php). Finally, the differences in mRNA expression levels were examined in 109 pairs of lung cancer tissues and adjacent normal tissues from the Cancer Genome Atlas (TCGA) dataset by using a paired t-test. Kaplan–Meier survival analysis was performed to assess the association between the mRNA expression level and survival probability (http://kmplot.com/analysis/index.php?p=service&cancer=lung).[31] All statistical analyses were performed with the SAS software (version 9.4; SAS Institute, Cary, NC) unless otherwise indicated.

Data availability

The datasets used for the analyses described in the present study were obtained from dbGaP (http://www.ncbi.nlm.nih.gov/gap) through dbGaP accession number phs000336.v1.p1 and phs000093.v2.p2.

Results

Associations between SNPs in the endosome-related pathway genes and NSCLC survival

The workflow chart of the present study is shown in Figure 1. The basic characteristics of 1,185 NSCLC patients from the PLCO trial and 984 NSCLC patients from the HLCS study have been described elsewhere.[32] In the discovery PLCO genotype dataset, a single-locus multivariate Cox regression analysis was performed for the selected 44,112 SNPs with adjustment for age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy, surgery and the first four principal components (Supporting Information Table S3). For multiple testing correction, none of the SNPs passed Bonferroni Correction ($p > 0.05$) or false discovery rate (>0.20). This is likely due to the high LD among the SNPs generated by imputation. Besides, our purpose of using this prescreening was to identify candidates for functional SNPs for further analysis. Therefore, we used the BFDP method as recommended by the authors of the method.[24] After multiple testing correction by BFDP ≤0.80, 821 SNPs were identified to be significantly associated with NSCLC OS ($p < 0.05$). All the significant SNPs identified from the PLCO trial were further validated by the HLCS genotype dataset, 14 SNPs remained significant. Subsequently, we performed the meta-analysis of these 14 newly identified SNPs in both PLCO and HLCS datasets and found that a better survival was associated with the *KIF16B* rs1555195 C>T ($p = 0.0007$), but a poor survival was associated with the other 13 SNPs, without heterogeneity between the two studies (Table 1).

Independent SNPs associated with NSCLC survival in the PLCO dataset

To identify independence of the 14 SNPs, we performed a multivariate stepwise Cox regression analysis with adjustment for demographic and clinical variables and the top four principal components in the PLCO dataset, and we used the Schwarz Bayesian Criterion (SBC)[33] for model selection to identify independent SNPs associated with NSCLC survival. When all the 14 validated SNPs were added to the model, only three SNPs were left and significantly associated with survival. After that, in the same model, we also adjusted for other 15 previously reported significant SNPs, and these three SNPs remained significantly associated with survival (Table 2). The results of selected SNPs are summarized in a Manhattan plot (Supporting Information Fig. S2) and the regional association plot of each of these three SNPs is shown in Supporting Information Figure S3.

In the PLCO dataset with available covariates for complete adjustment, patients with the rs1555195T allele had a decreased risk of death ($p_{trend} = 0.003$ for OS and $p_{trend} = 0.003$ for disease-specific survival [DSS]), while patients with both rs11660748G and rs73440898G alleles had an increased risk of death ($p_{trend} < 0.0001$ for OS and $p_{trend} = 0.0003$ for DSS; $p_{trend} = 0.001$ for OS and $p_{trend} = 0.015$ for DSS; respectively; Table 3). Compared to the reference genotype in a dominant genetic model, *KIF16B* rs1555195 CT + TT genotypes were associated with a better survival (HR = 0.81, 95% CI = 0.71–0.94, $p = 0.005$ for OS and HR = 0.80, 95% CI = 0.69–0.94, $p = 0.005$ for DSS), while *NEDD4L*

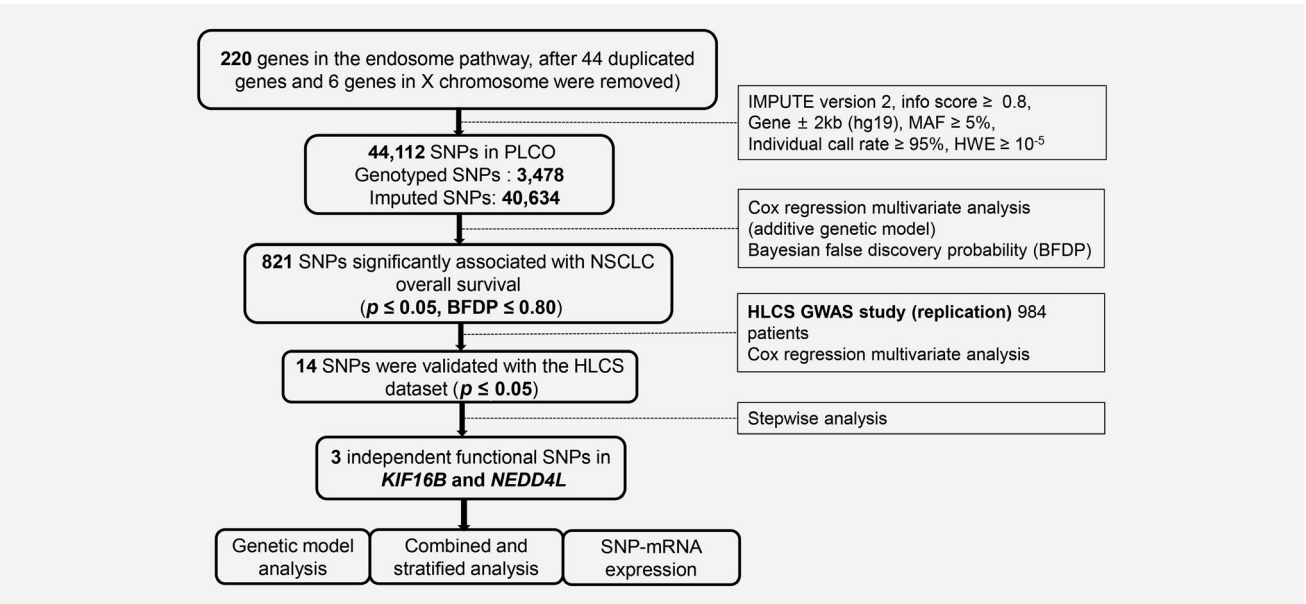

Figure 1. The flowchart of the present study. Abbreviations: SNP, single-nucleotidepolymorphism; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screeningtrial; NSCLC, non-smallcell lung cancer; HLCS, Harvard lung cancer susceptibility study; KIF16B, kinesin family member 16B; NEDD4L, neural precursor cell expresseddevelopmentally downregulated gene 4-like.

Table 1. The 14 validated and survival-associated significant SNPs in two previously published NSCLC GWAS datasets

			PLCO (n = 1,185)					HLCS (n = 984)			Combined-analysis			
SNP	**Allele[1]**	**Gene**	**FDR**	**BFDP**	**EAF**	**HR (95% CI)[2]**	p^2	**EAF**	**HR (95% CI)[3]**	p^3	$p_{het}{}^4$	I^2	**HR (95% CI)[5]**	p^5
rs1555195	C>T	*KIF16B*	0.29	0.66	0.26	0.84 (0.74–0.94)	0.003	0.28	0.89 (0.79–1.00)	0.049	0.520	0	0.86 (0.79–0.94)	0.0007
rs71355689	T>C	*NEDD4L*	0.29	0.23	0.12	1.32 (1.14–1.54)	0.0003	0.12	1.38 (1.16–1.63)	0.0003	0.722	0	1.34 (1.20–1.50)	2.9E−7
rs11660199	G>A	*NEDD4L*	0.29	0.17	0.12	1.33 (1.15–1.55)	0.0002	0.12	1.36 (1.15–1.61)	0.0003	0.834	0	1.34 (1.20–1.50)	2.2E−7
rs11665627	T>C	*NEDD4L*	0.29	0.33	0.18	1.25 (1.09–1.42)	0.0009	0.18	1.25 (1.08–1.44)	0.002	0.991	0	1.25 (1.13–1.38)	7.1E−6
rs9957736	G>A	*NEDD4L*	0.37	0.75	0.17	1.21 (1.05–1.38)	0.007	0.17	1.24 (1.07–1.44)	0.004	0.792	0	1.23 (1.11–1.35)	7.2E−5
rs60605848	C>G	*NEDD4L*	0.29	0.56	0.17	1.23 (1.08–1.40)	0.002	0.18	1.23 (1.06–1.42)	0.005	0.999	0	1.23 (1.12–1.35)	2.5E−5
rs12960902	T>A	*NEDD4L*	0.29	0.66	0.17	1.22 (1.07–1.39)	0.003	0.17	1.23 (1.06–1.42)	0.005	0.951	0	1.22 (1.11–1.35)	4.6E−5
rs60418930	G>A	*NEDD4L*	0.29	0.56	0.17	1.23 (1.08–1.40)	0.002	0.17	1.22 (1.06–1.41)	0.006	0.961	0	1.23 (1.11–1.35)	3.1E−5
rs59402591	A>G	*NEDD4L*	0.29	0.56	0.17	1.23 (1.08–1.40)	0.002	0.17	1.22 (1.06–1.41)	0.006	0.960	0	1.23 (1.11–1.35)	3.2E−5
rs17064520	C>T	*NEDD4L*	0.29	0.56	0.17	1.23 (1.08–1.40)	0.002	0.17	1.22 (1.06–1.41)	0.006	0.960	0	1.23 (1.11–1.35)	3.2E−5
rs11660748	A>G	*NEDD4L*	0.29	0.05	0.11	1.36 (1.17–1.58)	8.2E-5	0.11	1.23 (1.03–1.48)	0.024	0.409	0	1.31 (1.16–1.47)	6.0E−5
rs73440898	A>G	*NEDD4L*	0.29	0.50	0.10	1.31 (1.11–1.55)	0.002	0.11	1.23 (1.02–1.47)	0.030	0.596	0	1.27 (1.12–1.44)	0.0001
rs7576673	A>G	*ERBB4*	0.34	0.75	0.18	1.21 (1.06–1.38)	0.006	0.19	1.17 (1.02–1.34)	0.026	0.734	0	1.19 (1.08–1.31)	0.0003
rs10932385	C>G	*ERBB4*	0.32	0.75	0.18	1.21 (1.06–1.38)	0.005	0.18	1.17 (1.02–1.34)	0.027	0.728	0	1.19 (1.08–1.31)	0.0003

[1]Reference>effect allele.
[2]Obtained from an additive genetic model with adjustment for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2, PC3 and PC4.
[3]Obtained from an additive genetic model with adjustment for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2 and PC3.
[4]p_{het}: p value for heterogeneity by Cochrane's Q test.
[5]Meta-analysis in the fixed-effects model.
Abbreviations: BFDP, Bayesian false discovery probability; CI, confidence interval; EAF, effect allele frequency; FDR, false discovery rate; GWAS, genome-wide association study; HLCS, Harvard Lung Cancer Susceptibility; HR, hazards ratio; NSCLC, nonsmall cell lung cancer; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screening trial; SNP, single nucleotide polymorphism.

Table 2. Three independent SNPs from multivariate Cox regression analysis of selected functional variables and previous published SNPs in the PLCO GWAS dataset

Variables	Category	Frequency	HR (95% CI)[1]	p^1	HR (95% CI)[2]	p^2
Age	Continuous	1,185	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	0.78 (0.67–0.91)	0.001	0.78 (0.66–0.91)	0.002
Smoking status	Never	115	1.00		1.00	
	Current	423	1.69 (1.26–2.26)	0.0004	1.94 (1.44–2.62)	‹0.0001
	Former	647	1.65 (1.26–2.18)	0.0003	1.89 (1.42–2.51)	‹0.0001
Histology	AD	577	1.00		1.00	
	SC	285	1.14 (0.95–1.38)	0.163	1.20 (0.99–1.46)	0.064
	Others	323	1.32 (1.11–1.56)	0.002	1.37 (1.14–1.63)	0.0006
Stage	I–IIIA	655	1.00		1.00	
	IIIB–IV	528	2.82 (2.32–3.43)	‹0.0001	3.00 (2.46–3.66)	‹0.0001
Chemotherapy	No	639	1.00		1.00	
	Yes	538	0.58 (0.49–0.69)	‹0.0001	0.58 (0.48–0.70)	‹0.0001
Radiotherapy	No	762	1.00		1.00	
	Yes	415	0.97 (0.82–1.14)	0.724	0.97 (0.82–1.15)	0.738
Surgery	No	637	1.00		1.00	
	Yes	540	0.21 (0.16–0.27)	‹0.0001	0.19 (0.15–0.25)	‹0.0001
KIF16B rs1555195 C›T	CC/CT/TT	640/466/79	0.83 (0.74–0.94)	0.002	0.86 (0.76–0.97)	0.017
NEDD4L rs11660748 A›G	AA/AG/GG	937/229/19	1.34 (1.15–1.56)	0.0002	1.26 (1.07–1.47)	0.005
NEDD4L rs73440898 A›G	AA/AG/GG	959/216/8	1.28 (1.08–1.51)	0.004	1.28 (1.07–1.52)	0.006

[1]Stepwise analysis included age, sex, smoking status, tumor stage, histology, chemotherapy, radiotherapy, surgery, PC1, PC2, PC3, PC4 and SNPs.
[2]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: CI, confidence interval; GWAS, genome-wide association study; HR, hazards ratio; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screening trial; SNP, single-nucleotide polymorphism.

rs11660748 AG + GG genotypes and rs73440898 AG + GG were associated with a worse survival (HR = 1.37, 95% CI = 1.16–1.63 and $p = 0.0003$ for OS and HR = 1.37, 95% CI = 1.14–1.65 and $p = 0.0006$ for DSS; and HR = 1.32, 95% CI = 1.11–1.58, $p = 0.002$ for OS and HR = 1.25, 95% CI = 1.03–1.51, $p = 0.022$ for DSS; respectively; Table 3).

Haplotype analysis of two SNPs in *NEDD4L* and NSCLC survival in PLCO

Since rs73440898 and rs11660748 were both in *NEDD4L*, we performed haplotype analysis to assess the association between different haplotypes and survival. As shown in Table 4, there were four *NEDD4L* haplotypes (A–A, A–G, G–A and G–G) of the rs73440898 and rs11660748 loci, with a frequency of 82.40%, 8.18%, 7.16% and 2.26%, respectively, and a significant NSCLC death-risk was associated with the G haplotypes (HR = 1.32, 1.27 and 1.46 for OS, respectively; and HR = 1.32. 1.20 and 1.43 for DSS, respectively, compared to the A-A haplotype) in a G-allele dose-dependent manner ($p_{trend} < 0.0001$ for OS and $p_{trend} = 0.001$ for DSS). In the dichotomized analysis, patients who had 1–3 death-risk alleles had an unfavorable survival, compared to those with the A–A haplotype (HR = 1.32, 95% CI = 1.16–1.50; $p < 0.0001$ for OS and HR = 1.28, 95% CI = 1.12–1.47; $p = 0.0004$ for DSS). These results are consistent with the observed death-risk associated with the *NEDD4L* rs11660748G and rs73440898G alleles.

Combined effects of the three independent SNPs in the PLCO dataset

We used the PLCO dataset to assess the combined effect of the three independent SNPs on NSCLC OS and DSS. First, we combined the unfavorable genotypes (i.e., *KIF16B* rs1555195 CC, *NEDD4L* rs73440898 AG + GG, *NEDD4L* rs11660748 AG + GG) into a genetic score as the number of unfavorable genotypes (NUGs). As shown in Table 3, the increased genetic score of the NUGs was associated with a worse effect on death in the multivariate analysis in the PLCO dataset ($p_{trend} < 0.0001$ for OS and $p_{trend} < 0.0001$ for DSS). Then, we dichotomized all the patients into a low-unfavorable group (0–1 scores) and a high-unfavorable group (2–3 scores). Compared to the low-unfavorable score group, patients in the high-unfavorable score group had a significantly worse survival (HR = 1.58, 95% CI = 1.33–1.87, $p < 0.0001$ for OS and HR = 1.48, 95% CI = 1.23–1.78, $p < 0.0001$ for DSS). Kaplan–Meier survival curves were presented to depict the associations between unfavorable genotypes and NSCLC OS and DSS (Figs. 2*a*–2*d*).

Table 3. Associations between three significantly independent SNPs and 10-year survival of NSCLC in the PLCO Trial

		OS[6]			DSS[6]		
Genotype	**Frequency**	**Death (%)**	**HR (95% CI)**	***p***	**Death (%)**	**HR (95% CI)**	***p***
KIF16B rs1555195 C>T[1]							
CC	636	432 (67.92)	1.00		391 (61.48)	1.00	
CT	460	306 (66.52)	0.83 (0.72–0.97)	0.016	277 (60.22)	0.82 (0.70–0.96)	0.016
TT	79	46 (58.23)	0.71 (0.53–0.97)	0.031	40 (50.63)	0.70 (0.50–0.96)	0.029
Trend test				0.003			0.003
Dominant							
CC	636	432 (67.92)	1.00		391 (61.48)	1.00	
CT + TT	539	352 (65.31)	0.81 (0.71–0.94)	0.005	317 (58.81)	0.80 (0.69–0.94)	0.005
NEDD4L rs11660748 A>G[2]							
AA	929	609 (65.55)	1.00		550 (59.20)	1.00	
AG	227	159 (70.04)	1.33 (1.11–1.60)	0.002	144 (63.44)	1.34 (1.11–1.61)	0.003
GG	19	16 (84.21)	1.98 (1.20–3.29)	0.008	14 (73.68)	1.89 (1.10–3.24)	0.021
Trend test				<0.0001			0.0003
Dominant							
AA	929	609 (65.55)	1.00		550 (59.20)	1.00	
AG + GG	246	175 (71.14)	1.37 (1.16–1.63)	0.0003	158 (64.23)	1.37 (1.14–1.65)	0.0006
NEDD4L rs73440898 A>G[3]							
AA	952	628 (65.97)	1.00		571 (59.98)	1.00	
AG	213	149 (69.95)	1.30 (1.09–1.56)	0.004	131 (61.50)	1.23 (1.02–1.49)	0.034
GG	8	6 (75.00)	2.05 (0.91–4.61)	0.083	5 (62.50)	1.91 (0.79–4.63)	0.154
Trend test				0.001			0.015
Dominant							
AA	952	628 (65.97)	1.00		571 (59.98)	1.00	
AG + GG	221	155 (70.14)	1.32 (1.11–1.58)	0.002	136 (61.54)	1.25 (1.03–1.51)	0.022
NUG[4,5]							
0	344	223 (64.83)	1.00		199 (57.85)	1.00	
1	591	387 (65.48)	1.19 (1.01–1.41)	0.099	357 (60.41)	1.24 (1.04–1.49)	0.016
2	203	144 (70.94)	1.71 (1.38–2.12)	<0.0001	125 (61.58)	1.64 (1.30–2.06)	<0.0001
3	35	29 (82.86)	2.07 (1.40–3.06)	0.0003	26 (74.29)	2.05 (1.35–3.09)	0.0007
Trend test				<0.0001			<0.0001
0–1	935	610 (65.24)	1.00		556 (59.47)	1.00	
2–3	238	173 (72.69)	1.58 (1.33–1.87)	<0.0001	151 (63.45)	1.48 (1.23–1.78)	<0.0001

[1]Ten missing date were excluded.
[2]Ten missing date were excluded.
[3]Twelve missing date were excluded.
[4]Twelve missing date were excluded.
[5]Unfavorable genotypes were *KIF16B* rs1555195 CC, *NEDD4L* rs73440898 AG + GG, *NEDD4L* rs11660748 AG + GG.
[6]Adjusted for age, sex, smoking status, histology, tumor stage, chemotherapy, surgery and principal components.
Abbreviations: CI, confidence interval; DSS, disease-specific survival; HR, hazards ratio; NSCLC, nonsmall cell lung cancer; NUG: number of unfavorable genotypes; OS, overall survival; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screening trial; SNP, single nucleotide polymorphism.

Stratified analysis for associations between NUGs and NSCLC survival

We performed stratified analysis to evaluate whether the combined effect of unfavorable genotypes on NSCLC OS and DSS was modified by age, sex, smoking status, histology, tumor stage, chemotherapy, radiotherapy and surgery in the PLCO dataset. As a result, no significant interactions were found when it was performed on both NSCLC OS and DSS ($p_{inter} > 0.05$ for all factors, Supporting Information Table S4).

The ROC curves and time-dependent AUC

We assessed the prediction performance of the three SNPs with time-dependent AUC and ROC curves for five-year and one-year survival in the PLCO dataset. Compared to the covariates model, the time-dependent AUC plot with the independent unfavorable genotypes did not improve prediction performance for five-year survival (Supporting Information Fig. S4). However, when we performed the time-dependent AUC and ROC curves for one-year survival in the PLCO dataset, the prediction performance of the

Table 4. Haplotype analysis of association between two SNPs in *NEDD4L* and NSCLC 10-year survival in PLCO

	Haplotype frequency		Multivariate analysis[3] for OS		Multivariate analysis[3] for DSS	
Haplotypes[1,2]	*n*	%	HR (95% CI)	*p*	HR (95% CI)	*p*
A–A	1,933	82.40	1.00		1.00	
A–G	192	8.18	1.32 (1.10–1.58)	0.002	1.32 (1.09–1.59)	0.004
G–A	168	7.16	1.27 (1.05–1.54)	0.013	1.20 (0.98–1.47)	0.081
G–G	53	2.26	1.46 (1.06–2.02)	0.022	1.43 (1.01–2.01)	0.043
Trend test				‹0.0001		0.001
A–A	1,933	82.40	1.00		1.00	
(A–G) + (G–A) + (G–G)	413	17.60	1.32 (1.16–1.50)	‹0.0001	1.28 (1.12–1.47)	0.0004

[1]The alleles in the haplotype were ranked in the SNP order of rs rs73440898A›G and rs11660748A›G.
[2]Twelve missing date were excluded.
[3]Adjusted for age, sex, stage, histology, smoking status, chemotherapy, radiotherapy, surgery, PC1, PC2, PC3 and PC4;
Abbreviations: CI, confidence interval; DSS, disease-specific survival; HR, hazards ratio; NSCLC, non-small cell lung cancer; OS, overall survival; PLCO, the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; SNP, single nucleotide polymorphism.

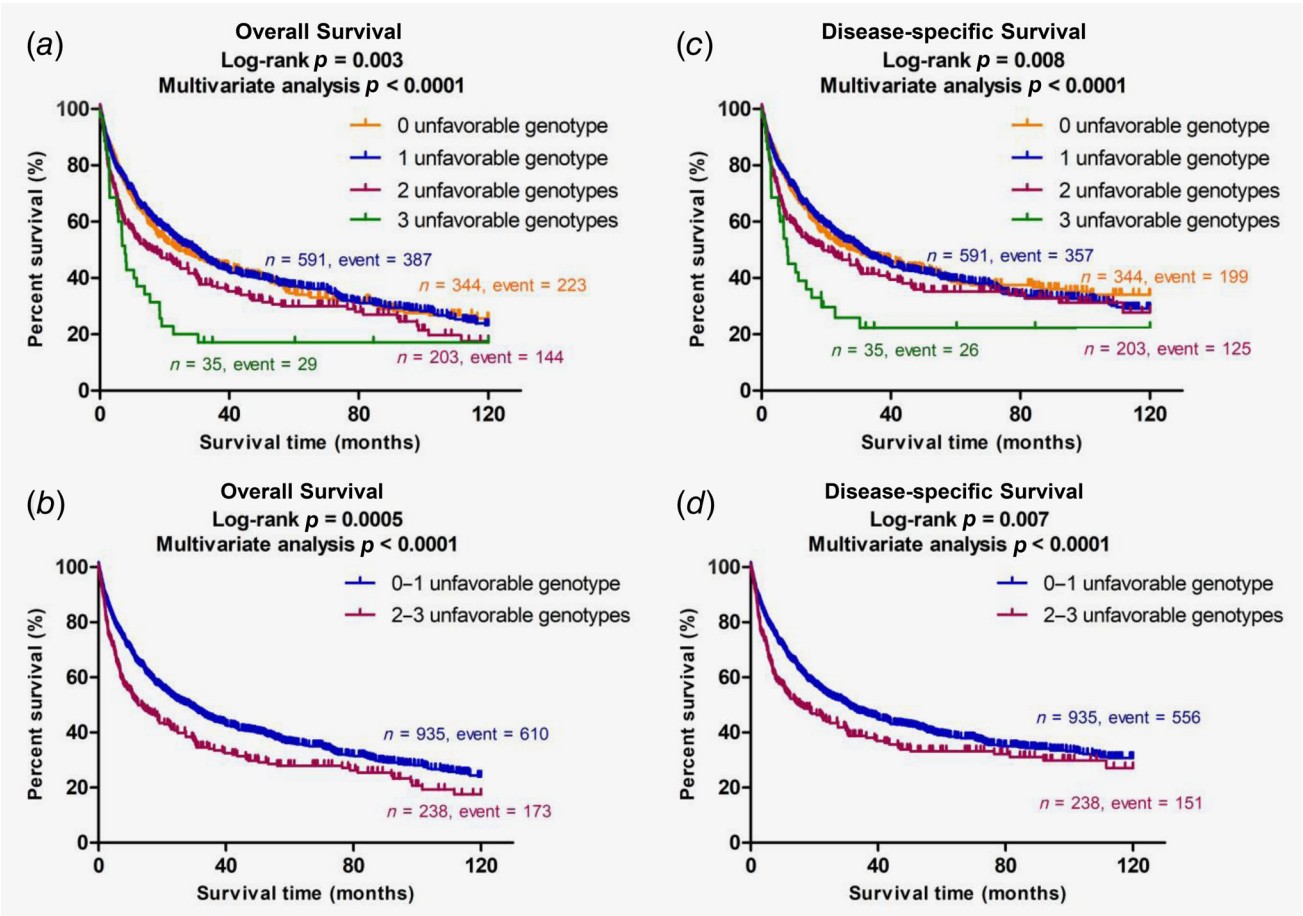

Figure 2. Prediction of 10-year survival with combined unfavorable genotypes and eQTL for KIF16B rs1555195. Kaplan–Meier survival curves for the 10-year OS in the PLCO dataset for (a) the combined unfavorable genotypes and (b) dichotomized groups of the NUGs; Kaplan–Meier survival curves for the 10-year DSS in the PLCO dataset for (c) the combined unfavorable genotypes and (d) dichotomized groups of the NUGs. One-year NSCLC OS prediction by ROC curve (e) and one-year NSCLC DSS prediction by ROC curve (f). The correlation of rs1555195 genotypes and corresponding mRNA expression levels in the GTEx Project was significant in(g) normal lung tissue ($P = 0.0009$) and (h) whole blood cells ($P = 0.005$). Abbreviations: eQTL, expression quantitative trait loci; OS, overall survival; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening trial; NUG, number of unfavorable genotypes; DSS, disease-specific survival; KIF16B, kinesin family member 16B; ROC, receiver operating characteristic curve. [Color figure can be viewed at wileyonlinelibrary.com]

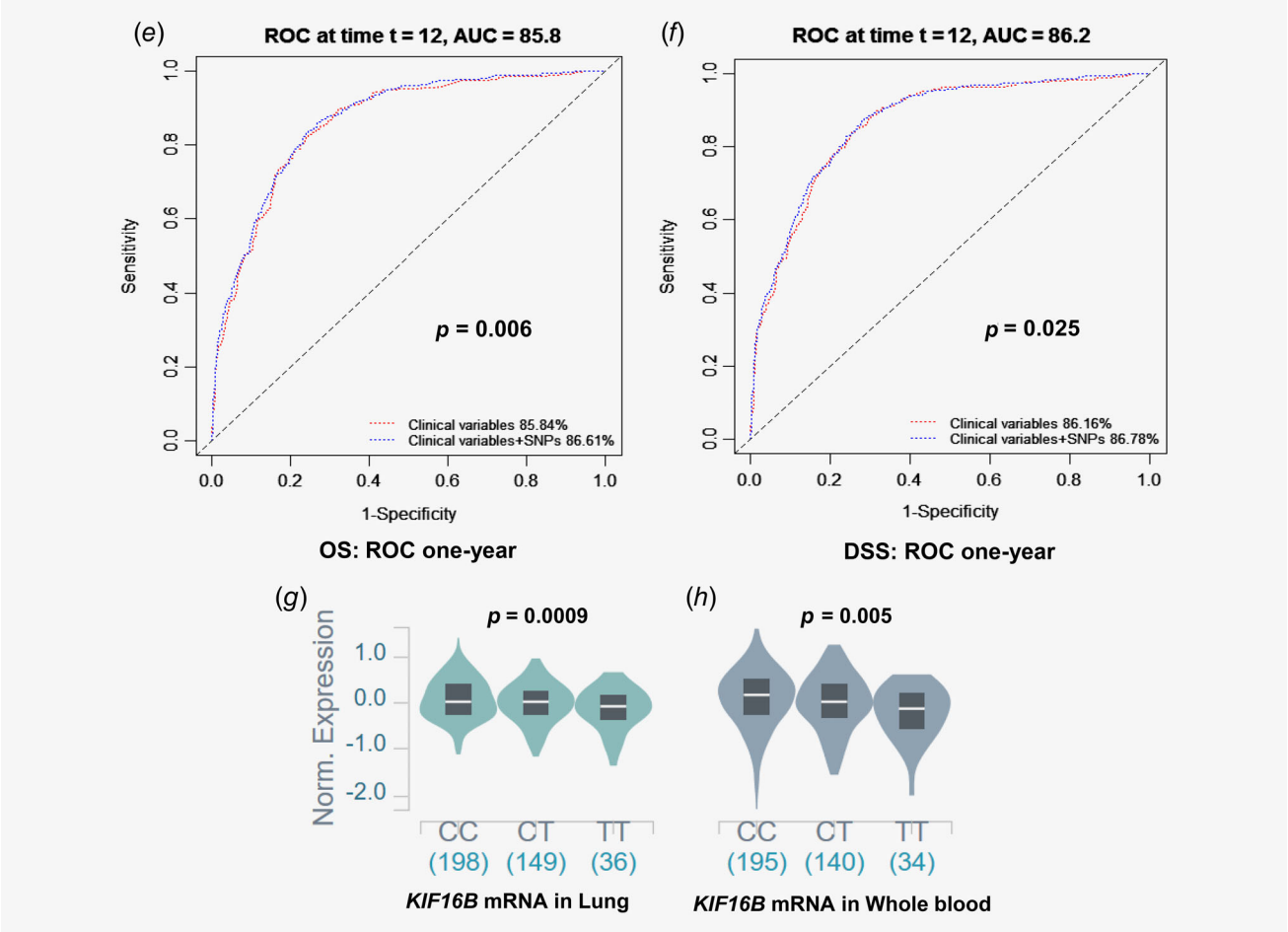

Figure 2. Continued. [Color figure can be viewed at wileyonlinelibrary.com]

model was improved significantly, and the AUCs changed from 85.84% to 86.61% (p = 0.006) for OS and from 86.16% to 86.78% (p = 0.025) for DSS (Figs. 2*e* and 2*f*).

The eQTL analyses

We performed the eQTL analysis to explore the correlations between genotypes of the three independent SNPs and their corresponding mRNA expression levels by using the RNA-Seq data of lymphoblastoid cell lines from 373 European descendants available in the 1000 Genomes Project and the data of 369 whole blood samples and 383 normal lung tissue from the GTEx Project. In the 1000 Genomes Project, all three SNPs showed no significant correlation with their corresponding mRNA expression levels (Supporting Information Fig. S5).[26] Then, we performed eQTL by using the expression data of the lung and whole blood from the GTEx project. We found that the *KIF16B* rs1555195T allele was associated with lower expression levels of *KIF16B* in both lung normal tissues and whole blood cells (p = 0.0009 and 0.005, respectively; Figs. 2*g* and 2*h*). For the *NEDD4L* rs11660748G and rs73440898G alleles, they were not significantly correlated with their corresponding mRNA expression levels (Supporting Information Fig. S6).[27] Finally, we performed functional prediction for these three SNPs with the online tools of SNPinfo,[28] RegulomeDB[29] and Haploreg[30] to predict their bioinformatics function. As a result, rs1555195 has an effect on enhancer histone marks, DNAse and motifs, while rs11660748 and rs73440898 have an effect on enhancer histone marks and motifs (Supporting Information Table S5 and Fig. S7).

Differential mRNA expression analysis

We assessed mRNA expression levels of the two genes in 109 pairs of NSCLC tumor and adjacent normal tissue samples available in the TCGA database. As shown in Supporting Information Figures 8*a*–8*c*, compared to adjacent normal tissues, the mRNA expression levels of *KIF16B* were not different in all tumor tissue samples (p = 0.449) but lower in lung adenocarcinoma (LUAD; p = 0.002) and higher in lung squamous cell carcinoma (LUSC) (p = 0.076). The higher expression levels of *KIF16B* mRNA were associated with a better survival in LUAD patients (Supporting Information Fig. 8*e*) but a worse survival in LUSC patients (Supporting Information Fig. 8*f*). Compared to adjacent normal tissues, mRNA expression levels of *NEDD4L* were lower in all tumor tissue samples as well as in LUAD and LUSC samples (p < 0.0001, p < 0.0001 and p < 0.0001, respectively; Supporting

Information Figs. 9*a*–9*c*). The higher expression levels of *NEDD4L* mRNA were associated with a better survival in LUAD patients but again a worse survival in LUSC patients (Supporting Information Figs. 9*e* and 9*f*).

Discussion

In the present study, we assessed associations between SNPs in the endosome-related gene-set and NSCLC survival by using available genotyping data from two published GWAS datasets. We identified and validated three independent SNPs (i.e., *KIF16B* rs1555195, *NEDD4L* rs11660748 and rs73440898) that were significantly associated with NSCLC survival in Caucasian populations. In subsequent eQTL analysis for functional genotype-mRNA expression correlation, we found that the *KIF16B* rs1555195T allele was associated with lower mRNA expression levels in normal lung tissues and whole blood cells.

Based on the TCGA database, *KIF16B* appears to be a potential oncogene, and we also found that the rs1555195T allele was associated with a lower risk of death and a lower mRNA expression level of *KIF16B*. However, this conclusion is consistent with the observation in LUSC but not in LUAD, and this discrepancy is likely due to small numbers of tumor samples included in the analysis or a difference at the transcriptomic level between LUSC and LUAD[34]. Other possible reasons may be differences in the molecular mechanisms of carcinogenesis[35–37] or therapies for these two tumors.[38]

Both rs11660748G and rs73440898G alleles in *NEDD4L* were found to be associated with a higher risk of death. However, we did not find eQTL evidence to support the relationship between the two SNPs and the mRNA expression levels of *NEDD4L*. According to the results from the differential mRNA expression analysis, *NEDD4L* is more likely to be a suppresser gene in LUAD, but also possibly an oncogene in LUSC, considering that a higher expression of *NEDD4L* was associated with a better survival in LUAD patients but a worse survival in LUSC patients. This differentiation may be due to the difference in tumor types as abovementioned for *KIF16B*. Additional functional investigations are needed to further explore the differences between these two types of NSCLC.

KIF16B, located on chromosome 20, encodes a member of the superfamily of kinesin proteins (KIF), which drives a variety of microtubule-dependent motility events.[39] A key feature of *KIF16B* is the PX domain at the C terminus that could target the motor at early endosomes by binding to PI(3)P, and through that, *KIF16B* could transport early endosomes to the plus end of microtubules in a process regulated by the small GTPase Rab5 and its effector.[40] *KIF16B* overexpression could relocate early endosomes to the cell periphery and inhibit the transport to the degradative pathway.[41] Conversely, expression of dominant-negative mutants or ablation of *KIF16B* by RNAi caused the clustering of early endosomes to the perinuclear region, delayed receptor recycling to the plasma membrane, and accelerated degradation.[41] These suggest that *KIF16B*, by regulating the plus end motility of early endosomes, modulates the intracellular localization of early endosomes and the balance between receptor recycling and degradation.[41] Overall, *KIF16B* expression affects the presentation of intracellular antigens by alternating early endosome location. However, few studies about *KIF16B* and lung cancer have been reported. One study reported that downregulation of *KIF16B* was found to be associated with brain metastasis in LUAD.[42]

NEDD4L, located on chromosome 18, encodes a ubiquitin ligase belonging to the NEDD4 family of E3 HECT domain ubiquitin ligases.[43,44] *NEDD4L* proteins are known to be involved in regulating many membrane proteins *via* ubiquitination and endocytosis.[45] *NEDD4L* binds through its WW domains to the PY motifs of the epithelial Na^+ channel (ENaC), leading to ENaC ubiquitylation, endocytosis to endosomes and multivesicular bodies, and degradation.[44] Overall, *NEDD4L* expression affects the presentation of intracellular antigens by alternating endosome forming and degradation. Few studies about *NEDD4L* and lung cancer have been reported. One study found that in NSCLC patients with low *NEDD4L* expression levels, their prognoses were significantly poorer than those with high *NEDD4L* expression levels.[46] It was found that miR-93 could promote TGF-β-induced epithelial-to-mesenchymal transition through downregulation of *NEDD4L* in lung cancer cells,[47] that *NEDD4L* acted as a tumor suppressor gene in NSCLC and that targeting EZH2 could upregulate *NEDD4L* expression.[48] There are no reports about the role of genetic variants of *NEDD4L* in the survival of NSCLC patients.

Although few studies about the relationship between *KIF16B* or *NEDD4L* and lung cancer have been reported, the relevant correlation between the endosome and immunotherapy for lung cancer have been well studied. For example, exogenous antigens including tumor antigens are taken up by antigen-presenting cells (APCs) and are degraded in endosome/lysosomes.[49] They are subsequently degraded to antigenic peptide and bound to MHC class II molecules. These antigenic peptide/MHC class II complexes are presented to CD4-positive T cells, generating helper T cell-based humoral immune responses. A part of the exogenous antigen is also carried onto MHC class I molecules *via* transferring from the endosome to cytosol or in an early endosomes to engender CTL-based cellular immune responses. This presentation process of exogenous antigen is known as "cross-presentation."[50] Therefore, the delivery of antigen into APCs in the body and the control of intracellular distribution of antigen in these cells for the induction of antigen-specific CTLs are crucially important to achieve cancer immunotherapy.

There are several limitations in the present study. First, although several genetic variants backed up with *in silico* functional evidence in the endosome-related genes were found to be associated with NSCLS survival, the exact molecular mechanisms of these SNPs underlying the observed associations are still unclear. Second, both discovery and validation datasets were from Caucasian populations; therefore, our results may not be generalizable to other ethnic populations. Third, though some clinical factors were available in the analysis for the PLCO but not HLCS datasets, there are still some information, such as the

performance status, nutritional status and specific treatments such as immunotherapy, that was not available for further adjustment. However, our findings provided new insights for additional functional studies to further support these genetic variants of endosome pathway genes as promising predictors of survival in NSCLC patients.

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