Genetic Variants of CLEC4E and BIRC3 in Damage-Associated Molecular Patterns-Related Pathway Genes Predict Non-Small Cell Lung Cancer Survival

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Accumulating evidence supports a role of various damage-associated molecular patterns (DAMPs) in progression of lung cancer, but roles of genetic variants of the DAMPs-related pathway genes in lung cancer survival remain unknown. We investigated associations of 18,588 single-nucleotide polymorphisms (SNPs) in 195 DAMPs-related pathway genes with non-small cell lung cancer (NSCLC) survival in a subset of genotyping data for 1,185 patients from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and validated the findings in another independent subset of genotyping data for 984 patients from Harvard Lung Cancer Susceptibility Study. We performed multivariate Cox proportional hazards regression analysis, followed by expression quantitative trait loci (eQTL) analysis, Kaplan-Meier survival analysis and bioinformatics functional prediction. We identified that two SNPs (i.e., CLEC4E rs10841847 G>A and BIRC3 rs11225211 G>A) were independently associated with NSCLC overall survival, with adjusted allelic hazards ratios of 0.89 (95% confidence interval=0.82-0.95 and P=0.001) and 0.82 (0.73-0.91 and P=0.0003), respectively; so were their combined predictive alleles from discovery and replication datasets (P_trend=0.0002 for overall survival). We also found that the CLEC4E rs10841847 A allele was associated with elevated mRNA expression levels in normal lymphoblastoid cells and whole blood cells, while the BIRC3 rs11225211 A allele was associated with increased mRNA expression levels in normal lung tissues. Collectively, these findings indicated that genetic variants of CLEC4E and BIRC3 in the DAMPs-related pathway genes were associated with NSCLC survival, likely by regulating the mRNA expression of the corresponding genes.

Keywords: non-small cell lung cancer, single-nucleotide polymorphism, variant, damage-associated molecular pattern-related pathway, survival
INTRODUCTION

Lung cancer remains one of the leading causes of cancer-related mortality in the United States. In 2021, it is estimated that there will be more than 235,000 new cases of and nearly 131,000 will die from lung cancer in the United States (1). Histologically, about 85% of lung cancer patients are classified as non-small cell lung cancer (NSCLC), and the majority of these cases present with local progression or distal metastasis at the time of diagnosis (2). Although there are some clinical predictors and newer treatment options for NSCLC, the clinical outcomes remain poor, largely because of the remarkable heterogeneity in the phenotypes of the early NSCLC, showing diverse innate aggressiveness. Some cases with an early stage of the disease have a favorable prognosis and could be spared the unnecessary therapy, while for other patients with an advanced stage, the five-year survival rate remains poor, despite the use of all the available targeted and immune therapies (3). Therefore, identification of additional prognostic factors for NSCLC-specific survival could add more value to precision medicine of NSCLC.

Damage-associated molecular patterns (DAMPs) are special molecules that are released from the damaged tissues or by the activated immune cells, alerting the organism about the incoming endogenous danger including cancer, inflammation, and tissue repair (4). There is some convincing evidence that DAMPs could recruit specific molecules of the innate and adaptive immune system to tumor microenvironment, ultimately inducing a tumor-targeting immune response (5, 6). Accumulating evidence has revealed the role of various DAMPs in NSCLC progression. For example, one study reported that the high mobility group box 1, one of DAMPs, was found to enhance NSCLC cell migration, leading to metastasis of NSCLC (7). Other studies showed that S100 family members, which also serve as DAMPs, drove NSCLC cell proliferation and invasion (8, 9). Recently, targeting the heat-shock protein family members showed anticancer therapeutic potential for NSCLC patients (10–12). Furthermore, increasing preclinical evidence has indicated that monitoring DAMPs in NSCLC patients could have potential prognostic value and some positive effects on the treatment outcomes (11, 13–15).

As we all know, innate immune interactions in the cancer context include recognition by innate cell populations, dendritic cells and macrophages in response to DAMPs (16). Dying tumor cells would express or release DAMPs for activation of immune cells. That suggests that DAMPs could be the trigger activating tumor innate immunity. But the role of DAMPs in tumor immunity is not completely understood and presents complicated activities of tumor immunity. For example, HMGB1 could trigger both antitumor immunity inflammation and immunotolerance (17).

However, the detection of single DAMP in NSCLC patients may be possible but not be accurate enough for predicting NSCLC prognosis or may cause conflicting results (18). Monitoring of DAMP-associated processes would be more accurate to predict prognosis of NSCLC patients but much difficult (19). Recent studies suggest that identification of single-nucleotide polymorphisms (SNPs) in certain pathway-related genes may help identify novel biomarkers for NSCLC progression and survival (20, 21). Such a post-genome-wide association study (GWAS) strategy with a pathway-based analysis is hypothesis driven, which uses available genotyping data from previously published GWAS datasets to identify functional genetic variants in the targeted biological pathway genes and may clarify possible molecular mechanisms underlying the observed associations with NSCLC survival (22, 23). Therefore, to better understand the value of DAMPs-associated processes in the prognosis of NSCLC survival, we hypothesize that genetic variants of DAMPs-related pathway genes are associated with NSCLC survival, and we tested this hypothesis using genotyping data from two publicly available NSCLC GWASs.

MATERIALS AND METHODS

Study Populations

The discovery genotyping data were derived from a GWAS dataset from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. The age of all the participants were between 55-74 years, who were recruited between 1993 and 2001 in the United States. These participants were randomly assigned to a screening group that received a trial intervention or a control group that received usual care and followed up for 13 years from the time of random assignment (24, 25). During the follow-up 1,185 patients in the screening group were diagnosed with NSCLC, and their detailed demographic data and clinical information on 984 Caucasian patients with histology-confirmed NSCLC from the Harvard Lung Cancer Susceptibility (HLCS) Study launched in 1992 (30). Genomic DNA was extracted from NSCLC patients for genotyping using the Illumina HumanHap240v1.0 and HumanHap550v3.0 (dbGaP accession: phs000093.v2.p2 and phs000336.v1.p1) (26–29). All the subjects provided a written informed consent under a protocol approved by the review board of each participating institutions. In contrast, the validation dataset comprised demographic data and clinical information on 984 Caucasian patients with histology-confirmed NSCLC from the Harvard Lung Cancer Susceptibility (HLCS) Study launched in 1992 (30). Genomic DNA from the HLCS NSCLC patients was genotyped using the Illumina HumanHap610-Quad array. The present study was approved by the Internal Review Board of Duke University School of Medicine (#Pro00054575) and was authorized to have the access so the GWAS datasets by the National Institutes of Health Data Access Committee (dbGaP, #6404). Supplementary Table S1 showed the detail of clinical characteristics between the PLCO trial
and the HLCS study. Since the PLCO discovery data comprised more available covariates than the HLCS validation dataset, we only used the PLCO dataset for further multivariate analyses in the present study.

**Gene Selection and SNP Genotyping**

We searched the Molecular Signatures Database of the Gene Set Enrichment Analysis (GSEA) website (http://software.broadinstitute.org/gsea/msigdb/search.jsp) for the genes of a defined pathway, and we identified 195 damage-associated molecular pattern-related pathway genes located only on the autosomes (Supplementary Table S2). We extracted SNPs within ± 2 kilobase flanking regions of 195 damage-associated molecular pattern-related pathway genes from the PLCO trial and performed imputation with IMPUTE2 using the reference from the 1,000 Genomes Project European data (phase 3). As a result, a total of 18,588 SNPs (1,657 genotyped and 16,931 imputed) remained for further analysis after quality control [presented in Figure 1: located within gene region ± 2kb (hg19), MAF ≥ 5%, HWE P ≥ 10^{-5}, individual call rate ≥ 95% (for genotyping SNPs), and imputation info score ≥ 0.8 (for imputed SNPs)]. The distribution of imputation score was presented in Supplementary Figure S1.

**Statistical Methods**

In the discovery PLCO dataset, we first assessed associations between all available candidate SNPs and NSCLC survival in a single-locus Cox proportional hazards regression analysis using the GenABEL package of R software. SNPs were coded under an additive genetic model according to the number of minor alleles. We performed Cox analyses with adjustment for available covariates in the PLCO trial (including age, sex, histology, smoking status, tumor stage, chemotherapy, surgery, radiotherapy and the first four principal components). The dependent variables included survival time and survival status recorded during the follow-up or at the endpoint of the PLCO trial. In consideration of the high level of linkage disequilibrium (LD) among these candidate SNPs, instead of using the stringent false discovery rate (FDR) method for multiple test correction, we also employed Bayesian false discovery probability (BFDP) with a cutoff value of 0.80 for multiple testing correction to lower the probability of potentially false positive results, as is recommended (31, 32). We assigned a prior probability of 0.10 and a detectable upper boundary HR of 3.0 for an association with variant genotypes or minor alleles of the SNPs with \( P < 0.05 \) (33).

By using the HLCS validation dataset, we replicated the associations of the discovered significant SNPs associated with NSCLC survival in multivariate Cox regression models comparably with a significance level of \( P < 0.05 \). To identify independent SNPs associated with NSCLC survival, we subsequently performed a multivariate stepwise Cox regression model with the PLCO trial. In addition to available clinical variables, other 28 previously published SNPs associated with NSCLC survival in the same PLCO dataset were also included in the model for further adjustment.

Next, we performed a meta-analysis to combine the identified SNPs from the PLCO trial with those in the HLCS dataset using the Cochran’s Q statistics and \( I^2 \) to assess the heterogeneity. Since no heterogeneity was observed between discovery trial and validation datasets (\( P_{het} > 0.10 \) and \( I^2 < 50\% \)), we implemented fixed-effects model for the meta-analysis. After that, we evaluated cumulative effects of all identified SNPs on NSCLC survival probability. For those remained in the final model, we combined
the protective alleles into one variable as a genetic score. NSCLC patients were categorized into four groups (i.e., 0, 1, 2, and 3-4) according to the number of their protective alleles (NPA). For the stratified analyses by subgroups, we calculated inter-study heterogeneity and evaluated possible effect modification or interaction. We constructed a survival prediction model by using the receiver operating characteristic (ROC) curve with the “survival” and “time ROC” packages of R software (version 3.6.2). Sensitivity, specificity, and time-dependent area under the curve (AUC) were used to measure the ability of survival models to predict NSCLC survival in association with both clinical and genetic variables (34). To evaluate the genotype-phenotype correlation between genotypes of identified SNPs and mRNA expression levels of the corresponding genes, we employed expression quantitative trait loci (eQTL) analyses with a general linear regression model using data from the 373 European descendants included in the 1,000 Genomes Project (Phase 3) (35), the genotype-tissue expression (GTEX) project (http://www.gtexportal.org/home; version V8) (36), and The Cancer Genome Atlas (TCGA) database. Additional bioinformatics functional prediction for the tagging SNPs were performed with SNPinfo (37), RegulomeDB (v2.0.3) (38) (http://www.regulomedb.org) and HaploReg V4.1 (39) (http://archive.broadinstitute.org/mammals/haploreg/haploreg.php).

Finally, we depicted the associations between the mRNA expression levels of genes where SNPs are located and NSCLC survival using the Kaplan-Meier (KM) survival curves from the online TCGA database (http://ualcan.path.uab.edu; last updated by 09/23/2019) and from Human Protein Atlas database (https://www.proteinatlas.org/; Version: 20.1). All statistical analyses were performed with SAS software (version 9.4; SAS Institute, Cary, NC), unless specified otherwise.

RESULTS

Associations Between SNPs in DAMPs-Related Pathway Genes and NSCLC in the PLCO Trial and the HLCS Study

Baseline characteristics of NSCLC patients from the PLCO trial and the HLCS study are described elsewhere (40), and an overall flowchart of the present study is presented in Figure 1. Among the acquired 18,588 SNPs in 195 DAMPs-related pathway genes, we found 315 SNPs to be significantly associated with the NSCLC overall survival (OS) in the PLCO trial in an additive model (Supplementary Table S3A), of which 9 SNPs remained significant as replicated in the HLCS dataset with multiple test correction by the BFDP (Supplementary Table S3B).

Identification of Independent SNPs Among the Nine Significant SNPs

In stepwise multivariable Cox regression analyses, we assessed effects of the nine validated SNPs on NSCLC survival in the PLCO trial. We then expanded this prediction model with adjustment for 28 previously reported SNPs in the PLCO trial. Finally, we identified two SNPs (CLECAE rs10841847 G>A and BIRC3 rs11225211 G>A) that remained significantly associated with NSCLC OS (P=0.019 and 0.012) (Table 1). The results of

**TABLE 1** | Two independent SNPs in a multivariate Cox proportional hazards regression analysis with adjustment for other covariables and additional 28 previously published SNPs for NSCLC in the PLCO Trial.

| Variables       | Category          | Frequency | HR (95% CI) | P   | HR (95% CI) | P   |
|-----------------|-------------------|-----------|-------------|-----|-------------|-----|
| Age             | Continuous        | 1185      | 1.04 (1.02-1.05) | <0.0001 | 1.04 (1.03-1.06) | <0.0001 |
| Sex             | Male              | 698       | 1.00         |      | 1.00         |      |
|                 | Female            | 487       | 0.77 (0.66-0.89) | 0.0005 | 0.71 (0.61-0.84) | 0.0001 |
| Smoking status  | Never             | 115       | 1.00         |      | 1.00         |      |
|                 | Current           | 423       | 1.70 (1.26-2.26) | 0.0004 | 1.88 (1.39-2.54) | 0.0001 |
|                 | Former            | 647       | 1.74 (1.32-2.29) | <0.0001 | 1.90 (1.43-2.54) | <0.0001 |
| Histology       | Adenocarcinoma    | 577       | 1.00         |      | 1.00         |      |
|                 | Squamous cell     | 285       | 1.20 (1.00-1.45) | 0.057 | 1.21 (1.00-1.48) | 0.053  |
|                 | others            | 323       | 1.31 (1.10-1.55) | 0.002 | 1.38 (1.15-1.65) | 0.0005 |
| Tumor stage     | I-IIIA            | 655       | 1.00         |      | 1.00         |      |
|                 | IIIB-IV           | 528       | 3.00 (2.46-3.65) | <0.0001 | 3.45 (2.82-4.23) | <0.0001 |
| Chemotherapy    | No                | 639       | 1.00         |      | 1.00         |      |
|                 | Yes               | 538       | 0.57 (0.48-0.68) | <0.0001 | 0.56 (0.47-0.68) | <0.0001 |
| Radiotherapy    | No                | 762       | 1.00         |      | 1.00         |      |
|                 | Yes               | 415       | 0.95 (0.81-1.12) | 0.569 | 0.99 (0.83-1.72) | 0.880  |
| Surgery         | No                | 637       | 1.00         |      | 1.00         |      |
|                 | Yes               | 540       | 0.21 (0.17-0.28) | <0.0001 | 0.20 (0.15-0.26) | <0.0001 |
| CLECAE rs10841847 G>A | GG/GA/AA       | 495/544/148 | 0.89 (0.80-0.98) | 0.019 | 0.88 (0.79-0.98) | 0.019  |
| BIRC3 rs11225211 G>A | GG/GA/AA       | 368/679/248 | 0.81 (0.71-0.93) | 0.003 | 0.83 (0.72-0.96) | 0.019  |

SNP, single-nucleotide polymorphism; NSCLC, non-small cell lung cancer; PLCO, the Prostate, Lung, Colorectal and Ovarian cancer screening trial; HLCS, Harvard Lung Cancer Susceptibility Study; HR, hazard ratio; CI, confidence interval.

*Adjusted for age, sex, tumor stage, histology, smoking status, chemotherapy, radiotherapy, surgery, and PC1, PC2, PC3, PC4.

**TABLE 2** | Associations between SNPs in DAMPs-related pathway genes and NSCLC survival in the PLCO trial. The results of...
meta-analysis for these two independent SNPs in each and combined datasets are presented in Table 2, showing the absence of heterogeneity across these two datasets.

Furthermore, as shown in Table 3, we noticed that the CLEC4E rs10841847 A allele and the BIRC3 rs11225211 A allele were protective alleles for NSCLC OS in the PLCO dataset ($P_{\text{trend}} = 0.012$ and $0.006$, respectively) with similar results for NSCLC disease-specific survival (DSS) in the PLCO dataset ($P_{\text{trend}} = 0.049$ and $0.017$, respectively). All identified SNPs in the present study are depicted in Manhattan plots for both PLCO and HLCS (Supplementary Figure S2) datasets, and regional association plots for these two independent SNPs (also including all SNPs located in their 100-kb flanking regions) are displayed in Supplementary Figure S3.

### Combined Protective Alleles of the Two Independent NSCLC Survival-Associated SNPs

To clarify collective effect of the two independent SNPs on NSCLC survival, we combined their protective alleles (i.e., CLEC4E rs10841847 A allele and BIRC3 rs11225211 A allele) into one variable as a genetic score. NSCLC patients were categorized into four groups (i.e., 0, 1, 2, and 3-4) according to the number of their protective alleles (NPA). Similarly, an

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**Table 2** Associations of significant SNPs with overall survival of patients with NSCLC in both discovery and validation datasets from two previously published GWASs.

| SNPs          | Allele a | Gene      | PLCO (n = 1185) | HLCS (n = 984) | Combined-analysis |
|---------------|----------|-----------|-----------------|----------------|-------------------|
|               | FDR      | BFDP      | EAF             | HR (95% CI)    | P (95% CI)        | P (95% CI)        |
| rs10841847    | G/A      | CLEC4E    | 0.61 0.79 0.44 0.88 (0.79-0.97) 0.012 | 0.42 0.89 (0.80-0.99) 0.039 | 0.070 0.89 (0.82-0.95) 1.41x10^{-3} |
| rs11225211    | G/A      | BIRC3     | 0.61 0.69 0.17 0.82 (0.72-0.96) 0.006 | 0.16 0.81 (0.68-0.98) 0.029 | 0.940 0.82 (0.73-0.91) 3.75x10^{-4} |

**Table 3** Associations of the protective alleles of two independent SNPs with OS and DSS of NSCLC in the PLCO Trial.

| Alleles | Frequency | OS a | DSS a |
|---------|-----------|------|-------|
|         | Death (%) | HR (95% CI) | P    | Death (%) | HR (95% CI) | P   |
| CLEC4E rs10841847 G>A | 361 | 248 (68.70) | 1.00 | 409 | 270 (66.01) | 0.80 (0.69-0.93) | 0.004 |
| BIRC3 rs11225211 G>A   | 812 | 559 (68.84) | 1.00 | 325 | 206 (63.38) | 0.79 (0.67-0.93) | 0.004 |
| NPA 0-1 | 246 | 176 (71.54) | 1.00 | 519 | 342 (65.90) | 0.83 (0.69-0.99) | 0.042 |
| NPA 1 | 519 | 342 (65.90) | 0.83 (0.69-0.99) | 0.042 | 323 | 219 (67.80) | 0.74 (0.60-0.91) | 0.004 |
| NPA 2 | 23 (62.16) | 0.82 (0.54-1.25) | 0.351 | 37 | 206 (63.38) | 0.79 (0.67-0.93) | 0.004 |
| NPA 3-4 | 86 | 51 (59.30) | 0.58 (0.42-0.80) | 0.0008 | 86 | 51 (59.30) | 0.58 (0.42-0.80) | 0.0008 |

SNP, single nucleotide polymorphisms; NSCLC, non-small cell lung cancer; GWAS, genome-wide association study; PLCO, the Prostate, Lung, Colorectal and Ovarian cancer screening trial; HLCS, Harvard Lung Cancer Susceptibility Study; EAF, effect allele frequency; HR, hazard ratio; CI, confidence interval; FDR, false discovery rate; BFDP, Bayesian false discovery probability; LD, linkage disequilibrium.

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|---------|-----------|------|-------|
|         | Death (%) | HR (95% CI) | P    | Death (%) | HR (95% CI) | P   |
| CLEC4E rs10841847 G>A | 361 | 248 (68.70) | 1.00 | 409 | 270 (66.01) | 0.80 (0.69-0.93) | 0.004 |
| BIRC3 rs11225211 G>A   | 812 | 559 (68.84) | 1.00 | 325 | 206 (63.38) | 0.79 (0.67-0.93) | 0.004 |
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| NPA 2 | 23 (62.16) | 0.82 (0.54-1.25) | 0.351 | 37 | 206 (63.38) | 0.79 (0.67-0.93) | 0.004 |
| NPA 3-4 | 86 | 51 (59.30) | 0.58 (0.42-0.80) | 0.0008 | 86 | 51 (59.30) | 0.58 (0.42-0.80) | 0.0008 |

SNP, single nucleotide polymorphism; NSCLC, non-small cell lung cancer; PLCO, Prostate, Lung, Colorectal and Ovarian cancer screening trial; HR, hazard ratio; CI, confidence interval; OS, overall survival; DSS, disease-specific survival; NPA, number of protective alleles.

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increased NPA was associated with better NSCLC OS and DSS in the PLCO dataset ($P_{\text{trend}}=0.0002$ and 0.002, respectively) after adjustment for available covariates (Table 3). Furthermore, we also dichotomized all NSCLC patients into two groups: 0–1 or 2–4 NPA. As shown in Table 3, compared with the 0–1 NPA group, the 2–4 group had a significantly favorable NSCLC OS and DSS in the PLCO dataset ($P_{\text{trend}}=0.004$ and 0.009, respectively). In addition, we further constructed KM survival curves to visualize the associations between NPA and NSCLC survival. As shown in Figures 2A, B, compared with the 2–4 NPA group, NSCLC KM survival curves of the 0–1 NPA group significantly declined (Log-rank $P=0.024$ for DSS and Log-rank $P=0.027$ for OS).

**Stratified Analysis for the Effect of NPA on NSCLC Survival**

To evaluate whether the effect of NPA on NSCLC survival was confounded by other clinical covariates, we performed stratified analysis in the PLCO dataset. Compared with NSCLC patients having 0–1 NPA, those with 2–4 NPA had a significantly better NSCLC survival, except for the subgroup with age >71, female, former smoking status, late tumor stage, and clinical therapy. No significant interactions between protective alleles and each covariate on NSCLC survival were observed (Supplementary Table S5).

**Time-Dependent AUC and ROC Curves of the Two Independent SNPs for NSCLC Survival Prediction**

To further evaluate predictive value of the two independent SNPs, time-dependent AUC and ROC curves were performed for NSCLC patients in the presence of available clinical covariates. In the PLCO trial, although the time-dependent AUC increased when protective alleles were added, the predictive performance of 60-month (5-year) NSCLC survival ROC curves was non-significantly improved ($P=0.074$ for DSS and $P=0.070$ for OS) (Supplementary Figures S4B, D). However, the predictive performance of 120-month (10-year) NSCLC survival ROC curves was significantly improved by adding protective alleles ($P=0.028$ for DSS and $P=0.046$ for OS) (Figures 2C, D).

**Bioinformatics Functional Prediction of the Two SNPs**

To assess specific biological functions of the two independent SNPs associated with NSCLC survival, we explored SNP-related genomics data using an online bioinformatics tool (HaploReg, https://pubs.broadinstitute.org/mammals/haploreg/haploreg. php). It appears that CLEC4E rs10841847 is located in a DNase district, and a G>A change may be involved in modifying several protein motifs; BIRC3 rs11225211 is predicted to be located in an enhancer histone marker district and a G>A change may be involved in disturbing several proteins’ expression (Supplementary Table S6). According to the data extracted from the Encyclopedia of DNA Elements (ENCODE) project, CLEC4E rs10841847 is probably located on the H3K4Me1 motif, while BIRC3 rs11225211 is located in the region with enriched H3K4Me1 and H3K4Me3 as well as the possible binding site of Txn factor (Supplementary Figure S5). These findings imply a strong possibility that these two SNPs may modulate their gene expression levels through transcriptional regulation.

**Two Independent SNPs and Their Corresponding mRNA Expression**

To further investigate molecular mechanisms underlying the associations between two identified SNPs and NSCLC survival, we explored correlations between protective alleles of two identified SNPs and their corresponding mRNA expression levels using eQTL analyses. In the RNA-Seq data on lymphoblastoid cell lines from 373 European descendants (obtained from the 1000 Genomes Project), the rs10841847 A allele showed a significant correlation with increased expression levels of CLEC4E mRNA in additive and dominant models ($P=0.001$ and $P=0.0002$, respectively) (Figures 2E, F), but not in recessive models (Supplementary Figure S6A). The rs11225211 A allele showed no correlation with BIRC3 mRNA expression in any of additive, dominant, or recessive models (Supplementary Figures S6B–D). Moreover, we also performed the eQTL analysis by extracting data from the GTEx Project. The results showed that rs10841847 A allele was significantly associated with elevated CLEC4E mRNA expression levels in normal whole blood samples ($P<0.0001$) (Figure 2G), but not in normal lung tissues (Supplementary Figure S6E). The rs11225211 A allele was significantly correlated with lower BIRC3 mRNA expression levels in normal lung tissues ($P=0.027$) (Figure 2H), but not in normal whole blood samples (Supplementary Figure S6F).

**CLEC4E and BIRC3 Expression Levels and Lung Cancer Survival**

Finally, we explored mRNA expression of CLEC4E and BIRC3 in normal lung tissues and primary lung cancer tissues available from the TCGA database (data obtained from http://uacancer.path. uab.edu/index.html). As shown in (Supplementary Figures S7A–D), compared with normal lung tissues, mRNA expression levels of CLEC4E were significantly lower in both primary lung adenocarcinoma tissues and lung squamous cell carcinoma tissues; mRNA expression levels of BIRC3 were significantly higher in primary lung adenocarcinoma tissues, but lower in lung squamous cell carcinoma tissues. Notably, as shown by the KM survival curve in Supplementary Figures S7G, H, lower mRNA expression levels of BIRC3 were significantly associated with better survival of lung adenocarcinoma in two different online databases. However, mRNA expression levels of CLEC4E showed no association with NSCLC survival in these databases (Supplementary Figures S7E, F, I).

**DISCUSSION**

In the present study, we evaluated the associations between 18,588 SNPs of a set of 195 DAMPs-related pathway genes and NSCLC survival by using available genotyping and clinical outcome data from two previously published NSCLC GWAS
datasets. Using this pathway approach, we identified two SNPs (i.e., CLEC4E rs10841847 G>A and BIRC3 rs11225211 G>A) that were independently associated with NSCLC survival. In addition, we found that the rs10841847 A allele and rs11225211 A allele were associated with significantly higher CLEC4E (in peripheral blood lymphocytes from 1000 Genomes) and lower BIRC3 mRNA expression (in normal lung tissues) levels, respectively. Moreover, we observed that levels of both CLEC4E and BIRC3
mRNA expression were altered in NSCLC tissues and that a higher expression level of BIRC3 mRNA was significantly associated with a poorer survival in NSCLC patients.

To date, there was no published report that described an association between genetic variants of CLEC4E and NSCLC survival. Because monitoring of the DAMPs-associated processes is difficult, we instead investigated SNPs in DAMPs-related pathway genes as independent prognostic biomarkers for NSCLC survival in a multivariate analysis. This multivariate model included adjustment for available covariates (i.e., age, sex, tumor stage, histology, smoking status, chemotherapy, radiotherapy, and surgery) as well as 28 previously published NSCLC survival–associated SNPs. As a result, we identified these two SNPs (i.e., rs1084147 and rs11225211) of the pathway genes, which collectively predicted prognosis of NSCLC patients.

CLEC4E is a C-type lectin domain family 4 member E (also known as MINCLE) that has been implicated in stimulating cell death-induced DAMPs, known to play a key role in antifungal and antibacterial immunity (41). It is known that CLEC receptors have potential regulatory effects on immune cell trafficking and modulatory effects on cancer cell activity in tumor microenvironment (TMB). But so far, there is no study about the role of CLEC4 as an immune regulator of TMB. Recently, it has been shown that CLEC4E is involved in enhancing the aggressiveness of urothelial cancer (42), and a new reported inhibitor of cancer cell invasion was identified based on its role in binding to the CLEC4E receptor (43). However, few studies have investigated the roles of CLEC4E in NSCLC. To our knowledge, the present study is the first to report an association between genetic variants of CLEC4E and NSCLC survival. Notably, the CLEC4E rs10841487 G>A showed a significant protective effect on NSCLC survival and a significant association with increased CLEC4E mRNA expression levels in both normal lymphoblastoid cells and whole blood cells. Moreover, CLEC4E mRNA expression was much more weakened in NSCLC tissues than in normal lung tissues. These observations imply that CLEC4E may function as a suppressor gene in NSCLC, but additional functional studies are needed to support this speculation.

BIRC3 (also known as baculoviral IAP repeat containing 3) encodes the protein c-IAP2, an inhibitor of an apoptosis–associated proteins family. BIRC3 has been shown to regulate the molecular signaling cell apoptosis, inflammatory signaling, cell proliferation, and migration (44, 45). Previous studies have revealed that accumulated BIRC3 contributes to tumor progression in several malignancies (46, 47), and the expression levels of BIRC3 have been shown to be correlated with prognosis of patients with different cancers (48, 49). In the present study, we evaluated associations between genetic variants of BIRC3 and NSCLC survival and found that the BIRC3 rs11225211 G>A had a significant protective effect on NSCLC survival. Interestingly, we also found that BIRC3 mRNA expression was accumulated conspicuously in lung adenocarcinoma tissues but weakened in lung squamous cell carcinoma tissues in the TCGA dataset. Additional studies are needed to explore if BIRC3 may serve as a potential biomarker of lung adenocarcinoma. Furthermore, the lower BIRC3 mRNA expression was obviously associated with a better survival in patients with lung cancer in the Human Protein Atlas database. A previous study suggested that BIRC3 might play a role of tumor suppressor, because its deficiency was associated with poor prognosis of the patients (50), which is consistent with our data. Taken together, these results suggest that BIRC3 may function as a suppressor gene in NSCLC, but this speculation also needs to be substantiated in additional molecular biology experiments and clinical studies in the future.

Despite the above-mentioned significant observations, there are several limitations in the present study. First, NSCLC patients in both discovery and validation datasets were recruited only from Caucasian populations, further validation in other NSCLC patient cohorts of different ethnicities should be pursued. Additionally, the PLCO discovery and HLCS replication datasets had differences in the distributions of baseline characteristics, which may partially influence the replication results, leading to fewer significant SNPs being identified. Finally, the sample sizes of these two datasets were still not large enough to perform the FDR test for multiple comparison correction; however, considering that nearly 91% of selected SNPs were imputed in the present study, the BFDP test might be more appropriate for these highly correlated SNPs under investigation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

Conception and design: QW, LHL, and DC. Development of methodology: QW, HL, and DC. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): QW, HL, and DC. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): QW, HL, LHL, and SL. Writing, review, and/or revision of the manuscript: All authors. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): QW, HL, LS, LJL, and DC. Study supervision: QW, HL, and DC. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. CA Cancer J Clin (2021) 71(1):17–33. doi: 10.3322/caac.21654

2. Pourhanifeh MH, Sharifi M, Reiter RJ, Davoodabadi A, Asemi Z, Melatonin and Non-Small Cell Lung Cancer: New Insights Into Signaling Pathways. Cancer Cell Int (2019) 19:1931. doi: 10.1186/s12935-019-0853-7

3. Massuti B, Sanchez JM, Hernando-Trancho F, Karachalou N, Rosell R. Are We Ready to Use Biomarkers for Staging, Prognosis and Treatment Selection in Early-Stage Non-Small-Cell Lung Cancer? Transl Lung Cancer Res (2013) 2(3):208–21. doi: 10.3978/j.issn.2218-6751.2013.03.06

4. Pandolfi F, Altamura S, Frosali S, Conti P. Key Role of DAMP in Inflammation, Cancer, and Tissue Repair. Clin Ther (2016) 38(5):1017–28. doi: 10.1016/j.clinthera.2016.02.028

5. Kroemer G, Galluzzi L, Kepp O, Zigovogel L. ImmuneGenic Cell Death in Cancer Therapy. Annu Rev Immunol (2013) 31:51–72. doi: 10.1146/annurev-immunol-031512-120008

6. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Damage-Associated Molecular Pattern-Related Pathway Genes and Lung Cancer Survival. cancers (2020) 12(12):860. doi: 10.3390/cancers12120860

7. Zhu J, Luo J, Li Y, Jia M, Wang Y, Huang Y, et al. HMGB1 Induces Human Alphavbeta3/FAK Through TLR4/NF-KappaB Signaling Pathway. Cell Res (2012) 22(1):81–91. doi: 10.1038/cr.2011.130

8. Stewart RL, Carpenter BL, West DS, Knudtson ML, Jazaeri AA, Schaid DJ, et al. Environment And Genetics in Lung Cancer Etiology (EAGLE) Study: An Integrative Population-Based Case-Control Study of Lung Cancer. BMC Public Health (2014) 14:961. doi: 10.1186/1471-2458-14-961

9. Naz S, Ranganathan P, Bodapati P, Shastri AH, Mishra LN, Kondiah P. Regulation of S100A2 Expression by TGFB-Induced MEK/ERK Signalling and Its Role in Cell Migration/Invasion. Biochem J (2012) 447(1):81–91. doi: 10.1042/BJ20120014

10. Rolfo C, Giovannetti E, Hong DS, Bivona T, Racz LE, Bronte G, et al. Novel Therapeutic Strategies for Patients With NSCLC That Do Not Respond to Treatment With EGFR Inhibitors. Cancer Treat Rev (2014) 40(8):990–1004. doi: 10.1016/j.ctrv.2014.05.009

11. Tsai MF, Wang CC, Chen JJ. Tumour Suppressor HLJ1: A Potential Antitumor Cancer Target. Curr Drug Targets (2011) 12(14):2001–8. doi: 10.2174/138945011798829339

12. Ota T, Kirita K, Matsuzawa R, Udagawa H, Matsumoto S, Yoh K, et al. Functional Genetic Variants in the TNF/TNFR Signaling Pathway Genes and Predictive Value of DAMPs and DAMP-Associated Processes in Cancer. Front Immunol (2015) 6:402. doi: 10.3389/fimmu.2015.00402

13. Hountis P, Matthaios D, Froudarakis M, Bouros D, Kokolakis S. S100A2 Protein and Non-Small Cell Lung Cancer. The Dual Role Concept. Tumour Biol: Int J Oncodevelop Biol Med (2014) 35(8):7327–33. doi: 10.1007/s13277-014-2117-4

14. Landi MT, Consonni D, Rotunno M, et al. Environment And Genetics in Lung Cancer Etiology (EAGLE) Study: An Integrative Population-Based Case-Control Study of Lung Cancer. BMC Public Health (2008) 8:203. doi: 10.1186/1471-2458-8-203

15. Zhai R, Yu X, Wei Y, Su I, Christiani DC. Smoking and Smoking Cessation in Relation to the Development of Co-Existing Non-Small Cell Lung Cancer With Chronic Obstructive Pulmonary Disease. Int J Cancer (2014) 134(4):961–70. doi: 10.1002/ijc.28414

16. Wakefield J. A Bayesian Measure of the Probability of False Discovery in Genetic Epidemiology Studies. Am J Hum Genet (2007) 81(5):679–91. doi: 10.1086/520019

17. Guo Y, Feng Y, Liu H, Luo S, Clarke JW, Crawford ED, et al. Design of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. Control Clin Trials (2000) 21(6 Suppl):273s–309s. doi: 10.1016/S0197-2456(00)00098-2

18. Gallagher MD, Chen-Potkin AS. The Post-GWAS Era: From Association to Function. Am J Hum Genet (2018) 102(5):717–30. doi: 10.1016/j.ajhg.2018.04.002

19. Landi MT, Consonni D, Naldi J, et al. Environment And Genetics in Lung Cancer Etiology (EAGLE) Study: An Integrative Population-Based Case-Control Study of Lung Cancer. BMC Public Health (2008) 8:203. doi: 10.1186/1471-2458-8-203

20. Chingensh M, Tishkoff SA, Handunnetti L, Lin B, et al. Environment And Genetics in Lung Cancer Etiology (EAGLE) Study: An Integrative Population-Based Case-Control Study of Lung Cancer. BMC Public Health (2008) 8:203. doi: 10.1186/1471-2458-8-203

21. Landi MT, Chatterjee N, Yu K, Goldin LR, Goldstein AM, Rotunno M, et al. A Genome-Wide Association Study of Lung Cancer Identifies a Region of Chromosome 3p15 Associated With Risk for Adenocarcinoma. Am J Hum Genet (2009) 85(5):679–91. doi: 10.1016/j.ajhg.2009.09.012

22. Gallagher MD, Chen-Potkin AS. The Post-GWAS Era: From Association to Function. Am J Hum Genet (2018) 102(5):717–30. doi: 10.1016/j.ajhg.2018.04.002

23. Hountis P, Matthaios D, Froudarakis M, Bouros D, Kokolakis S. S100A2 Protein and Non-Small Cell Lung Cancer. The Dual Role Concept. Tumour Biol: Int J Oncodevelop Biol Med (2014) 35(8):7327–33. doi: 10.1007/s13277-014-2117-4

24. Landi MT, Consonni D, Rotunno M, et al. Environment And Genetics in Lung Cancer Etiology (EAGLE) Study: An Integrative Population-Based Case-Control Study of Lung Cancer. BMC Public Health (2008) 8:203. doi: 10.1186/1471-2458-8-203

25. Zhai R, Yu X, Wei Y, Su I, Christiani DC. Smoking and Smoking Cessation in Relation to the Development of Co-Existing Non-Small Cell Lung Cancer With Chronic Obstructive Pulmonary Disease. Int J Cancer (2014) 134(4):961–70. doi: 10.1002/ijc.28414

26. Wakefield J. A Bayesian Measure of the Probability of False Discovery in Genetic Epidemiology Studies. Am J Hum Genet (2007) 81(2):208–27. doi: 10.1086/519024

27. Yerby BF, Yerby AF, Tulassay Z. The Problem of Multiple Testing and Solutions for Genome-Wide Studies. Bioinformatics (2007) 23(10):1294–6. doi: 10.1093/bioinformatics/btm108

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.717109/full#supplementary-material
34. Chambless LE, Diao G. Estimation of Time-Dependent Area Under the ROC Curve for Long-Term Risk Prediction. *Stat Med* (2006) 25(20):3474–86. doi: 10.1002/sim.2299
35. Lappalainen T, Sammeth M, Friedlander MR, T Hoen PA, Monlong J, Rivas MA, et al. Transcriptome and Genome Sequencing Uncovers Functional Variation in Humans. *Nature* (2013) 501(7468):506–11. doi: 10.1038/nature12531
36. Consortium GT. Human Genomics. The Genotype-Tissue Expression (GTEx) Pilot Analysis: Multitissue Gene Regulation in Humans. *Sci (New York NY)* (2015) 348(6235):648–60. doi: 10.1126/science.1262110
37. Xu Z, Taylor JA. SNPinfo: Integrating GWAS and Candidate Gene Information Into Functional SNP Selection for Genetic Association Studies. *Nucleic Acids Res* (2009) 37(Web Server issue):W600–5. doi: 10.1093/nar/gkp290
38. Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub M, Kasowski M, et al. Annotation of Functional Variation in Personal Genomes Using RegulomeDB. *Genome Res* (2012) 22(9):1790–7. doi: 10.1101/gr.137323.112
39. Ward LD, Kells M. HaploReg V4: Systematic Mining of Putative Causal Variants, Cell Types, Regulators and Target Genes for Human Complex Traits and Disease. *Nucleic Acids Res* (2016) 44(D1):D877–81. doi: 10.1093/nar/gkv1340
40. Wang Y, Liu H, Ready NE, Su L, Wei Y, Christiani DC, et al. Genetic Variants in ABCG1 Are Associated With Survival of Nonsmall-Cell Lung Cancer Patients. *Int J Cancer* (2016) 138(11):2592–601. doi: 10.1002/ijc.29991
41. Yamazaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle Is an ITAM-Coupled Activating Receptor That Senses Damaged Cells. *Nat Immunol* (2008) 9(10):1179–88. doi: 10.1038/ni.1651
42. Roperto S, Russo V, Esposito I, Cecarelli DM, Paciello O, Avallone L, et al. Mincle, an Innate Immune Receptor, Is Expressed in Urothelial Cancer Cells: these terms. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction in which does not comply with these terms.
43. Jacobsen KM, Keiding UB, Clement LL, Schaffert ES, Rambaruth ND, Johannsen M, et al. The Natural Product Brartemicin Is a High Affinity Ligand for the Carbohydrate-Recognition Domain of the Macrophage Receptor Mincle. *MedChemComm* (2015) 6(4):467–52. doi: 10.1039/c4md00512k
44. Silke J, Meier P. Inhibitor of Apoptosis (IAP) Proteins-Modulators of Cell Death and Inflammation. *Cold Spring Harbor Perspect Biol* (2013) 5(2):a008730. doi: 10.1101/cshperspect.a008730
45. Silke J, Vucic D. IAP Family of Cell Death and Signaling Regulators. *Methods Enzymol* (2014) 545:35–65. doi: 10.1016/B978-0-12-801430-1.00002-0
46. Chakravarti A, Noll E, Black PM, Finkelstein DF, Finkelstein DM, Dyson NJ, et al. Quantitatively Determined Survivin Expression Levels Are of Prognostic Value in Human Gliomas. *J Clin Oncol: Off J Am Soc Clin Oncol* (2002) 20(4):1063–8. doi: 10.1200/JCO.2002.20.4.1063
47. Nie Z, Du MQ, McAllister-Lucas LM, Lucas PC, Bailey NG, Hogaboam CM, et al. Conversion of the LIMA1 Tumour Suppressor Into an Oncogenic LMO-Like Protein by API2-MALT1 in MALT Lymphoma. *Nat Commun* (2015) 6:5908. doi: 10.1038/ncomms6908
48. Ponnelle T, Chapusot C, Martin I, Bonithon-Kopp C, Bouvier AM, Plenchette S, et al. Subcellular Expression of C-IAP1 and C-IAP2 in Colorectal Cancers: Relationships With Clinicopathological Features and Prognosis. *Pathol Res Pract* (2003) 199(11):723–31. doi: 10.1078/0344-0338-00488
49. Wang D, Berglund A, Kenchappa RS, Forsyth PA, Mule JJ, Etame AB. BIRC3 Is a Novel Driver of Therapeutic Resistance in Glioblastoma. *Sci Rep* (2016) 6:21710. doi: 10.1038/srep21710
50. Frazzi R. BIRC3 and BIRC5: Multi-Faceted Inhibitors in Cancer. *Cell Biosci* (2021) 11(1):8. doi: 10.1186/s13578-020-00521-0

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