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## Comprehensive functional annotation of susceptibility variants identifies genetic heterogeneity between lung adenocarcinoma and squamous cell carcinoma

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### Conflict of interest

Na Qin, Yuancheng Li, Cheng Wang, Meng Zhu, Juncheng Dai, Tongtong Hong, Demetrius Albanes, Stephen Lam, Adonina Tardon, Chu Chen, Gary Goodman, Stig E. Bojesen, Maria Teresa Landi, Mattias Johansson, Angela Risch, H-Erich Wichmann, Heike Bickeboller, Gadi Rennert, Susanne Arnold, Paul Brennan, John K. Field, Sanjay Shete, Loic Le Marchand, Olle Melander, Hans Brunnstrom, Geoffrey Liu, Rayjean J. Hung, Angeline Andrew, Lambertus A. Kiemeny, Shan Zienoldiny, Kjell Grankvist, Mikael Johansson, Neil Caporaso, Penella Woll, Philip Lazarus, Matthew B. Schabath, Melinda C. Aldrich, Victoria L. Stevens, Guangfu Jin, David C. Christiani, Zhibin Hu, Christopher I. Amos, Hongxia Ma, and Hongbing Shen declare that they have no conflict of interest.

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## Abstract

Although genome-wide association studies have identified more than eighty genetic variants associated with non-small cell lung cancer (NSCLC) risk, biological mechanisms of these variants remain largely unknown. By integrating a large-scale genotype data of 15,581 lung adenocarcinoma (AD) cases, 8,350 squamous cell carcinoma (SqCC) cases, and 27,355 controls, as well as multiple transcriptome and epigenomic databases, we conducted histology-specific meta-analyses and functional annotations of both reported and novel susceptibility variants. We identified 3,064 credible risk variants for NSCLC, which were overrepresented in enhancer-like and promoter-like histone modification peaks as well as DNase I hypersensitive sites. Transcription factor enrichment analysis revealed that USF1 was AD-specific while CREB1 was SqCC-specific. Functional annotation and gene-based analysis implicated 894 target genes, including 274 specific for AD and 123 for SqCC, which were overrepresented in somatic driver genes ( $ER=1.95$ ,  $P=0.005$ ). Pathway enrichment analysis and Gene-Set Enrichment Analysis revealed that AD genes were primarily involved in immune-related pathways, while SqCC genes were homologous recombination deficiency related. Our results illustrate the molecular basis of both well-studied and new susceptibility loci of NSCLC, providing not only novel insights into the genetic heterogeneity between AD and SqCC but also a set of plausible gene targets for post-GWAS functional experiments.

## Keywords

Lung cancer; genome-wide association study; function annotation; immune; homologous recombination repair deficiency; genetic heterogeneity

## Introduction

Lung cancer is the leading cause of cancer morbidity and mortality worldwide[1]. Non-small cell lung cancer (NSCLC) is the main type of lung cancer, accounting for ~85% of all lung cancer cases[2]. Adenocarcinoma (AD) and squamous cell carcinoma (SqCC) represent the two major histological subtypes of NSCLC. Although tobacco smoking is generally considered as the major cause of lung cancer, genetic factors also play

an important role in the development of lung cancer. Genome-wide association studies (GWASs) have previously identified 81 lung cancer susceptibility variants in 51 loci that robustly associated with lung cancer risk[3, 4]; however, only a relatively small proportion of lung cancer heritability (0.7%~2.4%) can be explained by the variants identified so far[5, 6]. Additionally, delineating the biological mechanism of susceptibility variants underlying the development of lung cancer has also lagged far behind[3].

The challenge of pinpointing predisposition genes in susceptibility loci lies in several aspects. First, most GWAS implicated variants are *tag* single-nucleotide polymorphisms (SNPs) which represent for all co-inherited SNPs in the same haplotype, and thus the direct inference of statistically associated SNPs rarely yields functional variants[7]. Second, as the vast majority (>80%) of the GWAS hits are located in the non-coding regions, distinguishing functional SNPs from non-functional ones can be a great challenge[8]. Third, as previous studies mainly focused on mixed NSCLC, the biological and genomic heterogeneity of lung AD and SqCC[9–11] also leads to a disparity of functional signals. Thus, further dissection of the genetic underpinnings of lung AD and SqCC is crucial for the understanding of lung cancer pathogenesis.

In recent years, the emergence of epigenomic datasets, such as the Encyclopedia of DNA Elements (ENCODE) project[12], the Functional Annotation of the Mammalian Genome (FANTOM) project[13], and the Roadmap Epigenomics project[14] provide a good opportunity to unveil the function of disease-associated signals from epigenomic level. By leveraging the wealth epigenomic data, many studies have attempted to illuminate the biological meanings of GWAS-implicated cancer risk loci. For example, a study from Michailidou *et al.*[15] examined the overlap of breast cancer variants with *cis*-regulatory elements (CREs) and observed a significant enrichment. Similar results were observed for the risk loci of colorectal, head and neck, ovary, and prostate cancers[16], and such CREs were active in disease-related cell lines[17]. Thus, incorporating functional information into association signals has the potential to improve our understanding of the biological consequences of human cancers.

In this study, we first conducted histology-specific genome-wide meta-analyses and then performed a comprehensive functional annotation of NSCLC susceptibility variants by integrating multiple in-house and publicly available databases. Our results first illustrate the molecular basis of all known susceptibility loci of lung cancer and provide novel insights into the genetic heterogeneity between lung AD and SqCC.

## Materials and Methods

### Study populations

We conducted a meta-analysis with 27,120 NSCLC cases (15,581 AD, 8,350 SqCC, and 3,189 other cases) and 27,355 controls. Of all subjects, 26,655 Chinese participants were from our previously published Nanjing Medical University (NJMU) lung cancer GWAS study[4] and 27,820 European participants were from the TRICL-ILCCO OncoArray project[18]. NJMU lung cancer GWAS study was consist of three datasets, including 19,546 participants (10,248 cases and 9,298 controls) from NJMU Global Screening Array

(GSA) project[4], 5,203 participants (2,126 cases and 3,077 controls) from NJMU GWAS project[19], and 1,906 participants (953 cases and 953 controls) from NJMU OncoArray project[4]. Informed consent was obtained from all the participants included in this study, and each study was approved by the corresponding institutional review board. Detailed demographic characteristics of included participants have been described in our previous study[4], and were provided in Table 1.

### Quality control and imputation

Detailed imputation process has been described in our previous study[4]. Briefly, we first excluded samples with genotype completion rates <95%, gender discrepancies, familial relationships, extreme heterozygosity rates (6 S.D. from the mean), or population stratification (>6 S.D. from the mean on any one of the top ten principal components). Then, we excluded duplicate markers or SNPs with call rates <95%, minor allele frequencies (MAFs) <0.01 or Hardy-Weinberg equilibrium (HWE)  $P$  value < $1 \times 10^{-7}$  in controls or HWE  $P$  value < $1 \times 10^{-12}$  in cases. We phased the haplotypes with SHAPEIT v2[20] and imputed with IMPUTE2[21]. The 1000 Genomes Project (the Phase III integrated variant set release, across 2,504 samples) was set as the reference.

### Identification of NSCLC risk loci

To perform functional annotation, we first conducted genome-wide meta-analyses for NSCLC, lung AD and SqCC respectively, and then derived a set of lung cancer risk associated index variants including both 81 previously reported SNPs (Table S1) and those with a genome-wide significant  $P$  value < $1 \times 10^{-6}$  in our overall NSCLC or histological meta-analyses. For 81 previously reported variants, index variants were defined as those met either of the following criteria: (1) MAF  $\geq 0.01$ ; and (2) SNPs in weak linkage disequilibrium (LD,  $r^2 < 0.6$ ) with each other. For other independent variants, index variants were defined if met one or more of the following criteria in our meta-analyses: (1) MAF  $\geq 0.01$ ; (2) with a genome-wide significant  $P$  value < $1 \times 10^{-6}$  in NSCLC, lung AD or SqCC meta-analysis; and (3) SNPs in weak LD with each other and previously reported variants ( $r^2 < 0.01$ ). If one SNP identified in histology-specific meta-analysis also showed association with the other histology of lung cancer (AD or SqCC) ( $P < 0.05$ ), it was considered as NSCLC related. Thus, 67 index variants derived from both 81 previously reported SNPs and those from our genome-wide meta-analyses were included in the following analysis. Then, we further mining SNPs in strong LD ( $r^2 \geq 0.6$ ) with above defined index SNPs and physically within 500 kb upstream or downstream of the index SNP. All above defined index SNPs and associated SNPs in strong LD were considered as credible risk variants (CRVs) (Figure 1A).

### Identification of target genes for NSCLC risk loci by functional annotation

To define candidate target genes for lung cancer risk loci, we performed functional annotation with an extended in silico prediction of GWAS targets (INQUISIT) strategy[15] and calculated a score for each gene-CRV pair representing for the coding impact or potential regulatory mechanisms (proximal or distal gene regulation) by integrating multiple lines of evidence (Table S2–3). Each target gene was scored based on distally regulation, proximally regulation, and coding sequence.

For the distally regulated gene, one score was given if: (1) the CRV was located in an enhancer element that predicted to physically interact with the promoter of the target gene by FANTOM5[13] or PreSTIGE [22]; (2) the enhancer element containing the CRV overlapped with the transcription factor binding sites (TFBS) of one transcription factor (TF) (two scores were given if overlapped with more than one TF); (3) the CRV is an expression quantitative trait loci (eQTL) for that gene in the Genotype-Tissue Expression (GTEx), The Cancer Genome Atlas (TCGA) or Nanjing Lung Cancer Cohort (NJLCC) [23] databases; or (4) the gene was listed as a lung cancer somatic driver gene (except for driver gene enrichment analysis). Additionally, two scores were added if the CRV was also located in an enhancer element that physically interact with the promoter of that gene based on Hi-C experiment. However, the score was down-weighted by multiplying by 0.05 if the target gene was separated from the CRV by topologically associating domain (TAD) boundaries, or down-weighted by multiplying by 0.1 if the gene was low expressed in the GTEx normal lung tissues (less than 1% samples with Transcripts Per Million [TPM] greater than 0.1), TCGA tumor/adjacent samples (less than 1% samples with Fragments Per Kilobase Million [FPKM] greater than 0.1) and NJLCC tumor/adjacent samples (less than 1% samples with TPM greater than 0.1).

Proximally regulated genes were defined as those with CRVs located 1 kb upstream and 100bp downstream surrounding the transcription start sites (TSSs). One score was given if: (1) the gene was overlapped with promoter histone modification peaks (H3K4me3 or H3K9ac); (2) the gene was listed as a lung cancer somatic driver gene (except for driver gene enrichment analysis); (3) the histone modification peak that the CRV resided was also intersected with the TFBS of TFs; or (4) the CRV is an eQTL for that gene in GTEx, TCGA or NJLCC databases. The down-weighted criteria were the same as that for distally regulated genes.

CRVs located in the exonic regions were evaluated for their impact on the protein function. Combined Annotation Dependent Depletion (CADD)[24], Functional Analysis through Hidden Markov Models (FATHMM)[25], LRT[26], MutationTaster[27], PolyPhen-2[28], and Sorting Tolerant from Intolerant (SIFT)[29] were used for the evaluation of missense variants. Other scoring strategy was the same as that for distally regulated genes.

Altogether, scores in the distal regulation category range from 0 to 7, in the promoter category from 0 to 4, and in the coding category from 0 to 3. We classified the candidate target genes into four levels based on the integrated scores: level 1 (distal score  $\geq 4$ , promoter score  $\geq 3$ , or coding score =3), level 2 (distal score 1–3, promoter score 1–2, or coding score 1–2), level 3 (any score greater than 0), and level 4 (score 0). Genes categorized into level 1–2 were considered as potential targets and were included in the following analysis.

### **Expression quantitative trait loci analysis based on NJLCC and TCGA data**

In addition to the GTEx project (v7), we also performed eQTL analysis with data from NJLCC and TCGA projects. The NJLCC[23] project included 90 Chinese NSCLC samples with available clinical information, gene expression data, copy number variation profiles and matched genotyping data. Gene expression data was available for 98 tumor/adjacent pairs. NJLCC samples were genotyped with whole-genome sequencing and the expression

data was quantified by RNA-seq (Illumina HiSeq 1500 platform)[23]. A systematic quality control (QC) procedure was performed to filter out samples with missing genotypes and duplicates. Principal component analysis (PCA) was also performed. Finally, all 90 NJLCC samples (55 lung ADs and 35 SqCCs) were included in the eQTL analysis.

Similarly, the TCGA project included 106 NSCLC samples with matched clinical information, gene expression data, copy number variation data, and genotyping data. TCGA samples were genotyped using Affymetrix Genome-Wide Human SNP Array 6.0 and the data was downloaded from the TCGA Firehose at the MIT Broad Institute. The RNA-seq (Illumina) based expression data of 106 TCGA matched adjacent normal samples was quantified by FPKM and obtained from the UCSC Xena website. For the genotyping data, we also performed a systematic QC procedure and PCA, and 3 TCGA samples with Asian ancestry were removed. As a result, 103 TCGA samples (55 lung ADs and 48 SqCCs) remained in the following analysis.

For eQTL analysis, we performed a linear regression using the R package Matrix eQTL[30] with default parameters. We set gene expression as the outcome, and SNP genotype as the covariate of interest with adjustment for age, gender, smoking status, the top ten principal components, and somatic copy number status.

### Functional enrichment analysis of defined CRVs

To investigate the enrichment or depletion in chromatin modification peaks, we estimated the distribution of above CRVs in active promoter and enhancer regions identified in normal lung tissues, lung fibroblasts (NHLF), and lung cancer (A549) cell lines by using Variant Set Enrichment (VSE)[31]. The same analysis was performed in three lung fibroblasts (IMR90, HPF and AG04450) and A549 cell lines to evaluate the overrepresentation of CRVs in DNase I hypersensitive sites (DHS) regions or TFBS. All the histone modification peaks of promoter and enhancer marks (H3K4me3, H3K9ac, H3K4me1 and K3K27ac), DHS, and TFBS data were downloaded from the UCSC Genome Browser.

### Gene-based analysis

We performed gene-based analysis with genome-wide gene-based association study (GWGAS) in MAGMA[32]. The *P* values from the GWAS meta-analyses for lung cancer, lung AD and SqCC were used as input, and all 19,427 protein-coding genes from the NCBI 37.3 gene definitions were used as the basis for GWGAS. We annotated all SNPs in our genome-wide meta-analyses to above genes, resulting in 18,233, 18,233 and 18,232 protein-coding genes that were represented by at least one SNP in the NSCLC, lung AD or SqCC meta-analyses, respectively. Genes with Benjamini-Hochberg (BH) adjusted *P* value <0.05 were considered as significant.

### Driver gene enrichment analysis

A gene was considered as lung cancer somatic driver gene if met one of the following criteria: (1) the gene was included in the COSMIC Cancer Gene Census (v78) and showed evidence to be lung cancer related[33]; (2) the gene was categorized as lung cancer-related mutational drivers, somatic copy number alteration (SCNA) drivers, or fusion drivers in

the IntOGen database[34]; or (3) the gene was identified as significantly mutated genes or SCNA-related genes in recent whole-genome or whole-exome sequencing studies[23, 35–39]. Finally, we listed a total 374 coding genes and 4 non-coding genes as lung cancer somatic driver genes. To evaluate the enrichment of these genes in our defined lung cancer target genes, we first re-scored genes implicated by INQUISIT, and then examined the overlap between this list of drivers and the target genes with different levels of evidence and performed fisher exact test to obtain the significance. To avoid the bias of non-coding genes, only protein-coding genes were included in this analysis.

### Pathway enrichment analysis

We performed pathway enrichment analysis on the above defined candidate target genes as well as genes identified by gene-based analysis to evaluate their potential function in the development of lung cancer. The analysis was conducted with the Reactome Pathway Database[40] using R package clusterProfiler[41] and pathways with BH adjusted  $P$  value  $<0.05$  were retained.

### Gene-Set Enrichment Analysis (GSEA)

The immune infiltration proportions[42] and homologous recombination deficiency (HRD) [43] index of TCGA lung AD and lung SqCC samples were downloaded from previously published studies. We first calculated the correlation coefficients between the proportions of six types of immune cells (B cell, CD4 T cell, CD8 T cell, Neutrophils, Macrophages and Dendritic cells) or HRD index and the expression of all protein-coding genes. Then, a ranked list of correlation coefficient was analyzed by Gene-Set Enrichment Analysis (GSEA) with our predefined lung AD and SqCC genes. This analysis was performed with R package clusterProfiler[41].

### Statistical analyses

Detailed description for the meta-analysis of GWAS data from Chinese and European populations was provided in our previous study[4]. Briefly, the association testing for each variant was performed using the SNPTEST software (v2.5.4) with adjustment for age, gender, and the principal components. Meta-analysis was performed with the fixed-effects inverse variance-weighting approach by using METAL [44]. Genetic variant with  $R^2 \geq 75\%$  or  $P$  value for Cochran's Q statistic  $\leq 1.0 \times 10^{-4}$  was considered with a high degree of heterogeneity, and was excluded from further analysis [45, 46]. The LD coefficients ( $R^2$  and  $D'$ ) was calculated with PriorityPruner, and the genotyping data of the East Asian and European populations from the 1000 Genomes Project (the Phase III integrated variant set release) were set as the reference. General analyses were performed with R software (version 3.5.1). All statistical tests were two-sided.

## Results

### Definition of credible risk variants for non-small cell lung cancer

We performed overall and histological GWAS meta-analyses (Figure 1A, Table 1). In order to clarify potential functional signals, we first defined 67 index SNPs based on the following criteria: (1) 58 index SNPs represented for 81 previously reported SNPs ( $r^2 \geq$

0.6) (Table S1); and (2) 9 independent index SNPs with a meta  $P$  value  $<1 \times 10^{-6}$  in our overall NSCLC or histological meta-analyses (Table S4). Of 9 SNPs with a meta  $P$  value  $<1 \times 10^{-6}$ , two were located in previously reported loci but showed weak LD ( $r^2 < 0.01$ ) with previously reported SNPs (6p22.1: rs1815741: OR=0.86, 95% CI=0.81–0.91,  $P=2.99 \times 10^{-7}$ ; 11q23.3: rs4938515: OR=1.07, 95% CI=1.04–1.10,  $P=3.46 \times 10^{-7}$ ), and the other seven SNPs included five for NSCLC (4p14: rs116205103: OR=0.83, 95% CI=0.77–0.89,  $P=1.82 \times 10^{-7}$ ; 15q24.1: rs76354137: OR=0.92, 95% CI=0.88–0.95,  $P=5.74 \times 10^{-7}$ ; 2q21.3: rs3217451: OR=0.90, 95% CI=0.87–0.94,  $P=7.35 \times 10^{-7}$ ; 4q27: rs35661893: OR=0.93, 95% CI=0.91–0.96,  $P=3.56 \times 10^{-6}$ ; 13q24: rs719739: OR=0.94, 95% CI=0.92–0.97,  $P=1.94 \times 10^{-5}$ ), one for AD (9q31.3: rs12006500: OR=1.12, 95% CI=1.07–1.16,  $P=8.43 \times 10^{-8}$ ), and one for SqCC (8p23.1: rs2945908: OR=0.89, 95% CI=0.85–0.93,  $P=7.92 \times 10^{-7}$ ) (Table S4, Figure S1).

Then, we defined additional CRVs for further functional annotation (SNPs with  $r^2 \geq 0.6$  with one of 67 independent index SNPs and within 500kb upstream or downstream of the corresponding index SNPs). Finally, we identified 3,064 CRVs in the following analysis, including 1,842 for NSCLC, 1,020 for AD and 220 for SqCC (Figure 1A).

### Enrichment analysis of NSCLC CRVs

Most of the defined CRVs were in the intronic (intronic and ncRNA intronic: 1489; 48.60%) or intergenic regions (intergenic, downstream and upstream: 1444; 47.13%) (Figure 1B). We systematically evaluated the enrichment of these variants in histone modification peaks. Interestingly, we observed a significant enrichment of NSCLC related CRVs in promoter-like (H3K4me3 and H3K9ac) and enhancer-like (H3K4me1 and H3K27ac) histone modification peaks in normal lung tissues, lung fibroblasts (NHLF) and lung cancer (A549) cell lines (Figure 1C), and most (7/11) of the enrichment degrees were greater for lung AD related CRVs (Figure 1C). Additionally, the defined CRVs were also enriched in DHS regions in lung fibroblasts (AG04450) and lung cancer (A549) cell lines (Figure 1C).

Then, we conducted TF enrichment analysis and strong signals were observed for the binding sites of ATF3, POLR2A, TCF12, MAX, YY1, CTCF, and MAFK in lung fibroblasts (IMR90 and AG04450) and lung cancer (A549) cell lines at the significant level of  $P_{BH} < 0.05$ . Of these TFs, CTCF (HPF, AG04450 and IMR90), RAD21 (IMR90) and USF1 (A549) were special for lung AD while TCF12 (A549) and CREB1 (A549) were for lung SqCC (Figure 1D).

Additionally, 22 of the 39 exonic variants were non-synonymous, including two nonsense and 20 missense variants (Table S5, Figure 1B). The index SNP rs11571833 (K3326\*, c.A9976T) in 13q13.1 was a nonsense variant in exon 27 of *BRCA2*. The T allele could significantly increase the risk of lung cancer (OR=1.50, 95% CI=1.26–1.78,  $P=4.63 \times 10^{-6}$ ). Of the missense variants, rs17121881 (c.T281A, I94N) in *AMICA1* was predicted with the highest CADD score (CADD score=23.5) and could lead to an isoleucine-to-asparagine change. Rs17121881 was in exon 3 of *AMICA1* and showed strong LD with the index SNP rs55768116 ( $r^2=0.78$ ) in 11q23.3. The A allele of rs17121881 could significantly increase the risk of lung cancer (OR=1.08, 95% CI=1.05–1.11,  $P=2.10 \times 10^{-9}$ ) as well as reduce the expression of *AMICA1* (GTEX:  $\beta=-0.10$ ,  $P=0.005$ ; NJLCC:  $\beta=-0.18$ ,  $P=0.02$ ). Interestingly, *AMICA1* also showed a decreased expression in both lung AD and SqCC

samples when compared to adjacent normal samples in TCGA (AD:  $P=1.03\times 10^{-17}$ ; SqCC:  $P=7.75\times 10^{-23}$ ) and NJLCC (AD:  $P=1.76\times 10^{-18}$ ; SqCC:  $P=6.58\times 10^{-18}$ ) data (Figure S2), suggesting a tumor suppressor role during the development of lung cancer.

### Systematic functional annotation of NSCLC CRVs

To link the candidate variants to genes, we then applied an extended INQUISIT functional annotation strategy and mapped CRVs to potential target genes by evaluating the impact on coding sequences, proximal promoter, and distal enhancer regulations (Figure 1A). Among all 3,064 CRVs, the coding impact evaluation strategy aligned CRVs to 25 genes, the proximal regulatory gene mapping strategy matched CRVs to 624 genes, and the distal regulatory gene mapping strategy annotated CRVs to 1,014 genes (Table S6). Above findings resulted in 1,047 unique mapped genes, among which 803 genes categorized as level 1 and 2 were considered as functional target genes and included in the following analysis (distal regulation strategy: 589 genes; proximal regulation strategy: 604 genes; coding impact strategy: 18 genes) (Figure 2A). Of these 803 genes, 395 were implicated by at least two mapping strategies, and 13 were implicated by all three (Figure 2B, Table S6). Additionally, 227 genes were implicated in lung AD samples while only 82 genes were in lung SqCC samples.

Of the newly identified genes, *CASP8* was in a locus defined in our previous study[4] and was implicated by all three mapping strategies. The index SNP rs3769821 at 2q33.1, located in the histone modification marks targeting both promoters and enhancers in A549 and NHLF cell types (Figure 2C–D), was confirmed as a *cis*-eQTL variant for *CASP8* in 383 GTEx lung tissues ( $P=1.09\times 10^{-37}$ ) (Figure 2E). Interestingly, we also identified a missense variant (rs3769823, c.41A>G, p.Lys14Arg), in strong LD ( $r^2=1$ ) with rs3769821, that was located in the first exon of *CASP8* isoform 7 (Figure 2D). This isoform was highly expressed in normal lung tissues and was found to be regulated by rs3769823 ( $P=2.39\times 10^{-10}$ ) (Figure 2F). Additionally, we identified *RAD52* as a lung SqCC related gene which was regulated by both distal and proximal elements (Figure S3). Rs11571376 (12p13.33) was located at the promoter region of *RAD52* and the C allele could significantly increase the risk of lung cancer (OR=1.10, 95% CI=1.05–1.14,  $P=2.27\times 10^{-5}$ ). Interestingly, C allele of rs11571376 was also associated with the expression of *RAD52* in normal lung tissues (beta=0.21,  $P=2.30\times 10^{-17}$ ), and the expression of *RAD52* was significantly elevated in lung SqCC samples (TCGA:  $P=1.71\times 10^{-4}$ ; NJLCC:  $P=6.27\times 10^{-5}$ ) (Figure S3). For lung AD, we also identified a novel gene *LIME1* with the highest INQUISIT score. Rs6122147, in strong LD with the index SNP rs41309931 ( $r^2=0.98$ ) in 20q13.3, was located in a distal enhancer element that physically interacted with the promoter of *LIME1* in A549 cell line (Figure S4A). The T allele of rs6122147 could significantly increase the risk of lung cancer (OR=1.06, 95% CI=1.02–1.11,  $P=4.42\times 10^{-3}$ ) and decrease the expression of *LIME1* in normal lung tissues ( $\beta=-0.14$ ,  $P=4.20\times 10^{-4}$ ) (Figure S4B).

### Gene-based analysis and driver gene enrichment analysis

To estimate the aggregated association of lung cancer, we performed GWGAS analysis using MAGMA and identified 154 lung cancer associated genes (Figure 3A, Table S7–9), of which 62 have been implicated in previous functional annotation analysis (Figure

2A). Of these 154 genes, 70 genes were specific for AD and 57 were specific for SqCC. When combined with 803 genes identified by INQUISIT, we implicated a total of 894 susceptibility genes for lung cancer (Figure 2A), of which 274 were specific for AD and 123 were for SqCC (Figure 3B).

Then, we evaluated the association between our defined target genes and previously known somatic driver genes (Table S10). To avoid the bias of non-coding genes, only protein-coding genes were included in this analysis. Among the genes implicated by INQUISIT (level 1–4), we observed an enrichment of our defined target genes in 374 established lung cancer somatic-driver genes (23 out of 374 genes, ER=1.95,  $P=0.005$ ), including *EGFR*, *CDKN2A*, *CHEK2*, and *TP53BP1*. The enrichment degree increased with the level of evidence (level 1: ER=14.05,  $P=6.90\times 10^{-5}$ ; level 2: ER=1.38,  $P=0.30$ ; level 3: ER=2.22,  $P=0.06$ ; level 4: ER=0.00,  $P=1.00$ ;  $P_{\text{trend}}=0.06$ ). Similar results were found when genes implicated by distal regulation (ER=1.97,  $P=0.005$ ;  $P_{\text{trend}}=0.95$ ), proximal regulation (ER=1.43,  $P=0.25$ ;  $P_{\text{trend}}=2.95\times 10^{-8}$ ), and coding impact (ER=4.64,  $P=0.08$ ;  $P_{\text{trend}}=0.13$ ) were included. For genes derived from gene-based analysis, we also observed a significant enrichment in lung cancer somatic-driver genes (ER=4.48,  $P=3.87\times 10^{-5}$ ). Additionally, four somatic drivers (*PTK6*, *CBL*, *MECOM*, and *SVEP1*) were implicated specially for lung AD (ER=2.48,  $P=0.09$ ), but no lung SqCC somatic drivers were detected.

### Pathway enrichment analysis

To further explore biological pathways involved in the process of lung tumorigenesis, we performed pathway enrichment analysis of our defined target genes. To avoid the influence of non-coding genes, we only included 592 protein-coding genes in this analysis. The result revealed the involvement of 29 pathways ( $P_{\text{BH}}<0.05$ ) in the development of NSCLC, including 20 pathways related to immune function, such as PD-1 signaling ( $P=1.09\times 10^{-13}$ ) and interferon gamma signaling pathway ( $P=1.15\times 10^{-10}$ ), and six pathways in the neuronal system that related to nicotinic acetylcholine receptors, and three pathways in the DNA repair system (Figure 3C, Table S11). We also performed the same analysis for lung AD and SqCC genes, and identified that lung AD related genes were specifically enriched in immune related pathways (Figure 3D, Table S12), while lung SqCC genes were enriched in homologous recombination (HR)-related repair pathways (Figure 3E, Table S13), suggesting diverse mechanisms underlying the development of lung AD and SqCC.

Above findings indicated the importance of immune function and HR repair in the carcinogenesis of lung cancer. Thus, we evaluated the association of defined lung cancer genes with immune infiltration proportions and HRD index in TCGA lung AD and SqCC samples. Interestingly, we identified that lung SqCC genes were overrepresented in HRD-related genes (NES=1.33,  $P=0.05$ ) (Figure 3F) while lung AD genes were significantly overrepresented in four types of immune cells-related genes (B cell: NES=1.39,  $P=0.002$ ; CD4 T cell: NES=1.32,  $P=0.01$ ; CD8 T cell: NES=1.31,  $P=0.04$ ; Neutrophil: NES=1.19,  $P=0.10$ ; Macrophage: NES=1.02,  $P=0.44$ ; Dendritic: NES=1.46,  $P=0.002$ ) while lung SqCC genes not (B cell: NES=1.22,  $P=0.15$ ; CD4 T cell: NES=1.14,  $P=0.26$ ; CD8 T cell: NES=-1.06,  $P=0.29$ ; Neutrophil: NES=0.94,  $P=0.62$ ; Macrophage: NES=-1.22,  $P=0.17$ ; Dendritic: NES=1.00,  $P=0.50$ ) (Figure 3G–J).

## Discussion

In this study, we performed a combined strategy of large-scale genome-wide meta-analysis and functional annotation to identify biological significance of lung cancer susceptibility loci and implicated a total of 894 candidate target genes. These predisposition genes could modify the risk of lung cancer by both shared and histology-specific transcriptional (enhancers, promoters and TF) or translational (missense and nonsense variants) regulations. Furthermore, pathway enrichment and GSEA analysis indicated the importance of immune and HR-related DNA repair during the carcinogenesis process of lung AD and SqCC. These findings provided both a rich set of plausible gene targets for further functional studies and novel insights into understanding the biological underpinnings underlying the development of different histology types of lung cancer.

Consistent with previous studies, NSCLC CRVs were primarily mapped to the non-coding regions and showed a strong enrichment in CREs such as enhancer elements and histone modification peaks, suggesting that these variants contributed to the development of NSCLC mainly through transcriptional regulation. Interestingly, TFBS enrichment analysis implicated some pathology-specific TFs. CREB1 (cyclic AMP response element-binding protein) is a transcriptional coactivator which plays important roles in the differentiation of bronchial epithelial cells and is overexpressed in NSCLC samples[47]. Many studies have identified that CREB1 could be activated by nicotine exposure[48, 49], and the activated CREB1 recruits additional transcriptional machinery elements and leads to tumorigenesis[49]. Thus, CREB1 could be a possible target for the pathobiological process of smoking induced lung SqCC. As a member of the basic helix-loop-helix leucine zipper family, USF1 (upstream transcription factor 1) functions as a cellular transcription factor[50] and regulates the expression of SP-A[51]. SP-A is a lung-specific gene, especially for type II cells which is the origin of lung AD[51]. Thus, USF1 may regulate the risk of lung AD by modifying the expression of SP-A. Although exact roles of these TFs in the susceptibility to lung cancer have not been comprehensively studied, the overrepresented binding sites provides an improved understanding of the transcriptional regulation mechanism in NSCLC etiology.

In previous studies, the development of lung AD and SqCC were found to share many genetic factors[3, 4]. However, in recent years, accumulating evidence suggested that these two types of lung cancer also had large discrepancy in terms of both germline variations and somatic alterations[18]. In this study, we identified a set of predisposition genes both shared by lung AD and SqCC and specific to each histology. One of the most interesting result is the identification of *CASP8* in 2q33.1, a NSCLC risk locus reported in our previous study[4]. *CASP8* encodes caspase 8, which is a multivalent controller of innate immune signaling and inhibits inflammasome activation in dendritic cells, interferon-regulatory factor 3 activation and pro-inflammatory cell death[52]. *CASP8* has been implicated as a susceptibility gene for multiple cancers except for lung cancer[53, 54]. In this study, we identified that the expression of *CASP8* is regulated by both a cis-eQTL in the enhancer element and a missense variant in the major isoform, suggesting a potential joint modification mechanism by which the susceptibility variants affect NSCLC risk[55]. Additionally, we identified some pathologically specific genes, such as *LIME1* for AD and

*RAD52* for SqCC. *LIME1* encodes a membrane raft-associated adaptor protein which is an organizer of immunoreceptor signaling[56] and involves in CD4 and CD8 coreceptor signaling[57]. Thus, we speculate that *LIME1* might regulate lung AD risk by modifying immune response. *RAD52* is a key member in HR repair[58], which functions in DNA repair during S phase of the cell cycle[59] and act as a regulator of genomic stability[60]. In this study, we identified that *RAD52* expression was elevated by cis-eQTLs in both proximal and distal regulatory elements in lung SqCC samples, suggesting a tumor-promoting role of *RAD52* in the development of lung SqCC[61]. Above results indicate an important role of immune response during lung AD carcinogenesis as well as HR deficiency during lung SqCC carcinogenesis.

Interestingly, pathway enrichment and GSEA analysis also provided evidence for the importance of immune system and HR-related DNA repair during the development of lung AD and SqCC. Tumor-prompting inflammation is defined as a tumor-enabling hallmark of cancer[62] and has previously been implicated in lung cancer[63, 64]. Our recent work also provided evidence for the specific association of immune infiltration with lung AD, and identified that inflammatory microenvironments formed in the early stage of lung AD[23], suggesting that immune infiltration occurring in the initial stage is the major risk factor for lung AD. As an indication of genomic instability[65], elevated somatic HRD level has also been reported in lung SqCC[43]. The strong association between lung SqCC susceptibility genes and HRD observed in this study provided additional evidence that the somatic differences may have genetic ancestry origins and could regulate lung SqCC risk. As cigarette smoking is the major causal factor for lung SqCC[66], we propose that the continuous exposure to tobacco may directly lead to an increased level of HRD[66], and finally lead to lung SqCC. Thus, the use of PD1/PD-L1 and PARP inhibitors, which target immune infiltration and HRD[67, 68], implicate significant potential for the prevention of lung AD and SqCC.

However, the interpretation of our findings needs to be considered within the limitations of the study. First, the aggregation of susceptibility variants identified in both European and Chinese populations prevented us from capturing the genetic heterogeneity within different ethnic and individuals. Second, as the annotation data used in this study were obtained from different sources of tissues and cell types, further biological experiments are needed to elucidate the exact molecular mechanisms of these variants underlying the development of lung cancer.

In conclusion, by integrating GWAS information, in-house and publicly available biological data, we illustrate the molecular basis of both well-studied and newly identified lung cancer susceptibility loci and provide novel insights into the understanding of genetic heterogeneity between lung AD and SqCC, which may serve as guides for post-GWAS functional experiments and clinical drug target testing.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

<b>NSCLC</b>	non-small cell lung cancer
<b>AD</b>	adenocarcinoma
<b>SqCC</b>	squamous cell carcinoma
<b>GWAS</b>	genome-wide association study
<b>SNP</b>	single-nucleotide polymorphism
<b>ENCODE</b>	Encyclopedia of DNA Elements
<b>FANTOM</b>	Functional Annotation of the Mammalian Genome
<b>CRE</b>	cis-regulatory element
<b>NJMU</b>	Nanjing Medical University
<b>GSA</b>	Global Screening Array
<b>MAF</b>	minor allele frequency
<b>HWE</b>	Hardy-Weinberg equilibrium
<b>LD</b>	linkage disequilibrium
<b>CRV</b>	credible risk variant
<b>TFBS</b>	transcriptional factor binding sites
<b>TF</b>	transcription factor
<b>eQTL</b>	expression quantitative trait loci
<b>GTE<sub>x</sub></b>	Genotype-Tissue Expression
<b>TCGA</b>	The Cancer Genome Atlas
<b>NJLCC</b>	Nanjing Lung Cancer Cohort
<b>TAD</b>	topologically associating domain
<b>TPM</b>	Transcripts Per Million
<b>FPKM</b>	Fragments Per Kilobase Million

<b>TSS</b>	transcription start site
<b>CADD</b>	Combined Annotation Dependent Depletion
<b>FATHMM</b>	Functional Analysis through Hidden Markov Models
<b>SIFT</b>	Sorting Tolerant from Intolerant
<b>QC</b>	quality control
<b>PCA</b>	principal component analysis
<b>NHLF</b>	lung fibroblasts
<b>VSE</b>	Variant Set Enrichment
<b>DHS</b>	DNase I hypersensitive sites
<b>GWAS</b>	genome-wide gene-based association study
<b>BH</b>	Benjamini-Hochberg
<b>SCNA</b>	somatic copy number alteration
<b>HRD</b>	homologous recombination deficiency
<b>GSEA</b>	Gene-Set Enrichment Analysis
<b>HR</b>	homologous recombination

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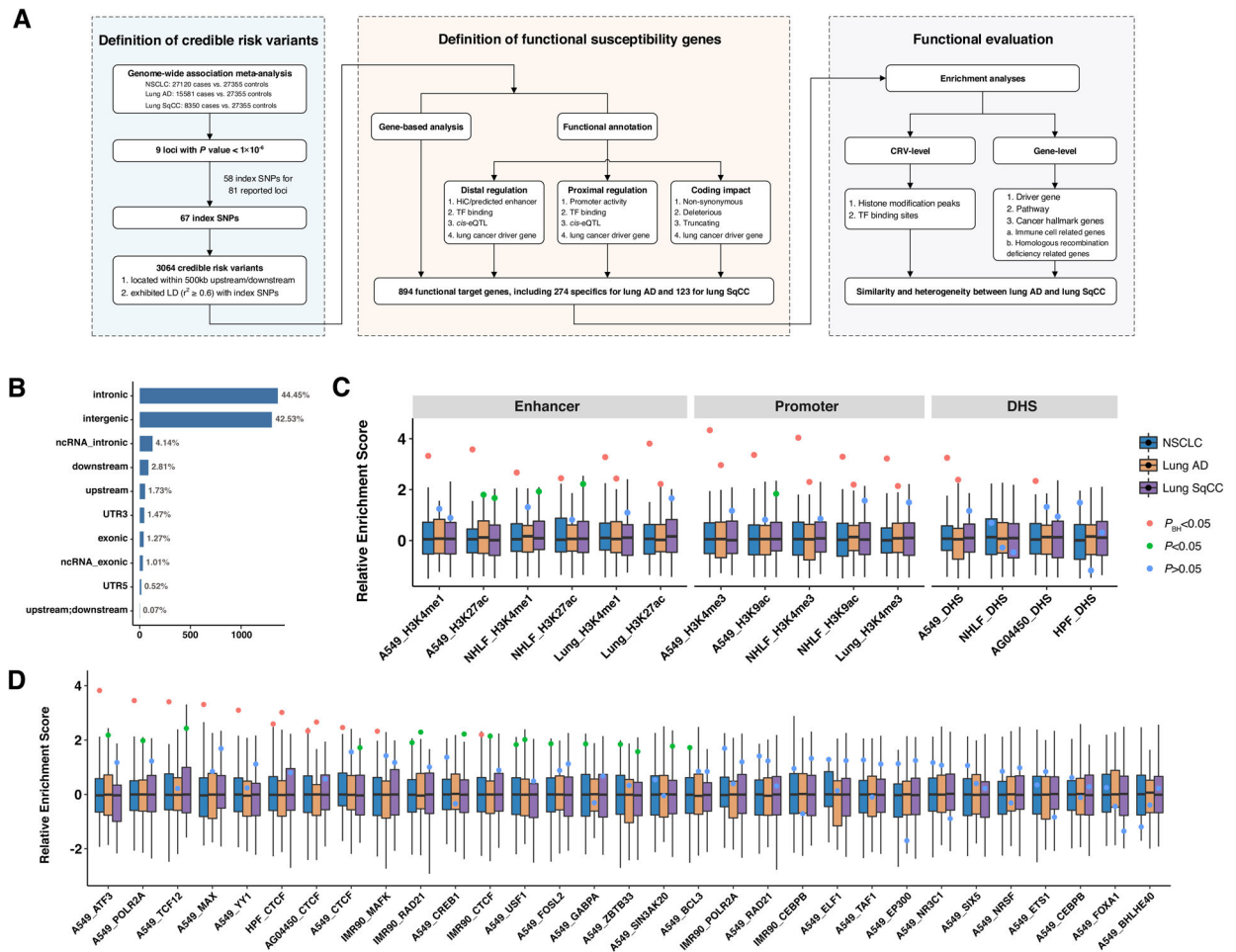
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**Figure 1. Functional evaluation of 3,064 lung cancer related credible risk variants (CRVs) defined in this study.**

A. Flowchart for the study design.

B. Genomic distribution of 3,064 lung cancer CRVs.

The x-axis indicates the number of CRVs included in the genomic region type.

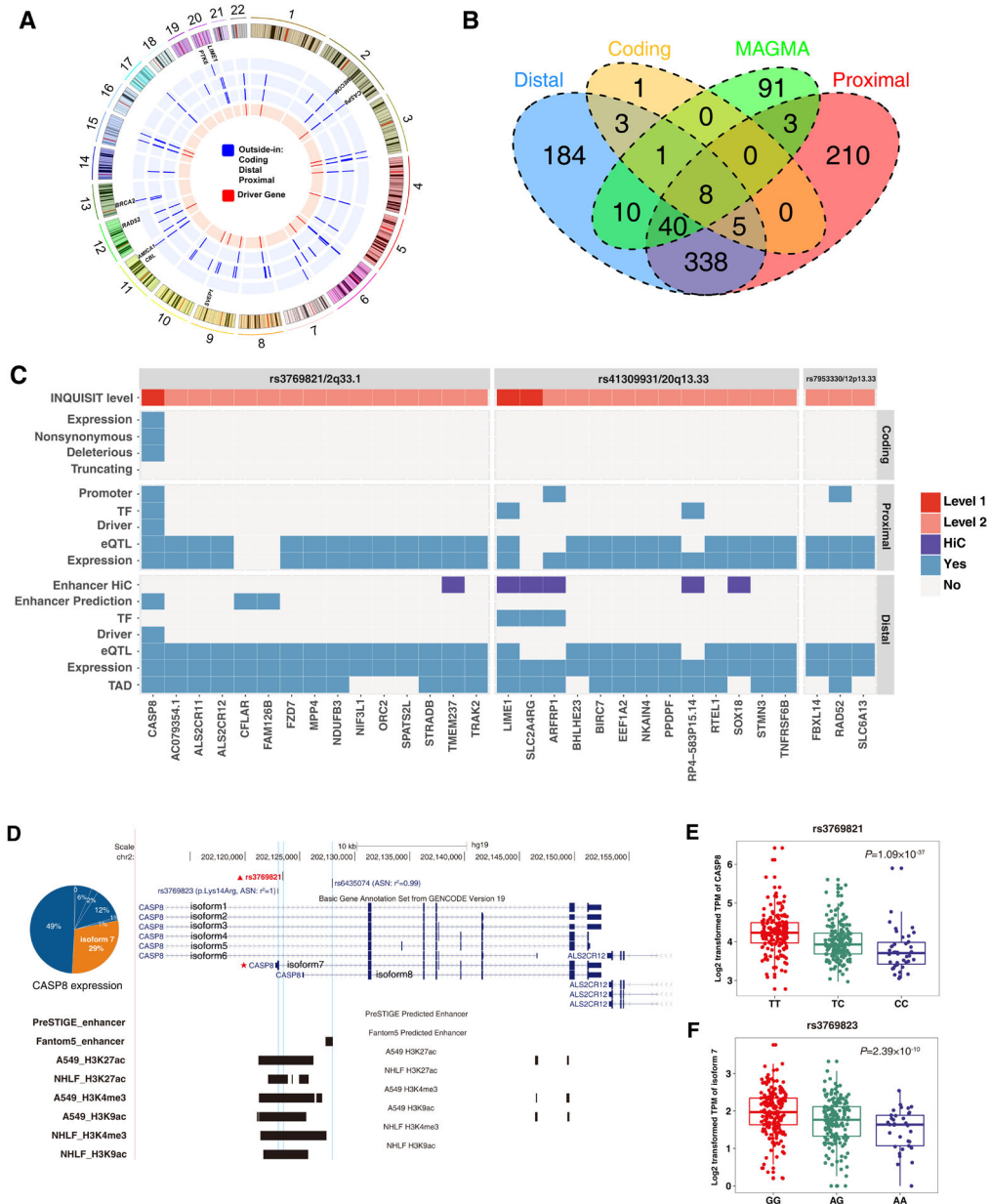
C. Enrichment of defined lung cancer CRVs (1,020 for lung AD and 220 for SqCC) in histone modification peaks and DNase I hypersensitive sites.

The x-axis indicates different types of modification peaks in lung cancer cell line types.

A549, lung AD cell line; NHLF, lung fibroblasts cell line; AG04450 and HPF, lung fibroblasts cell lines; Lung, normal lung tissue.

D. Enrichment of defined lung cancer CRVs (1,020 for lung AD and 220 for SqCC) in transcriptional factor binding sites.

The x-axis indicates binding sites of different transcriptional factors. IMR90, lung fibroblasts cell line.



**Figure 2. Implicated lung cancer target genes by functional annotation.**

A. Circos plot showing 803 implicated genes by distal mapping, promoter mapping and coding mapping strategies.

Blue indicates the mapping strategy (from inside to outside: distal, promoter, and coding mapping) and red indicates if the implicated gene is a driver gene.

B. Venn diagram showing the number of overlapped genes implicated by distal mapping, promoter mapping, coding mapping strategies, and GWAS.

C. Detailed functional annotation results for three risk loci of lung cancer.

The x-axis indicates the implicated genes, and y-axis indicates the annotation evidence types.

D. Genomic region of *CASP8* in 2q33.1.

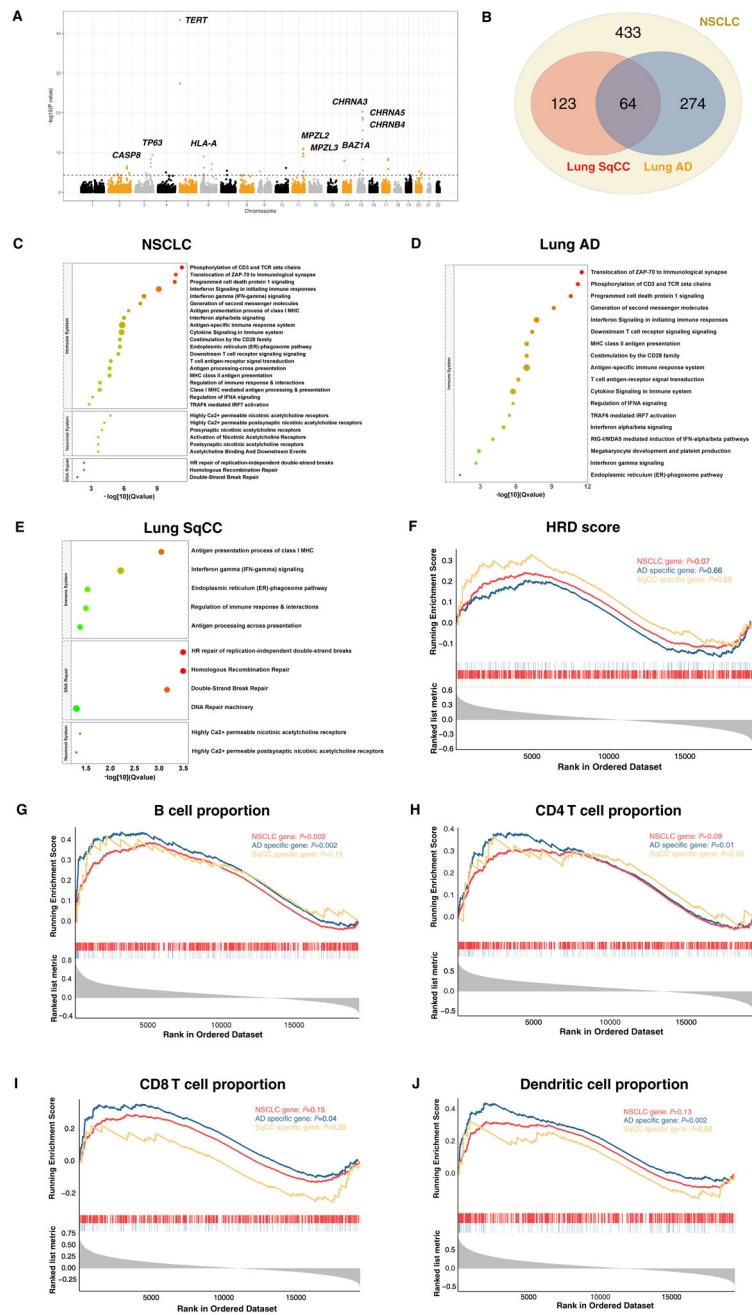
E-F. eQTL analysis of two CRVs (rs3769821 and rs3769823) and *CASP8* expression in 383 GTEx lung tissues.

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**Figure 3. Implicated lung cancer target genes by gene-based and pathway enrichment analyses.**

A. Manhattan plot of the GWGAS analysis for NSCLC meta-analysis.

The y axis shows the  $-\log_{10}$  transformed two-tailed  $P$  value of each gene from a linear model, and chromosomal position is shown on the x axis.

B. Venn diagram showing the overlap of genes implicated by INQUISIT and MAGMA in NSCLC, lung AD and SqCC.

C. Pathway enrichment analysis of all genes implicated by INQUISIT and GMAMA for NSCLC.

D. Pathway enrichment analysis of lung AD genes.

- E. Pathway enrichment analysis of lung SqCC genes.
- F. GSEA analysis of NSCLC, lung AD and SqCC genes with homologous recombination deficiency.
- G. GSEA analysis of NSCLC, lung AD and SqCC genes with B cell proportion.
- H. GSEA analysis of NSCLC, lung AD and SqCC genes with CD4 T cell proportion.
- I. GSEA analysis of NSCLC, lung AD and SqCC genes with CD8 T cell proportion.
- J. GSEA analysis of NSCLC, lung AD and SqCC genes with dendritic cell proportion.

**Table 1.**

Demographic characteristics of lung AD, SqCC and non-cancer controls included in this study

	NJMU project						TRICL-ILCCO OncoArray project					
	Lung AD		Lung SqCC		Control		Lung AD		Lung SqCC		Control	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
<b>Total</b>	8762	100.0	3860	100.0	13328	100.0	6819	100.0	4490	100.0	14027	100.0
<b>Age (Mean±S.D.)</b>	58.63±10.52		61.16±9.42		59.31±10.42		63.57±10.80		64.84±9.62		61.77±10.29	
<b>Gender</b>												
Male	4650	53.1	3470	89.9	8605	64.6	3626	53.2	3489	77.7	8638	61.6
Female	4112	46.9	390	10.1	4723	35.4	3192	46.8	1001	22.3	5386	38.4
Missing value	n/a	n/a	n/a	n/a	n/a	n/a	1	0.0	n/a	n/a	3	0.0
<b>Smoking status</b>												
Ever smoker	3364	38.4	3172	82.2	5606	42.1	5771	84.6	4276	95.2	9339	66.6
Life-long non-smoker	5397	61.6	688	17.8	7720	57.9	974	14.3	156	3.5	4412	31.5
Missing value	1	0.0	n/a	n/a	2	0.0	74	1.1	58	1.3	276	1.9

Abbreviations: AD, adenocarcinoma; SqCC, squamous cell carcinoma.

n/a: No patients with missing information.