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Original Research Article

LncRNA *RNF144A-AS1* gene polymorphisms and their influence on lung cancer patients in the Chinese Han population

Hongjiao Wu [a,b,c], Yuning Xie [a], Ang Li [a], Xiyao Liu [b], Liwen Guo [b], Fengjun Wu [b], Zhenbang Yang [d], Zhi Zhang [e], Xuemei Zhang [a,b,c,*]

[a] School of Public Health, North China University of Science and Technology, Tangshan, 063210, China
[b] College of Life Science, North China University of Science and Technology, Tangshan, 063210, China
[c] Hebei Key Laboratory of Occupational Health and Safety for Coal Industry, Tangshan, 063210, China
[d] School of Basic Medical Sciences, North China University of Science and Technology, Tangshan, 063210, China
[e] Affiliated Tangshan Gongren Hospital, North China University of Science and Technology, Tangshan, 063000, China

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ABSTRACT

Lung cancer is primarily classified as NSCLC, which is distinguished by a wide range of genetic variations. This study focused on *RNF144A-AS1*, a relatively unexplored lncRNA, to explore the impact of its genetic polymorphisms on the susceptibility to NSCLC. We detected *RNF144A-AS1* expression and its correlation with prognosis and clinical pathological features using bioinformatics analysis. The association between *RNF144A-AS1* polymorphism and NSCLC susceptibility was evaluated using case-control methods. This investigation featured a cohort of 700 NSCLC individuals and 700 healthy controls. The genotype of genetic variation was detected by PCR-RFLP and iMLDR, followed by subsequent calculation of *OR* and 95 % *CI*. Our data show that *RNF144A-AS1* exhibits high expression levels in LUAD tissues and its expression is closely linked to LUAD progression and prognosis. Carrier of *RNF144A-AS1* rs3806609 TT genotype increased NSCLC susceptibility compared to carrier of rs3806609 CC genotype ($OR = 2.21$, 95%$CI = 1.57–3.13$). Our study identifies *RNF144A-AS1* genetic variants as potential susceptibility markers in NSCLC. *RNF144A-AS1* promotes cell proliferation and migration in LUAD through the IFN-γ/JAK2/STAT1 signalling pathway. Collectively, these findings pave the way for developing targeted therapies and diagnostic tools based on *RNF144A-AS1* and its variants.

1. Introduction

Lung cancer is widely occurring cancer [1]. In recent years, there has been a rise in both the occurrence and death rate of lung cancer in China [2]. Lung adenocarcinoma (LUAD) represents a primary subtype within non-small cell lung cancer (NSCLC). In recent years, lung cancer treatment has seen notable advancements due to targeted therapy and immunotherapy; however, the prognosis and acquired drug resistance are still not optimistic. Lung cancer's onset and progression are determined by the intricate interplay between and genetic and environmental. Multiple genome-wide association study (GWAS) and research on candidate genes have revealed that genetic polymorphisms significantly affect an individual's genetic susceptibility and prognosis for lung cancer [3–5].

Long non-coding RNAs (LncRNAs) are found in the nucleus or cytoplasm, with poor interspecies conservation, and their expression typically exhibits spatial, temporal, and tissue-specificity patterns [6]. LncRNA can affect tumor cell phenotype by regulating the expression of genes related to tumor [7,8]. Antisense lncRNAs are the most common type of lncRNAs, which are the antisense lncRNA that are encoded by genes. For example, *AGAP2-AS1* has been identified as an oncogene that promotes the characteristics of lung cancer cells. It also inhibits cell apoptosis by suppressing tumor suppressors *LATS2* and *KLF213* [9]. The localization of antisense lncRNA determines its regulatory effects on target genes. For instance, *HOTAIR*, predominantly found in the nucleus, controls the cell cycle and boosts resistance to Gefitinib by recruiting the epigenetic regulator EZH2 to p16 and p21 [10]. Meanwhile, *SBF2-AS1*, primarily located in the cytoplasm, enhances *GRB2* expression by interacting with miR-362-3p in NSCLC [11].

SNPs in lncRNAs have an impact on many diseases [12,13]. LncRNA HOTAIR rs4759314, rs12826786 [14] and lncRNA PVT1 rs13254990 variants [15] have an impact on lung cancer susceptibility.

* Corresponding author. College of Life Science, North China University of Science and Technology, Tangshan, 063210, China.
E-mail address: zhangxuemei@ncst.edu.cn (X. Zhang).

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RNF144A-AS1, also known as *GRASLND*, inhibits the IFN-γ signalling pathway and is an important regulator of cartilage formation [16]. This pathway is involved in inhibiting lung cancer through mechanisms like JAK-STAT, Ras/Raf, and cell cycle arrest [17,18]. Elevated levels of *RNF144A-AS1* have been observed in cases of gastric cancer, promoting metastasis, angiogenesis, and proliferation [19]. The study also indicated that *RNF144A-AS1* function as a predictive indicator for cancer prognosis [20–22]. The relationship between genetic variations in *RNF144A-AS1* and cancer susceptibility has not been explored. This study investigated the influence of *RNF144A-AS1* SNPs on susceptibility to NSCLC through a case-control study. This study may help identify new biomarkers to identify individuals with higher susceptibility to lung cancer, which will affect cancer prevention and screening policies.

2. Materials and methods

2.1. Study populations in case-control

This study was case-control research involving 700 patients with NSCLC and 700 healthy individuals. All patients were from Tangshan GongRen Hospital without preoperative radiotherapy or chemotherapy. The controls came from individuals who underwent a physical examination in Tangshan city and had no history of tumor. General demographic and clinical information were gathered through medical records and questionnaires. All subjects provided informed consent and provided 2 ml of peripheral venous blood for genomic DNA extraction. The Ethics Committee of North China University of Science and Technology has granted approval for this study (approval number: 2019021).

2.2. Bioinformatics analysis

Transcriptome data and clinical data were obtained from 59 normal and 539 LUAD tissues from The Cancer Genome Atlas (TCGA). Differential lncRNA in LUAD was identified using the "DESeq" and "edgeR" R packages with criteria of $|\text{Log2FC}|\geq 2$ and $FDR<0.01$. The "ggplot2" package was used to visualize up-regulated and down-regulated lncRNA. Immune genes sourced from Molecular Signatures Database (MsigDB) were used for co-expression analysis of immune-related lncRNAs (IRlncRNAs), with screening criteria set at $|R|>0.4$. Cox regression analyses were carried out on differentially expressed IRlncRNAs (DEIRlncRNAs) to identify those linked to the prognosis of LUAD. Using "survival" package to study overall survival times of LUAD patients in two distinct groups and to assess the impact of DEIRlncRNAs on patient survival outcomes. The prognostic model's accuracy was assessed through the receiver operating characteristic (ROC) curve and the area under the curve (AUC).

Kaplan-Meier Plotter database was developed to assess survival rates associated with different genes in 21 different types of cancer [23]. The purpose of this study was to examine the impact of lncRNA on the survival rate of patients. "Limma" package was used to analyze *RNF144A-AS1* expression in normal and LUAD tissues. The evaluation of the association between *RNF144A-AS1* and clinicopathological features was conducted through the utilization of R wilcox.test. Expression data of for *RNF144A-AS1* (GSE19804 and GSE18842) were downloaded from Gene Expression Omnibus (GEO).

2.3. SNP selection and genotyping

Using Ensembl, dbSNP and lncRNASNP database, we screened the SNPs in *RNF144A-AS1* with a minimum allele frequency (MAF) greater than 0.05. The *RNF144A-AS1* rs3806609 C/T variant was analyzed through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The sense primer for PCR amplification is 5′-CGA GCA CTT GCC TTC TAA T-3′, the antisense primer is 5′- TGC AGA ACG ATT TTC ATC C-3′, and the amplification product is 140bp. In the 6 μl PCR reaction system, the upstream and downstream primers were 1 μmol each, 1 × Taq PCR mixture, and the genomic DNA was about 1∼20 ng. The PCR reaction procedure was pre-denaturation at 94 °C for 3min, and 35 cycles were performed at 94 °C 30s, 59 °C 30s, 72 °C 30s, and finally 72 °C for 5min. PCR products were digested with the restriction endonuclease *BsaJ I* (New England Biolabs, UK) in a 37 °C water bath, and then separated by electrophoresis on a 3.5 % agarose gel. The PCR product with rs3806609C allele produced 119bp and 21bp bands, while the product with the rs3806609T allele produced only a 140bp band.

Rs12373785, rs1375337, rs5829088 and rs6718980 were genotyped using improved multiplex ligation detection reaction (iMLDR) by Genesky Biotechnologies Inc (Shanghai, China). First, multiple PCR reactions were performed. In the 20 μl PCR reaction system, 2ul of 10 × PCR buffer, 2.4ul of dNTP (2.5 mM), 1.2ul of $MgCl_2$ (25 mM), 1.0ul of Probe Mix, 0.2ul of HotStarTaq (5U/ul), 5ul of ddH_2O and approximately 1 μl of genomic DNA (1∼20 ng) were used. The PCR reaction procedure was 95 °C 2min, 11 cycles × (94 °C 20sec, 65°C-0.5 °C/cycle 40sec, 72 °C 1min30sec), 24 cycles × (94 °C 20sec, 59 °C 30sec, 72 °C 1min30sec), 72 °C 2min. Multiple PCR product purifications were then performed by adding SAP enzyme (5U) and Exonuclease I enzyme (2U). The mixture was incubated in a warm bath at 37 °C for 1 h, followed by inactivation at 75 °C for 15 min. Next, 10x ligase buffer (1 μl), Labelp Mix (0.25 μl), Ligase Primer Mix(1uM) (0.4 μl), DNA ligase(50U/ul) (1 μl) were mixed with the purified multiple product (4 μl) and ddH_2O (3.35μl). The connection procedure is 38 cycles × (94 °C 1min, 56 °C 4min). After diluting the 1 μl connection product, it was combined with Liz500 size standard (0.5 μl) and Hi-Di (8.5 μl). The mixture was then denatured at 95 °C for 5 min before being loaded onto the ABI3730XL sequencer. The data obtained from the sequencer was processed using GeneMapper 4.1. Randomly select 10 % samples for genotyping again to determine consistency, and the genotyping repeatability was 100 %.

2.4. Cell culture and transfection

Human normal lung epithelial cells (BEAS-2B) and NSCLC cells (A549) were cultured using RPMI 1640 (GIBO, USA) supplemented with 10 % fetal bovine serum (GIBO, USA), l00U/ml penicillin and 100 μg/ml streptomycin (GIBO, USA). Cells were cultured in a 37 °C incubator containing 5%CO_2. Cells were seeded into 6-well plates. When the confluence of cells reached about 70%–80 %, transfection system was configured according to the instructions. Lipofectamine 2000 (Invitrogen, USA) was used to transfect overexpressed plasmids and Lipofectamine RNAiMAX (Invitrogen) was used to transfect siRNA.

2.5. The constructure of RNF144A-AS1 overexpressed vector and the synthesis of siRNA

The *RNF144A-AS1* overexpressed plasmid were synthesized by Genechem (Shanghai, China). Briefly, *RNF144A-AS1* (2388 bp) was amplified using primer pairs: 5′- ACGG GCCC TCTA GACT CGAG ACGC CATT CTCC TGCC TCAG CCTC CC -3′ and 5′- TTAA ACTT AAGC TTGG TACC CTTT CATT CAAC AAAT TTTT ATG -3'. The PCR product with *XhoI/KpnI* cutting site (underlined part in primers) was recombined into the GV219 vector to generate the *RNF144A-AS1* overexpression plasmid, which was verified by direct sequencing. GV219 vector was used as a control.

The siRNA was synthesized from Genepharma Technology (Shanghai, China). The sequences of si-RNF144A-AS1 were 5′-GCG CGC AGA UGA UGU GCA ATT-3′ and 5′-UUG CAC AUC AUC UGC GCG CTT-3'. The sequences of siRNA control were 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and 5′ACG UGA CAC GUU CGG AGA ATT-3'.

2.6. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific, USA) and the first strand of cDNA was synthesized

using reverse transcription kit (Thermo Fisher Scientific, USA). The qRT-PCR primers for *RNF144A-AS1* were 5 ′- GTG GAC ATC CTG AGA CCT GC-3′ and 5 ′- GTG TGT TCC GGT GTA GG-3’. For *GAPDH* amplification, the qRT-PCR primers are 5′-CTG GGC TAC ACT GAG CAC C-3′ and 5′-AAG TGGTCG TTG AGG GCA ATG-3’. The reaction mixture (5 μL) contains 0.2 μL each primer, 100 ng cDNA, 1 × SYBR Green Mix. The reaction procedure is pre-denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15s, 60 °C for 30s.

2.7. Cell viability assay

The effect of *RNF144A-AS1* on the proliferation of NSCLC cells was detected using CCK-8 kit. NSCLC cells (5000/well) from each group were inoculated into 96 well plates with 3 replicates in each group. At 24h, 48h and 72h time point, 10 μl CCK-8 reagent was added to each well and incubated for 1h. The absorbance at 450 nm was measured by Infinite 200Pro (Tecan).

2.8. Transwell assay

For cell migration and invasion assay, after transfected with over-expressed plasmid or siRNA, cells were collected and re-suspended in 1640 medium to 5×10^5 cells/mL. cell suspension (200 μL) was added to the upper chamber of transwell (migration: not coated with matrix glue; Invasion: coated with matrix glue), complete medium (600 μL) was added to the inferior chamber. After 24 h, remove the lower chamber and use a cotton swab to wipe off the cells that have not passed through the membrane. Cells in the low chamber were fixed with 4 % para-formaldehyde and stained with 0.1 % crystal violet. The migrated and invaded cells were observed under microscope (Olympus).

2.9. Western blot

A549 cells transfected with *RNF144A-AS1* overexpression plasmid for 48h were lysed and total protein was extracted. The protein samples were run on an 8 % or 10 % SDS polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) film. The PVDF membrane was sealed with 5 % skim milk powder for 2 h and incubated with each monoclonal antibody overnight at 4 °C. The membrane was cleaned with TBST, then incubated with the corresponding horseradish peroxidase (HRP) conjugate at room temperature for 1h, and then cleaned with TBST. Development of specific proteins using enhanced chemiluminescence (ECL) luminescence reagents. Anti-Beta Catenin (66379-1-Ig, 1:500), Anti-IFN Gamma (15365-1-AP,1:2000), Anti-STAT1 (82016-1-RR, 1:1000) from ProteinTech Group (Chicago, USA); Anti-JAK2 was purchased from Abcam (Cambridge, UK).

2.10. Statistical analysis

The statistical analysis was performed utilizing the SPSS (v23.0) software. Using χ^2 test to assess variation in the distribution of sex, age, and smoking status between two groups. Using logistic regression to analyze the impact of *RNF144A-AS1* SNPs on susceptibility to NSCLC. Evaluate the expression of RNF144A-AS1 in LUAD and adjacent normal tissues using a paired *t*-test. The relative expression quantity of qRT-PCR was calculated by $2^{-\Delta\Delta Ct}$ method. The two-tailed Student’s t-test was utilized for the analysis of the other experimental data.

3. Results

3.1. Subject characteristics in case-control study

According to the information provided in Table 1, there are no disparities in the sex and age distribution among the two groups. In the case group, there were 313 smokers (44.7 %), while in the control group, there were 192 smokers (27.4 %). Cumulative smoking distribution did not differ between the two groups ($P = 0.600$). There were 413 cases (59.0 %) of adenocarcinoma, squamous cell carcinoma for 264 cases (37.7 %), and other pathologic types for 23 cases (including 9 adeno-squamous carcinoma, 11 large cell carcinoma, and 3 bronchoalveolar carcinoma cases).

Table 1
Frequency distribution of select characteristics.

Variables	Controls (n = 700)		Cases (n = 700)		*P* value[a]
	No	(%)	No	(%)	
Gender					0.220
Male	439	62.7	461	65.9	
Female	261	37.3	239	34.1	
Age					0.914
≤60	399	57.0	400	57.1	
>60	301	43.0	300	42.9	
Smoking status					0.000
Non-smoker	508	72.6	387	55.3	
Smoker	192	27.4	313	44.7	
Pack year of smoking					0.600
≤30	118	61.5	185	58.2	
>30	74	38.5	128	40.3	
Histological types					
Adenocarcinoma	–	–	413	59.0	
Squamous cell	–	–	264	37.7	
Other carcinomas	–	–	23	3.3	

3.2. RNF144A-AS1 expressed in LUAD tissues and its impact on prognosis

Through DESeq differential analysis, a total of 1460 lncRNAs were identified, including 272 down-regulated and 1188 up-regulated lncRNA (Fig. 1A). Additionally, the edgeR package was used for differential analysis, and 1616 lncRNAs were screened, consisting of 206 down-regulated l and 1410 up-regulated) (Fig. 1B). A total of 1062 immune-related genes were extracted by MSigDB, and further analysis identified 4795 IRlncRNAs. Subsequently, the VennDiagram tool was utilized to overlap the groups of differentially expressed lncRNAs (DElncRNAs) and IRlncRNAs, and 687 DEIRlncRNAs were ultimately identified (Fig. 1C). Further determine whether these lncRNAs have an impact on LUAD patient prognosis by conducting an analysis using univariate cox regression. 178 lncRNAs were identified to have an impact on the overall survival of LUAD patients (Supplement Table 1). Through multivariate Cox regression analysis, 67 lncRNAs were ultimately selected as independent factors that may affect patient survival (Supplement Table 2). The ROC curve’s area for 3 years is 0.878, while for 5 years it is 0.863 (Fig. 1D and E). Evaluation of the impact on patient survival by dividing LUAD patients into high and low-risk groups revealed that individuals in the low-risk group had a longer survival rate compared to those in the high-risk group ($P < 0.0001$) (Fig. 1F). Effect of the expression of 67 lncRNA on LUAD patient prognosis was evaluated by the survival package. A total of 33 lncRNAs affected LUAD patient survival ($P < 0.05$), and further Kaplan-Meier Plotter analysis found that 7 lncRNAs affected patient survival. Patients with expression of these 7 lncRNAs had poor prognosis (Supplementary Fig. 1). Two lncRNA (*LINC01116* and *RNF144A-AS1*) were finally screened out by intersection of the above screening results. Both *RNF144A-AS1* and LINC01116 are lncRNAs that have been implicated in the progression of various cancers. Specifically, LINC01116 has been well-documented in lung adenocarcinoma [24,25], with studies showing that it is overexpressed in lung adenocarcinoma tissues, and its high expression is significantly associated with increased proliferation and metastasis. Additionally, LINC01116 has been reported to mediate gefitinib resistance in NSCLC cells by regulating IFI44 expression [26]. In contrast, the specific role of *RNF144A-AS1* in lung cancer has not been explicitly detailed in previous studies. However, its involvement in other cancer types suggests that *RNF144A-AS1* may also play a role in lung cancer, which we aimed to

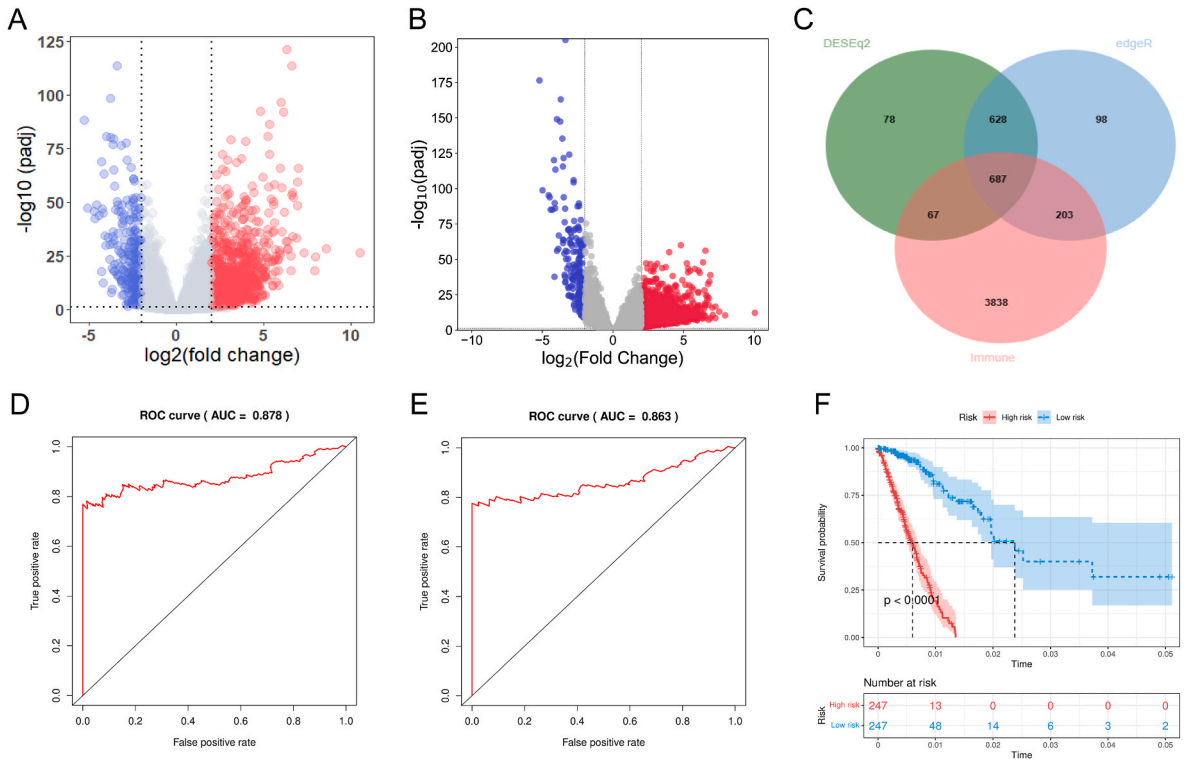

Fig. 1. LUAD related innate immune lncRNA prognostic model. **(A)** Differential lncRNAs were screened using the DESeq package. **(B)** Differential lncRNAs were screened using the edgeR package. **(C)** Venn diagram was drawn to select differentially expressed immune-associated lncRNAs. **(D)** ROC curve of 3-year survival rate. **(E)** ROC curve of 5-year survival rate. **(F)** Survival curve of LUAD patients in high and low risk groups.

investigate in this study.

Based on data from TCGA and GEO databases, it was observed that *RNF144A-AS1* expression in LUAD tissues was elevated compared to adjacent normal tissues ($P < 0.05$) (Fig. 2A-D). Patients exhibiting elevated levels of *RNF144A-AS1* demonstrate decreased survival rates compared to individuals with lower expression levels ($HR = 1.67$, 95% $CI = 1.31–2.13$) (Fig. 2E). *RNF144A-AS1* expression gradually increased from clinicopathological stage I to stage IV ($P = 0.006$) (Fig. 2F). We also conducted the longitudinal analysis tracking the expression of *RNF144A-AS1* and its potential effect on the prognosis of LUAD patients. Our findings indicate that *RNF144A-AS1* expression did not significantly affect the 1-year survival period ($HR = 1.41$, 95%$CI = 0.82–2.42$) (Fig. 2G). However, as the follow-up period extended, a higher expression of *RNF144A-AS1* was associated with a lower survival rate in LUAD patients (3-year survival: $HR = 1.45$, 95%$CI = 1.01–2.08$; 5-year survival: $HR = 1.50$, 95%$CI = 1.09–2.08$) (Fig. 2H and I).

3.3. Association of RNF144A-AS1 Variants with the risk of NSCLC

The Ensembl database provides essential details about the SNPs involved (Table 2). In the control group, genotype distribution of all SNPs adheres to Hardy-Weinberg genetic equilibrium ($P > 0.05$), suggesting that the research objects are well representative. Genotype differences between the two groups were analyzed after adjusting for sex, age and smoking (Table 3). For the rs3806609 polymorphism, NSCLC has a higher likelihood of carrying at least one T allele in comparison to the CC genotype (TT: $OR = 2.21$, 95%$CI = 1.57–3.13$; CT: $OR = 1.54$, 95%$CI = 1.22–1.94$). Other *RNF144A-AS1* genetic variants had no effect on NSCLC susceptibility ($P > 0.05$).

3.4. Integration analysis of RNF144A-AS1 and NSCLC biomarkers

In this study, we constructed a prognostic model by analyzing 19 molecular targets relevant to NSCLC, along with *RNF144A-AS1*. Using univariate Cox regression analysis, we initially identified seven molecular targets associated with overall survival in LUAD (Fig. 3A). Further multivariate Cox regression analysis allowed us to refine the model, ultimately selecting *KRAS*, *ALK*, and *RNF144A-AS1* as independent factors influencing patient survival (Fig. 3B). Additionally, we also explored the impact of mutations in these molecular targets on *RNF144A-AS1* expression. Our results indicated that while *ALK* mutations did not affect *RNF144A-AS1* expression (Fig. 3C), *KRAS* mutations were associated with increased *RNF144A-AS1* expression (Fig. 3D). We identified copy number variations in *RNF144A-AS1* in lung adenocarcinoma, with approximately 28.9 % of cases exhibiting an increase in copy number. This amplification is strongly associated with elevated *RNF144A-AS1* mRNA expression (Fig. 3E). Our findings suggest that *RNF144A-AS1* may play a significant role in the development and progression of lung adenocarcinoma, particularly in the context of *KRAS* mutations.

Moreover, our analysis revealed that in patients with *KRAS*-mutated LUAD, high *RNF144A-AS1* expression is associated with poor prognosis, advanced disease, and lymph node metastasis (Fig. 3F-I). In contrast, in patients with non-mutated *KRAS* LUAD, *RNF144A-AS1* expression had no significant effect on prognosis, while advanced disease, lymph node metastasis, and distant metastasis remained independent predictors of poor outcomes (Fig. 3J-M). These findings suggest that *RNF144A-AS1* may play an important role in the progression and prognosis of *KRAS*-mutated LUAD and could be a valuable component of a multi-marker prognostic model.

3.5. RNF144A-AS1 expression promotes the proliferation, migration and invasion of NSCLC cells

Subsequently, we investigated the role of *RNF144A-AS1* in the growth of NSCLC cells. By detecting the expression of *RNF144A-AS1* in lung normal epithelial cells (BEAS-2B) and lung adenocarcinoma cells (A549), we found that *RNF144A-AS1* was highly expressed in A549 cells

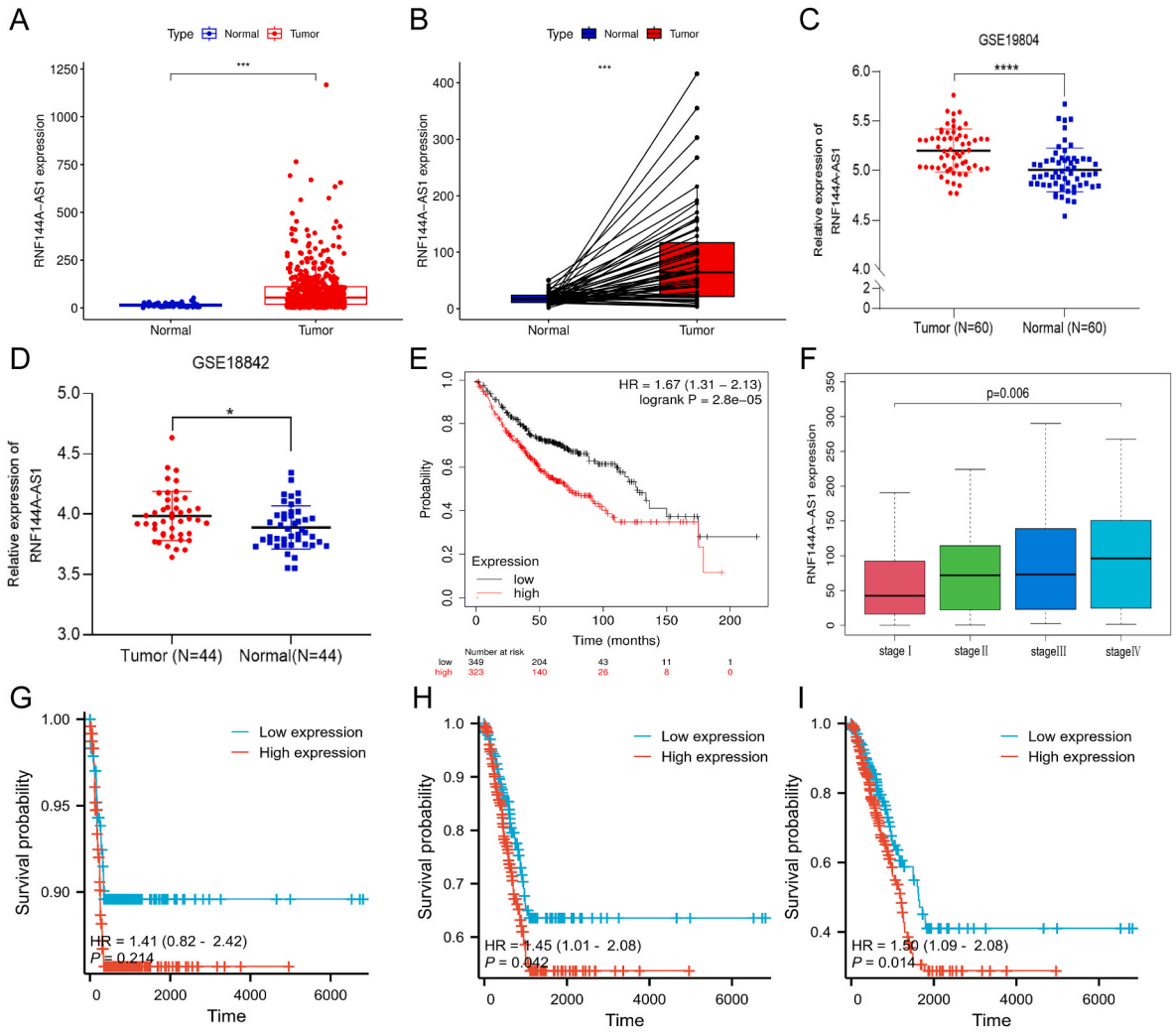

Fig. 2. Expression and clinical significance of *RNF144A-AS1* in LUAD. **(A)** *RNF144A-AS1* is highly expressed in LUAD tissues. **(B)** *RNF144A-AS1* is highly expressed in paired LUAD tissues. **(C–D)** The high expression of *RNF144A-AS1* in LUAD tissue was verified by GEO data sets. **(E)** The expression of *RNF144A-AS1* affects the prognosis of LUAD patients. **(F)** The expression of *RNF144A-AS1* affects the clinicopathological stage of LUAD patients. **(G–I)** Longitudinal analysis of the potential effect of RNF144A-AS1 expression on prognosis in patients with LUAD. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Table 2
General information of SNPs and Hardy–Weinberg test.

SNP	Allele gene	MAF	*P* value
rs1375337	G/A	0.22	0.464
rs6718980	T/G	0.36	0.676
rs3806609	T/C	0.41	0.781
rs5829088	Del/T	0.45	0.117
rs12373785	T/A	0.11	0.348

(Fig. 4A). After transfection of *RNF144A-AS1* siRNA, *RNF144A-AS1* was decreased in A549 cells (Fig. 4B). Transfected with *RNF144A-AS1* plasmid, *RNF144A-AS1* was significantly increased by 26.23 times in A549 cells (Fig. 4C). To determine whether *RNF144A-AS1* affects the proliferation of A549 cells, we performed CCK-8 detection and found that knockdown of *RNF144A-AS1* inhibited A549 cell proliferation (Fig. 4D), while overexpression of *RNF144A-AS1* promoted A549 cell proliferation (Fig. 4E).

In addition, we investigated the effects of *RNF144A-AS1* on migration and invasion of NSCLC cells. Transwell migration experiment showed that the number of cells passing through the chamber was significantly higher in *RNF144A-AS1* overexpression group than that in the control group, whereas the number of cells passing through the

Table 3
Genotype frequencies of SNPs in *RNF144A-AS1* and their association with NSCLC.

Genotypes	Controls (n = 700)		Cases (n = 700)		*OR* (95%*CI*)[a]	*P* value
	No	(%)	No	(%)		
rs1375337						
GG	525	75.0	544	77.7	1.00(Ref)	
GA	165	23.6	146	20.9	0.88(0.68–1.14)	0.331
AA	10	1.4	10	1.4	0.96(0.39–2.36)	0.923
rs6718980						
GG	244	34.9	239	34.2	1.00(Ref)	
TG	334	47.7	330	47.1	0.95(0.75–1.21)	0.695
TT	122	17.4	131	18.7	1.11(0.81–1.51)	0.522
rs3806609						
CC	328	46.9	240	34.3	1.00(Ref)	
CT	300	42.8	347	49.6	1.54(1.22–1.94)	<0.001
TT	72	10.3	113	16.1	2.21(1.57–3.13)	<0.001
rs5829088						
T/T	562	80.3	572	81.7	1.00(Ref)	
Del/T + Del/Del	138	19.7	128	18.3	0.95(0.72–1.24)	0.540
rs12373785						
TT	652	93.1	668	95.4	1.00(Ref)	
TA + AA	48	6.9	32	4.6	0.66(0.42–1.06)	0.086

[a] Adjusted for age, gender, and smoking status.

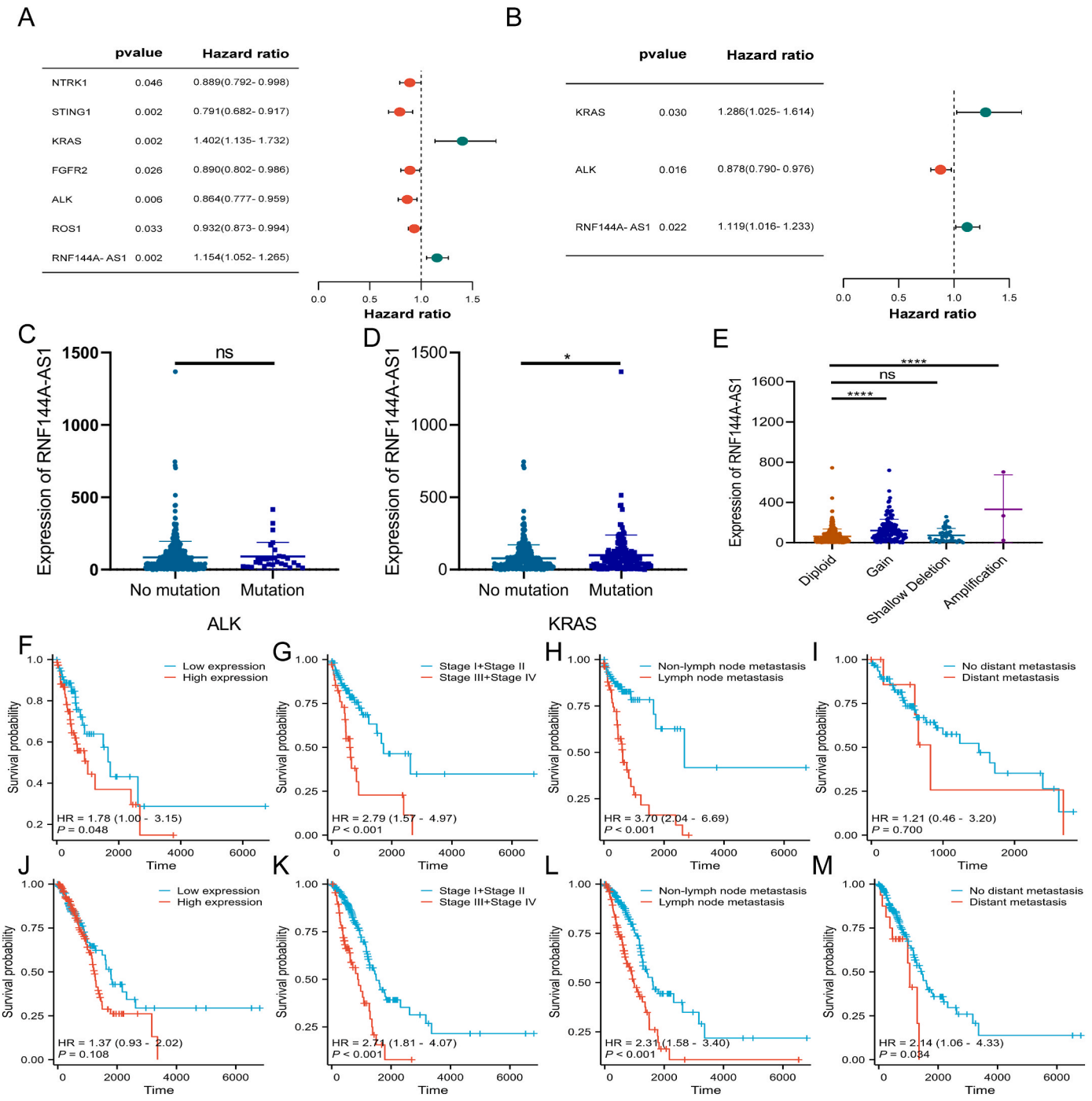

Fig. 3. Integration analysis of *RNF144A-AS1* and NSCLC biomarkers. **(A)** Univariate COX analysis of *RNF144A-AS1* and NSCLC biomarkers. **(B)** Multivariate COX analysis of *RNF144A-AS1* and NSCLC biomarkers. **(C)** Effect of *ALK* mutation on *RNF144A-AS1* expression. **(D)** Effect of *KRAS* mutation on *RNF144A-AS1* expression. **(E)** Effect of *RNF144A-AS1* copy number variation on its expression. **(F–I)** Independent predictors of prognosis were analyzed in LUAD patients with *KRAS* mutations. **(J–M)** Independent predictors of prognosis were analyzed in non-mutated *KRAS* LUAD patients.

chamber was significantly lower in *RNF144A-AS1* knockdown group than that in the control group (Fig. 4F and G). Transwell invasion assay showed that the overexpression of *RNF144A-AS1* promoted cell invasion, while knockdown of *RNF144A-AS1* inhibited cell invasion (Fig. 4F and G).

The expression of *RNF144A-AS1* is induced by TGF-β1 and hypoxia, suggesting that *RNF144A-AS1* may play a significant role in the tumor microenvironment. In this study, we used Western blot to detect the impact of *RNF144A-AS1* overexpression on the expression of key proteins in the IFN-γ/JAK2/STAT1 pathway. The results revealed that the expression levels of IFN-γ, JAK2, and STAT1 proteins decreased after overexpression of *RNF144A-AS1* (Fig. 4H). These findings indicate that *RNF144A-AS1* may influence the biological behavior of lung adenocarcinoma cells by regulating the IFN-γ/JAK2/STAT1 pathway.

4. Discussion

Lung adenocarcinoma is prevalent form of cancer. Patients with middle and advanced lung cancer often have poor prognosis, and the lack of LUAD diagnostic biomarkers are important reasons. LncRNAs are

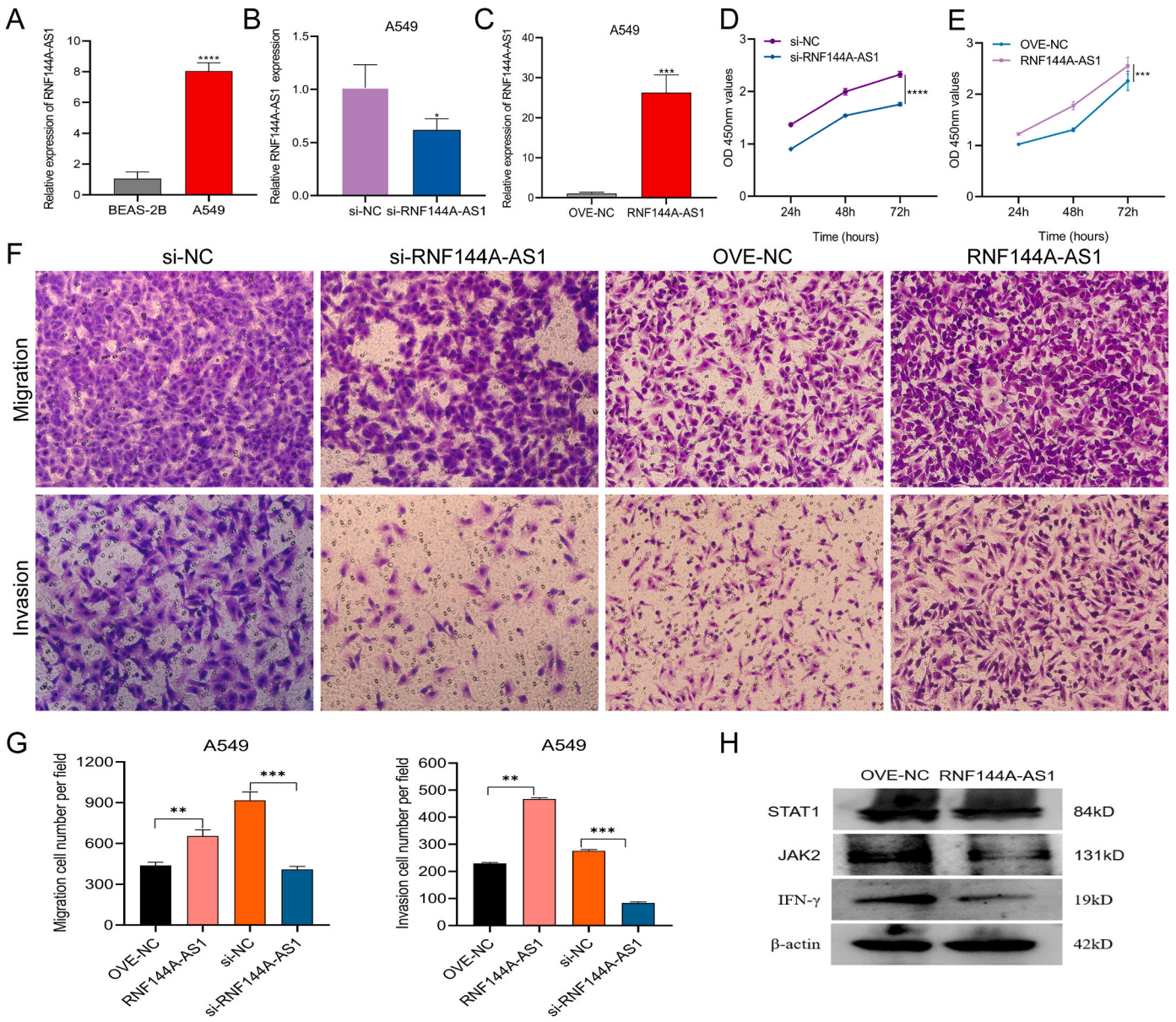

Fig. 4. Biological function of *RNF144A-AS1* in A549 cells. **(A)** Expression of *RNF144A-AS1* in BEAS-2B and A549 cells. **(B)** Detecting the knockdown efficiency of *RNF144A-AS1* in A549 cells. **(C)** Assessing the Overexpression Efficiency of *RNF144A-AS1* in A549 Cells. **(D)** Effect of *RNF144A-AS1* knockdown on proliferation of A549 cells. **(E)** Effect of overexpression of *RNF144A-AS1* on proliferation of A549 cells. **(F–G)** Effects of *RNF144A-AS1* on migration and invasion of A549 cells. **(H)** Effect of overexpression of *RNF144A-AS1* on key protein expression of IFN-γ/STAT1/JAK2 pathway.

important in the development of cancer [27,28]. There are many abnormally expressed lncRNA in LUAD, which play a certain role in diagnosis and prognosis prediction as a patient-specific biomarker of LUAD [29,30]. LncRNAs are involved in regulating immune cell function and immune response. IRlncRNAs have been significantly linked to the prognosis of various malignant tumors, such as LUAD. This indicates their promise as potential markers for early diagnostic and targets for treatment [31–33]. In this study, we focused on the differentially expressed immune-related lncRNA and constructed a prognostic model that effectively predicts the prognosis of LUAD.

Our data provides compelling evidence that *RNF144A-AS1* was markedly upregulated in LUAD tissues. This finding aligns with emerging researches which highlighting the role of lncRNA in cancer biology [8]. High expression of *RNF144A-AS1* seems to play a key factor contributing to the poor prognosis of lung adenocarcinoma patients, which suggests that *RNF144A-AS1* could be a critical player in the progression of this malignancy. Patients exhibiting higher levels of *RNF144A-AS1* might be at an increased risk of adverse outcomes, which might guide more personalized treatment strategies. Our longitudinal analysis of *RNF144A-AS1* expression and its impact on LUAD patient prognosis reveals a time-dependent relationship. In the short term, specifically within the first-year post-diagnosis, we found that *RNF144A-AS1* expression levels did not significantly influence patient survival. However, as the follow-up period extended, a clear association emerged between higher *RNF144A-AS1* expression and poorer patient outcomes. The increasing prognostic significance of *RNF144A-AS1* over time aligns with the concept of tumor evolution and the accumulation of genetic alterations during cancer progression [34]. By highlighting the time-dependent nature of *RNF144A-AS1*'s impact on LUAD prognosis, these findings support the use of longitudinal approaches in biomarker studies [35].

Our study's construction of a prognostic model for LUAD, integrating *RNF144A-AS1* with established molecular targets, provides novel insights into the complex interplay of genetic factors influencing patient

outcomes. The identification of *KRAS*, *ALK*, and *RNF144A-AS1* as independent prognostic factors through rigorous statistical analyses underscores the potential of this lncRNA as a valuable addition to existing biomarker panels in LUAD. Our results demonstrate that mutations in *KRAS*, but not *ALK*, are associated with increased *RNF144A-AS1* expression, highlighting the importance of *KRAS* in the regulation of this lncRNA. *KRAS* is one of the most frequently mutated oncogenes in LUAD, and its mutations are often associated with poor prognosis and resistance to targeted therapies [36,37]. Stratifying patients by *KRAS* mutation status highlighted the prognostic significance of *RNF144A-AS1* expression. The positive correlation between *KRAS* mutations and *RNF144A-AS1* expression suggests a potential functional relationship between these two factors. It is possible that *RNF144A-AS1* might be involved in the downstream signaling pathways of mutant *KRAS* or contribute to the aggressive phenotype associated with *KRAS*-mutant LUAD. Conversely, the lack of association between *ALK* mutations and *RNF144A-AS1* expression indicates that the prognostic value of *RNF144A-AS1* might be independent of *ALK* status.

RNF144A-AS1 has been shown to regulate the miR-30c-2-3p/LOX pathway, thereby promoting tumor metastasis, proliferation, and angiogenesis [19]. Additionally, *RNF144A-AS1* has been found to enhance *HMGA1* expression by targeting miR-665, contributing to glioma cell development [38]. Although *RNF144A-AS1* has been implicated in various types of tumors, its role in NSCLC, particularly in LUAD, remains largely unexplored. In the present study, we investigated the effects of *RNF144A-AS1* on the biological functions of LUAD cells. Our results demonstrate that overexpression of *RNF144A-AS1* promotes the proliferation, migration, and invasion of LUAD cells, whereas knockdown of *RNF144A-AS1* inhibits these processes. These findings suggest that *RNF144A-AS1* may be a key player in the progression of LUAD and that targeting this lncRNA could disrupt cancer progression pathways, offering a novel approach to treatment. The limitation of our study is the absence of in vivo experiments to validate *RNF144A-AS1*'s role in lung cancer progression. While our in vitro findings demonstrate that *RNF144A-AS1* promotes cell proliferation and migration in lung adenocarcinoma through the IFN-γ/JAK2/STAT1 signaling pathway, these results are confined to cell culture models. In vivo studies would provide a more comprehensive understanding of *RNF144A-AS1*'s effects on tumor growth and metastasis in a complex biological environment. Future research should incorporate animal models to strengthen the causal link between *RNF144A-AS1* and lung cancer, thereby enhancing the clinical relevance of our findings.

There is currently no research published on *RNF144A-AS1* polymorphism, various suggest its involvement in cancer occurrence, progression, and prognosis. Studies have indicated that certain lncRNA SNPs are linked to an increased risk of cancer and could serve as potential indicators for predicting cancer risk. These SNPs may occur in lncRNA transcripts or across regions, which may alter the structure, and expression of lncRNA and further contribute to cancer progression [39]. *MALAT1* rs3200401 polymorphism is related to drug efficacy and side effects, and it can serve as an indicator of drug toxicity [40]. The genetic susceptibility to NSCLC might be linked to the mutation rs7248320 in the LncRNA AC008392.1 [41]. The genetic variations of *PRNCR1* rs1456315 and *CCAT2* rs6983267 have been linked to an increased susceptibility to the development of lung cancer [42]. In our research, we chose specific genetic variations of *RNF144A-AS1* (rs1375337, rs6718980, rs3806609, rs5829088, and rs12373785) to investigate their risk of susceptibility to lung cancer. We demonstrated that individuals carrying *RNF144A-AS1* rs3806609 C > T variant increased the risk, while other genetic variations did not affect the susceptibility of patients to lung cancer.

The limitation of our study is the lack of functional assays specifically addressing how *RNF144A-AS1* polymorphisms, such as rs3806609, contribute to NSCLC at the molecular level. The rs3806609 polymorphism is located within an intronic region of *RNF144A-AS1*, making it challenging to directly assess its impact on the lncRNA's function. Future research should incorporate advanced techniques, such as allele-specific expression analysis or chromatin interaction assays, to investigate the potential regulatory effects of intronic polymorphisms on lncRNA function. Our research is still limited to the polymorphisms of *RNF144A-AS1* in lung cancer and has not yet explored broader range of genetic variations or other cancer types. Although we conducted copy number analyses for *RNF144A-AS1* in NSCLC, these efforts do not capture the full range of potential genetic alterations that could impact its function. Future studies should expand the scope to include a wider array of genetic variations, such as structural variants, or epigenetic changes, to provide a more comprehensive understanding of *RNF144A-AS1*'s role in cancer. Furthermore, investigating *RNF144A-AS1* across various cancer types would help determine whether similar genetic alterations are relevant to different malignancies.

Our case-control study offers strong evidence for the role of *RNF144A-AS1* in lung cancer susceptibility. However, the study's focus on the Chinese Han population limits the generalizability of our findings. Genetic variations across different populations may affect the applicability of these results to other ethnic groups. Future studies should include more diverse populations to validate and expand upon these findings, ensuring broader relevance and enhancing the potential for clinical application on a global scale.

CRediT authorship contribution statement

Hongjiao Wu: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yuning Xie:** Methodology, Data curation. **Ang Li:** Software, Methodology. **Xiyao Liu:** Validation, Methodology. **Liwen Guo:** Methodology. **Fengjun Wu:** Methodology. **Zhenbang Yang:** Software. **Zhi Zhang:** Supervision, Investigation. **Xuemei Zhang:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization.

Conflict of interest Statement

The authors affirm that they have no conflicts of interest.

Ethical approval

The Ethics Committee of North China University of Science and Technology approved all study procedures.

Consent

Informed consents were obtained from all individual participants included in the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.10.008.

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