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NCAPH Is a Prognostic Biomarker and Associated With Immune Infiltrates in Lung Adenocarcinoma

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Research Article

Keywords: NCAPH, biomarker, lung cancer, immune infiltration, prognosis

Posted Date: January 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1228934/v1

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Abstract

Non-SMC Condensin I Complex Subunit H (NCAPH) plays regulatory role in various cancers. However, its role on prognosis and immune infiltrates in lung adenocarcinoma (LUAD) remains unclear. This study try to explore expression of NCAPH in tumor tissues and its association with immune infiltrates and prognostic roles in LUAD patients. Patients characteristics were from the cancer genome atlas (TCGA). Integrated analysis of TCGA showed NCAPH was over-expression across cancers including LUAD. NCAPH expression was verified by quantitative polymerase chain reaction and western blotting in 20 LUAD matched tissues. NCAPH-high expression was significantly related to T, N, M, pathologic stage, primary therapy outcome and smoke by using Wilcoxon rank sum test. Cox- and Kaplan–Meier analysis showed NCAPH-high group was associated with shorter OS. PFI and DSS in NCAPH-high group were significantly decrease. Multivariate analysis showed NCAPH was independent prognosis factor. Gene set enrichment analysis demonstrated G2M checkpoint, ncRNA metabolic, memory B cells, KRAS, E2F targets and MIER1 process were significantly associated with NCAPH expression. Single-sample Gene Set Enrichment Analysis indicated NCAPH expression was associated with levels of Th2 and Mast cell. Impact of NCAPH on malignant phenotype were evaluated by MTT, transwell, cell-cycle and apoptosis assay in vitro. Malignant phenotype of LUAD cells was inhibited if NCAPH was knocked down. In conclusion, this research indicates that NCAPH could be a potential factor for predict prognosis and a new biomarker in LUAD.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide, with more than 1760000 deaths each year [1]. Non-small cell lung cancer (NSCLC) accounts for almost 85% of lung cancer. LUAD is the most common type of NSCLC and accounts for approximately 40% of NSCLC. In past decades, with advent of small molecule tyrosine kinase inhibitors and immunotherapy, survival of selected LUAD patients is significantly prolonged [2]. However, the overall cure and survival rates for lung adenocarcinoma remain limited depending on stage and regional differences [3]. LUAD is a highly heterogeneous malignancy. Current biomarkers such as EGFR mutation, BRAF mutation, HER2 amplification and microsatellite instability cannot completely clarify the different prognosis or therapeutic response of LUAD [4]. Since the molecular mechanisms of LUAD tumorigenesis and development are still not fully clear, numerous studies of targeted therapy and experiments try to elucidate the pathogenesis and improve the prognosis of LUAD patient [5]. Therefore, The development of novel biomarkers for diagnosis and treatment targets in LUAD is critical.

Non-SMC Condensin I Complex Subunit H (NCAPH) encoded by gene located on chromosome 2q11.2, belong to barr gene family and a regulatory subunit of the condensin complex [6]. NCAPH was revealed to be important for viability and to play a key function in mitotic chromosomal architecture and segregation in previous studies [7,8]. Recent studies demonstrated that NCAPH involved in various malignancies. In microarray analysis, NCAPH was found high expression and contributed to carboplatin resistance in

serous ovarian cancer patients [9]. In hepatocellular carcinoma, NCAPH was highly expressed in tumor tissues compared to normal non-cancerous tissues and associated with poor prognosis. In addition, NCAPH overexpression promoted tumor proliferation, migration and invasion in vitro and in vivo [10]. These findings suggested that NAPCH has notable roles in cancer progression and development. Moreover, the exact functions and mechanisms of of NCAPH in tumor progression and tumor immunology have not been well characterized.

To better explore role of NCAPH in LUAD progression, we applied RNA-seq data from the TCGA and GEO datasets. Statistical and bioinformatics ways, such as differentially expressed genes (DEG) analysis, Kaplan–Meier (KM) survival analysis, Cox & Logistic regression analysis, nomogram, Gene Ontology (GO) analysis, Gene Set Enrichment Analysis (GSEA), single-sample Gene Set Enrichment Analysis (ssGSEA) were utilized. Moreover, NCAPH was also knocked down in vitro to see how it affected LUAD proliferation, invasion, and migration in vitro.

Methods

Data source and preprocessing

LUAD patients' clinical information and gene expression (included 535 tumor and 59 normal tissues) were from TCGA (https://portal.gdc.cancer.gov/). Exclusion criteria were OS less than 30 days and normal tissues. Then, HTSeq-FPKM information of level 3 has been transformed into Transcripts Per Million (TPM); then TPM information of 513 lung adenocarcinoma samples was applied for the next analyses. 22 samples were excluded for lack of clinical variables.

NCAPH differential expression in LUAD tissues in the TCGA database

By using disease state (normal or tumor) as variable, scatter plots and boxplots were performed to estimate different expression levels of NCAPH. Receiver operating characteristic (ROC) curves were conducted to estimated the diagnostic value of NCAPH. NCAPH expression above or below the median value was defined as NCAPH-high or NCAPH-low, respectively.

Identification of differently expressed genes (DEGS) between NCAPH-high and -low expression LUAD groups

Analysis of gene expression between NCAPH-high and NCAPH-low patients from TCGA LUAD datasets was conducted using the DESeq2 (4.0 package). Genes with the adjusted *P* value <0.05 and the absolute FC larger than 1.5 were considered to be statistically significant. All significant DEGs were presented in volcano plots and heat map by using R software.

Function enrichment and infiltration of immune related cell evaluation

Enrichment of NCAPH relevant DEGs by pathway and process was analyzed by Metascape (http://metasape.org). Enrichment factor >1.5, a minimum count of three and P<0.01 were regarded as statistically significance. By using GSEA, we investigated differences in signal pathways between the NCAPH-high and NCAPH-low groups to predict the NCAPH-related phenotypes and signal pathway. The significantly changed pathways were identified by permutation test with 1,000 times. FDR <0.25 and adjusted P < 0.01 were recognized as significantly associated genes. R package cluster Profiler (4.0) was used to analysis and graphical plotting [11]. The relative tumor infiltration levels of 24 immune cell types were analyzed by ssGSEA to research expression levels of genes in published signature gene lists [12]. The signatures included multiple set of innate well as adaptive immune relative cell type and comprised 509 genes in total. To evaluate the association between the infiltration levels of immune cells and NCAPH, Spearman correlation and Wilcoxon test were used.

Risk prognosis model construction, model construction and estimation

All statistical analyses were performed in R package (V3.6.2). Using logistic regression and the Wilcoxon signed-rank sum test, the link between clinicopathological features and NCAPH was investigated. The clinical pathologic variables linked to 10-year overall survival (OS), disease-specific survival (DSS) and progression-free interval (PFI) in TCGA database were analyzed by using the Kaplan-Meier method and Cox regression. Univariate and Multivariate Cox analysis was utilized to investigate the effect of NCAPH level on survival along with other clinical variables. The cut-off value for NCAPH expression was determined as the median level. P<0.05 was considered statistically significant. The differences of OS, DSS as well as PFI between NCAPH-low and -high groups were analyzed by KM method with a log-rank test. Independent prognostic indicators were employed to generate nomograms for predicting the prognosis for one year based on the results of multivariate Cox analysis. We created nomograms with calibration plots and relevant clinical factors using the RMS package (https://cran.r-project.org/web/packages/rms/index.html). The calibration curves were pictorial evaluated by drafting the nomogram measuring likelihood versus actual occurrence and the 45 degrees line indicated the best predicting values. A concordance index (C-index) was calculated and used to evaluate the model's discrimination using a bootstrap method with 1,000 resamples. The C-index was used to assess the nomogram's each prognostic features and predictive accuracy.

Experimental verification of NCAPH differential expression in LUAD tissues by quantitative polymerase chain reaction (qPCR) and western blotting

From January 2018 to January 2019, 20 matched LUAD tissues and neighboring non-cancerous tissues were taken from patients who received surgery at the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital. All cases were pathologically confirmed. The current study was approved by the Ethic Committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital and conducted in according to standard of Helsinki declaration. All of the patients signed informed consent forms. Total RNA was extracted from tissues by using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The extracted RNA was reverse-transcribed into cDNA by using Takara PrimeScript RT Reagent Kit (Takara, Nanning, China). RT-PCR was performed by using LightCycler 480 Real-time PCR System (Roche, Shanghai, China). For normalizing NCAPH, 18s rRNA was used as an internal reference. The relative expression of NCAPH mRNA was calculated with the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: Sense: 5'-ATGTTGCTGATGGAAGTG-3' and antisense: 5'-GTTCTGCTCA ATAGTTCTGT-3' for NCAPH; Sense: 5'-AGGCGCGCAAATTACCCA ATCC-3' and antisense: 5'-GCCCTCCAATTGTTCCTCGTT AAG-3'for 18s rRNA.

Total protein was extracted from frozen tissues. Protein concentrations were tested by BCA protein assay kit. Protein samples were separated on 10% sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis gel. The separated proteins were transferred to Immun-Blot polyvinylidene fluoride membrane (Bio-Rad) using a wet transfer system (Bio-Rad) and then incubated with primary antibody at 4°C overnight, followed by incubation with horseradish peroxidase-linked anti-rabbit immunoglobulin G (Merck Millipore) in a dilution of 1:10,000 for 1 h at room temperature. Antibodies were applied in the experiment: NCAPH (1:1,000 dilution; Proteintech, China) and β-actin (1:2,000 dilution; Proteintech, China). Relative NCAPH protein expression levels was normalized by β-actin.

For cell migration assay, 1×10^4 cells in 200μL of medium without serum were trypsinizated, suspended and seeded in the upper chamber(8-μm pore size; Millipore, Zurich, Switzerland). Subsequently, the medium (600μL) containing with 20% FBS was added into the bottom compartment of chamber. Then, the chamber was placed in an incubator at 37°C. After incubation for 48h, cells were fixed by methanol and stained 0.1% crystal violet (Sigma - Aldrich). Then, the non-migrated cells were removed by scraping. Finally, migrated cells were counted by using a microscope (Nikon Corporation, Tokyo, Japan). Transwell invasion assay was similar to migration assay. The difference was that the upper chamber of Transwell invasion assay was covered with Matrigel matrix. These experiments were repeated three times. Relative migration or invasion (%) was calculated by average number of migrated (invaded) cells in transfection group/average number of migrated (invaded) cells in control group×100%.

Cell cycle distribution and apoptosis assay were conducted by flow cytometric. H2122 and H3122 cells were digested with trypsin, resuspended in phosphate-buffered saline (PBS) and then fixed by 70% ethanol. Cells were washed by PBS and treated by 100μg/ml RNase for 30 min. Then, DNA was stained with propidium iodide (50μg/ml) and analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptosis was assessed by using Annexin V FITC/PI apoptosis kit (KeyGen Biotech,

Nanjing, China). The samples were measured and analyzed by using a flow cytometer (Beckman Coulter, Brea, CA, USA).

Statistical analysis

Statistical analysis was performed with student's two tailed t-test using SPSS (version 22). Values of $P < 0.05$ were considered statistically significant.

Results

Abnormal Expression Level of NCAPH in Pan-Cancers and LUAD

By using Wilcoxon rank sum test, pan-cancer analysis was conduct to compare NCAPH levels in tumor samples of GTEx combined with TCGA and matched normal samples. NCAPH abnormally expressed in bladder urothelial carcinoma (BLCA), Breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma(ESCA), head and neck squamous cell carcinoma (HNSC), renal chromophobe cell carcinoma (KICH), renal clear cell carcinoma (KIRC), renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma(LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma(LUSC), pancreatic cancer (PAAD), prostate cancer (PRAD), rectum adenocarcinoma(READ), gastric cancer (STAD), thyroid cancer (THCA), endometrial cancer (UCEC) ($P < 0.05$)(Figure 1A and Figure 1B). NCAPH levels in 59 normal samples and 535 LUAD samples were compared in TCGA LUAD dataset. In LUAD samples, NCAPH expression was substantially increase ($P < 0.001$) (Figure 1C). Furthermore, NCAPH expression in 57 LUAD samples and matched normal samples were significantly different ($P<0.001$) (Figure 1D). According to these findings, we used QPCR and western blotting to quantity NCAPH level on 20 paired LUAD samples and matched normal samples. QPCR and western blotting results showed NCAPH expression elevated in LUAD tissues ($P<0.05$) (Figure 1E and Figure 1F). Furthermore, receiver operating characteristic (ROC) was used to evaluate diagnostic value of NCAPH between LUAD and normal lung tissue. The area under the curve (AUC) of NCAPH was 0.967(Figure 1G). These results meant NCAPH might be a potentially good diagnostic maker for LUAD.

Identification of NCAPH associated DEGs in LUAD

DEGs analysis involved 267 LUAD NCAPH-high samples and 268 NCAPH-low samples (control group). 1592 DEGs were identified and included 1167 upregulated genes and 425 downregulated genes (adjusted P-value<0.05, Log2-fold change>1.5) (Figure 1H). Then, DEGs in HTSeq-Counts were further analyzed by DESeq2 package. The genes of top 20 DEGs in the between two groups were presented in Figure 1I.

Function Enrichment and Analysis of NCAPH Related Genes in LUAD

To further study functional enrichment information of NCAPH related genes, Metascape was utilized for GO enrichment analysis. NCAPH-related genes play roles in various biological processes. (BPs), cellular compositions (CCs) and molecular functions (MFs), including distal axon, neuron projection terminus,

axon terminus, integrator complex, pre-miRNA processing, RNA 3'−end processing and miRNA catabolic process (Figure 2A).

Potential Mechanism of NCAPH in Progression of LUAD

To explore roles of NCAPH in LUAD pathway, we utilized GSEA to analyze differences between NCAPH-high and -low cohorts (adjusted P<0.05, FDR P value<0.25) (c2.cp.biocarta and hall. v6.1 symbols). According to normalized enrichment score (NES), top significant enriched pathways associated with NCAPH high expression were selected. G2M checkpoint, ncRNA metabolic process, memory B cells, KRAS signaling, E2F targets and MIER1 process were significantly enriched in patients with NCAPH (Figure 2B-G).

Correlation Between NCAPH Expression and Immune Infiltration.

We further analyzed the correlation between NCPAH expression and immune cell infiltrating by ssGSEA and spearman correlation. The relationship between NCAPH expression and immune cell infiltration was quantified by ssGSEA and analyzed by spearman correlation. The expression of NCAPH was positively associated with the level of acquired immunocyte [Th2 cells (R = 0.790, P< 0.001)] and negatively correlated with the abundance of innate immunocyte [Mast cell (R = -0.510, P< 0.001)] (Figures 3A-E).

The Correlation Between Expressions of NCAPH and Clinicopathologic Characteristics

513 patients' characteristics and NCAPH expression data were collected from TCGA to explore the relationship between NCAPH expression and clinicopathologic perimeters. Table 1 and Figure 4A-F shown high expression of NCAPH was significantly related to T stage (T1&T2/T3&T4 vs normal, P < 0.001), N stage (N0&N1/N2&N3 vs normal, P < 0.001), M stage (M1&M0 vs normal, P < 0.001), pathologic stage (stages III&IV vs. stage I, P < 0.001), primary therapy outcome (CR&PR vs normal, P < 0.001) and smoker (Yes vs normal, P < 0.001). Furthermore, we conducted univariate analysis to investigate NCAPH expression whether a dependent variable is associated with poor prognostic clinicopathological characteristics (Table 2). Higher expression of NCAPH in LUAD was positively correlated to T stage (OR = 1.932 for T2&T3&T4 vs. T1), pathologic stage (OR = 1.574 for Stage III& Stage IV vs. Stage I & Stage II), gender (OR = 1.671 for Male vs. Female), number of packs smoked per year (OR = 1.7566 for Mild and Severe vs. None), vascular invasion (OR = 1.05 for yes vs. no), race (OR = 1.07 for ≥40 vs. <40) and smoker (OR = 2.566 for Yes vs. No) (all P<0.05). High expression of NCAPH in LUAD was negatively correlated to primary therapy outcome (OR = 0.562 for PR&CR vs. PD&SD, P = 0.012).

High NCAPH expression Associated with Poor Prognosis of LUAD patients.

A univariate logistic regression was used to explore NCAPH role in LUAD patient prognosis. OS of LUAD NCAPH-high expression was significantly shorter (HR = 1.92, 95%CI:1.37-2.70). In addition, PFI and DSS in NCAPH high expression group were significantly lower in compared with NCAPH low expression (Figure

5A-H). We also performed subgroup analysis of prognosis. Subgroup analysis results shown the survival of high NCAPH level was poor in T1&T2, T3&T4, N0&N1, M0 and M1 group. In order to further evaluate NCAPH role in LUAD prognosis, multivariate regression was applied with T stage, N stage, M stage, pathologic stage, primary therapy outcome, gender and smoker. In multivariate analysis, high NCAPH expression was still an independent prognostic factor (Table 3).

NCAPH--related prognostic nomogram

To predict the prognosis value of NCAPH in LUAD, we established a nomogram and risk classification for predicting 1 year survival (Figure 6A). According to clinical relevance and multivariate Cox analysis results, variables in nomogram were selected. With the adjusted range from 1 to 100, points of each variable were added up and total scores were calculated. By delineating a direct line down from the total score line to the outcome line, the probable prognosis of each LUAD patients at 1years were defined. For example, a LUAD patient with high NCAPH risk (56 points), T3&T4(98 points), N2&N3(100 points), primary therapy outcome (100 points), smoker (30 points) account for 384 points. The probability of 1 year survival was about 56% (Figure 6A). The efficacy of the nomogram were also evaluated, and the result showed that the ability of prediction efficiency of the nomogram was moderately accurate (Figure 6B).

Knockdown of NCAPH suppress malignant phenotype of lung adenocarcinoma in vitro

H2122 and H3122 cell line were chosen to research the role of NCAPH in LUAD. Three NCAPH siRNAs were transfected into cells. NCAPH mRNA expressions were tested to evaluate three NCAPH siRNAs knockdown efficiency. Among these siRNAs, siRNA showed most significant inhibition ratio and was selected for further experiment. MTT assay data indicated that siRNA targeting NCAPH significantly reduced cell growth rates. Transwell assay revealed that NCAPH-targeted siRNAs transfection notably reduced migration and invasion in both cell lines. By using flow-cytometry analysis, the cell-cycle distribution of NCAPH siRNA transfected cells was increasing in G_1/G_0 cell population in both cell lines. Besides, the apoptosis of H2122 and H3122 cells remarkable increased in NCAPH siRNA treatment group, by using the Annexin V-FITC/PI double staining technique. These data were showed in Figure 7 and Figure 8.

Discussion

Condensin is a highly conserved multiprotein complex that regulates chromosomal assembly and separation during mitosis [13]. Condensin I and II are two forms of condensin complexes found in many eukaryotic cells, and both share the identical pair of structural maintenance of chromosome (SMC)2 and 4 subunits. Condensin I complex is comprised by SMC2-SMC4 proteins and three non-SMC proteins including subunits H (NCAPH), G (NCAPG) and D2 (NCAPD2) [14]. Former research shown that

phosphorylation of NCAPH at Ser70 by Aurora B kinase was indispensable in the recruitment of condensin I to mitotic chromosomesis [15]. Intriguingly, bioinformatics analyses of potential molecular mechanisms shown that NCAPH as a key gene involved in lung and prostate tumorigenesis [16,17]. Although previous study found overexpression of NCAPH was associated with LUAD pathogenesis, the function and molecular mechanism still remain unclear. Herein, we try to elucidate oncogenic impact of NCAPH in LUAD development.

According to our findings, the expression and prognostic values of NCAPH were assessed. We found that NCAPH expressions were abnormal in various tumors including LUAD in databases. Furthermore, NCAPH may serve as a good biomarker for high ROC score with AUC of 0.967 for LUAD. Generally, NCAPH expressed different in tumor and normal samples. Further studies are needed to fully research diagnostic value of NCAPH in LUAD.

To further study functional enrichment information of NCAPH related genes, Metascape was utilized for GO enrichment analysis. NCAPH related genes involved in in many biological processes (BPs), cellular compositions (CCs), and molecular functions (MFs), including distal axon, neuron projection terminus, axon terminus, integrator complex, pre-miRNA processing, RNA 3'−end processing and miRNA catabolic process.

We also revealed that G2M checkpoint, ncRNA metabolic process, memory B cells, KRAS signaling, E2F targets and MIER1 process were significantly associated with NCAPH expression. In former vitro study, cell proliferation, cell-cycle, colony formation, migration and invasion were inhibited when NCPAH was knockdown [18]. This study directly proves our result. KRAS signaling pathway and E2F were proved to play an important role in the progression and development of LUAD [19-21]. These studies and our results indicated that NCAPH might contribute to LUAD initiation and development by modulating E2F, cell cycle and KRAS pathway. The associations of NCAPH expression with memory B cells, ncRNA metabolic and MIER1 process were firstly reported. The molecular mechanisms needed to be further researched.

Previous clinical studies found that tumor-infiltrating lymphocytes (TILs) had vital impact on several cancers [22-24]. Strong infiltration of TILs was associated with a positive clinical outcome in several cancers, including lung cancer [25]. Our study shown NCAPH was positively associated with the level of acquired immunocyte (Th2 cells) and negatively correlated with the abundance of innate immunocyte (Mast cell). Th2 cells are defined by the expression of their signature cytokines IL-4, IL-5, and IL-13 which are important components against extracellular pathogens [26]. Cytokines such as IFN-γ, TNF-α and IL-2 produced by Th1 cells were found to be vital factors inhibiting tumor growth [27,28]. In contrast, cytokines IL-10, IL-4 and TGF-β from Th2 cells were proved to promote tumor cells dissemination and metastasis in various cancers [29]. Therefore, maintaining Th1/Th2 immune cell balance was considered to be very critical. Enhancing Th1 response and inhibiting Th2 effect may help to prevent disseminated cancer cells, recurrence and metastasis [30]. Consistent with these findings, our results revealed NCAPH may be associated with Th2 immune response in LUAD. In several studies, Mast cell were found to a predictor of

poor outcome [31-33]. However, a study of 175 patients with non-small-cell lung cancer also demonstrated Mast cell presence was a good prognosis factor [34]. The prognosis role of Mast cell was still uncertain. But the association of NCAPH expression with Mast cell was the first to be reported.

Our research indicated that NCAPH high expression was associated with clinical pathological characteristics and poor prognosis in LUAD. NCAPH expression was significant related to T stage、N stage、M stage、pathologic stage and smoke status. In univariate logistic regression, OS of LUAD patients with high NCAPH expression was significantly shorter. After regulating clinicopathological factors, our study found NCAPH could perform as an independent poor prognostic factor for LUAD. Then, we conduct clinical nomogram with NCAPH expression and other clinical factors. Based on the calibration plot, there was a favorable consistency between the actual and predicted values for 1 year OS. Our model could be a new method to estimate the prognosis in the future.

We also investigate the function of NCAPH in proliferation, invasion, migration, cell-cycle and apoptosis of LUAD in vitro. The malignant phenotype of LUAD cells was inhibited when NCAPH was knocked down.

In this study, we firstly reported that the high expression of NCAPH was significantly associated with the progression, poor survival and immune infiltration of LUAD, which might promote tumorigenesis through abnormal inflammation and immune response. NCAPH may be a potential factor for predict prognosis and a new biomarker. Vitro study demonstrate that NCAPH is functions as an oncogene in lung cancer.

Declarations

Ethics approval and consent to participate

The current study was approved by the Ethic Committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital. Signed informed consents were obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by Basic research project of Shenzhen knowledge innovation plan(JCY20170307112807219), Hospital Research Project(SZ2020QN002 and SZ2020QN020) and

Shenzhen High-level Hospital Construction Plan.

Authors' contributions

LC conceived and designed this study. Data analysis and paper writing was conducted by LC under the guidance of MJ and ZTT. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

Availability of data and materials

LUAD patients' clinical information were collected from TCGA (https://portal.gdc.cancer.gov/). Metascape (http://metasape.org) was applied to analysis the enrichment pathway and process.

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Tables

Table 1 The correlation between clinicopathological variables and NCAPH expression.

Characteristic	Low expression of NCAPH	High expression of NCAPH	p
n	256	257	
T stage, n (%)			0.003
T1	102 (20%)	66 (12.9%)	
T2	118 (23.1%)	158 (31%)	
T3	25 (4.9%)	22 (4.3%)	
T4	9 (1.8%)	10 (2%)	
N stage, n (%)			0.133
N0	173 (34.5%)	157 (31.3%)	
N1	43 (8.6%)	52 (10.4%)	
N2	31 (6.2%)	43 (8.6%)	
N3	0 (0%)	2 (0.4%)	
M stage, n (%)			0.100
M0	176 (47.7%)	168 (45.5%)	
M1	8 (2.2%)	17 (4.6%)	
Pathologic stage, n (%)			0.105
Stage I	148 (29.3%)	126 (25%)	
Stage II	58 (11.5%)	63 (12.5%)	
Stage III	36 (7.1%)	48 (9.5%)	
Stage IV	9 (1.8%)	17 (3.4%)	
Primary therapy outcome, n (%)			0.015
PD	22 (5.2%)	46 (10.8%)	
SD	19 (4.5%)	18 (4.2%)	
PR	3 (0.7%)	3 (0.7%)	
CR	168 (39.4%)	147 (34.5%)	
Gender, n (%)			0.005
Female	154 (30%)	122 (23.8%)	
Male	102 (19.9%)	135 (26.3%)	

Characteristic	Low expression of NCAPH	High expression of NCAPH	p
Race, n (%)			0.538
Asian	4 (0.9%)	3 (0.7%)	
Black or African American	23 (5.2%)	29 (6.5%)	
White	202 (45.3%)	185 (41.5%)	
Age, n (%)			0.015
<=65	106 (21.5%)	132 (26.7%)	
>65	143 (28.9%)	113 (22.9%)	
Residual tumor, n (%)			0.202
R0	166 (46%)	178 (49.3%)	
R1	6 (1.7%)	7 (1.9%)	
R2	0 (0%)	4 (1.1%)	
Anatomic neoplasm subdivision, n (%)			1.000
Left	100 (20.1%)	99 (19.9%)	
Right	149 (29.9%)	150 (30.1%)	
Anatomic neoplasm subdivision2, n (%)			0.412
Central Lung	33 (17.5%)	29 (15.3%)	
Peripheral Lung	58 (30.7%)	69 (36.5%)	
number_pack_years_smoked, n (%)			0.012
<40	92 (26.2%)	82 (23.4%)	
>=40	69 (19.7%)	108 (30.8%)	
Smoker, n (%)			< 0.001
No	51 (10.2%)	23 (4.6%)	
Yes	197 (39.5%)	228 (45.7%)	
Age, median (IQR)	68 (60, 74)	64 (58, 71)	0.003

Table 2 NCAPH expression association with clinical pathological characteristics (logistic regression).

Characteristics	Total(N)	Odds Ratio(OR)	*P* value
T stage (T2&T3&T4 vs. T1)	510	1.932 (1.329-2.822)	<0.001
N stage (N1&N2&N3 vs. N0)	501	1.444 (0.997-2.099)	0.053
M stage (M1 vs. M0)	369	2.226 (0.963-5.582)	0.070
Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II)	505	1.574 (1.029-2.427)	0.038
Primary therapy outcome (PR&CR vs. PD&SD)	426	0.562 (0.357-0.878)	0.012
Gender (Male vs. Female)	513	1.671 (1.179-2.375)	0.004
Race (Black or African American&White vs. Asian)	446	1.268 (0.276-6.500)	0.758
Age (>65 vs. ≤65)	494	0.635 (0.444-0.904)	0.012
Residual tumor (R1&R2 vs. R0)	361	1.710 (0.636-5.059)	0.301
Anatomic neoplasm subdivision (Right vs. Left)	498	1.017 (0.710-1.456)	0.927
Anatomic neoplasm subdivision2 (Peripheral Lung vs. Central Lung)	189	1.354 (0.737-2.499)	0.330
number_pack_years_smoked (≥40 vs. <40)	351	1.756 (1.151-2.690)	0.009
Smoker (Yes vs. No)	499	2.566 (1.531-4.420)	<0.001

Table 3 Univariate and multivariate Cox regression analyses of NCAPH mRNA expression for overall survival (OS) in patients with LUAD from The Cancer Genome Atlas (TCGA) data set.

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
T stage	523				
T1	175	Reference			
T2	282	1.521 (1.068-2.166)	**0.020**	1.356 (0.822-2.236)	0.233
T3&T4	66	3.066 (1.950-4.823)	**<0.001**	2.192 (1.077-4.458)	**0.030**
N stage	510				
N0	343	Reference			
N1	94	2.382 (1.695-3.346)	**<0.001**	1.750 (1.096-2.793)	**0.019**
N2&N3	73	2.968 (2.040-4.318)	**<0.001**	2.502 (1.017-6.153)	**0.046**
M stage	377				
M0	352	Reference			
M1	25	2.136 (1.248-3.653)	**0.006**	1.631 (0.623-4.266)	0.319
Pathologic stage	518				
Stage I&Stage II	411	Reference			
Stage III&Stage IV	107	2.664 (1.960-3.621)	**<0.001**	1.006 (0.400-2.531)	0.990
Primary therapy outcome	439				
PD&SD	108	Reference			
PR&CR	331	0.377 (0.268-0.530)	**<0.001**	0.360 (0.237-0.549)	**<0.001**
Gender	526				
Female	280	Reference			
Male	246	1.070 (0.803-1.426)	0.642		
Smoker	512				
Yes	440	Reference			

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
No	72	1.119 (0.742-1.688)	0.591		
NCAPH	526				
Low	263	Reference			
High	263	1.92 (1.37-2.70)	**<0.001**	1.347 (0.898-2.019)	0.150

Figures

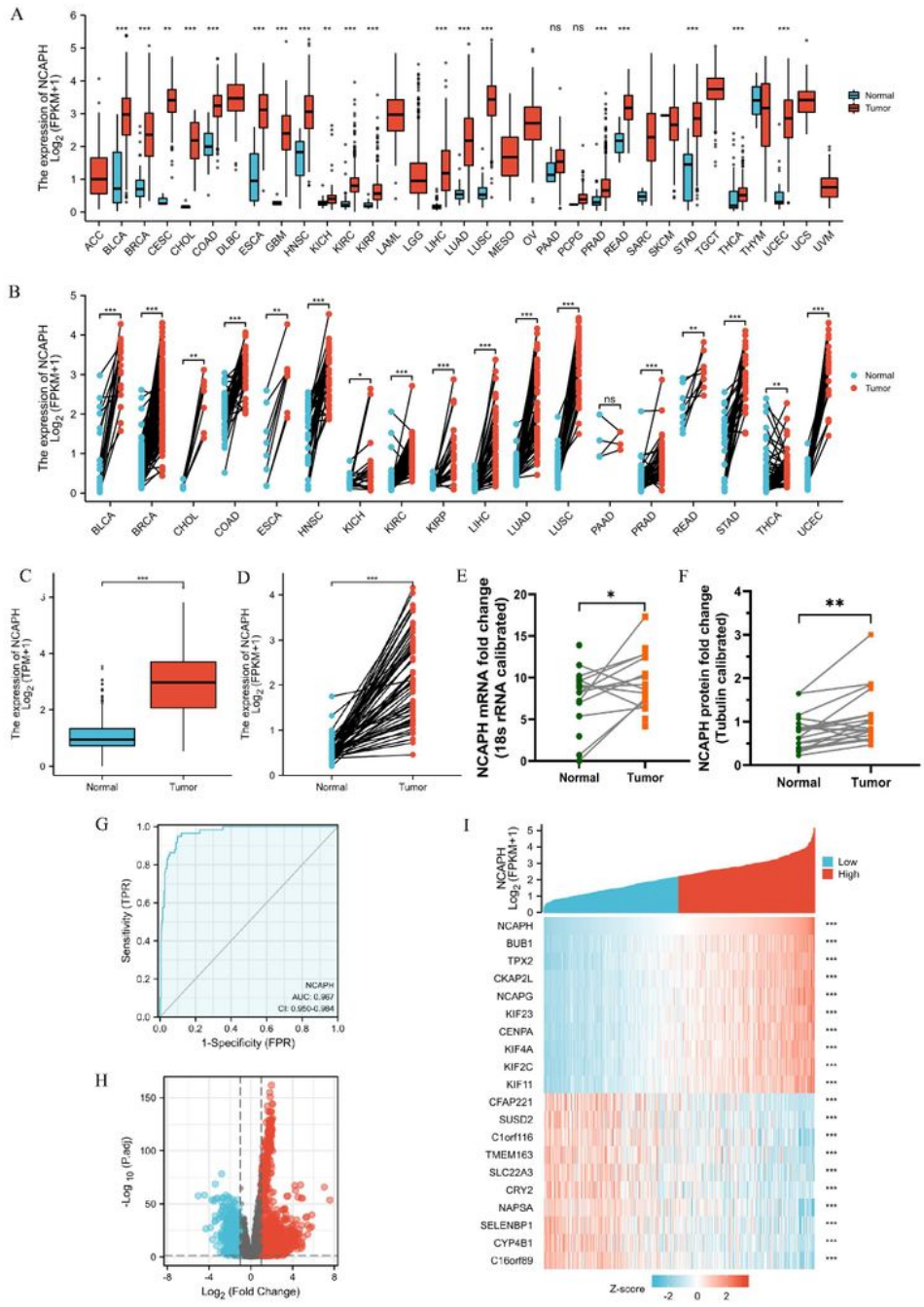

Figure 1

Expressions of NCAPH in tumors and NCAPH-related DEGs. (A,B) Abnormal expression of NCAPH in cancers in TCGA. (C,D) Levels of NCAPH in LUAD. (E,F) The mRNA and protein expression of NCAPH in 20 LUAD samples and matched normal samples. (G) ROC curve to test the value of NCAPH to identify LUAD tissues. (H,I) Volcano plots of the DEGs and heat map showing the top 20 DEGs.
*P<0.05,**P<0.01,***P<0.01

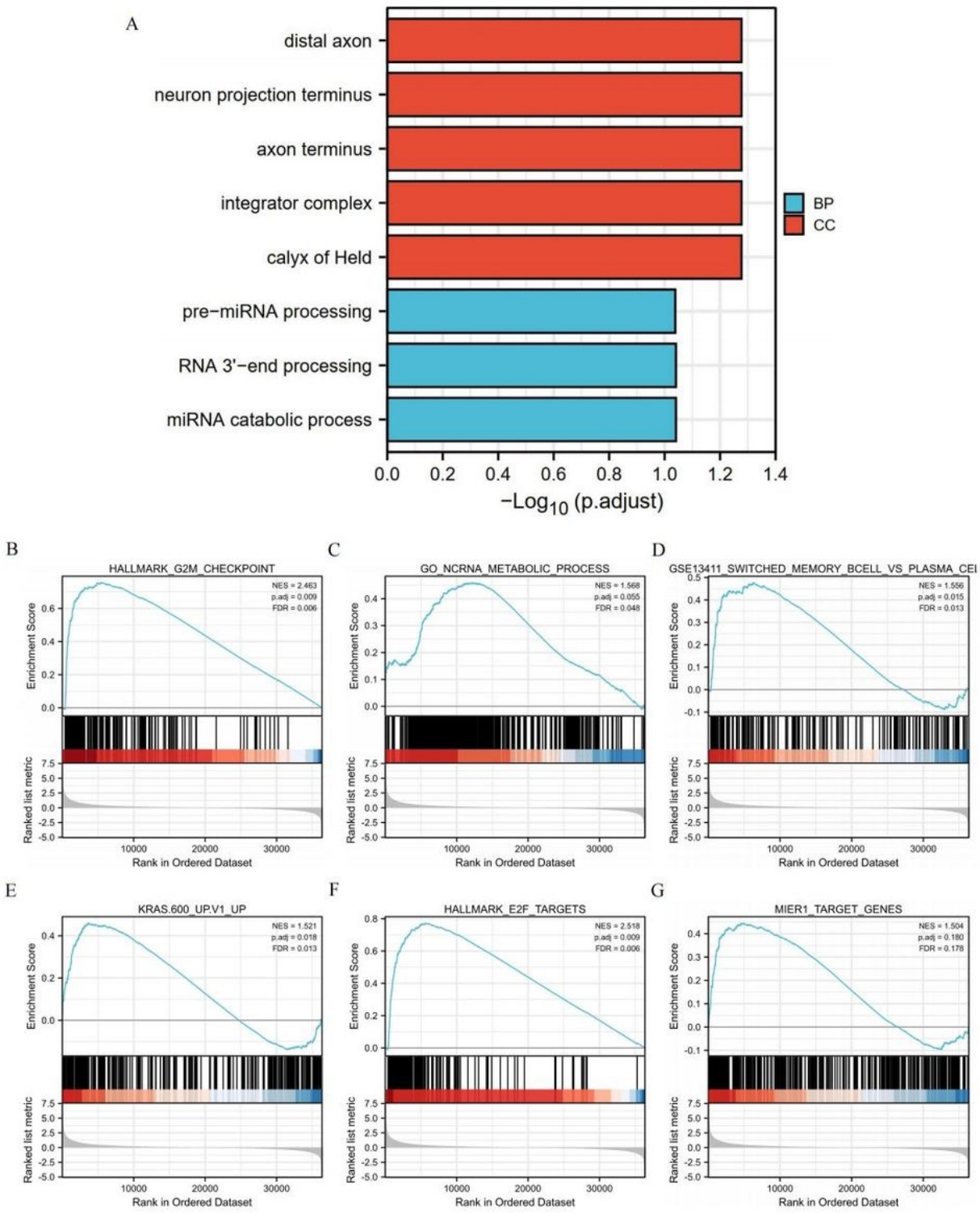

Figure 2

Significantly enriched GO annotations of NCAPH related genes in LUAD. (A) Top 8 of biological process enrichment related to NCAPH related genes with bar graph. (B-G) Enrichment plots from the gene set enrichment analysis (GSEA). Several pathways and biological processes were deferentially enriched in NCAPH-related LUAD including G2M checkpoint, ncRNA metabolic process, memory B cells, KRAS signaling, E2F targets and MIER1 pathway. BP, biological processes; CC, cellular composition.

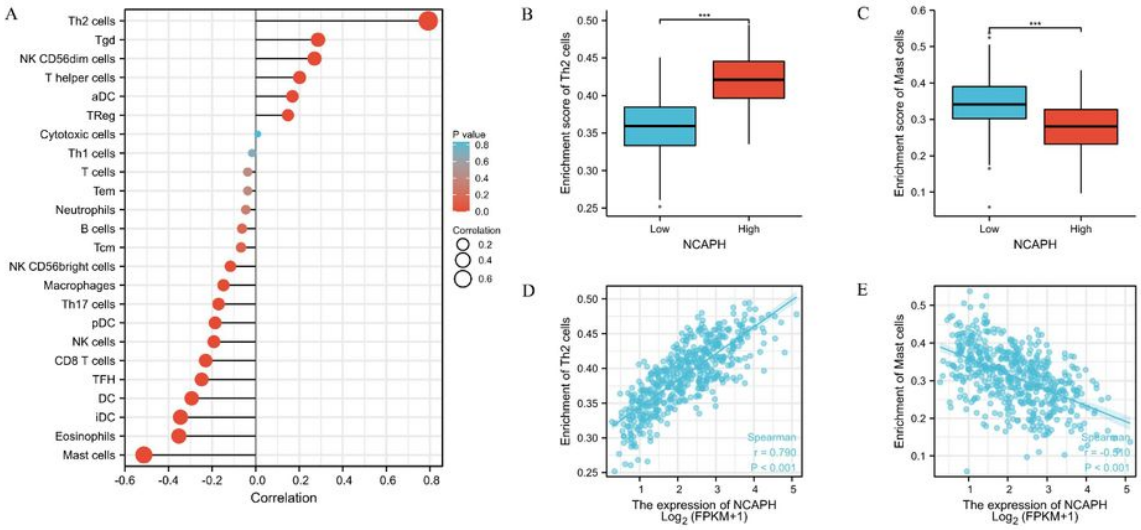

Figure 3

The expression of NCAPH was related to the immune infiltration in the tumor microenvironment. (A) Association between the NCAPH expression level and relative abundances of 24 immune cells. The size of dots demonstrates the absolute value of Spearman R. (B-E) Correlation diagrams and scatter plots indicating the differentiation of Th2 cells and Mast cells infiltration level between high and low groups of NCAPH expression. *P<0.05,**P<0.01,***P<0.01

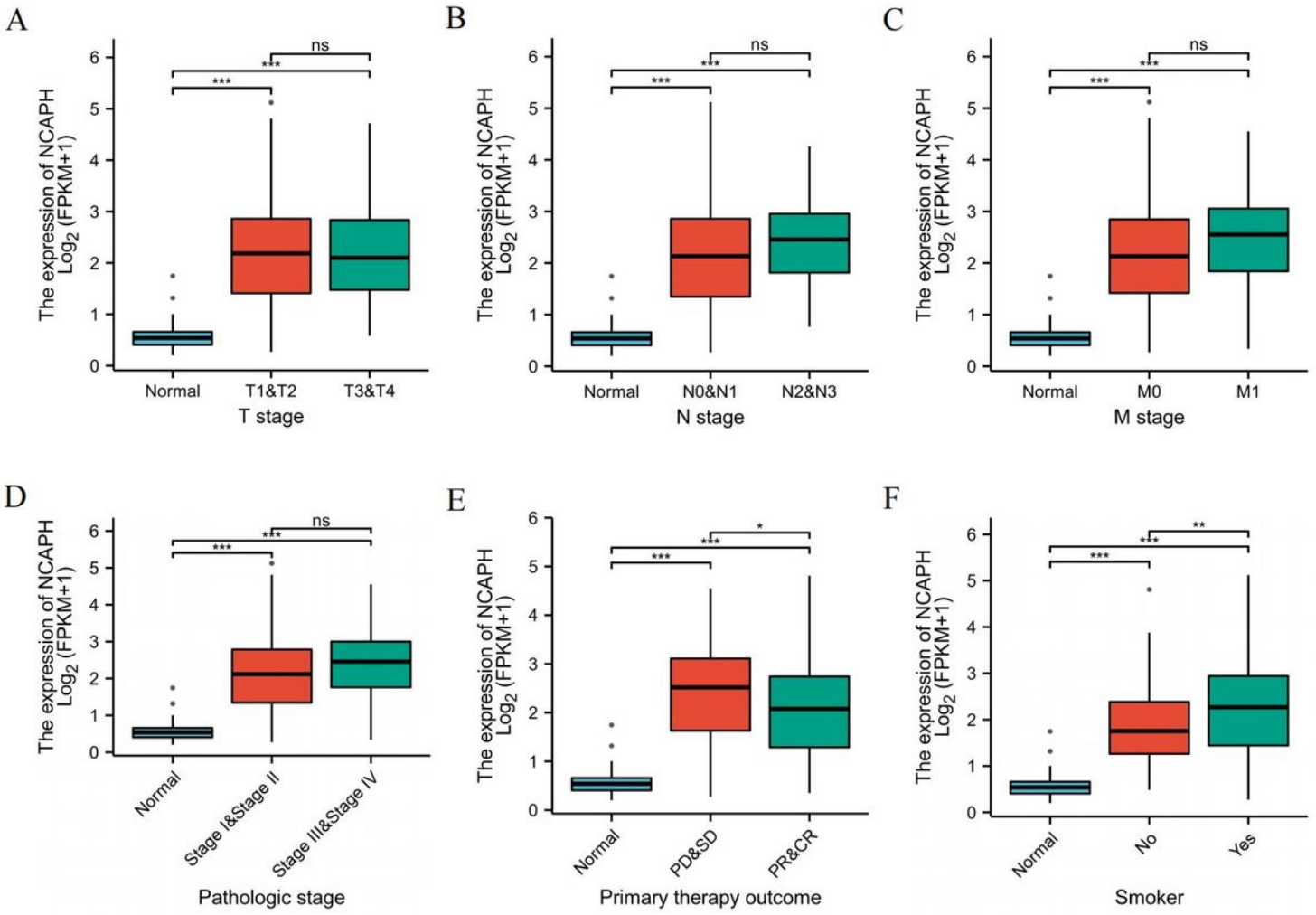

Figure 4

Correlation with NCAPH expression and clinicopathological characteristics, including (A) T stage, (B) N stage, (C) M stage, (D) pathologic stage, (E) primary therapy outcome, (F) smoke status in LUAD patients in TCGA cohort.*P<0.05,**P<0.01,***P<0.01

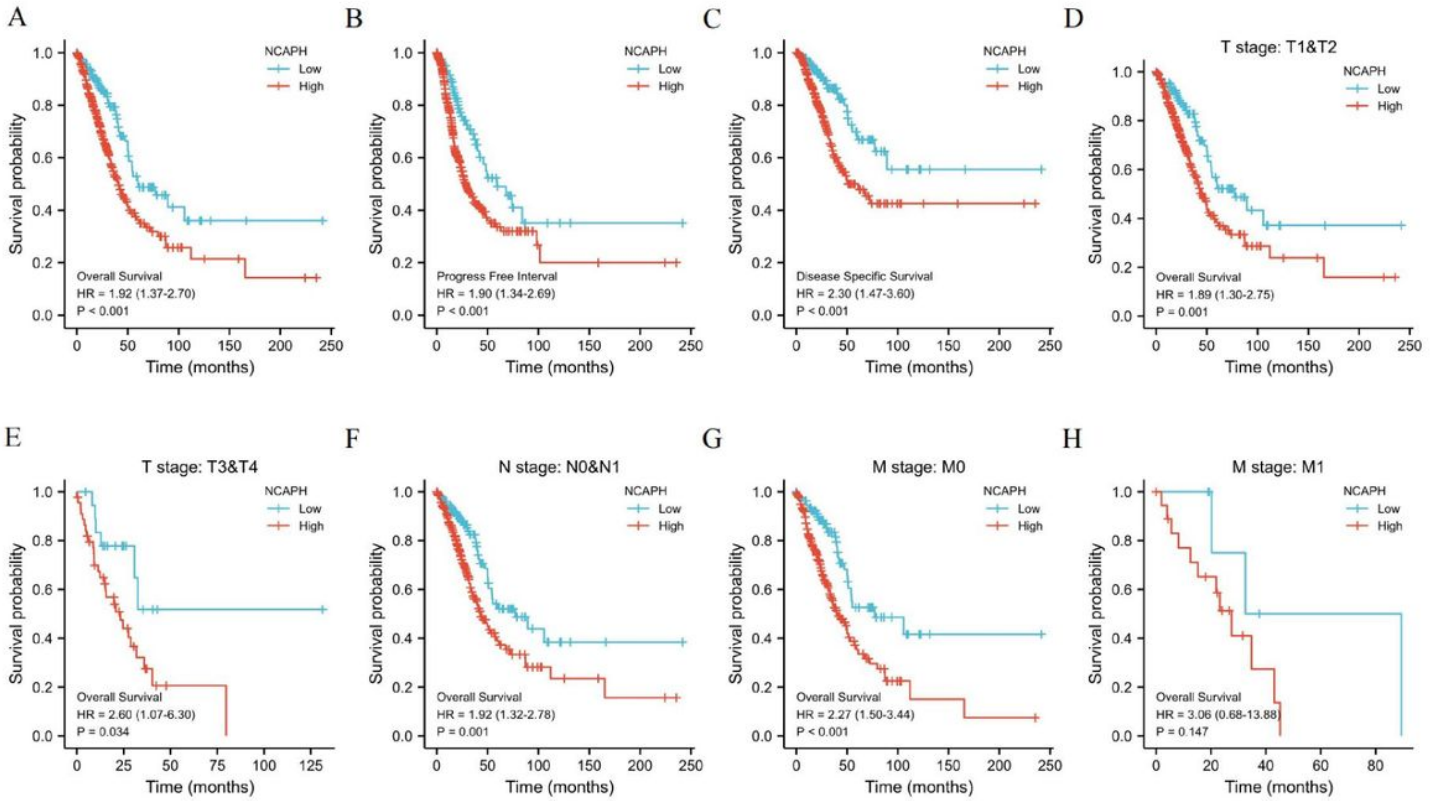

Figure 5

Kaplan-Meier survival curves comparing the high and low expression of NCAPH in LUAD. (A-C) Survival curves of OS, PFI and DSS between NCAPH-high and -low patients with LUAD. (D–H) OS survival curves between NCAPH-high and -low patients with LUAD in T1&T2, T3&T4, N0&N1, M0 and M1 sub-group. OS, overall survival; DSS, disease specific survival; PFI, progression free interval.

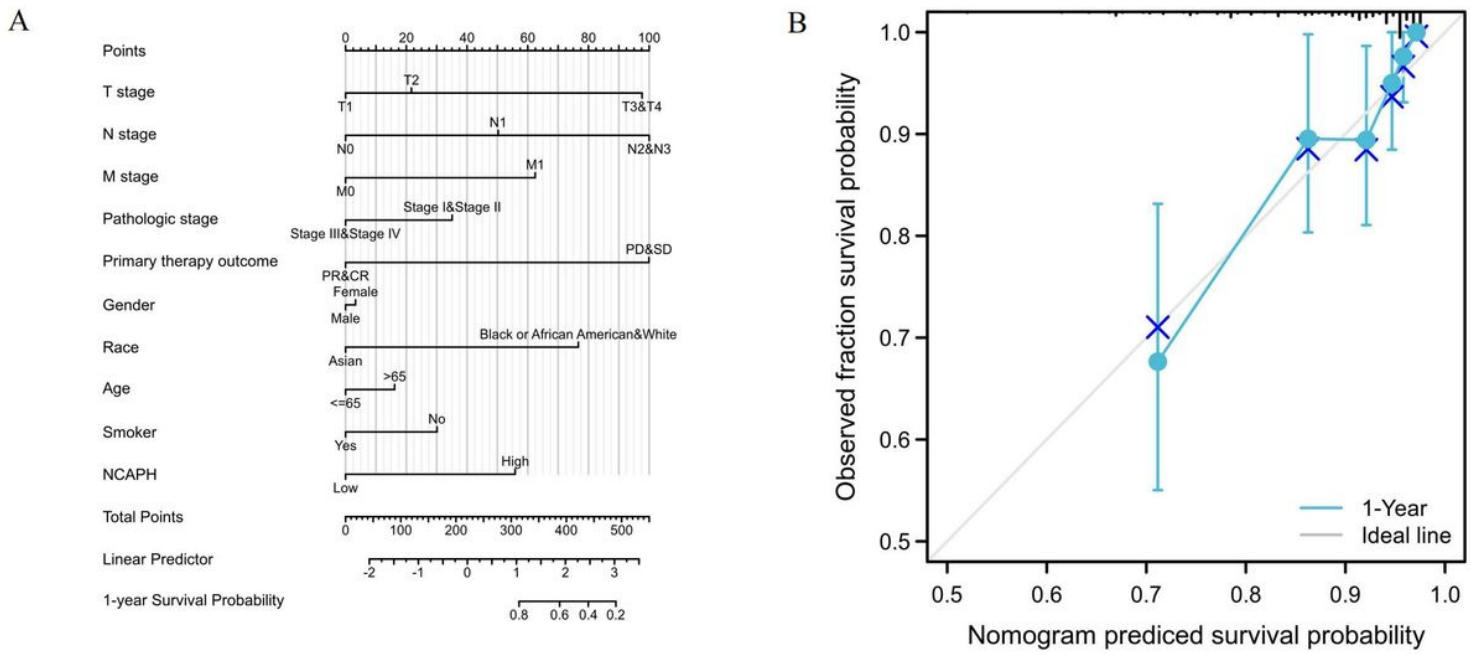

Figure 6

A quantitative method to predict LUAD patients' probability of 1 year OS. (A) A nomogram for estimating the probability of 1 year OS for LUAD patients. (B) Calibration plots of the nomogram for evaluating the probability of OS at 1 year.

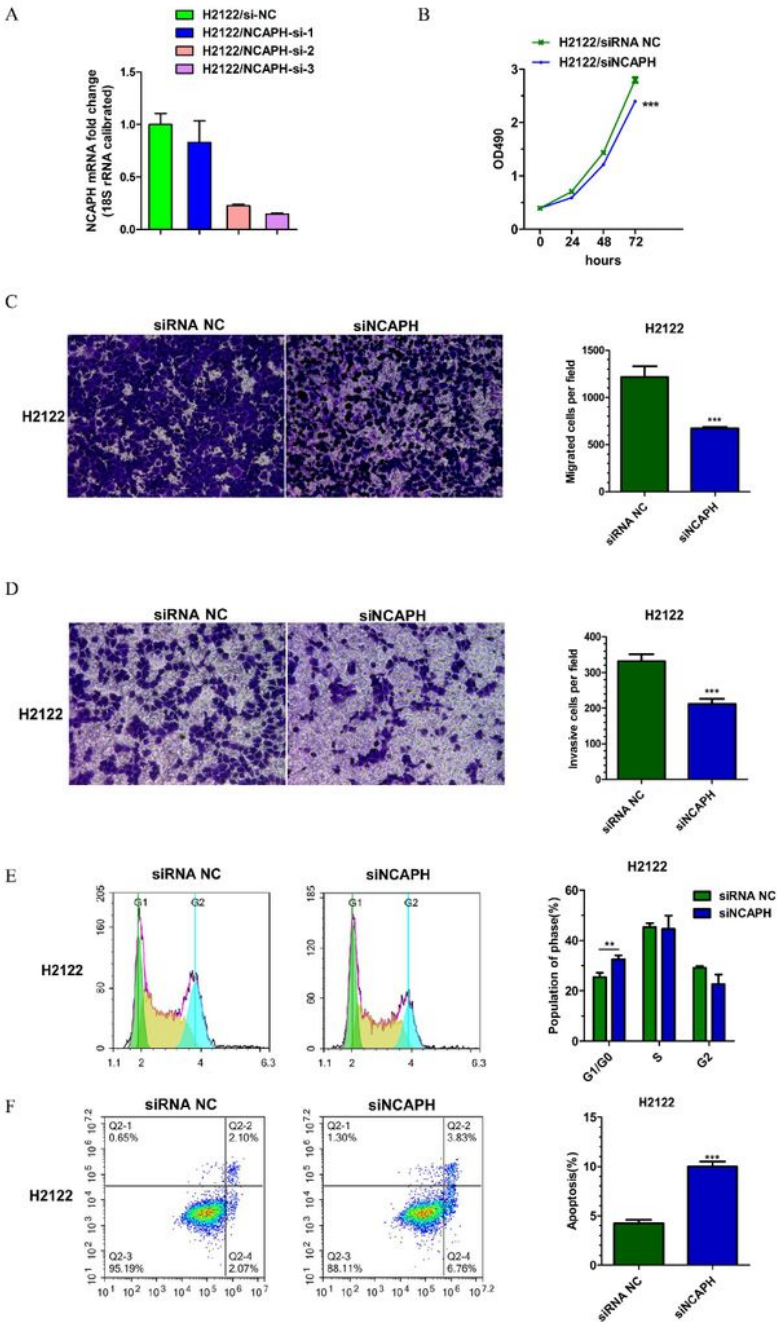

Figure 7

Knockdown of NCAPH by siRNA treatment regulate proliferation, migration, invasion, cell cycle and apoptosis of LUAD cells in H2122: (A) Knockdown efficiency of three different siRNAs for NCAPH; (B) MTT assay for cell proliferation; (C) Migration assay; (D) Invasion assay; (E) Cell cycle image and data; (F) Apoptosis assay.*P<0.05,**P<0.01,***P<0.01

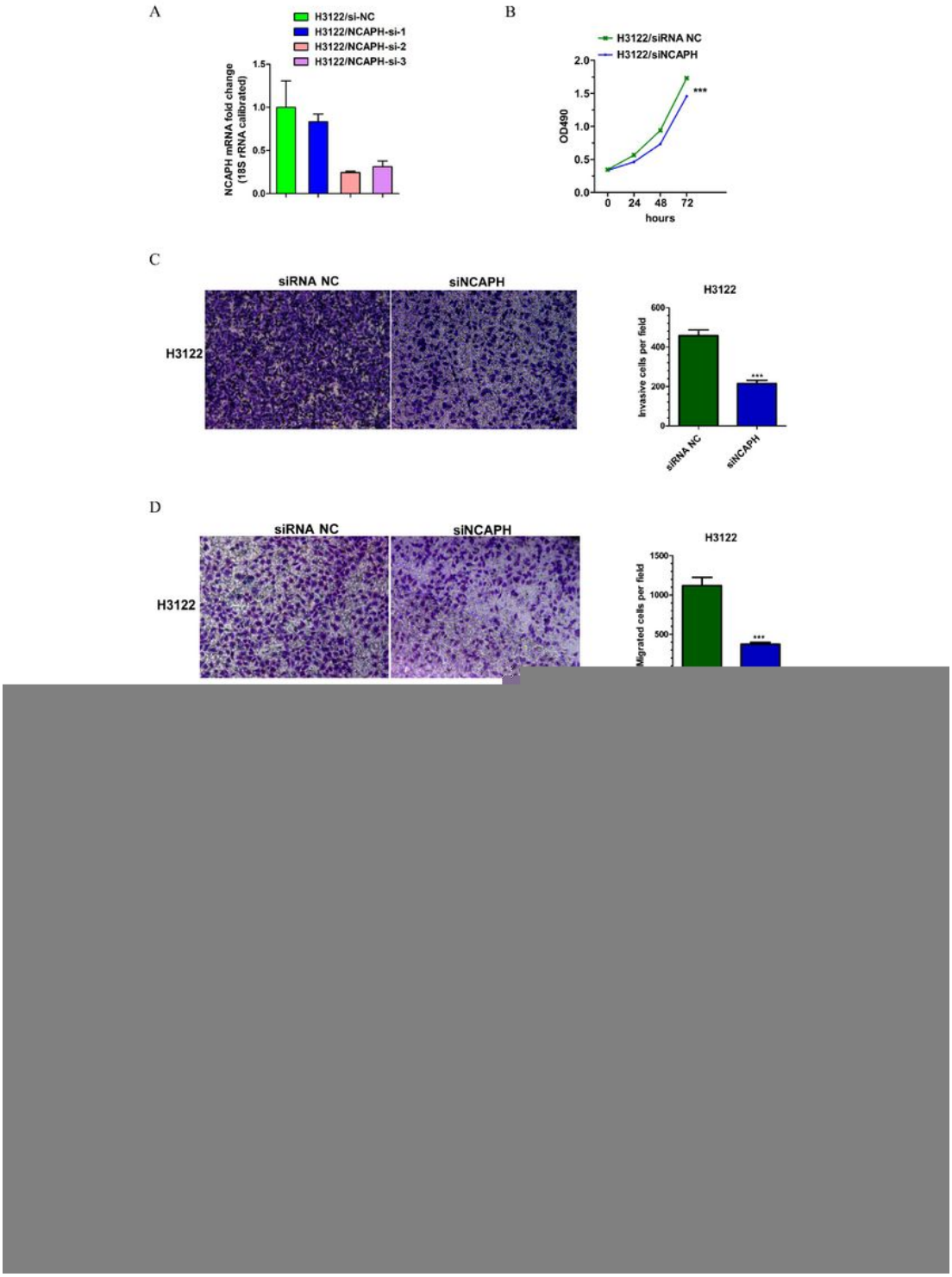

Figure 8

Knockdown of NCAPH by siRNA treatment regulate proliferation, migration, invasion, cell cycle and apoptosis of LUAD cells in H3122: (A) Knockdown efficiency of three different siRNAs for NCAPH; (B) MTT assay for cell proliferation; (C) Migration assay; (D) Invasion assay; (E) Cell cycle image and data; (F) Apoptosis assay.*P<0.05,**P<0.01,***P<0.01