Received: 16 July 2023 | Accepted: 20 February 2024

DOI: 10.1002/cai2.122

CANCER INNOVATION

ORIGINAL ARTICLE

Identification of NR3C2 as a functional diagnostic and prognostic biomarker and potential therapeutic target in non-small cell lung cancer

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Abstract

Background: Non-small cell lung cancer (NSCLC), including the lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) subtypes, is a malignant tumor type with a poor 5-year survival rate. The identification of new powerful diagnostic biomarkers, prognostic biomarkers, and potential therapeutic targets in NSCLC is urgently required.

Methods: The UCSC Xena, UALCAN, and GEO databases were used to screen and analyze differentially expressed genes, regulatory modes, and genetic/epigenetic alterations in NSCLC. The UCSC Xena database, GEO database, tissue microarray, and immunohistochemistry staining analyses were used to evaluate the diagnostic and prognostic values. Gain-of-function assays were performed to examine the roles. The ESTIMATE, TIMER, Linked Omics, STRING, and DAVID algorithms were used to analyze potential molecular mechanisms.

Results: NR3C2 was identified as a potentially important molecule in NSCLC. NR3C2 is expressed at low levels in NSCLC, LUAD, and LUSC tissues, which is significantly related to the clinical indexes of these patients. Receiver operating characteristic curve analysis suggests that the altered NR3C2 expression patterns have diagnostic value in NSCLC, LUAD, and especially LUSC patients. Decreased NR3C2 expression levels can help predict poor prognosis in NSCLC and LUAD patients but not in LUSC patients. These results have been confirmed both with database analysis and real-world clinical samples on a tissue microarray. Copy number variation contributes to low NR3C2 expression levels in NSCLC and LUAD, while promoter DNA

Abbreviations: ATCC, American Type Culture Collection; AUC, area under the curve; CNV, copy number variation; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NR3C2, nuclear receptor subfamily 3, group C, member 2; NSCLC, non-small cell lung cancer; OS, overall survival; ROC, receiver operating characteristic; TMA, tissue microarray; TME, tumor microenvironment.

Yuan-yuan Sun and Hai-cheng Gao contributed equally to this study and shared the first authorship.

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Funding information
Natural Science Foundation of Chongqing Municipality, Grant/Award Number: cstc2020jcyj-msxmX0565; National Natural Science Foundation of China, Grant/Award Number: 82073137; Natural Science Foundation of Jilin Province, Grant/Award Number: 20200201353JC

methylation is involved in its downregulation in LUSC. Two NR3C2 promoter methylation sites have high sensitivity and specificity for LUSC diagnosis with clinical application potential. NR3C2 may be a key participant in NSCLC development and progression and is closely associated with the tumor microenvironment and immune cell infiltration. NR3C2 co-expressed genes are involved in many cancer-related signaling pathways, further supporting a potentially significant role of NR3C2 in NSCLC.

Conclusions: NR3C2 is a novel potential diagnostic and prognostic biomarker and therapeutic target in NSCLC.

KEYWORDS
diagnosis and prognosis, expression regulation, function, immune cell infiltration, non-small cell lung cancer, NR3C2, therapeutic target, tumor microenvironment

1 | INTRODUCTION

Lung cancer is a type of malignant tumor that originates in the bronchial mucosa and alveoli. Globally, lung cancer remains the most common malignant tumor type and has a high morbidity and mortality [1]. In 2020, there were 2,206,771 new lung cancer cases worldwide, accounting for approximately 11.6% of all tumor cases. There were 1,796,144 deaths from this disease, which was about 18.4% of all tumor-related deaths [2, 3]. According to the latest cancer data in 2021, lung cancer deaths account for approximately 25% of tumor-related deaths [4]. Lung cancer is categorized into two main subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [5]. NSCLC, consisting of lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and lung large cell carcinoma, is the most common subtype and accounts for over 80% of lung cancer cases [6]. Because of the high degree of patient deterioration, limited clinical treatments, and diagnosis at middle and late stages, about 75% of NSCLC patients will die within 5 years [6]. This is primarily caused by a lack of effective early diagnostic biomarkers and powerful therapies developed from an in-depth understanding of pathogenesis. Therefore, screening for new diagnostic biomarkers and exploring the pathogenic mechanism of NSCLC to identify effective therapeutic methods are urgently required.

The nuclear receptor subfamily 3, group C, member 2 (NR3C2) is a type of ligand-dependent transcription factor that plays important roles in mediating aldosterone to regulate saline balance [7]. NR3C2 gene encodes the mineralocorticoid receptor, which binds to the mineralocorticoid response element to activate its target genes in *trans* [8, 9]. Recent studies have revealed that NR3C2 acts as a tumor suppressor [9–18]. In pancreatic cancer, NR3C2 suppresses the tumor cell growth, inhibits the epithelial to mesenchymal transition, and enhances the sensitivity to gemcitabine, and decreased NR3C2 expression is related to the low survival rate of the patients [10, 11]. NR3C2 can also act as a tumor suppressor in liver cancer by inhibition of the Wnt/β-catenin pathway [12]. NR3C2, as a target of miR-454, plays tumor inhibitory roles by affecting cell proliferation, apoptosis, migration, and invasion in oral squamous cell carcinoma, and NR3C2 low expression is associated with poor prognosis of the patients [14]. In colorectal cancer, NR3C2 is regulated by miR-4709 and can repress tumor development via inhibiting expressions of vascular endothelial growth factor A (VEGFA) and its receptor 2 (KDR), and decreased NR3C2 expression is associated with poor prognosis of the patients [13, 15]. In renal cell carcinoma, NR3C2 can repress the growth and migration of tumor cells, and the patients with high NR3C2 expression frequently have longer overall survival (OS) and disease-free survival [16]. In breast cancer, NR3C2 as a direct target of miR-301b-3p can inhibit the proliferation, migration, and invasion of tumor cells and high NR3C2 expression is associated with extended OS, disease-specific survival, and progression-free survival of the patients [17, 18]. Although a recent study performing a genome-wide lethal screening of NSCLC cells has suggested that NR3C2 is a potential tumor suppressor [19], the clinical significance, biological function, and molecular mechanism of NR3C2 are still largely unclear in lung cancer.

We previously identified NR3C2 as a gene expressed at a very low level in NSCLC (including LUAD and LUSC), suggesting that NR3C2 is possibly an important candidate for diagnosis and therapy in NSCLC. In this study, we performed further systematic analyses of NR3C2 in NSCLC. The association analyses indicated

that reduced NR3C2 expression could predict poor prognosis of NSCLC patients, with this altered expression pattern having a good diagnostic value in NSCLC and its subtype LUAD and LUSC. The area under the curve (AUC) values were more than 82%, reaching more than 98% in LUSC. Our experimental results reveal that NR3C2 can significantly inhibit tumor cell proliferation by affecting cell cycle progression, as well as suppress tumor cell migration and invasion. Low NR3C2 expression in tumors may be caused by copy number variation (CNV) in NSCLC and LUAD, while it is caused by promoter methylation in LUSC. Mechanistically, NR3C2 expression is closely associated with tumor microenvironment (TME) and immune cells in NSCLC, LUAD, and LUSC, with its co-expressed genes being involved in many cancer-related signaling pathways and connected with a variety of key tumorigenesis-associated molecules. The results of our study suggest that NR3C2 may be a new diagnostic and prognostic biomarker for NSCLC, as well as an important molecule for developing an effective therapeutic method.

2 | MATERIALS AND METHODS

2.1 | Analyses of publicly available datasets

NR3C2 gene expression, CNV data, methylation data, and clinical characteristics of patients with NSCLC were downloaded from the UCSC Xena database (https://xenaucsc.edu/). The Genomic Data Commons (GDC) and Cancer Genome Atlas (TCGA) LUAD and LUSC cohorts were used as patient's gene data sets. NR3C2 gene expression information data, copy number, methylation beta value, and clinicopathological indicators were extracted, and then the corresponding data were matched. Data from 1027 NSCLC patients (526 LUAD and 501 LUSC) with NR3C2 expression information and 108 corresponding cases of adjacent samples, 1018 NSCLC patients (520 LUAD and 498 LUSC) with NR3C2 copy number data, and 862 NSCLC patients (486 LUAD and 376 LUSC) with NR3C2 methylation beta values were used in the study.

2.2 | RNA sequencing (RNA-seq) data analysis in TCGA

Using the vlookup function matching and integration of NR3C2 expression data downloaded from UCSC Xena and the corresponding clinical data, 526 LUAD samples and 501 LUSC samples were obtained. The transcriptional level of NR3C2 in each sample was calculated from $\log_2$ (FPKM+1) of FPKM value. The Mann–Whitney non-parametric test was used to estimate the p value for the difference in gene expression and the relationship between gene expression and clinical parameters in NSCLC and the two main subtypes. The chi-square test was used to evaluate the difference in gene expression in different clinical parameters, with the continuous-corrected chi-square test being used for cases with a sample size of less than 5. All statistical analyses were performed using GraphPad Prism v8 software (GraphPad Software). A p value <0.05 was considered statistically significant. In addition, the differential expression of NR3C2 in cancer and adjacent normal tissues was analyzed using the online website UALCAN (http://ualcan.path.uab.edu/index.html).

2.3 | Receiver operating characteristic (ROC) curve analysis

The diagnostic value of NR3C2 was explored by examining expression levels in NSCLC, LUAD, and LUSC and normal samples. The x-axis and y-axis of the ROC curve represented specificity and sensitivity, respectively. The AUC value was calculated to evaluate the predictive ability of NR3C2 expression.

2.4 | Kaplan–Meier (KM) plotter

The online database KM Plotter tool (https://kmplot.com/analysis/index) [20] was used to evaluate the prognostic value of NR3C2 in NSCLC, LUAD, and LUSC patients. To analyze the effect of NR3C2 on OS, the patients were divided into high and low NR3C2 groups based on the median expression, then the log-rank test was used to evaluate the difference between KM survival curves. A log-rank $p < 0.05$ was considered statistically significant.

2.5 | Tissue microarray (TMA), ROC, and survival analyses

A cohort of 92 LUAD and 88 adjacent normal samples with clinicopathological and prognostic information on a TMA was provided by Shanghai Biochip Company Ltd. An antibody against NR3C2 (PA5-116990, Thermo Fisher Scientific) was used for immunohistochemistry (IHC) staining of NR3C2 in these samples. All core biopsies on the TMA were reviewed by two independent pathologists. Staining was considered positive when ≥10% of tumor cells were immunoreactive. The staining intensity

was divided into four levels: negative (score of 0), weak (score of 1), moderate (score of 2), and strong (score of 3). The percentage of positive cells was divided into five groups according to the degree of immunostaining: score of 0 with <10%, score of 1 with 10%–25%, score of 2 with 26%–50%, score of 3 with 51%–75%, and score of 4 with >76% of positive cells. NR3C2 protein expression was determined by multiplying the score of staining intensity and the score of positive cell percentage, with a score ≥8 being considered as high expression. The ROC and survival analyses were performed as described above.

2.6 | CNV analysis

NR3C2 copy number data and corresponding clinical data of 1018 NSCLC, 520 LUAD, and 498 LUSC samples were extracted and integrated from UCSC Xena. CNV classification was based on the copy number (gistic2_thresholded) standards from the TCGA gene copy database. A copy number of −1 or −2 was defined as a missing copy number, a copy number of 0 was defined as a normal copy number, and a copy number of 1 or 2 was defined as an elevated copy number. The chi-square test was used to evaluate the categorical variables of copy number in different clinical data. The Mann–Whitney non-parametric test was used to calculate the difference in copy number deletion/amplification and clinical characteristics, as well as the difference in NR3C2 expression in the copy number deletion group and normal group. Spearman's rank correlation was used to analyze the association of copy number with NR3C2 expression. A p value <0.05 was considered statistically significant.

2.7 | Methylation analysis

The methylation data measured using the Illumina Human Methylation 450 platform was downloaded from the UCSC Xena TCGA database. NR3C2 expression and methylation beta value data of 862 NSCLC patients (486 LUAD and 376 LUSC) were obtained through matching integration of the vlookup function. The average methylation beta value of all probe sites was calculated. The promoter region of NR3C2 (the 2000 bp region before the transcription start site) was analyzed using NCBI (https://www.ncbi.nlm.nih.gov/). Spearman's rank correlation was used to analyze the association of methylation with NR3C2 expression. The Mann–Whitney non-parametric test was used to calculate the correlation between NR3C2 methylation and clinicopathological parameters, as well as the difference in NR3C2 expression in the hypermethylated and hypomethylated groups. A p value <0.05 was considered statistically significant.

2.8 | Cell culture

The NSCLC cell lines A549 and H520 were purchased from the American Type Culture Collection (ATCC). The A549 and H520 cells were cultured in F12K and RPMI-1640 media, respectively, with 10% fetal bovine serum (FBS) and following the cell culture instructions from ATCC (37°C, 5% CO_2).

2.9 | Virus infection experiment

A549 and H520 cell models were constructed by infecting with an NR3C2 lentiviral vector. The cells were seeded evenly in a 12-well plate and incubated for 24 h, and then infected with NR3C2 lentiviral vectors at the optimal multiplicity of infection (MOI). An optimal MOI of 30 was determined with preliminary experiments. The cell morphology and fluorescence intensity were observed at 72 h and 96 h after infection.

2.10 | Cell proliferation assay

The infected A549 and H520 cells were evenly seeded in 96-well plates. Cell proliferation was detected by the CCK-8 colorimetric method according to the kit instructions (Biyuntian Biotechnology) at 24 h, 48 h, and 72 h after seeding. The optical density (OD) values were obtained at 450 nm. All tests were performed at least three times independently.

2.11 | Cell cycle assay

The infected A549 and H520 cells were seeded evenly in six-well plates. The cells were digested and collected 72 h later, then washed twice with pre-cooled PBS and fixed overnight in 70% ethanol at 4°C. The cell cycle of the fixed cells was measured by flow cytometry after staining according to the kit instructions (C1052, Biyuntian Biotechnology). All tests were performed at least three times independently.

2.12 | Transwell assay

The infected A549 and H520 cells were seeded evenly in transwell chambers (Corning). Cell invasion and migration rates were detected using transwell chambers with

and without matrigel (BD Biosciences), respectively. The cells that invaded and migrated were fixed in 4% paraformaldehyde at 24 h after seeding, stained with crystal violet, and photographed under a microscope. Five different fields of view were randomly selected for photograph and statistical analyses. All tests were performed at least three times independently.

2.13 | Correlation between NR3C2 expression and TME

The immune cell infiltration scores of TME were analyzed in the NR3C2 high and low expression groups. The immune and stromal scores (two main cell types of nontumor components) were calculated according to the ESTIMATE algorithm for quantification of immune and stromal components in tumors, which can predict the infiltration of nontumor cells. The data were obtained from the ESTIMATE online database (https://bioinformatics.mdanderson.org/estimate/index.html), then RNA-seq file was subjected to the ESTIMATE algorithm, and the relationship of each immune and stromal cell score with NR3C2 expression was analyzed. The Mann–Whitney non-parametric test was used to compare the score differences between the high and low NR3C2 groups. The infiltration status of six immune cell types (B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells) was obtained from the TIMER online database (https://cistrome.shinyapps.io/timer/). Pearson's correlation analysis was used to evaluate the correlation between these immune cells and NR3C2 expression. A p value <0.05 was considered statistically significant.

2.14 | Enrichment and network analyses

LUAD and LUSC cohorts named TCGA_LUAD and TCGA_LUSC in the HiSeq RNA platform were selected as target datasets. Pearson correlation test was used to analyze the NR3C2 co-expressed genes in LinkedOmics (http://www.linkedomics.org/login.php), and these co-expressed genes were explored using the Link Finder module and presented in volcano maps and heat maps.

The top 200 co-expressed genes of NR3C2 with official symbols were selected to perform gene ontology (GO) biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using David (https://david.ncifcrf.gov/). The protein molecules interacting with NR3C2 were predicted by constructing an interacting protein–protein interaction network using STRING (https://string-db.org/).

3 | RESULTS

3.1 | NR3C2 expression is significantly decreased in NSCLC and its subtypes

To identify new diagnostic biomarkers for NSCLC, we previously screened the differentially expressed genes between NSCLC and adjacent normal tissues in the TCGA database. NR3C2 was expressed at low levels in NSCLC tissues, making it a potential key differential gene (Figure 1a). NR3C2 expression was analyzed in 1027 tumors and 108 adjacent normal tissues of NSCLC, indicating significantly lower in tumor tissues ($p < 0.01$, Figure 1a). Furthermore, NR3C2 expression was significantly reduced in cancerous tissues of its two main subtypes, LUAD and LUSC ($p < 0.01$, Figure 1a). To verify these results, the microarray gene profile data set (GSE19188) was downloaded and analyzed from the Gene Expression Omnibus (GEO) database. NR3C2 expression was also significantly decreased in both LUAD ($p = 3.58 \times 10^{-12}$) and LUSC ($p = 2.87 \times 10^{-25}$, Figure 1a). These results show that NR3C2 expression is significantly downregulated in NSCLC, LUAD, and LUSC.

3.2 | Altered NR3C2 expression is closely related to several clinical indexes of NSCLC and its subtypes

To evaluate the clinical significance of lower NR3C2 expression, we obtained the clinical and expression data of NSCLC (including LUAD and LUSC) in TCGA cohort from UCSC Xena website (https://xena.ucsc.edu/), then analyzed the relationships between NR3C2 expression and clinical features of NSCLC ($N = 1027$), LUAD ($N = 526$), and LUSC ($N = 501$) patients. NR3C2 expression was significantly associated with gender ($p < 0.01$), smoking status ($p < 0.01$), primary tumor (T, $p < 0.01$), regional lymph nodes (N, $p < 0.01$), and clinical stage ($p < 0.01$) of NSCLC patients (Table 1 and Figure 1b). In LUAD and LUSC, NR3C2 expression was significantly related to the age ($p < 0.05$), gender ($p < 0.05$), regional lymph nodes ($p < 0.01$), and clinical stage ($p < 0.01$) of LUAD patients (Figure 1c and Supporting Information S1: Table 1), while it was only significantly related to the age ($p < 0.01$) and clinical stage ($p < 0.05$) of LUSC patients (Figure 1d and Supporting Information S1: Table 2). These data reveal that NR3C2 expression is significantly associated with several clinical indexes, including disease stages of NSCLC, LUAD, and LUSC.

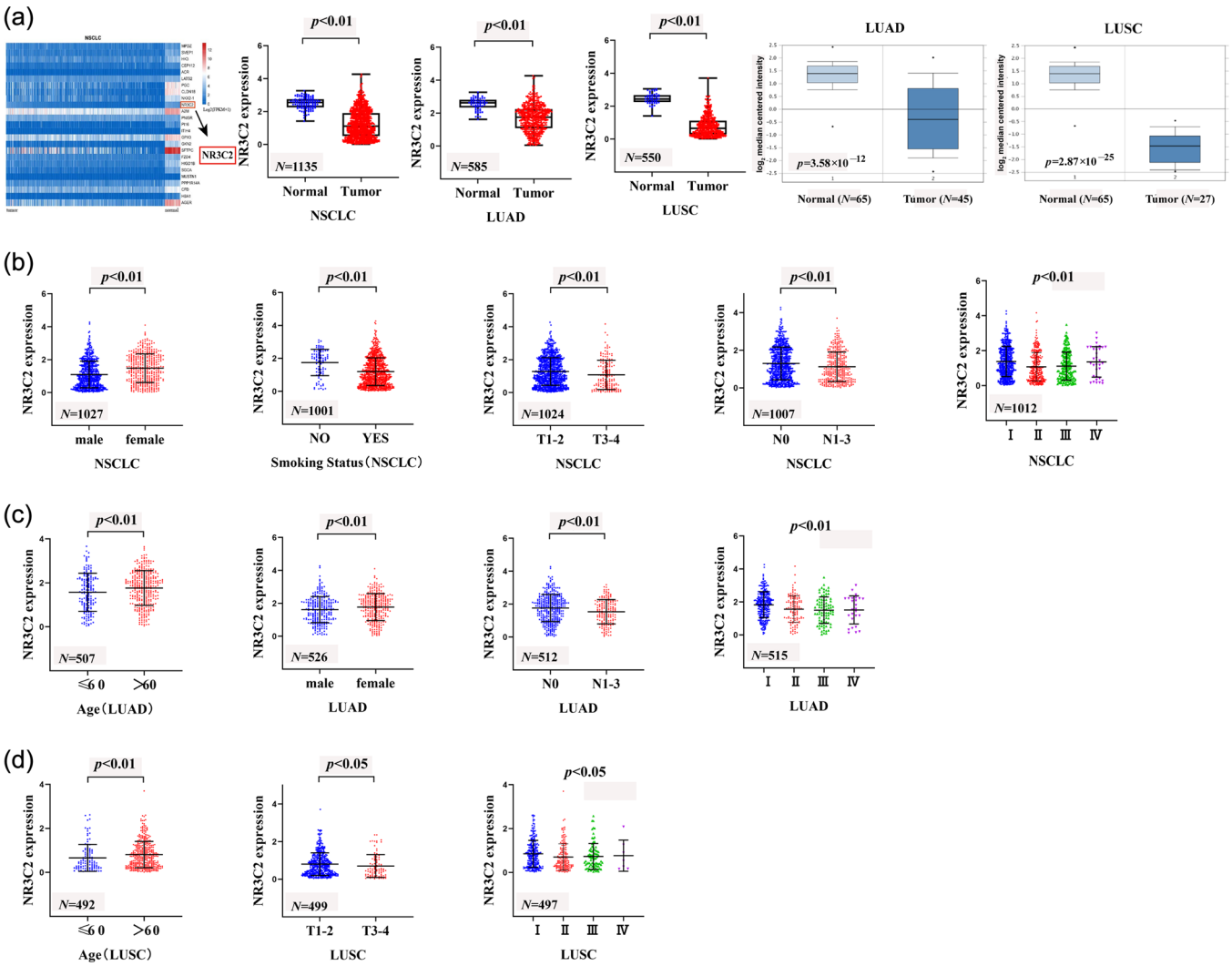

FIGURE 1 NR3C2 expression levels are significantly decreased and related to clinical indexes in non-small cell lung cancer (NSCLC), lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC) patients. (a) The differentially expressed genes and candidate expression in the cancer and adjacent normal tissues of NSCLC patients. NR3C2 mRNA expression levels were significantly downregulated in NSCLC, LUAD, and LUSC tissues from The Cancer Genome Atlas (TCGA) and independent data of the GSE19188 data set. (b) The relationships between NR3C2 expression levels and clinical indexes of NSCLC patients. NR3C2 expression levels were significantly related to the gender, smoking status, tumor size (T), regional lymph nodes (N), and clinical stage of NSCLC patients. (c) The relationships between NR3C2 expression levels and clinical indexes of LUAD patients. NR3C2 expression levels were significantly related to the age, gender, regional lymph nodes (N), and clinical stage of LUAD patients. (d) The relationships between NR3C2 expression levels and clinical indexes of LUSC patients. NR3C2 expression levels were significantly related to the age, tumor size (T), and clinical stage of LUSC patients. The two-tailed Mann–Whitney *U* test was used in these analyses.

3.3 | Altered NR3C2 expression has diagnostic value in NSCLC, LUAD, and LUSC

To determine the possible diagnostic significance of altered NR3C2 expression, we performed ROC curve analyses in NSCLC, LUAD, and LUSC patients. The AUC value of NR3C2 was 0.8987 (95% confidence interval (CI) = 0.879–0.9185, $p < 0.0001$) in NSCLC, with specificity and sensitivity values of 0.944 and 0.776, respectively (Figure 2a). The AUC value of NR3C2 was 0.8278 (95% CI = 0.7875–0.882, $p < 0.0001$) in LUAD, with specificity and sensitivity values of 0.932 and 0.62, respectively (Figure 2b). In LUSC, the AUC value of NR3C2 was 0.9806 (95% CI = 0.9699–0.9913, $p < 0.0001$), with specificity and sensitivity values of 0.98 and 0.94, respectively (Figure 2c). These data suggest that NR3C2 is a potential diagnostic marker for NSCLC, LUAD and LUSC, especially for LUSC with an AUC value of over 0.98.

TABLE 1 The relationships between clinical factors and NR3C2 mRNA expression levels in NSCLC patients ($N = 1027$).

Clinical factor	Clinical factor	NR3C2 expression		Censor	*p* value
		High ($N = 513$)	Low ($N = 514$)		
Age (yr)					
≤60	271	124	147	28	0.154
>60	728	370	358		
Gender					
Male	615	256	359	0	**0.000**
Female	412	257	155		
Smoking status					
No	93	72	21	26	**0.000**
Yes	908	428	480		
Clinical stage					
I	528	298	230	15	**0.000**
II	283	113	170		
III	168	73	95		
IV	33	21	12		
T (primary tumor)					
T1–2	863	446	417	3	**0.0128**
T3–4	161	66	95		
N (regional lymph nodes)					
N0	660	351	309	20	**0.0047**
N1–3	347	152	195		
M (distant metastases)					
M0	765	386	379	9	0.151
M1	32	12	20		

Note: The difference of categorical variables was analyzed by the chi-square test. Bolded values indicate statistical significance ($p < 0.05$).

3.4 | NR3C2 expression is associated with the prognosis of NSCLC and LUAD patients but not of LUSC patients

KM survival curves were generated to determine the prognostic value of NR3C2 expression in NSCLC, LUAD, and LUSC. As shown in Figure 3a, low NR3C2 expression was significantly correlated with poor OS of NSCLC (Logrank $p = 2.3 \times 10^{-16}$) and LUAD (Logrank $p = 3 \times 10^{-11}$) patients, while NR3C2 expression was not associated with OS (Logrank $p = 0.78$) of LUSC patients. To determine whether specific clinical information affects the correlation between NR3C2 expression and patient prognosis, we examined the relationships between NR3C2 expression and the prognosis of NSCLC and LUAD patients with different clinical characteristics. As shown in Figure 3b, low NR3C2 expression was significantly associated with poor OS of the NSCLC patients at clinical stage I (Logrank $p = 1.7 \times 10^{-15}$) but was not associated with OS of the NSCLC patients at clinical stage II or clinical stage III. In LUAD, low NR3C2 expression was significantly associated with poor OS of the patients at clinical stage I (Logrank $p = 4.8 \times 10^{-8}$) and clinical stage II (Logrank $p = 0.00097$), but was not associated with OS of the patients at clinical stage III (Figure 3c). In addition, low NR3C2 expression was significantly associated with poor OS of the LUAD patients without lymph node metastasis (N0) (Logrank $p = 0.0048$), but was not associated with OS of the LUAD patients with lymph node metastasis (N1, Figure 3d). The correlations between NR3C2 expression and prognosis were not significantly different in these patients with other clinical characteristics examined. These results reveal that NR3C2 expression can affect the OS of

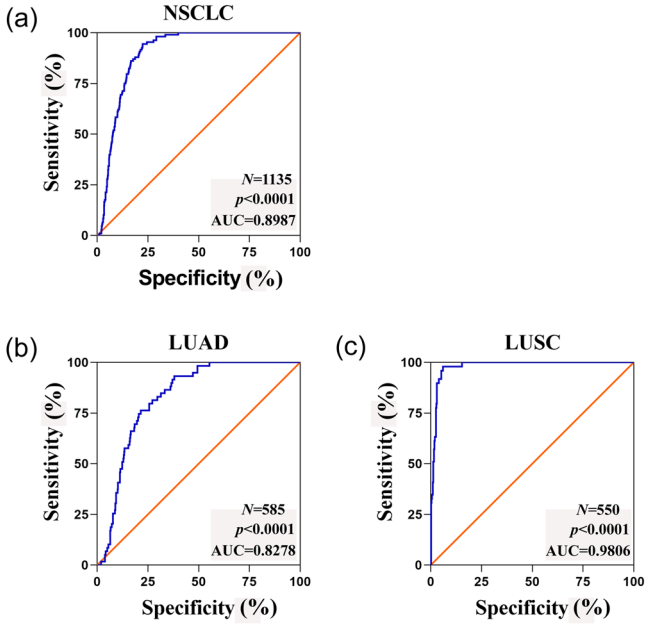

FIGURE 2 Altered NR3C2 expression patterns have diagnostic value in non-small cell lung cancer (NSCLC), lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC) patients. Receiver operating characteristic (ROC) curve analysis was performed to estimate the diagnostic value of NR3C2 expression in NSCLC (a), LUAD (b), and LUSC (c). The red and blue lines represent the ROC curve and random performance of NR3C2, respectively. AUC, area under the curve.

NSCLC and LUAD patients, suggesting a prognostic value.

3.5 | NR3C2 protein expression is also associated with OS and has a diagnostic value in LUAD

To further evaluate the clinical significance of NR3C2 expression, 92 LUAD samples were subjected to IHC analysis using a TMA. The staining intensity and positive percentage of NR3C2 protein scores were determined and then multiplied to calculate the NR3C2 protein expression level. The patients were divided into two groups: high NR3C2 (score of ≥8 and ≤12) and low NR3C2 (score of <8) (Figure 4a). When analyzing the relationships with clinicopathological characteristics, NR3C2 protein expression was found to be significantly associated with gender ($p = 0.0006$) and regional lymph nodes ($p = 0.0221$, Table 2). To clarify whether NR3C2 protein expression in these samples was related to the prognosis of patients, we generated KM survival curves to examine OS rates. The patients with low NR3C2 protein expression had significantly shorter survival times compared with the patients with high NR3C2 protein expression ($p = 0.0353$, Figure 4b). Furthermore, the AUC value of NR3C2 was 0.8472 (95% CI = 0.7888–0.9056, $p < 0.0001$), with specificity and sensitivity being 0.92 and 0.667, respectively (Figure 4c). These data suggest that NR3C2 protein expression may also serve as a prognostic and diagnostic biomarker for LUAD.

3.6 | Low NR3C2 expression may result from CNV in NSCLC and LUAD, but not in LUSC

To determine the mechanism behind the downregulation of NR3C2 in NSCLC, we analyzed the relationship between CNV (often manifests as copy number deletion or amplification) and NR3C2 expression. NR3C2 expression in NSCLC was significantly correlated with copy number deletion ($p < 0.0001$), but not with copy number amplification (Figure 5a). Similarly, NR3C2 expression in LUAD was also significantly correlated with copy number deletion ($p < 0.0001$), but not with copy number amplification (Figure 5b). Unexpectedly, NR3C2 expression in LUSC was not correlated with copy number deletion or copy number amplification (Figure 5c). To further verify whether loss of NR3C2 gene copy number affects its expression, we next divided the patients into the copy number deletion group and normal copy number group for NSCLC ($N = 927$) and LUAD ($N = 464$). The NR3C2 expression in the copy number deletion group was significantly lower than the normal copy number group in both NSCLC and LUAD ($p < 0.0001$, Figure 5d).

NR3C2 CNV was usually detected in NSCLC patients with a deletion rate of 44.99% and an expansion rate of 8.25% (Figure 6a). To evaluate potential associations, we then determine the connection of NR3C2 CNV with different clinical information in NSCLC patients. NR3C2 CNV distribution was altered in the patients with different gender and smoking status. Compared with females, male NSCLC patients had significantly higher NR3C2 CNV and copy number deletion rates ($p = 0.0033$, Figure 6a). Additionally, NSCLC patients who smoked had significantly higher NR3C2 CNV and copy number deletion rates compared with non-smokers ($p < 0.0001$), and this pattern for smokers was observed in both LUAD and LUSC ($p < 0.05$, Figure 6a). However, NR3C2 CNV distribution was not different between patients with different other clinical information, such as regional lymph nodes (N), distant metastases (M), and clinical stage (Supporting Information S1: Figure 1). Overall, NR3C2 copy number deletion was significantly related to gender ($p < 0.01$) and smoking status ($p < 0.01$), but not with primary tumor (T), regional lymph nodes, distant metastases (M), or clinical stage in NSCLC patients (Supporting Information S1: Figure 2A and Table 3). NR3C2 deletion was only

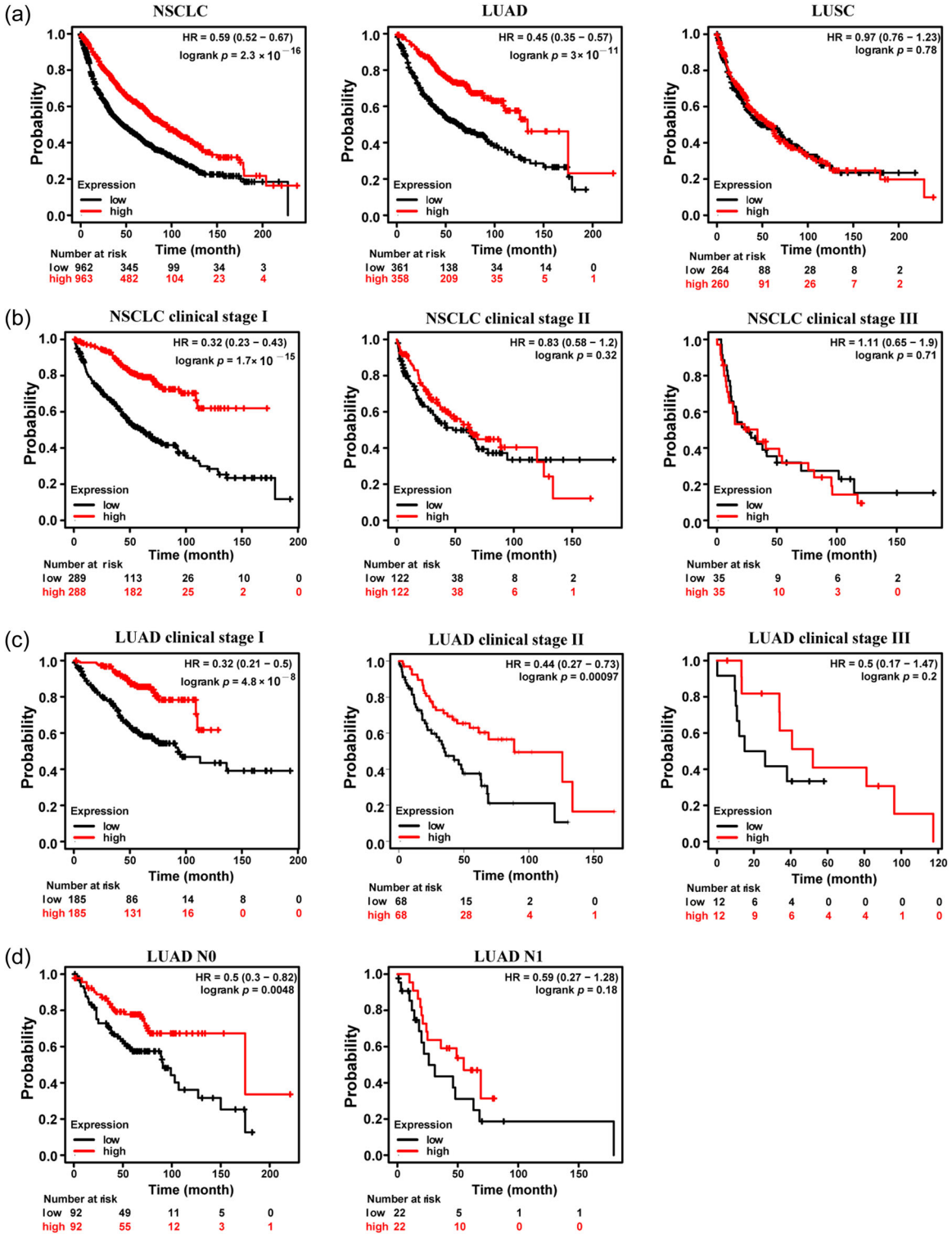

FIGURE 3 NR3C2 expression is significantly associated with the prognosis of non-small cell lung cancer (NSCLC) and lung adenocarcinoma (LUAD) patients but not of lung squamous cell carcinoma (LUSC) patients. (a) Kaplan–Meier (KM) survival analyses of NSCLC, LUAD, and LUSC patients. KM survival curves were generated to evaluate the prognostic value of NR3C2 expression in 596 NSCLC patients, 244 LUAD patients, and 70 LUSC patients. (b) KM survival analyses of NSCLC patients at different clinical stages. KM survival analysis was performed to determine the prognostic value of NR3C2 expression in NSCLC patients at clinical stages I, II, and III. (c) KM survival analyses of LUAD patients at different clinical stages. KM survival analysis was performed to determine the prognostic value of NR3C2 expression in LUAD patients at clinical stages I, II, and III. (d) KM survival analyses of LUAD patients with different N1 stages. KM survival analysis was performed to evaluate the prognostic value of NR3C2 expression in LUAD patients with N0 and N1 stage disease.

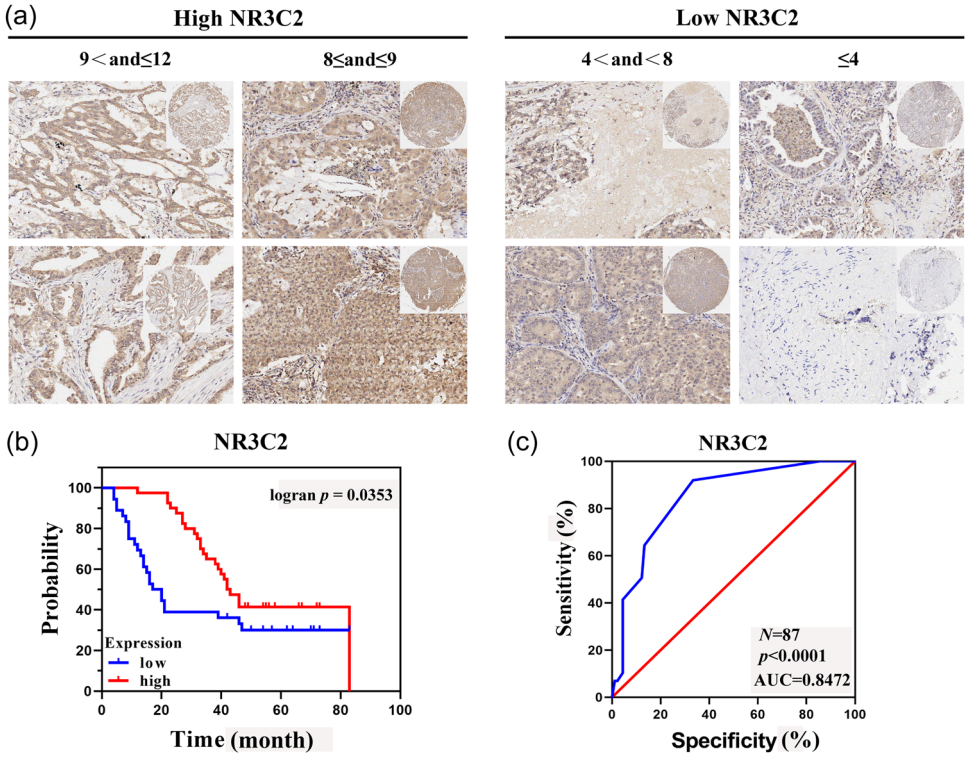

FIGURE 4 NR3C2 protein expression levels are associated with patient prognosis and diagnosis. (a) Representative images of different score levels of NR3C2 protein expression. The NR3C2 low expression group included patients with a score of <8. The NR3C2 high expression group included patients with a score of ≥8 and ≤12. (b) Kaplan–Meier survival analyses of patients with different NR3C2 protein expression levels. Patients in the NR3C2 high expression group had a better survival rate than those in the low expression group. (c) The diagnostic value of NR3C2 protein expression for patients. Receiver operating characteristic (ROC) curve analysis showed high sensitivity and specificity values of NR3C2 protein expression levels. AUC, area under the curve.

significantly related to gender ($p < 0.05$) in LUAD (Supporting Information S1: Figure 2B and Table 4) and to none of the clinical parameters examined in LUSC (Supporting Information S1: Figure 2C and Table 5). These data suggest that copy number deletion may regulate NR3C2 expression in NSCLC and LUAD but not in LUSC.

3.7 | NR3C2 expression is significantly associated with DNA methylation of the gene promoter region in LUSC

To further examine the mechanism controlling NR3C2 expression in LUSC, we analyzed the relationship between NR3C2 gene methylation and its expression levels. NR3C2 expression was significantly negatively related to the average methylation level at all sites examined in LUSC ($p = 0.0124$, Figure 6b). Because DNA methylation of the promoter region is often closely related to the gene's transcriptional activation, we then evaluated the connection between NR3C2 expression and the DNA methylation level of its promoter region. Probes corresponding to NR3C2 promoter region sequence were obtained from NCBI. The average methylation beta values of these probes were analyzed, and then the relationship between NR3C2 expression and promoter DNA methylation was examined. As shown in Figure 6b, NR3C2 expression was negatively correlated with the average methylation level of NR3C2 promoter region in LUSC ($p < 0.0001$). To further confirm this observed association, we analyzed the data from the UALCAN online database, and NR3C2 promoter region methylation level was significantly higher in LUSC than in adjacent normal samples ($p < 0.0001$, Figure 6c). The above results were further confirmed using an independent data set ($p < 0.05$, Figure 6d). These data suggest that DNA methylation of the NR3C2 gene promoter region may regulate its expression patterns in LUSC tissues.

To study its clinical significance, we analyzed the relationships between NR3C2 DNA methylation and clinical information of LUSC patients. NR3C2 DNA full-site methylation level was only significantly associated with gender ($p < 0.001$, Supporting Information S1: Table 6). We then selected two specific probes in the NR3C2 promoter region (cg13373360 and cg16692923) that were negatively associated with NR3C2 expression ($p < 0.05$, Figure 6e) and found that the methylation level of cg13373360 was

TABLE 2 The relationships between clinical factors and NR3C2 protein expression levels in lung adenocarcinoma (LUAD) patients ($N = 92$).

Clinical factor	Clinical factor	NR3C2 expression		Censor	*p* value
		High ($N = 60$)	Low ($N = 30$)		
Age (yr)					
≤60	37	23	14	0	0.4488
>60	53	37	16		
Gender					
Male	49	25	24	0	**0.0006**
Female	41	35	6		
Clinical stage					
I	20	11	9	35	0.1615
II	15	8	7		
III–IV	20	16	4		
T (primary tumor)					
T1–2	58	39	19	13	0.5987
T3–4	19	14	5		
N (regional lymph nodes)					
N0	33	17	16	21	**0.0221**
N1–3	36	28	8		

Note: The difference of categorical variables was analyzed by the chi-square test. Bolded values indicate statistical significance ($p < 0.05$).

significantly related to patient age ($p = 0.0041$) and primary tumor ($p = 0.0378$), while the methylation level of cg16692923 was significantly related to patient age ($p = 0.003$), primary tumor ($p = 0.0492$), and regional lymph nodes ($p = 0.0493$, Figure 7a,b). Using the median methylation beta value, the patients were divided into hypermethylation and hypomethylation groups for the NR3C2 promoter region, and the diagnostic value of NR3C2 promoter methylation was then calculated. The AUC values of both methylation sites were above 0.75, with respective sensitivity and specificity of 0.881 and 0.857 for the cg13373360 site (Figure 7a) and 0.559 and 0.881 for the cg16692923 site (Figure 7b). These results suggest that NR3C2 expression is associated with DNA methylation of its promoter region, and the methylation sites have high sensitivity and specificity for diagnosis in LUSC.

3.8 | NR3C2 is a key participant in NSCLC development

To clarify the role of NR3C2 in NSCLC, we constructed A549 and H520 cell models of NR3C2 overexpression (Figure 8a) and determined cell proliferation and metastasis abilities. The cell cycle of tumor cells was significantly arrested in the G1 phase resulting in clear reduction of tumor cell proliferation (Figure 8b,c), suggesting that NR3C2 may inhibit tumor cell proliferation by affecting cell cycle progression. To further examine the possible role of NR3C2 on tumor metastasis, the effects of NR3C2 expression on the migration and invasion abilities of tumor cells were analyzed using transwell assays. The results with and without matrigel showed that tumor cell invasion and migration rates were significantly decreased after NR3C2 overexpression (Figure 8d). These data reveal that NR3C2 significantly inhibits tumor cell proliferation, invasion, and migration, suggesting NR3C2 is a key participant in NSCLC development and progression.

3.9 | NR3C2 expression levels are associated with the TME and immune cell infiltration in NSCLC, LUAD, and LUSC

We then determined whether NR3C2 expression is related to TME using ESTIMATE (https://bioinformatics.mdanderson.org/estimate/index.html). The stromal score, immune score, and tumor purity of TME were significantly

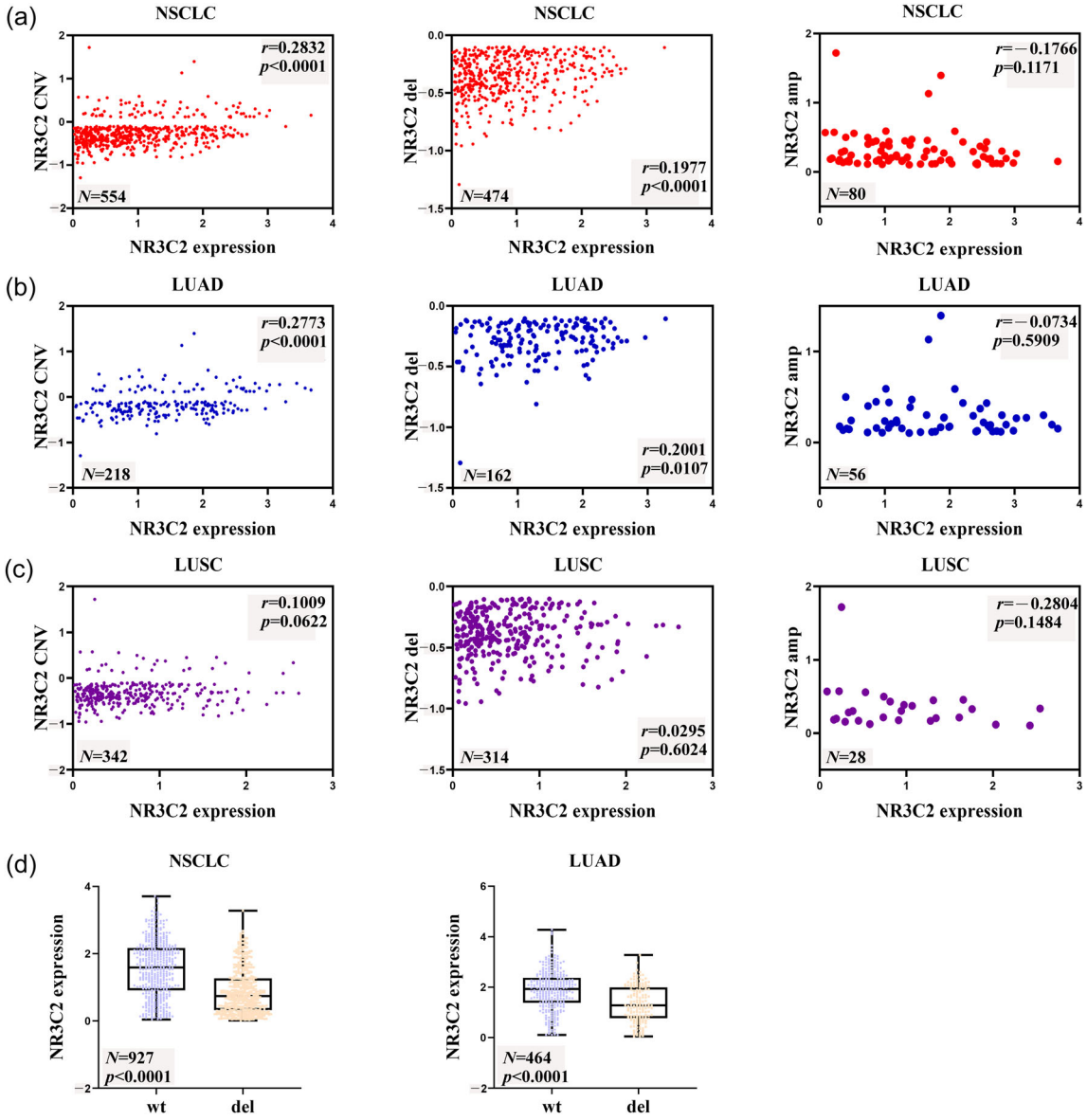

FIGURE 5 The relationships between copy number variation (CNV) and NR3C2 expression levels in non-small cell lung cancer (NSCLC) and lung adenocarcinoma (LUAD). (a) The correlations between CNV/deletion/amplification and NR3C2 expression levels in NSCLC samples. (b) The correlations between CNV/deletion/amplification and NR3C2 expression levels in LUAD samples. (c) The correlations between CNV/deletion/amplification and NR3C2 expression levels in lung squamous cell carcinoma (LUSC) samples. (d) NR3C2 expression in NSCLC patients with normal copy number and deletion and in LUAD patients with normal copy number and deletion.

different between the high and low NR3C2 groups (Figure 9a). In NSCLC, lower immune score ($p < 0.0001$), stromal score ($p < 0.0001$), and ESTIMATE score ($p < 0.0001$) were observed in the low NR3C2 group (Figure 9a). In LUAD, lower stromal score ($p = 0.0029$) and ESTIMATE score ($p = 0.0141$) were found in the low NR3C2 group (Figure 9b). In LUSC, lower immune score ($p < 0.0001$), stromal score ($p < 0.0001$), and ESTIMATE score ($p = 0.0003$) were observed in the low NR3C2 group (Figure 9c). These results show that NR3C2 expression levels are closely associated with the TME in NSCLC, LUAD, and LUSC.

Immune cell infiltration data were further obtained using the TIMER online database (https://cistrome.shinyapps.io/timer/), and Pearson correlation analysis was used to evaluate the connection between NR3C2 expression and each immune cell type. NR3C2 expression was positively correlated with B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells in NSCLC, LUAD, and LUSC ($p < 0.01$, Figure 9b–d). These results suggest that NR3C2 expression is also significantly associated with the tumor immune microenvironment in NSCLC, LUAD, and LUSC.

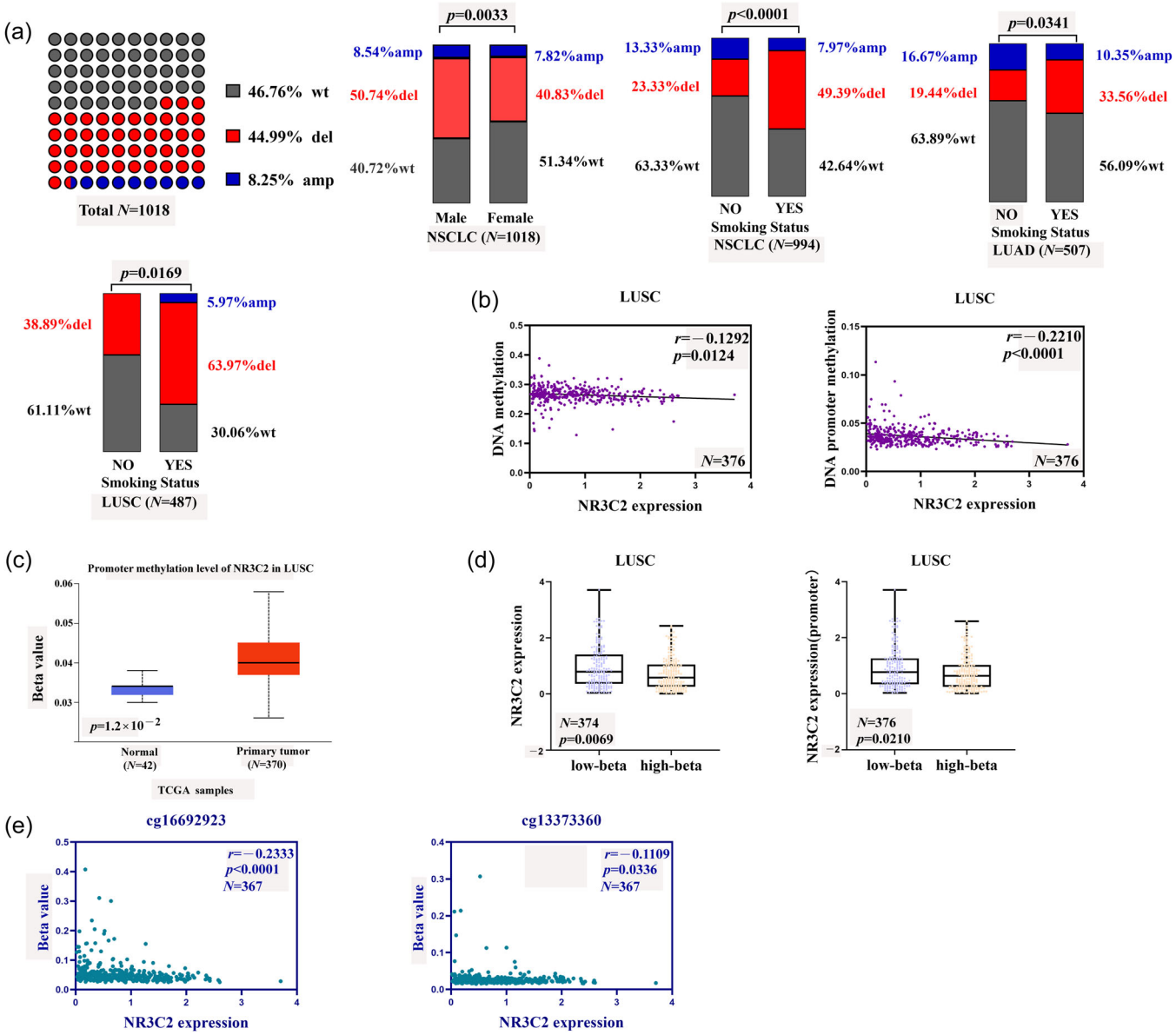

FIGURE 6 The clinical features of NR3C2 copy number variation (CNV) in non-small cell lung cancer (NSCLC) and lung adenocarcinoma (LUAD) patients and the connection of NR3C2 promoter methylation with its expression in lung squamous cell carcinoma (LUSC) patients. (a) The percentages and characteristics of NR3C2 wild type, deletion, and amplification in NSCLC. The proportions of NR3C2 wild type, deletion, and amplification differed in NSCLC patients by gender and smoking status, in LUAD patients by smoking status, and in LUSC patients by smoking status. (b) The correlation of NR3C2 expression levels with DNA methylation and promoter methylation in LUSC patients. (c) NR3C2 promoter methylation levels in LUSC and adjacent normal tissues analyzed using the UALCAN website. (d) NR3C2 expression levels in the NR3C2 hypermethylation and hypomethylation groups of LUSC patients. (e) The correlations of NR3C2 expression levels with probe cg13373360 and cg16692923 methylation in LUSC.

3.10 | Possible NR3C2-related mechanisms are determined by enrichment analysis of its co-expressed genes and interacting proteins

To identify the possible mechanisms of NR3C2 in LUAD and LUSC, we used the Link Finder module in the Linked Omics web portal to examine the genes co-expressed with NR3C2 in the TCGA-LUAD and TCGA-LUSC cohorts. Figure 10a/b displays the genes positively and negatively related to NR3C2 in LUAD and LUSC, respectively. In LUAD, NR3C2 co-expressed genes were primarily involved in sister chromatid cohesion, spindle microtubule, and kinesin complex processes, and enrichments of these co-expressed genes in the cell cycle, DNA replication, oocyte meiosis, p53, and base excision repair signaling pathways (Figure 10a). In LUSC, NR3C2 co-expressed genes were mainly involved in basolateral

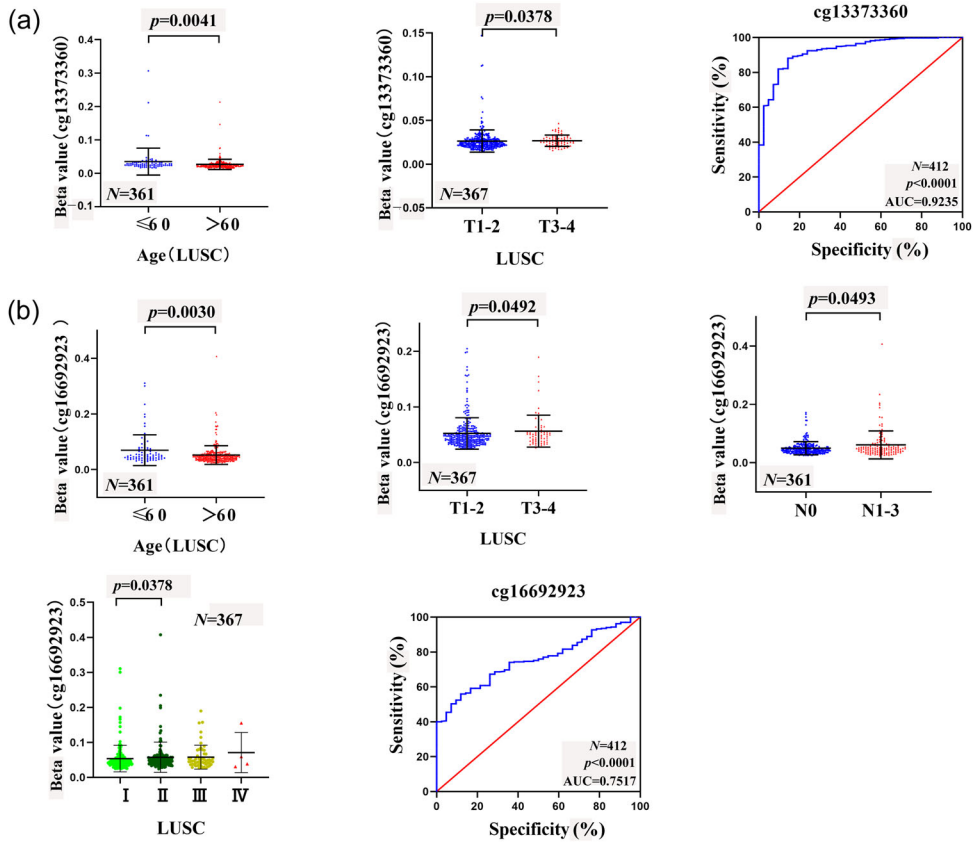

FIGURE 7 The clinical features and diagnostic value of NR3C2 methylation in lung squamous cell carcinoma (LUSC) patients. (a) The clinical features and diagnostic value of NR3C2 methylation at the cg13373360 site. The methylation beta value of the cg13373360 probe was different in LUSC patients by age and tumor size. The diagnostic value of the cg13373360 specific methylation site was analyzed in LUSC patients. (b) The clinical features and diagnostic value of NR3C2 methylation at the cg16692923 site. The methylation beta value of the cg16692923 probe was different in LUSC patients by age and tumor size. The diagnostic value of the cg16692923 specific methylation site was analyzed in LUSC patients.

plasma membrane, apical plasma membrane, and proteinaceous extracellular matrix processes, and enrichments of these co-expressed genes in protein digestion and absorption, arrhythmogenic right ventricular cardiomyopathy, hypertrophic cardiomyopathy, cell adhesion, Ras, and Rap1 signaling pathways (Figure 10b).

The main proteins interacting with NR3C2 were predicted using the STRING database. These predicted proteins were mainly involved in the cell cycle, transcriptional regulation, and cancer pathways (Figure 10c). To further verify these results, all the predicted NR3C2-interacting proteins were input into the DAVID Online annotation tool for enrichment analyses. The interacting proteins were primarily involved in the cell cycle, apoptosis, negative transcriptional regulation of RNA polymerase II promoter, and protein threosylation processes (Figure 10c). The interacting proteins were mainly involved in the PI3K–Akt signaling pathway and cancer-related signaling pathways (Figure 10c).

4 | DISCUSSION

NSCLC is the most common cancer type and has the highest morbidity and mortality [1, 6]. Identifying new useful early diagnostic and prognostic biomarkers and studying the mechanisms are critical for the development of more effective therapeutic methods for NSCLC. Current studies have shown that NR3C2 is closely associated with depression, pseudo-aldosteronism, and several cancer types [10–16]. However, the clinical value, diagnostic and prognostic significance, biological function, and associated mechanisms of NR3C2 are still unclear in NSCLC. In this study, we reported the expression, clinical significance, functions, and possible mechanisms of NR3C2 in NSCLC for the first time. NR3C2 expression is significantly downregulated in NSCLC, with this altered expression pattern being related to the clinical characteristics, survival time, and diagnosis of NSCLC, LUAD, and LUSC patients. Furthermore, the low NR3C2 expression levels in NSCLC

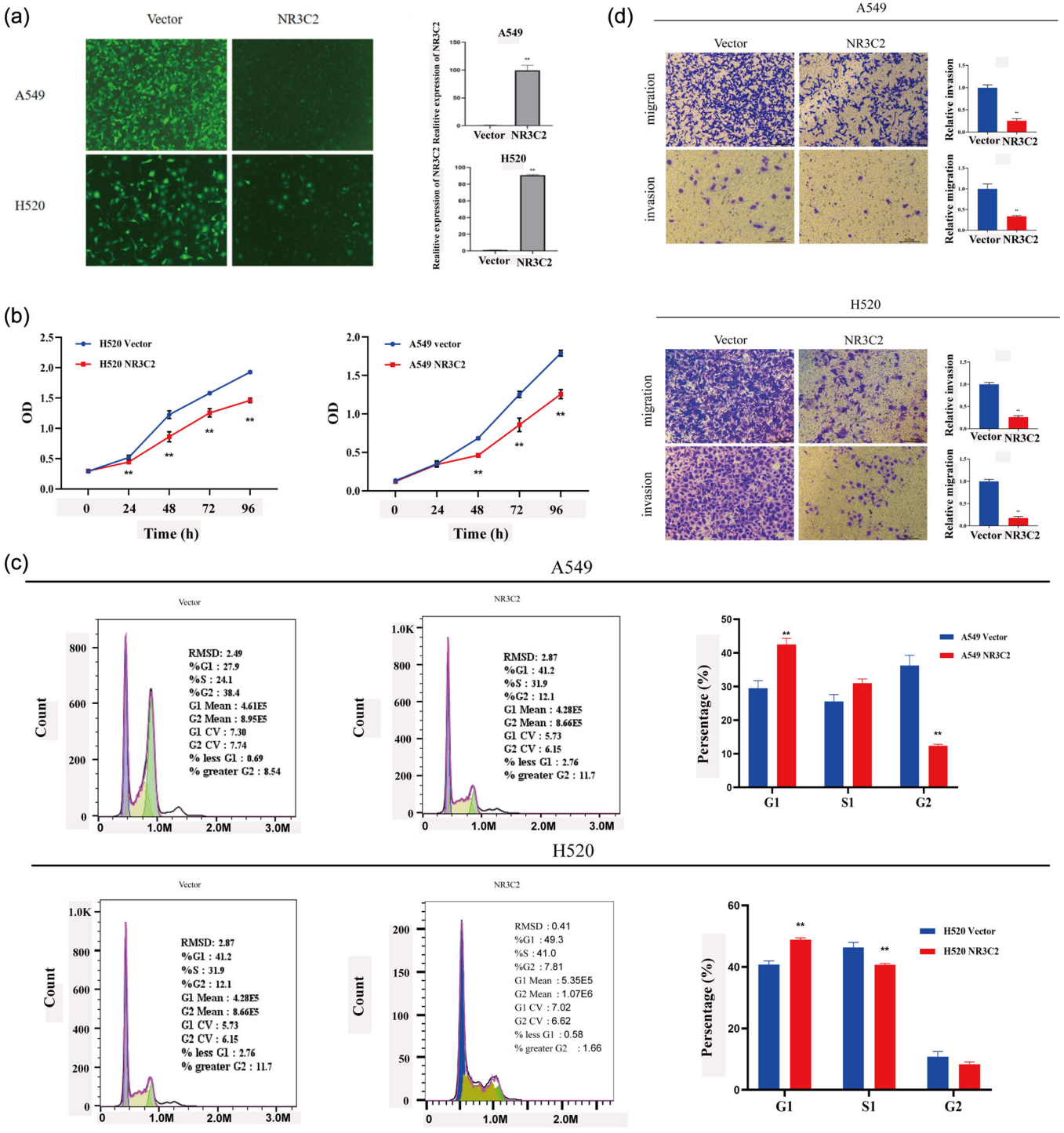

FIGURE 8 NR3C2 is a key participant in non-small cell lung cancer (NSCLC) development and progression. (a) Identification of NR3C2 expression levels by fluorescence and qPCR in A549 and H520 cells at 96 h after NR3C2 overexpression or control virus infection. (b) The effects of NR3C2 overexpression on tumor cell proliferation and growth, as detected by CCK8 assays. (c) The effects of NR3C2 overexpression on tumor cell cycle progression, as analyzed by flow cytometry. (d) The effects of NR3C2 overexpression on tumor cell migration and invasion, as analyzed by Transwell assays. $^{**}p < 0.01$.

and LUAD may be caused by gene copy number deletion, while they may be regulated by gene promoter DNA methylation in LUSC. Functionally, NR3C2 is an important participant in NSCLC development and progression. Moreover, NR3C2 is closely related to the TME and involved in the infiltration of a variety of immune cell types. Mechanically, NR3C2 is involved in many cancer-related signaling pathways in NSCLC, such

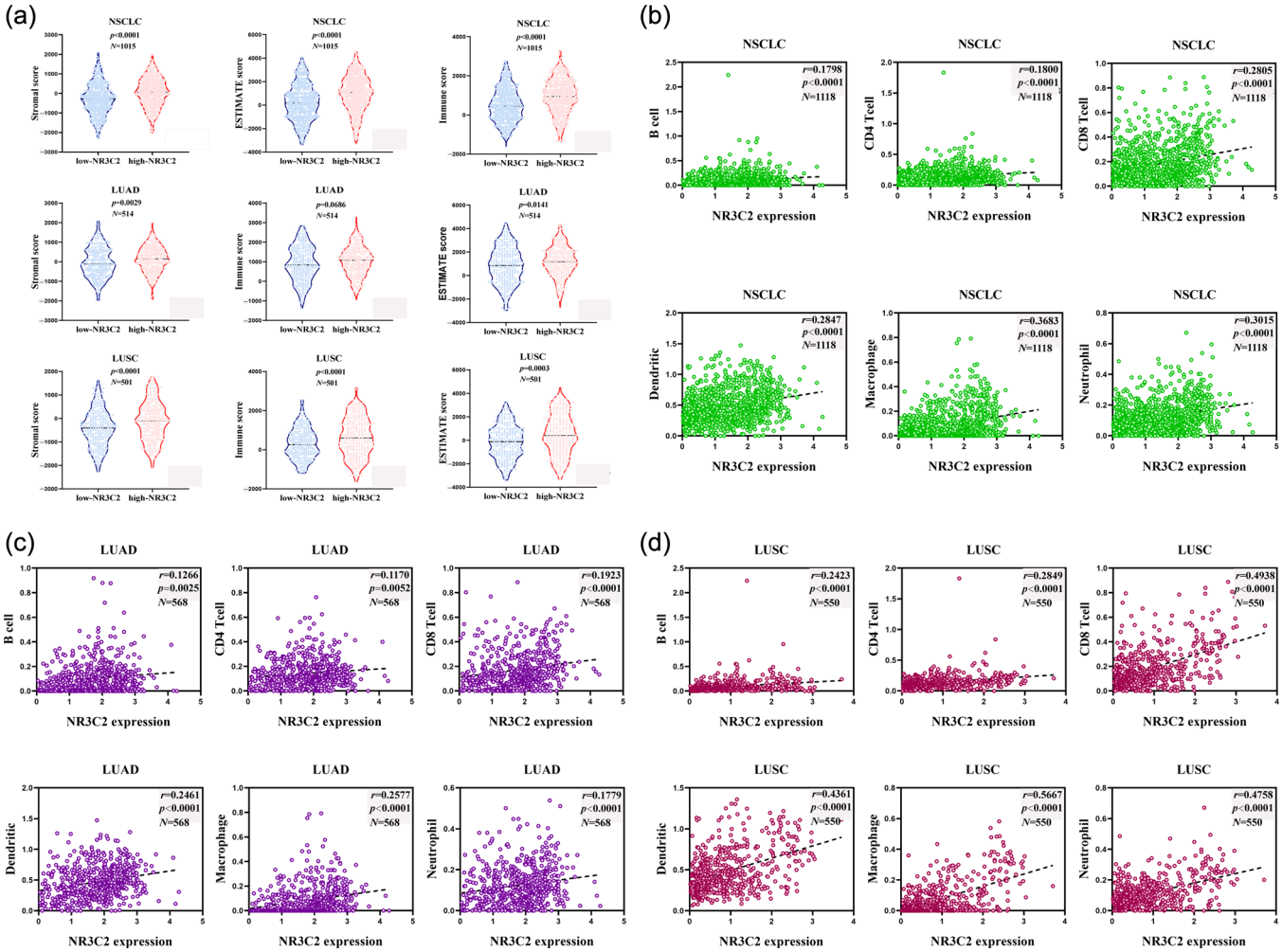

FIGURE 9 The association of NR3C2 expression levels with the tumor microenvironment and immune cell infiltration in non-small cell lung cancer (NSCLC), lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC). (a) The relationships of NR3C2 expression levels with the immune score, stromal score, and ESTIMATE score in NSCLC, LUAD, and LUSC. The patients in the low NR3C2 expression group had a lower immune score, matrix score, and ESTIMATE score compared with those in the high NR3C2 expression group. (b) The associations of NR3C2 expression levels with immune cell infiltration in NSCLC patients. (c) The associations of NR3C2 expression levels with immune cell infiltration in LUAD patients. (d) The associations of NR3C2 expression levels with immune cell infiltration in LUSC patients. The immune cells include B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells.

as the cell cycle, homologous recombination, DNA replication, and P53 signaling pathway.

NR3C2 can slow angiogenesis and the progression of colorectal tumors [13], as well as play inhibitory roles by regulating various signaling pathways in pancreatic, hepatocellular, and renal cancers [12, 13, 16]. In the present study, we observe significantly lower NR3C2 expression levels in tumor tissues. NR3C2 expression is significantly related to the age, gender, smoking status, TNM pathological stage, and clinical stage of NSCLC patients. In LUAD, it is related to the age, gender, N stage, and clinical stage, while it is related to the age, T stage, and clinical stage of LUSC patients. These results suggested that the altered NR3C2 expression levels may have different clinicopathological significance in NSCLC, LUAD, and LUSC. The OS of patients with high NR3C2 expression is longer. NSCLC and LUAD patients in the high NR3C2 group at an early clinical stage and without lymph node metastasis also have longer OS. These results indicate that NR3C2 expression may be a prognostic biomarker for NSCLC and LUAD patients. The prognostic value of NR3C2 expression was further confirmed by IHC staining in a TMA containing 92 LUAD samples. NR3C2 protein expression in these samples significantly correlated with patient age, N stage, and clinical stage, which was consistent with our database analysis findings. These results suggested that NR3C2 may act as a prognostic biomarker and tumor suppressor in NSCLC.

To further explore the regulatory mechanism responsible for the low NR3C2 expression in NSCLC, LUAD, and

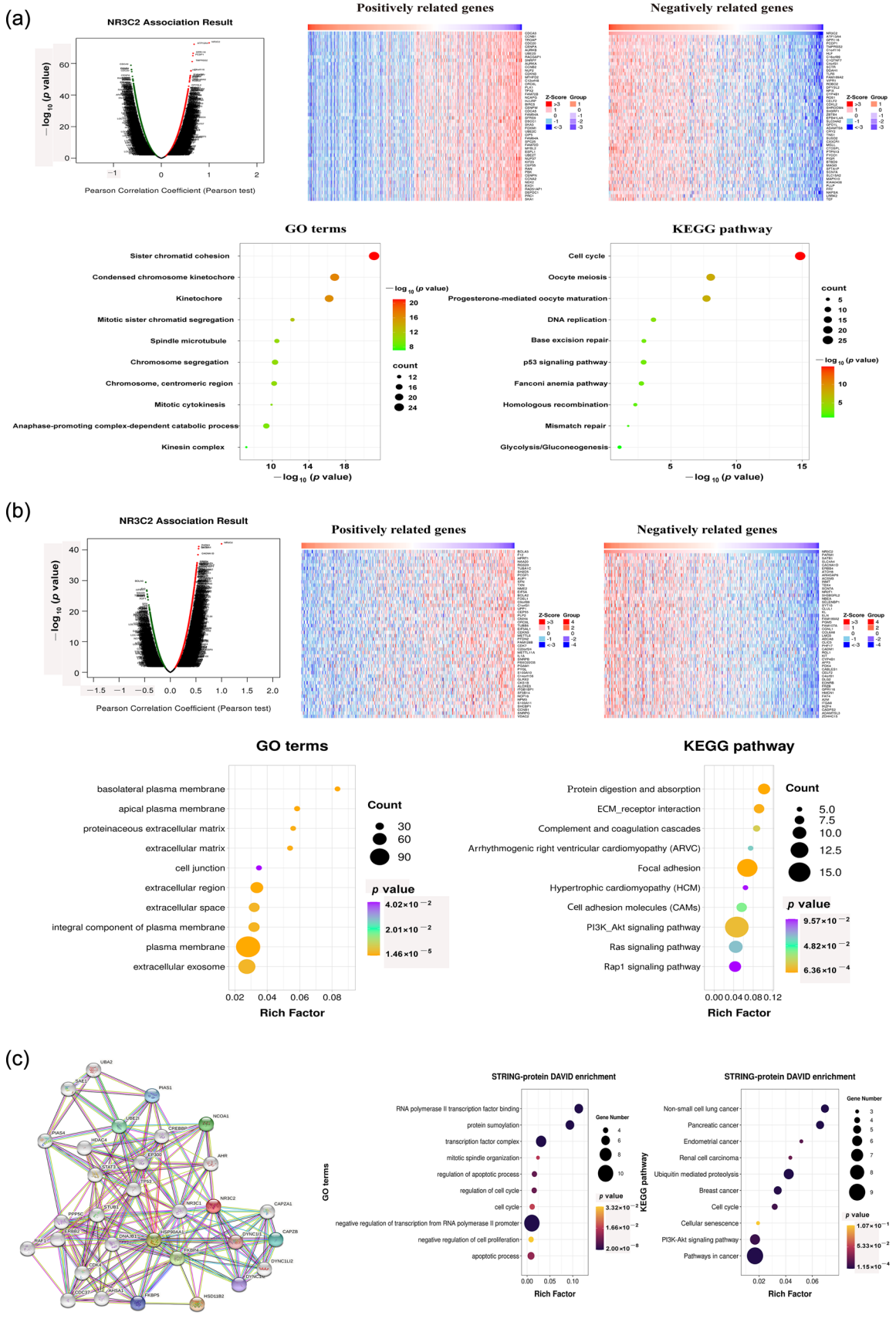

FIGURE 10 (See caption on next page).

LUSC, we analyzed the effects of NR3C2 CNV and DNA methylation on its expression. Low NR3C2 expression is regulated by copy number deletion in NSCLC and LUAD. Moreover, the proportion of NR3C2 CNV deletions is the highest in NSCLC and LUAD, suggesting that detecting NR3C2 deletions may be a useful method for predicting the occurrence of NSCLC and LUAD. However, low NR3C2 expression in LUSC is not controlled by CNV but rather by promoter hypermethylation of the NR3C2 promoter region. This finding may provide a new way to predict LUSC occurrence by examining NR3C2 promoter methylation.

Studying the biological functions and possible mechanisms of certain molecules can provide a deeper understanding of tumor characteristics and pathogenesis, as well as provide an effective reference for tumor therapy development. To determine the specific roles of NR3C2 in NSCLC, NR3C2 was overexpressed, and functional experiments were performed in two cell lines. NR3C2 overexpression significantly inhibits the proliferation of these cells by affecting cell cycle progression. Overexpressing NR3C2 can also suppress tumor cell migration and invasion. These data demonstrate that NR3C2 is an important participant in NSCLC development.

The TME includes tumor cells, immune cells, and related molecules, stromal cells, as well as microvessels and cytokines. Among these, the immune cells constitute the tumor immune microenvironment. Immune score has been reported to be an independent low-risk prognostic factor for cancer patients. Higher immune scores and increased numbers of infiltrating immune cells in the TME can lead to stronger antitumor immunity and better prognosis in patients. The value of these immune components of the TME has been stated to have an even greater value than TNM staging for evaluating prognosis [21, 22]. Tumor development can be regulated by the immune system, with B cells secreting cytokines or presenting antigens to support T cells with antitumor immunity and inhibiting tumor progression. T cells have a major role in antitumor immunity, and tumor cells can interact with T cells to increase antitumor immunity. In the present study, NR3C2 can inhibit tumor metastasis and tumor infiltration is closely associated with tumor metastatic state, suggesting that NR3C2 may be involved in immune cell infiltration in the TME. When analyzing the relationship between NR3C2 expression and the TME, higher immune and matrix scores in the high NR3C2 group are observed, indicating that NR3C2 may be closely related to the tumor immune microenvironment in NSCLC. Moreover, the proportions of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells were also higher in the patients with high NR3C2 expression. These data reveal that NR3C2 may be involved in certain immune processes to increase antitumor immunity and inhibit tumor growth. However, further research is required to examine this in more detail.

In summary, our data suggest that NR3C2 is expressed at lower levels in NSCLC, LUAD, and LUSC. This downregulation may be regulated by copy number deletion in NSCLC and LUAD, and by promoter hypermethylation in LUSC. These altered NR3C2 expression patterns have clear diagnostic and prognostic values in NSCLC. NR3C2 acts as an important participant in tumor development, with the involvement of many cancer-related signaling pathways. Moreover, NR3C2 may participate in immune processes and antitumor immunity. This study provides evidence that NR3C2 is a potential diagnostic and prognostic biomarker and therapeutic target in NSCLC, LUAD, and LUSC. However, this study still has shortcomings. Some of these results still require validation using a larger cohort of biopsies. The possible mechanisms were predicted by computer algorithms, and precise functional and mechanistic experiments are required for further verification.

AUTHOR CONTRIBUTIONS

Yuan-yuan Sun: Data curation (equal); formal analysis (lead); investigation (equal); methodology (equal); writing—original draft (lead). **Hai-cheng Gao**: Conceptualization

FIGURE 10 Analysis of potential NR3C2-related mechanisms by enrichment of its co-expressed genes and interacting proteins. (a) Enrichment analysis of the genes associated with NR3C2 in lung adenocarcinoma (LUAD). The volcano map of the genes associated with NR3C2 in LUAD is shown. The heat map shows the top 50 genes positively related to NR3C2 and the top 50 genes negatively related to NR3C2 in LUAD. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of NR3C2 co-expressed genes were performed in the LUAD cohort. (b) Enrichment analysis of the genes associated with NR3C2 in lung squamous cell carcinoma (LUSC). The volcano map of the genes associated with NR3C2 in LUSC is shown. The heat map shows the top 50 genes positively related to NR3C2 and the top 50 genes negatively related to NR3C2 in LUSC. GO and KEGG enrichment analyses of NR3C2 co-expressed genes were performed in the LUSC cohort. (c) The proteins predicted to interact with NR3C2 and the enrichment analysis of these proteins. The NR3C2 interacting proteins were predicted using the STRING database. The query protein (NR3C2) is highlighted in red, and the network nodes represent the interacting proteins. GO and KEGG enrichment analyses of the predicted NR3C2 interacting proteins were performed using the DAVID online feature annotation tool.

(equal); data curation (equal); investigation (equal); methodology (equal); validation (lead). **Peng Guo**: Data curation (equal); formal analysis (equal); funding acquisition (equal); methodology (equal); software (equal). **Na Sun**: Data curation (equal); formal analysis (equal); investigation (equal); validation (equal). **Chan Peng**: Data curation (equal); formal analysis (equal); methodology (equal); validation (equal). **Zhi-hua Cheng**: Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal). **Jing Gu**: Data curation (equal); formal analysis (equal); validation (equal). **Jin-yi Liu**: Conceptualization (equal); investigation (equal); resources (equal); supervision (equal); visualization (equal); writing—review and editing (equal). **Fei Han**: Conceptualization (lead); funding acquisition (lead); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing—review and editing (lead).

ACKNOWLEDGMENTS

We are very grateful for the public availability of the various databases.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of the present study are available from the MS, online supplementary information, and the corresponding author upon request.

ETHICS STATEMENT

Not applicable.

INFORMED CONSENT

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Sun Y-y, Gao H-c, Guo P, Sun N, Peng C, Cheng Z-h, et al. Identification of NR3C2 as a functional diagnostic and prognostic biomarker and potential therapeutic target in non-small cell lung cancer. Cancer Innov. 2024;3:e122. https://doi.org/10.1002/cai2.122