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ORIGINAL ARTICLE

Multiomics analyses and machine learning of nuclear receptor coactivator 6 reveal its essential role in hepatocellular carcinoma

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Abstract

Nuclear receptor coactivator 6 (NCOA6), a coactivator of numerous nuclear receptors and transcription factors, regulates multiple critical cellular functions. Nuclear receptor coactivator 6 is dysregulated in various cancers, including hepatocellular carcinoma (HCC); however, its role remains largely unknown. Here we reported that NCOA6 was highly expressed in HCC compared to the adjacent liver tissue, and NCOA6 overexpression was significantly correlated with poor HCC prognosis. Experiments revealed that the knockdown of NCOA6 damaged the proliferation, migration, and invasion of HCC cells. Multiomics and immune infiltration analyses showed a close relationship between NCOA6 expression, multiple cancer-related malignant pathways, and the immunosuppressive microenvironment. Finally, we established an effective NCOA6-related microRNA (miRNA) signature to distinguish HCC from hepatitis\liver cirrhosis patients. To the best of our knowledge, this study is the first to provide a comprehensive analysis of NCOA6 expression in HCC. We found that NCOA6 plays an important role in HCC development and has a potential mechanism of action. Establishing an NCOA6-related miRNA signature will help develop novel diagnostic strategies for HCC patients.

KEYWORDS
hepatocellular carcinoma, machine learning, multiomics analysis, NCOA6, prognosis

Abbreviations: AFP, alpha-fetoprotein; AUC, area under the receiver operating characteristic curve; CI, confidence interval; CNV, copy number variation; CPTAC, Clinical Proteomic Tumor Analysis Consortium; DEG, differentially expressed gene; EMT, epithelial–mesenchymal transition; GEO, Gene Expression Omnibus; GSEA, Gene Set Enrichment Analysis; GSVA, Gene Set Variation Analysis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ICGC, International Cancer Genome Consortium; IL, interleukin; LASSO, least absolute shrinkage and selection operator; MDSC, myeloid-derived suppressor cell; miRNA, microRNA; NCOA6, nuclear receptor coactivator 6; OS, overall survival; PD-1, programmed cell death-1; PD-L1, PD-1 ligand; PLC, primary liver cancer; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor-β; Th, T helper; Treg, regulatory T cell; WGCNA, weighted gene coexpression network analysis.

Yinghao Fang, Yuyan Xu, and Wei Liao contributed equally to this work and share first authorship.

1 | INTRODUCTION

Primary liver cancer is one of the most lethal malignancies worldwide, and its incidence has continued to increase in recent years.[1,2] Hepatocellular carcinoma, the most common form of PLC, accounts for approximately 90% of PLC cases.[3] Due to the limited early diagnosis and few available treatment options for advanced-stage HCC, most HCC patients have poor outcomes, with a 5-year survival rate of less than 20%.[4,5] Hepatocellular carcinoma is a complicated cancer with a multifactorial, multistage, complex genetic nature, and high heterogeneity. The pathogenesis of HCC has not been fully elucidated. Therefore, substantial efforts are needed to reveal the complicated molecular pathogenic and developmental mechanisms of HCC.

Nuclear receptor coactivator 6, also known as NRC, AIB3, ASC2, PRIP, TRBP, and RAP250,[6-11] was first identified in human breast tumors[7] and is a coactivator of numerous nuclear receptors and transcription factors.[12] Nuclear receptor coactivator 6 can interact with multiple nuclear receptors (including estrogen receptor-α, peroxisome proliferator-activated receptor-γ, retinoid X receptor, retinoic acid receptor, thyroid hormone receptors, glucocorticoid receptor, liver X receptor, vitamin D receptor, and androgen receptor) and several transcription factors (such as CREB, AP-1, NF-B, SRF, CEBP, and E2F1) and can promote their transcriptional activity.[13,14] In addition, NCOA6 is also an integral and specific unit of a Set1-like complex named ASCOM (for ASC-2 complex), which contains MLL3 or MLL4 and undergoes H3K4 methylation modification.[15] Previous studies have shown that YAP interacts with NCOA6 and recruits the histone methyltransferase complex to activate the transcription of downstream target genes.[16] Nuclear receptor coactivator 6 is essential for embryonic development. Knockdown of NCOA6 affected the invasion and migration of human placenta-derived trophoblast cells[17] and germline KO of NCOA6 in mice caused embryonic lethality.[18] A previous study reported that NCOA6 is amplified in multiple cancer types, such as breast, colon, and lung cancers.[8] In the present study, we found that NCOA6 was highly expressed in HCC compared to adjacent liver tissue. However, whether NCOA6 will promote cancer development, and its underlying mechanism remains unclear.

In this study, we comprehensively investigated the function of NCOA6 in HCC using multiomics analyses (including genomics, transcriptome, and proteome) based on public databases and various experiments. Additionally, we established an effective NCOA6-related miRNA signature for HCC detection using machine learning. These findings enhance our understanding of HCC and help develop a novel, precise, and convenient HCC detection.

2 | MATERIALS AND METHODS

2.1 | Tissue specimen collection and cell culture

Patients with HCC who underwent radical resection between November 2010 and November 2020 at Nanfang Hospital, Southern Medical University, were enrolled in this study. Hepatocellular carcinoma and adjacent liver tissues were obtained immediately after the resection. The specimens were stored in liquid nitrogen or paraformaldehyde-fixed and paraffin-embedded. Samples were obtained with the consent of the patients and the hospital ethics committee (approval document number: NFEC-2018-004). After discharge from the hospital, patients were monitored for survival analysis through regular outpatient follow-up. Relapse was diagnosed based on increased postoperative serum alpha-fetoprotein levels and the presence of typical space-occupying lesions in or out of the liver using at least one imaging examination. Follow-up began on the date of surgery and terminated on August 31, 2021.

The human HCC cell lines Hep3B and Huh-7 were purchased from the Type Culture Collection of the Chinese Academy of Sciences. All cell lines were cultured in DMEM (C11995500BT; Gibco) with 10% FBS (A3160801; Gibco) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.2 | Cell transfection

The cells were cultured in 6-well plates for 24 h and then transfected with siRNA. All transfections were undertaken using Lipofectamine 2000 reagent (11668-019; Invitrogen) according to the manufacturer's instructions. The NCOA6 siRNA oligonucleotides were synthesized by RiboBio. The siRNA duplex sequences used were si NCOA6-1 (5′-GCCCAUUGUUGGUCAACUUAUTT-3′) and si NCOA6-2 (5′-AGGACAGUGAUUGCCAGAAUUTT-3′).

2.3 | RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from HCC cell lines or clinical samples using TRIzol reagent (15596018; Thermo Fisher Scientific) in accordance with the manufacturer's instructions. For quantitative RT-PCR, total RNA was reverse-transcribed to cDNA using an Evo M-MLV RT kit (AG11711; Accurate Biology). Next, cDNA was quantified by real-time PCR using SybrGreen qPCRmasterMix (4309155; Thermo Fisher Scientific) on a StepOnePlus real-time PCR system (Applied Biosystems). The primer sequences were as follows: NCOA6, 5′- AAAACGTGCCCAATTTGTTACAC-3′ (forward) and 5′-CAATCTGAACGGAGAGAATCCC-3′ (reverse); 18S rRNA, 5′-GTAACCCGTTGAACCCCATT-3′ (forward) and 5′-CCATCCAATCGGTAGTAGCG-3′ (reverse). The 18s rRNA was used as the reference gene, and the $2^{-\Delta\Delta Ct}$ method was applied to analyze the expression of the target genes.

2.4 | Immunohistochemistry staining

Clinical samples were fixed with 4% formalin and cut into 4 μm-thick sections. Paraffin slides were deparaffinized and subjected to antigen retrieval using sodium citrate solution (pH 6.0) for immunohistochemical staining. The slides were then incubated in TBS

containing 1% BSA and 0.5% Triton X-100 for 1 h at room temperature. Slides were incubated with primary Abs against NCOA6 (A300-410A; Bethyl) at 1:1000 dilution overnight at 4°C. Endogenous peroxidase activity was blocked in a 3% hydrogen peroxide/methanol buffer for 15 min at room temperature. Bound Abs were detected using a streptavidin–peroxidase kit (sp-9001; ZSGB-BIO). Immunohistochemistry was independently carried out using the quick score method. Each specimen was assigned a score according to the intensity of the nuclear staining (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining), and the proportion of positive cells was estimated and given a percentage score on a scale from 1 to 3 (1, 1%–25%; 2, 25%–75%; and 3, 75%–100%). The final immunoreactive score was determined by multiplying the intensity score by the extent of the score for stained cells, ranging from a minimum of 0 to a maximum of 9.

2.5 | Cell proliferation assay, plate colony-forming assay, Transwell migration, and invasion assays

Cell viability was determined using the CCK-8 (CK04; Dojindo Laboratories) assay. In addition, cell migration and invasion abilities were evaluated using the Transwell assay. All experiments were repeated three times. For the CCK-8 assay, the cells (3000 cells/well) were seeded in 96-wells plates. After adhesion, cell viability was assessed at 0, 24, 48, 72, 96, and 120 h, according to the manufacturer's protocol. For Transwell assays, cells were harvested, suspended in serum-free media, and seeded (5×10^4/well) into the upper compartments of chambers (3422) coated without (for migration) or with Matrigel (at a dilution of 1:8 with serum-free medium, 354,277; Corning), and complete medium was added to the lower chambers. After incubation for 24–36 h, cells that migrated and invaded the other side of the membranes were fixed, stained with 0.5% crystal violet, and digitally imaged under a microscope (Olympus).

2.6 | Western blot analysis

Cells were collected, washed once with ice-cold PBS, and lysed on ice for 30 min using a cell lysis buffer (p0013; Beyotime) according to the manufacturer's protocol. Protein quantification was undertaken using a BCA Protein Assay Kit (ab102536; Abcam). A total of 30 μg protein was denatured in loading buffer at 95°C for 5 min, and western blotting was carried out using a Bio-Rad system (TGX 10%–15% gels). Transfer onto PVDF membranes was undertaken using a Trans-Blot turbo system (Bio-Rad). Images were acquired using the Bio-Rad Imaging ChemiDoc XPS+ system. Secondary anti-rabbit and anti-mouse HRP-conjugated Abs were purchased from Beyotime (A0208 and A0216, respectively). Proteins were detected using the following Abs: NCOA6 (A300-410A; Bethyl), E-cadherin (20874-1-AP; Proteintech), N-cadherin (22018-1-AP; Proteintech), vimentin (5741; Cell Signaling Technology), and β-actin (20536-1-AP; Proteintech).

2.7 | Bio-information analyses

The methods for bio-information analyses are listed in Appendices S1 and S2.

3 | RESULTS

3.1 | *NACOA6* gene expression and clinical characteristics

The TCGA database was used for analysis to explore *NCOA6* gene expression in pan-cancer. The results showed that the mRNA of NCOA6 was significantly higher in most cancers than in the relevant normal tissues, including cholangiocarcinoma and liver HCC, whose relevant normal tissue had the lowest NCOA6 mRNA expression (Figure 1A). To verify the low expression of NCAO6 in the liver, the GTEx database was analyzed, and the results indicated that NCOA6 was expressed at the lowest level in the liver compared to other organs (Figure 1B). Next, a univariate Cox regression analysis based on the TCGA database was carried out to investigate the relationship between NACOA6 expression and the prognosis of cancer patients. The results indicated that high expression of NCOA6 was a risk factor for short OS time in patients with HCC, pheochromocytoma, or paraganglioma (Figure 1C). The significant differences in NCOA6 expression in the liver and HCC (Figure 1D,E) and its clinical significance in prognostic prediction indicate that NCOA6 might play an important role in promoting HCC. Thus, we focused on HCC to explore the potential mechanism of action of NCOA6 in the development of cancer.

To further investigate the relationship between NCOA6 and HCC, we analyzed the clinical characteristics of HCC with NCOA6 expression based on the TCGA database. We found that NCOA6 expression was closely related to the TNM stage and differential grade of HCC. Specifically, NCOA6 was more highly expressed in the TNM stage III/IV group than in the TNM stage I/II group (Figure 1F), and the expression of NCOA6 was also significantly higher in the poorly differentiated HCC samples than in the well-differentiated samples (Figure 1G). Furthermore, NCOA6 expression was significantly higher in the T 3/4 stage of TNM classification than in the T 1/2 stage group (Figure 1H), and there was a trend that the HCC samples with lymph node invasion had higher NCOA6 expression than samples without lymph node invasion (Figure 1I). However, this trend was not seen with the characteristics of distant metastasis, which could be due to the small sample size of HCC patients with distant metastasis in the TCGA database (Figure 1J). These results suggest that NCOA6 might promote HCC development and metastasis.

3.2 | Bio-information analyses prove high expression of NCOA6 to be a prognostic risk factor for HCC

To comprehensively explore the prognostic value of NCOA6 in HCC, three mRNA sequencing databases (TCGA-HCC, ICGC-HCC, and

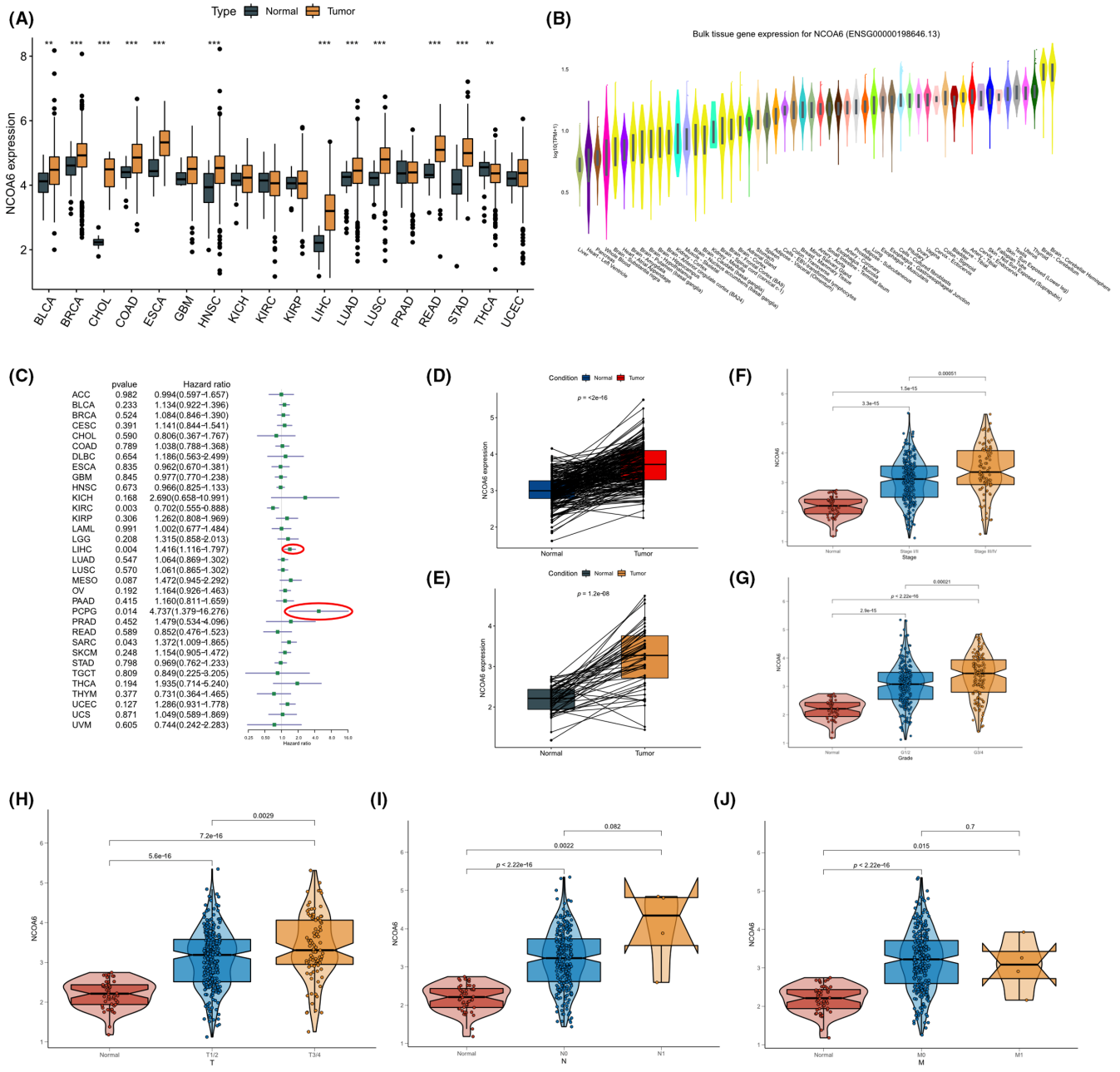

FIGURE 1 mRNA expression of nuclear receptor coactivator 6 (NCOA6) in pan-cancer and its relationship with clinical cancer characteristics. (A) mRNA expression of NCOA6 in pan-cancers and the relevant normal tissues from The Cancer Genome Atlas (TCGA) database. (B) mRNA expression of NCOA6 in normal tissues from the GTEx database. (C) Univariate Cox regression analysis of NCOA6 based on the TCGA database. Items with $p < 0.05$ are marked by a red circle. (D,E) mRNA expression of NCOA6 in hepatocellular carcinoma HCC samples and adjacent liver tissue from the TCGA (D) or International Cancer Genome Consortium (ICGC) databases (E). (F) mRNA expression of NCOA6 in normal liver tissue and HCC samples of different TNM stages from the TCGA database. (G) mRNA expression of NCOA6 in normal liver tissue and HCC samples of different tumor differential grades. (H–J) mRNA expression of NCOA6 in normal liver tissue and HCC samples of different T, N, and M stages from the TCGA database. $^{**}p < 0.01$, $^{***}p < 0.001$.

HBV-HCC cohorts) were used for the Kaplan–Meier analysis. The results confirmed that patients with high expression of NCOA6 in the HCC sample had significantly shorter OS than those with low expression of NCOA6 (Figure 2A–C). In addition, the univariate and multivariate Cox regression analyses of NCOA6 with other clinical parameters in HCC patients from the TCGA database revealed that high expression of NCOA6 was an independent prognostic risk factor for HCC patients (Figure 2D,E). To validate the prognostic value of NCOA6, three mRNA sequencing cohorts mentioned above with another three ChIP sequencing datasets (GSE116174, GSE14520, and GSE76427) from the GEO database were combined and used for meta-analysis by the "meta" R package. As shown in Figure 2F, the pooled hazard ratio was 1.53 (95% CI, 1.28–1.82) with minor heterogeneity ($I^2 = 0\%$, $p = 0.89$) among the six datasets. Hence, we

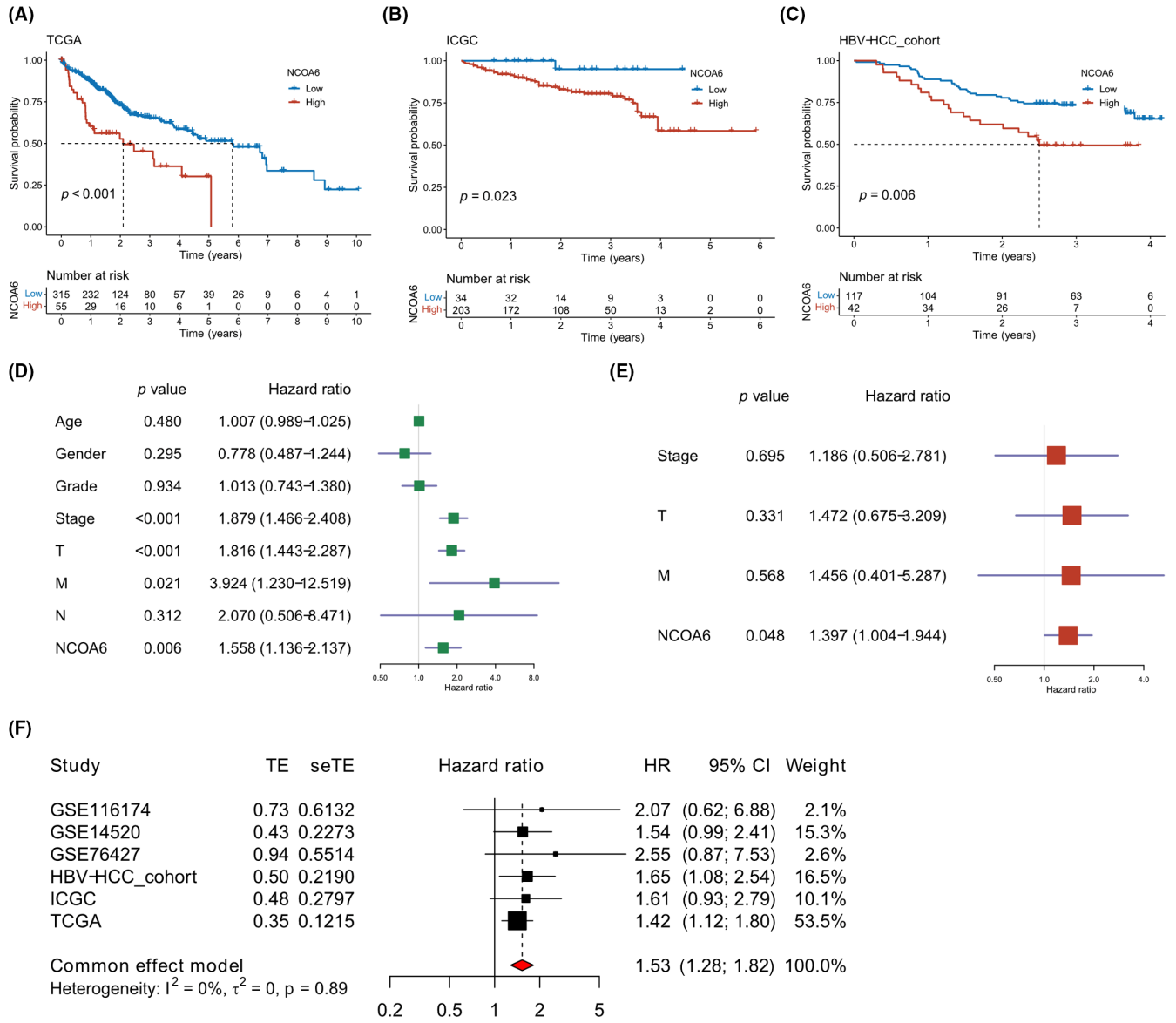

FIGURE 2 Relationship between the expression level of nuclear receptor coactivator 6 (NCOA6) and the overall survival of hepatocellular carcinoma (HCC) patients. (A–C) Kaplan–Meier analysis on HCC patients with high or low expression of NCOA6, data from The Cancer Genome Atlas (TCGA) (A) and International Cancer Genome Consortium (ICGC) (B) databases, and hepatitis B virus (HBV)-HCC cohort (C). (D,E) Univariate (D) and multivariate (E) Cox regression analyses of NCOA6 with other clinical parameters on HCC patients from the TCGA database. (F) Forest plot of meta-analysis for the relationship between NCOA6 expression and overall survival in HCC patients from six datasets. CI, confidence interval; HR, hazard ratio; seTE, The standard error of each effect size; TE, The calculated effect size of each study.

can confidently conclude that NCOA6, as a prognostic factor, can significantly affect the prognosis of HCC patients.

3.3 | Expression of NCOA6 in clinical HCC samples and its prognostic value

The above analyses implicated the high expression of NCOA6 in HCC and its prognostic value. To further verify these results, clinical HCC samples were used for analysis. As shown in Figure 3A,B, the mRNA expression of NCOA6 in 85% of the HCC samples (20 cases) was significantly higher than that in the adjacent normal liver tissue. Furthermore, immunohistochemical analysis of 15 pairs of HCC samples and adjacent liver tissues confirmed that NCOA6 was significantly highly expressed in the tumor tissues (Figure 3C,D). In addition, Kaplan–Meier analysis (Figure 3E), univariate (Figure 3F), and multivariate (Figure 3G) Cox regression analyses based on our HCC specimen bank indicated that patients with high expression of NCOA6 had a shorter tumor-free survival time than those with low expression of NCOA6 after curative hepatectomy, and high expression of NCOA6 was an independent risk factor for tumor-free survival time. These results were consistent with the above analyses, highlighting the importance of NCOA6 in HCC prognosis.

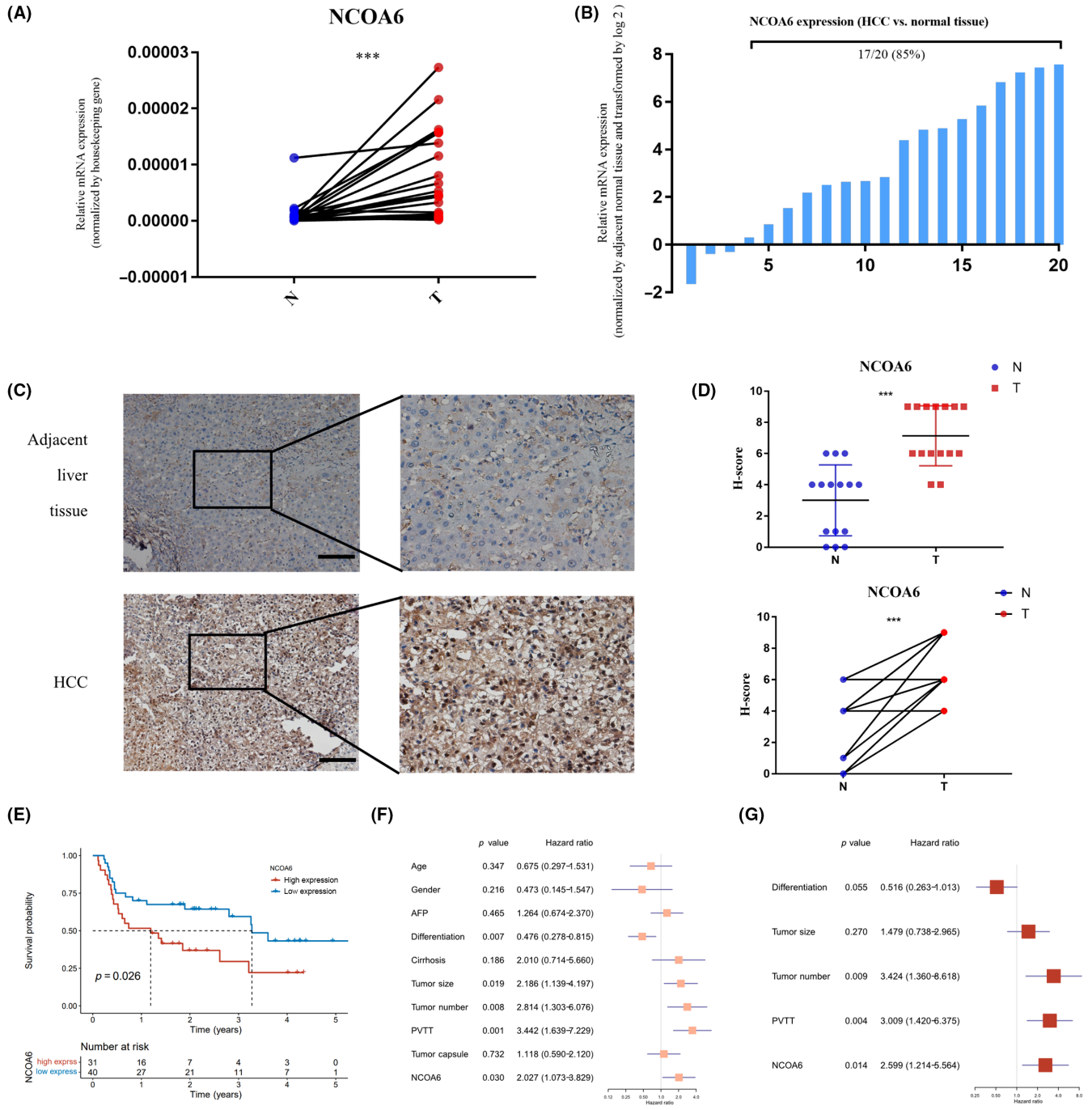

FIGURE 3 Expression of nuclear receptor coactivator 6 (NCOA6) in clinical hepatocellular carcinoma (HCC) samples and its prognostic value. (A,B) mRNA expression of NCOA6 in HCC samples (T) and adjacent liver tissues (N). (C) Immunohistochemistry of NCOA6 on HCC samples and adjacent liver tissues. (D) Immunohistochemistry score of NCOA6 in HCC samples and adjacent liver tissues (of total 15 pairs), presented in groups (upper) or pairs (below). (E) Kaplan–Meier analysis of NCOA6 on HCC from our HCC sample bank. (F,G) Univariate (F) and multivariate (G) Cox regression analyses of NCOA6 with other clinical parameters on HCC from our HCC sample bank. AFP, albumin; PVTT, portal vein tumor thrombus. Scale bar, 100 μM. ***$p < 0.001$.

3.4 | Knockdown of NCOA6 impacts proliferation, migration, and invasion of HCC cells in vitro

To further explore the impact of NCOA6 on HCC cells, we successfully constructed NCOA6 low-expression HCC cell models by siRNA (Figure 4A). As shown in Figure 4B,C, knockdown expression of NCOA6 in Hep3B and Huh-7 cells significantly inhibited HCC cell proliferation. In addition, migration (Figure 4D) and invasion (Figure 4E) experiments showed that in both Hep3b and Huh-7 cells, the number of low NCOA6 expression HCC cells that

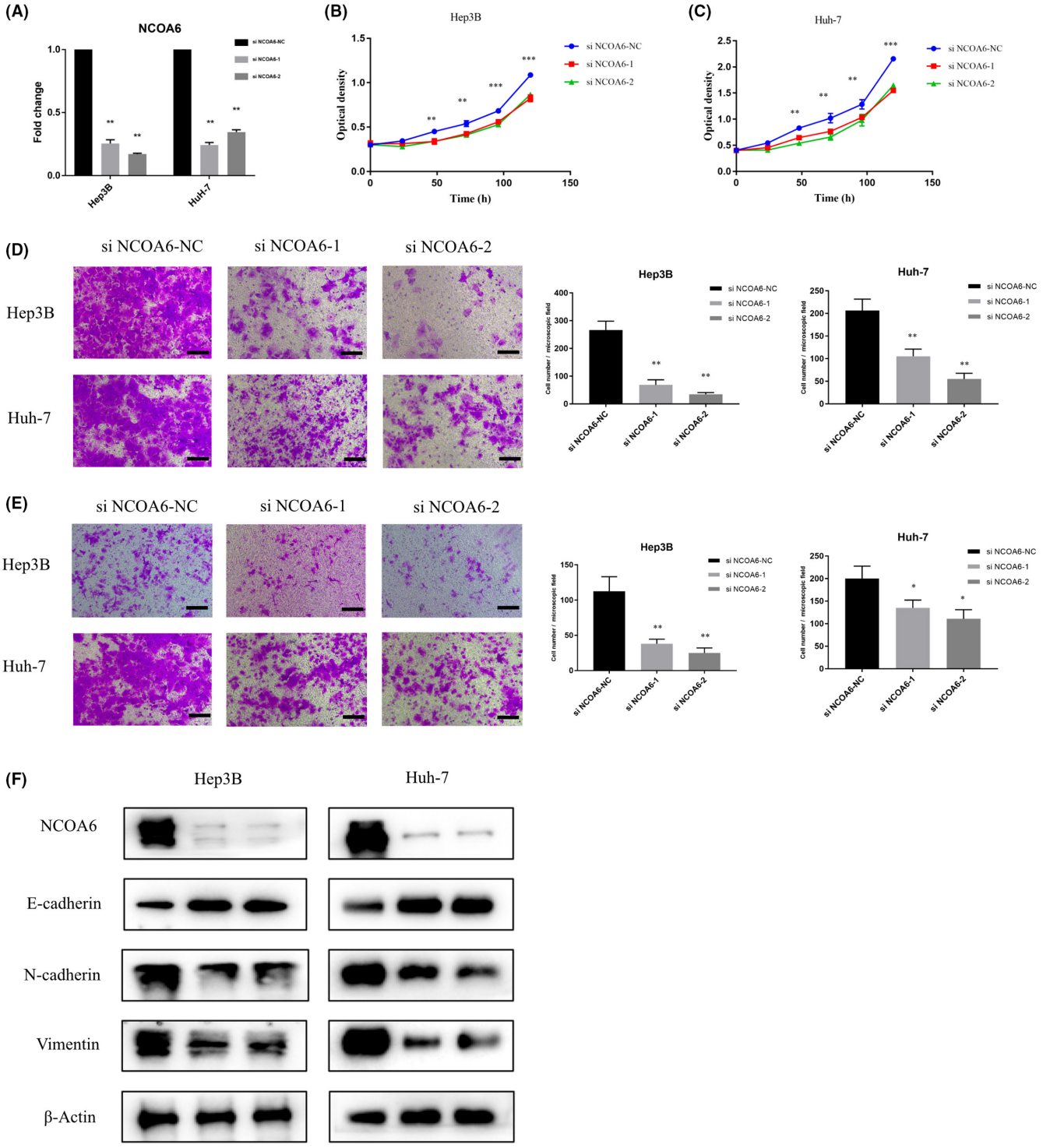

FIGURE 4 Impact of nuclear receptor coactivator 6 (NCOA6) on proliferation, migration, and invasion of hepatocellular carcinoma (HCC) cells. (A) mRNA expression of NCOA6 in Hep3B and Huh-7 cells treated with siRNA for 8 h. (B,C) Optical density curves of CCK-8 of Hep3b (B) and Huh-7 (C) cells treated with siRNA for 8 h. Measurement time points were 0, 24, 48, 72, 96, and 120 h. (D,E) Migration experiment (D) and invasion experiment (E) of Hep3B and Huh-7 cells treated with siRNA for 8 hours. Cells were stained with crystal violet. (F) Western blot of EMT markers (E-cadherin, N-cadherin, and vimentin) after depleting the expression of NCOA6; β-Actin is the internal reference. Scale bar, 100 μM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with negative control (NC) group.

passed through the basement membrane was significantly lower than that of the negative control. Next, we detected the protein levels of EMT markers. The results showed that depletion of NCOA6 downregulated the expression of N-cadherin and vimentin and increased the expression of E-cadherin, suggesting that depletion of NCOA6 impaired the EMT of HCC.

3.5 | Genetic and epigenetic alterations regulating the expression of NCOA6

The above analyses revealed the essential role of NCOA6 in HCC. However, the cause of the abnormal NCOA6 expression in HCC remains unclear. To investigate the potential mechanism underlying the dysregulation of NCOA6, we analyzed the genetic and epigenetic alterations in HCC using the TCGA database. As shown in Figure 5A, genetic mutations in NCOA6 were common among various tissues, and approximately 2.7% of HCC cases experienced genetic mutations. We also examined somatic CNVs in NCOA6 in patients with HCC. As shown in Figure 5B, CNV gain was the major alteration (30.7% of HCC cases), and NCOA6 expression in this group was significantly higher than that in the normal group (diploid group).

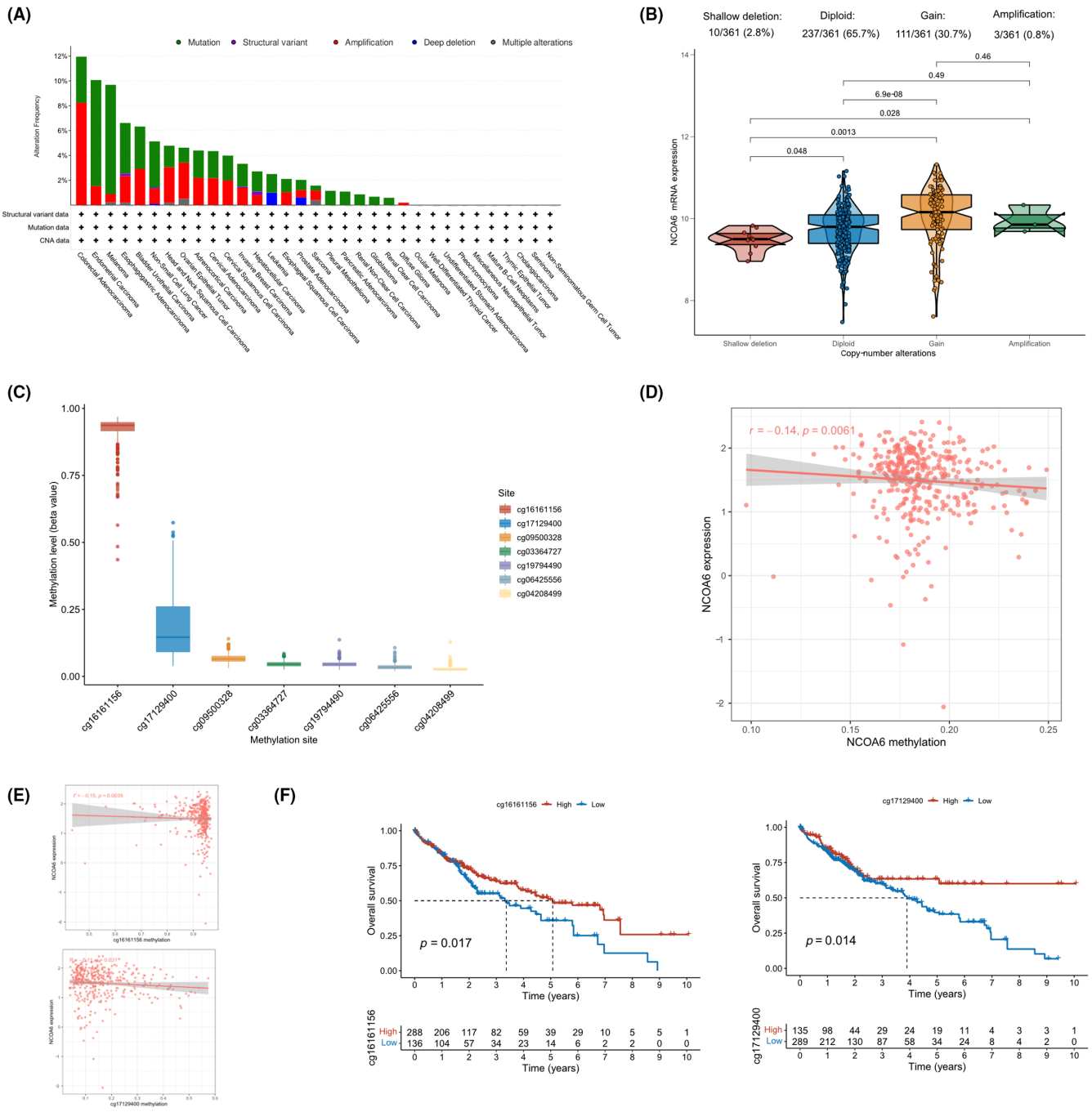

FIGURE 5 Impact of genetic and epigenetic alterations on nuclear receptor coactivator 6 (NCOA6) expression in The Cancer Genome Atlas (TCGA) database. (A) Genetic mutation of NCOA6 in different cancers. CNA, copy number alteration. (B) NCOA6 expression in hepatocellular carcinoma (HCC) with different somatic copy number variations. (C) Methylation level of six methylation sites in the *NCOA6* gene. (D) Correlation analysis between NCOA6 expression and total methylation level of the *NCAO6* gene. (E) Correlation analysis between NCOA6 expression and methylation level of two methylation sites of the *NCAO6* gene. (F) Kaplan–Meier analysis on HCC patients with high or low methylation levels of two NCOA6 methylation sites.

DNA methylation is an important mechanism that regulates gene expression.[19] Thus, we further analyzed the NCOA6 methylation level. The results showed that there were two methylation sites (cg16161156 and cg17129400) that were significantly methylated compared to the other four methylation sites, and the methylation level on cg16161156 was far higher than that of the other five sites (Figure 5C). Correlation analysis implied that NCOA6 expression was negatively correlated with the total gene methylation level ($r = -0.14$, $p = 0.0061$) (Figure 5D), and it was also negatively correlated with the gene methylation level on cg16161156 ($r = -0.15$, $p = 0.0039$) and cg17129400 ($r = -0.11$, $p = 0.031$), respectively (Figure 5E). Furthermore, survival analysis of the methylation levels of these two sites revealed that patients with low levels of methylation at these two sites had a worse prognosis (Figure 5F), and this result confirmed that gene methylation of NCOA6 could influence the function of NCOA6 in HCC. The above analyses clarified that CNV alterations and DNA methylation were two partial causes of the dysregulated expression of NCOA6 in HCC.

3.6 | Protein phosphorylation could regulate the function of NCOA6

Phosphorylation plays a critical role in regulating the function of multiple oncoproteins, and an in-depth understanding of oncoprotein phosphorylation is helpful for the development of new anticancer drugs.[20,21] An online tool, PhosphoSitePlus (https://www.phosphosite.org/homeAction), was used, and the results showed various potential phosphorylation sites in the NCOA6 protein (Figure 6A). Next, we analyzed previous public phosphorylation information of the NCOA6 protein in HCC patients (data from the report of Gao et al.).[22] The results indicated that six specific

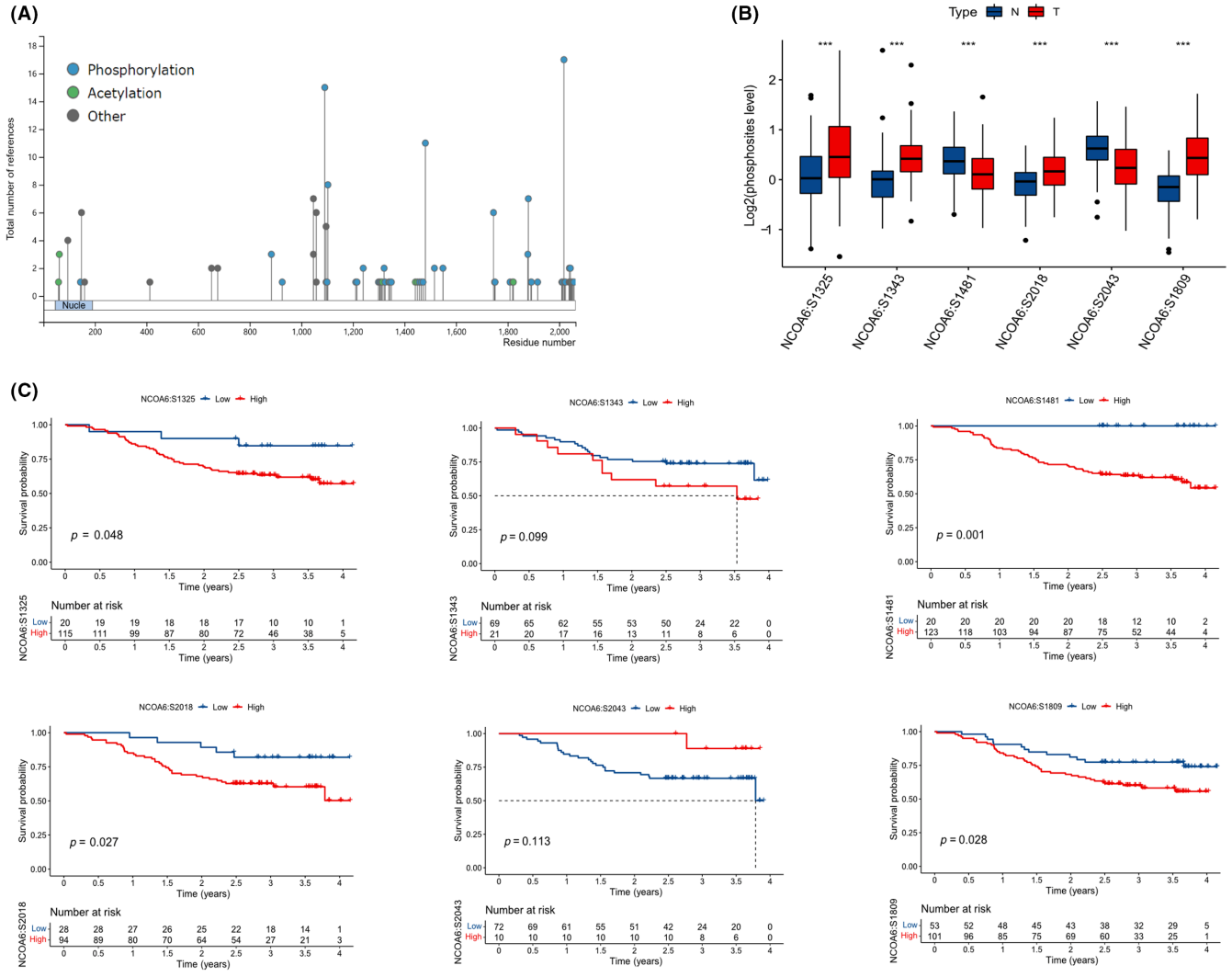

FIGURE 6 Analyses of phosphorylation of nuclear receptor coactivator 6 (NCOA6) protein in hepatocellular carcinoma (HCC). (A) Prediction of potential phosphorylation sites of NCOA6 protein by PhosphoSitePlus. (B) Phosphorylation level on six sites of NCOA6 protein in HCC (T) and adjacent liver tissues (N). (C) Kaplan–Meier analysis on HCC patients with high or low phosphorylation levels on six sites of NCOA6 protein.

phosphorylation sites were detected, and a distinct dysregulation existed in the phosphorylation levels of these sites between adjacent liver and HCC tissues (Figure 6B). Specifically, the phosphorylation sites S1325, S1343, S2018, and S1809 had significantly higher levels in HCC tissues than in adjacent liver tissues. However, the phosphorylation levels of S1481 and S2043 in the adjacent liver tissues were significantly higher than those in HCC. Furthermore, we analyzed the influence of NCOA6 protein phosphorylation on the prognosis of patients with HCC. The results showed that patients with higher phosphorylation levels at S1325, S1481, S2018, and S1809 had a worse survival probability than patients with lower phosphorylation levels at these sites (Figure 6C). This result implies that the phosphorylation of these protein sites might regulate the function of NCOA6 and provide a potential target for the development of anti-HCC drugs.

3.7 | Analyzing the potential downstream mechanism of NCOA6 at the transcriptional and translational level

To identify the potential downstream mechanisms by which NCOA6 influences HCC development, we first analyzed the translational data of HCC from the TCGA database. The GSVA enrichment analysis based on the cancer hallmark pathway gene set showed that HCC with high expression of NCOA6 was enriched in NOTCH/TGF-β/ WNT/PI3K/MYC signaling pathways and the cell cycle. In contrast, HCC with low expression of NCOA6 was enriched in metabolism-related pathways, including the metabolism of xenobiotics, bile acids, and fatty acids (Figure 7A). In addition, we used the GSEA enrichment method based on gene sets from the Kyoto Encyclopedia of Genes and Genomes to analyze the translational data of HCC from the TCGA database. The results indicated that HCC with high expression of NCOA6 was also enriched in the gap junction, IL-17 signaling pathway, mTOR signaling pathway, p53 signaling pathway, PD-L1 expression and PD-1 checkpoint pathway, and Th1 and Th2 cell differentiation (Figure 7B).

To confirm the results of the downstream analyses, we used the translational data of HCC from the CPTAC database to explore the potential downstream mechanisms of NCOA6. Weighted gene coexpression network analysis was undertaken using HCC protein profiles to establish a scale-free coexpression network. A total of 13 protein modules were generated with the optimal soft threshold set at a power of 4, and the merge cut height was set to 0.75 (Figure 7C,D). Among these gene modules, the MEgreen module shows the highest correlation with NCOA6 expression and was considered the "NCOA6-related protein module" (which contains 361 proteins; $r = 0.23$, $p = 0.003$). This protein module was significantly negatively correlated with the overall survival of HCC patients and positively correlated with the HCC thrombus, which indicated that the NCOA6-related protein module was related to the prognosis and metastasis of HCC (Figure 7E). Furthermore, we undertook an enriched biological pathway analysis on the NCOA6-related protein module using Metascape, an effective and efficient tool for experimental biologists to comprehensively analyze and interpret omics-based studies in the big data era, and the results confirmed the above results from transcriptional data. As shown in Figure 7F, the function of this protein module was enriched in the TGF-β signaling pathway, EMT, wound healing, and cell cycle and was also enriched in mRNA processing and splicing.

3.8 | Expression of NCOA6 associated with immune infiltration of HCC

The above analyses pointed out that HCC with NCOA6 high expression was enriched in the IL-17 signaling pathway, PD-L1 expression and PD-1 checkpoint pathway, and Th1 and Th2 cell differentiation, indicating the potential relationship between NCOA6 and the immune microenvironment. To verify this hypothesis, we first used the online tool TIMER 2.0 (http://timer.cistrome.org) to explore the influence of NCOA6 in HCC. As shown in Figure 8A, NCOA6 expression was significantly positively correlated with tumor purity and immune infiltration by MDSCs, Tregs, and M2 macrophages. To verify this result, we used the ImmuCellAI platform (http://bioinfo.life.hust.edu.cn/web/ImmuCellAI/) to calculate the infiltration of different immune cells in HCC from the TCGA database, and Pearson correlation analysis was used to calculate the relationship with NCOA6. The results showed that NCOA6 was significantly positively correlated with the infiltration of CD4 naïve T cells, CD8 naïve T cells, and Tregs and significantly negatively correlated with the infiltration of natural killer cells and CD8 T cells (Figure 8B). Gene coexpression analysis was undertaken in HCC to explore the relationship between NCOA6 expression and immunosuppressive genes. The results revealed that NCOA6 expression was positively correlated with most immunosuppressive genes, except for *KIR2DL1* and *LAG*. These results strongly suggested that high NCOA6 expression is associated with an immunosuppressive environment in HCC. TIDE is a predictor of immune checkpoint blockade therapy for appropriate candidates[23] and is widely used and strongly recommended to evaluate the immune response. Thus, we further analyzed the correlation between the TIDE score and NCOA6 expression. The results showed that NCOA6 expression was significantly positively correlated with the TIDE score, MDSC infiltration, and exclusion immune subtype (Figure 7D,E), consistent with the above results.

3.9 | Nuclear receptor coactivator 6-related serum miRNA signature shows high diagnostic value for HCC detection

Abundant evidence has proven the great application value of serum miRNA signatures in cancer detection.[24,25] As a coactivator of numerous transcription factors, NCOA6 can influence RNA production.[12] Analysis of the above mechanism at the translational level revealed

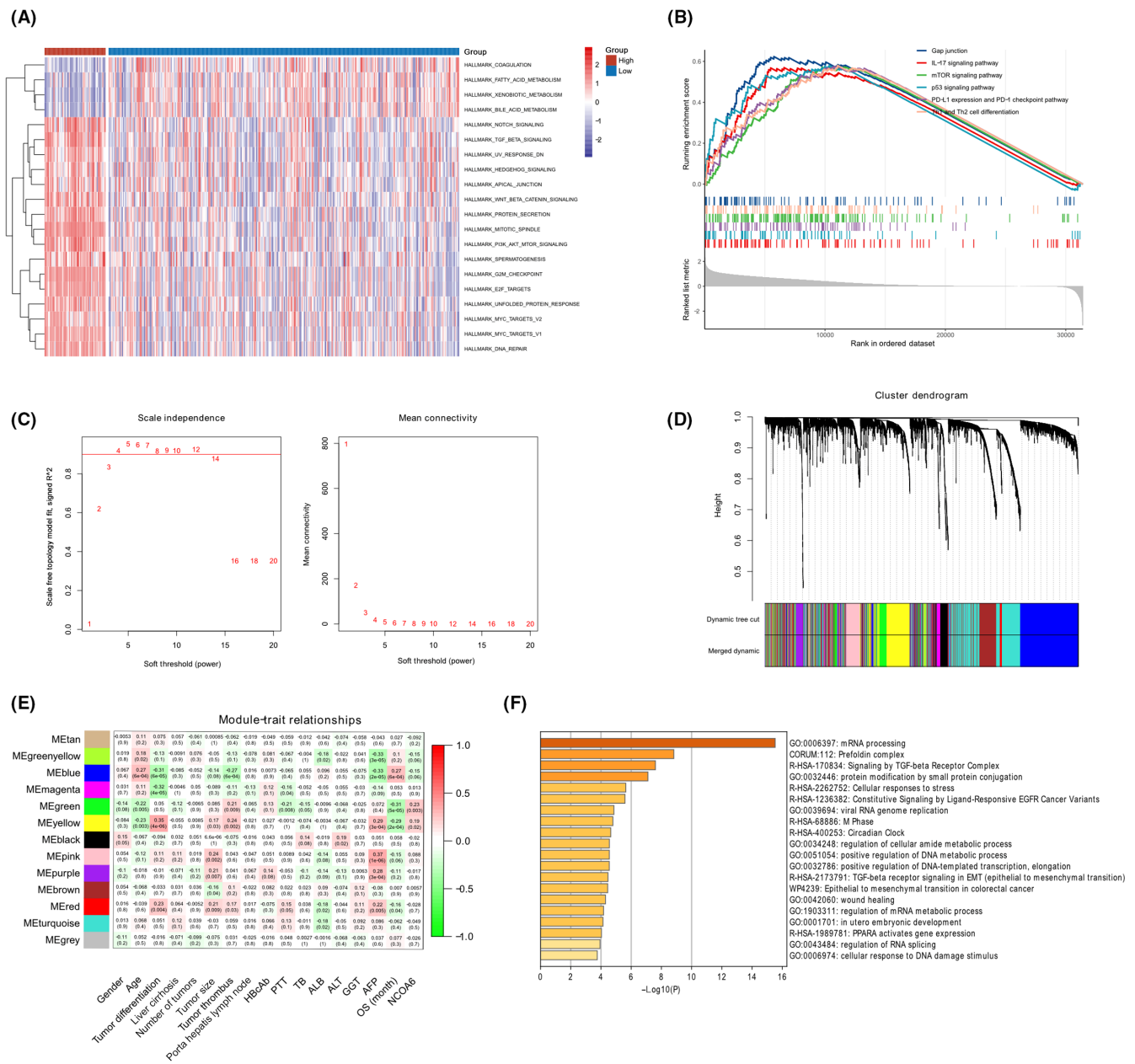

FIGURE 7 Downstream analyses of nuclear receptor coactivator 6 (NCOA6) at the transcriptional and translational levels. (A) Gene Set Variation Analysis (GSVA) enrichment analysis demonstrates the activation states of the cancer hallmark pathway between high or low NCOA6 expression hepatocellular carcinoma (HCC) groups. Data from The Cancer Genome Atlas database. Red and blue represent activated and inhibited pathways, respectively. (B) GSVA enrichment analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets shows the activated biological pathways in high NCOA6 expression HCC samples. (C) Analysis of the scale-free fit index for various soft-thresholding powers in weighted gene coexpression network analysis. (D) Cluster dendrogram of proteins in HCC. Each branch in the figure represents one protein; each color below represents one coexpression module. (E) Relationships between pathological parameters (including NCOA6 protein expression) and various gene modules. (F) KEGG enrichment analysis on the NCOA6-related protein module. AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; OS, overall survival; PTT, partial thromboplastin time; TB, total bile acid.

an NCOA6-related protein module performing a function in the regulation of RNA splicing, which indicated a close association between NCOA6 and miRNA production. Thus, to improve the clinical detection value of NCOA6 in HCC, we established an NCOA6-related serum miRNA signature using a machine learning algorithm. First, Spearman's correlation analysis was used in the TCGA-HCC cohort, which found 108 miRNAs that were closely related to NCOA6 expression; the "limma" R package was used in the TCGA-HCC cohort to identify the DEGs between the high and low NCOA6 groups, and 300 DEGs were identified. There were 42 miRNAs in the intersection of these two parts of miRNAs, and these 42 genes were named NCOA6-related miRNAs (Figure 9A). Thirty-nine candidate NCOA6-related

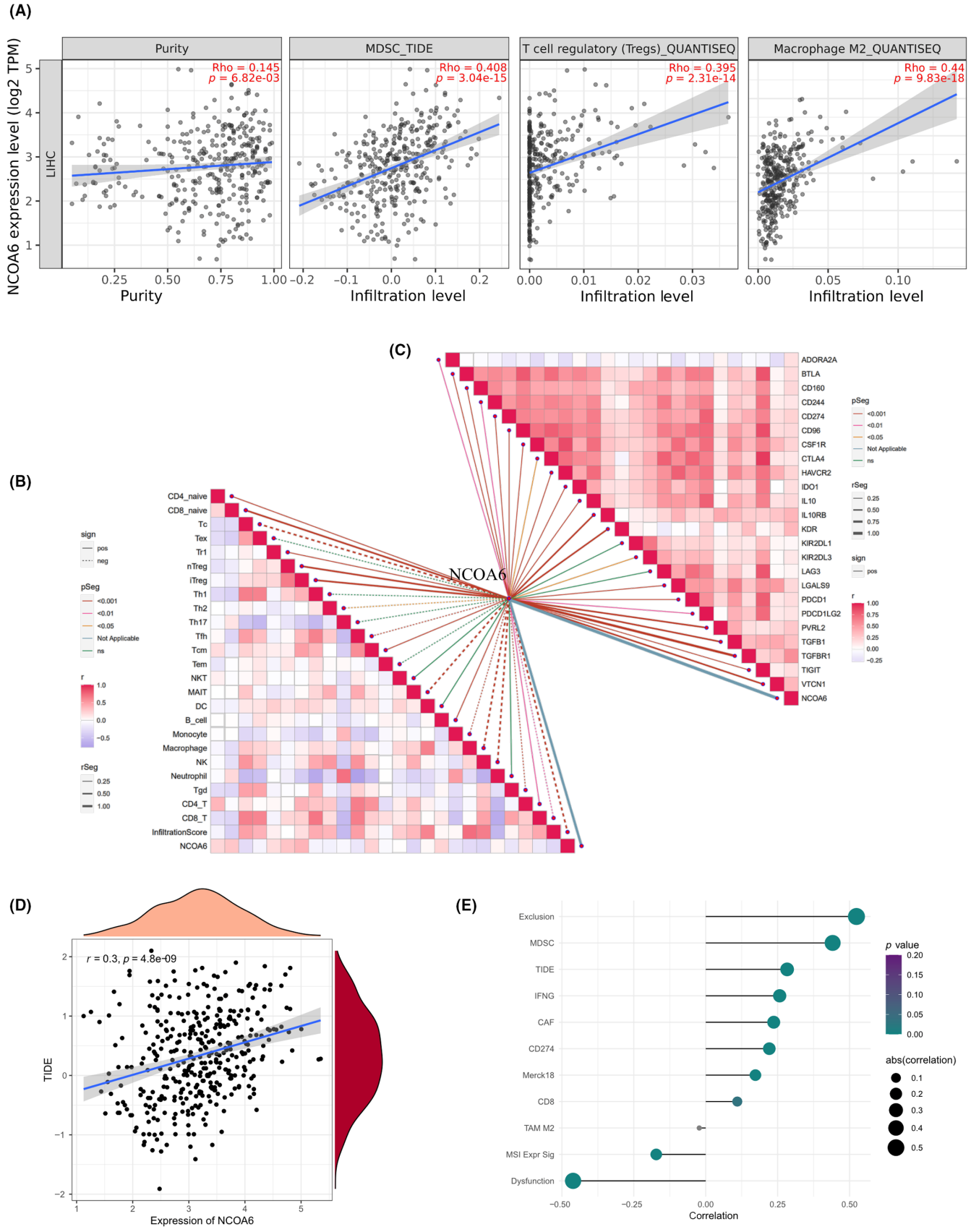

FIGURE 8 Analyses between nuclear receptor coactivator 6 (NCOA6) expression and immune infiltration in hepatocellular carcinoma from The Cancer Genome Atlas database. (A) Correlation of NCOA6 expression and immune infiltration of myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and M2 macrophages, analyzed by TIMER 2.0. (B) Correlation of NCOA6 expression and the infiltration of different immune cells. Cell infiltrations were calculated by the ImmuCellAI platform. (C) Correlation analysis between NCOA6 expression and immune-suppressive genes. (D,E) Correlation analyses between NCAO6 expression and immune features calculated by the TIDE method.

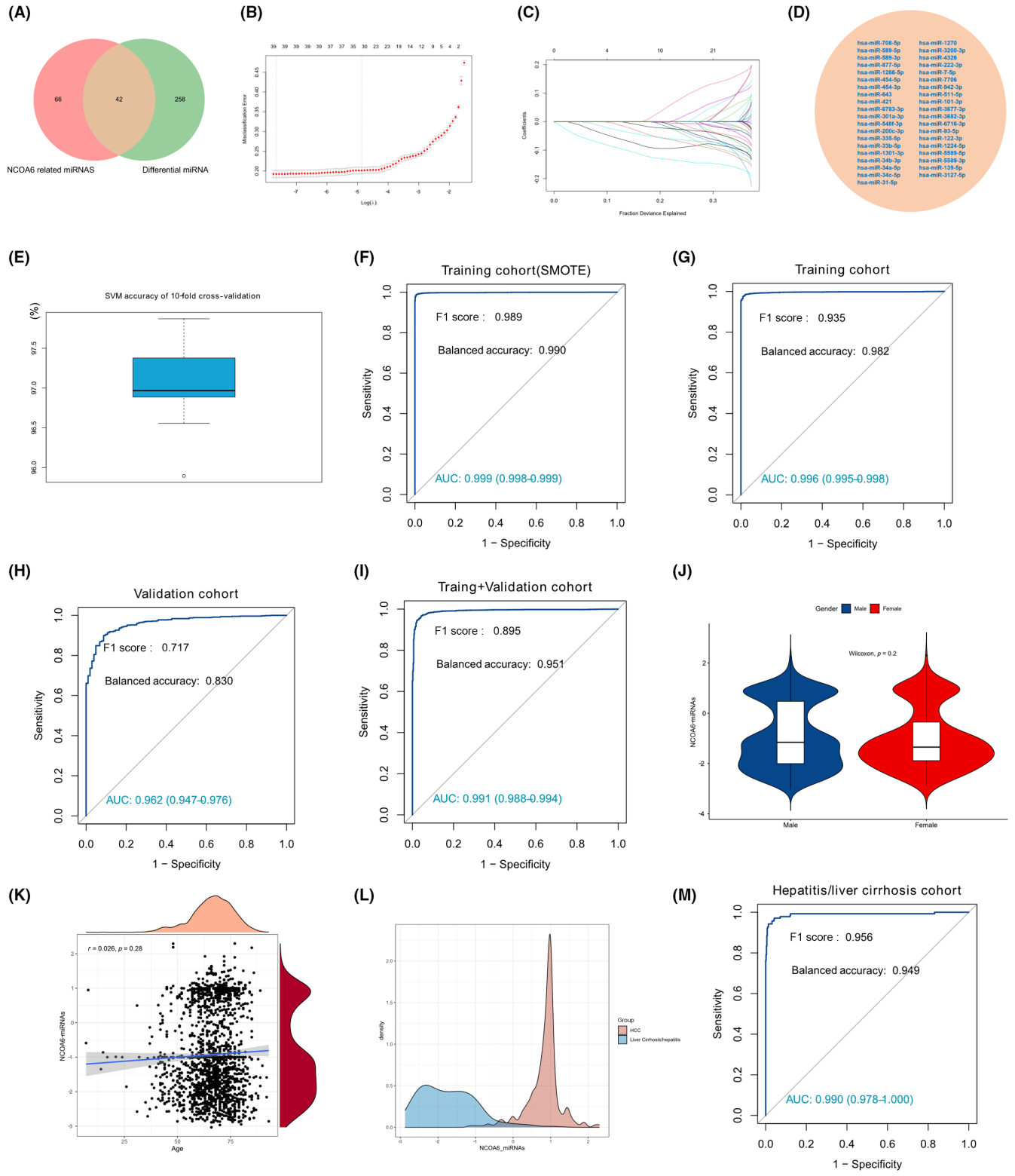

FIGURE 9 Establishing nuclear receptor coactivator 6 (NACO6)-related serum microRNA (miRNA) signature for hepatocellular carcinoma (HCC) diagnosis using a machine learning algorithm. (A) Venn diagram shows 108 miRNAs significantly correlated with NCOA6 expression (left circle) and 300 differentially expressed genes between high or low NCOA6 expression HCC groups (right circle) and 42 miRNAs in the intersection. (B,C) Least absolute shrinkage and selection operator regression were carried out to screen the candidate NCOA6-related miRNAs on the minimum criteria. (D) Thirty-nine candidate serum NCOA6-related miRNAs screened by LASSO. (E) Accuracy of 10-fold cross-validation in construction of a diagnostic signature. (F–I) Predictive value of NACO6-related serum miRNA signature in SMOTE-ameliorated training cohort, original testing cohort, training cohort, and the combined cohort. (J) Output strength of NCOA6 miRNA signature in male and female groups; results show no significance. (K) Correlation analyses between patient age and NCOA6 miRNA output strength. (L) Density of HCC and hepatitis/liver cirrhosis samples at different NCOA6 miRNA output strength. (M) Predictive value of NACO6-related serum miRNA signature in identifying HCC samples or hepatitis/liver cirrhosis samples (area under the receiver operating characteristic curve [AUC] = 0.990).

miRNAs were obtained using the LASSO method to establish a serum diagnostic signature for HCC detection (Figure 9B–D).

Serum samples (HCC = 516; noncancer control = 4008) with miRNA expression data (named the combined cohort) from HCC patients and noncancer controls were randomly split into two cohorts at a ratio of 8:2 (training and validation cohorts). Before LASSO regression and machine training, we used the SMOTE algorithm to solve the imbalanced class problem existing in the training cohort and obtained the SMOTE-ameliorated training cohort. Then, the support vector machine algorithm was used to construct a diagnostic signature (named NCOA6-miRNAs signature) for HCC detection in the SMOTE-ameliorated training cohort with 10-fold cross-validation (the total accuracy was 0.970, as shown in Figure 9E). The NCOA6-miRNA signature showed high diagnostic power in distinguishing HCC samples from noncancer controls in the SMOTE-ameliorated training cohort, with AUC of 0.999 (95% CI, 0.998–0.999), F1 score of 0.989, and balanced accuracy of 0.990 (Figure 9F). To further verify the HCC detection value of the NCOA6 miRNA signature, we applied it to the original training, validation, and combined cohorts. The results showed a satisfactory diagnostic value of the NCOA6 miRNA signature, with AUC values in the training (Figure 9G), validation (Figure 9H), and combined cohorts (Figure 9I) of 0.996, 0.962, and 0.991, respectively. The F1 score were 0.935, 0.717, and 0.895 in these three cohorts, respectively; the balanced accuracy was 0.982, 0.830, and 0.951 in these three cohorts, respectively. To reveal the influence of patient gender and age on the diagnostic efficacy of the NCOA6 miRNA signature, the output strength of the m6A miRNA signature in different genders was carried out, and there was no significant difference between the male and female groups; in addition, there was no significant correlation between patient age and the NCOA6 miRNA output strength (Figure 9J,K). These results suggest that our established NCOA6 miRNA signature is an independent biomarker for distinguishing HCC from controls and is not affected by the patient's sex and age. In contrast, considering that hepatitis B and C virus infection-induced cirrhosis is one of the main causes of HCC, which often interferes with the diagnosis of HCC, we examined the ability of the NCOA6 miRNA signature to distinguish HCC patients from those with chronic hepatitis and liver cirrhosis. We found that the value range of the output strength of the NCOA6 miRNA signature has only a small overlap between patients with HCC or hepatitis/liver cirrhosis (Figure 9L), and the AUC was 0.990 (95% CI, 0.978–1.000), the F1 score was 0.956, and balanced accuracy was 0.949 (Figure 9M). The above results indicated that the NCOA6 miRNA signature's diagnostic performance might not be affected by cirrhosis.

4 | DISCUSSION

Nuclear receptor coactivator 6, a coactivator of multiple transcriptional factors, plays an important role in regulating various cell functions, such as H3K4 methylation,[26] invasion, and migration of human placenta-derived trophoblast cells, and embryonic development.[17,18] A previous study revealed a common dysregulation of NCOA6 in diverse cancers, including breast, colon, and lung cancers.[27] Moreover, Kong et al.[28] reported that HBV X protein (a protein encoded by HBV, which induces cirrhosis and is one of the main causes of HCC) could stabilize NCOA6 to regulate gene transcription in liver cancer cells. These studies suggest that NCOA6 might play a significant role in tumor development, including HCC.

In this study, the transcriptional analyses of NCOA6 based on several databases and the detection of clinical HCC samples revealed significantly higher expression of NCOA6 in tumor tissues compared to adjacent liver tissues. High NCOA6 expression could be a risk factor for the poor prognosis of HCC patients. These findings suggest that NCOA6 could promote the development of HCC. To verify this hypothesis, we undertook cell experiments, and the results proved that depletion of NCOA6 in HCC cells would damage its essential functions, such as proliferation, migration, and invasion. These results highlight the crucial role of NCOA6 in HCC development.

To further explore the mechanism underlying the dysregulation of NCOA6 and its promotion of HCC development, we used multiomics analyses. Analyses at the genetic and epigenetic levels, copy number variations, and DNA methylation that can directly influence gene expression[8,29] revealed that 30.7% of HCC patients experienced CNV gain of NCOA6, and this part of the HCC sample had higher NCOA6 expression than other groups. Furthermore, the DNA methylation levels of cg16161156 and cg17129400 negatively correlated with NCOA6 expression. These results partially explained the cause of NCOA6 dysregulation in HCC. Considering that posttranslational phosphorylation can provide a molecular basis for determining the ability of various transcriptional coactivators to distinguish transcription factors,[30] we analyzed the phosphorylation of the NCOA6 protein. Six phosphorylation sites were found in the NCOA6 protein. Interestingly, the phosphorylation patterns of NCOA6 protein were distinct between HCC and normal liver tissue, and the phosphorylated levels of four of the six phosphorylation sites were significantly correlated with the prognosis of HCC patients, indicating that phosphorylation could influence the protein function of NCOA6. To investigate the potential mechanism by which NCOA6 affects HCC development, GSVA, GSEA, and WGCNA, based on transcriptional or translational databases, were used. The results indicated that high NCOA6 expression was correlated with the activation of multiple cancer malignant pathways, such as EMT and NOTCH/TGF-β/WNT/PI3K/MYC signaling pathways,[31] and also correlated with immune-related pathways, such as the IL-17 signaling pathway, PD-L1 expression and PD-1 checkpoint pathway, and Th1 and Th2 cell differentiation. We analyzed the relationship between NCOA6 expression and immune cell infiltration using a transcriptional database based on this information. We found that high NCOA6 expression was correlated with increased infiltration of immunosuppressive cells, including Tregs, M2 macrophages, and MDSCs, and was significantly positively correlated with the expression of multiple immunosuppressive genes, indicating a close

relationship between NCOA6 and the immunosuppressive tumor microenvironment.

MicroRNAs are noncoding RNAs with a length of 19–25 nt, which can reflect the specific molecular signatures of the cells from which they originate and can be discharged into the serum by cells through exosomes.[32] Tumor-derived exosomes contain tumor-specific miRNAs, making circulating exosomal miRNAs function as diagnostic and prognostic markers for various malignancies.[33,34] Presently, biomarkers for HCC are unsatisfactory, including the traditional biomarker AFP (performance of AFP: AUC, 0.65; specificity, 51.4%; sensitivity, 73.3%).[35] Therefore, it is worthwhile to develop new markers for HCC diagnosis. Nuclear receptor coactivator 6 is a coactivator of multiple transcriptional factors that can influence transcription, and WGCNA showed that the NCOA6-related protein module is functionally enriched in regulating RNA splicing, indicating a close relationship between NCOA6 and miRNAs. Thus, we screened 39 NCOA6-related miRNAs and established an NCOA6-related serum miRNA signature using a machine-learning algorithm. Interestingly, the diagnostic value of this miRNA signature for HCC is high, and the AUC for distinguishing HCC from hepatitis\liver cirrhosis was 0.990. This provides a new, accurate, and convenient method for diagnosing HCC.

5 | CONCLUSION

In this study, our findings revealed the prognostic value of NCOA6 in HCC and confirmed its regulation of HCC proliferation, migration, and invasion. Multiomics and immune infiltration analyses revealed the potential mechanism by which NCOA6 promotes HCC. Based on the NCOA6-related miRNAs, we established an miRNA signature using a machine learning algorithm, which performed powerfully in detecting HCC. In summary, our study enhanced our understanding of NCOA6 in HCC development and helped develop precise diagnostic strategies for patients with HCC. However, there is a limitation in this study. The potential mechanism of NCOA6 promoting HCC explored above needs experimental verification in future study.

AUTHOR CONTRIBUTIONS

Conception and design: DHY and MXP. Development of methodology: DHY, MXP, WL, YYX, and YHF. Acquisition of data: WL, YYX, and YHF. Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): YYX, WL, and YHF. Writing, review, and/or revision of the manuscript: WL, YYX, YHF, DHY, and MXP. Administrative, technical, or material support: LYY, TJ, and LHL. Study supervision: MXP, DHY. All authors contributed to the article and approved the submitted version.

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DISCLOSURE

None of the authors or their spouses or other immediate family members have financial conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request. The raw data of WB and code for constructing the diagnostic signature are provided as supporting information.

ETHICS STATEMENT

Approval of the research protocol: Clinical samples were obtained with the consent hospital ethics committee (approval document number: NFEC-2018-004).

Informed consent: Clinical samples were obtained with the consent of the patients.

Registry and registration no. of the study/trial: N/A.

Animal studies: N/A.

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SUPPORTING INFORMATION

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

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