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NCAPG is a prognostic biomarker associated with vascular invasion in hepatocellular carcinoma

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Abstract. – OBJECTIVE: Vascular invasion is closely associated with tumor recurrence and poor patient outcomes in individuals diagnosed with hepatocellular carcinoma (HCC). In this study, we explored the potential value of NCAPG as a prognostic biomarker of vascular invasion in HCC patients.

MATERIALS AND METHODS: Two Gene Expression Omnibus (GEO) datasets (GSE14520 - GPL3721 Subset; GSE67140 - GPL8786) were utilized to explore the relationship between genes and HCC-associated vascular invasion. Hub genes associated with vascular invasion were identified through analyses of Cytoscape using the Cytohubba plugin, and relationships between specific genes and patient survival outcomes were assessed through univariate and multivariate analyses of the TCGA-Liver Hepatocellular Carcinoma (TCGA-LIHC) database.

RESULTS: In total, 10 hub genes were associated with vascular invasion in the two analyzed GEO datasets. Importantly, non-SMC condensin I complex subunit G (NCAPG) overexpression was correlated with poor prognosis for patients in the TCGA-LIHC database. NCAPG was identified as an independent predictor of HCC patient overall survival (OS) (HR 2.543; 95% CI 1.224, 5.285; $p = 0.012$) and disease-free survival (DFS) (HR 2.034; 95% CI 1.160, 3.566; $p = 0.013$) in a multivariate Cox analysis. NCAPG expression status, vascular invasion status, tumor status, and AJCC T stage were independent predictors of OS, with a concordance index (c-index) value of 0.713 (95% CI, 0.671, 0.756). NCAPG expression levels were related to hypomethylation status and were positively correlated with tumor immune cell infiltration and immune cell-related biomarker expression.

CONCLUSIONS: NCAPG upregulation is associated with poor prognosis in hepatocellular carcinoma patients with vascular invasion.

Key Words:
Biomarker, Hepatocellular carcinoma, NCAPG, Prognosis, Vascular invasion.

Introduction

Hepatocellular carcinoma (HCC) is the second and sixth leading cause of cancer-related mortality in East Asian and Western nations, respectively[1,2]. Affected patients exhibit high rates of postoperative tumor recurrence, with a 77-100% 5-year cumulative recurrence rate and with 80-95% of patients exhibiting liver recurrence associated with a < 15% 5-year survival rate[3]. Vascular invasion is a key predictor of tumor recurrence owing to its association with the dissemination of tumor cells and with poor patient survival outcomes[4,5]. Advances in preoperative imaging can effectively enable the visualization of macroscopic lesions arising as a consequence of such vascular invasion, but microscopy invasion still remains difficult to detect and often can only be identified upon pathological analysis of resected tissue sections. The mechanisms governing HCC onset, progression, and vascular invasion thus warrant further study to better predict and/or prevent such invasion in affected patients. The development of novel molecular approaches to diagnosing HCC patients and evaluating their prognosis is vital to prolonging the survival and quality of life of these individuals[6,7].

Non-SMC condensin I complex subunit G (NCAPG) is a 1015 amino acid-long 114.1 kDa mitotic protein encoded by the NY-MEL-3 gene on chromosome 4p15.32 that plays a role in chromosome condensation[8]. High levels of NCAPG expression have previously been linked to poor prostate cancer patient outcomes[9], and inhibiting the expression of this gene can reduce Wnt/β-catenin signaling to impair the growth of endometrial cancer cells[10]. NCAPG functions as a promoter of tumor cell proliferative, migratory, and angiogenic activity that is commonly overexpressed in a variety of cancers including breast[11], gastric[12], and ovarian cancer[13]. In gastric cancer, NCAPG upregulation is associated with poorer overall survival (OS) and

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disease-free survival (DFS) outcomes and more advanced disease with respect to TNM staging, pathological grade, metastatic progression, and vascular invasion[12]. PI3K/Akt pathway activation in the context of the development of cardia adenocarcinoma has also been found to be partially associated with NCAPG-related stimulation[14]. Other preliminary data also suggest NCAPG to function as a promoter of HCC tumor growth[15], yet its role in the context of HCC vascular invasion remains to be clarified.

Herein, we conducted an analysis of potential prognostic genes associated with HCC vascular invasion (Figure 1). To that end, genes that were differentially expressed between normal samples (n=20) and samples from HCC patients with (n=20) and without vascular invasion (n=20) in the Gene Expression Omnibus (GEO) database were identified. In addition, 27 microRNAs (miRNAs) associated with vascular invasion were identified using a separate GEO dataset, and miRNA-mRNA relationships were used to identify potential target genes likely to be associated with HCC vascular invasion. Functional enrichment analyses were then conducted to probe the potential roles of these vascular invasion-associated mRNAs in the TGGA-LIHC database. Ten hub genes were identified, among which NCAPG was established as an important regulator of HCC-related disease parameters. GO and KEGG analyses were used to probe the potential roles of NCAPG in HCC, while the analyses of immune infiltration and methylation were conducted for additional studies of these possible regulatory relationships. Taken together, our findings suggest that upregulation of NCAPG correlates with poor prognosis and tumor immune infiltration in HCC patients with vascular invasion.

Materials and Methods

Data Processing

The GSE77509 dataset, which included 20 HCC tumor, 20 portal vein tumor thrombosis, and 20 paracancerous normal tissue samples (platform, GEO: GPL16791), was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) and used to identify genes associated with vascular invasion. The GSE67140 dataset, which included 177 HCC samples of which 83 exhibited positive vascular invasion (VI+) and 94 exhibited negative vascular invasion (VI-) (platform, GEO: GPL8786), was additionally downloaded to identify vascular invasion-related miRNAs. Potential miRNA target genes including 5765 mRNAs were identified using TargetScan (v 7.2, http://www.targetscan.org/) and miRDB (http://www.mirdb.org/). Gene expression patterns and clinical data pertaining to HCC patients were downloaded

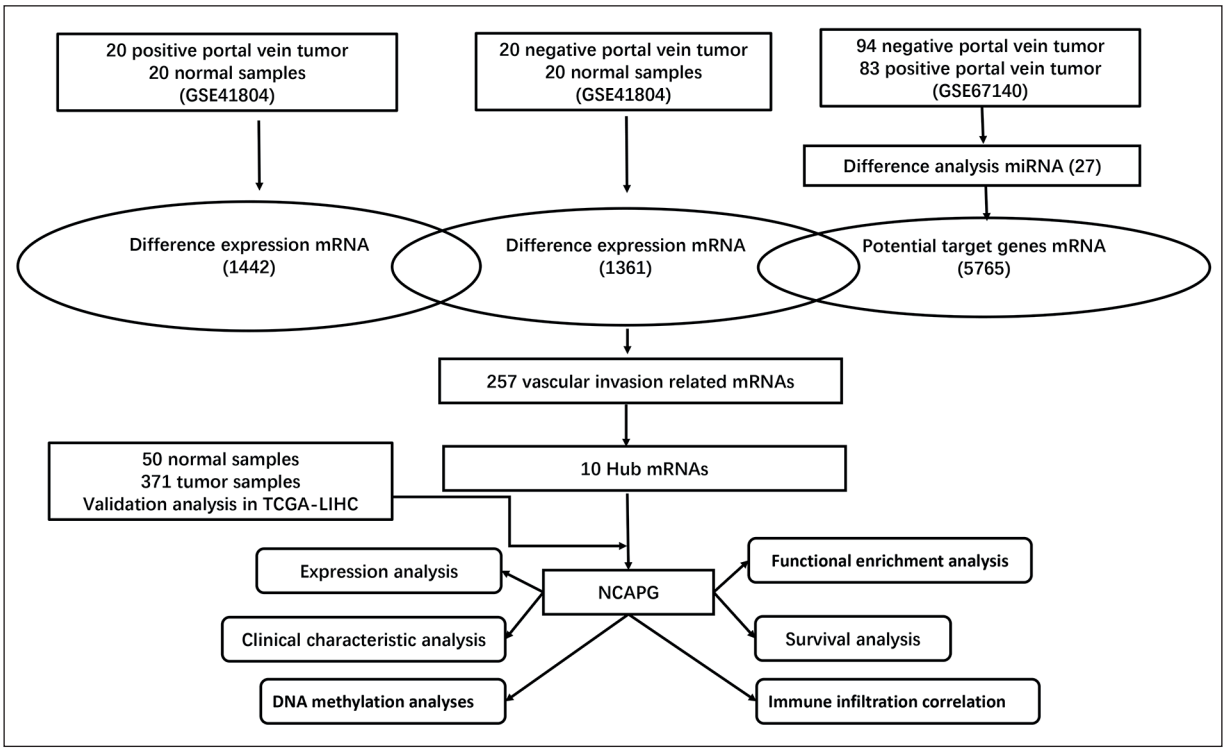

Figure 1. Study workflow.

from the TCGA database (https://portal.gdc.cancer.gov/). In total, RNA-seq data were downloaded for samples acquired from 371 HCC patients, including 50 for which paired samples were available. Raw RNA-seq data were normalized, and target genes were compared with the list of differentially expressed mRNAs (DEmRNAs) using the R package, with overlapping mRNAs being retained for further analysis. The Human Protein Atlas (HPA, http://www.proteinatlas.org/) was queried to assess NCAPG protein levels in samples of interest, NCAPG mutation status was evaluated with the cBioPortal for Cancer Genomics (http://www.cbioportal.org/). The Institutional Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University approved this study.

Differentially Expressed Gene Identification

DEmRNAs were identified by comparing samples with and without portal vein invasion at a detection threshold of $|\log FC| > 2$ and $p < 0.05$, while differentially expressed miRNAs (DEmiRNAs) were identified at a detection threshold of $|\log FC| > 0.5$ and $p < 0.05$. R (v 3.6) was used to generate volcano plots displaying these differentially expressed miRNAs, and mRNA networks were visualized using Cytoscape (v 3.7.0, https://www.cytoscape.org/). The cytoHubba plugin was further utilized for hub gene identification.

Functional Enrichment Analysis

Differentially expressed genes identified above, together with the top 200 NCAPG-related genes in the TCGA-LIHC cohort, were subjected to both GO and KEGG enrichment analyses conducted by employing Metascape and with the aid of ggplot2 and R clusterprofile packages were visualized. $p < 0.05$ was the threshold for significant enrichment.

Survival Analyses and Prognostic Model Development

Survival outcomes were analyzed via the Kaplan-Meier method using log-rank tests for patients in the TCGA HCC cohort. The prognostic relevance of individual DEmRNAs was assessed via this approach, and univariate Cox regression analyses were conducted to explore relationships between specific genes and patient OS in an effort to identify prognostic biomarkers. Subsequent multivariate Cox regression analyses were used to identify factors that were independently associated with HCC patient prognosis. All analyses were performed using R (v 3.6) and GraphPad Prism 8.3 (La Jolla, CA, USA), with $p < 0.05$ as the threshold of significance.

The Relationship between NCAPG Expression and DNA Methylation Analyses

The expression of NCAPG in HCC tissues and normal liver tissues or adjacent tissues was analyzed with the Human Protein Atlas (www.proteinatlas.org/) and the Hepatocellular Carcinoma Database (HCCDB, http://lifeome.net/database/hccdb). DNA methylation status is a key epigenetic determinant of gene expression that is regulated by the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, which can thus influence cancer cell behavior[16]. We, therefore, assessed the expressions of these three DNA methyltransferases within patients exhibiting low and high levels of NCAPG expression in the TCGA database. The UALCAN (http://ualcan.path.uab.edu/) and DiseaseMeth v 2.0 (http://bio-bigdata.hrbmu.edu.cn/diseasemeth/) databases were then leveraged to assess NCAPG expression in HCC tumors and paracancerous tissues. By taking advantage of MEXPRESS (https://mexpress.be), relationships between NCAPG expression and DNA methylation status were examined. Additionally, for multivariate survival analyses exploring different CpG islands in the context of such DNA methylation, MethSurv (https://biit.cs.ut.ee/methsurv/) was utilized.

Immune Cell Infiltration and NCAPG Expression Analyses

TIMER (https://cistrome.shinyapps.io/timer/) is an online database used to gauge intratumoral immune cell infiltration based on gene expression data. Herein, this database was utilized to explore the association between NCAPG expression levels and intratumoral infiltration by different immune cell populations. Specifically, the relationship between NCAPG expression and the abundance of specific immune cell populations within tumors (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells [DCs]) was assessed. Moreover, the relationship between these variables and NCAPG copy number were assessed in individuals with HCC, as was their prognostic relevance. Correlations between NCAPG expression and gene markers associated with these six different tumor-infiltrating immune cell types were also assessed.

Statistical Analysis

Clinicopathological variables associated with NCAPG expression were analyzed using Pearson chi-squared tests and Fisher's exact test, as appropriate. DFS was defined as the amount of time between

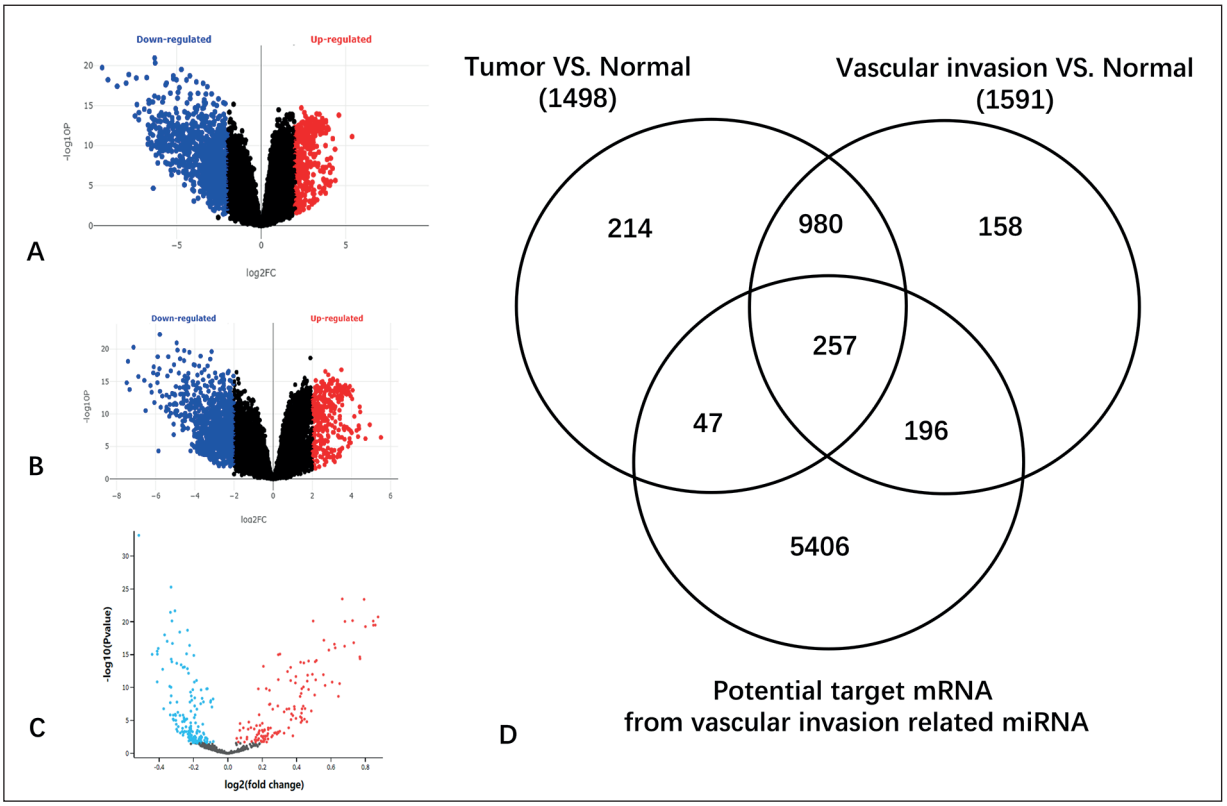

Figure 2. Comparison of differentially expressed mRNAs and miRNAs between HCC tissues with and without vascular invasion (VI+ and VI-) and normal tissues. Red and blue respectively correspond to genes that were upregulated and downregulated. Volcano plots were generated representing (**A**) differentially expressed mRNAs (|log2(FC) | > 2 and adjusted *p*-value < 0.05) between VI+ and normal, (**B**) VI- and normal, (**C**) differentially expressed miRNAs between VI+ and VI- (|log2(FC)| > 0.5 and adjusted $p < 0.05$). **D**, A Venn diagram of DEmRNAs and potential target mRNAs of vascular invasion-related miRNAs.

surgery and disease recurrence, whereas OS was defined as the amount of time from diagnosis to mortality or most recent follow-up. Patients lacking events or mortality as of most recent follow-up were eliminated. Survival outcomes were compared via Kaplan-Meier curves with the log-rank test. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for DFS and OS using univariate Cox proportional hazards regression analyses, with those variables that were significant in univariate analyses ($p < 0.05$) being incorporated into a multivariate analysis. A two-sided $p < 0.05$ was the significance threshold for this study, and SPSS (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

Results

Identification of Differentially Expressed Genes Associated with HCC Vascular Invasion

The identification of mRNAs and miRNAs that are differentially expressed between HCC patients with and without vascular invasion (VI+ *vs.* VI-) has the potential to facilitate the identification of prognostic biomarkers associated with vascular invasion status. To that end, we utilized datasets from the GEO database to identify differentially expressed miRNAs and mRNAs (DEmiRNAs and DEmRNAs) in the samples of HCC by comparing VI+ and VI- samples and adjacent paracancerous tissues, using a *p*-value < 0.05 and a |log2(fold-change [FC])| > 2 (DEmRNAs) or a |log2(fold-change [FC])| > 0.5 (DEmiRNAs) as criteria for differential expression. Overall, this approach led to the identification of 1591 DEmRNAs when comparing VI+ HCC tumors to normal tissues (1112 upregulated, 479 downregulated), 1498 DEmRNAs when comparing VI- HCC tumors to normal tissues (990 upregulated, 508 downregulated), and 27 DEmiRNAs when comparing VI+ and VI- HCC tumors. Volcano plots and heatmaps were then used to depict these DEmRNA and DEmiRNA distributions (Figure 2A-C).

Hub Gene Identification

To develop an mRNA network for HCC samples, we analyzed the VI+, VI-, and normal hepatic tissue samples. Initially, potential target genes associated with identified DEmiRNAs were identified using starBase databases (https://starbase.sysu.edu.cn/). Based on the intersection between these target genes and HCC tissue sample gene expression, 27 miRNAs were selected for further analyses. The miRDB and TargetScan databases were then utilized to select downstream target mRNAs of these DEmiRNAs, with only target genes identified by both databases being retained for further analysis. Finally 257 mRNAs (170 upregulated and 87 downregulated) were utilized to generate an HCC-specific miRNA-mRNA regulatory network using the Cytoscape program. Functional enrichment analyses were conducted using Metascape, revealing these DEmRNAs to be enriched for developmental processes, cellular processes, and growth (Figure 3A). To clarify the mRNAs most closely associated with HCC patient prognosis, the network developed above was analyzed using the cytoHubba plugin, which led to the identification of ten hub DEmRNAs (score > 2.0) (NCAPG, MELK, KIF11, MCM10, DTL, NCAPH, WDHD1, RAD51AP1, CDC25A, and GINS1) (Figure 3B).All ten hub genes were significantly upregulated in HCC tissue samples relative to levels in paracancerous tissues (Figure 4A). The expression of all of these mRNAs was additionally assessed in 50 pairs of HCC tissue samples from the TCGA cohort (Figure 4B), yielding largely consistent results.

Analysis of the Prognostic Relevance of NCAPG Overexpression in HCC Tumors

To assess the potential functional relevance of NCAPG in HCC, the HCC database was queried (hccdb, http://lifeome.net/database/hccdb/), revealing that NCAPG was overexpressed in HCC tumors as compared to normal hepatic tissue samples at the mRNA level (Figures 5A). Similarly, overexpression of NCAPG was also evident at the protein level in samples subjected to immunohistochemical (IHC) staining (Figure 5B) in the Human Protein Atlas, with corresponding patient data being compiled in Table I. Given the observed upregulation of NCAPG in tumor tissues, we additionally evaluated the clinical relevance of such NCAPG overexpression in HCC, revealing higher levels of NCAPG expression to be associated with poorer HCC patient OS (Figure 5C).

To explore the potential mechanistic basis for the upregulation of this gene, we evaluated NCAPG copy number variations in HCC by using cBioPortal to assess the TCGA-HCC dataset (Figure 5D). Approximately 2% of HCC samples exhibited NCAPG amplifications, and we did not detect any clear correlation between NCAPG copy number and the expression of this gene at the mRNA level in HCC patient samples (Figure 5E-F). Together, these data suggest that NCAPG is overexpressed in HCC through a mechanism largely independent of copy number amplification. To establish whether changes in NCAPG expression were associated with the proganosis of HCC patients, we conducted a series of analyses revealing NCAPG levels to be positively correlat-

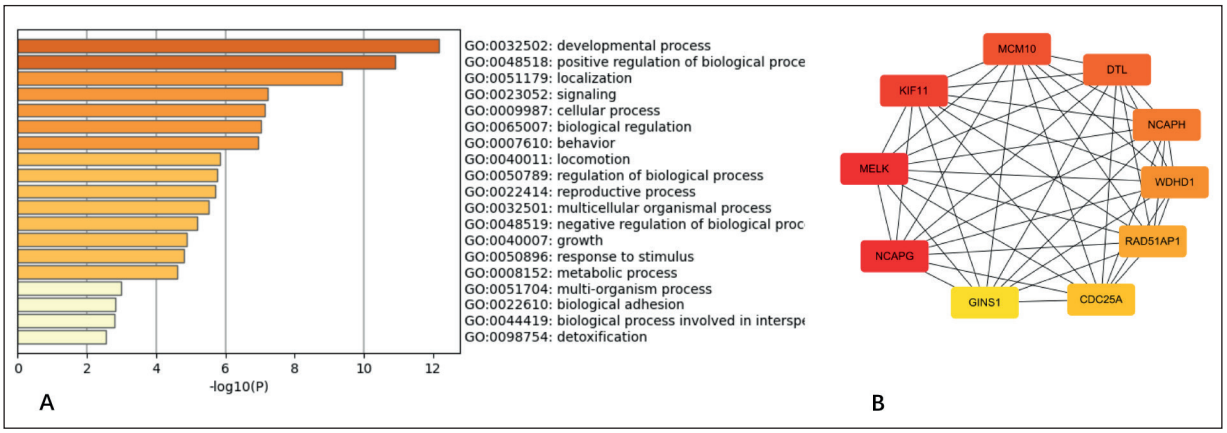

Figure 3. Enrichment analyses and protein-protein interaction network construction. **A**, Bar graph of enriched terms across input genes lists (257 vascular invasion-associated genes), colored by *p*-value. **B**, Protein-protein interaction network and MCODE components identified for the 10 hub genes.

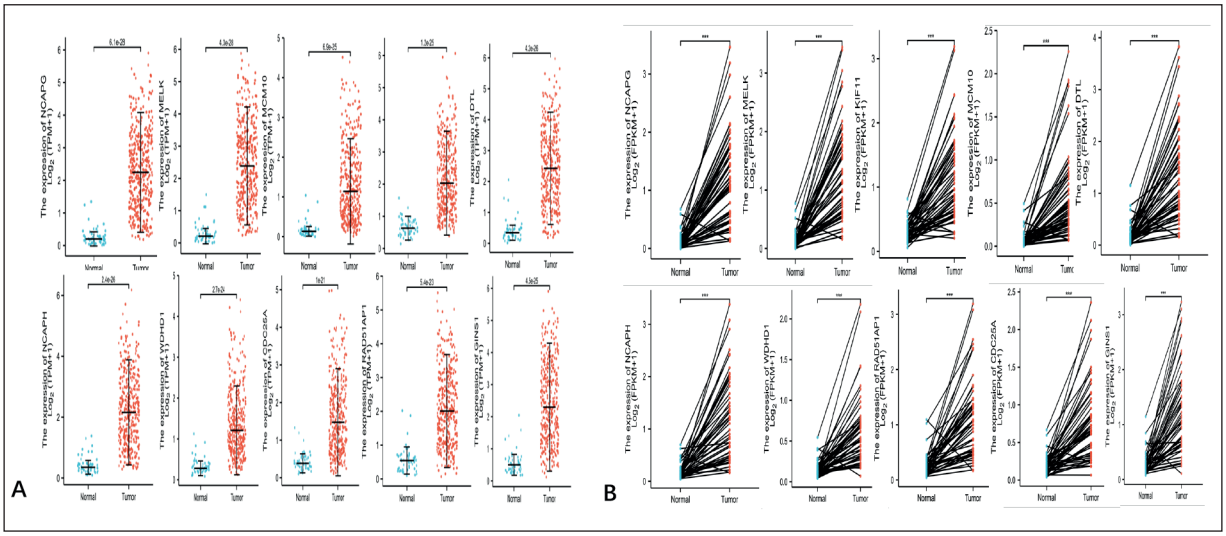

Figure 4. Hub gene expression. The expression of 10 hub genes (NCAPG, MELK, KIF11, MCM10, DTL, NCAPH, WDHD1, RAD51AP1, CDC25A, and GINS1) in the TCGA-LIHC dataset was assessed. **A**, Comparisons between tumors and normal tissues. **B**, Comparisons between tumors and normal tissues for 50 pairs of tissue samples.

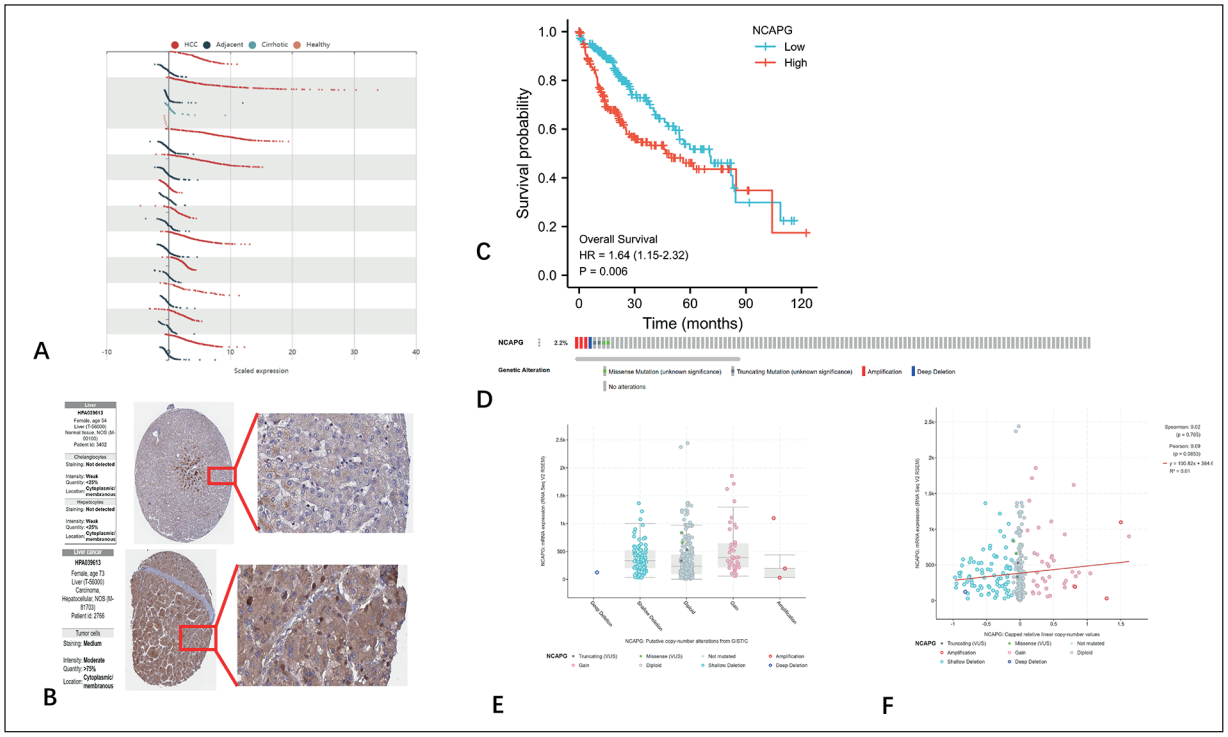

Figure 5. Analysis of NCAPG expression in human HCC. **A**, NCAPG expression in a different HCC database (HCCDB, http://lifeome.net/database/hccdb/). **B**, NCAPG protein level expression as assessed via immunohistochemical staining in the Human Protein Atlas database. **C**, Overall survival for patients with low (n = 185) and high (n = 184) levels of NCAPG expression was compared using Kaplan-Meier curves. **D**, Distributions of NCAPG genomic alterations in the TCGA-HCC cohort were represented with an OncoPrint plot from cBioPortal. Relationships between NCAPG copy number and mRNA expression levels were represented with dot plots (**E**) and correlation plots (**F**) from cBioPortal.

ed with histologic grade ($p < 0.001$), T stage ($p = 0.002$), pathologic stage ($p = 0.002$), tumor status ($p = 0.013$),and AFP ($p < 0.001$) (Table I). For additional exploration of the prognostic relevance of these different clinical parameters, a series of univariate and multivariate Cox regression analyses

Table I. NCAPG expression and clinicopathologic findings for individuals in the TCGA-LIHC cohort.

Characteristic	Low expression of NCAPG	High expression of NCAPG	p
n	187	187	
Etiology, n (%)			
Hepatitis	77(49.0)	80(51.0)	0.505
Alcohol consumption	56(48.7)	59(51.3)	0.538
Alcohol and hepatitis	23(47.9)	25(52.1)	0.512
Other	67(48.6)	71(51.4)	0.517
T stage, n (%)			0.002
T1	109 (29.4%)	74 (19.9%)	
T2	37 (10%)	58 (15.6%)	
T3	34 (9.2%)	46 (12.4%)	
T4	4 (1.1%)	9 (2.4%)	
N stage, n (%)			0.693
N0	121 (46.9%)	133 (51.6%)	
N1	1 (0.4%)	3 (1.2%)	
M stage, n (%)			0.573
M0	129 (47.4%)	139 (51.1%)	
M1	3 (1.1%)	1 (0.4%)	
Pathologic stage, n (%)			0.002
Stage I	102 (29.1%)	71 (20.3%)	
Stage II	35 (10%)	52 (14.9%)	
Stage III	33 (9.4%)	52 (14.9%)	
Stage IV	4 (1.1%)	1 (0.3%)	
Tumor status, n (%)			0.013
Tumor free	114 (32.1%)	88 (24.8%)	
With tumor	65 (18.3%)	88 (24.8%)	
Gender, n (%)			0.047
Female	51 (13.6%)	70 (18.7%)	
Male	136 (36.4%)	117 (31.3%)	
Age, n (%)			0.055
<=60	79 (21.2%)	98 (26.3%)	
>60	108 (29%)	88 (23.6%)	
BMI, n (%)			0.352
<=25	84 (24.9%)	93 (27.6%)	
>25	85 (25.2%)	75 (22.3%)	
Residual tumor, n (%)			0.588
R0	166 (48.1%)	161 (46.7%)	
R1	8 (2.3%)	9 (2.6%)	
R2	1 (0.3%)	0 (0%)	
Histologic grade, n (%)			< 0.001
G1	40 (10.8%)	15 (4.1%)	
G2	102 (27.6%)	76 (20.6%)	
G3	40 (10.8%)	84 (22.8%)	
G4	2 (0.5%)	10 (2.7%)	
Adjacent hepatic tissue inflammation, n (%)			0.179
None	66 (27.8%)	52 (21.9%)	
Mild	47 (19.8%)	54 (22.8%)	
Severe	12 (5.1%)	6 (2.5%)	
AFP(ng/ml), n (%)			< 0.001
<=400	121 (43.2%)	94 (33.6%)	
>400	20 (7.1%)	45 (16.1%)	
Albumin(g/dl), n (%)			0.721
<3.5	35 (11.7%)	34 (11.3%)	
>=3.5	125 (41.7%)	106 (35.3%)	
Child-Pugh grade, n (%)			0.646
A	120 (49.8%)	99 (41.1%)	
B	11 (4.6%)	10 (4.1%)	
C	1 (0.4%)	0 (0%)	

Table continued

Table I. *(Continued).* NCAPG expression and clinicopathologic findings for individuals in the TCGA-LIHC cohort.

Characteristic	Low expression of NCAPG	High expression of NCAPG	p
Fibrosis ishak score, n (%)			0.739
0	44 (20.5%)	31 (14.4%)	
1/2	15 (7%)	16 (7.4%)	
3/4	14 (6.5%)	14 (6.5%)	
5/6	43 (20%)	38 (17.7%)	
Vascular invasion, n (%)			0.139
No	116 (36.5%)	92 (28.9%)	
Yes	51 (16%)	59 (18.6%)	
OS event, n (%)			0.039
Alive	132 (35.3%)	112 (29.9%)	
Dead	55 (14.7%)	75 (20.1%)	
DSS event, n (%)			0.022
Alive	153 (41.8%)	134 (36.6%)	
Dead	30 (8.2%)	49 (13.4%)	
PFI event, n (%)			0.039
Alive	106 (28.3%)	85 (22.7%)	
Dead	81 (21.7%)	102 (27.3%)	

were used to identify predictors of patient OS and DFS. In univariate analyses, T stage, tumor status and NCAPG were associated with OS ($p < 0.05$; Table II) and DFS ($p < 0.05$; Table III) in the TCGA-HCC cohort. In a multivariate Cox analysis, NCAPG overexpression was independently associated with a poorer patient OS (HR = 2.543; 95% CI 1.224, 5.285; $p = 0.012$; Table II) and DFS (HR = 2.034; 95% CI 1.160, 3.566; $p = 0.013$; Table III). The concordance index (C-index) value for OS was 0.713 (95% CI, 0.671, 0.756), while that for DFS was 0.749 (%95 CI 0.729, 0.775). NCAPG expression may thus be a valuable independent predictor of HCC patient outcomes.

DNA Hypomethylation and NCAPG Overexpression

To gain additional insight into the mechanisms governing altered NCAPG expression in HCC, we assessed correlations between the expression of this gene and its methylation. We began by comparing the expression of the DNMT1, DNMT3A, and DNMT3B DNA methyltransferases between tumors with high and low levels of NCAPG expression, revealing all three to be upregulated in the context of higher NCAPG expression (Figure 6A). A UALCAN analysis further revealed DNMT1 to trend towards increased methylation in the typical tissues of the liver relative to the tissues of HCC ($p = 0.101$, Figure 6B), while an analysis performed using DiseaseMeth v 2.0 revealed NCAPG methylation to be significantly reduced in HCC tumors relative to paracancerous tissues ($p < 0.0001$; Figure 6C). Moreover, two key methylation sites (cg10333701 and cg03604819) in the NCAPG sequence were negatively correlated with the expression of this gene (Figure 6D). Hypermethylated regions were present within the 3'- and 5'-UTR

Table II. Univariate and multivariate Cox proportional hazard analyses of NCAPG expression and overall survival (OS) for patients with HCC in the TCGA-LIHC cohort.

Characters	Univariate analysis HR(95% CI)	p	Multivariate analysis HR(95% CI)	p
Sex (female/male)	0.772(0.529,1.127)	0.18		
Age(<=60/>60)	1.228(0.843,1.787)	0.285	2.598(1.417,4.765)	**0.002**
Tumor stage	1.139(0.782,1.657)	0.498		
T stage	2.517(1.730,3.661)	**<0.001**		
Vascular invasion	1.322(0.853,2.048)	0.212	1.957(1.030,3.721)	**0.040**
Tumor Status	1.489(1.009,2.197)	**0.045**	2.376(1.378,4.096)	**0.002**
AFP	1.3(0.729,2.319)	0.374		
NCAPG	2.557(1.715,3.813)	**<0.001**	2.543(1.224,5.285)	**0.012**

Abbreviations: CI, confidence interval; HR, hazard ratio; AFP alpha-fetoprotein. C-index = 0.713 (%95 CI 0.671,0.756).

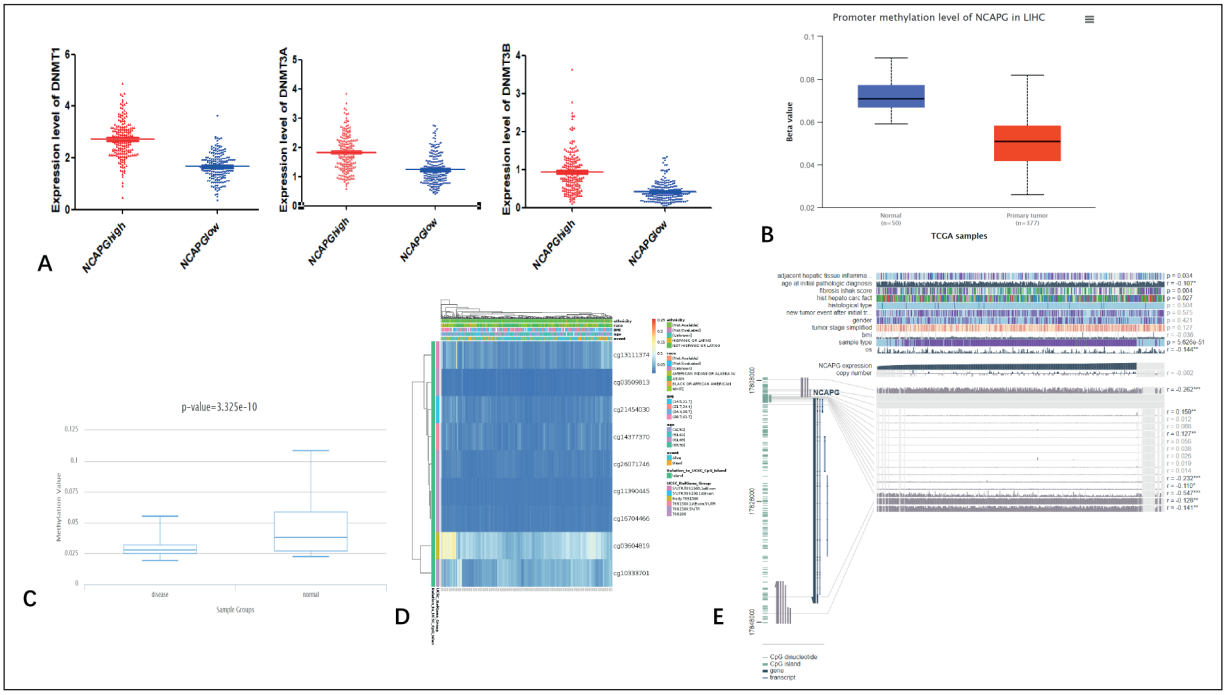

Figure 6. Assessment of the relationship between NCAPG and methylation. **A**, Analysis of the expression of the DNMT1, DNMT3A, and DNMT3B DNA methyltransferases. **B**, UALCAN was used to assess methylation status. **C**, DiseaseMeth v 2.0 was used to assess methylation status. **D**, NCAPG DNA methylation and the relationship between such methylation and gene expression was assessed with MEXPRESS. NCAPG expression levels are represented with a blue line, while Pearson's correlation coefficient values and *p*-values for particular sites of methylation and analyzed gene expression levels are demonstrated to the right. **E**, Differentially methylated regions related to NCAPG, which were represented using a heatmap generated with MethSurv.

regions, whereas the TSS1500 and TSS200 regions tended to be hypomethylated. These differential patterns of NCAPG expression were also represented using heatmaps (Figure 6E).

Evaluation of the Association between Immune Cell Infiltration and NCAPG Expression in HCC

In general, tumor-infiltrating lymphocytes (TILs) predict survival and lymph node metastasis in many cancers[17]. Accordingly, we utilized the TIMER database to explore potential links between NCAPG expression levels and such immune cell infiltration in HCC. An initial 'SCNA' module analysis revealed several infiltrating immune cell populations that were not associated with changes in NCAPG gene copy number in HCC including CD4+ T cells, DCs, B cells, and macrophages (Figure 7A). When we examined the association between such immune cell infiltration and HCC patient prognosis, we found

Table III. Univariate and multivariate Cox proportional hazard analyses of NCAPG expression and disease-free survival (DFS) for patients with HCC in the TCGA-LIHC cohort..

Characters	Univariate analysis HR(95% CI)	*p*	Multivariate analysis HR(95% CI)	*p*
Sex (female/male)	0.835(0.598,1.166)	0.290		
Age(<=60/>60)	0.929(0.665,1.298)	0.666		
Tumor stage	1.097(0.798,1.510)	0.568		
T stage	2.369(1.696,3.310)	<0.001	2.435(1.404,4.222)	0.002
Vascular invasion	1.842(1.281,2.647)	0.001		
Tumor Status	3.605(2.602,4.993)	<0.001	5.988(3.682,9.738)	<0.001
AFP	1.246(0.764,2.209)	0.378	2.164(1.197,3.912)	0.011
NCAPG	2.029(1.415,2.911)	<0.001	2.034(1.160,3.566)	0.013

Abbreviations: CI, confidence interval; HR, hazard ratio; AFP alpha-fetoprotein. C-index=0.749 (%95 CI 0.729, 0.775).

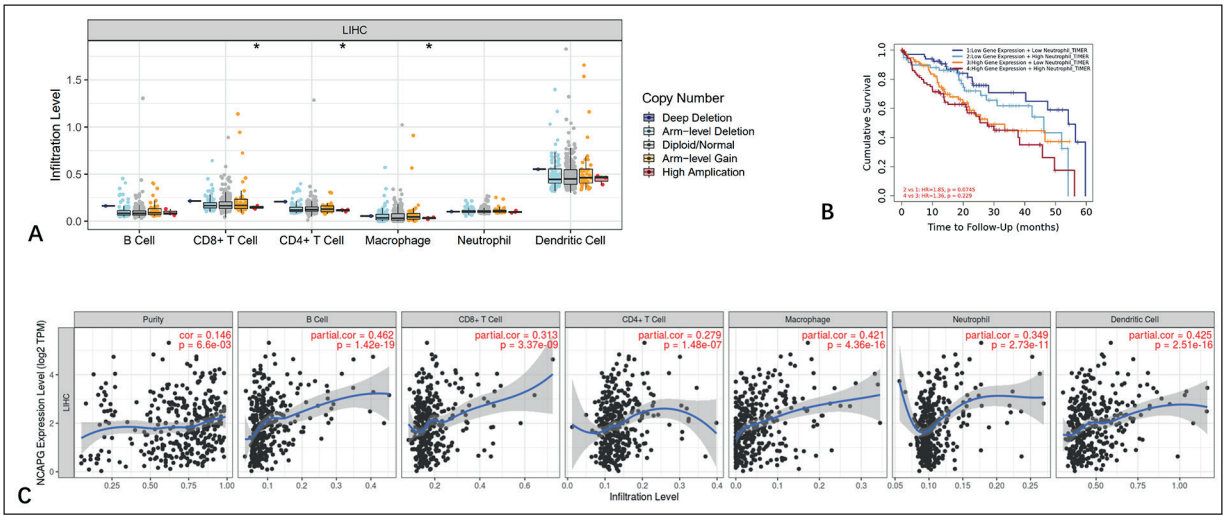

Figure 7. Evaluation of the relationship between NCAPG expression and immune cell infiltration in HCC. **A**, The relationship between NCAPG copy number and immune cell infiltration in HCC. **B**, Associations between immune infiltration and HCC patient OS were assessed with Kaplan-Meier plots. **C**, Correlations between NCAPG expression levels and immune infiltration in HCC. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

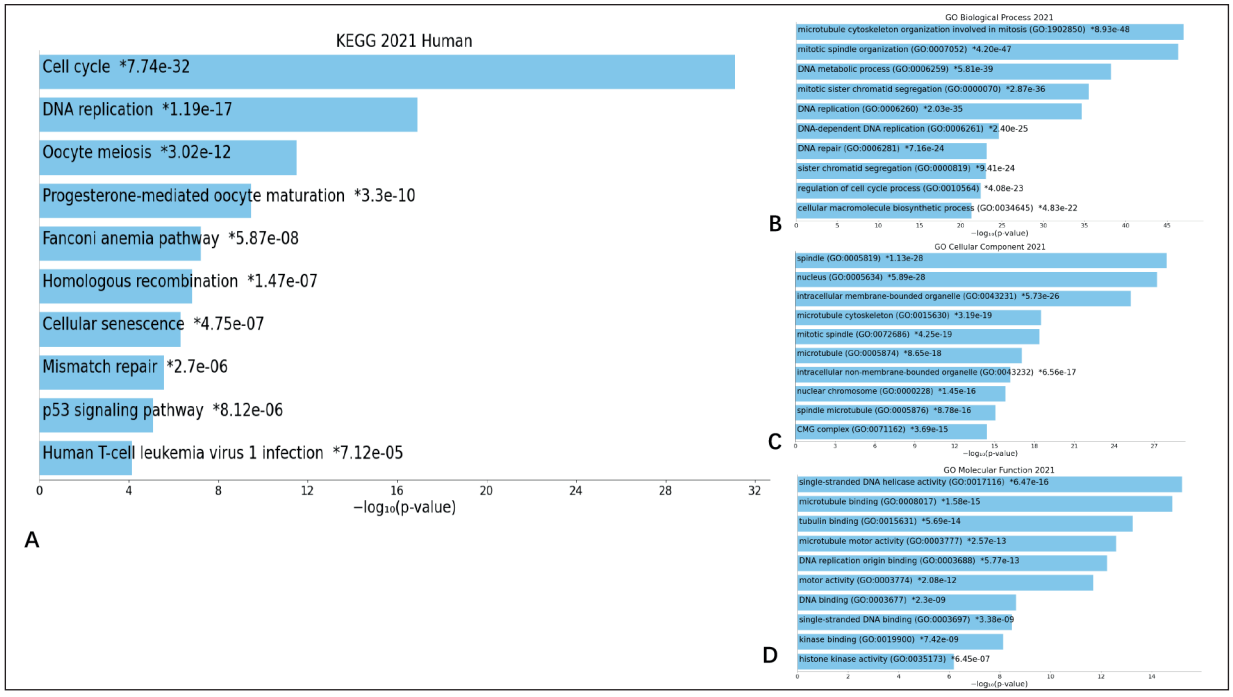

Figure 8. NCAPG functional enrichment assessment. KEGG enrichment (**A**) and GO analyses (**B** to **D**) of NCAPG-related genes in HCC were performed.

increased neutrophil cell infiltration to be associated with poorer HCC patient survival outcomes, with an OS of 60 months ($p = 0.019$, HR=5.114, 95% CI 1.306,20.032, Figure 7B). A subsequent 'Gene' module analysis revealed no correlations between the expression of NCAPG and tumor purity, although it was positively correlated with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and DC infiltration in HCC (Figure 7C). The obtained findings suggest that NCAPG may impact HCC patient prognosis and clinical outcomes in part by modulating intratumoral immune cell infiltration.

To additionally confirm the link between NCAPG expression and immune cell infiltration, we examined the relationship between such expression and the levels of immunological marker genes associated with 6 cell types. This approach revealed that five of these marker genes (CD19, IRF5, ITGAM, ITGAX) associated with B cells, M1 macrophages, neutrophils, and DCs were positively correlated with the expression of NCAPG in analyzed HCC samples (Table IV). As such, the interplay between NCAPG and these immune cell populations may shape HCC patient prognosis.

Analysis of the Functional Roles of NCAPG in HCC

Lastly, GO and KEGG enrichment assessments were executed for the top 200 genes that were most correlated with NCAPG expression in HCC to better explore its functional role in this oncogenic setting. This approach revealed NCAPG to be associated with the P53 signaling pathway (Figure 8A), and with GO terms including "DNA replication," "microtube cytoskeleton," and "histone kinase activity"(Figure 8B-D).

Discussion

Vascular invasion is a primary predictor of metastatic progression, tumor recurrence, and poor survival outcomes in HCC patients[3,4]. As such, the elucidation of the mechanistic basis for HCC vascular invasion has the potential to guide the identification of novel biomarkers and therapeutic targets that can be leveraged to improve outcomes for patients diagnosed with this form of cancer[2]. However, there have been relatively few studies to date exploring mRNAs related to the prognosis of vascular invasion in HCC.

Herein, we initially employed an *in silico* approach to develop a network composed of 257 mRNAs. Enrichment analyses revealed this network to be primarily associated with developmental, cellular, and reproductive processes. Ten hub genes within this network were subsequently identified and were found to be overexpressed in HCC and linked to HCC patient prognosis. A search of the PubMed database revealed that one of these hub genes, NCAPG, had been studied in detail in oncogenic contexts[17-20]. Sun et al[21]

Table IV. Correlation analysis of the relationship between NCAPG and immune cell biomarkers in HCC.

Immune Cell	Gene	Correlation Coef.	*p*
B cell	**CD19**	**0.26928767**	**1.23E-07**
	CD79A	0.108683427	0.035638416
CD8+ T cell			
	CD8A	0.16540517	0.00134393
	CD8B	0.159708829	0.001947048
CD4+ T cell	CD4	0.164780548	0.001402223
M1 macrophage			
	NOS2	-0.084955461	0.100916288
	IRF5	**0.32337945**	**1.91E-10**
	PTGS2	0.012444166	0.810436917
M2 macrophage			
	CD163	0.030633654	0.554632557
	VSIG4	0.042360396	0.413848183
	MS4A4A	0.029464696	0.569845371
Neutrophil	CEACAM8	0.159715136	0.001946233
	ITGAM	**0.285689954**	**2.16E-08**
	CCR7	0.001159209	0.982167473
Dendritic cell	HLA-DPB1	0.12087236	0.019419822
	HLA-DQB1	0.122723517	0.017626348
	HLA-DRA	0.132122436	0.010578512
	HLA-DPA1	0.08758435	0.090757871
	CD1C	0.054062997	0.297044825
	NRP1	0.124323264	0.016194786
	ITGAX	**0.27742742**	**5.58E-08**

Abbreviations: CI, confidence interval; HR, hazard ratio; AFP alpha-fetoprotein. C-index=0.749 (%95 CI 0.729, 0.775).

previously reported that NCAPG overexpression is associated with gastric cancer prognosis, making it a promising therapeutic target in this cancer type. Moreover, Jiang et al[22] determined that NCAPG can function as a prognostic biomarker and therapeutic target associated with overcoming resistance to trastuzumab treatment in HER2+ breast cancer. Zhang et al[23] further established NCAPG as an important mitotic protein involved in the promotion of HCC cell proliferative and migratory activity. We found that HCC tumors consistently exhibited higher levels of NCAPG expression as compared to normal tissues, with high levels of NCAPG expression being linked to poor survival outcomes.

It has been recognized that hepatitis is one of the initiating factors of liver cancer[24]. The antiviral treatment is closely associated with predictive epigenetic factors[25]. No correlations between the abnormal overexpression of NCAPG and viral factors or alcohol consumption were identified herein (Table I). Additionally, no correlations between the abnormal overexpression of NCAPG and copy number changes were evident in HCC. DNA methylation status can play a key role in determining the relative efficacy of targeted treatments for colorectal and hepatobiliary tumors[26,27]. We thus explored patterns of DNA methylation with the potential to account for abnormal NCAPG expression and ultimately found NCAPG to be hypomethylated in HCC tumors as compared to paracancerous tissues, Consistently, multiple DNA methyltransferases were upregulated in HCC samples including DNMT1, DNMT3A, and DMNT3B. Moreover, higher levels of NCAPG expression were associated with the increased expression = of these three DNA methyltransferases, providing a potential mechanism whereby this gene may be upregulated in HCC. We also found certain methylation sites to be negatively correlated with HCC patient prognosis. We further examined associations between the expression of NCAPG and genome-wide methylation patterns, revealing increased hypomethylation proximal to open sea regions (Figure 6E). These results suggest abnormal methylation patterns to be potentially associated with poor HCC patient prognosis.

Prior work suggests that immune cell infiltration is a key determinant of cancer patient prognosis [28,29]. Herein, we did not find NCAPG gene copy number to be correlated with B cell, CD4+ T cell, CD8+ T cell, macrophage, neutrophil, and DC infiltration using the TIMER database. We found NCAPG expression levels to be closely correlated with HCC tumor immune infiltration, and many tumor-infiltrating immune cells are associated with HCC patient prognosis[30,31]. Significant positive correlations between NCAPG expression levels and markers of certain immune cell subsets were detected in HCC, suggesting that this gene may shape the composition of the tumor immune microenvironment in the context of HCC development.

In order to more fully clarify the potential role of NCAPG as a regulator of oncogenic processes, we performed functional enrichment analyses revealing this gene to be associated with cellular proliferation, kinase activity, and the P53 signaling pathway.

There are multiple limitations to this study. Firstly, all *in silico* analyses of potential relationships between miRNAs and mRNAs warrant further experimental validation. Secondly, as TCGA is an open-access and large-scale database including many tumor samples, this dataset can provide multidimensional molecular profiles for only a relatively low number of *in vivo* HCC samples with vascular invasion information of HCC. Lastly, additional research regarding the mechanistic role of NCAPG is required.

Conclusions

Together, the results of the present analysis highlight the value of NCAPG as a prognostic biomarker associated with vascular invasion in patients with HCC. As our results were reproducible in a large TCGA patient dataset, they are robust and are thus likely to be closely associated with key disease-related parameters. Future research exploring the mechanisms whereby NCAPG influences HCC oncogenic progression is thus warranted.

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Authors' Contributions

Drs. Guo and Zhu proposed the concept and designed the study, and Dr. Jiang contributed to the acquisition of data. All authors provided input to the manuscript. All authors read and approved the final manuscript. Conceptualization: Ziyi Guo; Data curation: Ziyi Guo; Formal analysis: Ziyi Guo; Methodology: Lipeng Jiang; Software: Ziyi Guo; Supervision: Zhitu Zhu. Validation: Lipeng Jiang; Writing – original draft: Ziyi Guo, Zhitu Zhu; Writing – review & editing: Ziyi Guo, Lipeng Jiang, Zhitu Zhu.

Conflicts of interest

The authors declare no conflicts of interest.

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