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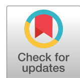
Original Article



E2F1-mediated Up-regulation of NCAPG Promotes Hepatocellular Carcinoma Development by Inhibiting Pyroptosis

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Abstract

Background and Aims: As a subunit of the condensin complex, NCAPG has an important role in maintaining chromosome condensation, but its biological function and regulatory mechanism in hepatocellular carcinoma (HCC) remains undefined. ***Methods:*** The prognostic ability of NCAPG in HCC patients was examined by univariate and multivariate Cox regression analysis. ROC curves were plotted to compare the predictive ability of NCAPG and AFP. Double luciferase reporter system, and ChIP were used to investigate transcriptional potential of E2F1 to NCAPG. Pyroptosis was observed by scanning electron microscopy. Protein expression of NCAPG, E2F1, and major proteins constituting NLRP3 inflammasome was determined by western blotting and ELISA. An *in vivo* tumor formation assay was conducted to verify the *in vitro* results. ***Results:*** Up-regulated NCAPG was identified in HCC tissues compared with adjacent tissue and high NCAPG was positively correlated with poor prognosis. Serum NCAPG mRNA level was a prognostic factor in HCC patients and also a diagnostic factor with higher predictive ability compared with AFP [AUROC 0.766 (95% CI: 0.650–0.881) vs. 0.649 (95% CI 0.506–0.793)]. HBx transfection resulted in concomitant up-regulation of E2F1 and NCAPG. E2F1 significantly increased the activity of luciferase reporter fused with NCAPG reporter, and the interaction of E2F1 and NCAPG gene was confirmed by ChIP. Silencing of E2F1 resulted in significant down-regulation of NCAPG. Knockdown of NCAPG promote pyroptosis mediated by NLRP3 inflammasome activation in multiple HCC cell lines and also suppressed tumorigenesis *in vitro*. ***Conclusions:*** We identified a novel role of NCAPG in the regulation of NLRP3 inflammasome-mediated pyroptosis, which was regulated by its upstream transactivator, E2F1. The role of E2F1-NCAPG-NLRP3 regulation of pyroptosis network may be a potential target in HCC treatment.

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Keywords: NCAPG; Hepatocellular carcinoma; Pyroptosis; NLRP3 inflammasome; Serum marker; circulating RNA.

Abbreviations: AFP, Alpha Fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; AUROC, Area under the ROC curve; ASC, apoptosis-associated speck-like protein containing CARD; CI, confidence interval; CHB, chronic hepatitis B; ChIP, chromatin immunoprecipitation; DAMP, damage-associated molecular patterns; E2F1, E2F Transcription Factor 1; ELISA, Enzyme-linked immunosorbent assay; GSEA, Gene set enrichment analysis; GEPIA, Gene Expression Profiling Interactive Analysis; HCC, hepatocellular carcinoma; HBV, Hepatitis B virus; HBx, hepatitis B virus x protein; HMGB1, High Mobility Group Protein 1; IL-1β, Interleukin (IL)-1β; NCAPG, Non-SMC Condensin I Complex Subunit G; NLRP3, NLR Family Pyrin Domain Containing 3; NC, negative control; qPCR, quantitative real-time; TCGA, the Cancer Genome Atlas; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling.

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Introduction

According to recent reports, liver cancer is projected to be the sixth most common cancer and the fourth leading cause of cancer death worldwide,[1] and HCC accounts for 80% of primary liver cancers.[2] Even though the treatment of HCC has greatly improved by interventional therapy and immunotherapy, surgical resection is still the most effective treatment for early HCC. Given the inconspicuous characteristics

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of HCC at an early stage, most patients have reached an advanced stage at the time of diagnosis and have lost the best opportunity for surgical treatment.[3] In addition, polykinase inhibitors such as sorafenib, regiffenil, and lenvatinib also have limited therapeutic effects on advanced HCC.[4–7]

At this time, NLRP3-inflammasome-dependent pyroptosis is well known for its function in type 2 diabetes and Parkinson's disease.[8,9] Some recent studies have focused on the role of pyroptosis in tumors. The roles of NLRP3 inflammasome were inconsistent depending on different cancer types. Its activation promoted gastric and lung cancer progression,[10,11] but inhibited colorectal cancer liver metastases.[12] NLRP3 is also expressed in hepatocytes.[13] The components of NLRP3 inflammasome are markedly downregulated in HCC, including NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), caspase-1 and interleukin (IL)-1β and their levels significantly correlate with advanced disease stages and poor pathological differentiation.[14] However, the underlying molecular mechanisms of how pyroptosis suppresses HCC remain largely unknown.

Research has shown that mitotic cells do not activate the NLRP3 inflammasome at the same time.[15] Non-SMC condensin I complex subunit G (NCAPG), one of the non-SMC subunits, is a mitosis-related chromosome condensation protein.[16,17] NCAPG has a crucial role in HCC proliferation and migration, and high expression is inversely associated with overall survival of cancer.[18–21] To date, neither the regulatory networks of NCAPG on pyroptosis nor the mechanisms underlying NCAPG deregulation have been disclosed. Our findings indicate that NCAPG inhibited NLRP3 inflammasome-mediated pyroptosis in HCC. After analyzing the promoter region of NCAPG, we found that E2F1 directly promoted the transcription of NCAPG. This study highlights a suppressive effect of NCAPG on pyroptosis and its underlying mechanism. Activity of the E2F1-NCAPG-NLRP3-gasdermin D regulatory axis in HCC, may have potential for development of novel therapeutic targets for the treatment of HCC in the coming future.

Methods

Patients and clinical specimens

All 200 paired HCC and adjacent nontumor liver tissues were obtained from patients undergoing HCC resection at the Third affiliated hospital of Sun Yat-sen University in Guangzhou, China. None of the patients received preoperative local or systemic anticancer treatment. Serum samples of healthy controls, chronic hepatitis B patients, cirrhotic patients, and HCC patients were also obtained from the Third Affiliated Hospital of Sun Yat-sen University, as previously described.[22] The patient characteristics are shown in Supplementary Table 1. This study was approved by the Institute Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University and informed consent was obtained from each HCC patient.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded specimens were prepared as previously described.[22] The sections were incubated with antibodies against NCAPG, 1:250 (ab56382, Abcam, U.K.), NLRP3, 1:500 (ab214185, Abcam, U.K.), gasdermin D, 1:500 (#96458, Cell Signaling Technology, USA).

Cell culture

HEK293FT and HCC cell lines MHCC-97L, SMMC-7721, QGY-7703 and HepG2 were cultured in Dulbecco's modified Eagle medium DMEM, (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). BEL-7402 cells were grown in RPMI 1640 (Gibco) containing 10% FBS.

Chromatin immunoprecipitation assay

ChIP experiments were performed with SimpleChIP\ Plus Enzymatic Chromatin IP kits (#9005, Cell Signaling Technology, USA) following the manufacturer's instructions. Briefly, 540 μL fresh 37% formaldehyde was added to 5×10^6 QGY-7703 cells transfected with E2F1 vector or control vector in 10 cm dishes to crosslink proteins with DNA. A glycine solution was used to terminate the reaction. Chromosome truncation to 100–150 bp was performed by enzymatic hydrolysis. Nuclear membranes were disrupted by sonication, and nuclear extracts were incubated with antibodies against E2F1 (#3742, Cell Signaling Technology, USA) or IgG (#2729, Cell Signaling Technology, USA) overnight at 4°C. PCR was performed with Tks Gflex DNA Polymerase Low DNA (R091S, Takara, Japan). Human NCAPG promoter primers used are shown in Supplementary Table 2.

Mouse xenograft model

Fourteen 4–5-week-old male B-NDG mice (NOD-*Prkdc*^{scid}*IL-2rg*^{tm1}/Bcgen, Biocytogen, China) were maintained in the laboratory of the Animal Center of Forevergen Biotechnology Co., Ltd (Guangzhou, China; http://www.forevergen.cn/). Mouse procedures were conducted under the guidelines and approved by the Animal Management Committee of Forevergen Medical Laboratory Animal Center. Scrabble vector-transfected or shNCAPG-transfected BEL-7402 cells (5×10^6) were suspended in 0.12 mL PBS and 0.12 mL of the suspended cells were injected subcutaneously into the posterior flanks of each B-NDG mice (life was scrabbled vector-transfected group and right was shNCAPG group). On the 11th day after inoculation, the length (L) and width (W) of the tumor were measured with a vernier caliper. Tumor volume = $(L\times W^2)/2$. Subcutaneous tumors were removed 28 days after inoculation

RNA oligoribonucleotides and plasmids

Small interfering RNA (siRNA) duplexes were obtained from Ribobio (Guangzhou, P.R. China). RNA oligonucleotides targeting human NCAPG (GenBank access No. NM_022346.4) and E2F1 (GenBank access No. NM_005225.2) are siNCAPG and siE2F1. The negative control (NC) RNA duplex for the siRNA was not homologous to any human genome sequences. The siRNA duplex oligonucleotides are listed in Supplementary Table 2. Lipofectamine RNAiMAX Reagent (Life Technologies, Thermo Fisher Scientific) was used to transfect the siRNA according to the manufacturer's instructions.

The full-length cDNA of human NCAPG gene was amplified by PCR and cloned into the pcDNA3.1 vector (Life Technologies, Thermo Fisher Scientific) by *Not1/XbaI* sites. The lentiviral shRNA sequence for NCAPG listed in Supplementary Table 2 was purchased from Forevergen Biotechnology Co., Ltd. pRP[Exp]-EGFP/Puro-CAG-E2F1[NM_005225.2] and pRP[Exp]-EGFP/Puro-CAG> hepatitis B virus x protein (HBx) were obtained from Vector Builder (https://en.vectorbuilder.com/). Vector transfections were performed with Lipofectamine 3000 (Life Technologies, Thermo Fisher Scientific) following the manufacturer's instructions.

Luciferase assay

For promoter analysis, QGY-7703 or BEL-7402 cells were used to characterize the NCAPG promoter and E2F1 targeted site. Human NCAPG promoter (−2 to +0.2 kb) overall length

Table 1. Univariate and multivariate analysis of factors associated with overall survival[a]

Clinical variable	Hazard ratio (95% CI[c])	*p*-value
Univariate analysis[b*]		
NCAPG (High vs. Low)	1.9 (1.1–3.1)	0.016
Sex (M vs. F)	1.3 (0.6–2.9)	0.504
Age-yr (> 45 vs. ≤ 45)	1.9 (0.8–4.1)	0.122
HBV (Positive vs. Negative)	1.2 (0.5–2.7)	0.727
Cirrhosis (Yes vs. No)	4.1 (1.5–11.2)	0.007
Ascites (Yes vs. No)	2.4 (1.2–5.1)	0.020
AFP (≥ 400 vs. < 400 ng/mL)	1.3 (0.8–2.1)	0.339
ALT (≥ 50 vs. < 50 U/L)	1.5 (0.9–2.5)	0.165
Tumor size (> 5 cm vs. ≤ 5 cm)	1.9 (1.0–3.4)	0.047
Multinodular (Yes vs. No)	1.9 (1.2–3.2)	0.011
Tumor capsule (None/incomplete vs. Complete)	2.0 (1.2–3.5)	0.010
Portal vein tumor thrombus (Yes vs. No)	1.2 (1.2–4.0)	0.015
Multivariate analysis[d*]		
NCAPG (High vs. Low)	2.4 (1.3–4.0)	0.003
Cirrhosis (Yes vs. No)	3.6 (1.3–9.9)	0.015
Tumor size (> 5 cm vs. ≤ 5 cm)	2.0 (1.1–3.7)	0.034
Multinodular (Yes vs. No)	2.0 (1.1–3.5)	0.019
Tumor capsule (None/incomplete vs. Complete)	2.0 (1.1–3.6)	0.018

*Significant values. [a]Analysis was performed on the entire cohort (n = 200). [b]Univariate analysis, Cox proportional hazards regression. [c]95% Confidence interval. [d]Multivariate analysis, Cox proportional hazards regression. NCAPG, Non-SMC Condensin I Complex Subunit G; AFP, Alpha-fetoprotein; ALT, alanine aminotransferase; HBV, Hepatitis B virus.

was purchased from GeneCopoeia (CS-HPRM24637-FR01). A series of promoter fragments with the same 5′ terminal was inserted into a pGL3-Basic plasmid (Promega) by *KpnI / HindIII* restriction site. All constructs were confirmed by DNA sequencing. Renilla luciferase was cotransfected with 200 ng each promoter-reporter construct by Lipofectamine 3000 (Life Technologies, Thermo Fisher Scientific) for cells cultured in 48-well.[23] After 48 h transfection, cells were lysed and a dual-luciferase reporter assay system (Promega) was used to measure luciferase reporter gene expression according to the manufacturer's protocol. The primers with different length of NCAPG promoter are shown in Table 1.

Analysis of gene expression

Western blotting and quantitative real-time (qPCR) were performed to analyze the expression of target genes. The qPCR primers are listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of High Mobility Group Protein 1 (HMGB1) and IL1β in the supernatant of QGY-7703 and MHCC-97L cells was assayed by ELISA. After transfection for 24 h, the medium was replaced by DMEM (Gibco, Thermo Fisher Scientific) containing 2% FBS (Gibco), and 48 h post-transfection, supernatants were harvested and assayed by HMGB1 (E-EL-H1554c, Elabscience, Hubei, China) and IL1β ELISA kits (RK00001, Abclonal, Hubei, China) following the manufacturer's instructions.

Bioinformatics analysis

Gene set enrichment analysis (GSEA) was conducted using GSEA software (www.broadinstitute.org/gsea). TCGA LIHC datasets containing RNA-seq of 371 HCC samples were divided into high and low groups for GSEA analysis. Gene set permutation mode was applied and pathways with false discovery rate (FDR) < 15% and p-values of < 0.01 were designated as the most significant pathways.

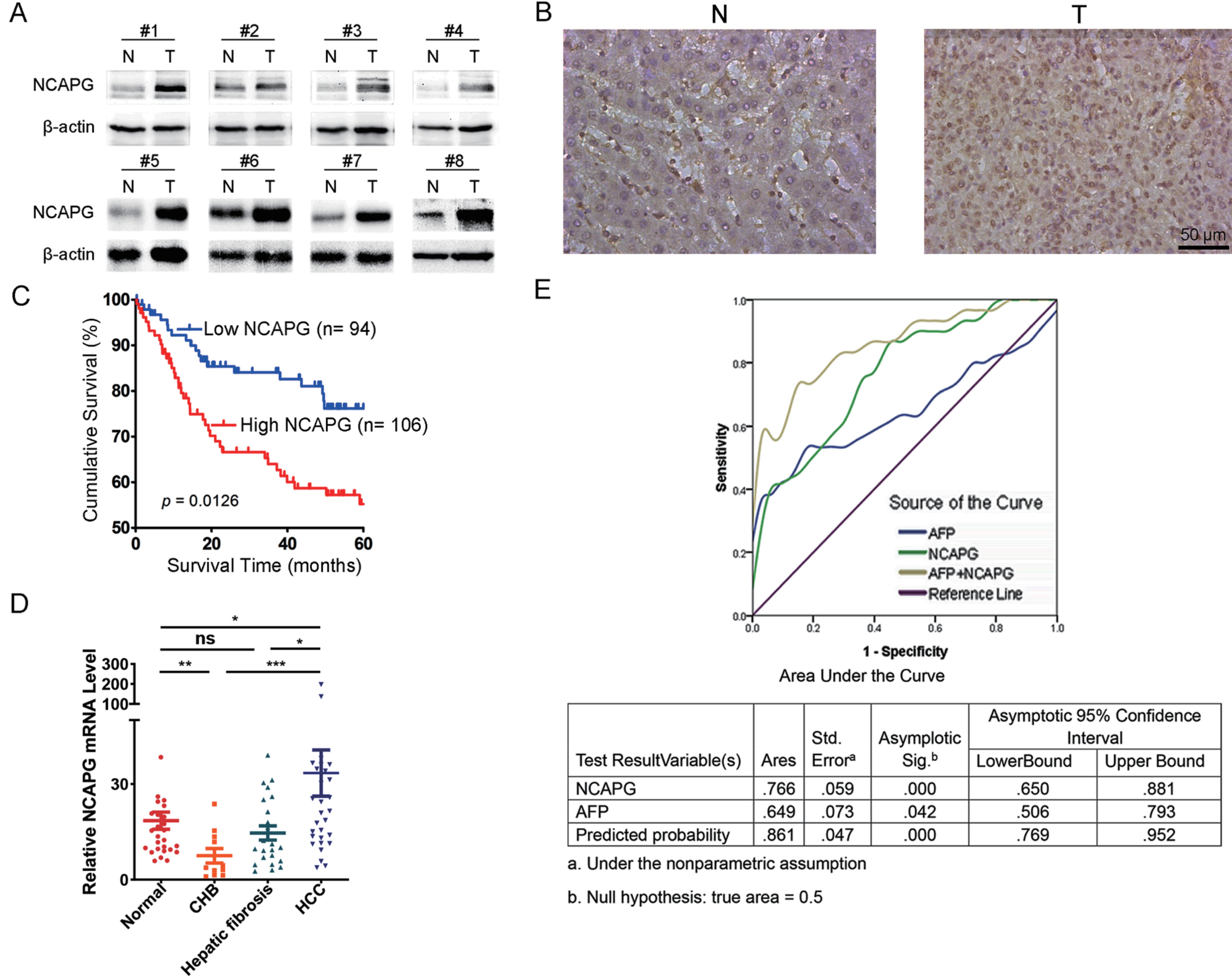
Statistical analysis

All *in vitro* experiments were repeated independently at least three times. Data were reported as means ± SEM and between-group differences were compared with student's t-tests and the Mann-Whitney U test or Kruskal–Wallis tests to compare more than two groups. Overall survival was estimated by the Kaplan–Meier method and was compared using log-rank tests using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). P-values < 0.05 were considered statistically significant. GraphPad Prism 5 was used to generate all graphs.

Results

NCAPG was up-regulated in HCC and positively correlated with poor prognosis in HCC patients

We observed an up-regulation of NCAPG in HCC tissues compared with the adjacent liver tissues at both protein and RNA levels (Fig. 1 and Supplementary Fig. 1A), which was in line with previous report.[24] HCC datasets, including 369 HCC tissue samples and 160 normal controls from the Gene Expression Profiling Interactive Analysis (GEPIA) database, yielded similar results. (p < 0.05; Supplementary Fig. 1B). To further investigate whether NCAPG could be a prognostic marker, we



Test ResultVariable(s)	Ares	Std. Error[a]	Asymplotic Sig.[b]	Asymptotic 95% Confidence Interval	
				LowerBound	Upper Bound
NCAPG	.766	.059	.000	.650	.881
AFP	.649	.073	.042	.506	.793
Predicted probability	.861	.047	.000	.769	.952

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Fig. 1. NCAPG was overexpressed in HCC. (A) Protein levels of NCAPG analyzed by western blotting in eight pairs of HCC tissues (T) and the matched adjacent nontumor tissues (N). (B) IHC staining of NCAPG in HCC tumors (right) and the corresponding nontumor tissues (left). Scale bar, 50 μm. (C) Kaplan–Meier survival curves showed that the overall survival rate was negatively associated with the expression of NCAPG in 200 patients with HCC. (D)Increased expression of NCAPG in the serum of HCC patients. Serum RNA was extracted from 28 healthy controls, 11 chronic hepatitis B patients, 23 cirrhosis patients, and 31 liver cancer patients, and NCAPG mRNA levels were compared by qPCR. β-actin was used as an endogenous reference. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (E) The diagnostic value of NCAPG for liver cancer is superior to AFP. AFP, Alpha Fetoprotein; NCAPG, Non-SMC Condensin I Complex Subunit G; HCC, hepatocellular carcinoma; IHC, Immunohistochemistry; qPCR, quantitative real-time.

analyzed the immunohistochemical staining of NCAPG in 200 patients with HCC. Using their NCAPG immunohistochemical scores, patients were divided into high and low expression groups by X-tile using the minimum *p*-value. High NCAPG levels were significantly associated with poor overall survival ($p < 0.05$; Fig. 1B, C). After univariate and multivariate Cox regression analysis, NCAGP was identified as an independent prognostic risk factor (Table 1). The results indicate that NCAPG has important clinical value.

Circulating NCAPG RNA levels were up-regulated in the serum of HCC patients

Our above results suggest that HCC patients with high expression of NCAPG had a poor prognosis. Next, we explored whether NCAPG could be used as a diagnostic marker of HCC. We collected serum from 28 healthy controls, 11 patients with chronic hepatitis B (CHB), 23 patients with hepatic fibrosis, and 31 patients with HBV-related HCC (Supplementary Table 1). Notably, the serum RNA of NCAPG was significantly higher in HCC patients than that in healthy individuals and in other patients with chronic hepatitis B or liver fibrosis. In addition, compared with patients with chronic hepatitis B, NCAPG was gradually overexpressed in liver fibrosis and HCC group (Fig. 1D).

Alpha-fetoprotein (AFP) is the most widely used diagnostic marker for HCC. To clarify the diagnostic value of NCAPG for HCC, we compared the predictive ability of NCAPG and APF in our HCC cohort. By comparing the area under the receiver operating characteristics curve (AUROC; Fig. 1E and Table 2), we found that the AUROC of NCAPG (0.766; 95% CI: 0.650–0.881) was larger than that of AFP (0.649; 95% CI: 0.506–0.793), indicating that NCAPG was superior to AFP for the diagnosis of HCC.[25] Using the best cutoff value of AFP in clinical practice (20 ng/mL), the sensitivity was 0.6 and the

Table 2. ROC curve analysis was used to analyze the detection of NCAPG and AFP

Diagnostic index	AUC	Standard error	95% Confidence interval
NCAPG	0.766	0.059	0.650–0.881
AFP	0.649	0.073	0.506–0.793
Logistic regression	0.861	0.047	0.769–0.952

ROC curve analysis was used to compare the sensitivity and specificity of NCAPG, AFP, NCAPG and AFP. NCAPG, Non-SMC Condensin I Complex Subunit G; AFP, Alpha-fetoprotein; ROC, Receiver operating characteristic.

specificity was 0.56. The optimal diagnostic cutoff value of NCAPG was 11.18, with a sensitivity of 0.87 and a specificity of 0.59. Combining NCAPG with AFP improved the diagnostic performance, and the combined AUROC rose significantly compared with AFP (0.853 vs. 0.640). In our study, 40% of the patients in the HCC cohort presented with normal AFP levels, among whom 91.6% presented with NCAPG levels higher than the best cutoff value (11.18), further demonstrating the higher sensitivity of NCAPG.

E2F1 transcriptionally activated the expression of NCAPG

To explore the reason for the high expression of NCAPG in HCC, we first assayed the expression of NCAPG DNA in HCC and adjacent tissues. The result showed that the expression of NCAPG at DNA level was comparable between HCC tissues and adjacent tissues (Supplementary Fig. 2A). We therefore tested whether the change in NCAPG protein and mRNA levels might occur at the transcription. We used three algorithmic tools to predict potential transcription factor binding sites within the NCAPG promoter using MatInspector (Alggen, Gene-Cloud of Biotechnology Information and Genomatix Software GmbH, Munich, Germany) and compared the results. Consequently, E2F1, a transcription factor of the E2F family, was selected for further study, and its binding sites within NCAPG promoter were identified. To evaluate the statistical correlation between NCAPG and E2F1, we analyzed their expression in HCC tissues and observed a positive correlation between them (Fig. 2A and Supplementary Fig. 2B). We divided an HCC cohort derived from 371 TCGA RNA-seq dataset into two groups by the medium value of NCAPG mRNA expression, and performed Gene set enrichment analysis (GSEA), which also found that NCAPG was positively correlated with E2F1 (Fig. 2B). To directly investigate the relationship of NCAPG and E2F1, we transfected siRNAs to inhibit the expression of E2F1 or plasmid to increase the expression of E2F1 in HCC cells (Supplementary Fig. 2C). E2F1 knockdown by siRNA downregulated NCAPG at both mRNA and protein levels (Fig. 2C and D), whereas overexpression of E2F1 enhanced the NCAPG level (Fig. 2E).

Reportedly, HBV promotes HCC development by regulating E2F1.[26] Hence, we speculated that HBV infection might also contribute to the up-regulation of NCAPG. To prove our hypothesis, HCC cells cultured in serum-free medium were treated with or without HBV-positive serum for 48 h. Both E2F1 and NCAPG levels were significantly higher in cells treated with HBV-positive serum than in controls (Fig. 2F). Similarly, overexpression of HBx, which was encoded by the smallest open reading frame of HBV, not only up-regulated the expression of E2F1 and NCAPG but also downregulated the gasdermin D level (Fig. 2G).

To identify which sequences accounted for the transcription activation of NCAPG, a 2,200 bp fragment at nucleotides −2,000 to +200 containing the proximal 5′-flanking region, the transcription start site, and a portion of exon 1 within NCAPG sequence was cloned and fused to the upstream of a promoter-less luciferase reporter gene (pGL3-basic-NCAPG). A series of 5′ deletions of the 2.2 kb NCAPG promoter were generated to identify their effect on reporter gene activity (Fig. 3A). These luciferase reporter constructs were transiently transfected into QGY-7703 and Bel-7402 cells, and luciferase activities were measured after 48 h. Significant transcriptional activity was observed with the p-(−500/+200bp) constructs (Fig. 3B). However, further truncations of the regions at nucleotides −500 to −250, and −250 to +200 (constructs p-(−500/−250), and p-(−250/+200) respectively) gradually increased the transcriptional activity, indicating that nucleotide −250 to +200 contained positive regulatory elements that were essential for basal promoter function.

In addition, our results showed that the expression of E2F1 was associated with a significant increase in p-(−250/+200) promoter activity (Fig. 3C). Accordingly, the promoter activity of the fragment decreased when endogenous E2F1 was inhibited (Fig. 3D). Within this region, two predicted E2F1 consensus binding sites were referred to sites A and B, respectively. Deletion and mutation analysis on the predicted E2F1 consensus binding sites revealed that removal of either site A (p-DB/ p-ΔB) or C (p-DA/ p-ΔA), attenuated p-(−0.25/0.2k) activity (Fig. 3E). Moreover, chromatin immunoprecipitation (ChIP) revealed the existence of direct interaction between E2F1 and the NCAPG promoter *in vitro* (Fig. 3F).

NCAPG inhibited the pyroptosis of HCC cells

To clarify the function of NCAPG in HCC development, we designed siRNAs targeting NCAPG to transfect HCC cell lines which showed an obviously decreased NCAPG expression in HCC cells compared with a negative control (NC; Supplementary Fig. 3A). Interestingly, after QGY-7703 cells were transfected with NCAPG siRNAs, we observed a typical pyroptosis morphology, which was characterized by cell swelling and membrane rupture by scanning electron microscopy (Fig. 4A). Consistently, aggravated DNA cleavage associated with NCAPG silencing was confirmed using terminal deoxynucleotidyl transferase-mediated nick-end labeling staining (Fig. 4B). Furthermore, NCAPG silencing triggered a significant increase in cell death in all four human HCC cell lines compared with that in NC through morphological examination (Fig. 4C). To further verify the findings from the loss-of-function analysis, gain-of-function analysis was further performed in the QGY-7703 and MHCC-97L cell lines. First, a pcDNA3.1-NCAPG (NCAPG) induced a sharply increased expression of NCAPG in QGY-7703 cells (Supplementary Fig. 3B), which encoded the entire coding sequence of NCAPG but lacked the 3′-untranslated region (UTR). In response to serum deprivation, NCAPG-overexpressing cells had obviously decreased cell death rates compared with the control cells (Fig. 4D).

NCAPG downregulated pyroptosis of cells with up-regulated NLRP3

Gasdermin D, the key effector of pyroptosis, is activated by inflammasomes and causes pyroptosis by forming membrane

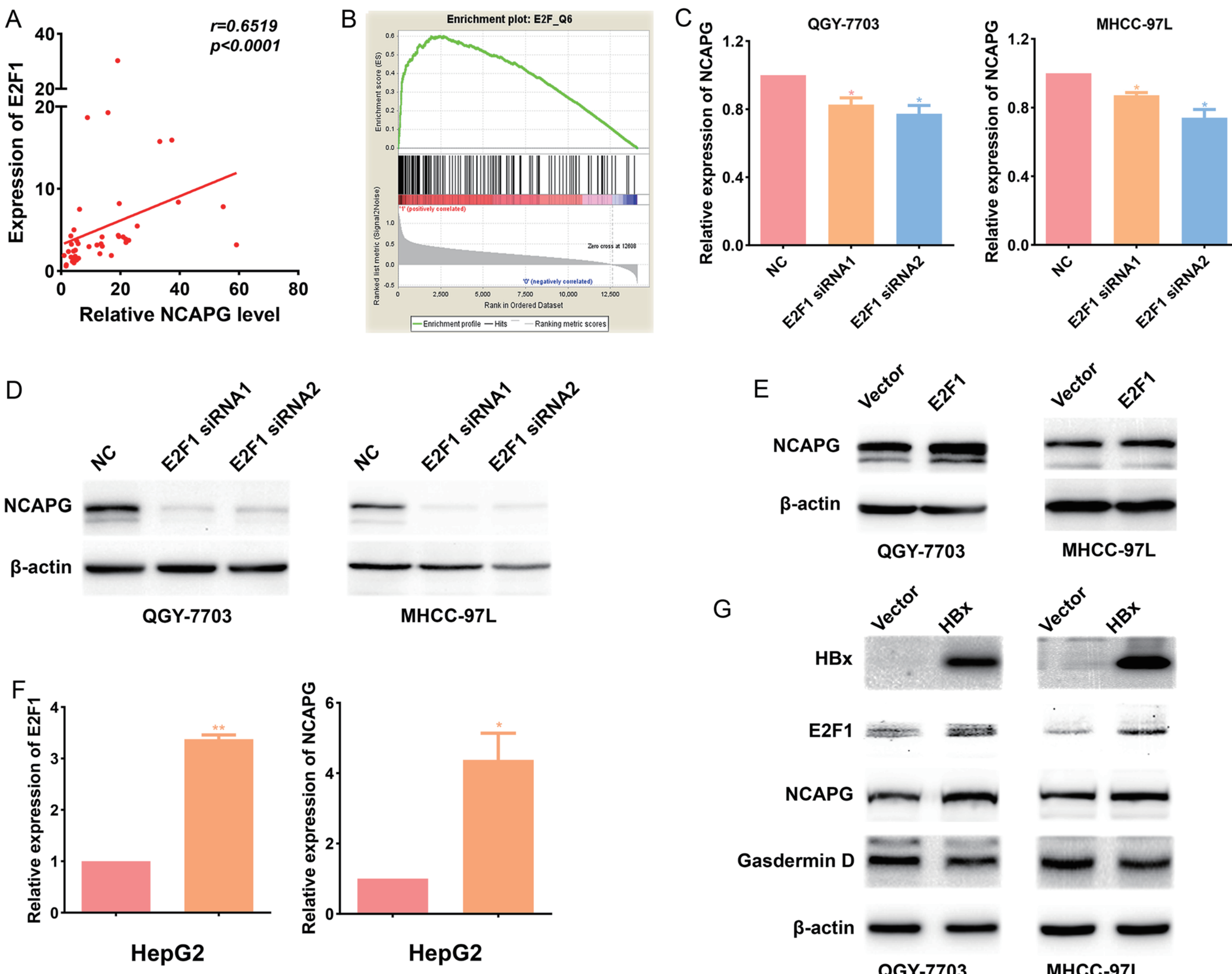


Fig. 2. HBV affected the expression of E2F1 and NCAPG. (A) Relationship between relative mRNA expression of NCAPG and E2F1 in tumor tissues of 44 HCC patients with Hepatitis B virus. (B) GSEA analysis showed that NCAPG was positively correlated with E2F1. (C, D) Inhibiting the expression of endogenous E2F1 reduces mRNA and protein levels of NCAPG. After QGY-7703 and MHCC-97L were transfected with NC or E2F1 siRNAs, the expression of NCAPG detected by qPCR (C) and western blotting (D). NC-transfected cells were set to 1. $^{*}p < 0.05$. (E) Overexpression of E2F1 up-regulated NCAPG expression. After inoculation and 24 h culture, QGY-7703 and MHCC-97L were transfected with pRP [Exp]-EGFP/Puro-CAG or pRP [Exp]-EGFP/Puro-CAG-E2F1 for 48h, then immunoblotting was used to detect the expression of NCAPG. (F) HBV up-regulated the expression of E2F1 and NCAPG. The serum of chronic hepatitis B patients was collected when HBV viral load greater than 10^8. HepG2 cells were treated with the serum. Total RNA of HepG2 cells was collected for 1 day and 3 days respectively. The expression of E2F1 and NCAPG was detected by qPCR. On the first day, the cell expression was set to 1 and the endogenous control was β-actin. (G) After HBx overexpression, E2F1 and NCAPG were up-regulated, while gasdermin D was downregulated. QGY-7703 and MHCC-97L cells were transfected with HBx overexpression plasmid or empty plasmid for 48 hours. Western blot was used to detect the expression of HBx, E2F1, NCAPG, and gasdermin D. HBV, Hepatitis B virus; HBx, hepatitis B virus x protein; qPCR, quantitative real-time; GSEA, Gene set enrichment analysis; E2F1, E2F Transcription Factor 1; NCAPG, Non-SMC Condensin I Complex Subunit G; HCC, hepatocellular carcinoma; GSEA, Gene set enrichment analysis; qPCR, quantitative real-time; NC, negative control.

pores. Firstly, pro-inflammatory mediators, like pro-IL1β and NLRP3, are transcriptionally generated. Then, the inflammasome is assembled and caspase-1 is activated. Activated caspase-1 proteolytically converts pro-IL1β into its active form and induces pyroptosis partially through cleavage of gasdermin D. Pyroptosis features rapid plasma membrane rupture resulting in the enhanced release of pro-inflammatory mediators and alarmins, including IL1β and HMGB1. NCAPG silencing significantly enhanced the expression of gasdermin D, ASC, NLRP3, cleaved-caspase-1, cleaved-IL1β, and HMGB1 proteins (Fig. 5A). Meanwhile, ELISA found that NCAPG siRNAs greatly increased the secret amounts of cleaved-IL-1β and HMGB1 into cell supernatants (Fig. 5B). On the contrary, overexpression of NCAPG inhibited the protein expression of gasdermin D and NLRP3, the key protein of pyroptosis and inflammasomes (Supplementary Fig. 3C). In brief, we used gain-and loss-of-function approaches to demonstrate the inhibitive actions of NCAPG on pyroptosis in HCC.

To validate the results of *in vitro* analyses, we first established stable Bel-7402 lines by infecting lentivirus-expressing control shRNA (nontarget scramble shRNA, scramble) or small hairpin RNA (shRNA) targeting the human NCAPG gene (shRNA shNCAPG), followed by selection with puromycin. NCAPG RNA expressin, as expected, was significantly decreased by NCAPG shRNA compared with control shRNA (Supplementary Fig. 3D). The cell clone with the highest knockdown efficiency

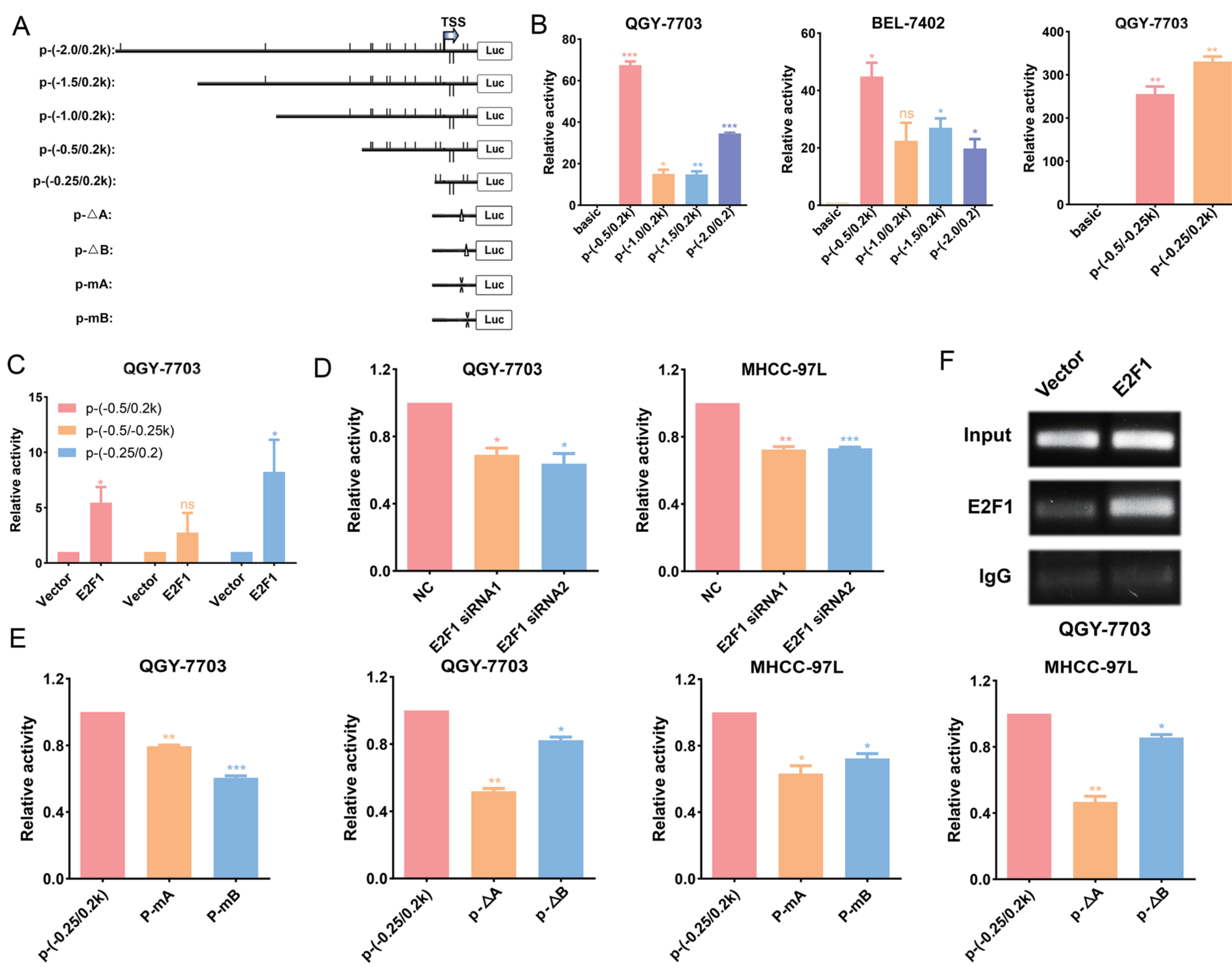


Fig. 3. Identification of the binding sites of E2F1 in NCAPG promoter region. (A) Schematic of the promoter region 2.0 kb upstream of NCAPG. Vertical short lines represent binding sites of E2F1. The triangle indicates the deleted E2F1 binding site. Point mutation of E2F1 binding sites were represented by crosses. These fragments were constructed on the luciferase vector of the firefly. (B) P-(−0.25/0.2kb) has the strongest activity. pGL3-basic; *p*-(−2.0/0.2kb); detailed delineation of the different promoter fragments activity. Cells were transfected with pRL-CMV and pGL3-basic or reporting vectors containing different luciferase promoter fragment lengths. Negative control group (pGL3-Basic and pRL-CMV) was set to 1. (C) The activity of p-(−0.25/0.2kb) increased most significantly, after overexpression of E2F1. The control plasmid or E2F1-expressing plasmid were cotransfected with *p*-(−0.25/0.2kb) and pRL-CMV, *p*-(−0.5/0.2kb) and pRL-CMV or *p*-(−0.5/−0.25kb) and pRL-CMV In QGY-7703 and MHCC-97L cells for 48 h, then we determined the promoter activity of each fragment by detecting the activity of double luciferase. (D) Inhibiting the expression of endogenous E2F1 by E2F1 siRNAs reduced the activity of NCAPG promoter. QGY-7703 and MHCC-97L co-transfection with p-(−0.25/0.2kb) and pRL-CMV for 48h after transfected with E2F1 siRNAs or NC for 24 hours, then detecting the activity of promoter by luciferase assay. (E) Mutation the binding site of E2F1 (p-ΔA, p-ΔB, p-mA, and p-mB) lead to decreased the activity of the promoter. QGY-7703 and MHCC-97L cells were co-transfected with pRL-CMV and p-(−0.25/0.2 kb) (WT) or p-ΔA or p-ΔB or p-mA or p-mB for 48h, followed by luciferase assay. P-ΔA and p-ΔB were deletion mutation of E2F1 binding site GTTGGCGGGCTG (52/64 region) and GCGGGCAG (27/35 region). P-mA and p-mB were point mutation of E2F1 binding site GTTGGCGGGCTG to CAACCGCCCGAC and GCGGGCAG to CGCCCGTC. WT group was set to 1. (F) Chromatin immunoprecipitation Polymerase Chain Reaction (ChIP-PCR) assay indicated the direct binding of E2F1 and NCAPG promoters. QGY-7703 cells were transfected with vector overexpression E2F1 or control vector for 48 hours, then applied to ChIP-PCR. The positive control was the input group, however, the IgG-incubated group was considered as a negative control. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. E2F1, E2F Transcription Factor 1; NCAPG, Non-SMC Condensin I Complex Subunit G; ChIP-PCR, Chromatin immunoprecipitation Polymerase Chain Reaction; NC, negative control.

by shNCAPG was inoculated subcutaneously into nude mice. We then observed a significant suppression of tumor growth in the shNCAPG group compared with the scramble group (Fig 5C–E). And as expected, gasdermin D, and NLRP3 were significantly increased in the shNCAPG tumors, as assessed by immunofluorescence staining and western blotting (Fig. 5F, G).

Discussion

We identified a novel pyroptosis-inhibiting gene, NCAPG, and its transcription factor E2F1. In addition, HBV infection upregulated the expression of NCAPG. Baseline serum NCAPG mRNA levels were significantly higher in HCC patients than in healthy control and high-risk patients. NCAPG was an independent prognostic factor for HCC with higher sensitivity than AFP. Our results are a new insight into the regulatory network of NCAPG, suggesting a critical role of NCAPG in the molecular etiology of HCC, and shedding the light on diagnosis and treatment of HCC.

Classical pyroptosis is mediated by the NLRP3 inflammasome and gasdermin D. NLRP3 inflammasome activation and gasdermin D form pores, causing pyroptosis.[27,28]

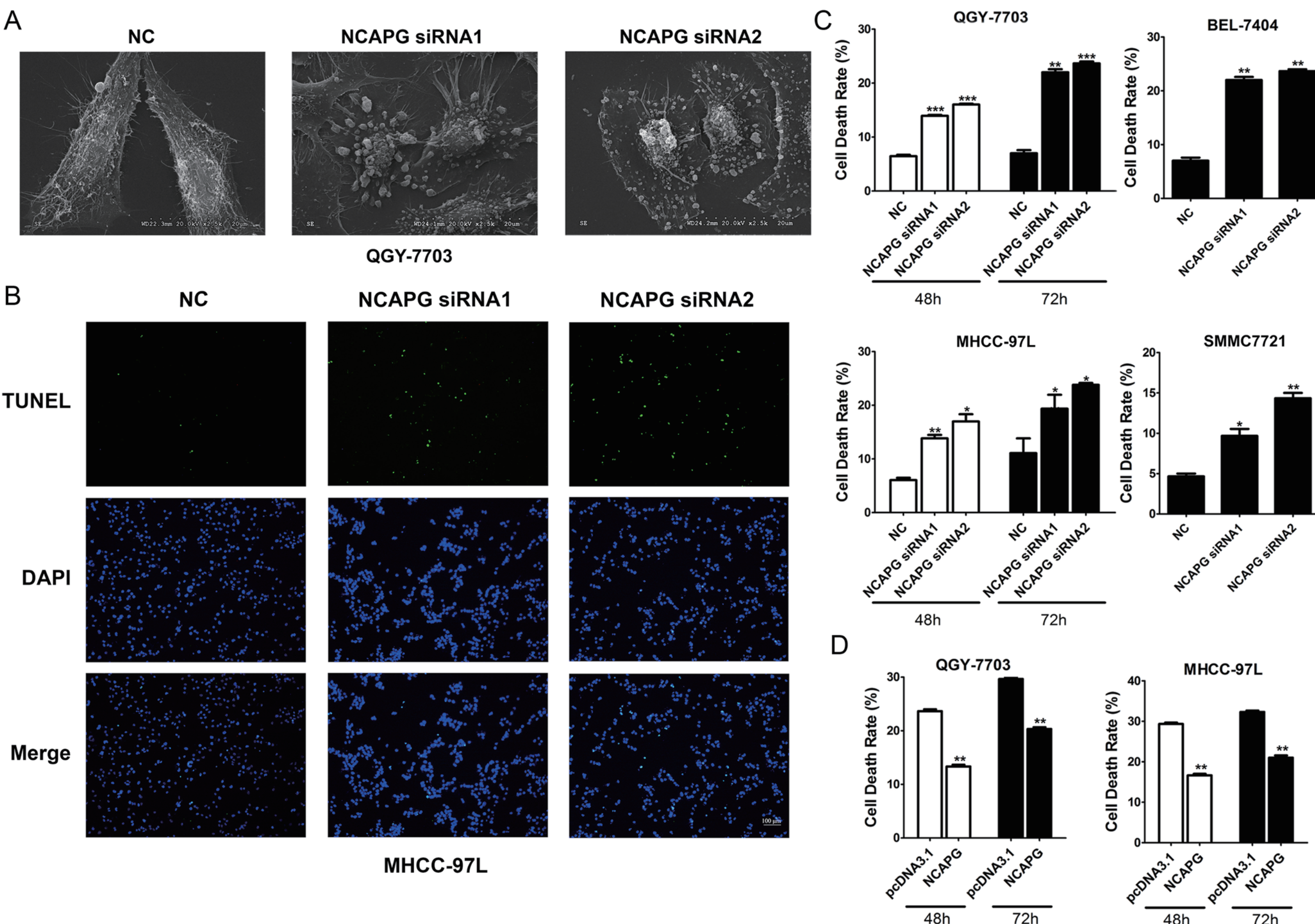


Fig. 4. NCAPG suppressed pyroptosis of HCC cells. (A) High-resolution SEM images exhibited that NCAPG knockdown promoted pyroptosis. Twenty-four hours after transfection with siNCAPG or NC, QGY-7703 cells were deprived of serum for 48 or 72 h. (B) Evaluation of cell death by TUNEL staining assay. Typical micrographs of TUNEL staining in transfected MHCC-97L. MHCC-97L cells were treated with NC or NCAPG siRNAs for 24 hours before serum deprivation for 48 hours, then cells were fixed for TUNEL staining. Scale bar, 100 μm. (C) NCAPG knockdown promoted cell death in HCC cell lines. Twenty-four hours after transfection, QGY-7703, MHCC-97L, BEL-7404, and SMCC7721 cells were deprived of serum for 48 or 72 h. (D) Overexpression of NCAPG decreased cell death rate. Twenty-four hours after transfection with NCAPG plasmid or the empty plasmid (negative control), QGY-7703 and MHCC-97L cells were deprived of serum for 72 h. DAPI staining was used for the analysis of cell death rates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. HCC, hepatocellular carcinoma; NCAPG, Non-SMC Condensin I Complex Subunit G; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling; NC, negative control.

Hepatocyte pyroptosis induced by NLRP3 inflammasome activation causes not only liver inflammation, but also results in liver fibrosis.[29,30] In HCC, expression of all the NLRP3 inflammasome components, including NLRP3, ASC, caspase-1 and IL1β are significantly downregulated compared to normal liver, and their down-regulation is inversely associated with advanced clinical stage and poor pathological differentiation.[14] However, the role of NLRP3 inflammasome-mediated pyroptosis in HCC development and progression remains controversial owing to inconsistent findings. In our study, scanning electron microscope showed that knockdown of NCAPG in HCC led to typical pyroptosis morphology with cell swelling and membrane rupture. In addition, silencing of NCAPG not only elevated the expression of the expression of gasdermin D and the components of NLRP3 inflammasome such as NLRP3 and ASC in HCC cells but also enhanced IL1β and HMGB1 levels in cell supernatants. *In vivo* experiments shows that gasdermin D and NLRP3 were significantly increased in the shNCAPG tumors through the mouse xenograft model. All the results suggest that NCAPG was closely associated with the expression and activation of NLRP3 inflammasome-gasdermin D-associated pyroptosis.

The activation of the NLRP3 inflammasomes is tightly controlled by a two-step process.[31] The first step requires PAMPs or DAMPs to activate pattern recognition receptors and subsequently promote the transcription of NLRP3 inflammasomes. In the second step, NLRP3 inflammasome assembly results in cleavage of pro-IL1β into active soluble IL1β and triggers pyroptosis. It is reported that NLRP3 expression is driven by NF-κB in the cultured hepatocytes.[32] Consistent with that study, our results indicate that NCAPG regulated NLRP3 mRNA through the NF-κB signaling pathway (data not shown). It is well known that nuclear DNA damage induced by ultraviolet B (UVB) irradiation triggers NLRP3 inflammasome activation.[33,34] To date, much effort has focused on exploring the activation of the inflammasome by DAMPs and not PAMPs.[34,35] DNA fragments could act as a kind of DAMPs to activate the NLRP3 inflammasome. TUNEL assays found that silencing of NCAPG caused DNA breaks and demonstrated that inhibition of NCAPG led to activation of the NLRP3 inflammasome. This suggests that DNA damage by NCAPG silencing may be another key mechanism to

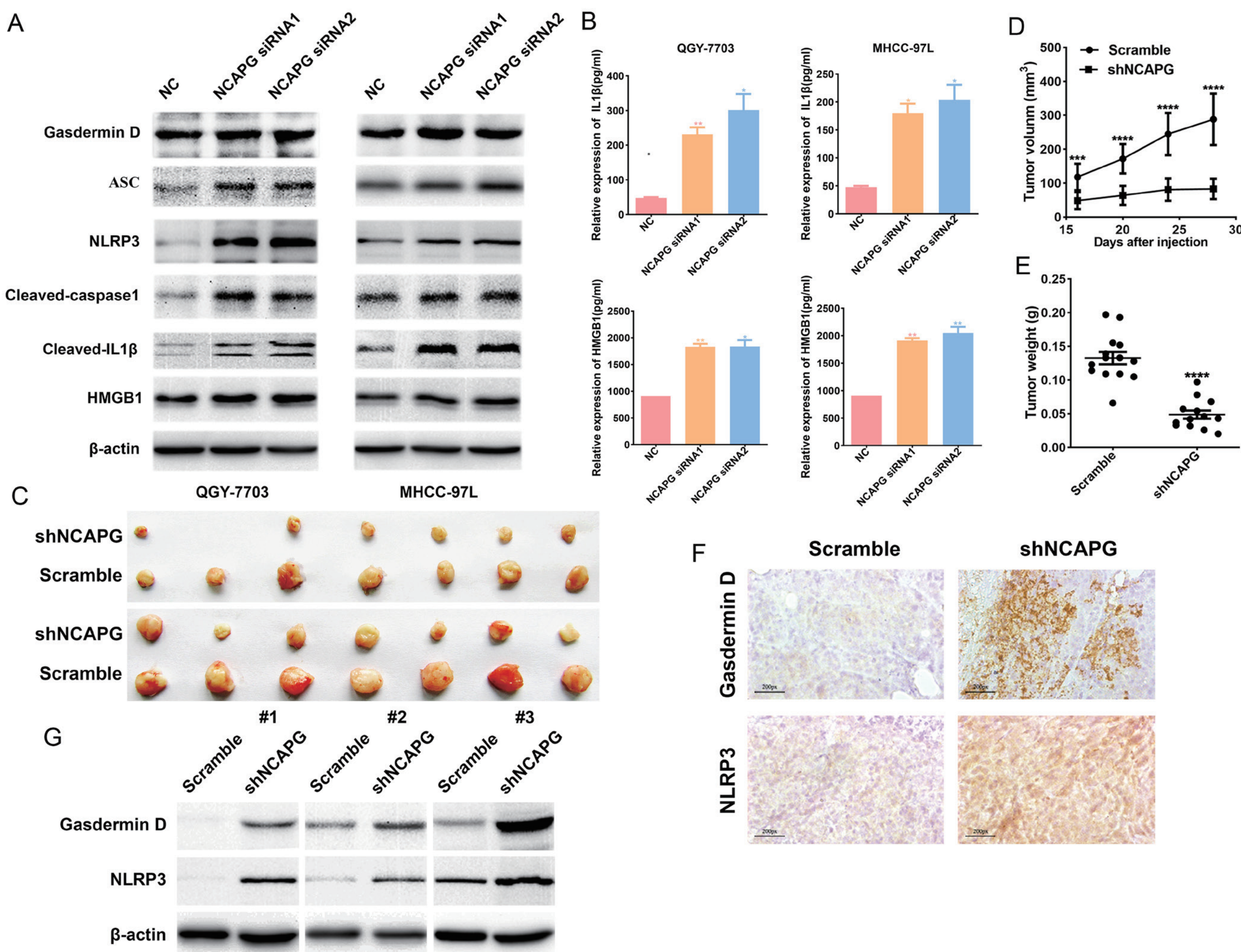


Fig. 5. NCAPG regulated the NLRP3 inflammasome-mediated pyroptosis. (A) NCAPG silencing increased the endogenous levels of gasdermin D, ASC, NLRP3, cleaved-caspase-1, cleaved-IL1β and HMGB1 in QGY-7703 and MHCC-97L cells. Forty-eight hours after transfection, endogenous protein levels were examined by western blotting. β-actin was an internal reference. After QGY-7703 and MHCC-97 L transfected with control or NCAPG siRNAs 24 hours, serum starvation for 48 h. (B) NCAPG silencing increased the levels of cleaved-IL1β and HMGB1 in cell supernatant. 48 h after transfection, ELISA was used to detect the proteins. (C, D, E, F, G) Bel-7402 cells infected with scramble or shNCAPG were subcutaneously inoculated in B-NDG mice (n = 14). Tumors were removed from the sacrificed mice after inoculation for 28 days. The size (C) and weight (D) of each paired tumor were compared. Weight was presented as means ± SEM. (E) Proliferation curves of infected BEL-7402 cells in B-NDG mice. (F) IHC and (G) western blot analysis of gasdermin D and NLRP3 expression xenograft tumors in B-NDG mice. Scale bar, 200 μm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p < 0.0001$. IHC, Immunohistochemistry; ELISA, Enzyme-linked immunosorbent assay; HMGB1; High Mobility Group Protein 1; NCAPG, Non-SMC Condensin I Complex Subunit G; NLRP3, NLR Family Pyrin Domain Containing 3; ASC, apoptosis-associated speck-like protein containing CARD; HMGB1, High Mobility Group Protein 1.

enable NLRP3 inflammasome activation. In brief, we found that NCAPG activated NLRP3 inflammasome-gasdermin D-mediated pyroptosis through increased activation of NLRP3 inflammasomes. Although NCAPG has been identified as a new therapeutic target for HCC by genome-wide by CRISPR cell growth screening,[18] much of the effort has been dedicated to identifying the effect of NCAPG on cell growth and migration rather than understanding the regulation of NCAPG. We identified E2F1, a crucial transcription factor that regulates the cell cycle, to be a transactivator of NCAPG transcription. Firstly, E2F1 and NCAPG were positively correlated with each other in HCC tissue samples, and E2F1 knockdown *in vitro* significantly reduced endogenous NCAPG expression. Secondly, two closely adjacent E2F1 consensus binding sites were located close to the TSS of the NCAPG gene, and either deletion or mutation of these sites caused a dramatic decrease in NCAPG promoter activity. Thirdly, E2F1 directly interacted with the NCAPG promoter *in vitro*.

Chronic HBV infection is a major risk factor of HCC.[36] It is of note that either HBV infection or HBx was shown upregulate the expression of NCAPG. E2F1 acts to regulate the cell cycle.[37] HBx and E2F1 also cooperatively work to promote reinitiation of DNA replication[38] or aberrant DNA methylation.[39] Our data reveal that HBx overexpression not only induced significant up-regulation of NCAPG but also increased E2F1 expression. Interestingly, we observed that HBx suppressed gasdermin D levels, suggesting that HBx may promote HCC development by regulating pyroptosis.

We found that up-regulation of NCAPG was a frequent event in HCC tissues and that high levels were associated with poor overall survival. NCAPG silencing dramatically suppressed the tumorigenicity of HCC cells. Recently, transient

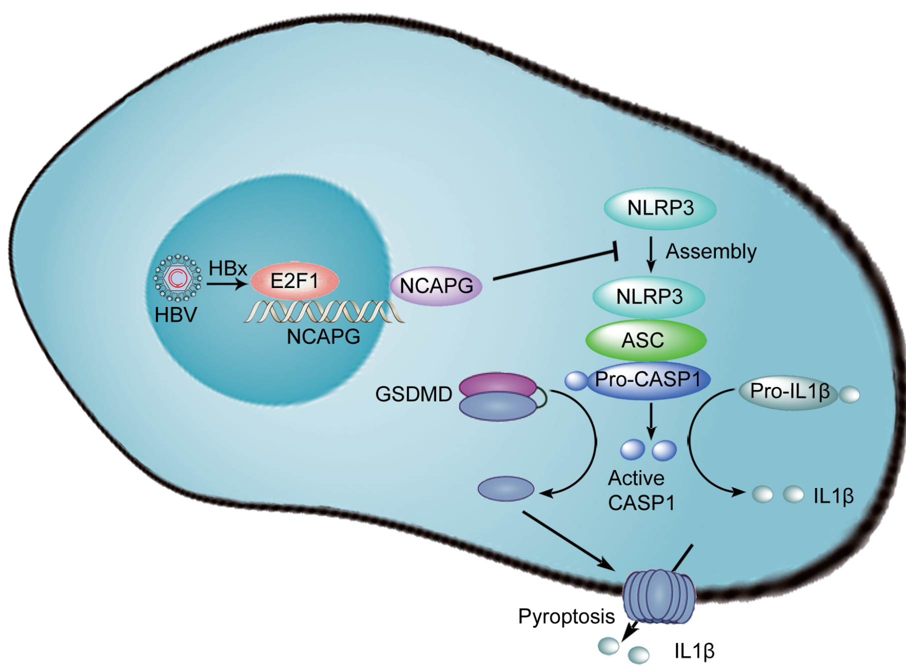


Fig. 6. Diagram of NCAPG-mediated pyroptosis in HCC. E2F1 inhibits NLRP3-mediated apoptosis of HCC by up-regulating NCAPG expression, which promotes the occurrence and development of HCC. High expression of NCAPG in the serum of patients with HCC has diagnostic value for HCC. E2F1, E2F Transcription Factor 1; NCAPG, Non-SMC Condensin I Complex Subunit G; NLRP3, NLR Family Pyrin Domain Containing 3; HCC, hepatocellular carcinoma.

inhibition of NCAPG was reported to result in a significant reduction in cell growth, migration, and the down-regulation of mitochondrial gene expression.[16,18,20,21] These studies emphasize the biomedical significance of NCAPG, whose up-regulation may contribute to malignant phenotypes, including uncontrolled proliferation and metastasis. Our findings are the first to show that up-regulation of NCAPG promoted tumorigenesis by decreasing pyroptosis. Activation of pyroptotic cell death pathways may be a novel target for cancer treatment. It has been reported that chemotherapy drugs, such as paclitaxel and cisplatin, induce pyroptosis by identifying endogenous molecules that may be helpful in the targeted treatment of cancer.[40,41]

In conclusion, our novel findings provide a comprehensive view of how NCAPG exerts its functions and how NCAPG gene expression is regulated in HCC. We identified a novel role of NCAPG in the transcriptional regulation of pyroptosis by an upstream transactivator, E2F1. The findings argue that NCAPG, together with gasdermin D, cleaved-caspase-1 and cleaved-IL1β, constitute functional networks that regulate pyroptosis of HCC cells, and E2F1 may further enhance the expression of NCAPG, which in turn may contribute to reduced pyroptosis within HCC, thus aggravate tumorigenesis (Fig. 6). The roles of both E2F1 and NCAPG warrant further study as potential targets in HCC treatment.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception and design (ZH, BH, QZ), performance of the experiments, analysis of the data, writing of the paper and made equal contribution (CX, JG and YJ), and provision of technical support (WL, YT, TL). All authors reviewed and approved the manuscript.

Ethical statement

This study followed the principles of the Declaration of Helsinki, and was approved by the Institute Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (Approval number: [2022]02-297-01) and informed consent was obtained from each HCC patient. The mouse experiments were conducted under the guidelines and approved by the Animal Management Committee of Forevergen

Medical Laboratory Animal Center (Approval number: GFAC-AEWC-003).

Data sharing statement

All datasets used and/or analyzed during the current study are available from the author upon reasonable request.

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