

NFKBIE is a predictive factor of survival and is correlated with immune infiltration and antigen processing and presentation in hepatocellular carcinoma

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Abstract. The important role of the nuclear factor κ B (NF κ B) pathway in tumour development has long been recognized; however, the role of the NF κ B inhibitor family in liver cancer has not been elucidated. Hepatocellular carcinoma (HCC) is a serious public health burden with a high incidence, poor prognosis, and early detection, especially in Asia, where hepatitis is prevalent. In the present study, the mRNA expression level of the NF κ B inhibitor family was assessed in HCC and normal tissues using the Metabolic Gene Rapid Visualizer, University of Alabama at Birmingham Cancer Data Analysis Portal, and the Tumor Immune Estimation Resource database (TIMER). Survival curves of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor (NFKBIE) were obtained using the Kaplan-Meier method. Genes co-expressed with NFKBIE in HCC samples were studied using data from the LinkedOmics and the Hepatocellular Carcinoma Databases. Protein-protein interaction networks, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment pathway analyses were used to assess the NFKBIE mechanism in HCC. Using the TIMER database, the association between immune infiltration

and NFKBIE was determined. RNA-sequencing (RNA-seq) was used to evaluate the function of NFKBIE in HCC and its impact on proliferation and migration. Western blotting was used to confirm the expression of NFKBIE in HCC cell lines. In addition, NFKBIE overexpression in HCC was demonstrated using tissue microarrays encompassing 80 pairs of HCC and normal liver tissues. NFKBIE was the only NF κ B inhibitor with high expression and an improved prognosis in HCC compared with other NF κ B inhibitors. NFKBIE was correlated with clinical characteristics, such as tumour grade, tumour protein P53 mutation status and tumour stage. Data obtained from Gene Set Cancer Analysis suggested that NFKBIE may inhibit the PI3K/AKT, RAS/MAPK, RTK and TSC/mTOR pathways. In addition, NFKBIE was significantly associated with B-cell immune infiltration and the RNA-seq data demonstrated that knockdown of NFKBIE significantly affected 'Antigen processing and presentation' and 'hepatocellular carcinoma' pathways. Immunohistochemistry of microarrays of tissue samples revealed that NFKBIE was overexpressed in several stages of HCC. Finally, inhibition of NFKBIE decreased the proliferation and migration of HCC cells. In conclusion, due to its prognostic value and overexpression in HCC, NFKBIE distinguished itself from other NF κ B inhibitors. As such, it may provide a novel prognostic indicator and immunotherapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common of all tumors worldwide and has the third highest mortality rate of all tumors. The curative effect of surgery, chemotherapy and radiotherapy was not satisfactory (1) and Few patients with advanced stages survive more than five years (2).

HCC is the most common form of liver cancer, accounting for approximately 75-85% of cases worldwide. The etiology

of HCC is multifactorial, with chronic hepatitis B virus (HBV) infection identified as a major risk factor, especially in endemic areas. Non-alcoholic fatty liver disease (NAFLD) has a global prevalence of 25% and is the leading cause of cirrhosis and hepatocellular carcinoma (3). Metabolic disorders, insulin resistance, and lipid deposition also contribute to the progression from NAFLD to HCC cirrhosis (3,4). Alcohol can enhance the risk of liver cancer by inducing direct hepatocyte toxicity, resulting in cellular damage and apoptosis (5), promoting oxidative stress and inflammatory response within the liver (6), interfering with carbon monoxide metabolism, thereby causing DNA damage and dysregulation of repair mechanisms (7) and other ways. Early diagnosis of HCC remains a major challenge, mainly due to the asymptomatic nature of the disease in its initial stages and the lack of specific biomarkers (8,9).

NF κ B is inactive when it binds to inhibitor of NF κ B at rest and is found in the cytoplasm. Activated NF κ B protein enters the nucleus and participates in inflammatory and immune responses (10) and NF κ B inhibitors are involved in the complex regulation of NF κ B (11). However, little is currently known about NF κ B inhibitors in HCC and their carcinogenic mechanism (12).

Deficiency of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor (NFKBIE) leads to hyperproliferation of a specific subset of B1 B-cells and results in increased activation of NF- κ B in these cells upon stimulation by Toll-like receptors. Furthermore, the deficiency of NFKBIE synergizes with mutant MYD88 signaling, thereby enhancing B-cell proliferation *in vitro* (13). NFKBIE mutations can facilitate the progression of chronic lymphocytic leukemia through various mechanisms, including bidirectional communication with the microenvironment and reduced responsiveness to BTK inhibitor therapy (14). However, the role of NFKBIE in HCC and its mechanism have not been reported.

Based on information from multiple open databases, the present study evaluated the NF κ B inhibitor family at multiple levels, including gene expression, prognosis, clinical features, and immune infiltration, to elucidate a new prognostic marker and treatment strategy for HCC.

Materials and methods

The cancer genome atlas (TCGA) database. All RNA expression patterns of NF κ B inhibitors in HCC and pan-cancer were taken from The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) and the Tumour Immune Estimation Resource (TIMER; <https://cistrome.shinyapps.io/timer/>), whose data were obtained from the TCGA database. TIMER (15,16) predicts tumour-infiltrating immune cells using gene expression patterns. In the present study, it was used to assess NFKBIE and immunological infiltration. UALCAN (<https://ualcan.path.uab.edu/index.html>) (17) is a resource that is available for free that can be used to evaluate TCGA gene expression data. In the present study, UALCAN was used to compare the NF κ B inhibitor family and its relationship with clinical and pathological characteristics and its prognostic value.

Kyoto encyclopedia of genes and genomes (KEGG). KEGG (18) is the main public database on pathways. Pathway

analysis of differential protein coding genes was performed using the KEGG database, and the significance of differential gene enrichment in each pathway entry was calculated using the hypergeometric distribution test. The fold change (FC) was set to 1.5, whilst adjusted $P < 0.05$ was statistically significant.

Survival analysis. Kaplan-Meier plots/Liver cancer mRNA-seq/Auto select best cutoff (<http://kmplot.com/analysis/>) were used to estimate HCC survival rates at 7/5/2024 (19).

Metabolic gene rapid visualizer (MERAV). Data from >4,000 microarrays are combined in MERAV (<http://merav.wi.mit.edu/>), enabling reliable cross-sectional comparisons of several tissues and cell lines (20).

The human protein atlas (HPA). NFKBIE protein expression information was obtained from the HPA (<https://www.proteinatlas.org/search/NFKBIE>). The HPA is a database based on proteomics data that contains protein expression in both normal and malignant tissues (21).

LinkedOmics. LinkedOmics (<https://linkedomics.org/>) (22) is a web-based analytical tool for comparing multiomics cancer datasets. This database was used for KEGG analysis and analysis of the TOP50 genes positively and negatively associated with NFKBIE.

STRING. STRING (<https://string-db.org/>, version 12.0) database is a protein interaction network database based on public database and literature information. It gathers several public databases, including UniProt, KEGG, NCBI, and Gene Ontology, to integrate these data and generate a comprehensive protein interaction network database (23-25).

The genomics of drug sensitivity in cancer. The Genomics of Drug Sensitivity in Cancer (GDSC, version 8.5, www.cancerRxgene.org) database currently encompasses data on drug sensitivity from nearly 75,000 experiments, detailing the responses of approximately 700 cancer cell lines to 138 anti-cancer drugs. Graphical representations of the data are provided throughout, along with links to related resources, and all datasets are fully downloadable. GDSC offers a unique resource, comprising an extensive collection of drug sensitivity and genomic datasets, to facilitate the discovery of new therapeutic biomarkers for cancer treatment (26).

The cancer therapeutics response portal (CTRP). CTRP (<https://portals.broadinstitute.org/ctrp>, version 2.1) integrates the intricate relationship between cancer cell line genetics, lineage, and other cellular characteristics with small molecule sensitivity to expedite the discovery of personalized cancer treatments. By seamlessly integrating comprehensive drug sensitivity data with detailed genomic information, CTRP serves as a robust resource for gaining profound insights into the complex interplay between cancer genetics and drug responses (27-29).

Hepatocellular carcinoma database (HCCDB). The gene expression data of ~4,000 clinical samples are contained in the HCCDB (version, <http://lifeome.net/database/hccdb/about>).

html), which is a database that provides accurate and reproducible differential expression profiles for HCC (30). The present study used this database to look at NFKBIE gene networks co-expressed with HCC as well as with normal tissues.

Gene set cancer analysis (GSCALite). GSCALite (<http://bioinfo.life.hust.edu.cn/web/GSCALite/>) is web server for dynamic analysis and visualization of cancer gene sets and drug sensitivity correlations that will be of wide practical value to cancer researchers. The present study used this database to analyze the signaling pathways of NFKBIE effects and studies related to drug resistance (31).

Tissue microarray and immunohistochemistry (IHC). An HCC tissue microarray (cat. no. HLivH160CS02) consisting of 80 matched tumours and normal samples was purchased from Shanghai Outdo Biotech Co., Ltd. Following the dewaxing of paraffin-embedded sections, antigens were repaired (20X Tris-EDTA antigen repair solution; Wuhan Servicebio Technology Co., Ltd.; cat. no. G1203), and endogenous peroxidase activity was quenched (3% hydrogen peroxide solution, incubated at room temperature for 25 min away from light). Subsequent steps included 3% BSA (Wuhan Servicebio Technology Co., Ltd.; cat. no. GC305010) at room temperature for 30 min blocking to prevent non-specific binding, incubation with the primary overnight at 4°C and (NFKBIE polyclonal antibodies 1:200; cat. no. 11273-1-AP; Proteintech Group, Inc.) secondary antibodies at room temperature (Wuhan Servicebio Technology Co., Ltd.; cat. no. GB23303; 1:500) and development of the signal using DAB chromogen (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1212). Finally, hematoxylin stained nucleus was used for comparison. The prepared slides were then digitized using a Panoramic MIDI scanner (3DHISTECH), ensuring high-resolution imaging of the histological details.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from cell samples with a total of 3×10^5 cells using the RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. RNA purity and quantity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Complementary DNA (cDNA) was synthesized from the isolated RNA using the PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.), followed by cDNA amplification with the SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.). The amplification reactions were conducted on an ABI StepOne Plus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). NFKBIE-Forward primer sequence: TCTGGCATTGAGTCTCTGCG; NFKBIE-Reversed primer sequence: AGGAGC CATAGGTGGAATCAG. GAPDH-Forward primer sequence: GAGAAGTATGACAACAGCCTCAA. GAPDH-Reversed primer sequence: GCCATCACGCCACAGTTT. The $2^{-\Delta\Delta C_q}$ method was used for quantitative analysis (32). The PCR cycling conditions were set as follows: initial denaturation at 94-95°C for 1-3 min; 25-35 cycles of denaturation at 94°C to 95°C for 15-30 sec, annealing at 4-5°C below the primer's melting temperature (~50-65°C) for 20-40 sec and extension at 72°C for ~1 min per 1 kb of target DNA. This was followed by a final extension at 72°C for 5-10 min, and the samples were

then held at 4°C. All protocols for RNA extraction, cDNA synthesis, and qPCR were conducted strictly following the manufacturers' guidelines. These experiments were replicated three times to ensure reliability and reproducibility of the results.

Cell culture and transfection. HEP-3B and PLC/PRF/5 cells were purchased from the American Type Culture Collection. Huh-7 cells were purchased from JCRB Cell Bank. HCCLM3 and MHCC97H cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences; Shanghai, China). All cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) or RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% (Bioind) and 1% ampicillin (Biosharp Life Sciences) at 37°C in a 5% CO₂ incubator.

Short-interfering (si)RNA technology was used to knock-down NFKBIE. The sequences of the siRNAs [General Biol (Anhui) Co., Ltd.] used were as follows: NFKBIE scrambled siControl: 5'-UUCUCCGAACGUGUCACGUTT-3' and 3'-ACGUGACACGUUCGGAGAATT-5'; NFKBIE siRNA-1: 5'-UCAAGGAACCACAGGAGAATT-3' and 3'-UUCUCCUGUGGUUCCUUGATT-5'; NFKBIE siRNA-2: 5'-GGA CCGGCAUGGUGACACATT-3' and 3'-UGUGUCACCAUG CCGGUCCTT-5'; and NFKBIE siRNA-3: 5'-GGAAACUGC UGCUGUGUACTT-3' and 3'-GUACACAGCAGCAGUUUC CTT-5'. Briefly, HCCLM3 and Huh-7 were first digested with Trypsin-EDTA Solution (Biosharp Life Sciences; cat. no. BL512A), then $\sim 2.5 \times 10^5$ cells were counted in each 6-well plate and, after the cells were attached to the wall, pre-configured NFKBIE-siControl (100 nM), NFKBIE-si1 (100 nM), and NFKBIE-si2 (100 nM), were added into the corresponding 6-well plates for 8 h at 37°C. The co-transfection reagent used was Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.). The culture medium was changed 8 h after transfection, and the NFKBIE protein level was measured by western blotting after 48 h. HCCLM3 and Huh-7 cells were transfected with the method and the NFKBIE siRNA knockdown effect was verified by western blotting, and then RNA-sequencing (RNA-seq) was performed by Shanghai OE Biotech Co., Ltd. (Shanghai, China). Wound healing and migration experiments were performed on HCCLM3 and Huh-7 cells after NFKBIE knockdown.

Western blotting. The same number of cells (1×10^6) were treated with cell lysate (Biosharp Life Sciences), the sample buffer (Bio-Rad 2X Laemmli Sample Buffer; Bio-Rad Laboratories, Inc.; cat. no. 1610737) was added and the proteins were denatured and stored at -80°C. The 5×10^5 cells yielded 100 μ l protein solution, 10 μ l per well. The proteins were separated on 10% SDS-PAGE gel and transferred onto a PVDF membrane (MilliporeSigma; 0.45 μ m). The membrane was blocked in skimmed milk at room temperature for 30-60 min and incubated with the primary antibody followed by the second antibody (OriGene Technologies, Inc.; cat. no. ZF-0516; 1:5,000) for 40-60 min at room temperature. ECL developer (Beyotime Institute of Biotechnology; cat. no. P0018FS) was used. In this study, protein concentration was determined using the BL367A assay kit provided by Biosharp Life Sciences. Initially, BCA Reagent A and BCA Reagent B

were mixed in specified ratios to prepare the working solution. Protein samples and a series of BSA standards with known concentrations were then prepared and added to a 96-well plate. The BCA working solution was added to each well, and the plate was incubated at room temperature for 30 min to 2 h. Following incubation, absorbance at 562 nm was measured using a spectrophotometer. Protein concentrations were calculated by comparing these measurements against a standard curve. ImageJ software (National Institutes of Health, version 1.48) was used to calculate grayscale values. NFKBIE polyclonal antibodies (1:1,000; cat no. 11273-1-AP; Proteintech Group, Inc.) and GAPDH monoclonal antibodies (1:10,000; cat no. 60004-1-Ig; Proteintech Group, Inc.) were used as primary antibodies.

Wound healing assays. A total of 3×10^5 HCCLM3/Huh-7 cells were initially cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO₂ atmosphere until reaching 90% confluence. A sterile 200 μ l pipette tip was employed to create a consistent scratch across the cell monolayer at near full confluence, focusing on maintaining uniform scratch width. After scratching, cells were washed with phosphate-buffered saline (PBS) to remove dislodged cells and debris, followed by the replacement of the culture medium with DMEM containing 1% FBS, to minimize proliferation and concentrate on migration. Photographic documentation was conducted immediately post-scratch (0 h) and at specified intervals (48 h), utilizing an inverted microscope with consistent magnification and focus. The extent of wound closure was assessed by measuring scratch width changes using ImageJ software (National Institutes of Health, version 1.48). The assay was replicated a minimum of three times to ensure data reliability. Statistical differences between groups were analysed using Student's t-test or one-way ANOVA with Tukey's multiple comparisons test, to determine the impact of the experimental conditions on the cells' migratory capabilities.

Migration experiment. The complete medium was prepared using DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biocind) and 1% penicillin-streptomycin (Biosharp). For both the experimental and control groups, cells were digested with trypsin and counted to achieve a concentration of 2×10^4 cells per 200 μ l. These cells were then seeded into culture chambers (Falcon) with serum-free medium inside the chambers and complete medium outside. The cells were incubated at 4°C in an atmosphere of 5% CO₂ for 48 h. After incubation, cells were fixed with 4% paraformaldehyde (Biosharp) and stained with crystal violet. Subsequently, the upper layer of cells in the chamber was gently wiped away, and the cells were observed and photographed under an inverted microscope. This experiment was repeated at least three times. Cell counting was performed using Image J, and data analysis was conducted using GraphPad Prism 8.0 software. Statistical significance between the experimental and control groups was assessed using t-tests or analysis of variance (ANOVA).

RNA-seq and differentially expressed genes (DEGs) analysis. The libraries were sequenced (TruSeq Stranded mRNA LTSample Prep Kit, Illumina, RS-122-2101; Agencourt

AMPure XP, BECKMAN COULTER, A63881; Qubit RNA Assay Kit, Life Technologies, Q32852; Qubit dsDNA Assay Kit, Life Technologies, Q328520; Bioanalyzer 2100 RNA-6000 Nano Kit, Agilent, 5067-1511; Bioanalyzer 2100 DNA-1000 Kit Agilent, 5067-1504; SuperScript II Reverse Transcriptase, Invitrogen, 18064014;) on an Illumina HiSeq X Ten platform (cat. no. DOE20221816; Illumina, Inc.) and 150 bp paired-end reads were generated. The Agilent 2100 bioanalyzer was used to measure the length and quality of the library. The base error rate in Illumina sequencing was determined by the Phred score, which was calculated by the model to predict the probability of error in base discrimination. Raw data (raw reads) in fastq format were firstly processed using Trimmomatic (version 0.36) (33) and the low-quality reads were removed to obtain the clean reads. Subsequently, ~14,000 clean reads for each sample were retained for analysis. The clean reads were mapped to the human genome (GRCh38) using HISAT2 (version 2.2.1.0) (34). The fragments per kilobase of transcript per million mapped reads (35) of each gene was calculated using Cufflinks (version 2.2.1) (36), and the read counts of each gene were obtained by HTSeq count (version 0.9.1) (37). The concentration of RNA samples can be quickly measured using NanoDrop spectrophotometers and the purity of RNA can be assessed by absorption ratios (A260/A280 and A260/A230). An A260/A280 ratio close to 2.0 usually indicates less protein contamination, while an A260/A230 ratio greater than 2.0 indicates less pollution from organic solvents or other impurities.

Differential expression analysis was performed using the DESeq (version 1.18.0) R package (38). $P < 0.05$ and $FC > 1.5$ or < 0.5 were set as the threshold for significantly differential expression. Hierarchical cluster analysis of DEGs was performed to demonstrate the expression pattern of genes in different groups and samples. Gene Ontology (<http://geneontology.org>) (39) enrichment and KEGG (18) pathway enrichment analysis of DEGs were performed respectively using R (version 4.3.3, Comprehensive R Archive Network) based on the hypergeometric distribution.

Statistical analysis. Kaplan-Meier plots were used to establish survival curves. Correlations between gene expression levels were assessed using Spearman's correlation coefficient. An unpaired Student's t test was used to evaluate the statistical significance of differences between two independent groups. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to assess the statistical significance between three independent groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Transcriptional levels of the NF κ B inhibitor family in HCC and across cancers. A total of five NF κ B inhibitors were identified and their transcript levels were compared at the pan-cancer level: NFKBIB, NFKBID and NFKBIE were significantly overexpressed in HCC tissue compared with normal tissue, and NFKBIA and NFKBIZ expression was not significantly different between HCC and normal tissues (Figs. 1 and S1).

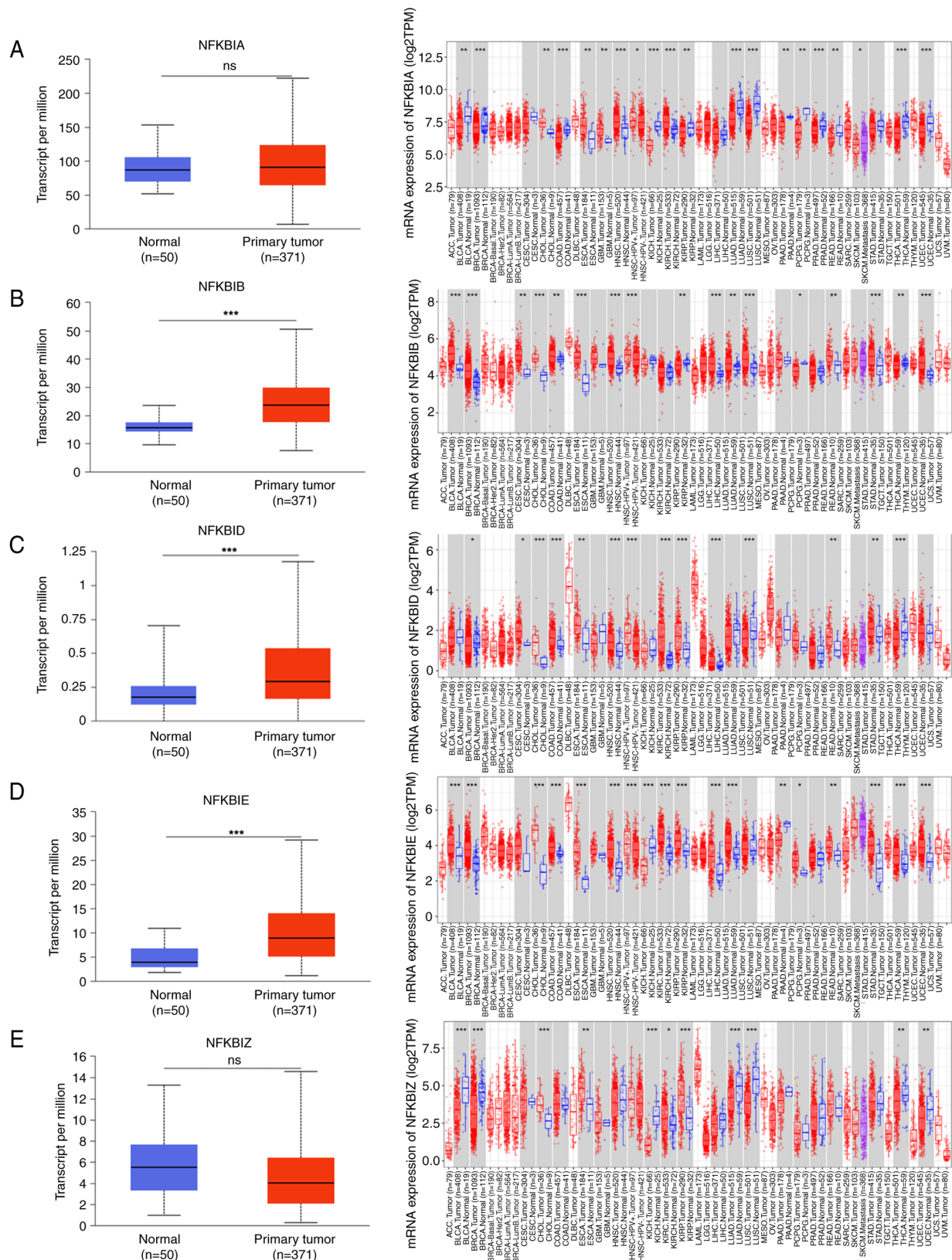


Figure 1. mRNA expression of the NFκB inhibitor family. Transcriptional levels of (A) NFKBIA, (B) NFKBIB inhibitors, (C) NFKBID, (D) NFKBIE and (E) NFKBIZ inhibitors in normal and hepatocellular carcinoma tissue samples. *P<0.05; **P<0.01; ***P<0.001. NFκB, nuclear factor κ B; NFKBI, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor; TPM, transcript per million; ns, not significant.

Correlation between NFκB inhibitor expression and prognosis. The predictive ability of NFκB inhibitors for HCC were analysed using Kaplan-Meier plots. According

to the results, there was no significant association between NFKBIA, NFKBIB, NFKBID and NFKBIZ expression and prognosis (Fig. 2A). However, a low expression of NFKBIE

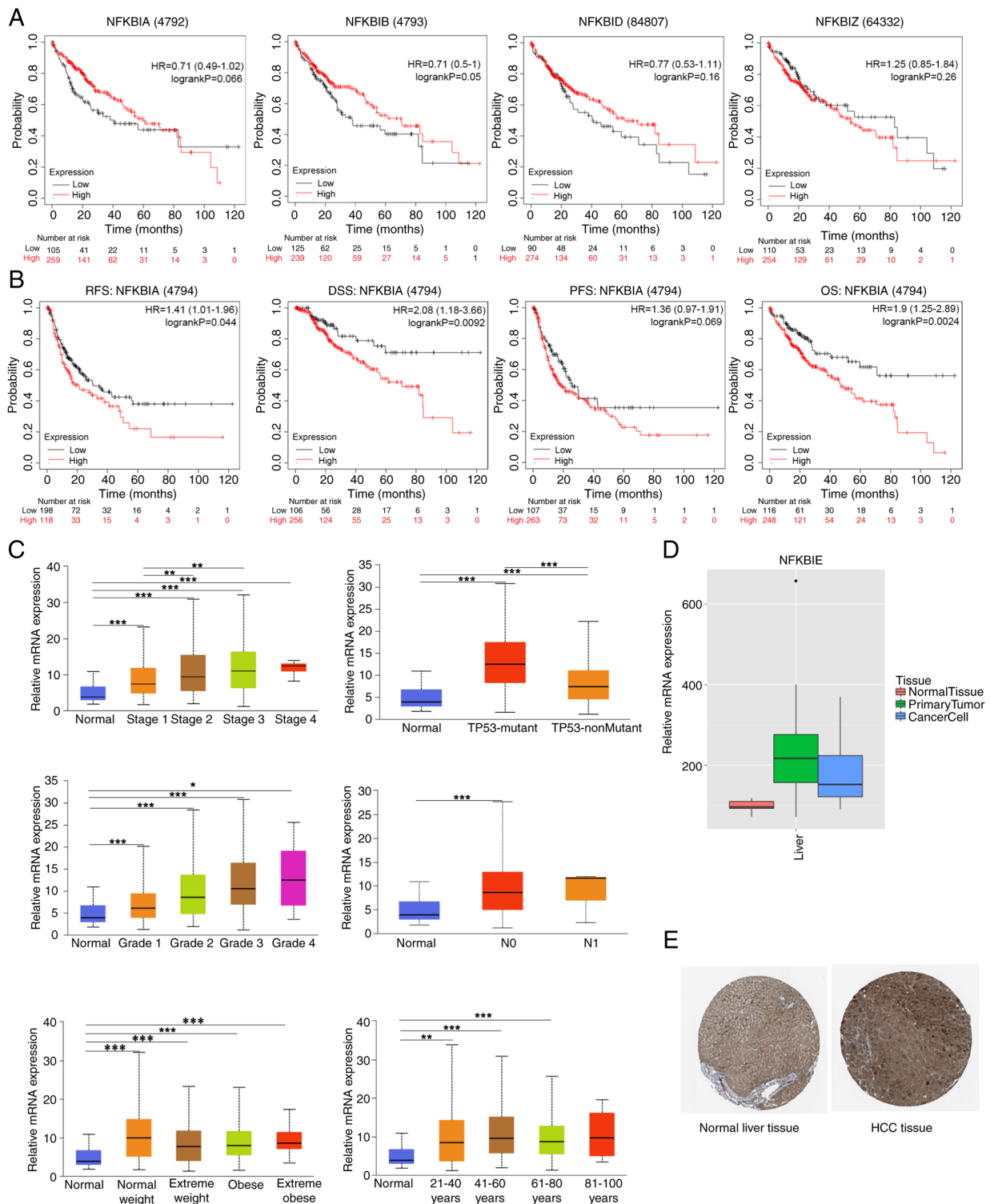


Figure 2. Low mRNA expression of NFKBIE in HCC is associated with a favourable prognosis compared with high expression. (A) Prognostic value of NFKBIA, NFKBIB, NFKBID and NFKBIZ in patients with HCC at the mRNA level. (B) Survival curve of NFKBIE in HCC (overall survival, disease specific survival, relapse-free survival, and progression-free survival). (C) NFKBIE mRNA expression is positively correlated with tumour grade, TP53 mutation status, tumour stage, lymph node metastatic status, patient weight, and age. (D) NFKBIE is overexpressed in HCC, according to data from the Metabolic Gene Rapid Visualizer. (E) NFKBIE expression in HCC and normal liver tissue was analysed using data from the Human Protein Atlas database. NFKBIE expression was higher in HCC than in normal liver tissue. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor; HCC, hepatocellular carcinoma; TP53, tumour protein P53; LIHC, liver HCC; TCGA, The Cancer Genome Atlas; HR, hazard ratio.

was significantly associated with a better prognosis for patients with HCC, regardless of overall survival, disease specific survival and relapse-free survival, compared with a high expression (Fig. 2B). Significant correlations were

demonstrated between the RNA expression level of NFKBIE and several clinical characteristics, including the stage of cancer and tumour protein P53 (TP53) mutation status, cancer stages, tumour grade, nodal metastasis status, patient's weight,

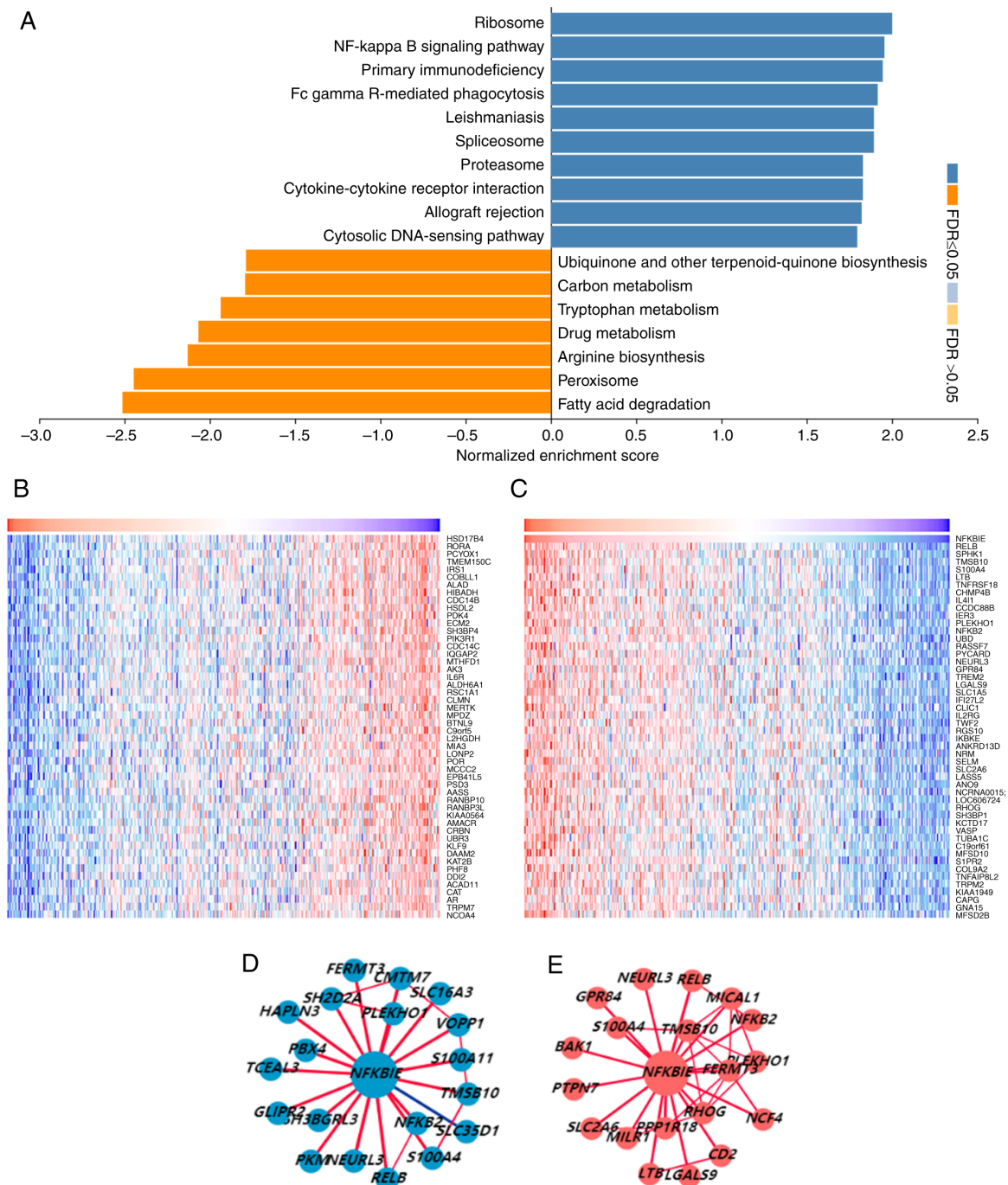


Figure 3. KEGG pathway analysis and positive and negative related genes analysis of NFKBIE. (A) Positively and (B) negatively associated genes of NFKBIE. (C) KEGG enrichment analysis using the LinkedOmics database. Network of genes co-expressed with NFKBIE in (D) hepatocellular carcinoma and (E) nearby liver tissue. KEGG, Kyoto Encyclopedia of Genes and Genomes; NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ϵ ; FDR, false discovery rate.

and patient's age (Fig. 2C). Data from MERAV and the HPA database also demonstrated that HCC had significantly higher transcriptional and protein levels of NFKBIE than normal tissue samples (Fig. 2D and E).

KEGG analyses and co-expressed genes. The 50 genes with the strongest associations were mainly enriched in the 'Ribosome', 'NF κ B signalling pathway' and 'Primary immunodeficiency' (Fig. 3A). Heatmaps were created to demonstrate all 50 genes that exhibited the strongest associations with

NFKBIE using the LinkedOmics database (Fig. 3B and C). Furthermore, the HCCDB indicated notable differences in the co-expression gene network between HCC and normal tissue (Fig. 3D and E).

Drug sensitivity, cancer pathway activity and the protein-protein interaction network. V-rel avian reticuloendotheliosis viral oncogene homolog A (RELA), v-rel avian reticuloendotheliosis viral oncogene homolog (REL), conserved helix-loop-helix ubiquitous kinase (CHUK),

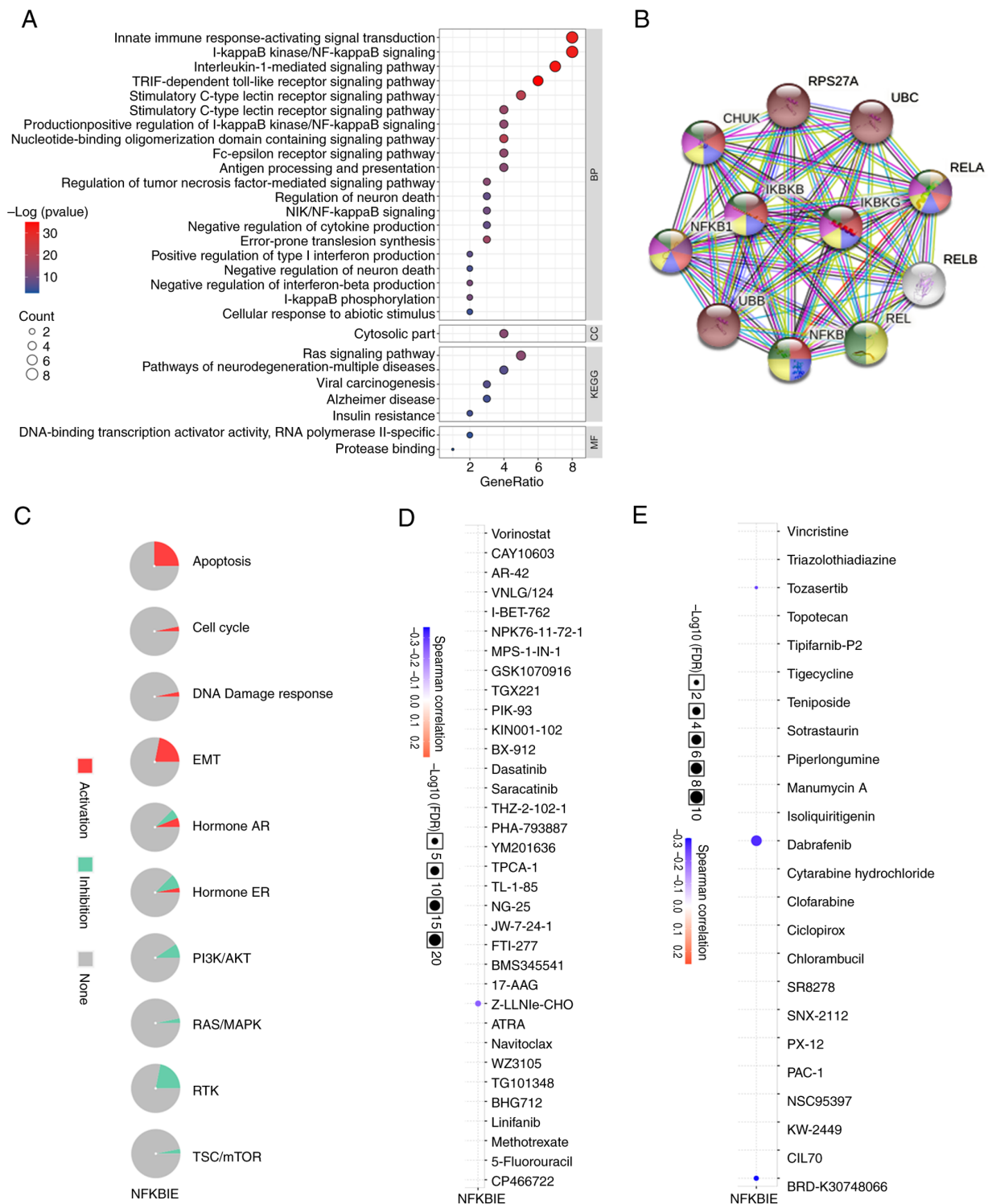


Figure 4. Drug sensitivity, cancer pathway activity and PPI network of NFKBIE. (A) Analyses of neighbouring genes of NFKBIE using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. (B) PPI of NFKBIE. (C) Effects of NFKBIE on several cell signalling pathways based on Gene Set Cancer Analysis. NFKBIE-related drug sensitivity analysis using data from the (D) GDSC database and (E) Cancer Therapeutics Response Portal databases. PPI, protein-protein interaction; NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ϵ ; GDSC, Genomics of Drug Sensitivity in Cancer.

inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKBKG), inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB), and v-rel avian reticuloendotheliosis viral oncogene homolog B (RELB) were mainly enriched in 'Shigellosis', 'Kaposi sarcoma-associated herpesvirus infection', 'C-type lectin receptor signaling

pathway', 'NF κ B signaling pathway', and 'antifolate resistance' (Fig. 4A). These proteins also had the strongest interactions with NFKBIE protein (Fig. 4B). In addition, data from Gene Set Cancer Analysis (GSCALite) indicated that NFKBIE suppression notably reduced the PI3K/AKT, TSC/mTOR, and other signaling pathways (Fig. 4C). Moreover, a drug

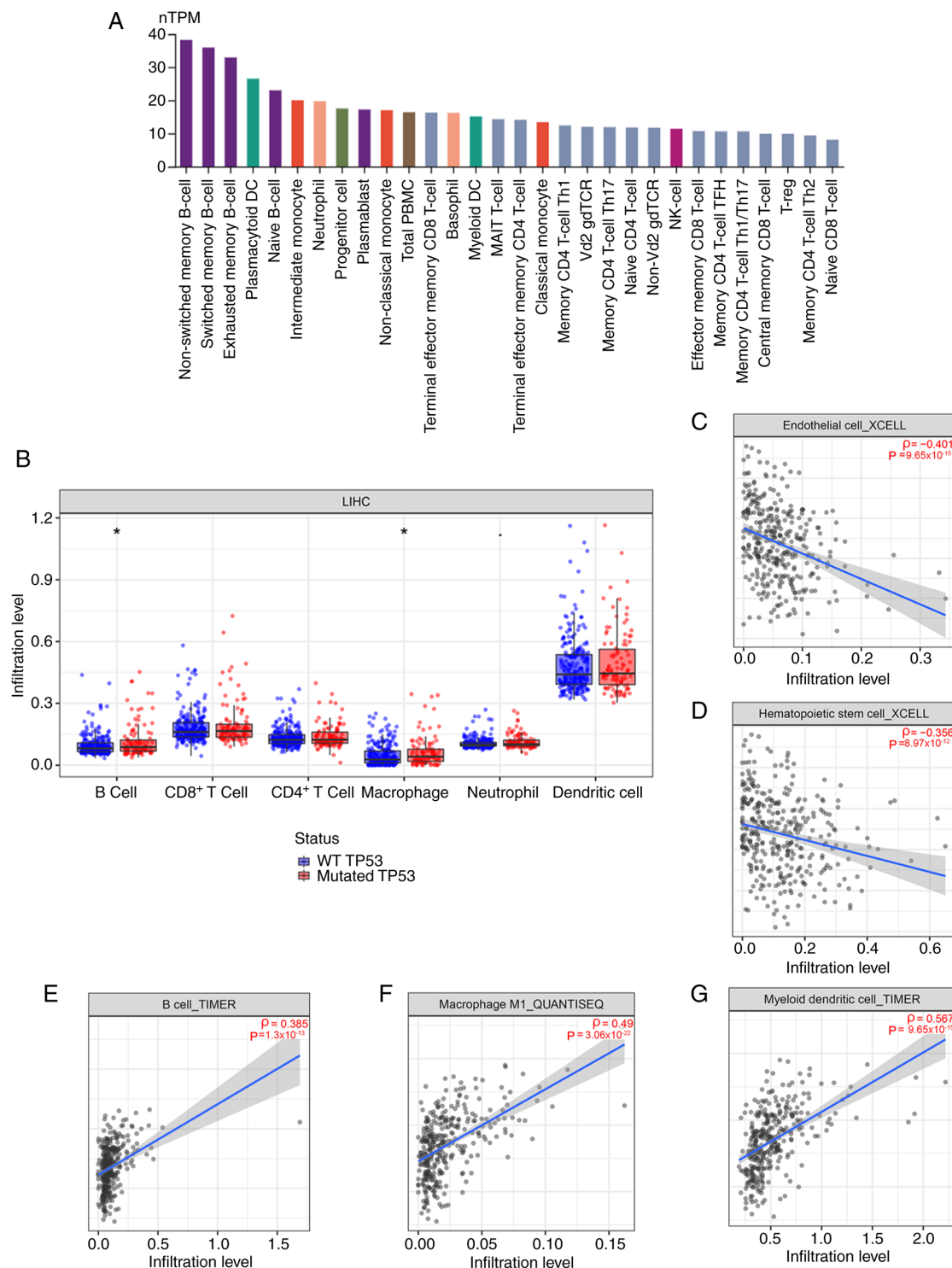


Figure 5. Association between NFKBIE and tumour immune infiltration. (A) NFKBIE is associated with immune infiltration in HCC samples from the Human Protein Atlas. (B) Association between infiltrating immune cells and TP53 mutations in HCC. NFKBIE is negatively correlated with (C) Endothelial cells and (D) Hematopoietic stem cells. $P < 0.05$. NFKBIE is positively correlated with (E) B cells, (F) Macrophages M1 and (G) Myeloid dendritic cells. NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ϵ ; HCC, hepatocellular carcinoma; LIHC, liver HCC; TP53, tumour protein P53; WT, wild type.

sensitivity analysis using the Genomics of Drug Sensitivity in Cancer and Cancer Therapeutics Response Portal databases revealed that low expression of NFKBIE was correlated with resistance to Z-LLNle-Cho (Fig. 4D) and dabrafenib (Fig. 4E). These results provide new therapeutic ideas for patients with HCC.

Correlation between NFKBIE and tumour immune infiltration. In HCC, memory B cells had the greatest expression of NFKBIE, whereas numerous T cells had the lowest expression of NFKBIE (Fig. 5A) (14). Using the TIMER database, B cells and macrophage cells with TP53 mutation had higher levels of immune infiltration. (Fig. 5B). NFKBIE was also

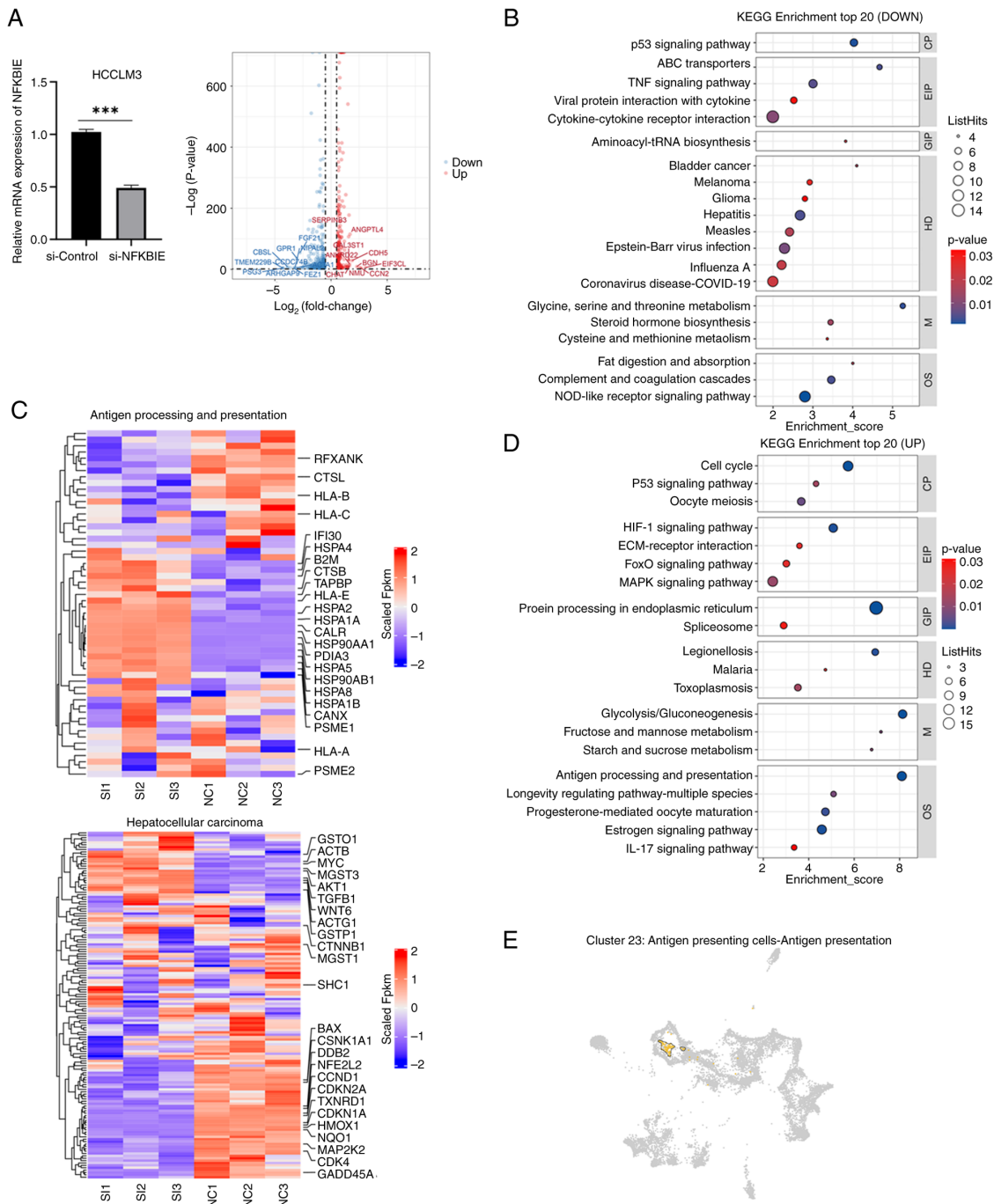


Figure 6. Silencing NFKBIE affects 'antigen processing and presentation' in hepatocellular carcinoma. (A) Knockdown of NFKBIE using siRNA technology (left panel) and volcano map of differential genes detected by line RNA-sequencing (right panel), grey indicates genes without nonsignificant differences in the volcano map, red indicates upregulated genes and blue indicates downregulated genes. (B) KEGG analysis of the top 20 genes that were downregulated. (C) Heatmaps of 'antigen processing and presentation' and 'hepatocellular carcinoma'. (D) KEGG analysis of the top 20 genes that were upregulated. (E) NFKBIE was associated with antigen presentation with high confidence Human Protein Atlas. NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ϵ ; siRNA, small interfering RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes. ***P<0.001.

significantly negatively associated with endothelial cell and haematopoietic stem cell immune infiltration. Conversely, B cell, M1 macrophage and myeloid dendritic cell immune infiltration were found to be significantly positively correlated with NFKBIE (Fig. 5C-G). Low expression of NFKBIE combined with immune cell infiltration has a better prognosis (Fig. S2).

Silencing NFKBIE regulates HCC through 'antigen processing and presentation'. To assess the role that NFKBIE serves in

HCC, siRNA was used to knockdown NFKBIE expression and then RNA-seq analysis was performed, and a volcanic map of DEGs was shown (Fig. 6A). Among the top 20 elevated genes, the 'P53 signalling pathway' was significantly enriched (Fig. 6B). It was demonstrated that knockdown of NFKBIE expression significantly affected 'antigen presentation' (HSA04612) and 'hepatocellular carcinoma' (HSA05225) (Fig. 6C). Additionally, among the top 20 elevated genes, the 'longevity-regulating pathway' was significantly enriched

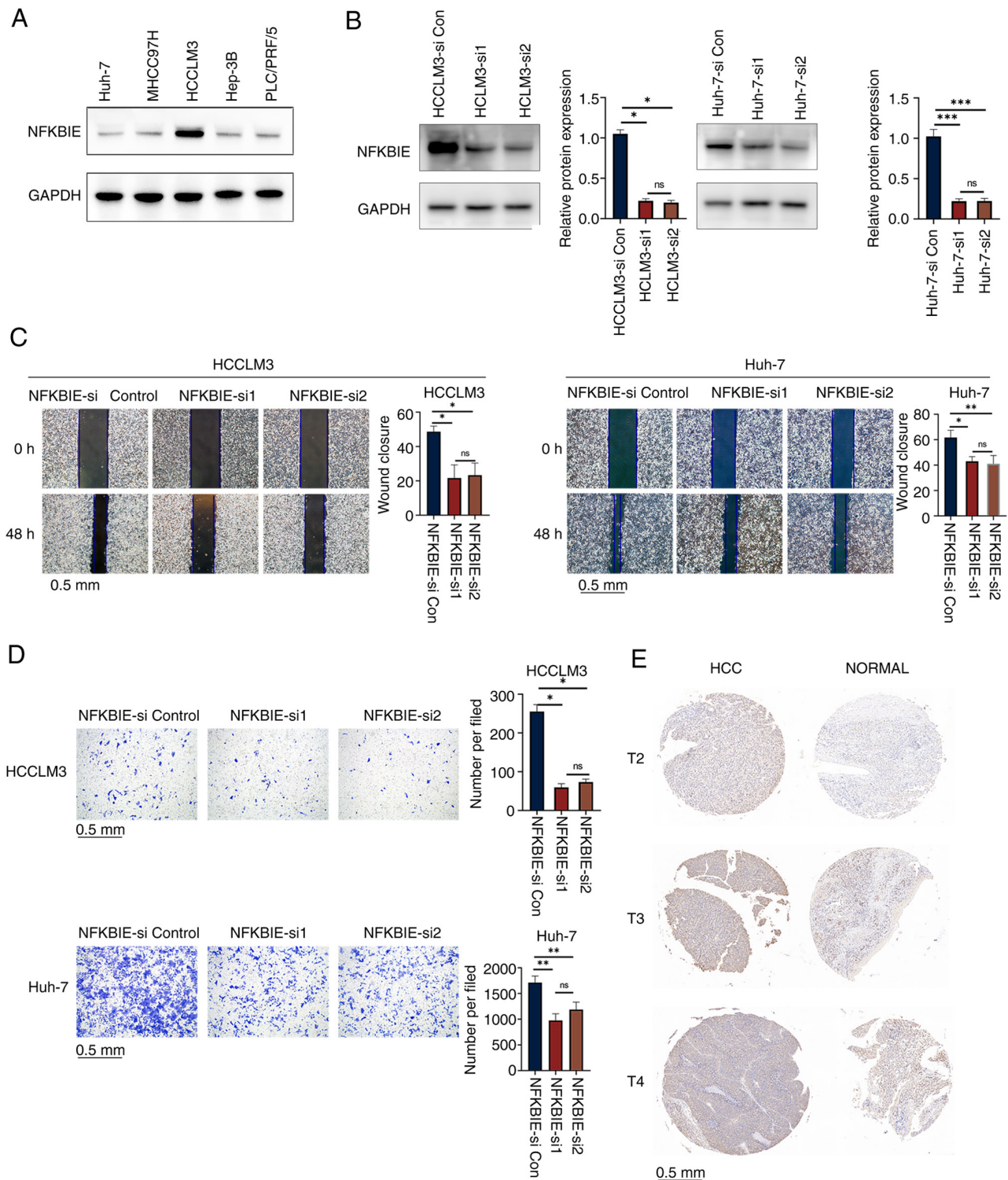


Figure 7. Silencing of NFKBIE affects the proliferation and migration of HCC cells. (A) NFKBIE expression in liver cancer and normal cells. (B) NFKBIE was silenced using siRNA technology. (C) Images representing the wound healing assays in the indicated cell lines. (D) Images representing the migration assays in the indicated cell lines. (E) Representative immunohistochemical images of NFKBIE in HCC tissues at different stages of progression. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NFKBIE, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ϵ ; siRNA, small interfering RNA; HCC, hepatocellular carcinoma; NC, negative control; ns, not significant.

(Fig. 6D). Similar results were demonstrated using data from the HPA database, and NFKBIE was notably associated with 'antigen presentation' with high confidence (Fig. 6E).

Silencing NFKBIE affects the proliferation and migration of HCC cells. The expression of NFKBIE was notably the lowest

in Huh-7 cells and the highest in HCCLM3 cells (Fig. 7A). After siRNA-mediated reduction in NFKBIE expression (Fig. 7B), the proliferation and migration of HCCLM3 and Huh-7 cells were significantly reduced, compared with the control (Fig. 7C and D). Furthermore, IHC of tissue microarray demonstrated NFKBIE overexpression in different stages of HCC (Figs. 7E and S3).

Discussion

Liver cancer is the fourth leading cause of cancer-related deaths worldwide and a global health threat. The main risk factors include infection, behavioural factors, metabolic factors, and aflatoxin (40).

The activation of NFκB is necessary for the formation and progression of HCC. In addition, the NFκB protein complex is essential for both conventional and atypical inflammatory activation and immunological development pathways (10,41,42). The NFκB inhibitor family regulates NFκB. Biologically, proteins encoded by the NFκB inhibitor family bind to a part of NFκB, blocking it from entering the nucleus and activating genes there (43,44). However, the role of the NFκB inhibitor family and associated carcinogenic processes in HCC are poorly understood. In the present study, most malignant tissues expressed more NFκB inhibitors than normal tissues and unpaired HCC samples had higher NFKBIB, NFKBID and NFKBIE expression than normal tissues (Fig. 1B-D).

The mechanism of NFKBIB, NFKBID and NFKBIE in HCC has not been reported to the best of the authors' knowledge; however, the RS3138053 NFKBIA polymorphism has been linked to an increased risk of HCC (45). NFKBID modulates the B-cell response, providing an antiparasitic antibody response (46). Targeting the NFKBID gene, lung tumour-derived exosome mir-3473b promotes the NFκB pathway in local lung fibroblasts, therefore promoting the pulmonary colonization of lung tumour cells (47). NFKBID encodes IκBNS, and its deletion affects B-cell development and function (48,49). Moreover, NFKBIE deficiency increases NFκB activation in cells and increases *in vitro* B-cell proliferation (13). In the present study, it was demonstrated that NFKBIB, NFKBID and NFKBIE were overexpressed in a range of malignancies. Furthermore, using Kaplan-Meier plots, the present study found that NFKBIA, NFKBID and NFKBIZ had no significant effect on prognosis. However, patients with HCC with low NFKBIE expression had a better prognosis; thus, NFKBIE was selected for future research.

NFKBIE is an NFκB suppressor, and it has been linked to several clinical characteristics of HCC. In the blood, deletion, and mutation of NFKBIE has been associated with several leukaemias (14,50). However, similar conditions have not been observed in HCC. Through the LinkedOmics database, it was demonstrated that NFKBIE was positively associated with the NFκB signalling pathway and primary immunodeficiency. The STRING database also verified the positive correlation between NFKBIE and the NFκB pathway. The NFκB family is known to comprise P50, P52, P65, C-REL, and RelB (51). From the STRING database, it was demonstrated that RELA, RELB, REL, NFKB1 and other genes interact with NFKBIE. RELA regulates its downstream target genes by activating apoptosis, and RELA/NFκB may be an important new therapeutic target for human liver cancer (52). In prostate cancer, RELB is essential for the differential radiosensitization of ascorbic acid (53). Moreover, according to GSCA database analysis, NFKBIE suppressed the PI3K/AKT, RAS/MAPK, RTK and TSC/mTOR signalling pathways.

Inflammation is a local protective response of the body to injury or infection, but chronic inflammation serves a pathogenic role in cancer (54). HCC is a useful tumour type to study the

association between tumours and inflammation as most HCC occurs in an inflamed liver (55). According to the results of the present study, the expression of NFKBIE was higher in B cells and lower in T cells. TP53 mutant samples also had higher levels of B-cell infiltration than wild-type TP53 samples. NFKBIE was negatively correlated with endothelial cells and haematopoietic stem cells, but positively correlated with B cells, M1 macrophages and myeloid dendritic cells. Furthermore, it was demonstrated that low levels of NFKBIE combined with High T cell CD4⁺ effector memory, macrophages and common lymphoid progenitors indicated a good prognosis. After the knockdown of NFKBIE, 'antigen processing and presentation' and the 'hepatocellular carcinoma' pathways were significantly affected, which is consistent with data from the Human Protein Atlas. Moreover, the results also demonstrated that the genes with increased expression were enriched in the P53 pathway and the longevity-regulating pathway. Additionally, the genes with reduced expression were enriched in non-alcoholic liver disease, which eventually progresses to liver cancer (56). These findings suggest that NFKBIE may have potential significance in treating this disease as the inhibition of NFKBIE significantly decreased both the proliferation and migration of HCC cells. In conclusion, NFKBIE is a promising immunotherapeutic target for HCC.

In the present study, the mechanism of NFKBIE was not deeply studied. In the future, the specific mechanism of NFKBIE promoting the proliferation and migration of liver cancer will be further explored.

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Availability of data and materials

The data generated in the present study may be found in the NCBI repository at the following URL: <https://www.ncbi.nlm.nih.gov/sra/PRJNA893825>.

Authors' contributions

LQ, YZ and HL designed the research. JT, JW, LZ, TD, SS and CT helped with the analysis. ZY wrote the manuscript. ZY and QL confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocol of the present study was established according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of

The First Affiliated Hospital of University of Science and Technology of China [approval no. 2020-SN(H)-023]. Written informed consent was obtained from individual participants or the guardians of participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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