NLRC5 promotes cell proliferation via regulating the AKT/VEGF-A signaling pathway in hepatocellular carcinoma

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Abstract

NLRC5, a newly found member of the NLR family and the largest member of nucleotide-binding, has been reported to regulate immune responses and is associated with hepatocellular carcinoma (HCC). We investigated the mechanisms and signaling pathways of NLRC5 in HCC progression. Increased expression of NLRC5, vascular endothelial growth factor-A (VEGF-A) were found in human HCC tissue. There was a positive correlation between NLRC5 and VEGF-A expression and cell proliferation were enhanced in NLRC5-overexpressing HepG2 cells, but inhibited in cells with NLRC5 silencing treatment. Interestingly, we found that up-regulation of NLRC5 also coordinated the activation of PI3K/AKT signaling pathway. An AKT inhibitor LY294002 blocked VEGF-A expression and AKT phosphorylation in HepG2 cells and NLRC5-overexpressing HepG2 cells. These results demonstrate that NLRC5 promotes HCC progression via the AKT/VEGF-A signaling pathway.

Keywords: NLRC5; VEGF-A; HCC; PI3K/AKT signaling pathway

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy and most lethal cancer in the world with the incidence and mortality increased rapidly [1-3]. The long-term survival and prognosis of HCC after conventional treatments including surgery, chemotherapy, radiation still remains generally poor [4, 5]. The development of HCC occurs as a multistage disease and hepatitis B or C virus infection, exposure to aflatoxin, and excessive intake of alcohol are the key risk factors in HCC development[6]. Thus, elucidating the molecular

mechanism of HCC is crucial for the prevention and treatment of the disease.

Recent studies have indicated that innate immunity is highly related to the HCC carcinogenesis [7]. As the sensors for innate immunity, pattern recognition receptors (PRRs) which can recognize virus bacteria and other pathogens to initiate immune responses, play dual roles in the regulation of tumor immunity. They can inhibit tumorigenesis through maintaining the host-microflora homeostasis and removing the death or mutant cells and recognize danger signals to induce immunogenic tumor cell death on one hand , they can also contribute to tumorigenesis in settings of chronic inflammation and induction of regulatory cell population and immunosuppressive cytokines to facilitate tumor growth on the other hand [8, 9]. Recent studies identified that the family of cytoplasmic PRRs consists of the retinoid acid-inducible gene I (RIG-I)-like receptors(RLRs), Toll-like receptors (TLRs), C-type lectin receptors(CLRs), and the nucleotide binding and oligomerizationdomain (NOD)-like receptors (NLRs) [10-12]. NLR family, CARD domain containing 5 (NLRC5; also known as NOD4 or NOD27) is a newly found member of the NLR family which contains more than 20 members in mammalian genome and even more in plant or some fish species [13, 14]. NLRC5 contains three structural domains including an N-terminal caspase activation and recruitment domain (CARD), a centrally located nucleotide-binding domain (NBD) and a C-terminal leucine-rich repeats (LRRs)[15]. Cell-specific analysis indicates that NLRC5was strongly expressed in immune cells and immune-related tissue such as B cells,

bone marrow, spleen, lung, thymus, and liver, suggesting that the molecule is biologically conserved in these tissues [16, 17]. Nevertheless, recent studies have revealed controversial and alternating roles of NLRC5 in innate and adaptive immunity [18]. Meanwhile, novel studies suggest that mutations of some NLRs such as NOD1, NOD2, NLRP1, NLRP3, NLRP7 and NLRP12 have been closely related to tumorigenesis [18-23]. Previous studies from our laboratory also have proved that NLRC5 plays a negative role in the regulation of cytokine secretion in RAW264.7 macrophages, but positive in LX-2 cells [24, 25]. We also demonstrated that NLRC5 promoted proliferation and activation of hepatic stellate cells during hepatic fibrosis[26]. In addition, there is a report depicting the expression and oncogenic role of NLRC5 in HCC. It has shown that NLRC5 regulates cell proliferation, migration and invasion in HCC by the Wnt/ β -catenin signaling pathway [27]. However, no further function study of NLRC5 in HCC has been reported. What's more, its relationship with vascular endothelial growth factor-A (VEGF-A), a critical player in angiogenesis, is still unknown, though VEGF-A has been implicated in the tumorigenesis including HCC [28].

Increasing evidence suggests that aberrant activation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is closely related with angiogenesis and hepatic cancer progression[29]and its activation leads to VEGF-A expression [30]. However, whether NLRC5 promotes hepatic carcinoma progression via AKT /VEGF-A signaling pathway warrants validation.

To declare these important issues we projected the study to elucidate whether NLRC5 modulated VEGF-A expression and cell proliferation via

PI3K/AKT signaling pathway in HCC. We first obtained the clinical samples of HCC and found that NLRC5 was up-regulated in HCC patients. Second, we show that NLRC5 expression levels were positively correlated with the increased cell proliferation rate and VEGF-A expression levels in vitro. Last, we indicated for the first time that the PI3K/AKT pathway is involved in the oncogenic function of NLRC5 regulated cell proliferation. This research suggested that NLRC5 maybe a potential therapeutic target toward HCC.

2. Materials and methods

2.1. HCC tissue samples collection

Human HCC and adjacent normal tissue (>2 cm away from the tumor) samples were acquired from patients going ahead partial liver resection in First Affiliated Hospital of Anhui Medical University (Table 1). The study accorded with the standards set by the Declaration of Helsinki, which was permitted by the Health Human Research Ethics Committee of Anhui Medical University. All participants signed informed consent. The experimental protocol was implemented in keeping with the approved guidelines.

2.2. Cell cultures

Human HCC cell lines HepG2, SMMC-7721 and BEL-7402 cells were purchased from the Cell Bank at Chinese Academy of Sciences (Shanghai, China). The same with human HCC cell lines, normal liver cell line (L02) which is usually used as control cell in liver cancer studies was obtained from Cell Bank at Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium

(DMEM, HyClone, USA) supplemented with penicillin (100 IU/mL), streptomycin (100 mg/mL) and 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

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

HepG2 cells were collected, and total RNA was extracted using TRIZOL total RNA isolation reagents (Invitrogen, USA), and the first-strand cDNA was synthesized using a Thermoscript RT-PCR synthesis kit (Fermentas, Canada) in accordance with the manufacturer's instructions. Real-time quantitative PCR analyses for messenger RNA (mRNA) of VEGF-A, NLRC5 and β -actin were disposed under a standard protocol using the following primers listed in Table 2. The fold-change for mRNA relative to β -actin was detected by the formula: $2^{-\Delta\Delta C_t}$. All experiments were performed in triplicate and repeated at least three times.

2.4. Generation of recombinant NLRC5 plasmid

Expression plasmid of the full-length coding region (1276 bp) NLRC5 was created by amplifying complementary DNA (cDNA) coding from pancreas cDNA and inserting cDNA coding for NLRC5 into destination vectors by Gateway cloning (Invitrogen). The following primers were put to use for amplification: forward: 5'-CCGGAATTCCGGATGGCCAGGAAGCTGGA-3'; reverse: 5' -GGGATCCCGTCACCTGAGTGTCTTCCCA-3'. The N-terminal region of the NLRC5 coding region containing the predicted CARD domain was cloned into pEGFP-C2 empty vector by using EcoRI and BamHI. The constructed plasmid was transfected into HepG2 cells with the LipofectamineTM2000 (Invitrogen, USA) according to the manufacturer's

manuals and observed under fluorescence microscopy assays. Overexpression of efficiency was identified by qRT-PCR and Western blot analysis.

2.5. Small interfering RNA

HepG2 cells were plated at a density of 0.6×10^5 cells/ml and cultured for 24 hours before experimentation. NLRC5 was knocked down with small interfering RNA (siRNA) purchased from GenaPharma Corporation (Shanghai, China) using LipofectamineTM2000 (Invitrogen, USA). The sequences of oligonucleotides used are as follows: NLRC5-siRNA (human), forward, 5'-AAGAACGAGAGACUCUGCCAACUGCdTdT-3' and reverse, 5'-CAGUUGGCAGAGUCUCUCGUUCUdTT-3'; negative scrambled siRNA, forward: 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. The RNA and protein were extracted and analyzed respectively.

2.6. Cell cycle analysis

For transfection for 48 h, Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) was used for cell cycle analysis. Briefly, cells were trypsinized, and then cells were fixed in 70% ethanol in PBS at 4°C overnight. After fixation, cells were washed with cold PBS and stained with 0.5 ml of propidium iodide (PI) staining buffer, which contains 200 mg/ml RNaseA and 50 µg/ml PI at 37°C for 30 min in the dark. The cell cycle was analyzed by flow cytometer (BD FACSVerse, USA). Cell debris and fixation artifacts (aggregates of cells) were gated out, and cell populations that were at the G0/G1, S, and G2/M phases were calculated

by the FlowJo data analysis software package (TreeStar, USA).

2.7. Protein extraction and Western blot assay

Human liver cancer tissues and HepG2 cells were lysed by RIPA lysis buffer (Beyotime, China) with 1 % phenyl methyl sulfonyl fluoride (PMSF) (Beyotime, China). Protein concentration of the extract was measured by a BCA protein assay kit (Boster, China). Total proteins (20–50µg) were electrophoresed to sodium dodecyl sulfate polyacryl-amide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membrane (Millipore, USA). After blockade with 5 % milk, nitrocellulose blot were incubated for overnight at 4 °C with primary antibodies diluted in Dilution Buffer (Beyotime, China). Rabbit monoclonal antibody recognizing NLRC5 (Abcam, UK) and pAKT (s473)(Cell Signaling, USA) were diluted at 1:500, rabbit polyclonal anti- VEGF(Biorbyt, UK) used at 1:200, rabbit monoclonal antibodies against AKT (Boster, China) were used at 1:400, mouse monoclonal antibody directed against β -actin (Santa Cruz, USA) was diluted at 1:200. After three washes in TBS/Tween-20, blots were incubation for 1 h in goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP, Santa Cruz, USA) conjugate antibody at 1:7500 dilution in TBS/Tween-20 containing 5 % skim milk. Stripes of protein were visualized with the ECL-chemiluminescent kit (ECL-plus, Thermo Scientific, USA). The variation in the density of bands was expressed as fold changes compared to the control in the blot after normalized to β -actin. Representative blots were from three independent experiments.

2.8. Inhibitor treatment

NLRC5-transfected HepG2 cells were treated with LY294002

(Beyotime, China) at 25 μ M for 48 h.

2.9. In vitro plate-colony formation assay

Cells were seeded at a density of 200 cells per well in a 24-well plates. After culturing for two weeks, colonies (>50cells/colony) were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal violet (Beyotime, China) for 15 min. Colonies were counted and colony formation efficiency was calculated according to the following formula: (number of colonies/number of cells inoculated) $\times$ 100%.

2.10. Proliferation assay

3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assay was used for detecting the proliferation rate of HepG2 cells. Briefly, HepG2 cells on long phase were cultivated at a 96-well plates at a density of 4×10^3 cells per well in 200 μ L suspension for 12h. Cells were transfected with siRNA expressing the siNLRC5 or negative control and plasmid expressing the pEGFP-C2-NLRC5 or Vector. After the transfection for 48, 20 μ l (5 mg/mL) MTT (Sigma, USA) was added to each well and incubated at 37 °C for another 4 h. Then, the medium was substituted for 150 μ l dimethylsulfoxide (DMSO) to dissolve the production of formazan crystals. The absorbance was detected with microplate reader (Bio-Tek EL, USA) at 490 nm. All experiments were texted in triplicate and should be repeated at least three times.

2.11. Enzyme-linked immunosorbent assay (ELISA)

The HepG2 cells culture medium was collected and centrifuged at 1000 $\times$ g and the supernatant was detected for secreted endogenous VEGF-A levels using Human VEGF-A ELISA Kit (Elabscience, China) according to the manufacturer's instructions.

2.12. Immunohistochemistry staining and evaluation of NLRC5 and VEGF-A expression

Fresh tumor tissues were fixed with 10 % neutral formalin for three days and then embedded in paraffin. The tissues were cut into slices at the thickness of 4 μ m. Immunohistochemistry staining was performed as previously described[31]. After sequential treatments, tissue sections were sequentially incubated with normal serum for 30 min, rabbit polyclonal antibody against VEGF(1:100, Biorbyt, UK) and NLRC5 (1:200, Abcam, UK) at 4 °C overnight and then horseradish peroxidase anti-rabbit IgG antibody (Cowin Bioscience, China) for 30 min. The diaminobenzidine tetrahydrochloride solution (DAB, Cowin Bioscience, China) staining was used for development of brown color to identify the expression of VEGF-A and NLRC5. All slices were observed under a fluorescence microscope and photographed.

2.13. Statistical analysis

All data were analyzed by the SPSS 17.0 Soft and presented as means $\pm$ SD. The statistical significance of differences was determined by the Student's *t* test for comparison between means or one-way analysis of variance (ANOVA). Data were regarded as to be statistically significant when $*p < 0.05$ and $**p < 0.01$.

3. Result

3.1. NLRC5 and VEGF-A are up regulated in human HCC tissues and human hepatoma carcinoma cell lines

To identify the change in the NLRC5 expression between HCC tissues and adjacent noncancerous tissues, the degree of liver carcinoma

was determined by H&E staining. Histopathological analysis showed that human HCC tissues displayed hepatic steatosis, cellular edema, focal nodular hyperplasia and inflammatory infiltration, while normal lobular architecture with central veins and radiating hepatic cords as control group (Fig. 1A). The immunohistochemical analysis prompted that NLRC5 and VEGF-A was positive for HCC cells. And immunohistochemical analysis also found NLRC5 existed in the cytoplasm. Western blot results indicated that the protein levels of NLRC5, VEGF-A were higher in human HCC tissues compared with corresponding normal liver tissues (Fig. 1A and B). We collected HCC cell lines including HepG2, SMMC-7721 and BEL-7402 for investigation. Meanwhile, L02 cell was included as the normal control. Results suggested that protein expressions of NLRC5 and VEGF-A in HCC cell lines were much higher in comparison with that in control normal L02 cell (Fig. 1C). The results suggested that NLRC5 and VEGF-A in human HCC were possibly correlated.

3.2. Liposome-Mediated transduction resulted in NLRC5 overexpression or knockdown in HepG2 cells

NLRC5 overexpression and knockdown were introduced into HepG2 cells by liposome. To confirm the effect of NLRC5 overexpression or knockdown, we detected the level of NLRC5 in the conditioned medium and the results are exhibited in Fig. 2. Transfection efficiency of pEGFP-C2-NLRC5 and NLRC5-siRNA were detected by inversed fluorescent microscope (Fig. 2A and D). In figure 2D, structures of cells transfected with NLRC5-siRNA were disorganized. After detecting NLRC5 expression by Western blot and qRT-PCR, we found

NLRC5 was increased by pEGFP-C2-NLRC5 in protein and RNA level (Fig. 2B and C). In contrast, NLRC5-siRNA induced an obvious decrease of NLRC5 protein and RNA level in HepG2 cells (Fig. 2E and F).

3.3. NLRC5 up regulation enhances VEGF-A expression and cell proliferation of HepG2 cells

NLRC5 overexpression increased the level of VEGF-A, respectively, by Western blot (Fig. 3A) and qRT-PCR (Fig. 3B). Result of ELISA assay (Fig. 3C) also showed that pEGFP-C2-NLRC5 had a positive role on VEGF-A secretion in HepG2 cells. Furthermore, NLRC5 overexpression promoted cell proliferation by MTT (Fig. 3D). The percentage of colonies in pEGFP-C2-NLRC5 HepG2 cells was higher compared with pEGFP-C2 cells (Fig. 3E). Cell cycle analysis illustrated that the proportion of cells arrested in the G1 phase amounted to 38.2% in pEGFP-C2-NLRC5 HepG2 cells, compared with 51.7% in pEGFP-C2 cells (Fig. 3F).

3.4. NLRC5 repression inhibits VEGF-A expression and cell proliferation of HepG2 cells

NLRC5-siRNA transfection was used to knock down NLRC5 expression in HepG2 cells. NLRC5-siRNA decreased the level of VEGF-A as detected by Western blot (Fig. 4A) and qRT-PCR (Fig. 4B). Further result of ELISA assay (Fig. 4C) also showed that NLRC5-siRNA had a negative role on VEGF-A secretion in HepG2 cells. A remarkable slower proliferation rate was observed in NLRC5-siRNA cells compared with control-siRNA cells (Fig. 4D). Silencing NLRC5 decreased the ability of HepG2 cells to form colonies (Fig. 4E). There were increased NLRC5-siRNA HepG2 cells arrested in G1 phase (61.44%) compared with

control-siRNA cells (54.34%) (Fig. 4F).

3.5. NLRC5 regulates VEGF-A activity through the PI3K/AKT signaling pathway

To investigate whether NLRC5-regulated VEGF-A expression was mediated through the PI3K/AKT signaling pathway, we measured the protein levels of Ser⁴⁷³-AKT when NLRC5 expression levels were modulated in HepG2 cells. Knocking down of NLRC5 by siRNA significantly decreased the protein levels of Ser⁴⁷³-AKT compared with that transfected with negative control in HepG2 cells (Fig. 5A). In contrast, the expressions of Ser⁴⁷³-AKT protein were dramatically up-regulated after transfection with pEGFP-C2-NLRC5 compared with that derived from pEGFP-C2 transfected HepG2 cells (Fig. 5B). Moreover, the expression of Ser⁴⁷³-AKT was also dramatically increased in human HCC tissues (Fig. 5C). In addition, we treated pEGFP-C2 transfected HepG2 cells or NLRC5-overexpressing cells with LY294002 (25 μ M) which was an AKT inhibitor for 48h. At 48 hours after LY294002 (25 μ M) treatment, the protein and mRNA level of phosphorylated Ser⁴⁷³-AKT decreased significantly in pEGFP-C2 transfected HepG2 cells or NLRC5-overexpressing cells, the same as VEGF-A mRNA (Fig. 5D and E). Result of ELISA assay (Fig. 5F) also indicated LY294002 (25 μ M) could inhibited the secretion of VEGF-A in pEGFP-C2 transfected HepG2 cells or NLRC5-overexpressing cells. These data suggest that NLRC5 regulates VEGF-A activity through the AKT signaling pathway in HepG2 cells.

4. Discussion

Hepatic cancer is the fifth most common neoplasm in the world and

its incidence is increasing due to infection of the extensively disseminated hepatitis B and C viruses [32]. The development and evolution of HCC are both a multifactorial and multistep biological process [1]. The survival of liver cancer patients has not observably improved over the past decades. It is therefore necessary to find the better treatments. It is also faced with a enormous challenge, and will potentially lie on the progressive in basic research for the management of HCC.

Innate immune system as the first line of host defense against exogenous organism invasion, continually monitor the environment for invading pathogens. It recognizes the invasion of foreign microorganism and cellular stress through PRRs, which are structurally related proteins that detect conserved microbial components, called pathogen-associated molecular patterns (PAMPs) [10]. The family of cytoplasmic PRRs is comprised of TLRs, NLRs, CLRs, and RLRs [33]. Notably a recent study showed that dramatical attentions have been acquired by the role of NLRs in innate immune and other diseases. On the other hand, evidence has emerged that the susceptibility to inflammatory disease is related to the mutations and polymorphisms in NLRs genes, including Nod1, Nod2, NLRP1 and NLRP3[34]. Additionally, it has been shown that the activation of NLRs has been implicated in numerous types of cancer, including colon cancer, breast cancer and hepatocellular carcinoma [35-37]. Similarly to most NLRs, NLRC5 has commonly been reported to play an important role in innate immunity. Previous studies demonstrated that NLRC5 negatively regulated NF- κ B signaling pathway by suppressing the phosphorylation of IKK α and IKK β [17, 38]. Novel studies suggest that NLRC5 interacts with RIG-1 and MDA5 and

negatively modulates type I interferon signaling [17]. Furthermore, NLRC5 acts as a transcriptional regulator in the MHC class I expression [39, 40]. NLRC5 regulates activation, proliferation and cytotoxicity of L. monocytogenes-specific CD8⁺ T cell [40]. However, the certain role of NLRC5 in immune response is still controversial and need to be further explored [18]. The studies from our lab indicate that NLRC5 overexpression promoted cell proliferation, migration and invasion in HCC by the Wnt/ β -catenin signaling pathway and it also showed that NLRC5 was a vital regulator of tumor angiogenesis[27], raising the question of the mechanisms and signaling pathways mediated by NLRC5 in hepatic cancer progression.

The present study revealed that NLRC5 expression was negative in normal liver tissues but positive in liver cancer tissues and HCC cell lines, including HepG2, Bel-7402, SMMC-7721. Immunofluorescence co-localization analysis demonstrated the positive correlation between the expression of NLRC5 and VEGF-A in HCC tissues. These results indicate that NLRC5 might interact with VEGF-A in liver cancer tumorigenesis and progression.

In order to better comprehending the functional mechanism of NLRC5 in liver cancer, we detected the effect of NLRC5 overexpression or silencing on VEGF-A expression and the proliferation of HepG2 cells. We transfected pEGFP-N2-NLRC5 into HepG2 cells for 48h and the result showed that transient transfection of pEGFP-N2-NLRC5 completely increased VEGF-A expression, promoted cell proliferation of HepG2 cells by MTT assay, colony formation assay. Moreover, the cell cycle was arrested in the G1 phase after NLRC5 silencing, while NLRC5

overexpression had the opposite effects. Overall, these data suggest that NLRC5 promotes liver cancer progression partially through enhancing cell proliferation regulation.

Types of molecular pathways, including PI3K/Akt/mTOR, JAK/STAT, Wnt/ β -catenin, Ras/Raf/MEK/ERK are implicated in the pathogenesis of HCC[29]. Being an essential component of the PI3K/AKT signaling pathway, AKT regulates a variety of cellular responses in managing angiogenic growth factors by activating VEGF-A expression [41, 42]. Angiogenesis is needful for the growth of solid tumors, and VEGF-A is the most closely connected angiogenic mediator [43]. Recently several studies have reported that there is a link between the activation of PI3K/AKT pathway and the increased expression of VEGF-A [44]. The present study indicated that *in vitro* overexpressing NLRC5 upregulated VEGF-A expression and activated AKT phosphorylation while silencing NLRC5 had the opposite effect. We also analyzed the relative phosphorylated Ser⁴⁷³-AKT expression in human HCC tissue and the phosphorylation level of AKT was higher in human HCC tissue than the control. These results suggested that NLRC5 regulates VEGF-A expression in HCC progression, possibly through the PI3K/AKT signaling pathway. To further confirm whether NLRC5-induced VEGF-A regulation was mediated by the AKT signaling pathway, LY294002, a PI3K/AKT inhibitor, was used to treat the normal and NLRC5-overexpressing HepG2 cells. The result showed that LY294002 (25 μ M) led to the decline of VEGF-A expression in both normal and NLRC5-overexpressing HepG2 cells. This supports our conclusion that NLRC5 regulates VEGF-A expression was mediated by

the AKT signaling pathway.

5. Conclusion

- NLRC5 overexpression enhances VEGF-A expression and cell proliferation of HepG2 cells.
- NLRC5 silencing inhibits VEGF-A expression and cell proliferation of HepG2 cells.
- High NLRC5 expression induces high AKT activation and VEGF-A expression in HCC.
- NLRC5 plays a critical role in HCC tumor proliferation via the PI3K/AKT signaling pathway.

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Conflict of interest statement

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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Figure legend

Fig. 1 NLRC5 and VEGF-A are over-expressed in HCC tissue and human hepatoma carcinoma cell lines. **(A)** Pathology observation of the HCC patient liver sections stained with hematoxylin and eosin (H&E) staining ($\times 100$). Normal liver tissue and HCC tissue were stained by immunohistochemistry with NLRC5 and VEGF-A. **(B)** The protein expression of NLRC5 and VEGF-A in normal liver tissue and HCC tissue. The bar graphs (mean $\pm$ SD) and representative images are shown. $**P < 0.01$ vs normal group. **(C)** The protein expression of NLRC5 and VEGF-A in normal liver cell line (L02) and HCC cell lines (HepG2, SMMC-7721 and BEL-7402). The bar graphs (mean $\pm$ SD) and representative images are shown. $**p < 0.01$ vs the L02 group. Representative views from each group are presented.

Fig. 2 Lipidosome-mediated transient transfection results in NLRC5 over-expression or knockdown in HepG2 cells. **(A, D)** HepG2 cells were transfected with plasmid pEGFP-C2-NLRC5 and NLRC5-siRNA. After 48 h, the cells were photographed by fluorescence microscope at $\times 920$ magnification. **(B, C)** After transient transfection of pEGFP-C2 and pEGFP-C2-NLRC5, NLRC5 protein and mRNA expressions were measured in HepG2 cells. $**p < 0.01$ vs pEGFP-C2 group. **(E, F)** After transient transfection of control-siRNA and NLRC5-siRNA, NLRC5

protein and mRNA expressions were measured in HepG2 cells. $**p < 0.01$ vs control-siRNA group. Each bar graphs (mean $\pm$ SD) and representative images are shown. Results were representative of three independent experiments, $*p < 0.05$, $**p < 0.01$.

Fig. 3 NLRC5 up regulation enhances VEGF-A expression and cell proliferation of HepG2 cells. (A) Increased protein level of VEGF-A in pEGFP-C2-NLRC5 group compared with pEGFP-C2 group by Western blot. $**p < 0.01$ vs pEGFP-C2 group. (B) The mRNA levels of VEGF-A were detected in HepG2 cells without transfection or transfected with pEGFP-C2 or pEGFP-C2-NLRC5. $**p < 0.01$ vs pEGFP-C2 group. (C) The analysis of ELISA confirmed increase secretion of VEGF-A using cell supernatant. $**p < 0.01$ vs pEGFP-C2 group. (D) Cell growth of HepG2 cells was determined after transfection with pEGFP-C2 and pEGFP-C2-NLRC5 by using MTT assay at different time points. $*p < 0.05$ vs pEGFP-C2 group. (E) Increased colony formation ability of pEGFP-C2-NLRC5 group compared with pEGFP-C2 group. $*p < 0.05$ vs pEGFP-C2 group. (F) Decreased cells arrested in the G1 phase in pEGFP-C2-NLRC5 group compared with pEGFP-C2 group by flow cytometric analysis. Each bar graphs (mean $\pm$ SD) and representative images are shown. Results were representative of three independent experiments, $*p < 0.05$, $**p < 0.01$.

Fig. 4 NLRC5 repression inhibits VEGF-A expression and cell proliferation of HepG2 cells. (A) Decreased protein level of VEGF-A in NLRC5-siRNA group compared with control-siRNA group by Western blot. $**p < 0.01$ vs control-siRNA group. (B) The mRNA levels of VEGF-A were detected in HepG2 cells without transfection or transfected

with control-siRNA or NLRC5-siRNA. $**p < 0.01$ vs control-siRNA group. (C) The analysis of ELISA confirmed reduced secretion of VEGF-A using cell supernatant. $**p < 0.01$ vs control-siRNA group. (D) Cell growth of HepG2 cells was determined after transfection with control-siRNA and NLRC5-siRNA by using MTT assay at different time points. $*p < 0.05$ vs control-siRNA group. (E) Reduced colony formation ability of NLRC5-siRNA group compared with control-siRNA group. $**p < 0.01$ vs control-siRNA group. (F) Increased cells arrested in G1 phase in NLRC5-siRNA group compared with control-siRNA group by flow cytometric analysis. Each bar graphs (mean $\pm$ SD) and representative images are shown. Results were representative of three independent experiments, $*p < 0.05$, $**p < 0.01$.

Fig. 5 NLRC5 regulates VEGF-A activity through the PI3K/AKT signaling pathway. (A) Cells were transfected with NLRC5-siRNA and control-siRNA, respectively. The protein level of phosphorylated Ser⁴⁷³-AKT was analyzed by western blot. $**p < 0.01$ vs control-siRNA group. (B) Cells were transfected with pEGFP-C2 or pEGFP-C2-NLRC5. The protein level of phosphorylated Ser⁴⁷³-AKT was analyzed by western blot. $**p < 0.01$ vs pEGFP-C2 group. (C) Relative phosphorylated Ser⁴⁷³-AKT protein expression in human HCC tissue. $**p < 0.01$ vs normal group. (D) pEGFP-C2-NLRC5 transfected cells were treated with 25 μ M LY294002 for 48 h. Expression of phosphorylated Ser⁴⁷³-AKT and VEGF-A was assessed by western blot. β -actin was used as the loading control. $**p < 0.01$ vs pEGFP-C2 group, $^{##} p < 0.01$ vs pEGFP-C2-NLRC5 group. (E) The mRNA level of VEGF-A was measured by qRT-PCR. $**p < 0.01$ vs pEGFP-C2 group, $^{##} p < 0.01$ vs pEGFP-C2-NLRC5

group. (F) The analysis of ELISA confirmed the result using cell supernatant. $**p < 0.01$ vs pEGFP-C2 group, $^{##}p < 0.01$ vs pEGFP-C2-NLRC5 group. Each bar graphs (mean $\pm$ SD) and representative images are shown. Results were representative of three independent experiments, $*p < 0.05$, $**p < 0.01$, $^{\#}p < 0.05$, $^{##}p < 0.01$.

Fig.6 Overview of NLRC5 on HCC.

Table1 Characteristics of the subjects enrolled in the study of HCC protein expression

Parameter	Patients
Case, <i>n</i>	9
Sex, <i>n</i> (%)	
Male	5(55.6%)
Female	4(44.4%)
Age, <i>n</i> ($\pm$ SD)	54.7($\pm$ 10.0)
Compensation/decompensation, <i>n</i> (%)	
Compensation	2(22.2%)
Decompensation	5(55.6%)
Unknown	2(22.2%)
Etiology, <i>n</i> (%)	
Alcoholic	3(33.3%)
HBV	4(44.4%)
HCV	1(11.1%)
Others	1(11.1%)

Table 2

Primers used for real time-PCR.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
Human	CTATCAACTGCCCTTCCACAA	
NLRC5	T	TCTCTATC TGCCCACAGCCTAC
VEGF	CGGGCAGGAGGAAGGAGCCT	GTGATGGTGTGGTGGCGGCA
β-actin	GCCAACACAGTGCTG TCTGG	CTCAGGAGGAGCAATGATCTTG

B

HCC

H&E

NLRC5

VEGF-A

NLRC5

VEGF-A

β-actin

Normal

HCC

C

NLRC5
 VEGF-A

Relative level of protein

NLRC5

VEGF-A

β-actin

LO2 HepG2 SMMC-7721 BEL- 7402

1

Fig.2

Fig.3

A

D

E

B

C

F

Fig.4

A

NLRC5-siRNA	control-siRNA
-	-
-	+
+	-

B

NLRC5-siRNA	control-siRNA
-	-
-	+
+	-

C

NLRC5-siRNA	control-siRNA
-	-
-	+
+	-

D

E

F

Fig.5

A

NLRC5-siRNA - - +
control-siRNA - + -

B

pEGFP-C2-NLRC5 - - +
pEGFP-C2 - + -

C

Normal HCC

D

pAKT(s473)

AKT

β-actin

pEGFP-C2-NLRC5 - - - + +
pEGFP-C2 - + + - -
LY294002 - - + - +

E

Relative mRNA expression of VEGF

pEGFP-C2-NLRC5 - - - + +
pEGFP-C2 - + + - -
LY294002 - - + - +

F

VEGF-A (pg/ml)

pEGFP-C2-NLRC5 - - - + +
pEGFP-C2 - + + - -
LY294002 - - + - +

Fig.6