Title

NLRC5 regulates cell proliferation, migration and invasion in hepatocellular carcinoma by targeting the Wnt/ β -catenin signaling pathway

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Abstract

NLRC5, the largest member of nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) family, has been reported to regulate immune responses and be associated with chronic inflammatory diseases. However, the biological function of NLRC5 in hepatocellular carcinoma (HCC) has not yet been well demonstrated. In this study, the role of NLRC5 in hepatocellular carcinoma cell proliferation, migration and invasion capacities were evaluated by using MTT, flow cytometry, wound healing, transwell assay, tumor formation assay in nude mice. Western blot analysis and qPCR assay were performed to assess NLRC5 interacting with the activation of Wnt/ β -catenin signaling pathway. Here, we demonstrate that NLRC5 was highly expressed in HCC. Knockdown of NLRC5 significantly inhibited cell proliferation, migration, invasion and the tumor formation in nude mice, and arrested the cell cycle at G0/G1 phase. Furthermore, overexpression of NLRC5 promoted the proliferation, migration and invasion of HCC cells *in vitro*. Interestingly, we found that up-regulation of NLRC5 not only positively correlates with the increase of β -catenin but also coordinates the activation of downstream Wnt/ β -catenin signaling pathway. Thus, our findings suggest that NLRC5 may play an important role in progression of HCC and provide a potential therapeutic value in this tumor.

Keywords NLRC5 •HCC • β -catenin •Wnt/ β -catenin signaling pathway •Therapeutic

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world, and its incidence and mortality increased rapidly in recent decades (Farazi and DePinho 2006). The long-term survival of HCC after surgical or chemotherapy

treatment still remains generally poor, for which the high recurrence and metastasis rates became the major obstacle (Yang et al. 2009). Hepatitis B or C virus infection is the key risk factors in HCC development (Marrero and Marrero 2007). Various molecular pathways, including Wnt/ β -catenin, JAK/STAT, PI3K/Akt/mTOR, Ras/Raf/MEK/ERK, Hedgehog pathways and others, are implicated in the pathogenesis of HCC (Hoshida et al. 2010). Moreover, increasing evidence suggests that aberrant activation of Wnt/ β -catenin signaling pathway may be associated with hepatocarcinogenesis (Nhieu et al. 1999). Previous reports showed that various target genes activated by Wnt/ β -catenin signaling, such as C-myc, cyclinD1, metalloproteinases, or VEGF, have been involved in cell proliferation, adhesion, migration, and tumorigenesis (Donnellan and Chetty 1998, Prins et al. 1993, Shtutman et al. 1999). However, the molecular mechanisms of HCC remain largely unknown. Thus, the exact mechanisms and effective therapeutic strategies have been always required for the prevention and treatment of HCC.

NLRC5 (NLR family, CARD domain containing 5, also called NOD4 or NOD27) is a member of the NLR protein family, whose members are named due to the tripartite domain characteristic structure, consists of an N-terminal caspase activation and recruitment domain (CARD), a centrally located nucleotide-binding domain (NBD) and a C-terminal leucine-rich repeats (LRRs) (Fritz et al. 2006). NLRC5 is expressed widely in various tissues and cell lines in both humans and mice, but is expressed predominantly in immune cells and immune-related tissues, such as bone marrow-derived macrophages (BMDMs), CD4⁺ T cells, CD8⁺ T cells and B cells, bone marrow, lymph node, spleen, lung, intestine and liver (Benko et al. 2010, Cui et al. 2010, Kuenzel et al. 2010). Meissner et al. observed that NLRC5 interacted with the promoters of the MHC class I genes and transactivates their expression (Meissner et al. 2010). Mammalian NLR family contains 23 members, which are mostly localized to the cytoplasm of the cells and are similar to each other not only in their protein structure but also in the function (Kanneganti et al. 2007, Rathinam et al. 2012). The mutations and single nucleotide polymorphisms (SNPs) in their genes are essential for human diseases which are reflected their important roles in host defense.

Moreover, the effect of NLRs is not restricted to immunity, as they also have the ability to influence reproduction and embryonic development (Fernandes et al. 2012, Murdoch et al. 2006, Tong et al. 2000). Mutations of several NLRs, including NOD1, NOD2, NLRP1, NLRP3, NLRP7 and NLRP12, have been implicated in tumorigenesis (Burdette et al. 2012, da Silva Correia et al. 2006, Hugot et al. 2001, Ohno et al. 2008, Villani et al. 2009, Zaki et al. 2011b). The relevance of NLRs to tumors has started to gain much attention in recent years. However, whether NLRC5 is involved in tumor progression, particularly in HCC, to our knowledge, is still unknown.

In this study, we investigated the potential involvement of NLRC5 in HCC cell proliferation, migration and invasion *in vitro* and *in vivo*. We elaborate whether Wnt/ β -catenin is involved in the biological activities of NLRC5 in the context of HCC. Here, we found that NLRC5 was up-regulated in HCC and its expression levels were positively correlated with the increased invasion and migration potential and proliferation rate in HCC cells. Our data suggested that NLRC5 may be a potential therapeutic target toward HCC.

2. Materials and methods

2.1 HCC tissue samples and Cell cultures

Some HCC and adjacent normal tissues were obtained from patients in First Affiliated Hospital of Anhui Medical University. The tissues were immediately snap-frozen in liquid nitrogen after surgical removal and stored at -80°C. All protocols were approved by the Ethics Committee of Anhui Medical University.

Human HCC (HepG2, SMMC-7721 and BEL-7402 cells) and normal liver cell line (L02) were obtained from the Cell Bank at Chinese Academy of Sciences (Shanghai, China). L02 is a normal liver cell line which is usually used as control cell in cancer research. All cells were stored in liquid nitrogen and cultured in 5% CO₂ at

37°C with Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with penicillin(100 IU/mL), streptomycin (100 mg/mL) and 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco, USA).

2.2 RNA interference analysis

Small interfering RNA (siRNA) oligonucleotides against NLRC5 genes or scrambled sequences were designed and synthesized by the Gema Pharma Corporation (Shanghai, China) and contained the following sequences: NLRC5 siRNA (sense: AAGAACGAGAGACUCUGCCAACUGCdTd T and antisense: GCAGUUGGCAG AGUCUCUCGUUCUdTdT); negative control with scrambled sequence (sense: UUCUCCGAACGUGUCACGUTT and antisense: ACGUGACACG UUCGGAGAA TT), which is no homologous to any human DNA sequence. Human HepG2, SMMC-7721 and BEL-7402 cells (5×10^5 cells) were cultured in 6 well plates with antibiotics-free DMEM for 24h and then subjected to transfection with siRNA using LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer's protocol. Knockdown efficiency was determined by qRT-PCR and Western blot analysis.

2.3 Plasmid Construction

The full-length coding region (1276 bp) of NLRC5 was amplified from human genomic DNA by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: forward: 5'-CCGGAATTCCGGATGGCCAGGAAGCTGGA-3'; reverse: 5'-GGGATCCCGTCACCTGAGTGTCTTCCCA-3'. The PCR products were digested with EcoR I/BamH I and were inserted into the pEGFP-C2 empty vector. The recombinant construct pEGFP-C2-NLRC5 was verified by direct DNA sequencing. Cell transfection was performed with the LipofectamineTM 2000 according to the manufacturer's manuals. Overexpression of efficiency was validated with qRT-PCR and Western blot analysis.

2.4 Western blot analysis

The knockdown or overexpression efficiency was validated with Western blot at 48 h post transfection. Total proteins were extracted with RIPA buffer (Beyotime, China). Whole extracts were prepared, and protein concentrations were measured using a BCA protein assay kit (Beyotime, China). Approximately 30 µg of protein from each sample was separated using a SDS- polyacrylamide gel and then transferred onto the PVDF membrane (Millipore, USA) and then blocked with 5% skimmed milk. The transferred membranes were incubated with rabbit antibody recognizing NLRC5 (1:400, Abcam, UK), rabbit anti-β-catenin, anti-c-myc, anti-cyclinD1 and anti-MMP- 7 (1:500, Cell Signaling, USA), and mouse antibody against β-actin (1:1 000, Santa Cruz, USA) overnight at 4 °C followed by incubation for 1 h with horseradish peroxidase (HRP) conjugated IgG (1:10 000, Santa Cruz, USA). After extensive washing in TBST (Boster, China), proteins were detected by Quantity-one software (Bio-Rad, Laboratories, Inc, USA) using the ECL-chemiluminescent kit (Thermo Scientific, USA).

2.5 RNA isolation and quantitative real-time PCR

The knockdown or overexpression efficiency was determined by quantitative real-time PCR at 24 h after transfection. Total RNA was extracted using Trizol total RNA isolation reagent (Invitrogen, USA) and reverse transcribed was conducted as followed with the RT-PCR synthesis kit (Fermentas, Canada), according to the manufacturer's instructions. The cDNA was stored at -20°C and performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) on the PikoReal 96 qPCR system (Thermo Scientific, USA). The average of three independent analyses for each gene and sample was calculated and normalized to the endogenous reference control gene β-actin. All samples were determined using the $2^{-\Delta\Delta CT}$ analysis method. The primers used in real-time PCR were as follows: β-actin forward, 5'-GCCAACACAGTGCTG

TCTGG-3'; β -actin reverse, 5'-CTCAGGAGGAGCAATGATCTTG-3'; NLRC5 forward, 5'-CTATCAACTGCCCTTCCACAAT-3'; NLRC5 reverse, 5'-TCTCTATC TGCCACAGCCTAC-3'; β -catenin forward, 5'- GCGTTCTCCTCAGATGGTGTGTC -3'; β -catenin reverse, 5'-CCAGTAAGCCCTCACGATGAT- 3'.

2.6 Immunohistochemistry

The tissues were dehydrated and then embedded in paraffin wax. The sections were dewaxed in xylene and dehydrated in alcohol. Antigen retrieval was done by incubating the slides in citric acid buffer for 10 min. After blocking with 3% hydrogen peroxide for 15 min and 5% bovine serum albumin, the slides were incubated overnight at 4°C with rabbit polyclonal antibody against NLRC5 (1:100, Abcam, UK). After rinsing with PBS, the sections were incubated with a horseradish peroxidase anti-rabbit IgG antibody (Cowin Bioscience, China) for 30 min at room temperature. NLRC5 expression was visualized by diaminobenzidine tetrahydrochloride solution (DAB, Cowin Bioscience, China) staining. The sections were finally counterstained with haematoxylin for 5min. All slides were observed under a fluorescence microscope and photographed.

2.7 MTT assay

The MTT assay was used to detect the proliferation rate of tumor cells. Cells of HepG2, SMMC-7721 and BEL-7402 were transfected with siRNA expressing the siNLRC5 or negative control and plasmid expressing the pEGFP-C2-NLRC5 or Vector. After the transfection for 24 h, cells were trypsinized and seeded in 96-well plates at a density of 4×10^3 cells/well in 200 μ l of complete medium [or containing 2 μ mol/L iCRT3 (Sigma-Aldrich, USA) in the inhibition assay]. 20 μ l of 5 mg/ml MTT (Sigma, USA) was put into each well at 0, 24, 46, and 72 h and incubated at 37°C for 4 h, and then the medium was replaced by 150 μ l of DMSO (Sigma, USA) and shaken at room temperature for 10 min. The absorbance was measured at 490 nm.

2.8 Cell cycle analysis

For transfection for 48 h, the cells were trypsinized and washed in PBS (Phosphate Buffered Saline), then fixed in 70% ethanol at 4°C overnight. Cell cycle analysis was prepared by a cell cycle detection kit (BD Corp., USA). After that, the cells were incubated in propidium iodide (PI) at 37°C for 30 min in the dark. The cell cycle was analyzed by flow cytometer (BD FACSVerse, USA).

2.9 Cell wound healing assay

Wound healing assay was used to assess cell migration. After the appropriate treatments, cells (1×10^5) were trypsinized and seeded into a 6-well plate and allowed to grow to 75% confluence in a complete medium, wounded by a sterile pipette tip, and then we washed with PBS for several times to remove cell debris and incubated for 48 h with serum-free medium (or containing 2 $\mu\text{mol/L}$ iCRT3 in the inhibition assay). During incubation at 37°C, cells migrated into wound surface which was considered as the process of in vitro healing. The wound healing in vitro was photographed by an inverted fluorescence microscope and assessed the rate of closure. The rate of wound healing = $[(\text{the wound width of 0 h} - \text{48 h}) / \text{0 h wound width}] \times 100\%$.

2.10 Cell transwell migration and invasion analysis

The transwell 24-well Boyden chamber (Corning, USA) with 8.0 μM pores size polycarbonate membrane was used for the migration and invasion assay according to the manufacturer's protocol. For migration assay, after the appropriate treatments, cells were trypsinized and seeded in the upper chamber at a density of 1×10^5 cells/well in 100 μl of serum-free medium. 600 μl complete medium was adding to the lower chamber as a chemoattractant. After incubation for 24 h at 37°C, cells

remaining at the upper surface of the membrane were removed with cotton swabs. The cells on the lower surface of the membrane are representative of the migrated cells. After fixation with 4% paraformaldehyde and staining with crystal violet solution, the cells passed through the filter were photographed by an inverted fluorescence microscope. The cell invasion assay was carried out similarly, except that 100 μ l of 1:8 DMEM-diluted Matrigel (BD, USA) was added to each well at 37°C for 6 h before cells were seeded onto the membrane, followed by incubating for 48 h.

2.11 Stable Cell Line Establishment Assay

HepG2 cells were transfected with the plasmid of pFU-GW-shNLRC5 (shNLRC5) or pFU-GW-Scramble (control) using LipofectamineTM2000 (Invitrogen, USA) according to the instructions. After 24 h, HepG2 cells were grown in culture medium containing 500 ng/ml puromycin (Invitrogen, USA) and after 14 days, puromycin-resistant clones were formed. Resistant clones were resuspended and cultured in 96-well plates, one cell in each well. After 15-30 days, resistant clones formed from one cell were resuspended and expanding culture in new culture dish. The plasmid of short hairpin RNAs (shRNA) against human NLRC5 (pFU-GW-shNLRC5) and the control nonsense shRNA (pFU-GW-Scramble) were purchased from Shanghai GenePharma. The sequences were as follows: shNLRC5, 5'-GATAGAGAATCTCAGCTTT-3'. Control, 5'-TTCTCCGAACGTGTCACGT-3'.

2.12 Tumor Formation Assay in Nude Mice

6-week-old male BALB/c nude mice (n = 10) were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and housed under pathogen-free conditions in animal experiment center of Anhui Medical University (Hefei, China). Cultured plenty HepG2 cells of control and shNLRC5, and then resuspended cells with PBS. Mice were randomly divided into two groups, five each

group. 6×10^6 cells (shNLRC5 or control) in 100 μ l PBS were inoculated subcutaneously into the left armpit of each mouse. After 25 days, tumor diameters were measured with a vernier caliper every three days and tumor volumes were calculated using the equation of tumor volume (cm^3) = $0.5 \times \text{tumor length (cm)} \times \text{tumor width}^2 (\text{cm}^2)$. After 6 weeks, mice were all sacrificed, and tumors were separated, taken a picture and weighed.

2.13 Statistics analysis

All statistical calculations were carried out with the SPSS 17.0 Software. The statistical significance of differences was determined by either the Student's *t* test for comparison between means or one-way analysis of variance (ANOVA). Data were considered to be statistically significant when $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1 NLRC5 is over-expressed in HCC tissue samples and cell lines

To clarify whether protein levels of NLRC5 are differed between normal liver tissues versus cancerous tissues, we performed immunohistochemistry on the liver tissues. As shown in (Fig. 1A), the significant high levels of NLRC5 were detected in tumors of livers compared with normal tissues. We collected three HCC cell lines including HepG2, SMMC-7721 and BEL-7402 for investigation. Meanwhile, the L02 cell was included as the normal control for liver cell lines. Expression of NLRC5, β -catenin, C-myc, cyclinD1 and MMP-7 protein extracted from HepG2, SMMC-7721 and BEL-7402 and L02 cells were quantified by Western blot. Results indicated that the protein expression of NLRC5 in HepG2, SMMC-7721 and BEL-7402 cell lines were much higher in comparison with that in control normal L02 cell (Fig. 1B). As

shown in Fig. 1C, the higher protein level of β -catenin, C-myc, cyclinD1 and MMP-7 was confirmed in HepG2, SMMC-7721 and BEL-7402, compared with those of L02 cells. The results indicated that NLRC5 was over-expressed in the three HCC cell lines.

3.2 NLRC5 promotes proliferation, migration and invasion of HCC cells

Next, the functional role of NLRC5 in HCC was further detected. The mRNA and protein expressions of NLRC5 in each cell were measured with quantitative PCR and Western blot. The tumor cells derived from siNLRC5-transfectants showed a much lower level of NLRC5 mRNA and protein in comparison with that derived from NC (negative control) or nontransfected HepG2, SMMC-7721 and BEL-7402 cells (Fig. 2A, C, E). However, compared with the cells which without transfection or transfected with Vector, the levels of NLRC5 mRNA and protein were greatly up-regulated in HepG2, SMMC-7721 and BEL-7402 cells transfected with NLRC5 (Fig. 2B, D, F). MTT assay was performed to assess the effect of NLRC5 on cell proliferation. The knockdown of NLRC5 by siRNA in HepG2, SMMC-7721 and BEL-7402 cells showed lower proliferation rate than the negative control cells (Fig. 3A, B, C). Conversely, enforced over-expression of NLRC5 of HepG2, SMMC-7721 and BEL-7402 cells exhibited higher proliferation rate, compared with control group (Fig. 3D, E, F). We also carried out cell cycle analysis to corroborate the data of proliferation. The results showed that reduced expression of NLRC5 was associated with G1 cell cycle arrest, as evidenced by the increased the percentage of G1 and the reduced percentage of S and G2/M (Fig. 3G, H, I). We next investigated the potential role of NLRC5 in modulating the ability of HCC cells to invade and migrate. The results of wound healing assays indicated that siNLRC5- transfectants showed slower rates of wound healing compared with that transfected with negative control in HepG2, SMMC-7721 and BEL-7402 cells (Fig. 4A, C, E), vice versa, NLRC5-transfectants of HepG2, SMMC-7721 and BEL-7402 cells displayed a tendency of faster healing compared with that transfected with Vector (Fig. 4B, D, F).

To further identify these observations, transwell assays without Matrigel exhibited that siNLRC5-transfectants of SMMC-7721 and BEL-7402 cells displayed a lower ability of migration, compared with that derived from negative control cells (Fig. 5A, C). In contrast, NLRC5-transfectants showed a higher ability of migration, compared with that derived from Vector transfected SMMC-7721 and BEL-7402 cells (Fig. 5B, D). Next, we determined the role of NLRC5 in tumor cell invasion. Transwell assays with Matrigel revealed that lower expression of NLRC5 exhibited significantly reduced rate of invasion compared with that derived from negative control transfected SMMC-7721 and BEL-7402 cells (Fig. 5E, G), however, over-expression of NLRC5 invaded faster compared with that derived from Vector transfected SMMC-7721 and BEL-7402 cells (Fig. 5F, H). Therefore, these results demonstrated that the NLRC5 expression levels are positively correlated with HCC cells migration, invasion and proliferation potentialities, suggesting that NLRC5 is implicated in tumor progression of HCC.

3.3 NLRC5 is associated with β -catenin in HCC cells

Then we investigated a potential mechanism for NLRC5 mediated HCC cell proliferation, migration and invasion. The Wnt/ β -catenin signaling pathway is known to stimulate cell proliferation, and the critical protein of β -catenin is abnormally altered in HCC (de La Coste et al. 1998). However, whether NLRC5 interacts with β -catenin in HCC remains elusive. We analyzed β -catenin expression using Western blot in L02, HepG2, and SMMC-7721, BEL-7402 cells. Of great interest, the NLRC5 level was positively correlated with β -catenin expression (Fig. 1B, C). Consistently, silencing of NLRC5 remarkably inhibited the mRNA and protein level of β -catenin in HepG2, and SMMC-7721, BEL-7402 cells, compared with those of control cells (Fig. 6A, C, E). More interestingly, we observed that the up-regulation of NLRC5 expression was positively correlated with the enhanced mRNA and protein levels of β -catenin in HepG2, and SMMC-7721, BEL-7402 cells (Fig. 6B, D, F). These

findings indicated that β -catenin may be a target downstream of NLRC5 in HCC cells.

3.4 NLRC5 promotes the activation of Wnt/ β -catenin signaling pathway

Some oncogenic signaling pathways, such as the Wnt/ β -catenin signaling pathway, are associated with tumor cells proliferation, invasion and migration (Klaus and Birchmeier 2008). To determine the mechanism underlying NLRC5-induced tumor cell proliferation, invasion and migration, we measured the protein levels of its downstream target C-myc, cyclinD1 and MMP-7 when NLRC5 expression levels were modulated in HepG2, SMMC-7721 and BEL-7402 cells. Knocking down of NLRC5 by siRNA significantly decreased the protein levels of C-myc, cyclinD1 and MMP-7, compared with that transfected with negative control in HepG2, SMMC-7721 and BEL-7402 cells (Fig. 7A, B, C). In contrast, the expression of C-myc, cyclinD1 and MMP-7 protein were dramatically up-regulated after transfection with NLRC5, compared with that derived from Vector transfected HepG2, SMMC-7721 and BEL-7402 cells (Fig. 7D, E, F). In the presence of specific β -catenin inhibitor iCRT3, up-regulation of NLRC5 in BEL-7402 cells transfected with NLRC5 had no more enhanced effects on the proliferation and migration. (Fig. 8A, B, C). Consequently, in BEL-7402 cells, such positive correlation between NLRC5 expression and the protein levels of β -catenin, C-myc, cyclinD1 and MMP-7 disappeared when the inhibitor iCRT3 was added (Fig. 8D). These data together demonstrated that the enhancement of NLRC5 is associated with the increased proliferation, invasion and migration of HCC cells, which may be dependent on the activation of β -catenin and the downstream target components of Wnt/ β -catenin signaling pathway. Therefore, our findings suggested the mechanism insight in NLRC5-promoted HCC progression was related with the Wnt/ β -catenin signaling pathway.

3.5 NLRC5 knock-down inhibited tumor growth *in vivo*

We discovered that NLRC5 knock-down could restrain the clone-forming ability of HepG2 cells *in vitro*. In order to prove the effect of NLRC5 on the tumor formation *in vivo*, We first established stable HepG2 cell line of shNLRC5 and the Scramble shRNA was used as a negative control (Fig. 9A). The shNLRC5 decreased the expression of NLRC5 significantly compared with control group (Fig. 9B). Nude mice received subcutaneous injection of shNLRC5 or control HepG2 cells to establish the tumor model. After 43 days, tumors were completely stripped and taken photos (Fig. 10A). Our result indicated that NLRC5 knock-down had antitumor activity *in vivo* as witnessed by the reduced tumor volume (Fig. 10B) and tumor weight (Fig. 10C). Western blot showed that NLRC5 knocked-down could inhibit the expression of β -catenin, c-myc and cyclinD1 compared with the control (Fig. 10D). These data together demonstrated that NLRC5 knock-down inhibited tumor growth *in vivo* by blocking the Wnt/ β -catenin signaling pathway in HepG2 cells.

4. Discussion

Hepatocellular carcinoma (HCC) is a rapidly fatal disease due to the rising incidence and high mortality (Imbeaud et al. 2010). HCC is an example of inflammation-related cancer as more than 90 % of HCC arise in the context of hepatic injury and inflammation (Matsuzaki et al. 2007). A key event leading to persistent hepatic injury is liver chronic inflammation, with the consequent of sequential development of fibrosis, cirrhosis, and eventually HCC (Sanz-Cameno et al. 2010). Although many preclinical and clinical researches underscore the importance of chronic inflammation in liver cancer, the clinical application of this knowledge has not been fully accomplished.

Within the past 10 years, significant attentions have been gained into the role of NLRs in host defense and disease. In addition to their roles in pathogen recognition,

new findings have showed that the NLR family members contributed either directly or indirectly to a variety of hallmarks associated with cancer, including inflammation, cell death, tumor growth, angiogenesis, invasion, and metastasis (Allen et al. 2010, Hu et al. 2010, Hu et al. 2013). For example, the activation of NLR inflammasome have been implicated in many types of cancer, including colon cancer, breast cancer and hepatocellular carcinoma (Inoue et al. 2014, Jin et al. 1997, Negash et al. 2013). NLRs resulted in the production of inflammatory cytokines and chemokines by inducing activation of NF- κ B, MAPK, and caspase-1, such as IL-1 β , IL-6, IL-8 and TNF- α (Zaki et al. 2011a). These inflammatory cytokines were shown to induce chronic hepatic inflammation (Capece et al. 2013, Naugler and Karin 2008, Prieto 2008). Chronic inflammation can participate in during all stages of tumorigenesis, depending on the type of inflammatory cytokines (Grivennikov et al. 2010). Similarly to most NLRs, NLRC5 has universally been reported to play a vital role in innate immunity and host physiology. Previous studies suggested that NLRC5 may also regulate innate immune responses through regulating NF- κ B activation and type I interferon (IFN) pathways (Kuenzel et al. 2010). Of note, NLRC5 may promote inflammasome- mediated IL-1 β processing in human and murine cells (Davis et al. 2011). We presumed that NLRC5 activation functions as an innate defense against localized transformation events in early pre-neoplastic situations. However, while clinical pathology is observed, the function of NLRC5 has failed to be detected. Deregulation of NLRC5 may lead to aberrant inflammation, which will further contribute to inflammatory diseases and tumorigenesis. The effect of NLRC5 on hepatocellular carcinoma has not yet to be studied. In this study, we provided evidence that NLRC5 is increased in HCC and may play an important role in controlling the proliferation, migration and invasion of HCC cells via Wnt/ β -catenin signaling pathway.

This study for the first time reveals that NLRC5 is overexpressed in both HCC cell lines and clinical HCC samples. The expression of NLRC5 is positively associated with the proliferation, migration and invasion of HCC. Importantly, we show that the mechanism by which increase of NLRC5 enhances HCC cells'

proliferation, migration and invasion is attributable to activation of Wnt/ β -catenin signaling pathway.

Inspired by the close correlation between NLRs and tumorigenesis, we supposed that NLRC5 may be also involved in tumor malignancy. The proliferation, migration and invasion capacity of cells are three the most important features of malignantly cell behavior. Interestingly, we have found that down-regulation of NLRC5 by siRNA resulted in reduced cell proliferation, decreased migration rate, and impaired invasion ability in HCC cells. Moreover, the cell cycle was arrested in the G0/G1 phase after NLRC5 silencing. Knocking down of NLRC5 caused identical cell phase arrests in different HCC cells. *In vivo*, We further investigated the effect of NLRC5 knock-down on tumor formation ability of HepG2 cells in nude mice. We found that the average weight of tumors formed by the NLRC5 knocked-down HepG2 cells were only half to that of the control cells, which was in agreement with the results *in vitro*. Meanwhile, our findings indicated that up-regulation of NLRC5 significantly promoted the proliferation, migration and invasion of HCC cells. All results confirmed that NLRC5 may play an important role in HCC progression.

Deregulation of Wnt/ β -catenin signaling pathway is considered as a marker of cancers including HCC (Llovet and Bruix 2008). It is well-known that β -catenin is involved in tumor progression through activation of Wnt/ β -catenin signaling pathway and its down-stream proteins (Park et al. 1999). Here, our data suggested that NLRC5 can promote tumor cell proliferation, migration and invasion by stimulating β -catenin transcription and translation through Wnt/ β -catenin signaling pathway in HCC cells. The conclusion is based on the following evidence. Firstly, NLRC5 overexpression not only markedly promoted the level of β -catenin expression, but also enhanced the proliferation, migration and invasion of HCC cells *in vitro*. Moreover, a positive relationship of NLRC5 expression with β -catenin level was found in HCC tissues and cells. Secondly, silencing of NLRC5 not only significantly suppressed β -catenin expression, but also attenuated the proliferation, migration and invasion of HCC cells. Thirdly, when the β -catenin was inhibited by its specific inhibitors, the positive correlation between the NLRC5 abundance and activation of β -catenin was abolished.

At last, *in vivo* NLRC5 knock-down not only inhibited tumor growth but also reduced the expression of β -catenin.

The coordinated up- and down- regulation of multiple genes converges to increase the migration and growth of tumor cell during liver cancer progression (Whittaker et al. 2010). CyclinD1, which can regulate the cell cycle G0/G1 checkpoint for entrance into the cell cycle, has been reported as overexpressed in varied human cancers (Donnellan and Chetty 1998). The C-myc oncogene is identified as a target gene in the Wnt/ β -catenin signaling pathway (He et al. 1998). MMP-7, a secreted protein implicated in destruction of extracellular matrix substrates, is highly expressed in hepatocellular carcinoma and capable to increase the invasive and metastatic potential of tumor cells (Gao et al. 2006, Ochiai et al. 2008, Oshima et al. 2008). In the present study, knockdown of NLRC5 strongly inhibited the proliferation, migration and invasion of HCC cells, which were related to a decrease of the expressions of C-myc, cyclinD1 and MMP-7 at proteins levels in HCC cells. In addition, stable HepG2 cell line of NLRC5 knock-down could inhibit the expression of C-myc, cyclinD1 compared with the control *in vivo*. Conversely, over-expression of NLRC5 increases the activation of C-myc, cyclinD1 and MMP-7 in HCC cells. Here, we demonstrated that up-regulation of NLRC5 is associated with enhanced of C-myc, cyclinD1 and MMP-7 expression at protein levels and proliferation and migration ability in BEL-7402 cell. In addition, β -catenin specific inhibitors iCRT3 suppressed the protein levels of C-myc, cyclinD1 and MMP-7 expression and attenuated NLRC5-mediated proliferation and migration of BEL-7402 cell. Therefore, these observations indicated that NLRC5 may promote HCC progression through Wnt/ β -catenin-dependent mechanisms and suggest that NLRC5 can be further evaluated as a potential therapeutic target to HCC.

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Conflict of interest

The authors declared that they have no competing interest.

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Figure legend

Fig. 1 NLRC5 is over-expressed in HCC tissue and cells. (A) Normal liver tissue (left panel) and HCC tissue (right panel) were stained by immunohistochemistry with NLRC5 (original magnification $\times 200$). (B) The protein expression of NLRC5 in normal liver cell line (L02) and HCC cell lines (HepG2, SMMC-7721 and BEL-7402). β -actin was used as an internal control. (C) Western blot analysis of β -catenin, C-myc, cyclinD1 and MMP-7 in the same cell lines as described in B. β -actin served as a loading control. The data were normalized to the expression level of these proteins in L02 cells. The bar graphs (mean $\pm$ SEM) and representative images are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the L02 group.

Fig. 2 The expression levels of NLRC5 in untreated HCC cells and HCC cells transfected with siNLRC5 or NLRC5 plasmid were determined by using qRT-PCR and Western blot. (A, C, E) The mRNA and protein levels of NLRC5 were detected in HepG2, SMMC-7721 and BEL-7402 cells without transfection (lane 1) or transfected with negative control (lane 2) or siNLRC5 (lane 3). * $p < 0.05$ or ** $p < 0.01$, compared with the NC group. NC: Negative Control. (B, D, F) Expression of NLRC5 mRNA

and protein were detected in HepG2, SMMC-7721 and BEL-7402 cells without transfection (lane 1) or transfected with vector (lane 2) or NLRC5 (lane 3). $**p<0.01$ or $***p<0.001$, compared with the Vector group. Data from at least 3 independent experiments were measured with the band density, which were normalized to β -actin.

Fig. 3 Effects of NLRC5 on proliferation ability and cell cycle distribution of HCC cells. (A, B, C) Cell growth of HepG2, SMMC-7721 and BEL-7402 cells was determined after transfection with siNLRC5 by using MTT assay at different time points. $*p<0.05$ or $**p<0.01$, compared with the NC group. NC: Negative Control. (D, E, F) The proliferation of HepG2, SMMC-7721 and BEL-7402 cells was determined after transfection with NLRC5 at different time points. $*p<0.05$ or $**p<0.01$, compared with the Vector group. (G, H, I) Knockdown of NLRC5 induced G0/G1 phase arrest in HepG2, SMMC-7721 and BEL-7402 cells. All images of FACS analyses on the upper panel were representative of at least three independent experiments. The graphs on the lower panel show quantification of the band intensity. $**p<0.01$, compared with the NC group. NC: Negative Control.

Fig. 4 Effects of NLRC5 on wound healing ability of HCC cells. (A, C, E) Wound healing assay indicated that NLRC5 down-regulation inhibited HepG2, SMMC-7721 and BEL-7402 cells migration. $**P < 0.01$, compared with the NC group. NC: Negative Control. (B, D, F) The migration of HepG2, SMMC-7721 and BEL-7402 cells was up-regulated after transfection with NLRC5. $*p<0.05$ or $**p<0.01$, compared with the Vector group. All images on the left panel were representative of at least three independent experiments. The graphs on the right panel show the quantification of the band intensity.

Fig. 5 Effects of NLRC5 on the cell migration and invasion ability of HCC cells. (A, C) Transwell migration assays showed that SMMC-7721 and BEL-7402 cells transfected with siNLRC5 inhibited migratory potentials. $**P < 0.01$, compared with the NC group. NC: Negative Control. (B, D) Overexpression of NLRC5 increased the migration rate of SMMC-7721 and BEL-7402 cells. $*p<0.05$ or $**p<0.01$, compared

with the Vector group. **(E, G)** Transwell invasion assays showed that SMMC-7721 and BEL-7402 cells transfected with siNLRC5 inhibited invasive potentials. $**P < 0.01$, compared with the NC group. NC: Negative Control. **(F, H)** Overexpression of NLRC5 increased the invasion rate in SMMC-7721 and BEL-7402 cells. $**p < 0.01$, compared with the Vector group. Cells were counted after staining with crystal violet. Representative images are shown on the upper panel. Graphs on the lower panel indicate the average number of cells per field 24 or 48 h after transfection. The data are representative of at least three independent experiments

Fig. 6 NLRC5 associates with β -catenin in HCC. **(A, C, E)** Western blot and qRT-PCR analysis were respectively used to detect the mRNA and protein levels of β -catenin in HepG2, SMMC-7721 and BEL-7402 cells without transfection (lane 1) or transfected with negative control (lane 2) or siNLRC5 (lane 3). $**P < 0.01$, compared with the NC group. NC: Negative Control. **(B, D, F)** Overexpression expression of NLRC5 increased the mRNA and protein expressions of β -catenin in HepG2, SMMC-7721 and BEL-7402 cells without transfection (lane 1) or transfected with Vector (lane 2) or NLRC5 (lane 3) were analyzed using qRT-PCR and Western blot. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with the Vector group. Data from at least 3 independent experiments were measured with the band density, which were normalized to β -actin. The graphs and representative images are shown.

Fig. 7 The effect of NLRC5 on Wnt/ β -catenin signaling pathway downstream molecules. **(A, B, C)** Western blot analysis of the expressions of cyclin D1, C-myc and MMP-7 proteins in HepG2, SMMC-7721 and BEL-7402 cells transfection with siNLRC5. $*P < 0.05$ or $**P < 0.01$, compared with the NC group. NC: Negative Control. **(D, E, F)** The expressions of cyclin D1, C-myc and MMP-7 were determined in HepG2, SMMC-7721 and BEL-7402 cells transfected with NLRC5 by using Western blot. $*p < 0.05$ or $**p < 0.01$, compared with the Vector group. Representative images from three independent experiments are shown on the upper panel. The graphs on the lower panel show the quantification of the band intensity.

Fig. 8 NLRC5 activates Wnt/ β -catenin signaling pathway in HCC cells. (A) The proliferation was determined in BEL-7402 cells transfected with NLRC5 in the presence of β -catenin specific inhibitor iCRT3 (2 μ mol/L) at different time points. (B, C) The migration was determined in BEL-7402 cells transfected with NLRC5 in the presence of β -catenin specific inhibitor iCRT3 (2 μ mol/L). (D) The expressions of β -catenin, C-myc, cyclinD1 and MMP-7 at protein level in the presence of β -catenin specific inhibitor iCRT3 (2 μ mol/L) were determined by immunoblotting of BEL-7402 cells after transfection with NLRC5. * p <0.05 or ** p <0.01, compared with the Vector group. Representative images were representative of at least three independent experiments. The graphs show the quantification of the bands intensity.

Fig. 9 Establishment of HepG2 stable cell line with down-regulated NLRC5. (A) Stable HepG2 cell lines of the control (upper panel) and shNLRC5 (lower panel) were observed by inversed fluorescent microscope. (B) The protein expression of NLRC5 in control group and shNLRC5 group. β -actin was used as an internal control. The bar graphs (mean $\pm$ SEM) and representative images are shown. * p <0.05, ** p <0.01, *** p <0.001 compared with the control group.

Fig. 10 NLRC5 knock-down inhibits *in vivo* tumor formation ability of HCC HepG2 cell line in nude mice. Stable HepG2 cell lines of the control and shNLRC5 were implanted subcutaneously in the left flank of nude mice. (A) After 43 days, mice were sacrificed and tumors were excised. (B) NLRC5 knock-down had antitumor activity *in vivo* as evidenced by the smaller tumor volumes, (C) as well as tumor weight (n=5) compared with the control. (D) Western blot showed the expression of β -catenin, c-myc and cyclinD1 in tumor tissues of control and shNLRC5 groups. β -actin was used as an internal control. The bar graphs (mean $\pm$ SEM) and representative images are shown. * p <0.05, ** p <0.01, *** p <0.001 compared with the control group.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5**A** **B** **C** **D**

Fig.5
E

F

G

H

Fig.6**A** **B** **C** **D** **E** **F**

Fig.7

Fig.8

Fig.8

Fig.9

Fig.10

A

Control

shNLRC5

B

C

Fig.10

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

Parameter	Patients
Case, <i>n</i>	11
Sex, <i>n</i> (%)	
Male	6(54.5%)
Female	5(45.5%)
Age, <i>n</i> ($\pm$ SD)	52.7($\pm$ 10.0)
Compensation/decompensation, <i>n</i> (%)	
Compensation	3(27%)
Decompensation	5(46%)
Unknown	3(27)
Etiology, <i>n</i> (%)	
Alcoholic	3(27%)
HBV	5(46%)
HCV	2(18%)
Others	1(9%)