Research paper

Aspirin targets P4HA2 through inhibiting NF-κB and LMCD1-AS1/let-7g to inhibit tumour growth and collagen deposition in hepatocellular carcinoma

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Abstract

Background: Abnormal construction of the extracellular matrix (ECM) is intimately linked with carcinogenesis and the development of solid tumours, especially hepatocellular carcinoma (HCC). As the major component of the ECM, collagen plays a pivotal role in carcinogenesis. P4HA2, the essential enzyme during collagen formation, becomes an important target in HCC treatment. Here, we tried to decipher whether aspirin (ASA), a classic anti-inflammatory drug, could improve the prognosis of HCC through targeting P4HA2.

Methods: Western blotting, qRT-PCR assay, immunofluorescence staining, luciferase reporter gene assay, and ChIP assay were applied to demonstrate the molecular mechanism of the regulation of P4HA2 expression by aspirin. A mouse xenograft model, cell viability assay, colony formation assay, and immunohistochemistry analysis were used to evaluate the anti-fibrosis effect of aspirin through targeting the NF-κB/P4HA2 axis and LMCD1-AS1/let-7g/P4HA2 axis in vitro and in vivo. The TCGA database was used to evaluate the correlation among P4HA2, let-7g, LMCD1-AS1 and overall survival of HCC patients.

Findings: In xenograft mice, aspirin was capable of targeting P4HA2 to decrease collagen deposition, resulting in the inhibition of liver tumour growth. TCGA database analysis revealed the close association between a higher P4HA2 concentration in HCC patients and shorter overall survival or a higher cancer stage and the pathological grade. Mechanistically, NF-κB can bind to the promoter of P4HA2 to activate its transcription. Moreover, IncRNA LMCD1-AS1 functions as a molecular sponge of let-7g to post-transcriptionally induce the target gene of let-7g, namely, P4HA2.

Interpretation: Our findings disclose the novel role and regulatory mechanism of aspirin in the suppression of HCC by disrupting abnormal collagen deposition.

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1. Introduction

Hepatocellular carcinoma (HCC) accounts for almost 90% of liver cancer. Regardless of aetiology, HCC is a typical inflammation-related cancer; chronic liver inflammation can transform to liver cirrhosis and eventually lead to the development of tumours [1–3]. It is worthy to note that the deposition of the extracellular matrix (ECM) plays a momentous role in the fibrosis process [4–6]. The biochemical and biomechanical properties of the ECM are pivotal for the integrity, nutrition and differentiation of tumour cells. Moreover, the increased deposition of the ECM and its stiffening lead to enhanced cell growth, survival, migration, adhesion, angiogenesis and consequently cancer progression [7,8]. The construction of the ECM contains various types of collagen as well as other non-collagenous constituents, such as elastin, fibronectin, laminin
Research in context

**Evidence before this study**

Chronic liver inflammation can transform to liver cirrhosis and eventually lead to hepatocellular carcinoma (HCC), a typical inflammation-related cancer. The deposition of the extracellular matrix (ECM), especially for collagen, takes great part in the fibrosis and the associated cancer. An essential enzyme for collagen formation, collagen prolyl 4-hydroxylase α2 (P4HA2) is implicated in the development of cancer. Recent research reveals the effect of a classic anti-inflammation drug, aspirin, on cancer including colorectal cancer, ovarian cancer, breast cancer, and prostate cancer. However, the role of aspirin in liver cancer treatment and its target remain unclear.

**Added value of this study**

In this study, we found that aspirin resisted the collagen deposition through targeting P4HA2, leading to the suppression of liver tumour growth. *In vitro and in vivo* experiments revealed that in aspirin-restrained HCC, NF-κB/p65, a classic downstream factor of aspirin is responsible for the P4HA2 activation. Meanwhile, lncRNA LMCD1-AS1 as a sponge quenched let-7g and eventually induced one target gene of let-7g, P4HA2. TCGA database indicated that the expression of P4HA2, let-7g and LMCD1-AS1 is closely correlated with overall survival of HCC patients.

**Implications of all the available evidence**

This study identifies that aspirin suppresses collagen deposition and subsequent liver tumour growth through targeting the axis of NF-κB/P4HA2 and LMCD1-AS1/let-7g/P4HA2. This study may provide positive evidence for the clinical use of aspirin in the field of HCC prevention and therapy.

and hyaluronic acid. The principle structural elements of the ECM are, however, formed by collagen [9,10]. Collagen consists of intimately related but characteristic extracellular constitutive proteins, of which collagen type I, II and III are major types in different organs. Collagen type I has been reported to participate in breast carcinoma of which collagen type I, II and III are major types in different organs. However, the role of aspirin in liver cancer treatment and its target remain unclear.

Over the past decade, new technologies have shown that the non-coding RNAs (ncRNAs) have a tremendous impact on the regulation of gene expression, the cell signalling pathway, and the development and therapy of diseases including cancer [34,35]. Long ncRNAs (lncRNAs) and microRNAs (miRNAs) are the most well-studied ncRNAs in cancers. The most extensive function of the miRNAs is the degradation of mRNA or the inhibition of gene translation by binding to the 3′UTRs of the mRNAs [36]. Additionally, the lncRNAs can modulate gene expression in a posttranscriptional manner by sequestering miRNAs to keep them away from their target genes. Studies have shown that aspirin regulates cell proliferation, apoptosis, migration and other aspects through interfering with some transcription factors, such as NF-κB, oestrogen receptor (ER) and p53 [37–39]. Meanwhile, an increasing number of studies indicated the important role of miRNAs such as miR-9 and miR-21 in aspirin-involved cancer treatment [40,41]. Based on the regulation of aspirin on non-coding RNAs and the important role of miRNAs in affecting gene expression at the posttranscriptional level, we wonder if there exists the potential lncRNA/miRNA axis during the process of aspirin inhibiting P4HA2 expression. In the present study, we are interested in searching for the aspirin-targeted transcription factors and ncRNAs in HCC development induced by collagen deposition via P4HA.

In this study, we aim to decipher the mechanism involved in the aspirin-induced inhibition of collagen deposition-associated HCC. We disclose the novel regulatory mechanism of P4HA2 expression which includes the transcriptional regulation by NF-κB and posttranscriptional regulation by LMCD1-AS1/let-7g. It is intriguing that aspirin could simultaneously decrease the level of P4HA2 via these two pathways. Our findings could probably provide an effective therapeutic strategy for HCC.

2. Materials and methods

2.1. Materials and cell lines

HepG2 and Huh-7 cell lines were acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2-pcDNA was stably transfectcd with pcDNA3.1 + and HepG2-P4 was stably transfected with pcDNA-P4HA2. All cell lines were cultivated in 10% foetal bovine serum (FBS, Gibco, Waltham, MA, USA) supplemented with DMEM media (Gibco) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO2. The reagents used in this study were aspirin (Sigma-Aldrich), PDTC (Sigma-Aldrich, St. Louis, MO, USA) and TNF-α (Peprotech, Suzhou, Jiangsu, China). The applied concentration of aspirin, PDTC and TNF-α was indicated within the following related method section.
2.2. Primary hepatocyte cells

The tumour sample was obtained from the surgical ward, and the information of the patient is supplied in Supplementary Table S2. The tumour sections were cut into tiny pieces and transferred to incubating dishes. The primary human hepatocytes were incubated in William’s medium E (Invitrogen) containing with penicillin (100 U/mL) and streptomycin (100 μg/ml), 2 mM of l-glutamine, and, additionally, with 10% FBS and 100 nM of dexamethasone for the first 12 h.

2.3. Plasmid construction and small interference RNAs (siRNAs)

The promoter region (from −1706 to −335) of P4HA2 was obtained and inserted into the pG3-L-Basic vector (Promega, Madison, WI, USA) using the KpnI/Xhol site, named pGL3-P0. To construct the luciferase reporter plasmids of the various truncated promoter regions of P4HA2, the regions (−1302/-335, −932/-335, −609/-335 and −553/-335) were inserted into the pGL3-Basic vector, named pGL3-P1, pGL3-P2, pGL3-P3 and pGL3-P4, respectively. The mutant sequence of pGL3-P0 carried a substitution of nucleotides within three binding sites of NF-κB/p65 that were constructed and named p65-mut 1 (site 1 mutated), p65-mut 2 (site 2 mutated), p65-mut 3 (site 3 mutated) and p65-mut 1 + 3 (site 1 and 3 dually mutated). All primers are listed in table S5. The 3’UTR sequence of P4HA2 was constructed into the FseI/Xhol site, which was the downstream of the luciferase gene in the pGL3-Control vector (Promega), named pGL3-P4-UTR-wt. The site-directed mutation of the let-7g target site in pGL3-P4-UTR-wt was named pGL3-P4-UTR-mut. All primers are listed in Supplementary Table S1. The P4HA2 stably overexpressed HepG2 cell line, i.e., HepG2-p65, was screened by G418 after transfection with the previously constructed plasmid pCDNA3-P4HA2 [33]. The pcDNA-LMCD1-A51 plasmid, all siRNAs, miRNAs, the let-7g inhibitor, and the miRNA control mimics are purchased from RiboBio and the sequences are listed in Supplementary Table S1.

2.4. Luciferase reporter gene assay

HepG2 cells were plated onto 24-well plates at a density of 4 × 10^4 cells per well. The cells were co-transfected with reporter gene plasmids (pGL3-P0, pGL3-P1, pGL3-P2, pGL3-P3, pGL3-P4, p65-mut 1, p65-mut 2, p65-mut 3, p65-mut 1 + 3, pGL3-P4-UTR-wt and pGL3-P4-UTR-mut) at a dose of 100 ng/well and pRL-TK plasmids (40 ng/well) (Promega) and corresponding siRNAs, miRNA and miRNA inhibitors. The cells were collected after 48 h and the luciferase activity was evaluated according to the manufacturer’s instructions provided by Promega. Each experiment was repeated at least three times.

2.5. Western blotting

The western blotting assay was performed as previously reported [42]. Total proteins were extracted from the cells after corresponding treatments, and the same amount of protein from each sample was analysed. The primary antibodies, namely P4HA2, Col I, Col IV, p65 and β-actin, were purchased from Proteintech (Chicago, IL, USA) and diluted as recommended by the instructions. Next, the secondary antibodies such as goat anti-rabbit (Sigma-Aldrich) or anti-mouse antibody (Sigma-Aldrich) were incubated with the blots and visualized using Bio-Rad GelDoc system.

2.6. RNA extraction, RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent as described previously [43]. Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega), according to the manufacturer’s protocols. For each sample, 1 μg RNA was reverse transcribed into cDNA. The mRNA levels were measured by RT-PCR and qRT-PCR using the SYBR PCR Master Mix (Takara, Dalian, China). The relative quantification of the mRNAs was performed according to the comparative method (2-ΔΔCT, Applied Biosystems User Bulletin no. 2P/N 4303859), and the ΔCT value for each sample was the average of triplicates. The specific primers used in these experiments are listed in Supplementary Table S1.

2.7. Immunofluorescence staining

Cells were cultured on gelatin-coated glass coverslips for 24 h and treated with aspirin (4 mM), PDTC (30 μM), TNF-α (20 ng/ml) or corresponding RNAs. Then, the cells were fixed in 4% paraformaldehyde for 20 min at 4 °C. After thrice washing the cells in PBS and permeabilizing them in 2% Triton X-100 (Sigma-Aldrich), the cells were blocked with 2% foetal bovine serum albumin (BSA, Sigma-Aldrich) for 30 min. Thereafter, the cells were incubated over night with 2% BSA and the P4HA2, Col I or p65 antibodies at 4 °C. After incubation, the cells were washed three times with PBS and incubated in 2% BSA with the anti-rabbit IgG-PE secondary antibody at room temperature for 30 min. The samples were counterstained with DAPI and analysed using fluorescence microscopy (BX63, Olympus).

2.8. Chromatin immunoprecipitation (ChIP) analysis

HepG2 cells were treated with aspirin (4 mM) or TNF-α (20 ng/ml) as indicated in the figure legends. The cells were fixed with 1% formaldehyde and lysed, and then the DNA was sheared by sonication. Next the DNA samples were precipitated and collected with normal mouse IgG as the negative control, anti-RNA polymerase II as positive control or anti-p65 antibody. Subsequently, the cross-linked DNA samples were reversed and purified as templates for further PCR analysis. The primers used in this experiment are listed in Supplementary Table S1. The ChIP-enriched DNA analysed using qRT-PCR was normalized to input DNA, and followed by subtracting the nonspecific binding determined by the control IgG.

2.9. Tumour xenograft assay

Five-week-old male BALB/c-nu/nu athymic nude mice were fed and housed. All animal procedures were performed under the procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tumours were established by subcutaneous injection of indicated cell lines at the concentration of 2 × 10^6 cells/ml in 0.2 ml of 1:1 saline/Matrigel (BD Biosciences) mixture. Daily oral administration of saline or aspirin at 75 mg/kg was initiated after the tumour size exceeded 100 mm^3 approximately 10–14 days after injection. Five mice were assigned to each group. The tumour volume and body weight of the mice were assessed every 3 days. The mice were sacrificed when the tumour size reached approximately 1000 mm^3. The tumour volume (V) was calculated using the following formula: (length × width^2)/2. No side effects or mortality were observed in this experiment.

2.10. Histological analysis

The tumours from the mice who received the indicated treatment were fixed with 4% paraformaldehyde and sectioned for further experiments. Masson’s trichrome staining was processed according to standard methods (Solarbio). For the immunohistochemical (IHC) assay, the tissues were incubated with primary antibodies diluted as indicated in the materials and methods sections. Digital images of 5 random regions were taken for each tissue section. The staining of each section was calculated using Image-Pro Plus software.
2.11. MTT

The cell proliferation ability was determined using the MTT assay. Cells were seeded onto 96-well plates with at least three replicates at a density of 3000 cells per well. After 10 h of incubation to form a confluent monolayer, the media were replaced with media containing aspirin (4 mM) and/or TNF-α (20 ng/ml) for another 24 h. Then, 10 μL MTT [5 mg mL⁻¹ in phosphate buffered saline (PBS)] was added to each well. Four hours later, the medium was removed, and the MTT formazan was dissolved in 150 μL DMSO per well. The absorbance values were measured at OD490nm using an absorbance reader.

2.12. Colony formation

For the clonogenicity analysis, the cells were seeded onto 12-well plates at a density of 500 cells per well. Twenty-four hours later, different treatments were administered. The cells were subsequently incubated for another 15–20 days. After the formation of monoclonal colonies, the cells were stained with crystal violet.

2.13. Statistical analysis

The results are expressed as the means ± standard deviation (SD). Multiple comparisons were performed using one-way ANOVA followed by the LSD as a post-hoc test. Significant differences between the two groups were analysed using the Student's t-test. In this study, we used Kolmogorov-Smirnov test and Shapiro-Wilk test to verify normal distribution. When the data did not fit the normal distribution, Kruskal-Wallis or Mann-Whitney test were applied. The non-significant difference was marked as N. The criteria for statistically significant differences were considered as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

2.14. Public database

Public TCGA (https://portal.gdc.cancer.gov/) data repositories for live hepatocellular carcinoma (LIHC) (Cancer Genome Atlas Network, 2014) and NCBI Gene Expression Omnibus (GEO) databases (GEO: GSE76427 is available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76427) were used as the sources for the sample data. For the analysis of the LIHC TCGA sets, we used mRNA expression (by RNA sequencing). The Kaplan-Meier method and log-rank test were used to compare survival between the indicated groups identified by the median of the mRNA levels. The primary clinical data of TCGA is provided in Supplementary Table S3.

3. Results

3.1. Aspirin reduces the collagen deposition-associated growth of liver tumour in mice

Aspirin is reported to exert an anti-proliferative effect in multiple cancers [21,44–46], yet its molecular mechanism needs to be further clarified. To decipher this question, we performed a xenograft experiment with the hepatocellular carcinoma (HCC) cell line HepG2, and treated the tumour-bearing mice with saline or aspirin (75 mg/kg/day). Administration with aspirin reduced the tumour size and weight (Fig. 1A and B) without harming the body weight of the BALB/c-nu/nu mice (Fig. 1C). Moreover, the decreased ratio of Ki 67 staining confirmed weakened proliferation under aspirin treatment (Fig. 1D). Collagen fibres are greatly implicated in the proliferation and metastasis of cancers [14]. In this current investigation, we are interested in whether the collagen deposition takes part in the aspirin-inhibited growth of liver cancer. We observed obvious collagen deposition in the HepG2 group using Masson's trichrome staining. Interestingly, aspirin treatment decreased the deposition of collagen (Fig. 1E). Among several members of the collagen family, collagen type I and IV were revealed to be closely associated with the progression of many cancers [14,47,48]. Immunoblotting and immunohistochemistry (IHC) staining revealed that aspirin could dampen the expression of collagen type I and IV in aspirin-treated group (Fig. 1F and G) and could also impair the level of a hepatocyte-derived marker of fibrogenesis, CTGF (Fig. 1H). Collectively, we conclude that aspirin interrupts collagen deposition and further fibrosis-associated liver tumour development.

3.2. Aspirin targets P4HA2 to hamper collagen synthesis in liver tumour growth

To determine the genes that play a critical role in the suppression of collagen deposition by aspirin, we analysed the expression of the P4HA family in 371 cases of HCC patients from the TCGA and 228 cases of HCC patients from GEO database. We found that among the P4HA family members (P4HA1, P4HA2 and P4HA3), a higher level of P4HA2 was significantly associated with a shorter overall survival period of liver tumour patients (Fig. 2A; Supplementary Fig. S1A). In 50 paired liver tumour tissues and para-tumour tissues among 371 cases of HCC patients from TCGA database, only the level of P4HA2 mRNA was upregulated (Supplementary Fig. S1B). Additionally, the level of P4HA2 mRNA was gradually increased with the cancer stage and pathological grade (Supplementary Fig. S1C and D). To study whether aspirin could affect the expression of P4HA2, we evaluated the level of P4HA2 in the tumour samples from mice of the aspirin-treated and control groups. Immunoblotting and IHC staining revealed that the expression of P4HA2 was decreased in mouse tumours treated with aspirin (Supplementary Fig. S1, E and F). Next, we investigated the effect of aspirin on P4HA2 in HCC cells. We treated hepatoma HepG2 and Huh-7 cells with elevated concentrations of aspirin (0, 2, and 4 mM). Our results revealed that the expression of both P4HA2 and its downstream protein, collagen type I declined at the levels of mRNA and protein (Fig. 2B and C; Supplementary Fig. S1, G and H). Immunofluorescence (IF) staining also showed that aspirin treatment could decrease P4HA2 expression and the deposition of collagen type I, the downstream protein of P4HA2 (Fig. 2D; Supplementary Fig. S11). These data validate P4HA2 as an attractive target for aspirin in HCC treatment.

To further confirm the role of P4HA2 in aspirin-reduced tumourigenesis, we subcutaneously implanted HepG2 and P4HA2-overexpressed HepG2 cell lines into the BALB/c-nu/nu mice, and then the mice were treated with aspirin or saline as the control. We found that the tumour size, volume and weight were elevated in the P4HA2 overexpression group compared with that in the HepG2 group, while this elevation could be retarded by oral gavage of aspirin (Fig. 2, E and F; Supplementary Fig. S1J). In accordance with the tumour growth curve, the staining of Ki 67 showed activated-cell proliferation in the P4HA2-overexpression group and impaired growth after administration of aspirin (Supplementary Fig. S1K). Masson's trichrome staining (Fig. 2G), western blotting assay (Fig. 2H) and IHC staining (Fig. 2I) indicated that P4HA2 overexpression induced the deposition of collagen and aspirin treatment tempered this process. MTT assay also indicated that aspirin could impair the enhanced proliferation of primary HCC cells triggered by P4HA2 overexpression (Supplementary Fig. S1L). Thus, our data imply that aspirin can destroy P4HA2-induced collagen deposition to depress liver tumour growth.

3.3. NF-κB/p65 is responsible for activating the promoter of P4HA2 in aspirin-regulated hepatoma

Taken a step further, we determined whether aspirin could regulate the promoter activity of P4HA2. We predicted the promoter region of P4HA2 (−1706 to −335) through the UCSC Genome Browser (https://genome.ucsc.edu/) and cloned this region into the pGL3-basic vector (named pGL3-P0). As shown in Fig. 3A, the luciferase activity critically declined along with the elevated concentration of aspirin in the HepG2 cell line. We next constructed four truncated promoter regions
shorter than pGL3-P0, including −1302/−335 (pGL3-P1), −932/−335 (pGL3-P2), −609/−335 (pGL3-P3) and −553/−335 (pGL3-P4). Compared with pGL3-P0, the inhibitory rate of aspirin on pGL3-P1, pGL3-P2 and pGL3-P3 activity was inhibited. In addition, pGL3-P4 activity showed a collapse under aspirin treatment, indicating that the fragments −1706/−1302 and −609/−553 were probably the key regions of the P4HA2 promoter upon aspirin treatment (Fig. 3B). Interestingly, the prediction of the transcription factors on the key regions using the JASPAR database and the ALGGEN website determined that NF-κB/p65, a known target of aspirin, was the promising candidate since it has three binding sites in the two core regulating regions. The use of PDTC, the NF-κB/p65 inhibitor, could dose-dependently inhibit the expression of P4HA2 at both mRNA and protein levels, similar to the aspirin-treated groups (Fig. 3C and Supplementary Fig. S2A). Moreover, administration of the NF-κB/p65 activator, TNF-α, led to the enhanced expression of P4HA2 and this enhancement could be inhibited by the addition of aspirin via RT-PCR, immunoblotting and IF assays (Fig. 3, D and E and Supplementary Fig. S2B). We further conducted a knockdown experiment to confirm the role of NF-κB/p65 in the regulation of P4HA2 activation. The efficiencies of p65 siRNAs were estimated using RT-PCR and western blotting (Supplementary Fig. S2. C), and the effective siRNA, si-p65 #2, was used to perform further experiments. The results of luciferase reporter gene assay (Fig. 3F), RT-PCR, western blotting and IF assays (Fig. 3, G and H) revealed that the knockdown of NF-κB/p65 by p65 siRNAs impaired the transcription and expression of P4HA2. Analysis of the TCGA database also indicated the positive association between the mRNA levels of P4HA2 and NF-κB/p65 (Supplementary Fig. S2D).

Next, we performed the ChIP assay to determine the direct binding of NF-κB/p65 to the P4HA2 promoter. The data of RT-PCR and qRT-PCR showed attenuated binding under aspirin treatment (Fig. 3I). TNF-α, the NF-κB/p65 activator, could dose-dependently strengthen the binding (Fig. 3J). Then, we tried to determine whether NF-κB/p65 binds to all three of the potential binding sites on the P4HA2 promoter. We cloned the promoter region containing mutated NF-κB/p65 binding sites individually (mut1, mut2 and mut3) as well as the dual mutation of mut1 and mut3 (mut1 + 3). All the constructs were transfected into the HepG2 cells. After using TNF-α, mut2 was capable of increasing the luciferase activity of the P4HA2 promoter while mut1 and mut3 could not (Fig. 3K), which indicated that NF-κB/p65 activated the P4HA2 promoter via binding to site1 and site3. All the above results demonstrate that NF-κB/p65 functions as the transcription factor to stimulate P4HA2 transcription and aspirin could inhibit P4HA2 expression by targeting NF-κB/p65.
Let-7g acts as a mediator of aspirin-reduced P4HA2 in liver tumour growth.

Other than the regulation of transcription factors, microRNA (miRNA) could modulate gene expression. Here, we predicted the microRNAs targeting P4HA2 through TargetScan (http://www.targetscan.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/) and microrna (http://microrna/home.do) (Supplementary Fig. S3A).

The qRT-PCR analysis identified that miR-495 and let-7g were the mostly altered miRNAs under aspirin treatment (Supplementary Fig. S3B). We also conducted an analysis of 371 cases of HCC patient data from the TCGA database. It’s notable that the lower expression of let-7g was significantly correlated with poor survival (Fig. 4A) and occurred in tumour tissues in comparison with their normal counterparts (Fig. 4B), while other predicted miRNAs including miR-30e, miR-494...
and miR-495 are not (Supplementary Fig. S3, C and D). Based on the website prediction and experimental analysis, let-7g and miR-495 were selected to perform a further study. We cloned the 3'UTR region of P4HA2 mRNA into the pGL3-control plasmid to detect whether let-7g and miR-495 target P4HA2. As shown in Fig. 4C, the activity of the luciferase reporter gene gradually decreased along with the elevated dose
of let-7g, while the administration of miR-495 did not change the luciferase activity, indicating that let-7g rather than miR-495 was the miRNA targeting P4HA2. In addition, we confirmed the regulation of let-7g on P4HA2 expression at mRNA and protein levels after the addition of let-7g mimic (Fig. 4, D and E and Supplementary Fig. S3E). Meanwhile, the inhibition of let-7g with its inhibitor led to restored P4HA2 expression (Fig. 4F and Supplementary Fig. S3F). Then, we cloned pGL3-P4-UTR-mut, which contained a mutated let-7g binding site (Supplementary Fig. S3 G). The application of let-7g suppressed the luciferase activity of pGL3-P4-UTR-wt compared to that of pGL3-P4-UTR-mut (Fig. 4G and Supplementary Fig. S3H). In parallel, the administration of the let-7g inhibitor could not change the activity of pGL3-P4-UTR-mut (Fig. 4H and Supplementary Fig. S3 I). Notably, the addition of the let-7g inhibitor counteracted the inhibition of pGL3-P4-UTR activity caused by aspirin (Fig. 4I and Supplementary Fig. S3 J). Altogether, these results imply that let-7g can target the 3’UTR of P4HA2 mRNA to impede P4HA2 expression in aspirin-suppressed HCC cells.

3.5. LncRNA LMCD1-AS1/let-7g axis participates in aspirin-decreased P4HA2 expression in liver tumour

As we have demonstrated the regulation of let-7g on P4HA2 mRNA expression, the mechanism of the upregulation of let-7g by aspirin was to be determined. In a previous study, several IncRNAs were mentioned to be altered by aspirin treatment [49]. We performed screening of those reported IncRNAs by analyzing the TCGA database, and the elevated expression of CFAP53, LMCD1-AS1 and GGT3P were related to an adverse prognosis in HCC patients (Fig. 5, A and Supplementary Fig. S4, A). We then detected the change in RNA levels of these three IncRNAs in HepG2 cells after aspirin treatment (Fig. 5B), and only the level of LMCD1-AS1 declined. Moreover, the prediction on the lncRNASNP website (http://bioinfo.life.hust.edu.cn/lncRNASNP/) showed binding between let-7g and LMCD1-AS1 (Fig. 5C) compared to the other two IncRNAs. The TCGA-based analysis also revealed that the level of LMCD1-AS1 was significantly higher in HCC tumour tissues compared with their nontumour counterparts (Supplementary Fig. S4 B). The HCC clinical stage and pathological grade were related to an elevated expression of LMCD1-AS1 (Supplementary Fig. S4, C and D). Thus, we hypothesize that IncRNA LMCD1-AS1 may be involved in the process of the upregulation of let-7g by aspirin. To validate this hypothesis, we conducted knockdown and overexpression experiments in the HepG2 cell line by transfecting si-LMCD1-AS1 (named si-lm5AS) and pcDNA-LMCD1-AS1 (named pcDNA-LMCD1-AS1). The knockdown and overexpression of the LMCD1-AS1 dose dependently elevated and decreased the level of let-7g (Fig. 5, D and E). Conversely, knockdown and overexpression of LMCD1-AS1 dose dependently impaired and stimulated the luciferase activity of pGL3-P4-UTR, respectively (Fig. 5, F and G), and accordingly changed the expression of P4HA2 at the mRNA and protein levels (Fig. 5, H and I). As shown in Fig. 5J, the introduction of LMCD1-AS1 could increase the activity of pGL3-P4-UTR and the expression of P4HA2, and this increase was blocked by the addition of let-7g. Moreover, the administration of the let-7g inhibitor could reverse the pGL3-P4-UTR reporter gene activity and P4HA2 expression inhibited by si-LMCD1-AS1 (Fig. 5K). Thus, we reveal that aspirin regulates the P4HA2 expression by the LMCD1-AS1/let-7g axis.

3.6. Aspirin bates the axis of NF-kB/P4HA2 and LMCD1-AS1/let-7g to block the growth of liver tumours in vitro and in vivo

Based on the findings of the role of NF-κB/p65 and LMCD1-AS1/let-7g in aspirin-repressed P4HA2, we next verified the effect of these two signals on cell proliferation using MTT and colony formation assays. We found that either TNF-α or LMCD1-AS1 was capable of accelerating cell proliferation of liver cancer, and then TNF-α combined with LMCD1-AS1 could lead to a greater promotion of cell proliferation. Intriguingly, aspirin significantly stifled the increase in cell proliferation induced by both TNF-α and LMCD1-AS1 (Fig. 6A). We observed that there was an increase in colony numbers in the TNF-α-treated or LMCD1-AS1-overexpressed group and more colonies existed in TNF-α/LMCD1-AS1 treated group. In a further experiment, the colony formation ability of liver cancer cells was decreased after aspirin administration (Fig. 6B). At the same time, the RNA or protein levels of NF-κB/p65, let-7g, and P4HA2 were evaluated (Fig. 6C). To further verify aspirin-regulated NF-κB or LMCD1-AS1/let-7g in the growth of HCC in vivo, we detected the levels of NF-κB/p65, let-7g and LMCD1-AS1 in the mice xenograft model. As expected, the decrease of NF-κB/p65 in tumours from the aspirin-treated group was revealed using IHC (Fig. 6D), western blotting (Fig. 6E) and real-time PCR assays (Fig. 6F). Additionally, the levels of let-7g and LMCD1-AS1 were correspondingly elevated (Fig. 6G) and decreased (Fig. 6H) in mouse tumours after aspirin administration, respectively. In conclusion (Fig. 6I), aspirin decreases the collagen deposition-associated growth of liver cancer through targeting P4HA2 at both transcriptional and post-transcriptional levels. On the one hand, aspirin restrains the transcription of P4HA2 by inactivating the transcriptional factor of P4HA2, namely, NF-κB. On the other hand, aspirin interferes with the interaction of IncRNA LMCD1-AS1 with miRNA let-7g to release let-7g, leading to a decrease in the expression of let-7g’s target gene, P4HA2, in liver cancer cells. Finally, a decrease in P4HA2 impends the maturity and function of collagen, which further affects cell proliferation in liver cancer.

4. Discussion

The development of HCC is frequently associated with inflammation and fibrosis [50]. As is known, fibrosis is a process of ECM deposition and tissue stiffening [6]. Meanwhile, as the key component of the ECM, collagen is capable of supporting tumour cell proliferation and impairing the therapeutic efficacy of clinical drugs by obstructing the drug delivery after it is thickened. Nevertheless, there is no specific anti-inflammatory pharmacological cure for liver conditions that is approved to be effective to date. Targeting the pivotal mechanisms of liver fibrosis may provide a novel solution for HCC treatment. Pathways involving extracellular matrix synthesis, deposition and stabilization could be one of such candidate mechanisms [51]. Hydroxylating enzyme prolyl-4-hydroxylase (P4H) and cross-linking enzyme lysyl oxidase (LOX) could be considered as markers for collagen formation [13]. The inhibition of LOX/L2, a LOX homolog, shows potential anti-fibrotic effect in liver fibrosis in animal experiments [52]. However, the humanized anti-LOX2 antibody simtuzumab does not have an impaired effect on liver fibrosis in clinical trials [53]. Our group found that one member of the P4HA family, P4HA2 is implicated in the development of liver fibrosis-associated HCC [33]. Hence, targeting the P4HA family may be an alternative therapeutic strategy for liver fibrosis-related HCC.
Considering the manifold studies of aspirin as an antitumour agent in colorectal cancer, ovarian cancer, breast cancer and prostate cancer, we tried to evaluate the function of aspirin in the development of HCC. We found that aspirin could suppress the growth of liver cancer in vivo and impair the expression of matricellular protein CTGF, which implied the change of ECM constituents. Interestingly, Masson’s trichrome staining showed that aspirin destroyed collagen deposition in the suppression of liver cancer growth in the mouse model. Immunoblotting and IHC assays revealed a decrease in the collagen level in the mouse tumour with aspirin treatment, which further confirmed our finding. Based on the role of the P4HA family in collagen maturation and fibrosis-associated liver cancer, we were wondering whether the P4HA family was involved in aspirin-depressed liver cancer. Our analysis of the TCGA database exposed that out of the three members of the P4HA family (P4HA1, P4HA2 and P4HA3), P4HA2 is upregulated in liver tumour tissues compared with the nontumour
counterparts. Only increased P4HA2 was correlated with decreased overall survival of 371 HCC patients and poor prognosis of HCC including the tumour stage and pathological grade. Moreover, in vivo and in vitro experiments further showed that aspirin could target P4HA2 and sabotage P4HA2-induced excessive growth of HCC along with collagen deposition. Thus, our finding supports that aspirin is able to target P4HA2 to reduce collagen deposition-associated HCC growth.

We were next interested in deciphering the underlying mechanism of the aspirin-resisted HCC growth by reducing P4HA2. Reports have revealed that aspirin can target some transcription factors, such as NF-κB, ER and p53 to modulate cell proliferation, apoptosis, or migration \([37–39]\). We observed that there were three binding sites of transcription factor NF-κB in the promoter of P4HA2. Our further investigation proved that as the transcription factor, NF-κB plays a pivotal part in the P4HA2 transcription reduction by aspirin. In recent years, novel targets including miRNAs and lncRNAs emerged as therapies for cancers \([36]\). Herein, we were wondering if there exist any noncoding RNAs involved in aspirin-inhibited collagen deposition in liver cancer progression. Sequence analysis, website predictions and in vitro experiments indicated that tumour suppressor miRNA let-7g was capable of regulating the expression of P4HA2 and aspirin treatment could enhance the level of let-7g. Our data later revealed that let-7g was able to bind to the 3’UTR of P4HA2 mRNA to suppress its expression. These results indicate that aspirin could restrain the expression of P4HA2 through augmenting the level of tumour suppressor let-7g.

We then proposed the question of how aspirin affected the let-7g expression. Since lncRNAs can serve as molecular sponges to regulate miRNAs through lncRNA-miRNA interaction, we focused on searching for the appropriate lncRNAs in aspirin-depressed HCC by let-7g. We first used the lncRNASNP website to search for several lncRNAs which...
Fig. 6. Aspirin abates the axis of NF-κB/P4HA2 and LMCD1-AS1/let-7g/P4HA2 to block the growth of liver tumour in vitro and in vivo. (A and B) MTT assay (A) and colony formation assay (B) of HepG2 and Huh-7 cell lines under treatment with the TNF-α, pcDNA-LMCD1-AS1, TNF-α + pcDNA-LMCD1-AS1, or TNF-α + pcDNA-LMCD1-AS1 + aspirin. (C) Quantitative real-time PCR, RT-PCR and western blotting assays were performed to detect the expression of let-7g, p65 and P4HA2 in HepG2 and Huh-7 cell lines after indicated treatment. (D and E) IHC (D) and western blotting (E) detection of the level of NF-κB/p65 in mice tumour tissues from saline- and ASA-treated groups. (F to H) Quantitative real-time PCR detection of NF-κB/p65 (F), let-7g (G), or LMCD1-AS1 (H) expression in mice tumour tissues from saline- and ASA-treated groups. (I) Graphic model of aspirin (ASA)-ameliorated liver tumour development. As a classic downstream factor of aspirin, NF-κB/p65 is able to activate the transcription of P4HA2. Meanwhile, lncRNA LMCD1-AS1 acts as a sponge to decrease the level of tumour suppressor miRNA let-7g and subsequently enhances the target gene of let-7g, i.e., P4HA2. Aspirin suppresses collagen deposition and further liver tumour growth through targeting the axis NF-κB/P4HA2 and LMCD1-AS1/let-7g/P4HA2. The data are representative of three independent experiments (means ± SD). ***P < 0.001; **P < 0.01; *P < 0.05.
were mentioned in public studies to be possible targets [49]. Evaluation of the structure of let-7g and those lncRNAs suggested that only one lncRNA, i.e., LMC1D-AS1 has the possibility for forming sequence complementary to the seed region of let-7g. Moreover, the TCGA analysis indicated that the levels of LMC1D-AS1 in tumour tissues were higher compared with that of their nontumour counterparts, and the elevated expression of LMC1D-AS1 was related to a poor prognosis. The further experiments testified that the knockdown and overexpression of LMC1D-AS1 could correspondently interfere with the level of let-7g and then the expression of its target gene, P4HA2 in liver cancer cells. Functional experiments in vitro and in vivo demonstrated that combined treatment with the NF-κB activator TNF-α and the ectopic expression of LMC1D-AS1 could significantly elevate the expression of P4HA2, leading to the accelerated-proliferation of HCC cells. Furthermore, the use of aspirin markedly reduced collagen accumulation and then retarded this accelerated-proliferation in liver cancer.

In summary, our study uncovers a novel mechanism for aspirin-ameliorated liver tumor development [Fig. 6I]. In the aspirin-treated HCC model, as a classic downstream factor, NF-κB/p65 is able to activate the transcription of P4HA2. Meanwhile, IncRNA LMC1D-AS1 acts as a sponge to decrease the level of tumour suppressor miRNA let-7g and subsequently enhances the target gene of let-7g, i.e., P4HA2. Aspirin suppresses collagen deposition and subsequent liver tumour growth through targeting the axis of NF-κB/P4HA2 and LMC1D-AS1/let-7g/P4HA2. Together with the analysis of the TCGA and GEO databases for human HCC which reveals the significant association of P4HA2, let-7g and LMC1D-AS1 with the prognosis of HCC patients, our study sheds light on the clinical use of aspirin in liver cancer therapy. 

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Author contributions
Tianjiao Wang designed the research methods, performed the experiments, analysed the data, and drafted the manuscript. Xuei Fu, Tianzhi Jin, Lu Zhang, Bowen Liu, Feifei Xu, Yue Wu, Xue Wang and Kai Ye participated in the experiments. Weiyang Zhang and Lihong Ye designed the research and revised the manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest
None.

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