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H19-Wnt/β-catenin regulatory axis mediates the suppressive effects of apigenin on tumor growth in hepatocellular carcinoma

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ABSTRACT

Hepatocellular Carcinoma (HCC) is one of the leading causes of cancer-related deaths in the world. However, the effective pharmacological approaches remain scanty in clinical practice. As a bioactive flavonoid, apigenin (API) is enriched in common fruits and vegetables. Although pharmacological activities of API have been widely investigated, its biological function in HCC remains obscure. In the present study, we found that API strongly suppressed cell growth and induced apoptosis in HCC cells. Using a xenograft mice model, API was demonstrated to inhibit the *in vivo* tumor growth. It is known that the long non-coding RNA H19, which is frequently elevated in HCC, plays a vital role in mediating tumorigenesis and cancer progression. Our results demonstrated that H19 was down-regulated by API, and thereby induced the inactivation of the canonical Wnt/β-catenin signaling. In conclusion, our results demonstrated that API was able to suppress tumor growth of HCC through H19-mediated Wnt/β-catenin signaling regulatory axis, suggesting that API may be a promising candidate for developing novel therapeutic approaches against liver cancer.

1. Introduction

Flavonoids are a class of natural polyphenols that are highly enriched in fruits and vegetables, and they have been widely applied for the prevention and treatment of a variety of human diseases. Apigenin (API), 4,5,7-trihydroxyflavone, is a flavonoid mainly derived from parsley and chamomile (Madunic et al., 2018), and recorded as a traditional Chinese medicine to interfere with the various disease progression such as cancer (Yan et al., 2017), inflammation (Ai et al., 2017; Kim and Lee, 2018), oxidation (Sanchez-Marzo et al., 2019) and so on. Especially, the anti-cancer properties of API have been reported in variety of previous studies. For instance, API inhibits *in vitro* and *in vivo* prostate cancer cell growth; and it suppresses cell viability and induces apoptosis in human prostate cancer cells (Singh et al., 2015). In addition, API also triggers autophagy of erythroleukemia TF1 cells, a so-called type 2 non-apoptotic cell death (Ruela-de-Sousa et al., 2010). Among all malignant tumors, hepatocellular carcinoma (HCC) is ranked as the fourth most frequent cause of cancer-related deaths in the world-wild scale (Siegel et al., 2019). Considering the anti-cancer potential of API, it is of great clinical importance to investigate the function of API in HCC.

Long non-coding RNAs (lncRNAs) are a kind of transcripts without protein-coding capacity, and their length is more than 200 nucleotides (Li et al., 2015). Previous studies have revealed that lncRNAs are important and powerful regulators in various biological activities and play a critical role in the regulation of a variety of cellular activities and disease progressions including carcinogenesis (Liu and Ding, 2017; Song et al., 2017; Huarte, 2015). lncRNA H19, encoded by an imprinted gene,

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was initially proposed to possess tumor-suppressive properties (Raveh et al., 2015; Ishiwata, 2018). However, recent studies have shown that H19 is up-regulated in tumors, and it possesses oncogenic properties (Zhang et al., 2019). Many types of cancers such as breast (Lottin et al., 2002), bladder (Lv et al., 2017; Luo et al., 2013), lung (Gong et al., 2016), oesophageal (Chen et al., 2019) and cervical carcinomas (Quaye et al., 2009; Song et al., 2009) all exhibit frequent elevation of H19. In HCC, H19 was reported to closely correlate with tumor grade and metastasis, suggesting it may be a potential marker of early recurrence (Zhang et al., 2013).

Although many studies have illuminated the anti-tumor effect of API, the detailed mechanisms have not been clarified. In the present study, we demonstrated that API suppressed tumor growth *via* down-regulation of H19, thus leading to the inactivation of the Wnt/β-catenin signaling, which provides a novel mechanism of API-mediated liver cancer progression. The information gained from this study suggest API may be developed as a promising candidate for the cancer patients in the coming future.

2. Materials and methods

2.1. Cell culture and treatment

The human immortalized hepatocyte LO2 and HCC cell lines SMMC-7721 and HepG2 were cultured with DMEM (Gibco, Carlsbad, CA) together with 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin (Hyclone, Pasching, Austria) at 37 °C with a humidity of 5% CO_2. API was purchased from Aladdin and dissolved in DMSO and stocked at -20 °C. All the cells were seeded and cultured in 60 mm culture dishes until the cells were grown to 70%~80%, and then the cells were treated with API. The DMSO (0.1%) treatment was used as control.

2.2. Cell viability assays

LO2, SMMC-7721 and HepG2 cells were seeded into 96-well plates at 5000 cells per well and cultured at 37 °C, 5% CO_2 for 24 h. Then, the cells were treated with API with different concentrations from 10μM to 40 μM. After 24, 48 and 72 h treatment with API, cell viabilities were measured by Cell Counting Kit-8 (Beyotime, Shanghai, China).

2.3. Colony formation assays

HepG2 and SMMC-7721 cells (200 per well) were seeded into 6-well plates and then maintained in 20 μM API culture medium for two weeks. The colonies were fixed with 4% paraformaldehyde for 20min and stained with 0.5% crystal violet for 15min. The number of colonies counted by ImmunoSpot analyzers (CTL).

2.4. Cell apoptosis assays

Both SMMC-7721 and HepG2 cells were seeded in six-well plates at 2 × 10^5 cells/well for 24 h and then incubated with 20 μM API for 48 h. Cells were collected and stained using a Cell Apoptosis PI Detection Kit (KeyGEN, Nanjing, China) as mentioned before (Pan et al., 2017). This measurement was performed using FC-500.

2.5. RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) examination

The total RNA was extracted by Trizol (Invitrogen, Carslbad, CA, USA), and the RNA quantity and integrity were examined by Nanodrop (Thermo Fisher, Waltham, MA, USA). After RNA extraction, cDNA was reversely transcribed from RNA samples by PrimeScript™ RT Reagent Kit (TaKaLa, Japan). Quantitative PCR reactions were set up in triplicates and performed on a LightCycler 480 system (Roche, Basel, Switzerland) using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher, USA) according to the manufacturer's protocol. Relative expression levels of candidate genes were calculated *via* the $2^{-\Delta\Delta Ct}$ method and normalized to the corresponding GAPDH values. The primer sequences for real-time PCR are listed in Table 1.

2.6. Cell transfection

The H19 overexpression plasmid (pH19) was generated according to previous study and the empty plasmids (pBabe)were used as control (Liang et al., 2015). The HepG2 and SMMC-7721 cells were seeded in six-well plates at a concentration of 1.0 × 10^5 per well. After incubation for 24 h, cells were transfected with pH19 and pBabe by using Lipofectamine 3000 (Invitrogen) respectively.

2.7. Luciferase assay

The HepG2 and SMMC-7721 cells were seeded on 24-well plates at about 60% confluence for 24 h, and then the cells were transfected with TOPflash plasmids *via* Lipofectamine 3000 according to the previous study (Fu et al., 2015). Luciferase assays were performed after API-treatment using Bright-Glo™ Luciferase Assay System (Promega, Madison, WI, USA).

2.8. Protein production and analysis

The cells were harvested and lysed in the lysis buffer (Thermo Fisher Scientific) to generate the total protein. And the nuclear and cytoplasmic fractions were isolated by using the Nuclear and Cytoplasmic Protein Extraction kit (Invent) according to the manufacturer's instructions. The concentration of extracted proteins was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Then loading equal amounts of protein on each well of the 10% SDS-PAGE gel (Epizyme, Shanghai, CHN) was separated using at 120 V for 80min and then transferred electrophoretically to a nitrocellulose membrane at 100 V for 90min. The transferred membranes were blocked by 5% skim milk for 1 h and then they were incubated with primary antibody included β-catenin (1:2000; Cell Signaling Technology, USA), Lamin B1 (1:2000; Cell Signaling Technology, USA) and GAPDH (1:2000; Cell Signaling Technology, USA) at 4 °C for 8–12 h. Subsequently, they were incubated with second antibody for 1 h, and exposed by the FluorChem R system (ProteinSimple, San Jose, CA, USA) with the help of Luminous fluid. Lamin B1 and GAPDH were used as the internal control of nuclear protein and total protein, respectively.

2.9. Immunofluorescence

The HepG2 and SMMC-7721 cells were cultured in 24-well plates (1 × 10^3 cells per well). With API treatment for 48 h, the immunofluorescence assays were conducted as previously described (Lv et al., 2019). The antibodies used in this study are β-catenin (1:100) and donkey anti-rabbit IgG-Alexa Fluor 594 (Absin, Shanghai, China). The nuclei were stained with DAPI.

2.10. Xenograft mouse model

The subcutaneous xenograft assay was performed under the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Southern Medical University (Guangzhou, China, approval No. L2018187). Male nude mice (3–4 weeks old) were purchased from the Laboratory Animal Center, Southern Medical University. 1.5 × 10^6 HepG2 cells were injected subcutaneously into the dorsal of nude mice. When the tumor was palpable, animals were randomly divided into four groups (n = 6 per group). API was suspended in vehicle material (0.5% sodium methylcellulose) by sonication for 30 min. Group 1 and group 2 were given API (50 mg/kg) *via* intraperitoneal and intragastric

Table 1
The primer sequences used in this study.

	Forward	Reverse
β-catenin	CCGTTCGCCTTCATTATGGA	GGCAAGGTTTCGAATCAATCC
CCND1	CTGGAGGTCTGCGAGGAACA	CCTTCATCTTAGAGGCCACGAA
CD44	TCAGAGGAGTAGGAGAGAGGAAAC	GAAAAGTCAAAGTAACAATAACAGTGG
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
OCT3/4	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
Axin2	CTTTCGCCAACCGTGGTT	GGATCGCTCCTCTTGAAGGA
H19	TGCTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGC
Linc-ROR	CTGGCTTTCTGGTTTGACG	CAGGAGGTTACTGGACTTGGAG
HOTTIP	CCTAAAGCCACGCTTCTTTG	TGCAGGCTGGAGATCCTACT
TINCR	TGTGGCCCAAACTCAGGGATACAT	AGATGACAGTGGCTGGAGTTGTCA
HULC	TTCACCAGGCTGATAGTCCG	ACACGTCCTTCCCATAAACCC
Gapdh	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA

administration, respectively. Group 3 and group 4 were served as control groups and given equal vehicle *via* the same administration modes. The treatment was performed every other day for 4 weeks and the tumor sizes were recorded twice a week. The tumor volumes (V) were calculated by following the formula: $V = \text{length} \times \text{width}^2/2$.

2.11. Immunohistofluorescence

Tumor specimens were fixed in 4% paraformaldehyde overnight and then embedded in paraffin. Sections were incubated with the Ki-67 antibody (Calbiochem, Darmstadt, Germany) and β-catenin antibody and stained according to the previous study (Wang et al., 2018).

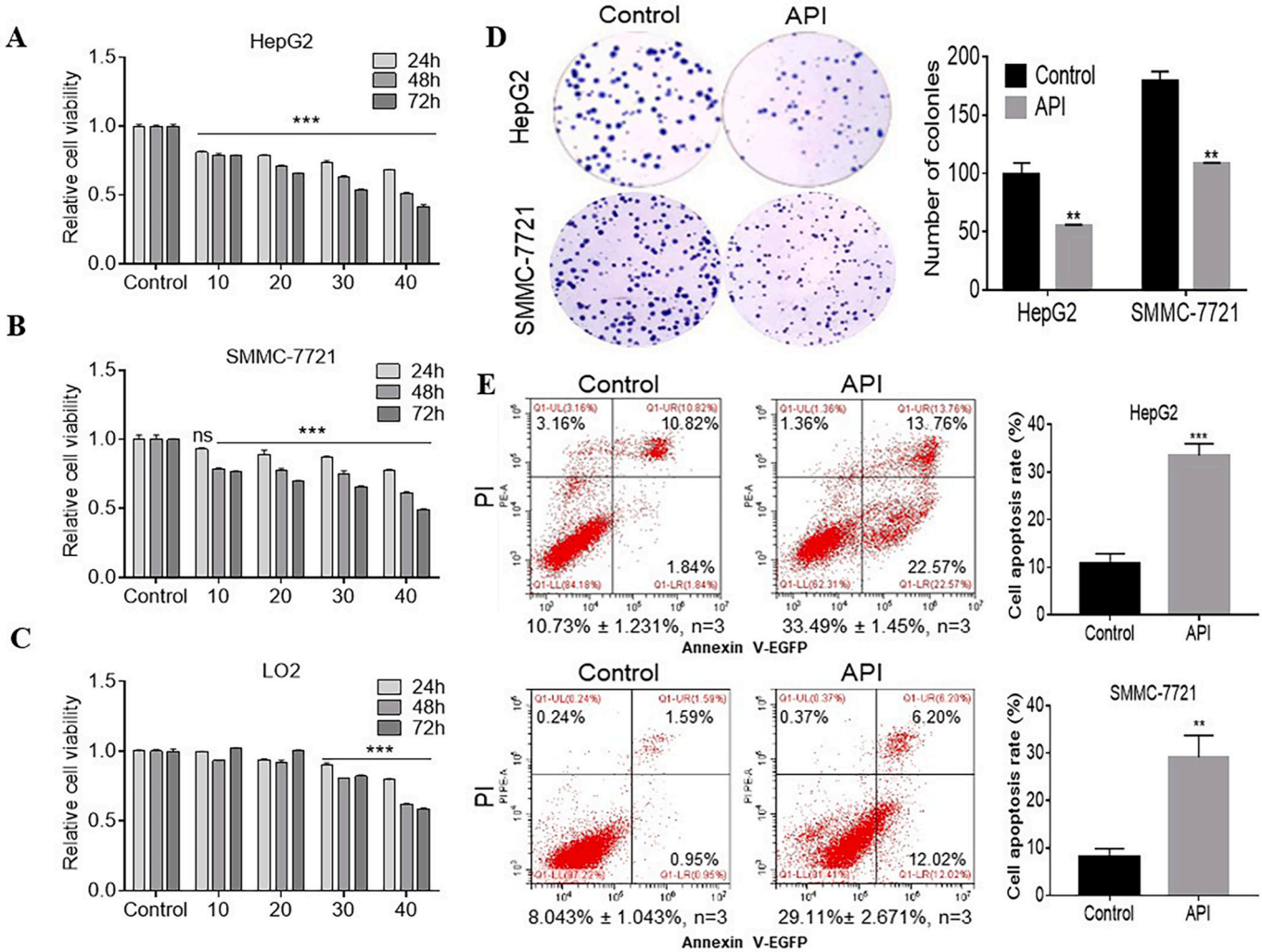

Fig. 1. API suppressed cell viability and induced apoptosis in HCC cells. A-C, The HepG2 (**A**), SMMC-7721 (**B**) and non-tumorigenic cell line LO2 (**C**) were treated with serial concentrations of API, and its effects on cell viability were measured by CCK-8 assays at 24, 48 and 72 h. ***, $P < 0.001$, compared with Control. **D**, the colony formation was examined for two HCC cells with 20 μM API treatment. **, $P < 0.01$, compared with Control. **E**, The two HCC cells were incubated with 20 μM API for 48 h, and the apoptotic cells were monitored by Annexin V-FITC and PI double staining. **, $P < 0.01$, ***, $P < 0.001$, compared with Control.

2.12. Statistical analysis

At least three times repeats were taken in each experiment and the data were expressed as the means ± S.D.. Differences between two independent groups were analyzed by Student's t-test and one-way ANOVA. The statistical differences were considered to be statistically significant when $P < 0.05$.

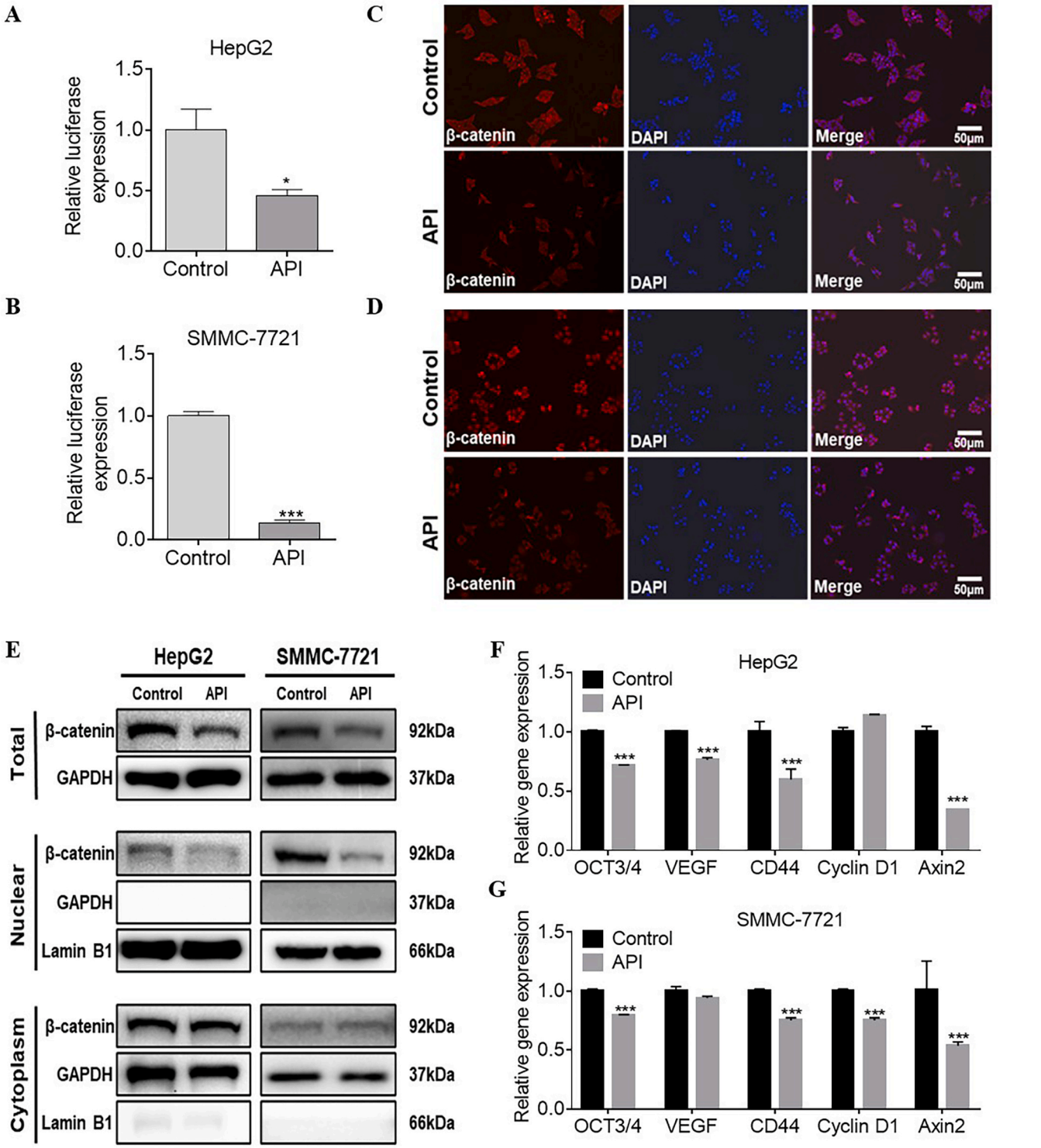

Fig. 2. API induced the inactivation of Wnt/β-catenin signaling in HCC cells. A-B, API significantly suppressed the TOPflash luciferase activity in HepG2 (**A**) and SMMC-7721 (**B**) cells. **C-D**, β-catenin was detected by immunofluorescence staining (× 100) in HepG2 (**C**) and SMMC-7721 (**D**) cells. **E**, The expression of total β-catenin, intranuclear β-catenin and intracytoplasmic β-catenin were examined by Western blotting in HepG2 and SMMC-7721 cells. **F-G**, the mRNA levels of several downstream targets of Wnt/β-catenin pathway was determined by qRT-PCR examination in HepG2 (**F**) and SMMC-7721 (**G**) cells. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared to Control.

3. Results

3.1. API suppressed cell viability and induced apoptosis in HCC cells

API has been reported to inhibit tumor growth in several cancer types. To validate its anti-cancer function in HCC, two HCC cell lines, SMMC-7721 and HepG2, and a non-tumorigenic cell line LO2 were used to evaluate the cell viability with API treatment. As shown in Fig. 1A-1 B, API with the concentration from 10 to 40 μM exhibited significant anti-tumor activity in a dose- and time-dependent manners in HepG2 cells (Fig. 1A) and SMMC-7721 cells (Fig. 1B). As for the non-tumorigenic LO2 cells, API showed no obvious effect on the cell viability in doses ranging from 10 to 20 μM, and it displayed the inhibitory effect on this LO2 cells with the concentration of over 20 μM (Fig. 1C). Therefore, 20 μM was selected for further investigation. Using this concentration, the results of colony formation assays further confirmed the suppressive effects of API on the two HCC cells (Fig. 1D). Furthermore, the next apoptotic examination illuminated that higher apoptotic rates were observed in API-treated HCC cells (Fig. 1E).

3.2. API alleviated the activation of Wnt/β-catenin signaling in HCC cells

We subsequently investigated the underlying mechanism of API in HCC cells. Considering that the canonical Wnt/β-catenin signaling plays a crucial role in tumorigenesis. We therefore examined whether this signaling was mediated by API in HCC. The Wnt signaling luciferase reporter TOPflash, which contains three binding sites for the key transcription factors TCF and β-catenin, was transfected into HCC cells, and the results showed that API significantly suppressed the luciferase activities in HepG2 cells (Fig. 2A) and SMMC-7721 cells (Fig. 2B). Moreover, the immunofluorescence staining analyses demonstrated the expression of β-catenin was decreased in API-treated HepG2 cells (Fig. 2C) and SMMC-7721 cells (Fig. 2D). It is well known that cellular accumulation of β-catenin and nuclear translocation are key points for Wnt/β-catenin signaling activation. As showed in Fig. 2E, the expression of total and nuclear β-catenin was significantly decreased by API in the two HCC cells while the cytoplasmic β-catenin expression remained unchangeable in both treated cells. Several downstream target genes of Wnt/β-catenin signaling such as OCT4, VEGF, CD44, cyclin D1 and Axin2 were also examined and their mRNA levels were suppressed by API treatment in HepG2 cells (Fig. 2F) and SMMC-7721 cells (Fig. 2G).

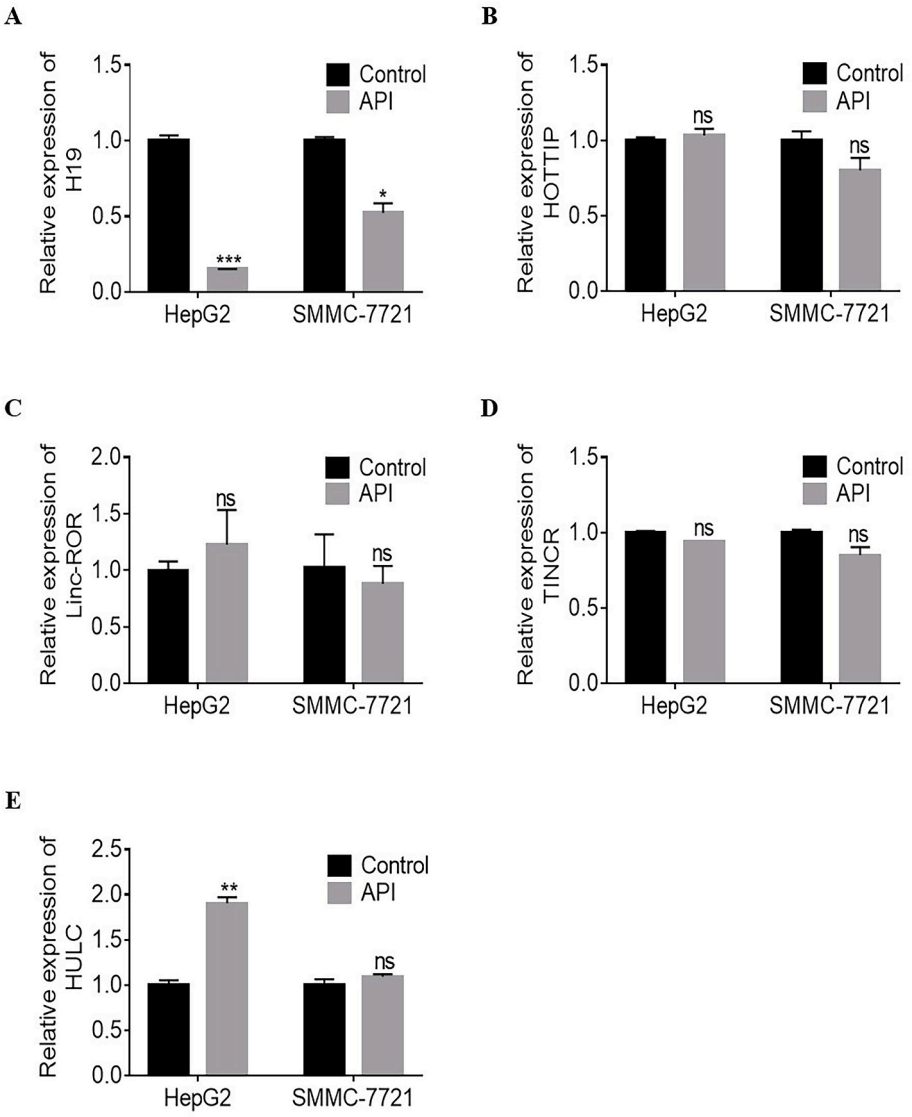

Fig. 3. H19 was down-regulated by API in HCC cells. Several lncRNAs including H19 (**A**), HOTTIP (**B**), Linc-ROR (**C**), TINCR (**D**) and HULC (**E**) were selected for investigating their expression profiling in API-treated HCC cells. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ compared to Control.

These results indicated that the API suppressed the nuclear β-catenin expression, and led to the disruption of the canonical Wnt/β-catenin signaling.

3.3. API induced H19 down-regulation in HCC cells

LncRNAs have been considered as significant regulators in tumor progression and a series of lncRNAs were reported to mediate the Wnt/β-catenin signaling. To identify the candidate lncRNA which participates in the API-mediated the inactivation of Wnt/β-catenin signaling, several lncRNAs related to this signaling such as H19 (Fig. 3A), HOTTIP (Fig. 3B), linc-ROR (Fig. 3C), TINCR (Fig. 3D) and HULC (Fig. 3E) were subjected to their expression examination. The results showed that among the expression profiling of all the lncRNAs above, only H19 was down-regulated by API in the two HCC cells.

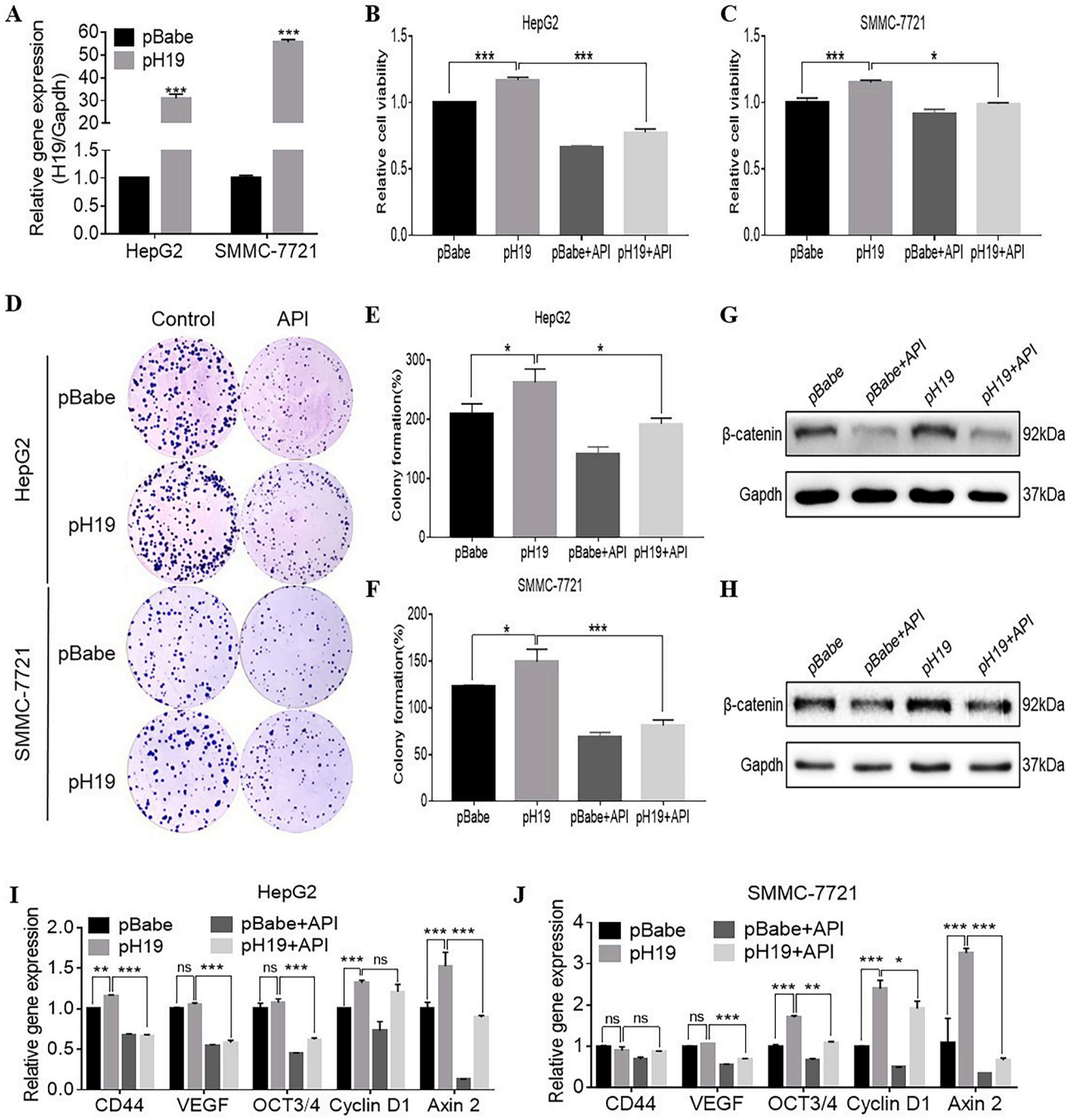

Fig. 4. API suppressed cell proliferation in HCC cells *via* H19-Wnt/β-catenin regulatory axis. A, The expression of H19 in the H19 overexpressing HepG2 and SMMC-7721 cells was measured by qRT-PCR examination. **, $P < 0.01$, ***, $P < 0.001$, compared to pBabe. **B–C**, The cell viabilities were examined in API treated the H19 overexpressing HepG2 (**B**) and SMMC-7721(**C**) cells. *, $P < 0.05$, ***, $P < 0.001$. **D-F**, The colony formation was measured with API treatment in H19 overexpressing HCC cells. *, $P < 0.05$, ***, $P < 0.001$. **G-H**, The protein level of β-catenin was examined by Western blotting after treatment with API in H19 overexpressing HepG2 (**G**) and SMMC-7721 (**H**) cells. **I-J**, Several downstream target genes of Wnt/β-catenin signaling were examined by qRT-PCR after API treatment in H19 overexpressing HepG2 (**I**) and SMMC-7721 (**J**) cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.4. API inhibited cell viability through the H19/β-catenin regulatory axis

To further confirm whether H19 participates in API-mediated anti-cancer activity, two H19-overexpressing HCC cell lines were generated, and qRT-PCR examination showed that H19 was obviously up-regulated in these H19-overexpressing HCC cells (Fig. 4 A). Subsequently, these cells were treated with API and the cell viability and colony formation were monitored. As shown in Fig. 4B– F, H19 overexpression partially attenuated the API-induced suppressive effects on cell viability and colony formation in HCC cells. We also investigated the inhibitory effect of API on Wnt/β-catenin signaling with elevated expression of H19, and the results showed that H19 overexpression significantly reversed the suppressive expression of total β-catenin (Fig. 4G–H, Fig. S1) and several downstream target genes of Wnt/β-catenin signaling (Fig. 4I–J), thus partially alleviated the API-induced inactivation of Wnt/β-catenin signaling in two HCC cells.

3.5. API inhibited the in vivo tumor growth

To elucidate the *in vivo* anti-cancer potential of API, a xenograft nude mouse model was applied and API was administrated *via* two models, intraperitoneal injection and oral gavage. Compared to the vehicle control groups (0.5% CMC-Na), smaller tumors were burdened in the two API-treated groups (Fig. 5A). Moreover, a significant inhibition in tumor weight (Fig. 5B–C) and tumor growth (Fig. 5D) was found in the API-treated groups with two models of administration. As a significant cellular marker of proliferation, Ki-67 expression was evaluated by immunofluorescence staining. A decreased Ki-67 expression (Fig. 5E) was observed in tumor specimens derived from API-treated groups, indicating an inhibitory effect of API on *in vivo* tumor growth. The β-catenin expression in tumor specimens was also found to be suppressed by API treatment *in vivo* (Fig. 5F).

4. Discussion

Plant active phytochemicals have been historically considered as an important source for developing the mature medical drug for clinical practice. Previous reports have demonstrated that phytochemicals contributed to the treatment of malignancy, and numerous phytochemicals like astragalin have been validated to be available for HCC patients (Liao et al., 2015; Li et al., 2017). API, a naturally-derived plant flavone, is present in numerous fruits, vegetables, and herbs. A study on the pharmacokinetics and metabolism of API exhibited slower absorption and metabolism in rats. Both the urine and feces served as the main routes of excretion, with approximately 63% of API being excreted *via* these two routes. Ten days post-treatment, around 24.8% API was still detectable in the rats, suggesting a slow metabolism (Gradolatto et al., 2005). Increasing evidence unanimously demonstrates that API acts as a significant inhibitor for malignancy progression (Sung et al., 2016). For instance, API induced the G2/M cell cycle arrest to cause growth suppression on human colorectal carcinoma (Lee et al., 2014); and it also inhibited cell migration and invasion by impairing focal adhesion kinase expression in human ovarian cancer cells (Hu et al., 2008).

HCC is the most common type of liver cancer and advanced HCC is frequently associated with poor prognosis, which makes it the third leading cause of cancer-related mortality. Sorafenib, as a novel multi-kinase inhibitor, targets on serine/threonine and receptor tyrosine kinases of the tumor cells and vasculature, leading to the blockage of the signal transconduction and the suppression of tumor growth (Miller et al., 2006). Sorafenib was approved by Food Drug Administration for the treatment of advanced renal cancer (RCC) in 2005. And now, it also acts as the first-line systemic therapy for liver cancer in clinical practice (Strumberg, 2012). However, the acquired drug resistance really becomes a major obstacle to its clinical application. Recent study showed that API could improve the sensitivity of sorafenib, and promote its anti-cancer effect in HCC(Sirin et al., 2020). Being aware of the inherent anti-tumor activity of API, we mainly focused on the effect of API in HCC in the present study. And the results demonstrated its anti-proliferative and pro-apoptotic potential in HCC cells. Furthermore, the underlying therapeutic mechanism of this phenomenon was illuminated, indicating that API may be a potential candidate for liver cancer.

To identify the anti-proliferative effect of API in HCC, the cell counting kit-8 and colony formation assays were applied. It was found that API inhibited the viability and colony formation in HCC cells, suggesting that the anti-proliferative potential of API in HCC cells. We further examined the *in vivo* function of API using a xenograft nude mouse model and found that API strongly suppressed tumor growth. The *in vitro* and *in vivo* combined data suggest that API may be a promising therapeutic candidate for HCC treatment.

In terms of anti-cancer effect of API on HCC, the related molecular mechanism was further elucidated. As well known, MAPK, PI3K/AKT, JAK-STAT, Wnt, growth factor-mediated angiogenesis, vascular endothelial growth factor (VEGF), Caspase-mediated apoptosis signaling are the main pathways for the evolution and progression of HCC. Wnt signaling is one of the most important pathways during the tumorigenesis, and our team has been committed to this signaling in the past decade. As a very complex pathway, this signaling plays critical roles in numerous cellular and developmental processes. The activated Wnt signaling frequently occurs in tumorigenesis and is highly associated with the tumor progression and recurrence (Anastas and Moon, 2013). The increased cytoplasmic expression of β-catenin indicated the poor outcome in breast cancer patients, and the accumulation of β-catenin was positively associated with tamoxifen-resistant character of breast cancer cells (Lopez-Knowles et al., 2010; Hiscox et al., 2006). In liver cancer, 40%–50% molecule mutations were found in the Wnt pathway in which the point mutations in exon 3 of β-catenin could stabilize β-catenin and cause intracellular recruitment (Miyoshi et al., 1998; Nakagawa et al., 2019). Recently, API has been demonstrated to suppress the proliferation and invasion of osteosarcoma and colorectal cancer by inhibiting the Wnt/β-catenin signaling (Liu et al., 2015). Based on these studies, we hypothesize that Wnt/β-catenin signaling may be involved in the API-mediated anticancer activity in HCC. Our results showed that API significantly suppressed the luciferase activity of Wnt/β-catenin TOPflash reporter in HCC cells. The expression of nuclear β-catenin was decreased by API, and its downstream target genes were inhibited as well. Therefore, these data suggest that API exerts the anti-cancer function *via* inducing the inactivation of Wnt/β-catenin signaling.

LncRNAs have been reported to play important roles in various biological activities and gene regulation (Qian et al., 2019). A number of lncRNAs have been demonstrated to exert their functions *via* mediating Wnt/β-catenin signaling. For instance, HOTAIR could decrease the expression Wnt-1 and then suppressed the activation of Wnt/β-catenin signaling to promote synoviocyte apoptosis (Mao et al., 2019). Several lncRNAs such as HOTTIP, TINCR, HULC, Linc-ROR, H19 were reported to mediate Wnt/β-catenin signaling and participate the tumorigenesis. The lncRNA HOTTIP has been considered as an oncogenic regulator in different cancers (Lian et al., 2016) and its knockdown inhibited cell proliferation and improved cell sensitivity to cisplatin *via* suppressing the Wnt/β-catenin pathway (Jiang et al., 2019). Placenta-specific protein 2 (PLAC2), also known as TINCR44 could promote cell proliferation and invasion *via* the activation of the Wnt/β-catenin pathway in oral squamous cell carcinoma(MJ Chen et al., 2019). HULC was identified as a liver-specific lncRNA, and its aberrant up-regulation was also confirmed in other cancer types, such as human pancreatic cancer, epithelial ovarian carcinoma and gastric cancer (Takahashi et al., 2020; Chen et al., 2017). Although Xin et al. reported that HULC overexpression suppressed the expression of β-catenin, most studies have demonstrated that excessive HULC promoted Wnt/β-catenin activities (Yang et al., 2020). Another lncRNA linc-ROR could induce the epithelial-to-mesenchymal transition (EMT) in ovarian cancer by increasing Wnt/β-catenin signaling (Lou et al., 2017). H19 is one of the

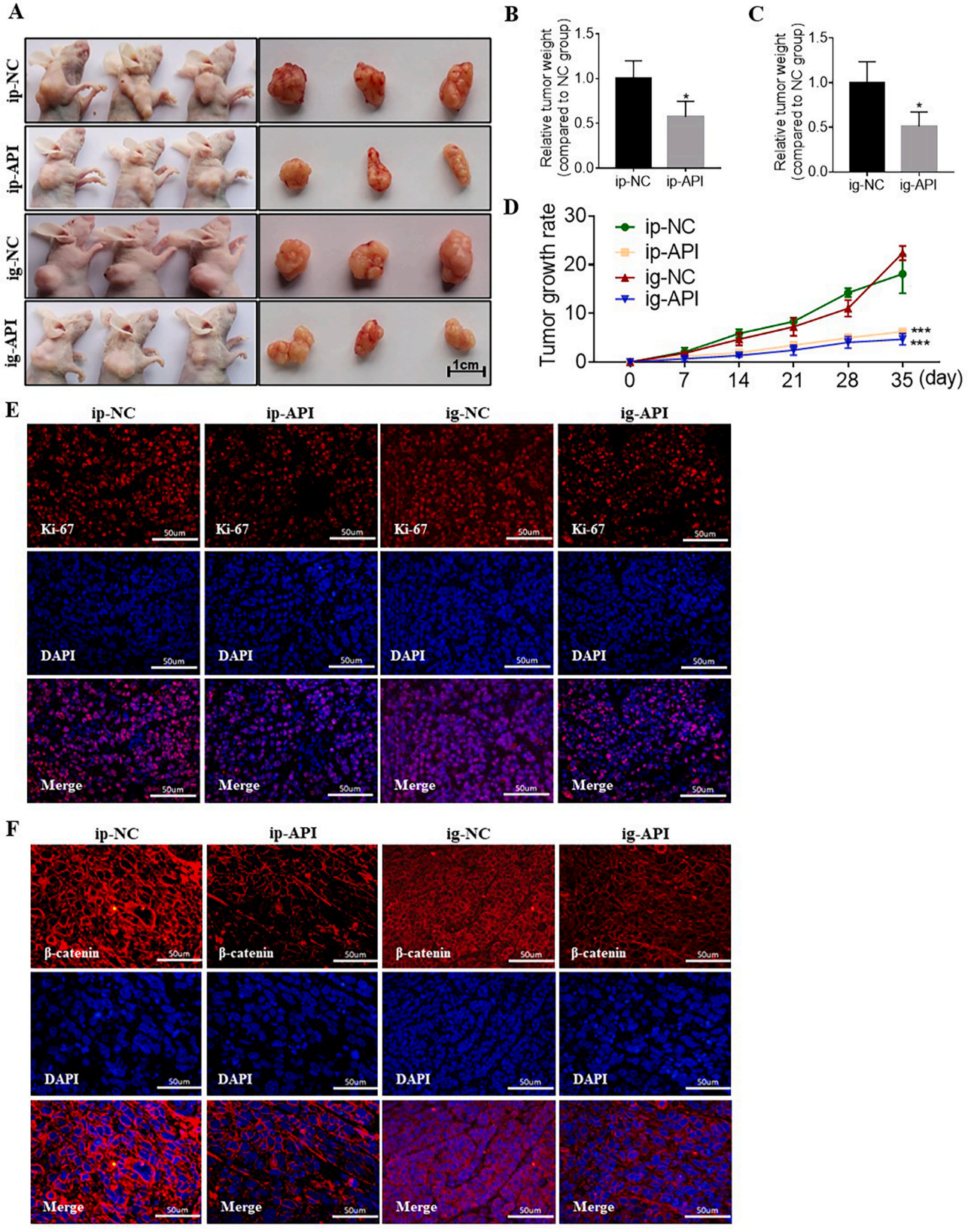

Fig. 5. API reduced the tumor growth of HCC cells *in vivo*. HepG2 cells were subcutaneously injected into the dorsal flank of nude mice, and API was administrated with two models, i.g or i.p (n = 6 per group). **A**, The representative images showed the two API-treated groups developed smaller tumor compared with their control groups, respectively. **B-D**, The tumor weight (**B**–**C**) and the growth curve of tumor volumes (**D**) were examined in the treated and NC groups. *, $P < 0.05$. **E**, The immunofluorescence of Ki-67 and β-catenin followed by counterstaining with DAPI were performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; vs ip-NC or ig-NC.

first identified imprinted non-coding RNA genes, and increasing studies have demonstrated that H19 is up-regulated in multiple cancers, and it possesses oncogenic properties (Matouk et al., 2007; Brannan et al., 1990). Our previous studies also showed that H19 could regulate epithelial to mesenchymal transition (EMT) and methotrexate resistance in colorectal cancer (Liang et al., 2015; Wu et al., 2017). In the present study, we found that H19 was down-regulated by API in two HCC cells, and the Wnt/β-catenin signaling was suppressed as well. H19 overexpression partially reversed the API-induced cell proliferative inhibition, and partially abolished the inactivation of Wnt/β-catenin signaling in HCC cells. These results indicated that the H19 was a potential therapeutic target of API in HCC.

In conclusion, our results demonstrated that API suppressed *in vitro* and *in vivo* tumor growth via down-regulation of H19 and decreased β-catenin expression, thereby leading to the inactivation of Wnt/β-catenin signaling.Therefore, the knowledge gained from this study may provide a novel mechanism of the API-mediated tumor supression and suggests API may be developed as a promising candidate for cancer patients soon.

Author contributions

Zhang J. and Fu W. designed and supervised the experiments. Pan F., Zheng X., Shi C. and Zhang F. conducted the experiments. Pan F., Fu W., and Zhang J. analyzed the data and prepared the manuscript.

CRediT authorship contribution statement

Fei-fei Pan: Visualization, Investigation, Writing - original draft. **Yan-Biao Zheng:** Data curation, Formal analysis, Validation. **Chuan-Jian Shi:** Validation, Methodology. **Feng-wei Zhang:** Resources, Methodology. **Jin-fang Zhang:** Supervision, Writing - review & editing. **Wei-ming Fu:** Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

All authors declared no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

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