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Research Article

# HBV precore G1896A mutation promotes growth of hepatocellular carcinoma cells by activating ERK/MAPK pathway

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is one of the leading causes of hepatocellular carcinoma (HCC). The HBV genome is prone to mutate and several variants are closely related to the malignant transformation of liver disease. G1896A mutation (G to A mutation at nucleotide 1896) is one of the most frequently observed mutations in the precore region of HBV, which prevents HBeAg expression and is strongly associated with HCC. However, the mechanisms by which this mutation causes HCC are unclear. Here, we explored the function and molecular mechanisms of the G1896A mutation during HBV-associated HCC. G1896A mutation remarkably enhanced the HBV replication *in vitro*. Moreover, it increased tumor formation and inhibited apoptosis of hepatoma cells, and decreased the sensitivity of HCC to sorafenib. Mechanistically, the G1896A mutation could activate ERK/MAPK pathway to enhanced sorafenib resistance in HCC cells and augmented cell survival and growth. Collectively, our study demonstrates for the first time that the G1896A mutation has a dual regulatory role in exacerbating HCC severity and sheds some light on the treatment of G1896A mutation-associated HCC patients.

## 1. Introduction

Chronic hepatitis B virus (HBV) infection is the most prominent risk factor for developing hepatocellular carcinoma (HCC), which accounts for approximately 50% of all HCC cases (Tang et al., 2018). Worldwide, it is estimated that more than 296 million people are chronically infected with HBV, which accounts for 820,000 deaths as a result of progressive liver diseases such as cirrhosis and HCC(Yardeni et al., 2023). In Southeast Asia and Sub-Saharan Africa, high hepatitis B surface antigen (HBsAg) seroprevalence is often linked to high levels of HBV-driven HCC incidence (Liu et al., 2022; Sivasudhan et al., 2022). However, the molecular mechanisms of HBV-related HCC are not fully elucidated.

HBV mutations are closely related to the malignant transformation of liver disease (Chotiyaputta and Lok, 2009). HBV is a hepatotropic virus with a 3.2 kb circular and partly double-stranded DNA genome. The genome contains four overlapping open reading frames (OFRs) called preS/S, polymerase, precore/core, and X, which encodes the surface protein (S), core protein, polymerase, and X protein, respectively (Seeger and Mason, 2000; Nguyen et al., 2008). Hepatitis B virus genome mutation rate is high due to the error-prone nature of reverse transcription, and mutations have been found in all four ORFs (Girones and Miller, 1989). Most frequent mutations occur in the basal core promoter (BCP) and precore (PC) regions. The BCP region (nucleotides 1742–1849) plays a vital role in HBV replication and morphogenesis by controlling the synthesis of precore mRNA and pregenomic RNA (pgRNA) (Quarleri, 2014). The PC region (nucleotides 1814–1900) encodes the precore protein, which is a precursor of HBeAg. Accumulated studies have shown that mutations in BCP and PC regions, such as A1613, T1653, G1727, A1757/T1764/G1766, S1753, T1773, T1766/A1768, A1896 and A1899 are related to progression to HCC, and those mutations individually and/or in combination are prognostic markers of HCC (Kumar, 2022). Our previous study identified six mutations (nucleotides 1896, 1915, 2134, 2221, 2245, and 2288) in precore/core regions that were associated with HBV-related HCC survival by multivariate analysis; among

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them, G1896A and C2288A can introduce mutations in amino acid residues (Xie et al., 2015). G1896A is one of the most commonly observed precore mutations in chronic HBV patients (Suppiah et al., 2015), and the prevalence of this mutation ranges between 20% and 95% in adults. The G1896A mutation stops the production of HBeAg by substitution of a tryptophan residue at amino acid position 28 with a premature stop codon (Tong et al., 2005). Although numerous epidemiologic studies have demonstrated that the G1896A mutation is significantly associated with progressive liver diseases (Liao et al., 2012; Kim et al., 2016; Al-Qahtani et al., 2018), the underlying molecular mechanisms of this mutation in the development of HBV-related HCC remain unknown.

Here, we investigated the effects of the G1896A mutation on HCC growth by performing a series of functional analyses. Our results showed that the G1896A mutation can enhance HBV replication and promote HCC cell growth *in vitro* and *in vivo*, and cells carrying the G1896A mutation were resistant to sorafenib treatment. The effect of the G1896A mutation on the development of HCC was closely related to the activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway. Our finding provides new insights into the mechanism of G1896A mutation on HBV-associated HCC.

## 2. Materials and methods

### 2.1. Cell culture

HepG2, Huh-7, PLC/PRF/5 and 293T cells were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). HCC cells were cultured in Eagle's Minimum Essential Medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Australia). 293T cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, CA, USA) with 10% fetal bovine serum. All the culture medium contained 100 U/mL penicillin and 100 μg/mL streptomycin, and cells were incubated at 37 °C in a humidified incubator containing 5% $CO_2$.

### 2.2. Plasmids and transfection

pCS-HBV1.3 and control plasmids were deposited in our laboratory, and G1896A mutated plasmid were generated using mutagenesis kit (Vazyme, Nanjing, China). For transfection, cells were seeded into 6-well plates to 80% confluence and transfected with plasmids using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols. Cells and supernatant were collected to analyze viral infection markers (i.e., HBsAg, HBeAg, HBV DNA, and pgRNA) as previous description (Chuai et al., 2014).

### 2.3. Generation of stable cell lines

The wild-type (WT) and G1896A mutated precore/core gene were synthesized by Sangon and cloned into the lentiviral expression vector pCDH. Lentiviruses were produced by cotransfection of pCDH and the packaging plasmids pLP1, pLP2 and pVSV-G into 293T cells. Then, pseudoviral particles were used to infect HCC cells, and cells stably expressing HBcAg and/or HBeAg were isolated using puromycin.

### 2.4. Immunofluorescence microscopy

HCC cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked in 10% goat serum. Then, the cells were incubated with HBc antibody (1:100, ab8638, abcam, Cambridge, UK) at 4 °C overnight and stained with Alexa Fluor 594-conjugated secondary antibody (1:200, ab150116, abcam, Cambridge, UK) for 1 h at room temperature, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The images were captured on a confocal laser scanning microscope with a 40× objective (Carl Zeiss AG, Germany).

### 2.5. Western blot

Total protein was extracted from HCC cells using RIPA buffer (89900, Thermo Fisher Scientific, MA, USA) supplemented with protease inhibitor and phosphatase inhibitor (K1007, K1015, ApexBio, MA, USA). Protein was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Next, the membranes were blocked followed by incubation with primary antibodies (HBc, abcam, Cambridge, UK; p-MEK1/2, t-MEK1/2, p-ERK1/2, t-ERK1/2, BAX, BCL2, c-myc and GAPDH antibodies, Proteintech, IL, USA) with a dilution of 1:1000 at 4 °C overnight and HRP-conjugated secondary antibodies (ab205718 and ab205719, abcam, Cambridge, UK) with a dilution of 1:5000 for 1 h at room temperature. The signals were detected using a chemiluminescence substrate (310208, ZETA LIFE, CA, USA).

### 2.6. Cell proliferation assay

HCC cell proliferation was measured by CCK8, colony formation and 5-ethynyl-2′-deoxyuridine (EdU) incorporation assays. For the CCK8 assay, HCC cells were seeded at $2 \times 10^3$ cells/well in 96-well plates and cultured for the indicated times. The cell number was determined by adding 10 μL of CCK8 solution (GK10001, GlpBio, CA, USA) and measuring the absorbance at 450 nm of each well. For the colony formation experiments, HCC cells were plated at $1 \times 10^3$ cells/well in twelve-well plates and incubated for 7 days. Cells were stained with crystal violet solution, and the colonies in each well were counted. For the EdU incorporation assay, HCC cells were inoculated in 6-well plates at $1 \times 10^5$ cells/well and cultured overnight. HCC cells were incubated with 10 μmol/L EdU (565456; BD Biosciences, NJ, USA) and then fixed and permeabilized. The working solution of the dye azide was added to each sample, the samples were incubated at room temperature, and the nuclei were counterstained with DAPI. The images were captured on a microscope with a 40× objective.

### 2.7. Apoptosis analysis

HCC cells were incubated for 48 h and used to assess cell apoptosis. APC Annexin V (550474; BD Biosciences, NJ, USA) and 7-amino-actinomycin D (7-AAD) (559925; BD Biosciences, NJ, USA) were added to cells resuspended in binding buffer, and then the cells were stained in the dark for 15 min and analyzed by flow cytometry (BD Biosciences,USA). Cells that were APC Annexin V positive and 7-AAD negative were considered at the early apoptosis phase. The apoptosis data were analyzed with FlowJo V10 software (FlowJo, LLC, OR, USA).

### 2.8. Drug sensitivity assay

HepG2 and PLC/PRF/5 cells seeded onto a 96-well plate ($5 \times 10^3$ cells/well) were treated with 0–60 μmol/L sorafenib (Y0002098, Sigma-Aldrich, Germany) for 48 h, and cell viability was determined by CCK8 assays. Concentration-response curves of sorafenib were fit to a nonlinear regression, and the $IC_{50}$ value was calculated with GraphPad Prism (GraphPad Software, MA, USA). Then, sorafenib sensitivity was compared among the three groups.

### 2.9. Mouse xenograft tumor model

Four-week-old male BALB/cA-nude mice were purchased from Beijing HFK Bioscience Co., Ltd. and were maintained under specific pathogen-free conditions. Two million HepG2 cells stably transfected with WT or G1896A precore/core gene were subcutaneously injected into the axilla of the nude mice. When masses developed after 18 days, the tumor volume was estimated every 7 days and was calculated as follows: volume = length × width$^2$/2. The mice were euthanized 46 days after implantation, and the tumors were weighed. The tumor tissue was

fixed with paraformaldehyde (4%), embedded in paraffin, and subjected to immunohistochemistry (IHC) to detect HBc, Ki67 and p-ERK1/2.

### 2.10. Statistical analysis

The statistical analysis was performed by using SPSS 25.0 (SPSS, Inc., Chicago, IL, USA). Student's *t*-test and one-way ANOVA were used to compare differences between two groups or among three or more groups, respectively. All experiments were performed independently and repeated at least three times, and data are presented as the means ± standard deviations (SD). A $P$ value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. G1896A mutation promotes HBV proliferation in HBV plasmid transfection model

To detect the effect of G1896A mutation on HBV replication, we constructed G1896A mutated plasmid based on HBV WT pCS-HBV1.3. Indicators of viral replication were examined after transfection of WT or G1896A mutant plasmids into HepG2 or Huh-7 cells respectively. G1896A mutation abrogated HBeAg expression (Fig. 1A), while HBsAg expression, HBV DNA and pgRNA (Fig. 1B–D) were all significantly increased in HCC cells transfected with G1896A mutant plasmid compared with WT group.

### 3.2. HBV G1896A mutation promoted HCC cell proliferation in vitro

To investigate whether the HBV G1896A mutation influence HCC cell proliferation, we overexpressed WT or G1896A mutated HBV precore/core gene in HepG2 and PLC/PRF/5 cells. HBcAg was successfully expressed in both HCC cell lines after WT or G1896A lentivirus transduction, as shown by immunostaining and Western blot assays (Fig. 2A and B), and HBeAg was expressed only in WT lentivirus-transduced HCC cells (Fig. 2C). This indicated that the precore/core gene was expressed in the same way as the HBV whole viral genome in cells.

Next, we performed a series of experiments on precore/core-overexpressing HCC cells and the corresponding control cells to evaluate whether HBV G1896A mutation can promote HCC cell proliferation. A significant increase in cell viability in HepG2 and PLC/PRF/5 cells after precore/core overexpression was detected by CCK8 assay, and we found that HCC cells with G1896A muation exhibited higher cell viability than WT HCC cells (Fig. 3A). G1896A HCC cells were more likely to grow into colonies than WT and control HCC cells (Fig. 3B). Furthermore, EdU incorporation assays showed a higher proportion of cell progressing through the S (DNA synthesis) phase of the cell cycle in the G1896A group than in the WT and control groups (Fig. 3C). These results indicated that the G1896A mutation enhanced the proliferative ability of HCC cells.

### 3.3. HBV G1896A mutation attenuated the sorafenib sensitivity of HCC cells

The multikinase inhibitor sorafenib is the approved first-line therapy for advanced hepatocellular carcinoma (Huang et al., 2020). It can prolong the survival of HCC patients, but its effect is limited due to acquired resistance (Fan et al., 2020; Zhang et al., 2023). The relationship between the G1896A mutation and the sensitivity of HCC cells to sorafenib was determined by cytotoxicity assay *in vitro*. We first established dose-response curves of cell growth inhibition by sorafenib. The $IC_{50}$ values following 48 h incubation were 5.24 (4.79–5.74) μmol/L and 5.97 (5.50–6.47) μmol/L for control HepG2 and PLC/PRF/5 cells, respectively (Fig. 4A). Then, we incubated control, WT or G1896A HCC

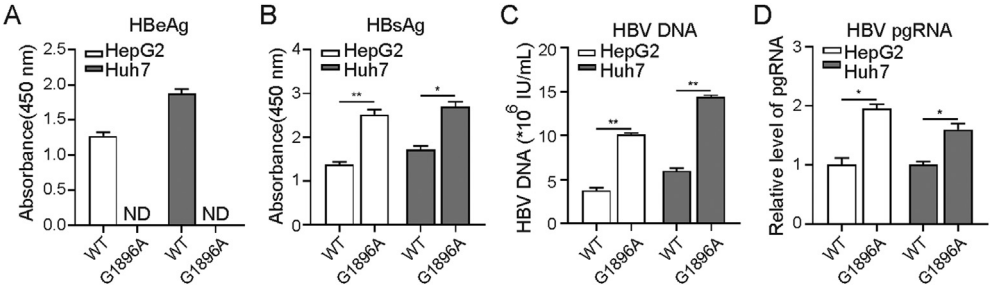


**Fig. 1.** G1896A mutation promotes HBV proliferation in transfection model. HBeAg (**A**), HBsAg (**B**), HBV DNA (**C**) and pgRNA (**D**) levels in the wild-type and G1896A mutated pCS-HBV1.3 transfected HCC cells were determined by ELISA and qPCR. The data are presented as the means ± standard deviations (SD). Statistical analysis was performed by student's *t*-test. $*P < 0.05$, $**P < 0.01$; ND, not detected.

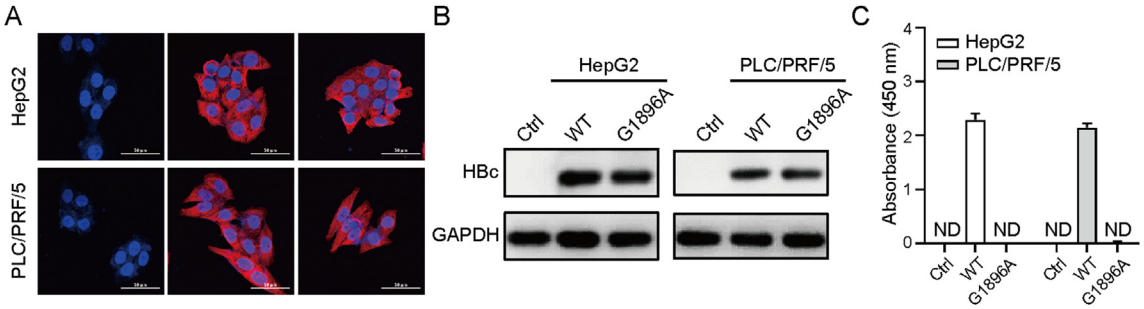


**Fig. 2.** Overexpression of wild-type and G1896A mutated HBV precore/core gene in human HCC cell lines. **A**, **B** Immunofluorescence and immunoblot analysis were employed to detect the expression of HBc in control (control lentiviruses infected), wild-type and G1896A HCC cells. Scale bar, 50 μm. Blue, DAPI; red, HBc. **C** HBeAg levels in the supernatant of control, WT and G1896A HCC cells were determined by ELISA. ND, not detected. One-way ANOVA was used for statistical analysis.

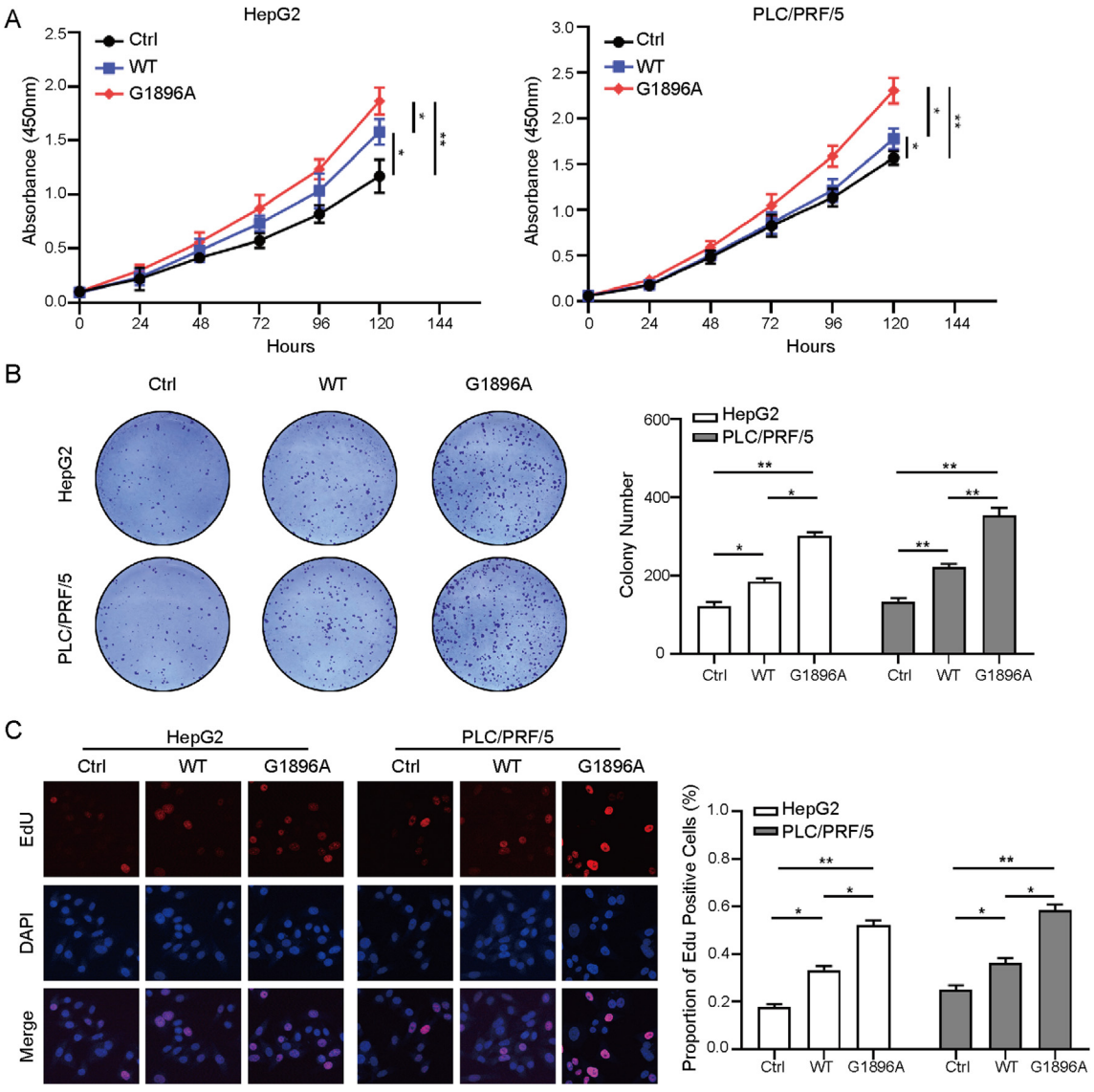


**Fig. 3.** G1896A mutation promotes HCC cell proliferation *in vitro*. Control (control lentiviruses infected), wild-type and G1896A HCC cells were used to detect cell proliferations. **A** CCK8 assay was performed to evaluate HCC cell viability. **B** Colony formation experiments and statistical analysis were performed to assess the long-term proliferation of HCC cells. **C** Representative images and statistical analysis of the EdU assay of HCC cells. Scale bar, 50 μm; One-way ANOVA was used for statistical analysis. $*P < 0.05$, $**P < 0.01$.

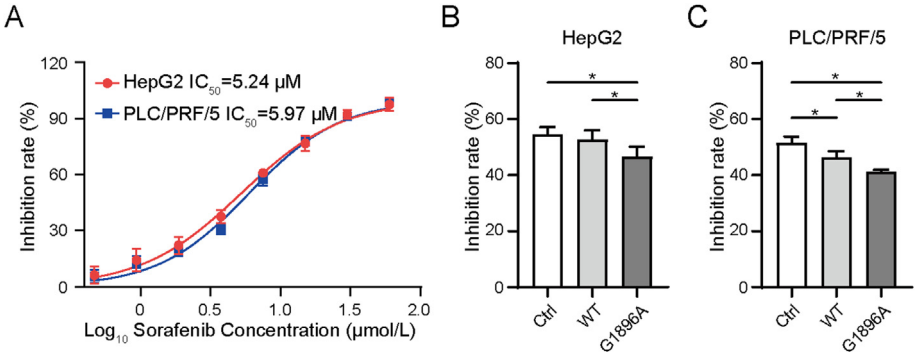


**Fig. 4.** G1896A mutation impairs the antiproliferative activity of sorafenib in HCC cells. Control (control lentiviruses infected), wild-type and G1896A HCC cells were used. **A** Dose-response curves of cell proliferation inhibition by sorafenib in HepG2 and PLC/PRF/5 cells. **B**, **C** Inhibition rate of sorafenib at $IC_{50}$ in control, wild-type and G1896A HCC cells. One-way ANOVA was used for statistical analysis. $*P < 0.05$.

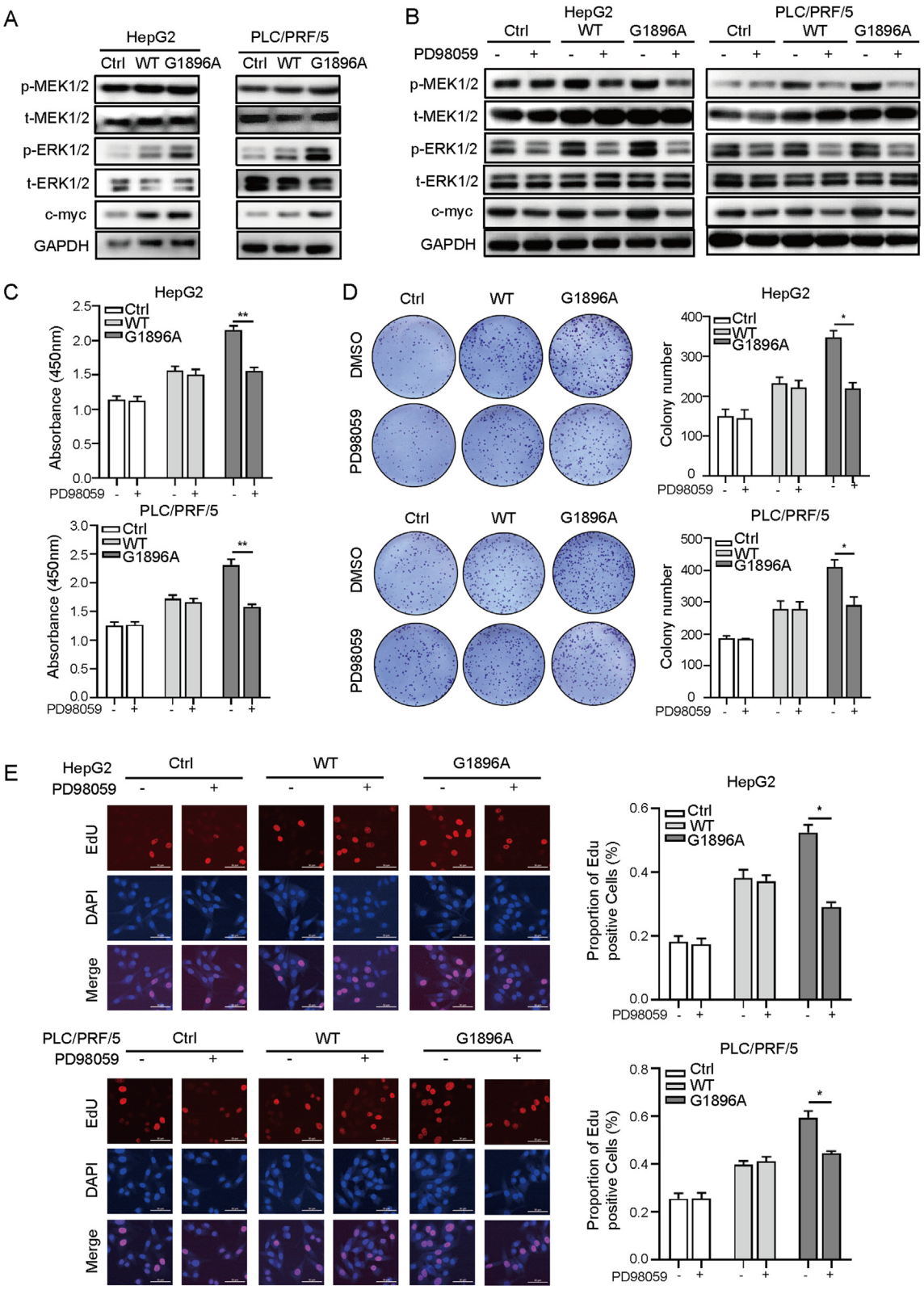


**Fig. 5.** HBV G1896A mutation promotes HCC cell proliferation by activating the ERK/MAPK signaling pathway. The results from control (control lentiviruses infected), wild-type and G1896A HCC cell groups were compared. **A** Immunoblot analysis of p-MEK1/2, t-MEK1/2, p-ERK1/2, t-ERK1/2 and c-myc in control, wild-type and G1896A HCC cells. **B** Lysates of control, wild-type and G1896A HCC cells treated with or without PD98059 were analyzed by immunoblotting using p-MEK1/2, t-MEK1/2, p-ERK1/2, t-ERK1/2 and c-myc antibodies. CCK8 assay (**C**), colony formation assay (**D**) and EdU incorporation assay (**E**) were conducted to assess the influence of PD98059 on control, wild-type and G1896A HCC cell proliferation. Scale bar, 50 μm. Student's *t*-test was used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

cells with sorafenib at $IC_{50}$ and found that the G1896A mutation alleviated the inhibitory effects of sorafenib on HCC cells (Fig. 4B and C).

### 3.4. HBV G1896A mutation promoted the proliferation of HCC cells by activating the ERK/MAPK signaling pathway

Since sorafenib is a receptor tyrosine kinase inhibitor that can block the proliferation of tumor cells by suppressing the RAF/MEK/ERK pathway (Meyer et al., 2017), which is one of the most important MAPK signaling pathways and is tightly associated with cellular transformation and carcinogenesis in HCC(Guo et al., 2020). Therefore, we focused on the relationship between the G1896A mutation and the ERK/MAPK pathway. The phosphorylation of MEK1/2 and ERK1/2 and the expression of downstream c-myc were all elevated in G1896A HCC cells (Fig. 5A). We then performed loss-of-function assays to investigate the functional role of ERK/MAPK signaling in G1896A mutation-induced cell proliferation. As shown in Fig. 5B, PD98059, a selective inhibitor of ERK/MAPK signaling, significantly downregulated the phosphorylation of MEK1/2 and ERK1/2 and expression of c-myc in control, WT or G1896A HCC cells. In addition, disruption of ERK/MAPK signaling reduced the viability of G1896A HCC cells but had no remarkable influences on control or WT HCC cells (Fig. 5C). A significant reduction in cell proliferation in G1896A HepG2 and PLC/PRF/5 cells after PD98059 treatment was also detected by colony formation assay and EdU incorporation assay, while only modest effects were found in control or WT HCC cells (Fig. 5D and E). These data suggested that ERK/MAPK signaling is indispensable in the accelerated cell proliferation caused by the G1896A mutation.

### 3.5. HBV G1896A mutation inhibited HCC cell apoptosis by activating the ERK/MAPK signaling pathway

To detect whether the G1896A mutation influences HCC cell apoptosis, we performed a flow cytometry to quantify the apoptosis rate in control, WT and G1896A HCC cells, and the results showed that the G1896A mutation decreased cell apoptosis (Fig. 6A). Then, we tested the protein levels of BCL2 and BAX, which are antiapoptotic and proapoptotic members, respectively, of apoptosis signaling pathways. The expression of BCL2 was significantly increased, while the expression of BAX was significantly decreased in G1896A HCC cells compared with WT HCC cells (Fig. 6B). Considering that ERK/MAPK signaling plays a crucial role in the apoptosis of tumor cells, we next examined the effect of interference with this signaling on cell apoptosis. After treatment with PD98059, the protein level of BCL2 decreased, while the BAX level increased significantly in G1896A HCC cells (Fig. 6C). Meanwhile, the apoptosis rate of G1896A HCC cells was also increased (Fig. 6D). These results indicated that ERK/MAPK signaling may serve as a connecting bridge through which the G1896A mutation suppresses HCC cell apoptosis.

### 3.6. HBV G1896A mutation promoted HCC cell proliferation in vivo

To explore the association between the G1896A mutation and HCC cell growth *in vivo*, HepG2 cells with or without the G1896A mutation were injected into different groups of nude mice. As revealed by the findings, precore/core overexpression effectively increased the volume and weight of tumors in mice, and the G1896A mutation strengthened this effect (Fig. 7A–D). IHC analysis revealed that HBc was expressed properly as *in vitro*, and Ki67, a biomarker for proliferating cells, was significantly increased in G1896A HCC group, while p-ERK was upregulated in G1896A cells compared with WT HCC cells (Fig. 7E). The above data demonstrated the significance of the G1896A mutation in promoting HCC cell survival and proliferation.

## 4. Discussion

Mutations in the precore region are closely related to the progression of HBV-related liver diseases. The G1896A mutation, which creates a premature stop codon and abolishes the synthesis of HBeAg, is one of the most frequently observed precore mutations in chronic HBV patients and is linked with high HCC risk and liver disease severity (Liao et al., 2012; Suppiah et al., 2015). However, how the G1896A mutation affects HCC progression remains unclear. In the current study, for the first time, we demonstrated that the G1896A mutation enhanced HBV replication and promoted the growth of human HCC cells both *in vitro* and *in vivo*, which may be due to the promotion of HCC cell proliferation and apoptosis

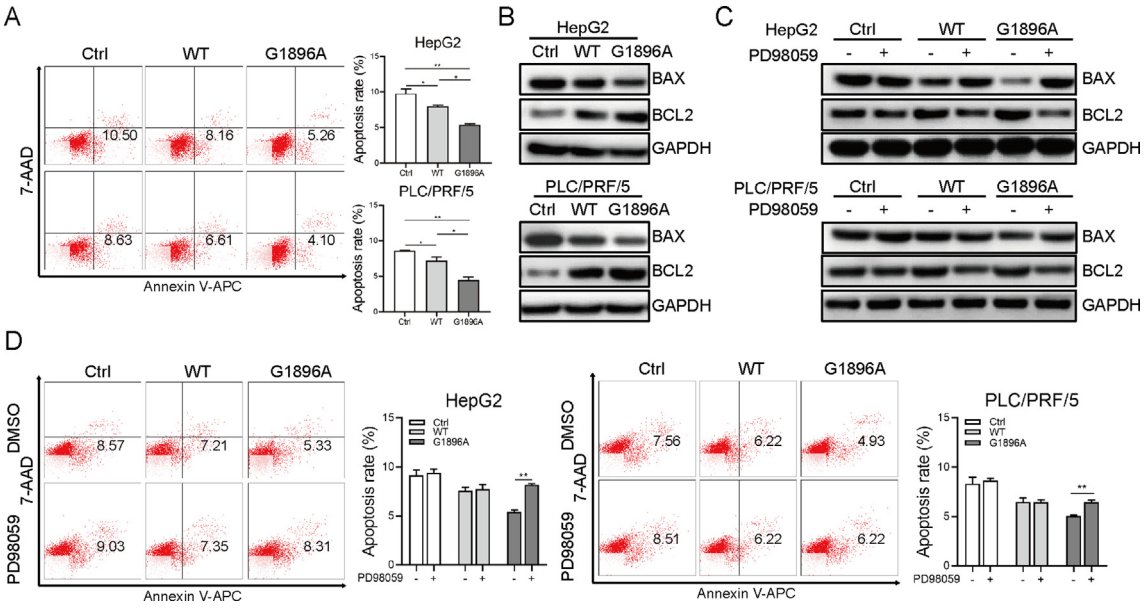


**Fig. 6.** HBV G1896A mutation suppressed HCC cell apoptosis by activating the ERK/MAPK signaling pathway. The results from control (control lentiviruses infected), wild-type and G1896A HCC cell groups were compared. **A** Apoptosis rate of control, wild-type and G1896A HCC cells were determined by flow cytometry. **B** Immunoblot analysis of BAX and BCL2 in control, wild-type and G1896A HCC cells. **C** Lysates of control, wild-type and G1896A HCC cells treated with or without PD98059 were analyzed by immunoblotting using BAX and BCL2 antibodies. **D** Flow cytometry was used to analyze the influence of PD98059 on control, wild-type and G1896A HCC cell apoptosis. Student's *t*-test and one-way ANOVA were used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

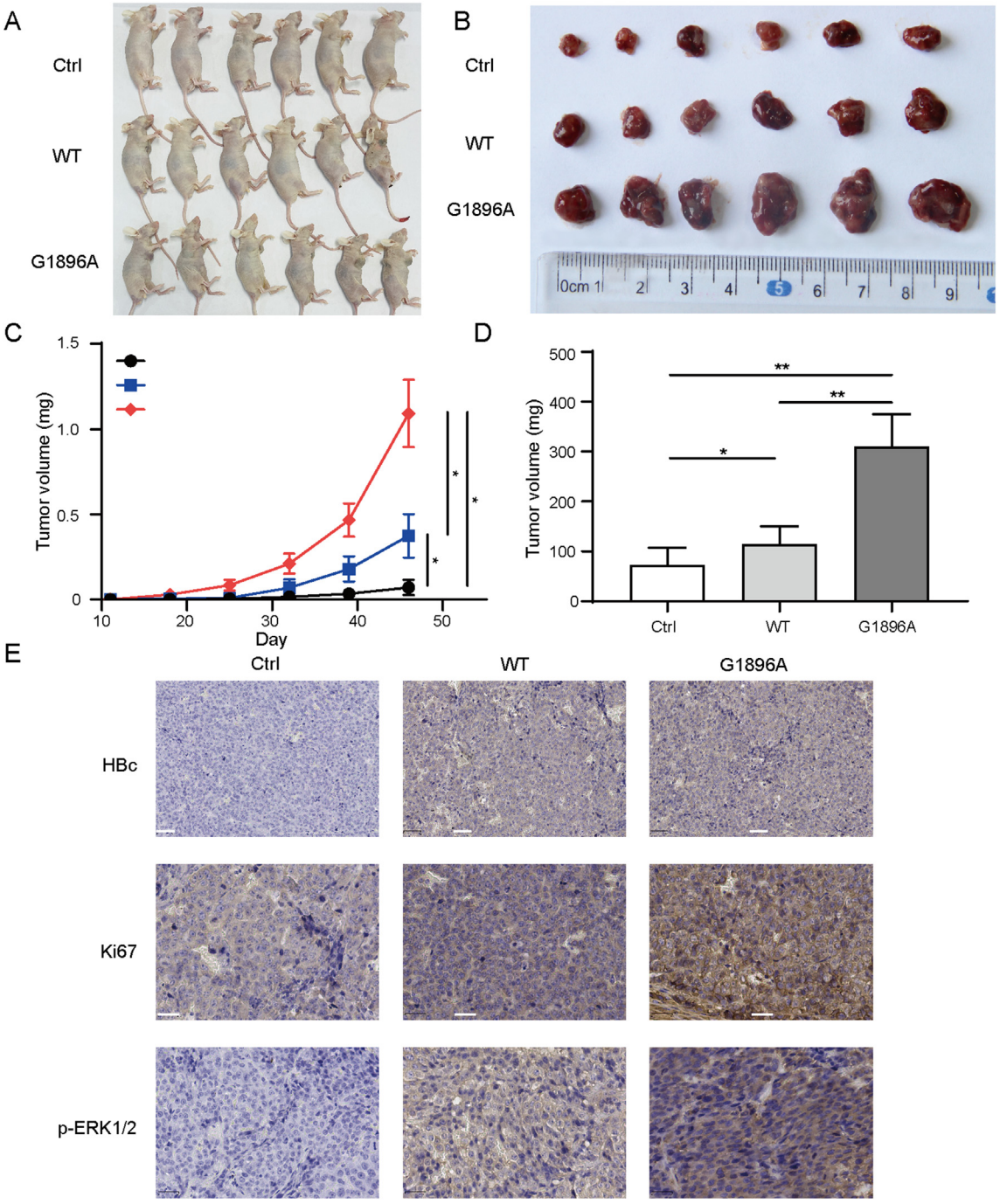


**Fig. 7.** G1896A mutation promotes HCC cell tumorigenicity *in vivo*. The results from control (control lentiviruses infected), wild-type and G1896A HCC cell groups were compared. **A, B** The xenograft mouse model and representative images of tumors formed in mice implanted with control, wild-type and G1896A HCC cells. **C, D** Changes in the tumor volume and weight were evaluated 46 days after implantation. **E** Representative IHC images of HBc, Ki67 and p-ERK1/2 staining of the xenografts. Scale bar, 30 μm. One-way ANOVA was used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$.

inhibition. Furthermore, G1896A HCC cells were resistant to sorafenib treatment. Mechanistically, the G1896A mutation affects HCC cell growth via the ERK/MAPK pathway (Fig. 8), a decisive and central pathway in the development of HCC (Xia et al., 2012; Li et al., 2016; Moon and Ro, 2021; Zhang et al., 2021).

Considering that HBV replication is a potential risk factor for HCC, and prognosis of HCC patients is strongly related with higher serum HBV DNA levels (Chen et al., 2009; Kubo et al., 2015), we examined the effect of G1896A mutation on HBV replication. Some similar studies have also shown that G1896A mutation promote HBV replication both *in vitro* an *in vivo* (Lee et al., 2015; Samal et al., 2015). The G1896A mutant plasmid represented higher levels of HBV DNA, pgRNA and HBsAg, suggesting that it is associated with higher replication efficiency, and this mutation may promote HCC progression by enhancing HBV replication. Considering the immunogenicity of HBeAg, the G1896A mutation may evade the attack of the host immune response by inhibiting HBeAg production, thus increasing the HBV replication level and contribute to the inflammatory responses in the progression to HCC (Arzumanyan et al., 2013). Our results were in agreement with the previous report that G1896A mutation increased HBV DNA replication efficiency when it was compared with the wild-type virus (Tian et al., 2016).

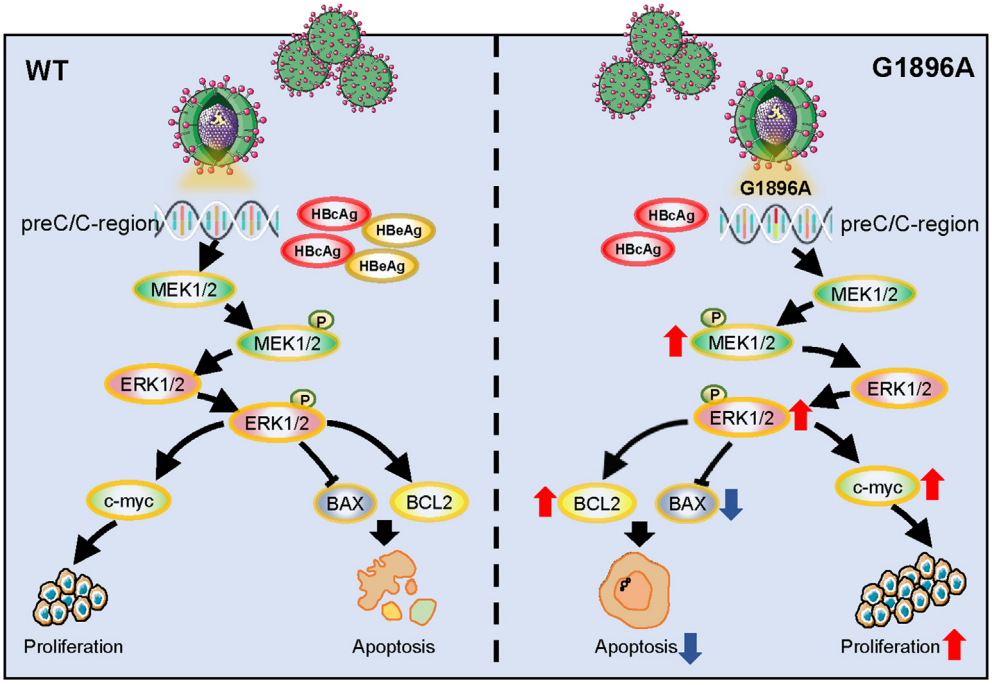


**Fig. 8.** Schematic representation of the proposed molecular mechanism, by which G1896A mutation promotes proliferation and inhibits apoptosis of HCC cells.

To characterize the roles of the G1896A mutation in HCC progression, we then generated stable clones of HepG2 and PLC/PRF/5 cells expressing either WT or G1896A mutated HBV precore/core gene. Both the G1896A HCC express HBcAg but do not express HBeAg. We found that overexpression of WT and G1896A mutated HBV precore/core can promote cell growth and enhance the colony formation ability of HepG2 and PLC/PRF/5 cells, and the G1896A group showed significantly higher pro-proliferation function than the WT group. Cells overexpressing the WT HBV precore/core gene can express both HBc and HBe antigens. HBc is the structural protein of the viral nucleocapsid and is involved in nearly every stage of the HBV life cycle and the pathogenesis induced by HBV (Jeong et al., 2014). Numerous studies have suggested that HBc is an oncogenic factor capable of promoting HBV malignant transformation (Lefeuvre et al., 2021). G1896A-mutant cells still expressed HBc, which significantly enhanced the proliferation-promoting effect of HBc in our study. Moreover, the G1896A mutation stops HBeAg expression, which is consistent with some clinical surveys showing that the G1896A mutant can be detected in up to 80% of HBeAg-negative patients (Bonino et al., 1991; Lok et al., 1995). Our results indicated that the G1896A mutation was also involved in anti-apoptosis in HepG2 and PLC/PRF/5 cells, and the apoptosis rate in the two HCC cell lines carrying the G1896A mutation was significantly lower than that of the WT and control groups. The expression of BAX was significantly decreased, while the expression of BCL2 was significantly increased in the G1896A HCC cells compared with WT HCC cells. Therefore, the G1896A mutation is involved in both the proliferation of HCC cells and the resistance of HCC cells to apoptosis, which contributes to the growth of HCC cells *in vitro*. To further evaluate the effect of the G1896A mutation on tumor growth *in vivo*, xenograft model were employed, and we found that the xenografts size of G1896A group was significantly increased compared with that of the control and WT groups. Furthermore, IHC staining revealed an increase in Ki67 staining in the G1896A tumors. Our results suggest that G1896A mutation plays a carcinogenic role in HCC cells, which could explain why patients with this mutation have a high risk of HCC as well as the progression of liver diseases.

Sorafenib is a targeted drug for HCC and is a tyrosine kinase inhibitor with the ability to inhibit tumor cell proliferation and angiogenesis (Huang et al., 2020). It inhibits the proliferation of tumor cells by inhibiting the RAF/MEK/ERK signaling pathway and inhibits vascular endothelial growth factor receptor 2/3 (VEGFR-2/3) and platelet-derived growth factor receptor (Llovet et al., 2008; Meyer et al., 2017). In addition, sorafenib also induces apoptosis in HCC cells (Wilhelm et al., 2004). However, approximately 70% of patients are resistant to sorafenib, which remains a huge challenge in HCC treatment (Llovet et al., 2008; Chen et al., 2015; Tang et al., 2020), and combination therapies could be used to sensitize HCC cells to sorafenib (Jiang and Dai, 2023; Rimassa et al., 2023). In our study, we observed that, compared with the control and WT group, G1896A HepG2 and PLC/PRF/5 cells are both resistant to sorafenib treatment. Our results suggest that in HBV-related HCC patients, the G1896A mutation may be one of the mechanisms of sorafenib resistance and provide valuable clues for the clinical treatment of HCC patients.

The ERK/MAPK signaling pathway is frequently activated in liver cancer and has been demonstrated to enhance tumor growth in multiple kinds of cancer, including HCC (Ito et al., 1998; Moon and Ro, 2021). Notably, the ERK pathway is a classical signaling pathway that promotes tumor proliferation and inhibits apoptosis (He et al., 2022). MEK1/2 are major components of the ERK/MAPK signaling pathway, which leads to phosphorylation of the downstream ERK1/2 (Dimri and Satyanarayana, 2020). Activation of ERK1/2 stimulate the expression of other proteins, such as c-myc and BCL2, thus regulating tumor proliferation and apoptosis (Deng and Karin, 1994; Xing et al., 1996). Our studies indicated that the precore G1896A mutation could accelerate proliferation and inhibit the apoptosis of HCC cells, and it was also resistant to sorafenib treatment, which was reported to inhibit the RAF/MEK/ERK signaling pathway. Therefore, we hypothesized that the G1896A mutation plays an important role in HCC through the ERK/MAPK signaling pathway. Previous studies have verified that c-myc is a proto-oncogene that plays a vital role in the carcinogenic transformation of normal cells and tumor proliferation (Zhu et al., 2019). It has been reported to play an important role in the development of HCC(Zhao et al., 2013). A study conducted by Zehua Z et al. (Zuo et al., 2023) showed that c-myc is a downstream effector of ERK signaling, and inhibition of the ERK-c-myc axis in tumor endothelial cells (ECs) may be an efficient strategy to inhibit tumor growth. Our data indicated that overexpression of precore G1896A could upregulate p-MEK, p-ERK and c-myc expression, while the

ERK inhibitor PD98059 inhibited HCC cell proliferation by down-regulating the expression levels of p-ERK and c-myc. PD98059 also enhanced G1896A HCC cell apoptosis through increasing BAX expression and decreasing BCL2 expression. These data suggested that the G1896A mutation promotes the growth of HCC partially by activating the ERK/MAPK pathway.

## 5. Conclusions

Overall, the current study identified a novel role of precore G1896A mutation in HCC carcinogenesis. It can promote HCC cells proliferation and inhibit apoptosis in *vitro* and increase HCC tumor growth *in vivo* by increasing HBV replication and activating the ERK/MAPK pathway. However, the mechanisms by which the G1896A mutation activates the ERK/MAPK pathway need further exploration.

## Data availability

All data generated or analyzed during this study are included in this published article.

## Ethics statement

This study was supervised and approved by the Ethics Board of the Animal Ethics Committee of Hebei Medical University. All experiments involving the use of mice were approved by the Ethics Committee of Hebei Medical University.

## Author contributions

Sandra Chiu and Xia Chuai: conceptualization and design. Baoxin Zhao, Hongxiu Qiao and Yan Zhao: methodology and software. Baoxin Zhao and Yan Cui: data curation, writing-original draft preparation. Zhiyun Gao and Jian Li: visualization, investigation and supervision. Weijie Wang: software, validation. Zhanjun Guo: writing-reviewing and editing. All authors read and approved the final version of the manuscript.

## Conflict of interest

Prof. Sandra Chiu is an editorial board member for *Virologica Sinica*, and was not involved in the editorial review or the decision to publish this article. The authors declare no conflicts of interests.

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## References

Al-Qahtani, A.A., Al-Anazi, M.R., Nazir, N., Abdo, A.A., Sanai, F.M., Al-Hamoudi, W.K., Alswat, K.A., Al-Ashgar, H.I., Khan, M.Q., Albenmousa, A., El-Shamy, A., Alanazi, S.K., Dela Cruz, D., Bohol, M.F.F., Al-Ahdal, M.N., 2018. The correlation between hepatitis B virus precore/core mutations and the progression of severe liver disease. Front. Cell. Infect. Microbiol. 8, 355.

Arzumanyan, A., Reis, H.M., Feitelson, M.A., 2013. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat. Rev. Cancer 13, 123–135.

Bonino, F., Brunetto, M.R., Rizzetto, M., Will, H., 1991. Hepatitis B virus unable to secrete e antigen. Gastroenterology 100, 1138–1141.

Chen, C.J., Yang, H.I., Iloeje, U.H., Group, R.-H.S., 2009. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. Hepatology 49, S72–S84.

Chen, J., Jin, R., Zhao, J., Liu, J., Ying, H., Yan, H., Zhou, S., Liang, Y., Huang, D., Liang, X., Yu, H., Lin, H., Cai, X., 2015. Potential molecular, cellular and microenvironmental mechanism of sorafenib resistance in hepatocellular carcinoma. Cancer Lett. 367, 1–11.

Chotiyaputta, W., Lok, A.S., 2009. Hepatitis B virus variants. Nat. Rev. Gastroenterol. Hepatol. 6, 453–462.

Chuai, X., Wang, W., Chen, H., Deng, Y., Wen, B., Tan, W., 2014. Lentiviral backbone-based hepatitis B virus replicon-mediated transfer favours the establishment of persistent hepatitis B virus infection in mice after hydrodynamic injection. Antivir. Res. 101, 68–74.

Deng, T., Karin, M., 1994. c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature 371, 171–175.

Dimri, M., Satyanarayana, A., 2020. Molecular Signaling Pathways and Therapeutic Targets in Hepatocellular Carcinoma. Cancers 12, 491.

Fan, G., Wei, X., Xu, X., 2020. Is the era of sorafenib over? A review of the literature. Ther Adv Med Oncol 12, 1758835920927602.

Girones, R., Miller, R.H., 1989. Mutation rate of the hepadnavirus genome. Virology 170, 595–597.

Guo, Y.J., Pan, W.W., Liu, S.B., Shen, Z.F., Xu, Y., Hu, L.L., 2020. ERK/MAPK signalling pathway and tumorigenesis. Exp. Ther. Med. 19, 1997–2007.

He, B., Guo, L., Hu, Y., Huang, H., Wan, L., Xu, K., Wang, F., Wen, Z., 2022. Desmocollin-2 inhibits cell proliferation and promotes apoptosis in hepatocellular carcinoma via the ERK/c-MYC signaling pathway. Aging (Albany NY) 14, 8805–8817.

Huang, A., Yang, X.R., Chung, W.Y., Dennison, A.R., Zhou, J., 2020. Targeted therapy for hepatocellular carcinoma. Signal Transduct. Targeted Ther. 5, 146.

Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., Hori, M., 1998. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 27, 951–958.

Jeong, H., Cho, M.H., Park, S.G., Jung, G., 2014. Interaction between nucleophosmin and HBV core protein increases HBV capsid assembly. FEBS Lett. 588, 851–858.

Jiang, Z., Dai, C., 2023. Potential treatment strategies for hepatocellular carcinoma cell sensitization to sorafenib. J. Hepatocell. Carcinoma 10, 257–266.

Kim, H., Lee, S.A., Do, S.Y., Kim, B.J., 2016. Precore/core region mutations of hepatitis B virus related to clinical severity. World J. Gastroenterol. 22, 4287–4296.

Kubo, S., Takemura, S., Tanaka, S., Shinkawa, H., Nishioka, T., Nozawa, A., Kinoshita, M., Hamano, G., Ito, T., Urata, Y., 2015. Management of hepatitis B virus infection during treatment for hepatitis B virus-related hepatocellular carcinoma. World J. Gastroenterol. 21, 8249–8255.

Kumar, R., 2022. Review on hepatitis B virus precore/core promoter mutations and their correlation with genotypes and liver disease severity. World J. Hepatol. 14, 708–718.

Lee, H., Kim, H., Lee, S.A., Won, Y.S., Kim, H.I., Inn, K.S., Kim, B.J., 2015. Upregulation of endoplasmic reticulum stress and reactive oxygen species by naturally occurring mutations in hepatitis B virus core antigen. J. Gen. Virol. 96, 1850–1854.

Lefeuvre, C., Le Guillou-Guillemette, H., Ducancelle, A., 2021. A pleiotropic role of the hepatitis B virus core protein in hepatocarcinogenesis. Int. J. Mol. Sci. 22, 13651.

Li, M., Zhou, Z.H., Sun, X.H., Zhang, X., Zhu, X.J., Jin, S.G., Gao, Y.T., Jiang, Y., Gao, Y.Q., 2016. Hepatitis B core antigen upregulates B7-H1 on dendritic cells by activating the AKT/ERK/P38 pathway: a possible mechanism of hepatitis B virus persistence. Lab. Invest. 96, 1156–1164.

Liao, Y., Hu, X., Chen, J., Cai, B., Tang, J., Ying, B., Wang, H., Wang, L., 2012. Precore mutation of hepatitis B virus may contribute to hepatocellular carcinoma risk: evidence from an updated meta-analysis. PLoS One 7, e38394.

Liu, T., Liu, A., Liu, Y., Cen, S., Zhang, Q., 2022. In vitro investigation of hbv clinical isolates from chinese patients reveals that genotype c isolates possess higher infectivity than genotype b isolates. Virol. Sin. 37, 398–407.

Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., De Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Greten, T.F., Galle, P.R., Seitz, J.F., Borbath, I., Haussinger, D., Giannaris, T., Shan, M., Moscovici, M., Voliotis, D., Bruix, J., Group, S.I.S., 2008. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 359, 378–390.

Lok, A.S., Akarca, U.S., Greene, S., 1995. Predictive value of precore hepatitis B virus mutations in spontaneous and interferon-induced hepatitis B e antigen clearance. Hepatology 21, 19–24.

Meyer, T., Fox, R., Ma, Y.T., Ross, P.J., James, M.W., Sturgess, R., Stubbs, C., Stocken, D.D., Wall, L., Watkinson, A., Hacking, N., Evans, T.R.J., Collins, P., Hubner, R.A., Cunningham, D., Primrose, J.N., Johnson, P.J., Palmer, D.H., 2017. Sorafenib in combination with transarterial chemoembolisation in patients with unresectable hepatocellular carcinoma (TACE 2): a randomised placebo-controlled, double-blind, phase 3 trial. Lancet Gastroenterol. Hepatol. 2, 565–575.

Moon, H., Ro, S.W., 2021. MAPK/ERK signaling pathway in hepatocellular carcinoma. Cancers 13, 3026.

Nguyen, D.H., Ludgate, L., Hu, J., 2008. Hepatitis B virus-cell interactions and pathogenesis. J. Cell. Physiol. 216, 289–294.

Quarleri, J., 2014. Core promoter: a critical region where the hepatitis B virus makes decisions. World J. Gastroenterol. 20, 425–435.

Rimassa, L., Finn, R.S., Sangro, B., 2023. Combination immunotherapy for hepatocellular carcinoma. J. Hepatol. https://doi.org/10.1016/j.jhep.2023.03.003.

Samal, J., Kandpal, M., Vivekanandan, P., 2015. Hepatitis B "e" antigen-mediated inhibition of HBV replication fitness and transcription efficiency in vitro. Virology 484, 234–240.

Seeger, C., Mason, W.S., 2000. Hepatitis B virus biology. Microbiol. Mol. Biol. Rev. 64, 51–68.

Sivasudhan, E., Blake, N., Lu, Z., Meng, J., Rong, R., 2022. Hepatitis B viral protein HBx and the molecular mechanisms modulating the hallmarks of hepatocellular carcinoma: a comprehensive review. Cells 11, 741.

Suppiah, J., Mohd Zain, R., Bahari, N., Haji Nawi, S., Saat, Z., 2015. G1896A precore mutation and association with HBeAg status, genotype and clinical status in patients with chronic hepatitis B. Hepat. Mon. 15, e31490.

Tang, L.S.Y., Covert, E., Wilson, E., Kottilil, S., 2018. Chronic hepatitis B infection: a review. JAMA 319, 1802–1813.

Tang, W., Chen, Z., Zhang, W., Cheng, Y., Zhang, B., Wu, F., Wang, Q., Wang, S., Rong, D., Reiter, F.P., De Toni, E.N., Wang, X., 2020. The mechanisms of sorafenib resistance in hepatocellular carcinoma: theoretical basis and therapeutic aspects. Signal Transduct. Targeted Ther. 5, 87.

Tian, Y., Kuo, C.F., Akbari, O., Ou, J.H., 2016. Maternal-Derived Hepatitis B Virus e Antigen Alters Macrophage Function in Offspring to Drive Viral Persistence after Vertical Transmission. Immunity 44, 1204–1214.

Tong, S., Kim, K.H., Chante, C., Wands, J., Li, J., 2005. Hepatitis B Virus e Antigen Variants. Int. J. Med. Sci. 2, 2–7.

Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., Mcnabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., Mchugh, M., Cao, Y., Shujath, J., Gawlak, S., Eveleigh, D., Rowley, B., Liu, L., Adnane, L., Lynch, M., Auclair, D., Taylor, I., Gedrich, R., Voznesensky, A., Riedl, B., Post, L.E., Bollag, G., Trail, P.A., 2004. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. 64, 7099–7109.

Xia, L., Tian, D., Huang, W., Zhu, H., Wang, J., Zhang, Y., Hu, H., Nie, Y., Fan, D., Wu, K., 2012. Upregulation of IL-23 expression in patients with chronic hepatitis B is mediated by the HBx/ERK/NF-kappaB pathway. J. Immunol. 188, 753–764.

Xie, Y., Liu, S., Zhao, Y., Zhang, L., Zhao, Y., Liu, B., Guo, Z., 2015. Precore/core region mutations in hepatitis B virus DNA predict postoperative survival in hepatocellular carcinoma. PLoS One 10, e0133393.

Xing, J., Ginty, D.D., Greenberg, M.E., 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273, 959–963.

Yardeni, D., Chang, K.M., Ghany, M.G., 2023. Current best practice in hepatitis B management and understanding long-term prospects for cure. Gastroenterology 164, 42–60 e46.

Zhang, C., Gao, Y., Du, C., Markowitz, G.J., Fu, J., Zhang, Z., Liu, C., Qin, W., Wang, H., Wang, F., Yang, P., 2021. Hepatitis B-induced IL8 promotes hepatocellular carcinoma venous metastasis and intrahepatic treg accumulation. Cancer Res. 81, 2386–2398.

Zhang, Y., Tan, Y., Liu, S., Yin, H., Duan, J., Fan, L., Zhao, X., Jiang, B., 2023. Implications of Withaferin A for the metastatic potential and drug resistance in hepatocellular carcinoma cells via Nrf2-mediated EMT and ferroptosis. Toxicol. Mech. Methods 33, 47–55.

Zhao, Y., Jian, W., Gao, W., Zheng, Y.X., Wang, Y.K., Zhou, Z.Q., Zhang, H., Wang, C.J., 2013. RNAi silencing of c-Myc inhibits cell migration, invasion, and proliferation in HepG2 human hepatocellular carcinoma cell line: c-Myc silencing in hepatocellular carcinoma cell. Cancer Cell Int. 13, 23.

Zhu, P., Li, Y., Li, P., Zhang, Y., Wang, X., 2019. c-Myc induced the regulation of long non-coding RNA RHPN1-AS1 on breast cancer cell proliferation via inhibiting P53. Mol. Genet. Genom. 294, 1219–1229.

Zuo, Z., Liu, J., Sun, Z., Cheng, Y.W., Ewing, M., Bugge, T.H., Finkel, T., Leppla, S.H., Liu, S., 2023. ERK and c-Myc signaling in host-derived tumor endothelial cells is essential for solid tumor growth. Proc. Natl. Acad. Sci. U. S. A. 120, e2211927120.