



ORIGINAL ARTICLE

α -Fetoprotein fragment synergizes with sorafenib to inhibit hepatoma cell growth and migration and promote the apoptosis

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Abstract

Alpha fetoprotein (AFP) is associated with hepatocellular carcinoma (HCC) by stimulating the proliferation, metastasis and drug resistance. The application of AFP fragments to inhibit the malignant behaviours induced by AFP is a new strategy for the treatment of HCC. In an effort to design, screen and discover drugs, we attempted to express different human AFP fragments (AFP²²⁰⁻⁶⁰⁹, AFP³⁹⁰⁻⁶⁰⁹ and AFP⁴⁶⁰⁻⁶⁰⁹) in a Bac-to-Bac system. We found that the AFP³⁹⁰⁻⁶⁰⁹ fragment was highly expressed in the system. Then, we assessed the bioactivity of the fragment in the human liver cancer cell line Bel7402, and the results indicated that the AFP fragment synergized with sorafenib to inhibit the hepatoma cell growth and migration and promote the apoptosis. This study provides a method to produce significant AFP fragments to screen AFP inhibitors for use in HCC therapy.

KEYWORDS

AFP inhibiting fragments, drug delivery, HCC therapy, protein expression, sorafenib

Qiujiiao Wang, Wei Li, Minni Zhang and Zijuan Zou contributed equally to this work and are the co-first authors.

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1 | INTRODUCTION

Human alpha fetoprotein (AFP) has a molecular mass of 70kDa, which consists of 609 amino acids with glycosylation sites that may link different structures of carbohydrate moieties in different tissues and diseases.¹ AFP contains three domains, each domain is composed of approximately 200 amino acids, and the domain3 is considered to play an important role in binding to signalling proteins and implementing the function.² AFP may interact with multiple signalling molecules, including membrane receptors, PTEN, caspase-3 and retinoid acid receptor- β (RAR- β), to mediate HCC cell proliferation, metastasis and apoptosis in oncogenic growth processes.^{3,4} The use of AFP fragments (derived from domain3) to prevent AFP from binding to its receptors or signal transduction molecules can inhibit the malignant behaviours mediated by AFP. Additionally, the use of AFP fragments that synergize with drugs can selectively destroy cancer cells.^{4,5}

Alpha fetoprotein fragments not only deliver drugs that selectively destroy cancer cells which highly express AFP receptors (AFPRs), but also inhibit the role of AFP in promoting malignant behaviours of cancer cells. For example, AFP-3BC (an AFP fragment derived domain3), which does not bind to non-stimulated lymphocytes, can be loaded with drugs to selectively accumulate in ovarian adenocarcinoma SKOV3 cells and human breast MCF7 cells, suppressing the proliferation of these cancer cells.⁶ Other fragments, rAFP3D and r3dAFP, which are also derived from AFP domain3, can deliver drugs to cancer cells with high AFPR expression. These AFP fragments can be combined with drugs to strengthen the therapeutic effect.^{7,8} These findings indicate that AFP fragments can be a promising new vector for delivering drugs or synergizing with drugs in selectively targeting and killing cancer cells.

The study was the first report of that different human AFP fragments (AFP²²⁰⁻⁶⁰⁹, AFP³⁹⁰⁻⁶⁰⁹ and AFP⁴⁶⁰⁻⁶⁰⁹) were expressed in a Bac-to-Bac system and the AFP³⁹⁰⁻⁶⁰⁹ fragment was the most highly expressed in the system. We also detected the bioactivity of the AFP³⁹⁰⁻⁶⁰⁹ fragment in the human liver cancer cell line Bel7402, which highly expresses AFPR.⁹ The results indicated that the AFP fragment synergized with sorafenib to inhibit HCC cell growth and migration and promote the apoptosis. The present study established a method for expressing and producing AFP fragments, which could be used to deliver drugs or synergize with drugs in the therapeutics of HCC.

2 | MATERIALS AND METHODS

2.1 | Expression vector construction

The four vectors constructed are shown in Table 1.

AFP (full-length AFP including residues 1–609) gene clone: AFP (NCBI: [NM001134](#)) cDNA (from base 45 to base 1872) was synthesized with a 6xHis tag sequence at the C-terminus. Synthetic cDNA was amplified by PCR using the following primer pair: 5'-CCGAAGC TTATGAAGTGGGTGGAATCAATTTTTT-3' and 5'-CGGGGATCCTC AGTGATGGTGGTGGTGAAGTCCCAAAG-3'.

AFP²²⁰⁻⁶⁰⁹ (AFP fragment including residues 220–609 with 6xHis) gene clone: AFP (NCBI: [NM001134](#)) cDNA (from base 705 to base 1872) was synthesized with ATG at the N-terminus and a 6xHis tag sequence at the C-terminus. Synthetic cDNA was amplified by PCR using the following primer pair: 5'-CCGAAGCTTATG CAACAT GCATGTGCAG TAATGAAAAATTTT-3' and 5'-CGGGGATCCTCAGT GATGGTGGTGGTGGTGAAGTCCCAAAG-3'.

AFP³⁹⁰⁻⁶⁰⁹ (AFP domain-3 fragment including residues 390–609 with 6xHis) gene clone: AFP (NCBI: [NM001134](#)) cDNA (from base 1215 to base 1872) was synthesized with ATG at the N-terminus and a 6xHis tag sequence at the C-terminus. Synthetic cDNA was amplified by PCR using the following primer pair: 5'-CCGAAGCTTATGCTTGAATGCCAAGAT-3' and 5'-CGGG GATCCTCAGTGGTGGTGGTGGTGAAGTCCCAAAG-3'.

AFP⁴⁶⁰⁻⁶⁰⁹ (AFP domain-3 fragment including residues 460–609 with 6xHis) gene clone: AFP (NCBI: [NM001134](#)) cDNA (from base 1425 to base 1872) was synthesized with ATG at the N-terminus and a 6xHis tag sequence at the C-terminus. Synthetic cDNA was amplified by PCR using the following primer pair: 5'-CCGAAGCTTATGTGTTGCCAACTCA-3' and 5'-CGGGGATCCTCAG TGATGGTGGTGGTGGTGAAGTCCCAAAG-3'.

The PCR products were digested with restriction enzymes (Takara Bio Inc.), and the restriction sites (*HindIII* and *BamHI*) are underlined. Then, the genes were ligated into the pFastBac-Dual expression vector (Invitrogen Inc.), and the fusion sequences were confirmed by sequencing. After that, the fusion pFastBac-Dual vectors were transformed into bacterial *DH10* cells, and the extracted bacmids were then transfected into Sf9 cells for 4 days to obtain passage 1 baculoviruses (P1 baculoviruses).¹⁰

TABLE 1 Constructed vectors

Vector name	Protein sequence
pFastBac-Dual-AFP	Full-length AFP including residues 1–609 with 6xHis at the C-terminus
pFastBac-Dual-AFP ²²⁰⁻⁶⁰⁹	AFP fragment including residues 220–609 with 6xHis at the C-terminus
pFastBac-Dual-AFP ³⁹⁰⁻⁶⁰⁹	AFP domain3 fragment including residues 390–609 with 6xHis at the C-terminus
pFastBac-Dual-AFP ⁴⁶⁰⁻⁶⁰⁹	AFP domain3 fragment including residues 460–609 with 6xHis at the C-terminus

2.2 | AFP and AFP fragment expression in a Bac-to-Bac baculovirus system

AFP and AFP fragments were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen Inc.). The process was as follows: (1) Sf9 insect cells were cultured in SFX-Insect (Cytiva Ltd.), SF900 II and Grace's medium (Thermo Fisher Ltd.) containing 10% foetal bovine serum (FBS) or without serum at a density of 1×10^6 cells/ml. (2) P1 baculoviruses were amplified to obtain P2 baculoviruses. The amplification process was as follows: 10 ml of P1 baculovirus was added to 100 ml of Sf9 cells cultured in SFX-Insect, SF900 II and Grace's medium and harvested at 72 h after infection. (3) Fifty milliliters of P2 baculovirus was added to 1 L of Sf9 cells, and secreted AFP and AFP fragments were harvested in the medium at 72 h after infection, as described previously.¹⁰

2.3 | Analysis of the expression of secreted AFP and AFP fragments

The insect medium containing secreted AFP and AFP fragments was collected and centrifuged at 4700 g for 5 min. Then, 200 μ l of 10 \times HBS buffer (10 mM HEPES [pH 7.2] and 150 mM NaCl) and 100 μ l of nickel-charged resin (Ni-NTA Agarose; Qiagen Ltd.) were added to 4 ml of supernatant. After the sample was mixed and shaken for 3 h at 4°C, it was centrifuged at 1200 g for 2 min and eluted with 200 μ l of 300 mM imidazole in HBS buffer. The eluted AFP and AFP fragments were analysed by SDS/PAGE. The reduced protein (denatured) SDS/PAGE sample contained a reducing buffer and was boiled for 10 min. The nonreduced protein sample did not contain a reducing buffer and was not heated.¹⁰

2.4 | Expression and purification of the AFP³⁹⁰⁻⁶⁰⁹ fragment in a Bac-to-Bac baculovirus system

Fifty millilitres of P2 baculoviruses with the AFP³⁹⁰⁻⁶⁰⁹ fragment gene was added to 1 L of Sf9 cells in SFX-Insect medium and harvested at 72 h after infection. Then, the medium was centrifuged at 4700 g for 5 min, and the supernatant was collected, filtered and concentrated to 100 ml with HBS buffer (10 mM Hepes [pH 7.2] and 150 mM NaCl) by cross-flow filtration (Millipore Corp.). The concentration was passed through nickel-charged resin (Ni-NTA Agarose; Qiagen Ltd.). The AFP³⁹⁰⁻⁶⁰⁹ fragment was captured by the resin and eluted with 500 mM imidazole. The eluted buffer was concentrated to 1 ml and then further purified by gel filtration chromatography with HBS buffer using a Superdex 200 Increase 10/300 column (Cytiva Ltd.).¹⁰ After affinity chromatography, it was centrifuged at 4700 g for 1 h by centrifugal filter units (Millipore Corp.) to obtain about concentration of 1 mg/ml protein.

2.5 | Western blotting

Western blotting was used for analysis of the expression of AFP and AFP fragments in a Bac-to-Bac system. The anti-AFP third domain antibodies used in this experiment were purchased from Beyotime Biotechnology. In addition, the anti-His tag antibodies were purchased from Sangon Biotech.

2.6 | Laser confocal microscopy observation AFP³⁹⁰⁻⁶⁰⁹ fragment uptake by HCC cells and normal hepatic cells

Firstly, cells were diluted with DMEM (Dulbecco's Modified Eagle Medium) without serum into a suspension of 2×10^4 cells/ml, and 500 μ l suspension cells were added into a chamber slide. Then add 30 μ l FITC-AFP³⁹⁰⁻⁶⁰⁹ fragment (FITC-AFP³⁹⁰⁻⁶⁰⁹ fragment link used HOOKDye Labeling Kit). Samples were culture at 37°C avoid light, with 5% CO₂ for 37 h. Secondly, the cells were washed three times (5 min each) with PBS, added 500 μ l 4% paraformaldehyde-fixed cell 30 min and used PBS wash three times. Thirdly, added 200 μ l DAPI for 10 min, wash with PBS for three times, then, added 50 μ l fluorescent quencher and observed with an Olympus FluoView FV1000 laser.

2.7 | MTT assay

Bel7402 cells were plated into 96-well plates and added with sorafenib or sorafenib + AFP fragments then, cultured in DMEM medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO₂ for 72 h. The viability of the cells was measured by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay as described. The cells viability = treated group A490/control group A490 \times 100%.

2.8 | RNA interference

Bel7402 cells were cultured until they reached 50% confluence. Then, they were transfected of siRNA-AFP Lentivirus (HitransG A; Shanghai Genechem Co., Ltd.) The cell lines Bel7402-siRNA-AFP which were interfered AFP expression by siRNA were screened by puromycin (Beyotime Biotechnology) after 72 h. The siRNA sequence is as follows: 5'-ccCTCTTGAATGCCAAGATAA-3', use vector GV493 (http://www.genechem.com.cn/service/index.php?ac=gene&at=vector_search&keyword=GV493).¹¹

2.9 | Scratch test

Cell motility was analysed by a scratch repair assay. One day before scratching, Bel7402 cells were treated with sorafenib (5 μ g/

ml); sorafenib (5 µg/ml) + AFP fragment (AFP³⁶⁰⁻⁶⁰⁹); and Bel7402-siRNA-AFP cells were treated with sorafenib (5 µg/ml), and were seeded into 6-well plates. A scratching wound was created by scraping the middle of the cell monolayer with a sterile micropipette tip. After all detached cells were washed away with PBS, the cells were cultured with medium containing 10% FBS, and images of cell migration into the wound area were captured at 0 and 72 h by an inverted microscope (100×) to record the distance migrated.¹¹

2.10 | Cell migration was detected by the transwell method

To measure cell migration, transwell chambers were used to observe cultured cell inserts (Transwell chamber; 8 mm pore size; Costar, Corning Co., Ltd). Bel7402 cells (5×10^4) were added to the upper chambers and cultured with serum-free DMEM and treated with sorafenib (5 µg/ml); sorafenib (5 µg/ml) + AFP fragment (AFP³⁶⁰⁻⁶⁰⁹); and Bel7402-siRNA-AFP cells were treated with sorafenib (5 µg/ml). In addition, the lower chamber was filled with complete medium containing 20% FBS. After 72 h of incubation, the cells in the upper chamber were carefully removed with a cotton swab, and those that had migrated through the membrane to the lower surface were fixed with 90% methanol and stained with 0.1% crystal violet. The number of cells that had migrated through the pores was quantified by counting five independent visual fields using a microscope (Olympus) with 20× objective. The migratory cell ratio = numbers of treated groups/numbers of untreated groups $\times 100\%$.¹¹

2.11 | Flow cytometry analysis

Bel7402, L-02 and PLC/PLF/5 cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. Then, the cells were treated with sorafenib (10 µg/ml), sorafenib (10 µg/ml) + siRNA-AFP or sorafenib (10 µg/ml) + AFP fragment (AFP³⁶⁰⁻⁶⁰⁹) for 24 h; harvested by trypsinization; washed with PBS; and resuspended and incubated in PE Annexin V solution (BD Biosciences) for apoptosis analysis. At least 20,000 live cells were analysed on a NovoCyte™ flow cytometer. Data were analysed using NovoExpress software.¹¹

2.12 | Statistical analysis

The results of multiple observations were presented as the mean \pm SD of three independent experiments. Statistical analysis was determined using one-way ANOVA and t-test. ** $p < 0.01$ and *** $p < 0.001$ were statistical significance.

3 | RESULTS

3.1 | Analysis of expression vectors

Figure 1A–D shows the four expression vectors, namely AFP, AFP²²⁰⁻⁶⁰⁹, AFP³⁹⁰⁻⁶⁰⁹ and AFP⁴⁶⁰⁻⁶⁰⁹. Figure 1A–D Lane M shows the DNA marker, and Lane 1 shows that the vectors were digested with *Bam*HI and *Hind*III restriction enzymes to release a band measuring approximately 2000 bp (A), 1200 bp (B), 660 bp (C) and 450 bp (D), confirming the presence of the recombinant human *afp* gene and the human *afp* fragment genes. DNA sequencing of the four recombinant vectors also confirmed the correct gene sequences. Figure 1A–D Lane 2 shows the recombinant vectors, and Lane 3 shows the correct homologous recombination that occurred in the bacmid.

3.2 | Analysis of AFP and fragments expression with 10% FBS or without serum

Sf9 cells cultured in SFX-Insect medium were transfected with P1 virus, and secreted recombinant AFP and fragments (the expressed protein secreted into the insect medium) were assessed by SDS/PAGE. The results showed that all four recombinant secreted proteins could be expressed and detected in the medium (Figure 2A: 1–8). The reduced AFP molecular mass was approximately 70 kDa, the AFP²²⁰⁻⁶⁰⁹ fragment molecular mass was approximately 43 kDa, the AFP³⁹⁰⁻⁶⁰⁹ fragment molecular mass was approximately 26 kDa, and the AFP⁴⁶⁰⁻⁶⁰⁹ fragment molecular mass was approximately 16 kDa, consistent with the sizes of the *afp* gene and the *afp* fragment gene. The nonreduced or native proteins were slightly lower than the reduced proteins in the SDS/PAGE gel because they were nonlinear and ran a little fast.

To test whether FBS (foetal bovine serum) could increase the expression level, 10% FBS was added to the medium, and it was found that 10% FBS did not increase the expression level (Figure 2B: 1–8); moreover, FBS affected the purification of protein.

To further confirm the bands are representing the corresponding AFP fragments, Western blotting was performed with anti-AFP third domain antibody and with anti-His tag antibody. The result showed the expressed proteins were correct (Figure 2C,D).

3.3 | Analysis of fragment expression in different media

To test different medium expression levels, P1 viruses with AFP³⁹⁰⁻⁶⁰⁹ and AFP⁴⁶⁰⁻⁶⁰⁹ fragments were transfected into Sf9 cells cultured in different media with or without 2% FBS. The secreted recombinant fragments extracted from the medium were assessed by SDS/PAGE, and the results showed that the AFP³⁹⁰⁻⁶⁰⁹

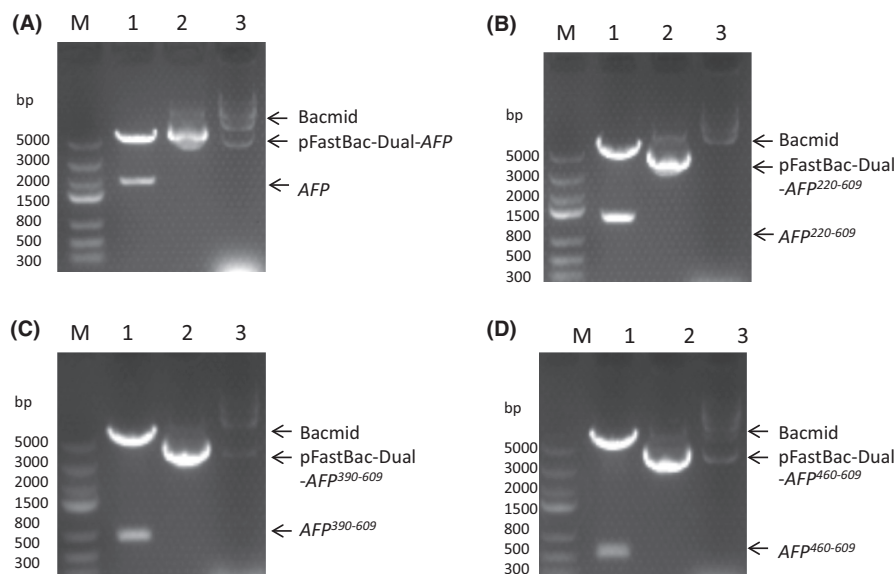


FIGURE 1 Agarose gel electrophoresis analysis of the recombinant vectors containing the human *afp* and fragment genes. (A) pFastBac-Dual-AFP (full-length AFP) vector, (B) pFastBac-Dual-AFP²²⁰⁻⁶⁰⁹ (AFP fragment including residues 220–609 with 6xHis) vector, (C) pFastBac-Dual-AFP³⁹⁰⁻⁶⁰⁹ (AFP domain3 fragment including residues 390–609 with 6xHis) vector and (D) pFastBac-Dual-AFP⁴⁶⁰⁻⁶⁰⁹ (AFP domain3 fragment including residues 460–609 with 6xHis) vector. Lane M, DNA marker; Lane 1, pFastBac-Dual-AFP and fragment vector digested by *Bam*HI and *Hind*III restriction enzymes; Lane 2, pFastBac-Dual-AFP and fragment vector; Lane 3, recombinant Bacmid.

fragment could be expressed in SFX-Insect medium with or without 2% FBS. However, the fragment could not be expressed in SF900 II medium without FBS (Figure 3A). The AFP⁴⁶⁰⁻⁶⁰⁹ fragment could be expressed in SFX-Insect and SF900 II medium with or without 2% FBS, but the expression in SFX-Insect medium was higher than that in the SF900 II medium (Figure 3A,B). Grace's medium did not express the fragments (data not shown). Therefore, it is better to express the AFP recombinant fragments in SFX-Insect medium.

3.4 | Purification of the AFP³⁹⁰⁻⁶⁰⁹ fragment

It was found that the AFP³⁹⁰⁻⁶⁰⁹ fragment was highly expressed, so we further expressed a certain amount and purified the protein in the system. Figure 4A shows the purification chromatography of the secreted AFP³⁹⁰⁻⁶⁰⁹ fragment from 1L of medium. The peak value was approximately 240mAU, which indicated that the purification process was successful and that a certain amount of the fragment protein was obtained. The Eluted fractions (from No.10 tube to No.24 tube) were collected and analysed by Coomassie-blue staining (Figure 4B); it was found that No.15–No.24 tube contained high concentration of AFP³⁹⁰⁻⁶⁰⁹ fragment. The fragment protein in No.15–No.24 tube was collected and further purified by gel filtration chromatography. The last purified AFP³⁹⁰⁻⁶⁰⁹ fragment was analysed by silver staining and Coomassie-blue staining (Figure 4C,D). The molecular mass was approximately 26kDa, which is consistent with the size of the *afp* gene fragment. The native protein formed two bands, the high band may be dimers, and the low band was monomer which ran slightly lower than the reduced proteins in the SDS/PAGE gel because they were nonlinear and ran a little fast.

The AFP³⁹⁰⁻⁶⁰⁹ protein was further analysed by LC/LC-MS/MS¹² and the results showed that the molecular weight of the purified sample was 25,834Da. In total, 88% of the protein sequence was determined and matched the sequence of AFP³⁹⁰⁻⁶⁰⁹ (Figure S1), indicating that AFP³⁹⁰⁻⁶⁰⁹ was correctly expressed and purified. The purified AFP³⁹⁰⁻⁶⁰⁹ fragment is soluble and its isoelectric point (IP) at pH 5.86.

3.5 | The AFP³⁹⁰⁻⁶⁰⁹ fragment (named AFP fragment briefly) uptake by Bel7402 cells

To test the AFP³⁹⁰⁻⁶⁰⁹ fragment uptake by HCC cells with high AFP receptor expressed, immunofluorescence and confocal microscope were observed using Bel7402 cells as model (HCC cells which highly expressed AFP receptor). Figure 5A showed that AFP³⁹⁰⁻⁶⁰⁹ fragment was uptake by Bel7402 cells, but it was not uptake by the L-02 cells (normal hepatic cells which did not expressed AFP receptor). It was also found that the uptake AFP³⁹⁰⁻⁶⁰⁹ fragment was mainly concentrated in the cell bulge that might affect the communication and growth between cells (Figure 5B).

3.6 | AFP³⁹⁰⁻⁶⁰⁹ fragment synergize with sorafenib to inhibit the growth of Bel7402

To test AFP³⁹⁰⁻⁶⁰⁹ fragment bioactivity, MTT assay was conducted. The results indicated that AFP³⁹⁰⁻⁶⁰⁹ fragment suppressed Bel7402 cell growth. The viability of Bel7402 cells decreased to 85% when treated with 200 µg/ml AFP³⁹⁰⁻⁶⁰⁹ fragment. However, the fragment

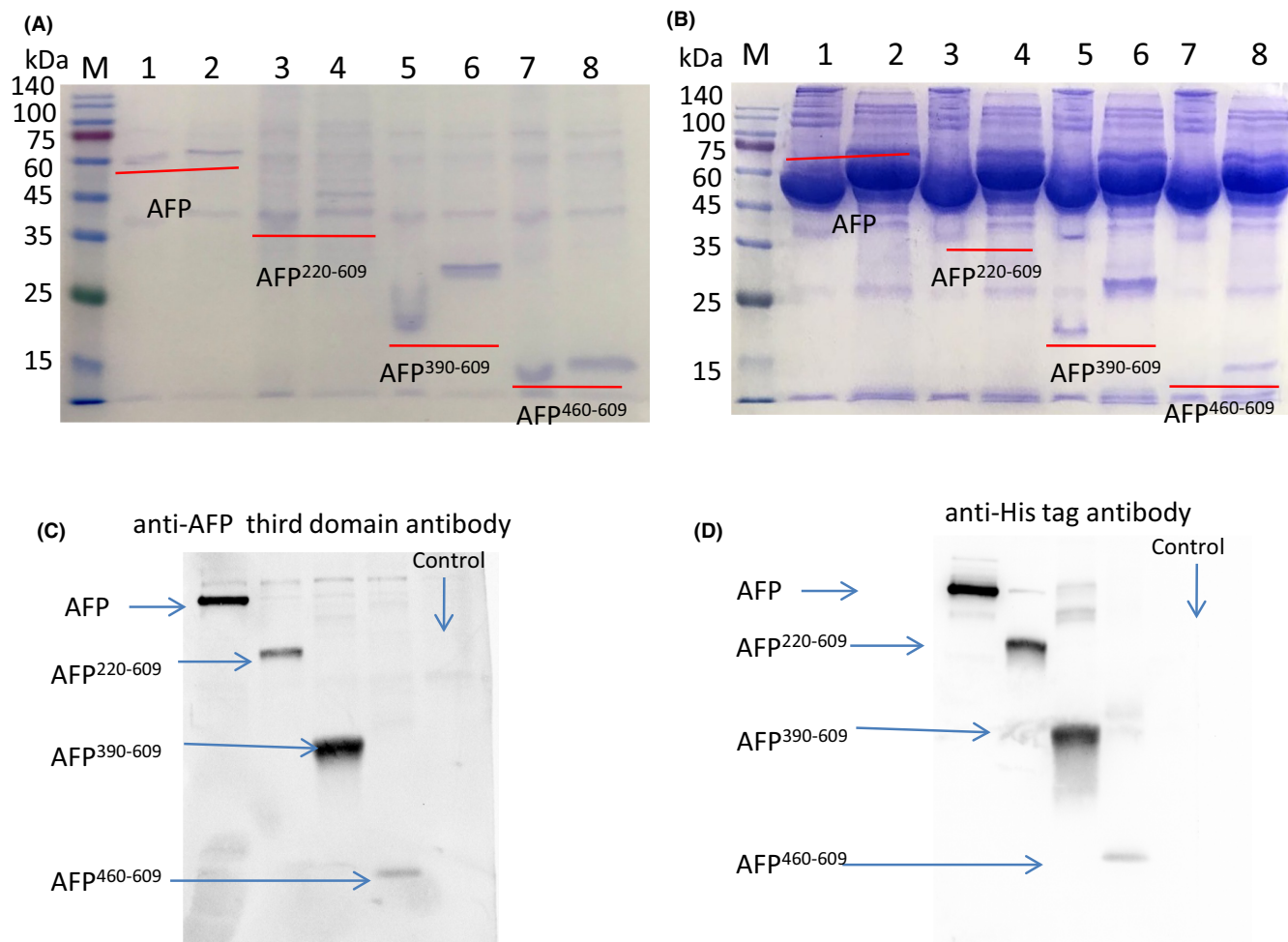


FIGURE 2 AFP and fragments expression in SFX-Insect medium. P1 virus-infected Sf9 cells were cultured in SFX-Insect medium for 72 h, and the medium was collected and analysed by SDS-PAGE and Western blotting. (A) Medium without serum. Lanes 1 and 2: AFP (Lanes 1 nonreduced or native AFP, Lanes 2 reduced rAFP); Lanes 3 and 4: AFP²²⁰⁻⁶⁰⁹ (Lanes 3 nonreduced or native AFP²²⁰⁻⁶⁰⁹, Lanes 4 reduced AFP²²⁰⁻⁶⁰⁹); Lanes 5 and 6: AFP³⁹⁰⁻⁶⁰⁹ (Lanes 5 nonreduced or native AFP³⁹⁰⁻⁶⁰⁹, Lanes 6 reduced AFP³⁹⁰⁻⁶⁰⁹); Lanes 7 and 8: AFP⁴⁶⁰⁻⁶⁰⁹ (Lanes 5 nonreduced or native AFP⁴⁶⁰⁻⁶⁰⁹, Lanes 6 reduced AFP⁴⁶⁰⁻⁶⁰⁹). (B) SFX-Insect medium with 10% FBS (foetal bovine serum). Lanes 1 and 2: AFP, Lanes 3 and 4: AFP²²⁰⁻⁶⁰⁹, Lanes 5 and 6: AFP³⁹⁰⁻⁶⁰⁹, Lanes 7 and 8: AFP⁴⁶⁰⁻⁶⁰⁹ fragment. (C) Western blotting with anti-AFP third domain antibody and (D) Western blotting with anti-His tag antibody. Control: medium collected from un-transfected cells.

did not appreciably affect normal L-02 cell growth, and the viability of L-02 cells decreased slightly to approximately 5% when exposed to corresponding concentration (Figure 6A).

To detect the AFP³⁹⁰⁻⁶⁰⁹ fragment in effect the anticancer drug activity, it affect sorafenib was analysis by MTT. The results indicated that Bel7402 and L-02 treated with different concentrations of sorafenib (2.5–20 µg/ml), the growth (viability) of these cells was significantly inhibited when sorafenib was present at ≥10 µg/ml, and the viability of Bel7402 cells was significantly suppressed synergizes with AFP³⁹⁰⁻⁶⁰⁹ fragment (Figure 6B), but L-02 cells were not significantly suppressed synergizes with AFP³⁹⁰⁻⁶⁰⁹ fragment (Figure 6C). This result indicated that AFP³⁹⁰⁻⁶⁰⁹ fragment was able to synergize with sorafenib to inhibit growth of Bel7402 which highly expressed AFP receptor, and when the AFP fragment concentration increased to 300 µg/ml, the synergized effect with sorafenib was similar to 200 µg/ml. So, we chose 200 µg/ml of AFP fragment in the follow functional assays.

3.7 | AFP³⁹⁰⁻⁶⁰⁹ fragment synergizes with sorafenib to suppress Bel7402 cell scratch repair and migration

To further detect the AFP³⁹⁰⁻⁶⁰⁹ fragment function in effect the drug activity, the effects of AFP³⁹⁰⁻⁶⁰⁹ fragment combine with sorafenib on scratch repair and the migration were also observed using Bel7402 cells as model. Because MTT showed that the viability of Bel7402 was significantly inhibited when sorafenib was present at concentration ≥10 µg/ml and not much effect at 5 µg/ml. The high concentration sorafenib (≥10 µg/ml) can cause the cells death and apoptosis that will affect the result in the scratch repair and migration experiment very much. So, we chose 5 µg/ml of sorafenib (the concentration does not much affect at the cell viability) and extended incubation period for 72 h in the scratch repair and migration experiments.

Scratch repair assays indicated that the control cells covered more than 85% scratch area. However, when Bel7402 cells were

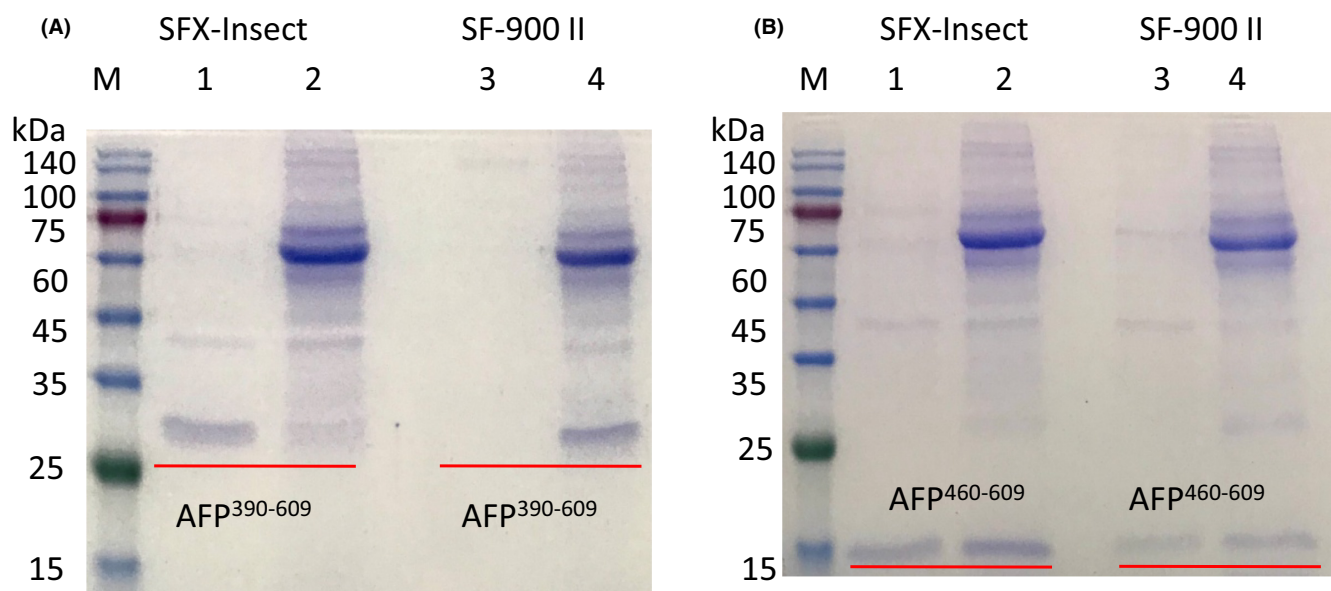


FIGURE 3 AFP³⁹⁰⁻⁶⁰⁹ and AFP⁴⁶⁰⁻⁶⁰⁹ fragments expressed in different media. (A) AFP³⁹⁰⁻⁶⁰⁹ fragment expressed in SFX-Insect and SF900 II media, Lanes 1 and 2: SFX-Insect medium, Lanes 3 and 4: SF900 II medium; Lanes 1 and 3: without FBS; Lanes 2 and 4: FBS concentration of 2%. (B) AFP⁴⁶⁰⁻⁶⁰⁹ fragment expressed in SFX-Insect and SF900 II media. Lanes 1 and 2: SFX-Insect medium. Lanes 3 and 4: SF900 II medium. Lane 1-3: without FBS; Lane 2 and Lane 4: FBS concentration of 2%.

treated with sorafenib (5 µg/ml) for 72h, the cells covered 45% of the scratch area, when Bel7402 cells were treated with sorafenib (5 µg/ml) + AFP fragment (AFP³⁹⁰⁻⁶⁰⁹), the cells covered only 30%.

The Bel7402-siRNA-AFP cells which were silenced AFP expression by siRNA (Figure S2) covered about 50% scratch area, when Bel 7402-siRNA-AFP cells treated with sorafenib (5 µg/ml), the cells covered 35% scratch area (Figure 7A,B). These results indicated that AFP³⁹⁰⁻⁶⁰⁹ fragment and siRNA-AFP harbour a function to synergize with sorafenib to suppress Bel7402 cells scratch repair.

The migration assays shown the similar result. When Bel7402 cells were treated with sorafenib (5 µg/ml) for 72h, the migration ratio of the cells was 70% compared with the control group, when Bel7402 cells were treated with sorafenib (5 µg/ml) + AFP fragment (AFP³⁹⁰⁻⁶⁰⁹), the migration ratio of the cells was 54%. In addition, the migration ratio of Bel7402-siRNA-AFP cells was 64%, when Bel7402-siRNA-AFP cells treated with sorafenib (5 µg/ml), the migration ratio of the cells was 56% (Figure 7C,D).

These results demonstrated that the AFP³⁹⁰⁻⁶⁰⁹ fragment could also synergize with sorafenib to inhibit migration in Bel7402 cells. The effect of AFP³⁹⁰⁻⁶⁰⁹ on migration and scratch repair of Bel7402 cells were similar to that of siRNA-AFP, suggesting that AFP³⁹⁰⁻⁶⁰⁹ may inhibit the Bel7402 cells (HCC with highly AFP receptor expressed) migration by regulating the AFP signalling pathway.

3.8 | AFP³⁹⁰⁻⁶⁰⁹ fragment synergizes with sorafenib to promote apoptosis of Bel7402 cells

To further detect the bioactivity of the AFP³⁹⁰⁻⁶⁰⁹ fragment in the present study, we also selected Bel7402 cells to examine the effects of

AFP³⁹⁰⁻⁶⁰⁹ on cellular apoptosis synergized with sorafenib. In the apoptosis experiment, it was found that cell apoptosis not much affected by 5 µg/ml of sorafenib (data not shown). So, it chose treatments 10 µg/ml of sorafenib for 24h that could get statistical significance of result.

Flow cytometric analysis demonstrated that when Bel7402 cells were treated with sorafenib (10 µg/ml) for 24h, the apoptosis ratio of the cells was 30%, and after treatment with sorafenib (10 µg/ml) + AFP³⁹⁰⁻⁶⁰⁹ fragment (200 µg/ml), the apoptosis ratio of the cells was increased to 45%.

The apoptosis ratio of Bel7402-siRNA-AFP cells was 33%, when Bel7402-siRNA-AFP cells treatment with sorafenib (10 µg/ml) the apoptosis ratio of the cells was 44%. These results demonstrated that the AFP³⁹⁰⁻⁶⁰⁹ fragment also synergized with sorafenib to promote apoptosis in Bel7402 cells (Figure 8A,B), and the effect of AFP³⁹⁰⁻⁶⁰⁹ on hepatoma cell apoptosis was similar to that of siRNA-AFP, also suggesting that the AFP³⁹⁰⁻⁶⁰⁹ fragment may promote apoptosis by inhibiting AFP signalling.

4 | DISCUSSION

Alpha fetoprotein is a crucial molecule that promotes proliferation or drug resistance in liver cancer cells. AFP promotes the growth of human HCC by antagonizing paclitaxel and suppressing the expression of activated caspase-3. AFP is involved in multidrug resistance (MDR) by activating the PI3K/AKT/mTOR signalling pathway, which leads to metabolic reprogramming of cancer stem cells, inhibition of the expression of apoptosis-related enzymes and resistance to tumour cell apoptosis.¹³⁻¹⁵

Alpha fetoprotein domain3 fragments can be used to inhibit AFP malignancy by completing AFP to interact with signalling molecules

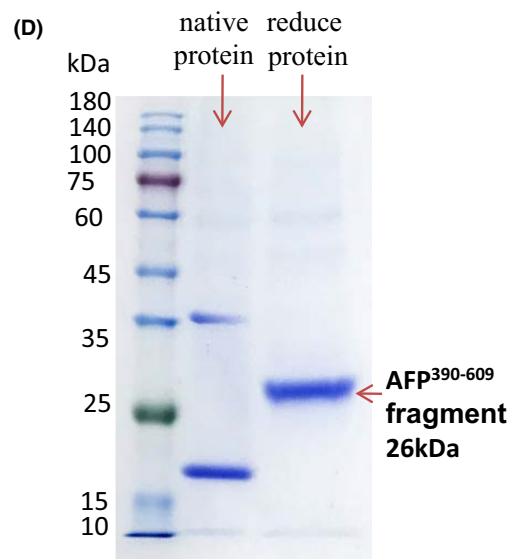
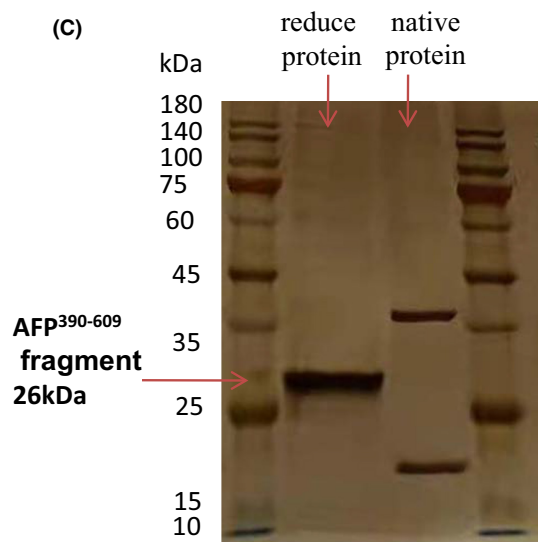
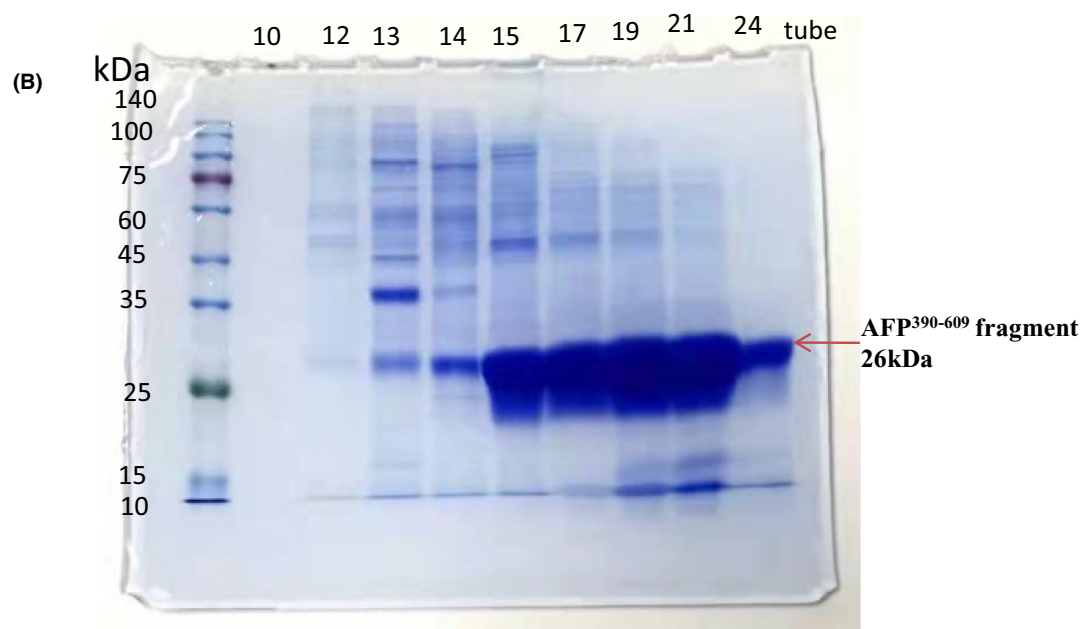
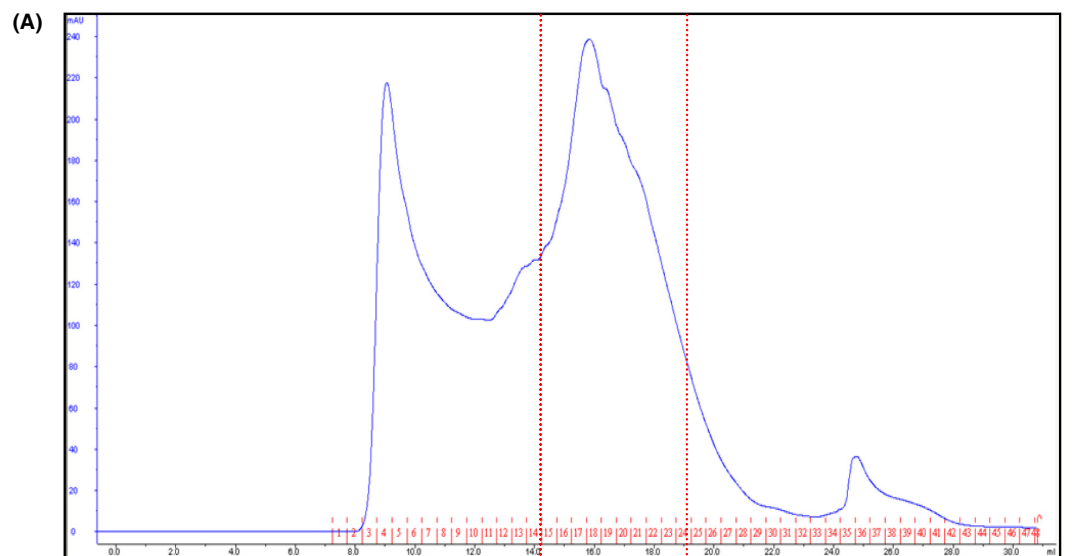


FIGURE 4 Analysis of the purified AFP³⁹⁰⁻⁶⁰⁹ fragment. (A) Gel filtration chromatography of the AFP³⁹⁰⁻⁶⁰⁹ fragment purification. (B) Coomassie-blue staining analysis of the AFP³⁹⁰⁻⁶⁰⁹ fragment eluted from Gel filtration chromatography (No.10 tube to No.24 tube fractions). (C) silver staining (the amount of protein loaded in each lane was 10 μ l). (D) Coomassie-blue staining analysis of purified the AFP³⁹⁰⁻⁶⁰⁹ fragment.

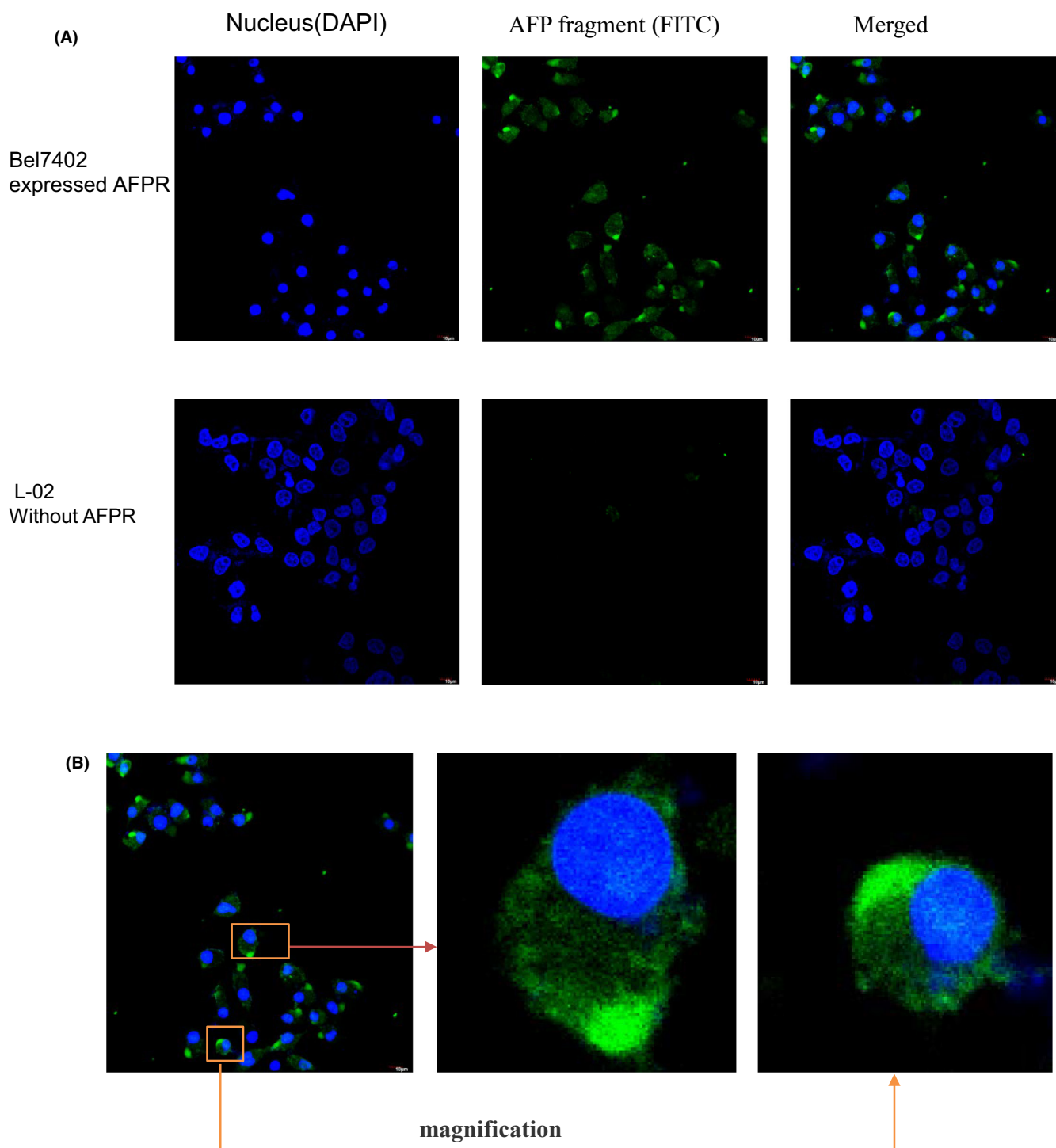


FIGURE 5 Localization of AFP³⁹⁰⁻⁶⁰⁹ fragment (or named AFP fragment) in Bel7402 and L-02 cells. (A) Bel7402 and L-02 cells were added with AFP fragment, followed by culturing at 37°C in a humidified atmosphere of 5% CO₂. The images were captured under laser confocal microscopy. Localization of AFP fragment was visualized in Bel7402 (HCC cells with high AFP receptor expressed) but not in L-02 cells (normal hepatic cell). (B) Localization of AFP fragment in Bel7402 was magnification. Nuclei are stained with DAPI (blue). AFP fragment was labelled with FITC (green). Three independent experiments were performed for these data.

and affect cancer cell growth, drug resistance and metastasis. For example, rAFP3D can recognize and bind to the AFPR of cancer cells, which can increase the intake and enhance the accumulation of drugs in cancer cells, and also strengthen the cytotoxic effect of drugs. rAFP3D delivery of doxorubicin (Dox) can increase the sensitivity of human ovarian carcinoma cells and breast cancer cells to Dox.⁸ Some recombinant fragment proteins of AFP domain3 have been reported, such as rAFP3D, which has residues 404–609⁸; r3dAFP, which has residues 357–590⁷; and AFP-3 BC, which has residues 473–596,⁶ these fragments can deliver drugs to destroy cancer cells.

Here, we report a new AFP domain3 fragment containing residues 390–609 with 6xHis that was expressed in a Bac-to-Bac system. The bioactivity of the fragment was detected in the HCC cell line Bel7402. These results indicated that the AFP fragment can synergize with sorafenib to inhibit the hepatoma cell growth and migration and promote the apoptosis.

Sorafenib is a multitarget drug for the treatment of inoperable HCC or distant metastasis. It has been one of the good choices for the first-line treatment of advanced HCC for decades. However, sorafenib can only extend the average life expectancy of patients

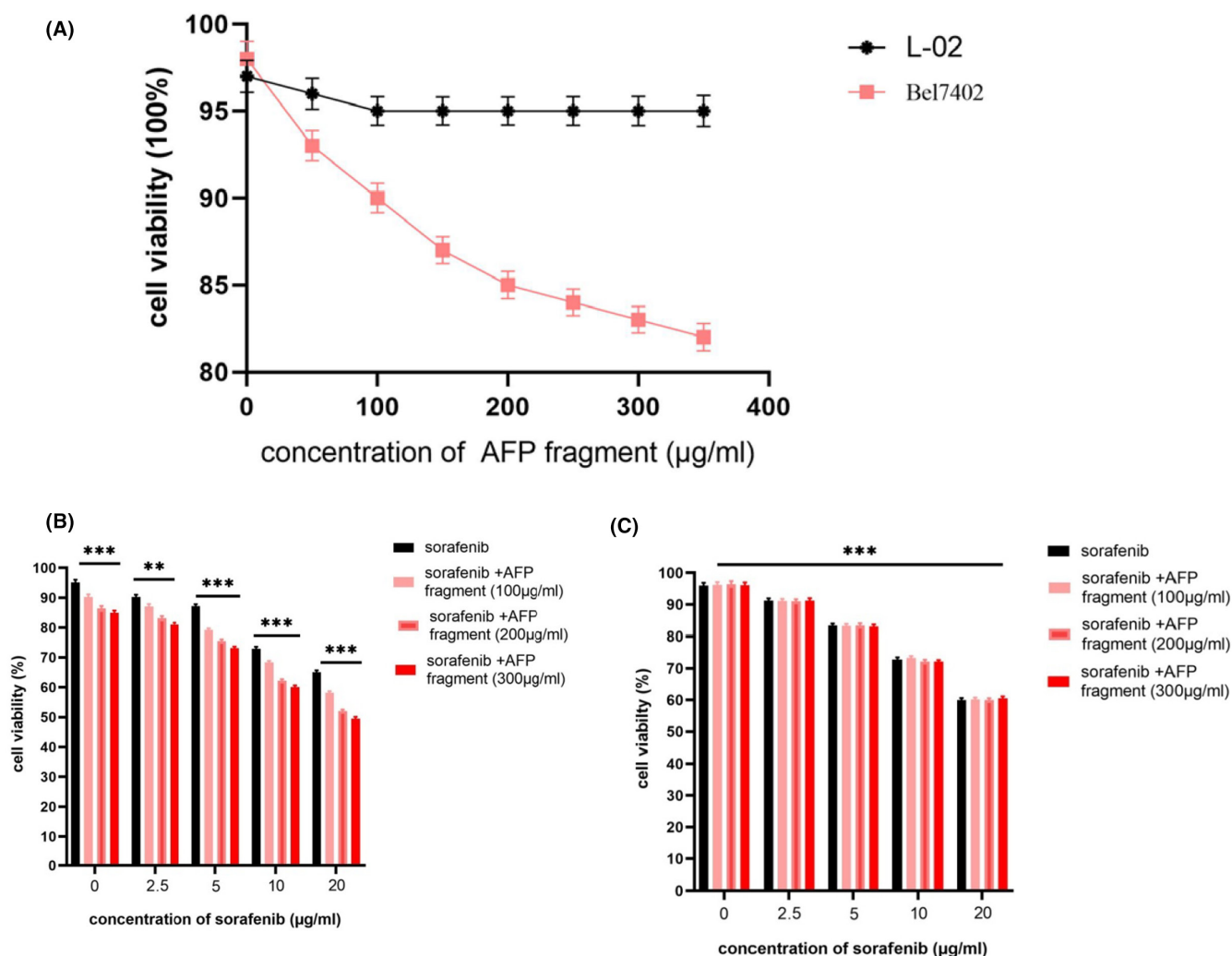
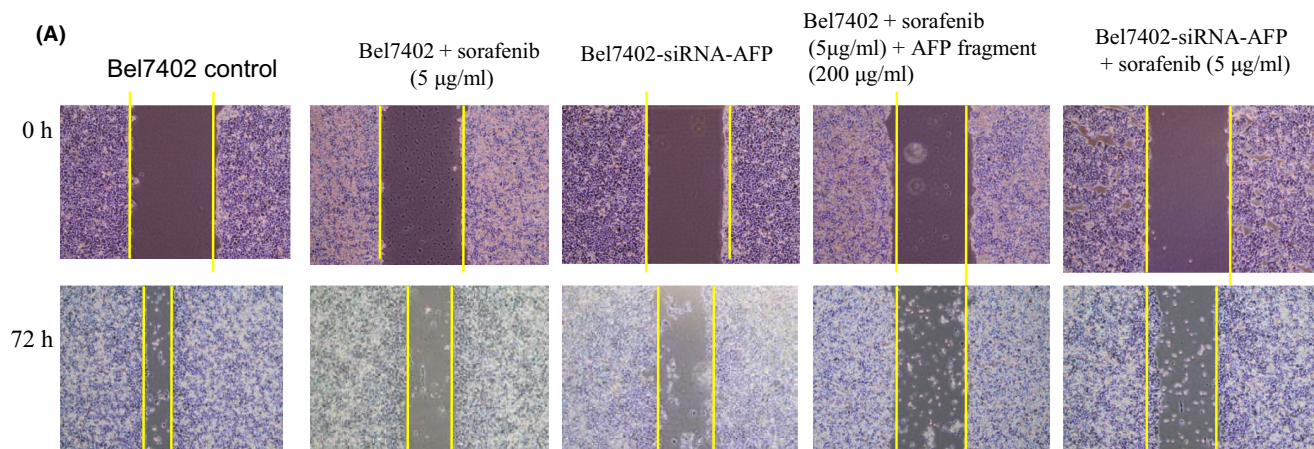
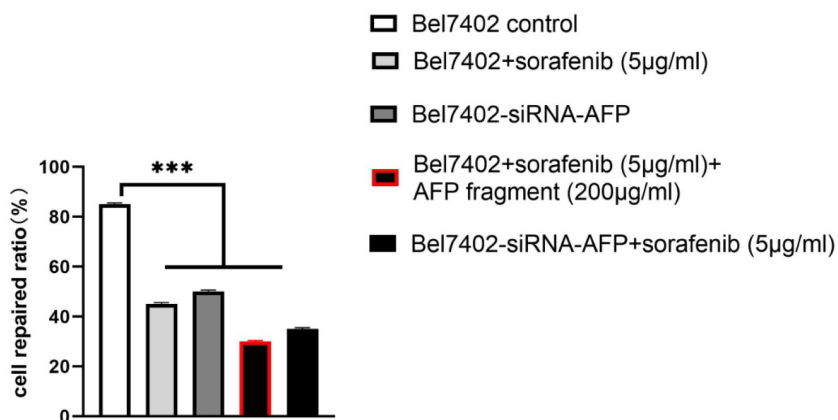
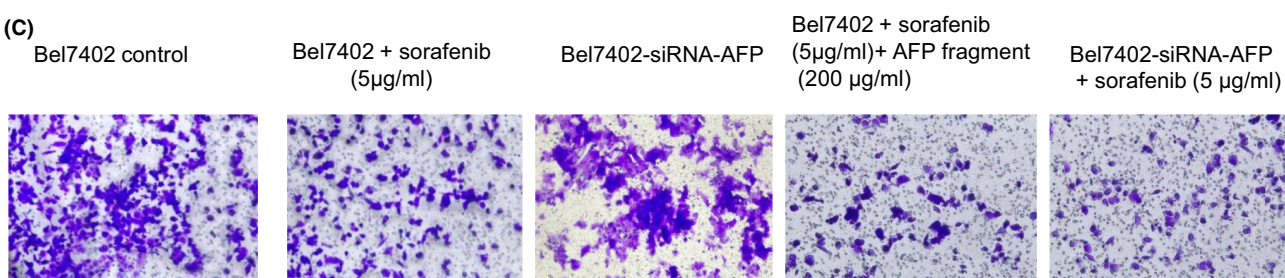
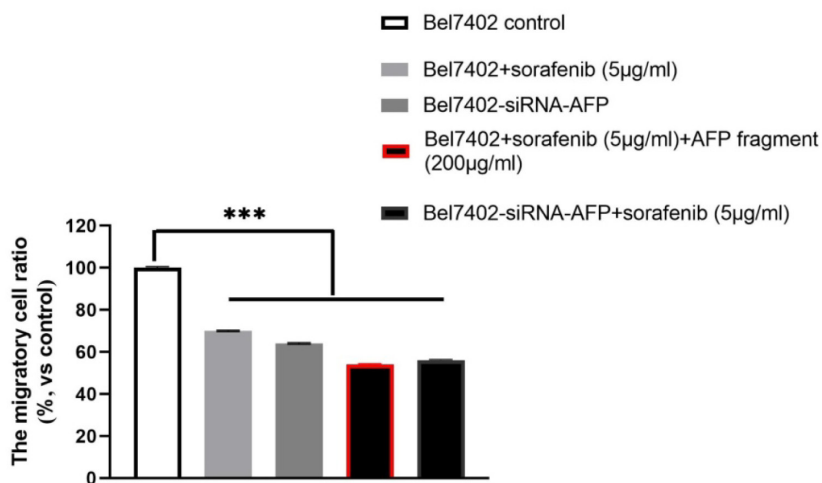


FIGURE 6 Effects of and AFP^{390–609} (AFP fragment) and sorafenib on the growth of human hepatoma cells Bel7402 and human normal liver cells L-02. (A) The human hepatoma cell line Bel7402 and normal hepatic cell L-02 were treated with AFP fragment at concentrations of 0–350 μg/ml. The viability of the cells was analysed by MTT. (B) Bel7402 and (C) L-02 cells were treated with sorafenib at concentrations of 2.5–20 μg/ml; at the same time synergized with AFP fragment at concentrations of 100, 200 and 300 μg/ml for 72 h. The viability of the cells was analysed by MTT. ***p* < 0.01 and ****p* < 0.001 versus untreated groups. *N* = 3.

FIGURE 7 AFP^{390–609} fragment synergizes with sorafenib to inhibit scratch repair and migration of Bel7402 cells. (A) Bel7402 cells were treated with sorafenib (5 μg/ml); sorafenib (5 μg/ml) + AP fragment (200 μg/ml); or Bel7402-siRNA-AFP cells were treated with sorafenib (5 μg/ml) for 72 h; and the scratch area of Bel7402 cells covered was detected in scratch repair experiment. (B) The column picture indicates the statistical analysis of the cell repair ratio. (C) The migration of Bel7402 cells and Bel7402-siRNA-AFP cells (treated as A) was detected in a transwell chamber. (D) The column picture indicates the statistical analysis of the migratory cell ratio. ****p* < 0.001 versus control groups. The images are representative of at least three independent experiments.

**(B)****(C)****(D)**

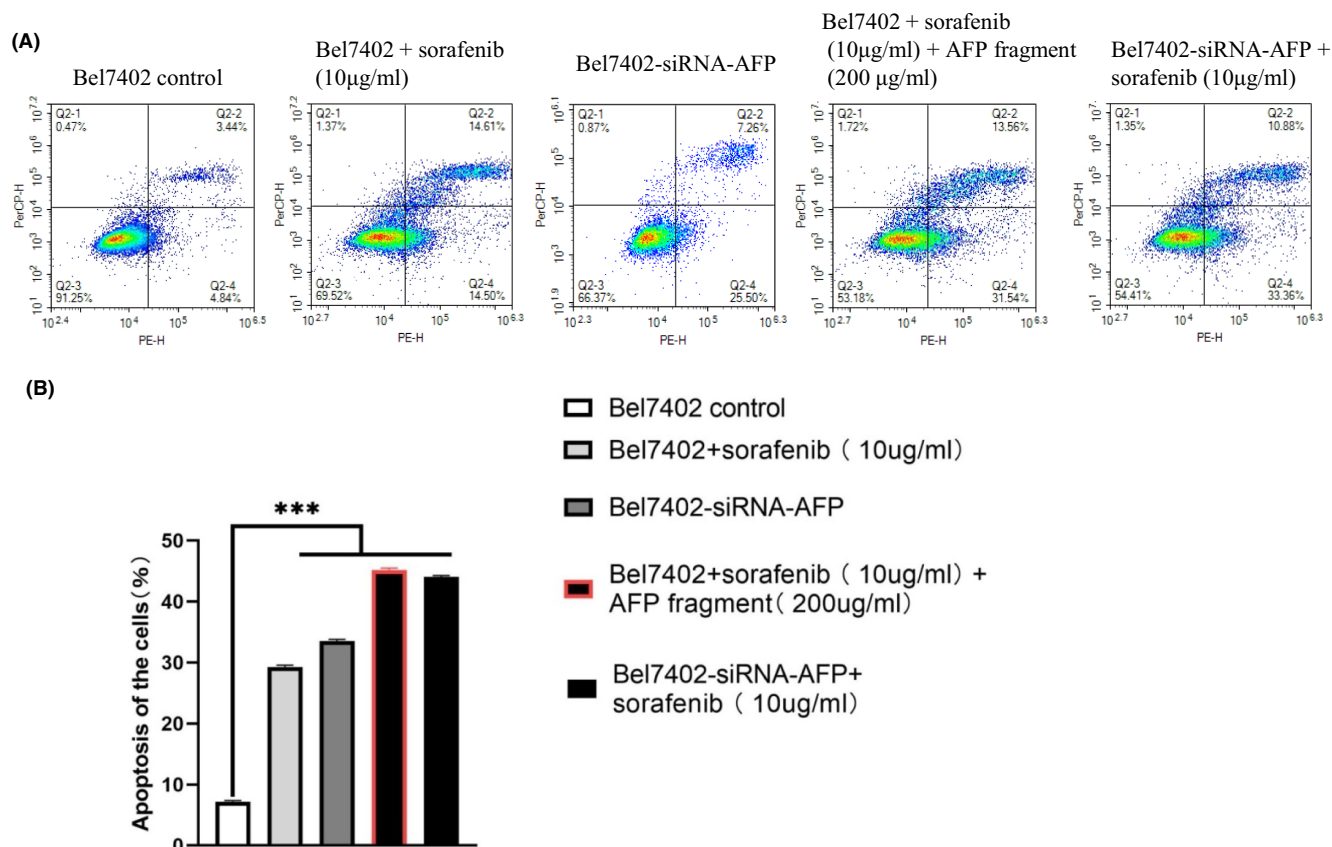


FIGURE 8 AFP³⁹⁰⁻⁶⁰⁹ fragment synergizes with sorafenib to promote the apoptosis of Bel7402 cells. (A) Bel7402 cells were treated with sorafenib (10 µg/ml), sorafenib (10 µg/ml) + AFP fragment (200 µg/ml) or Bel7402-siRNA-AFP cells treated with sorafenib (10 µg/ml) for 24 h; the apoptosis of Bel7402 cells was analysed by flow cytometry. (B) The column picture shows the statistical analysis of the apoptosis ratios; *** $p < 0.001$ versus control groups. The images are representative of at least three independent experiments.

by approximately 1 year, and drug resistance is usually acquired within 6 months, suggesting that HCC easily acquires resistance to sorafenib.^{16,17} Drug resistance to sorafenib remains incompletely elucidated. AFP may neutralize sorafenib-induced death signals and abnormalities in signalling proteins such as AKT, VEGF and EGFR,¹⁸ promotes cell growth and inhibit tumour cell apoptosis.¹⁹ High expression of AFP in HCC could induce drug resistance result in sorafenib treatment failure. In the 2018 EASL (European Association for the Study of the Liver)²⁰ clinical practice guidelines, it suggests that AFP can be used as an indicator for the diagnosis and prognosis of advanced HCC. AFP can activate the PI3K/AKT signalling, stimulates the transcription cofactor mTOR, STAT3, HIF-1 α and Bcl-2, which regulates the expression of oncogenes, as well as promotes angiogenesis and the growth of the hepatoma cells. Many HCC patients with elevated AFP expression may have drug resistance and a poor prognosis.^{4,21} Therefore, it is urgent to find a way to inhibit AFP malignancy in treatment of HCC.

In this report, it was found that a new AFP fragment can synergize with sorafenib to inhibit HCC line Bel7402 growth and migration and promote the apoptosis. The effects of AFP fragments (AFP³⁹⁰⁻⁶⁰⁹) on Bel7402 apoptosis and migration were similar to those of siRNA-AFP, suggesting that AFP³⁹⁰⁻⁶⁰⁹ fragments may inhibit the signalling pathway mediated by AFP. We also found similar results in other

HCC cells, such as PLC/PLF/5, which highly expressed AFPR (data not shown). However, the fragment did not have much effect on normal hepatocyte L-02 cells with low AFPR expression. This indicated that the AFP³⁹⁰⁻⁶⁰⁹ fragment may compete with AFP to bind to AFPR or be a single molecule to prevent AFP malignant behaviour. The AFP fragment could inhibit PI3K/AKT signalling which induced by AFP,¹³ and sorafenib could block the RAF/MEK/ERK, MAPK and EMT pathway.²² These pathways play key roles in the development of HCC. This also indicated that AFP fragment could synergize with sorafenib to block the important pathways that involve in tumour growth, proliferation and metastasis, result in inhibit angiogenesis, and promote apoptosis in HCC. These studies show that the AFP fragment can be a promising vector to deliver drugs or can act directly as a drug to synergize with sorafenib in the treatment of HCC.

AUTHOR CONTRIBUTIONS

Bo Lin: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal). **Qiujiao Wang:** Conceptualization (equal); data curation (equal); methodology (equal). **Wei Li:** Conceptualization (equal); data curation (equal); formal analysis (equal). **Minni Zhang:** Conceptualization (equal); data curation (equal); formal analysis (equal); software (equal); supervision

(equal); visualization (equal); writing – original draft (equal). **Zijuan Zou:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Xu Dong:** Conceptualization (equal); investigation (equal); validation (equal); visualization (equal). **Yi Chen:** Formal analysis (equal); methodology (equal); project administration (equal). **Junnv Xu:** Investigation (equal); methodology (equal); project administration (equal). **Mingyue Zhu:** Conceptualization (equal); data curation (equal); funding acquisition (equal). **Mengsen Li:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare that this article content has no conflict of interest. All authors of this paper have read and consented to publication of the final version

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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