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# p55PIK regulates alpha-fetoprotein expression through the NF-κB signaling pathway

Guoguo Ye, Ge Sun, Zhikui Cheng, Lei Zhang, Kanghong Hu, Xianmin Xia*, Yin Zhou*

*Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei Provincial Cooperative Innovation Center of Industrial Fermentation, Hubei Key Laboratory of Industrial Microbiology, School of Food and Biological Engineering, Hubei University of Technology, Wuhan 430068, China*

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ABSTRACT

*Aims:* Alpha-fetoprotein (AFP) is regarded as a diagnostic and prognostic biomarker and a potential therapeutic target for hepatocellular carcinoma (HCC). However, the regulation of AFP expression in HCC remains poorly understood. This study aimed to investigate the mechanism by which AFP expression is regulated by p55PIK, an isoform of PI3K.

*Main methods:* Human HCC cell lines (HepG2 and Huh-7) were treated with p55PIK specific competitive inhibitor or shRNA, or p55PIK overexpression vector, in the absence or presence of NF-κB inhibitor PDTC. AFP expression was detected by quantitative real-time PCR and Western blotting. NF-κB responsive elements in AFP enhancer region were characterized by luciferase reporter assay.

*Key findings:* p55PIK significantly stimulated the expression of AFP by activating NF-κB signaling pathway in HCC cells. Furthermore, two NF-κB binding sites in AFP enhancer region were identified to be primarily responsible for p55PIK mediated upregulation of AFP expression.

*Significance:* p55PIK/NF-κB signaling plays an important role in the upregulation of AFP expression in HCC.

## 1. Introduction

The alpha-fetoprotein (AFP) gene encodes a serum protein which is mainly expressed in the yolk sac and fetal liver during gestation. In adults, the expression level of AFP is extremely low in the liver; however, AFP expression can be reactivated during hepatocyte proliferation such as liver regeneration and hepatocellular carcinomas (HCC) [1]. AFP is a diagnostic and prognostic biomarker for HCC [2]. AFP plays a critical role in promoting metastasis of HCC, and becomes a novel therapeutic target in HCC [3]. Therefore, it is important to elucidate the mechanism underlying the regulation of AFP expression.

AFP is a regulated by numerous transcription factors. The upstream region of AFP gene contains five regions: two enhancers, two silencers and a promoter [4]. Transcriptional activators and repressors of AFP gene regulate its expression through binding to transcriptional regulatory elements in the promoter and enhancer(s), generating a tissue-specific expression pattern. A tissue-specific promoter is located in the region from − 182 to + 112 bp of AFP gene [5]. Multiple binding sites in AFP promoter region are recognized by various transcription factors, including hepatocyte nuclear factor 1 (HNF1), nuclear factor 1 (NF1), and CAAT/enhancer binding protein (C/EBP) [6]. HNF1, a liver-specific factor, could be one of the regulators of AFP expression in the differentiated hepatocyte [7]. HNF1 interacts with a large family of hepatic genes, and HNF1-binding sites may exist in the promoter region of these genes [8]. The AFP promoter is relatively weak; therefore, regulation of AFP expression is largely dependent on the enhancer sites [9,10]. The two human AFP enhancers have been designated as enhancer A (− 4120 to − 3756), and enhancer B (− 3492 to − 3300) [11]. The enhancer region between − 3.3 and − 3.5 kb upstream of the transcription initiation site contains three binding sites for C/EBP, one for HNF1 and one for HNF3, while the enhancer region between − 3.7 and − 4 kb upstream of the transcription initiation site contains two binding sites for C/EBP, one for HNF1 and one for HNF4 [12,13]. Two silencers, located between the promoter and the enhancers, have a highly conserved sequence 5′-CTTCATAACTAATACTT-3′, which prevents the function of the enhancers in promoting promoter activity [14].

NF-κB is an ubiquitous transcription factor and commonly activated in numerous malignancies [15,16]. NF-κB activation is a frequent and early event in viral and non-viral causes of HCC [17]. In the canonical NF-κB pathway, NF-κB activation depends on IκBα phosphorylation and degradation by I-kappa B kinase (IKK). In addition to phosphorylating IκBα, IKKs phosphorylate p65 at serine residues (for example, Ser536) [18]. Recent reports had reported that NF-κB regulates AFP expression

* Corresponding authors at: School of Food and Biological Engineering, Hubei University of Technology, No. 28, Nanli Road, Wuhan 430068, China.
*E-mail addresses:* xianminxia@hbut.edu.cn (X. Xia), zhouyin@hbut.edu.cn (Y. Zhou).

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during liver tumor formation and embryonic development. Repression of an inhibitor of NF-κB kinase-β (IKK2) activity promotes down-regulation of AFP expression in HCC, and genetic disruption of the RELA subunit represses AFP expression during embryonic liver development [19,20]. However, the exact mechanism by which NF-κB regulates AFP expression remains unclear.

Phosphatidylinositol 3 kinases (PI3Ks) play crucial roles in diverse cellular activities, including metabolism, proliferation, survival, and polarity. Class IA PI3Ks, the most extensively studied, consist of heterodimers of a p110 catalytic subunit and a regulatory subunit. The regulatory subunits determine PI3K activity through regulating its subcellular location, binding partners, and the activities of the catalytic subunits [21]. PI3K regulatory isoform p55PIK plays an important role in cancer cell proliferation, and activates NF-κB signaling through promoting the phosphorylation of Ser536 of p65 [22,23]. TAT-N24 was synthesized by the fusion of a cell-penetrating peptide TAT and the N-terminal 24 amino acids of p55PIK and acted as a specific inhibitor of p55PIK to inhibit cell cycle progression in several cancer cell types, including colorectal and thyroid cancer cells [24]. TAT-N24 also inhibited NF-κB signaling pathway in colorectal cancer cells by inhibiting the phosphorylation of p65 (Ser536) [23].

Considering that p55PIK could activate NF-κB signaling pathway and NF-κB signaling regulates AFP expression, we wondered whether p55PIK regulates AFP expression. In present study, we investigated the mechanism by which AFP expression is regulated by p55PIK mediated signaling pathway. We found that p55PIK significantly stimulated the expression of AFP by activating NF-κB signaling pathway in HCC cells. Furthermore, two NF-κB binding sites in AFP enhancer region were identified to be primarily responsible for p55PIK mediated upregulation of AFP expression.

## 2. Materials and methods

### 2.1. Cell culture, treatment and cell transfection

Human HCC cell lines (HepG2 and Huh-7) were purchased from China Center for Type Culture Collection (CCTCC; Wuhan, China), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) at 37 °C in a humidified chamber supplemented with 5% $CO_2$. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with plasmid at cell density of 80–90%. P15 was synthesized in our laboratory as a fused polypeptide with a cell-penetrating peptides TAT and the conserved 15 amino acid sequence of N24 (MPYSTELIFYIEMDP) [24]. NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma (St. Louis, MO, USA). Cells were treated with P15 or PDTC for the indicated concentration or time period.

### 2.2. Real-time PCR

Total RNA was isolated from HepG2 cell using TRIzol regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and were quantified with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) [25]. First-strand cDNA was synthesized using the PrimeScript RT Reagent kit (Takara, Dalian, Japan). Real-time PCR was performed with SYBR Green premix Ex Taq (Takara) on Roche Lightcycler 480 (Roche Scientific, Indianapolis, IN, USA). The primers were as follows: AFP forward 5′ CACGGATCCAACTTGAGGCTGTCATTGC 3′ reverse 5′ CGGAATTCGATAAGGAAATCTCACATAAAAGTC 3′; β-actin 5′ forward CGTGACATTAAGGAGAAGCTG 3′ reverse 5′ CTAGAAGCATTTGCGGTGGAC 3′. β-actin was used as an internal control. All reactions were run in triplicate and the fold changes of genes were calculated by the 2-ΔΔCt method [26].

**Table 1**
Primers used for the generation of AFP promoter deletion constructs.

| Primer name | Sequence (5′ to 3′) |
|---|---|
| PGL3-AFP (−5184/+29)-forward | AAAGGTACCGAATTCTTAGAAATATGGGGGTAG |
| PGL3-AFP (−3330/+29)-forward | CCCGGTACCACTTATTTGACAGTATTATTGCGA |
| PGL3-AFP (−1820/+29)-forward | CCCGGTACCCTTATATAGTTTGCTTCATAAAACTC |
| PGL3-AFP (−180/+29)-forward | AAAAGGTACCCCCAAAGAGCTCTGTGTC |
| PGL3-AFP-shared reverse | GGGCTCGAGTGTTATTGGCAGTGGTGGAAGC |

### 2.3. Plasmid constructs

The oligonucleotides encoding short hairpin RNAs (shRNA) targeting p55PIK (5′-GGACUUGCUUUAUGGGAAA-3′) were synthesized and inserted into pSUPER vector, and designated as pSUPER-shRNA-p55PIK. The oligonucleotides encoding shRNA targeting firefly (5′-CGCTGAGTACTTCGAAATG-3′) were used for control shRNA, and designated as pSUPER-nc. The cDNA encoding the full-length human p55PIK was subcloned into vector pCDNA3.1 by using primers (forward 5′ CGGCTAGCATGTACAATACGGTGTGGAGTAT 3′ and reverse 5′ CCCTCGAGTTATCTGCAAAGCGAGGGCAT 3′).

The 5′ region between −5184 and +29 relative to transcription start site of AFP was used as the major promoter region for 5′ truncations analysis, and a series of 5′ truncations of this region (−3330 to +29, −1820 to +29, and −180 to +29) were amplified from genomic DNA of HepG2 cells and subcloned into pGL3-control vector (Promega, Madison WI, USA) at *Kpn*I and *Xho*I sites. The primers for 5′-series deletion of human AFP promoter were listed in Table 1. The luciferase vectors were named as (−5184/+29)-AFP-pGL-3, (−3330/+29)-AFP-pGL-3, (−1820/+29)-AFP-pGL-3, and (−180/+29)-AFP-pGL-3, respectively.

For NF-κB binding site mutation analysis, site directed mutagenesis of three NF-κB binding sites was achieved by overlap-extension PCR using the primers listed in Table 2.

### 2.4. Western blotting

Cells were washed twice in cold PBS and lysed in RIPA buffer for 30 min at 4°C. Cell lysates were centrifuged, and protein concentration was determined by the Bradford method. The samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% milk and 0.1% Tween-20 in PBS for 2 h at room temperature, then incubated with primary antibody at 4 °C overnight. Primary antibodies were against AFP and p55PIK (Santa Cruz Biotechnology), p65 and phospho-p65 (S536), IκBα and phospho-IκBα (S36) (Abcam) as well as GAPDH and β-actin (Abcam). After washing, blots were incubated with horseradish peroxidase conjugated goat anti-Mouse or anti-Rabbit secondary antibody (1:10,000 dilution; Abclonal) for 1 h. The labeled blot was detected using a C-DiGit Blot Scanner (LI-COR Biosciences).

### 2.5. Prediction of NF-κB binding site

The NF-κB binding sites in the AFP promoter region were predicted by TRANSFAC database (http://gene-regulation.com) [27].

### 2.6. Luciferase reporter assay

For luciferase assay, HepG2 cells (60% confluent) were seeded into 96-well plates. After culturing for 24 h, 100 ng AFP promoter luciferase reporter constructs and 20 ng Renilla vector (pRL-TK) (Promega, Madison, WI, USA) were co-transfected into HepG2 cells using

**Table 2**
Primers used for site directed mutagenesis of NF-κB binding sites in AFP promoter.

| Name | Deletion site | Primer | Sequence (5′ to 3′) |
|---|---|---|---|
| AFP-Mut1 | CACAGGAATC | forward | GTTCGGG ACACAAAGATGAGTCTTTGAATTTAAGAAG/ |
| | | reverse | CTCATCTTTGTGTCCCGAACAAAACATTTGTC |
| AFP-Mut2 | AGGAATTTTC | forward | GAGATCACAACCCACACGTATAG/ |
| | | reverse | GTGTGGGTTGTGATCTCTTATCTCACTGTTTCTGAG |
| AFP-Mut3 | AGGAATTCCC | forward | CTTTGCCAATATCTAGTATTTTCTATTAAACTTTGTG/ |
| | | reverse | CTACTCTTCCTCAAGGTCAAATATATGAAAGCCAAAGTATTTG |

Lipofectamine 2000. Luciferase activity levels were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized using the corresponding Renilla luciferase activity. In addition, HepG2 cells were treated with P15 (120 μg/ml) or PDTC (60 μM) before the co-transfection and luciferase assay. Data are representative of three independent experiments. Error bars indicate standard deviation.

### 2.7. Statistical analysis

Statistical analysis (Student's *t*-test) was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). In all tests, the data represent mean ± standard deviation. **P < 0.01 was considered statistically significant.

## 3. Results

### 3.1. p55PIK specific inhibitor or shRNA decreased AFP expression

To understand the role of p55PIK in the regulation of AFP expression, first we employed a loss of function approach to inhibit or knockdown p55PIK in HCC cells and examined the effects on AFP expression. HepG2 cells were treated with different concentrations of p55PIK inhibitor P15 (50, 100, and 150 μg/ml) for 48 h and real-time PCR results indicated that treatment with P15 induced a dose-dependent decrease in the mRNA expression of AFP (Fig. 1). Similar results that P15 treatment led to a decrease in AFP expression were observed in another HCC cell line Huh7 (data not shown).

To further confirm the effects of p55PIK on AFP expression, we used shRNA-p55PIK to knockdown p55PIK expression in HepG2 cells. The results indicated that p55PIK knockdown led to significant reduction of AFP protein level compared with cells transfected with control shRNA (shRNA-sc) (Fig. 2).

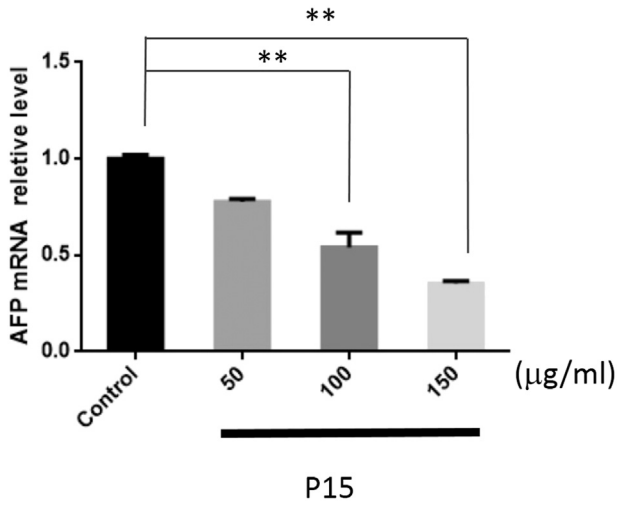


**Fig. 1.** p55PIK inhibitor P15 decreased the mRNA expression of AFP in HepG2 cells. HepG2 cells were treated with P15 at the indicated concentrations for 48 h and the mRNA level of AFP was determined by real-time PCR. Data represent mean ± standard deviation (n = 3). **P < 0.01.

### 3.2. p55PIK inhibitor inhibited NF-κB signaling pathway

To examine whether p55PIK regulated NF-κB signaling pathway, we treated HepG2 cells with P15 (120 μg/ml) or NF-κB inhibitor PDTC (60 μM) for 48 h, and detected protein levels of p65, phosphorylated p65 (Ser536), IκBα and phosphorylated IκBα (Ser36). P15 treatment had no significant effect on p65 protein expression, but significantly decreased the phosphorylation of p65 (Ser536) and IκBα (Ser36) in HepG2 cells. Moreover, P15 treatment led to a significant increase in the expression of IκBα (Fig. 3A–E). As positive control, PDTC treatment significantly decreased the levels of p-p65 (Ser536), IκBα, and p-IκBα (Ser36) and also decreased p65 expression level (Fig. 3A–E).

### 3.3. Blocking NF-κB signaling led to decreased AFP expression

Next we wondered whether p55PIK regulated AFP expression via NF-κB pathway. HepG2 cells were treated with NF-κB inhibitor PDTC at different concentrations (60, 120, and 240 μM) for 48 h and real-time PCR results showed that treatment with PDTC induced a dose-dependent decrease in AFP mRNA level (Fig. 4A). Moreover, treatment with PDTC (60 μM) for 48 h significantly decreased AFP protein expression in HepG2 cells (Fig. 4B, C). In addition, treatment with P15 (120 μg/ml) for 48 h led to a significant decrease in AFP protein expression (Fig. 4B, C). These data suggest that p55PIK may regulate AFP expression in HCC cells via NF-κB signaling.

### 3.4. p55PIK mediated upregulation of AFP expression was attenuated by NF-κB inhibitor

To confirm that p55PIK regulated AFP expression via NF-κB signaling, we overexpressed p55PIK in HCC cells and found that overexpression of p55PIK led to significant increases in both mRNA and protein levels of AFP (Fig. 5A–D). However, increases in AFP expression levels due to p55PIK overexpression were significantly attenuated by treatment with PDTC (60 μM) (Fig. 5E–G). These data indicate that the upregulation of AFP expression by p55PIK is dependent on the activation of NF-κB pathway.

### 3.5. AFP enhancer region (−5184/−1820) is essential to the regulation of AFT expression by NF-κB

To locate NF-κB responsive region in AFP promoter, a series of AFP promoter and enhancers truncations linked to a firefly luciferase reporter were constructed: the whole transcriptional regulation region (−5184 to +29 bp); downstream of two enhancers region (−3330 to +29); two silencers and a core promoter region (−1820 to +29); core promoter region (−180 to +29) (Fig. 6). The results of luciferase assays indicated that the promoter activity of (−3330/+29)-AFP-pGL-3 was much lower than that of (−5184/+29)-AFP-pGL-3, indicating the importance of the two enhancers for AFP expression. Moreover, P15 and PDTC significantly repressed the promoter activity of the (−5184/+29)-AFP-pGL-3 and (−3330/+29)-AFP-pGL-3 constructs, while they had no effect on transcription from (−1820/+29)-AFP-pGL-3 and

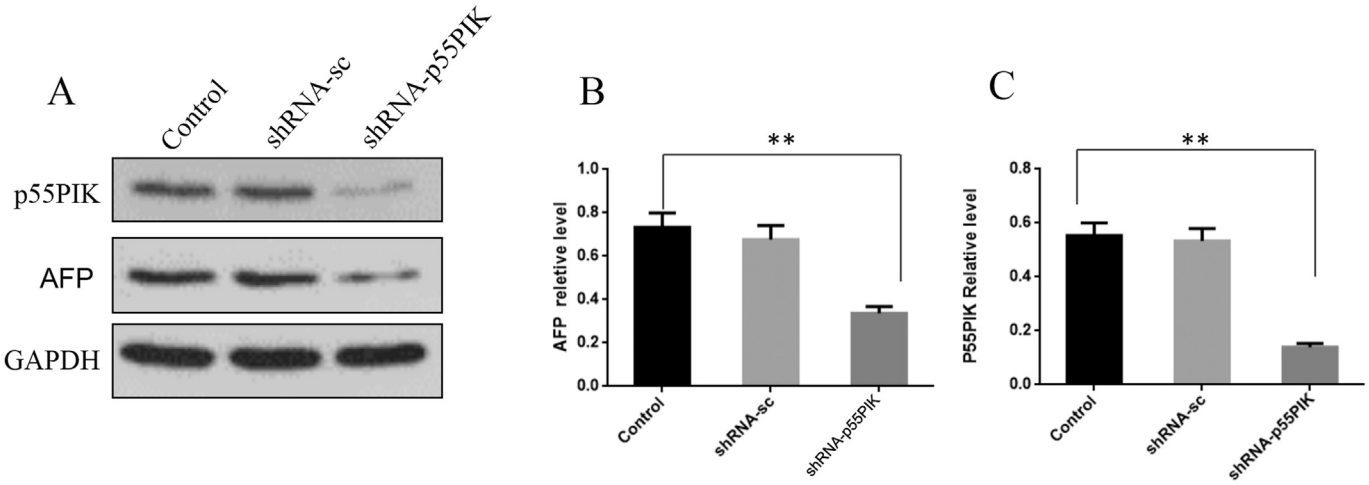


**Fig. 2.** p55PIK knockdown decreased the protein expression of AFP in HepG2 cells. HepG2 cells were transfected with control Psuper-shRNA or Psuper-shRNA-p55PIK, and the protein level of AFP was analyzed by Western blot analysis. (A) Representative blots. (B) Quantification of band intensity of p55PIK. (C) Quantification of band intensity of AFP. Data represent mean ± standard deviation (n = 3). **P < 0.01.

(− 180/+29)-AFP-pGL-3 in HepG2 cells (Fig. 6). These results suggest that a NF-κB responsive region was located between 5184 bp and 1820 bp upstream of the ATG start codon of AFP.

### 3.6. Identification of the two functional NF-κB binding sites in AFP enhancer region

To further illustrate the regulation mechanism of p55PIK and NF-κB signaling on AFP expression, it is necessary to identify NF-κB binding sites in AFP promoter region. Previous study showed that NF-κB regulates target gene expression by binding with the conserved NF-κB DNA sequences (GGGRNNTYCC) in the promoter regions of the target genes [28]. Since NF-κB responsive region may be located in the region between positions − 5184 and − 1820 bp of AFP promoter, we predicted putative NF-κB binding sites in this region using TRANSFAC. The results indicated three putative binding sites for NF-κB, at positions − 4726 to − 4717 bp, − 4381 to − 4372 bp, and − 3180 to − 3171 bp, respectively. The first two NF-κB binding sites are in the upstream region of enhancer A and B of AFP, while the third NF-κB binding site is in the downstream region of enhancer A and B of AFP.

To characterize candidate NF-κB binding sites on AFP promoter, we performed site directed mutagenesis of the three NF-κB binding sites and inserted them into pGL3-Basic luciferase vector to make Mut1 (− 4726/− 4717), Mut2 (− 4381/− 4372), and Mut3 (− 3180/− 3171) constructs. Luciferase assays showed that the promoter activities of Mut1 and Mut2 were reduced significantly compared with wild-type constructs, while the promoter activity of Mut3 were not significantly different from that of wild-type construct.

Furthermore, HepG2 cells were treated with P15 and PDTC to further confirm that the effects of p55PIK and NF-κB on AFP expression are mediated by the binding of these NF-κB binding sites. Luciferase assays demonstrated that P15 and PDTC reduced the promoter activity of Mut3 construct significantly compared with the control, but had no significant effect on the promoter activities of Mut1 and Mut2 (Fig. 7). These results indicate that once the region of − 4726 to − 4717 bp or − 4381 to − 4372 bp in AFP promoter is deleted, AFP expression would fail to respond to the inhibition by P15 and PDTC. Collectively, these data suggest that the regions of − 4726 to − 4717 bp and − 4381 to

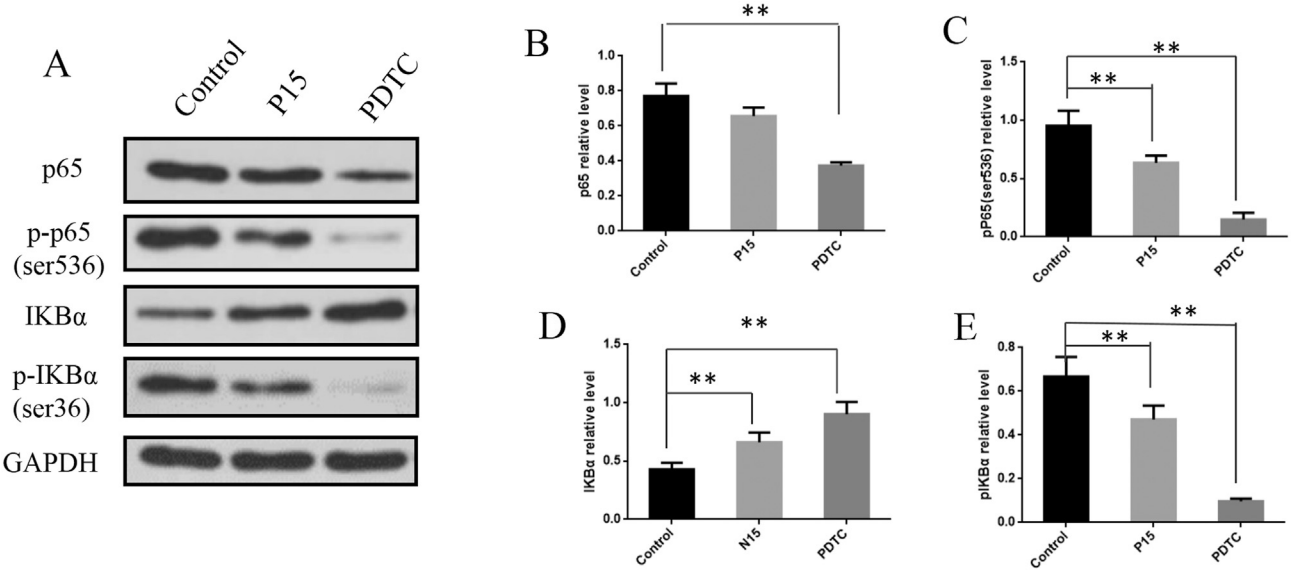


**Fig. 3.** p55PIK inhibitor inhibited NF-κB signaling in HepG2 cells. (A) HepG2 cells were treated with P15 (final concentration: 120 μg/ml) or PDTC (final concentration: 60 μM) for 48 h, and the protein levels of p65, p-p65 (Ser536), IκBα, and p-IκBα (Ser36) were analyzed by Western blot analysis, GAPDH was used as loading control. (B-E) Quantification of band intensities of p65, p-p65 (Ser536), IκBα, and p-IκBα (Ser36), respectively. Data represent mean ± standard deviation (n = 3). **P < 0.01.

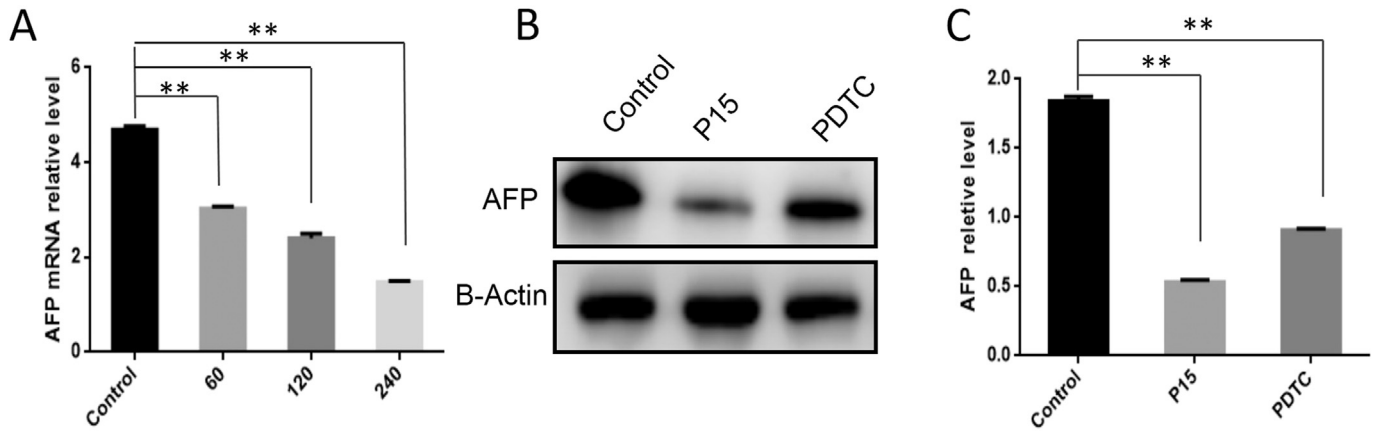


**Fig. 4.** NF-κB inhibitor PDTC repressed the expression of AFP in HepG2 cells. (A) HepG2 cells were treated with PDTC at the indicated concentrations (in μM) for 48 h, and the AFP mRNA level was determined by real-time PCR. Data represent mean ± standard deviation (n = 3). **P < 0.01. (B) HepG2 cells were treated with P15 (final concentration: 120 μg/ml) or PDTC (final concentration: 60 μM) for 48 h, and the protein level of AFP was analyzed by Western blot analysis. β-actin was used as loading control. (C) Quantification of band intensity of AFP. Data represent mean ± standard deviation (n = 3). **P < 0.01.

− 4372 bp in AFP promoter are two NF-κB binding sites that are responsible for transcriptional activation of AFP by NF-κB.

## 4. Discussion

HCC is a major cause of cancer-related death worldwide, and understanding of molecular mechanisms underlying HCC is important to develop cancer prevention strategies. AFP is a marker for HCC and is used to assess hepatic masses in patients at particular risk of developing hepatomas [1]. Overexpression of AFP may be of significance in the development of liver tumors [2]. High AFP expression in HCC cells can promote cell proliferation, migration, and invasion [29]. p55PIK, a regulatory subunit of PI3K, is overexpressed in many cancers, and has important roles in several oncogenic processes, including cell cycle regulation, cell growth, differentiation, metastasis, and angiogenesis [22–24]. In this study, we found that p55PIK inhibitor or shRNA downregulated AFP expression while p55PIK overexpression upregulated AFP expression in HCC cells, and this was mediated by NF-κB signaling. These findings suggest that p55PIK is a novel therapeutic target for HCC.

AFP expression is regulated by several tissue-specific and ubiquitous transcriptional factors. There are several transcription factor binding sites in the promoter region of AFP, and liver-enriched and ubiquitous transcriptional factors determine tissue specificity of AFP expression in

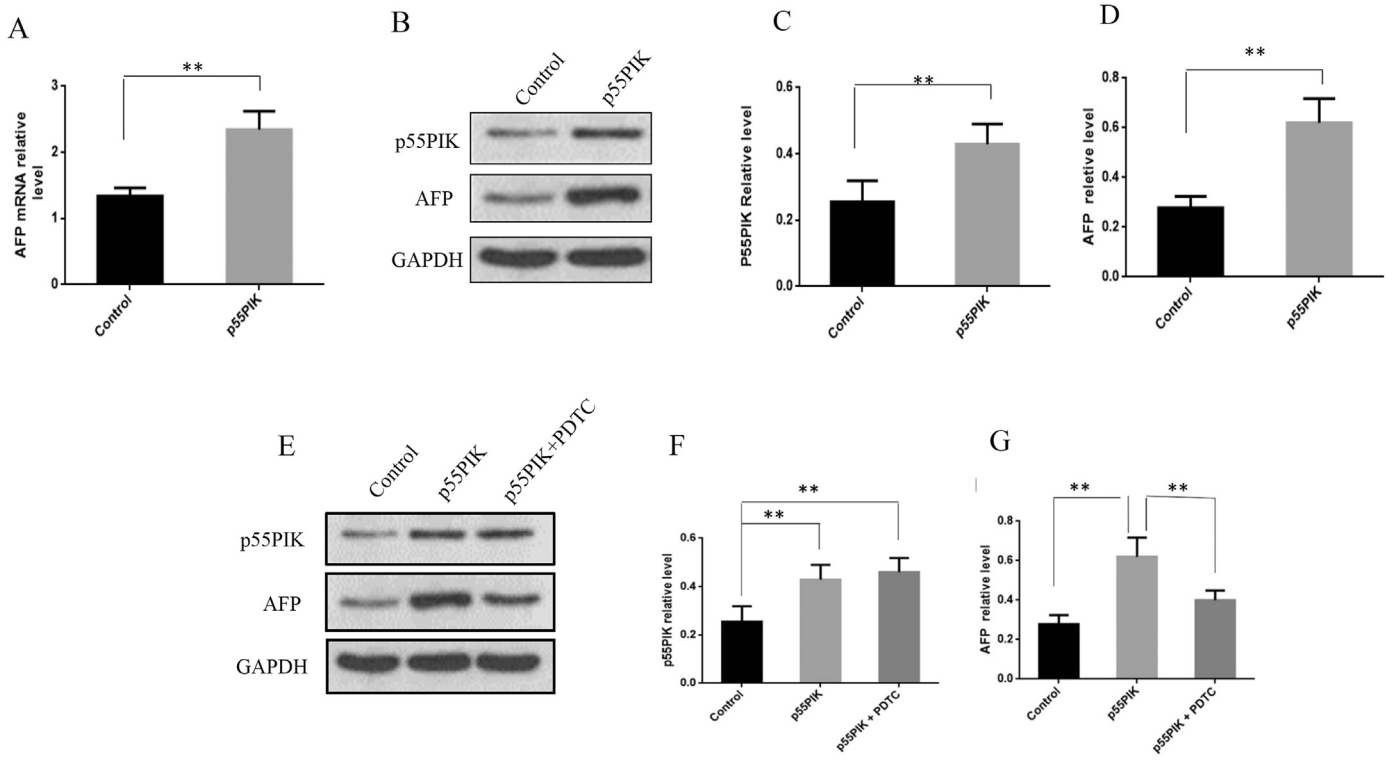


**Fig. 5.** PDTC abrogated p55PIK induced AFP upregulation in HepG2 cells. HepG2 cells were transfected with pCDNA3.1-p55PIK or control pCDNA3.1 vector, and AFP mRNA level was determined by real-time PCR (A), AFP and p55PIK protein levels were detected by Western blot analysis with GPDH as loading control (B), band intensity of p55PIK was analyzed (C), and band intensity of AFP was analyzed (D). HepG2 cells were transfected with pCDNA3.1-p55PIK or control pCDNA3.1 vector and treated with PDTC (final concentration: 60 μM) for 48 h, and then the protein levels of p55PIK and AFP were detected by Western blot analysis with GAPDH as loading control (E), band intensity of p55PIK was analyzed (F), and band intensity of AFP was analyzed (G). All data represent mean ± standard deviation (n = 3). **P < 0.01.

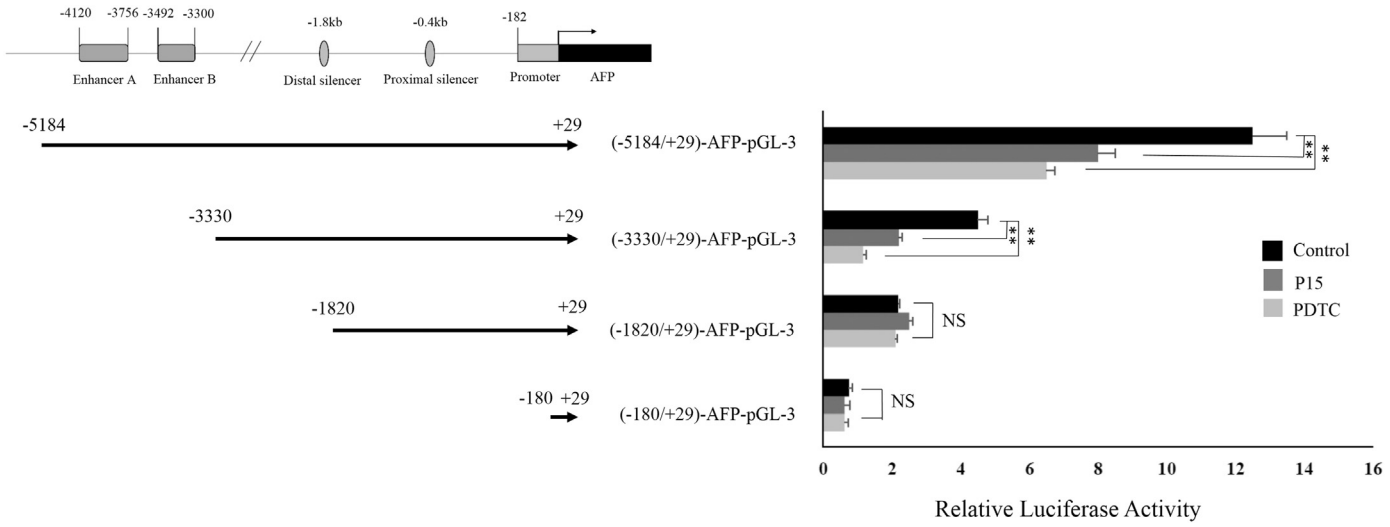


**Fig. 6.** Characterization of NF-κB responsive region in AFP promoter.
Left panel: Schematic diagram of AFP promoter-luciferase reporter constructs. Numbers indicate the base pairs upstream of the ATG translation initiation codon. Right panel: Luciferase assays of luciferase activity in HepG2 cells transfected with (−5184/+29)-AFP-pGL-3, (−3330/+29)-AFP-pGL-3, (−1820/+29)-AFP-pGL-3 or (−180/+29)-AFP-pGL-3 in the absence or presence of treatment with P15 (120 μg/ml) or PDTC (60 μM). All data represent mean ± standard deviation (n = 3). **P < 0.01.

the liver [7]. NF-κB is a ubiquitous transcriptional factor and commonly activated in various malignancies including HCC [17]. A previous study reported that AFP expression levels were repressed by an inhibitor of NF-κB kinase-β (IKK-2) or by genetic disruption of the RELA subunit [19]. PDTC is an inhibitor of NF-κB signaling and could decrease the phosphorylation levels of p65 and IκBα while increase IκBα level [30]. In current study, our results showed that PDTC repressed the expression of AFP, consistent with previous studies that NF-κB signaling could regulate AFP expression.

The phosphorylation of p65 on Ser536 is necessary for NF-κB activation. Our previous studies demonstrated that p55PIK activates NF-κB signaling through promoting the phosphorylation of Ser536 of p65 [22,23]. P15, a peptide inhibitor of p55PIK, can block NF-κB signaling in HepG2 cells by repressing the phosphorylation of Ser536 of p65 and Ser36 of IκBα. In this study, we provide several lines of evidence indicating that p55PIK regulates the expression of AFP through NF-κB signaling pathway. As p55PIK is a regulatory subunit of PI3K, it recruits catalytic subunit p110 to specific cellular targets and enable catalytic function, and its N-terminal 24 amino acid had been confirmed to bind Rb and PCNA. One possible mechanism how p55PIK

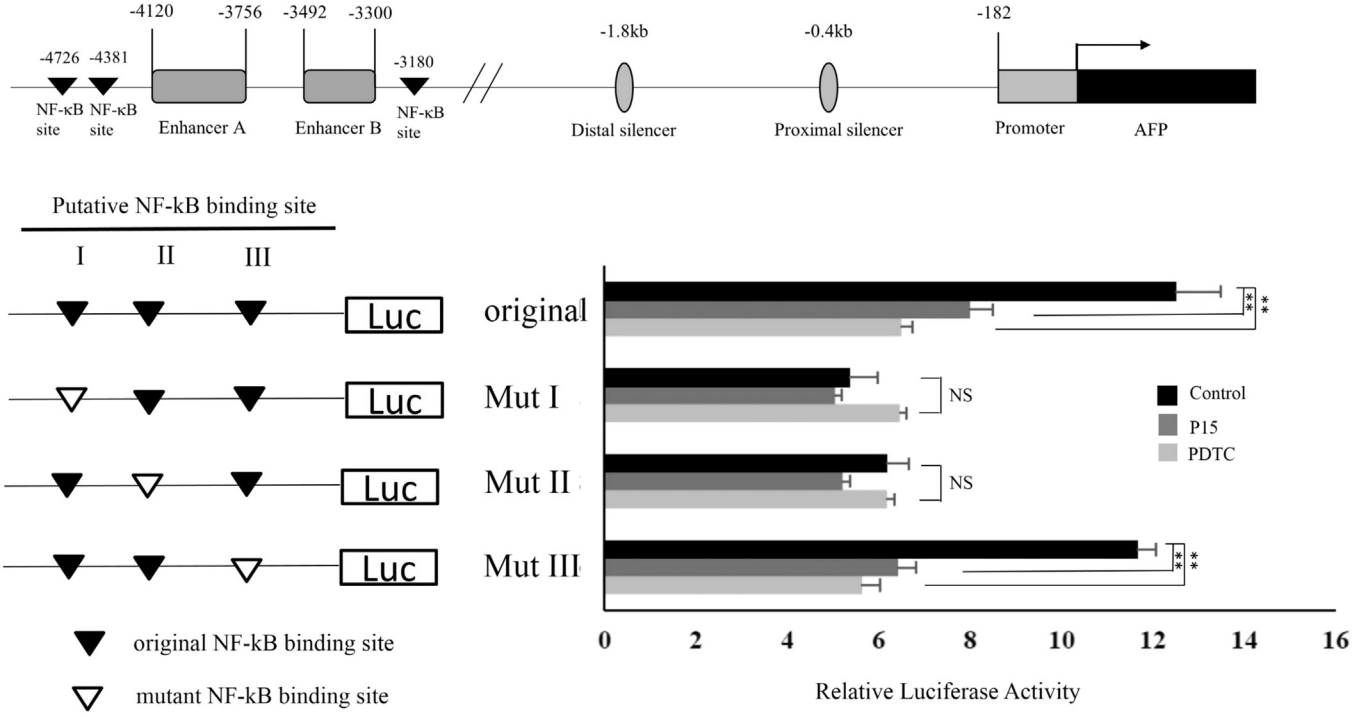


**Fig. 7.** Identification of NF-κB binding sites in AFP promoter.
Left panel: Schematic diagram of the three putative NF-κB binding sites in AFP promoter region. Numbers indicate the base pairs upstream of the ATG translation initiation codon. Right panel: Luciferase assays of luciferase activity in HepG2 cells transfected with Mut1 (−4726/−4717)-AFP-pGL-3, Mut2 (−4381/−4372)-AFP-pGL-3 or Mut3 (−3180/−3171)-AFP-pGL-3 in the absence or presence of treatment with P15 (120 μg/ml) or PDTC (60 μM).
All data represent mean ± standard deviation (n = 3). **P < 0.01.

regulates NF-κB might be that p55PIK binds NF-κB through its unique N-terminal domain, and further studies are needed to test it.

The liver-specific transcriptional factors regulate AFP transcription through binding to enhancer A and B [31]. In this study, our results indicate that a NF-κB responsive region is likely to be located in the −5184 to −1820 bp region upstream of AFP enhancer. The canonical NF-κB binding sequence is GGGRNNTYCC (where R is G or A, Y is C or T, and N is any nucleotide) [28]. In this study, three predicted NF-κB binding sites were identified in the upstream region of AFP promoter, and two of them were confirmed to be functional for NF-κB regulation. The sequences of the three NF-κB binding sites were: CACAGGAATC (Site 1), AGGAATTTTC (Site 2), and AGGAATTCCC (Site 3). None of the three NF-κB cis-acting elements were highly conserved and our results indicate that the first two sites are regulated by NF-κB in HepG2 cells while the third site is not responsive to NF-κB. Based on the results of truncation analysis of AFP promoter, the region of −3330 to −1820 could be also responsive to NF-κB regulation, but we did not identify any functional NF-κB binding sites in this region. It is likely that some less conserved NF-κB binding sites exist in this region and could not be predicted by software.

## 5. Conclusions

In summary, NF-κB regulates AFP expression through binding with two NF-κB binding sites (−4726/−4717, −4381/−4372) in the 3.3-kb (−5184/−1820) enhancer region of AFP. Furthermore, p55PIK upregulates AFP expression through the activation of NF-κB signaling. p55PIK is a promising therapeutic target for HCC.

## Abbreviations

| | |
|---|---|
| NF-κB | nuclear factor-kappa B |
| DMEM | Dulbecco's modified Eagle medium |
| HCC | hepatocellular carcinoma |
| PBS | phosphate-buffered saline |
| PDTC | pyrrolidine derivative of dithiocarnamate |
| PVDF | Polyvinylidene fluoride |
| RIPA | radio immunoprecipitation assay |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| shRNA | short hairpin RNA |

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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