



## Molecular cloning, expression and metabolic activity assay of CYP3A46 gene

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### ABSTRACT

The present study is to study drug metabolic characteristics of CYP3A46 gene. *Sus scrofa* CYP3A46 gene from Bama miniature pig liver was obtained by PCR method and cloned in pCDNA3.1, the recombinant plasmid pCDNA-CYP3A46 was obtained. The recombinant plasmid and empty plasmid were transfected into HepG<sub>2</sub> which designated as HepG<sub>2</sub>-CYP3A46 and HepG<sub>2</sub>-CDNA3.1 respectively. The stably expressing cell lines HepG<sub>2</sub>-CYP3A46 and HepG<sub>2</sub>-pCDNA3.1 were achieved by the selection of gentamycin (G418), and were incubated with the probe drug nifedipine (NF) in optimal conditions. After the cells were incubated for 60 minutes, the high performance liquid chromatography (HPLC) was utilized to detect the change of metabolites in cells. The results showed that the sequence of cloned *Sus scrofa* CYP3A46 gene was correct, and the stably expressing cell lines transfected with pCDNA-CYP3A46 and pCDNA3.1 were established successfully. The activity analysis demonstrated that the cell line transfected with pCDNA-CYP3A46 had the significantly metabolic activities of nifedipine ( $P < 0.001$ ).

**Key words:** gene cloning, expression, metabolic activity, CYP3A46

### INTRODUCTION

Currently, there are more than 20 minipig strains or varieties worldwide. In foreign countries, such as Minnesota hormel minipig in USA, Oh mini Bud and Clawn minipigs in Japan, and Gottingen miniature pig in Germany; In China, such as Guizhou miniature pig, Yunnan Banna miniature pig, Bama miniature pig and Wuzhishan miniature pig [1].

Comparing with rodents, miniature pig is not only similar to human in anatomy, physiology and disease model, but also exists economic and ethical advantages in using miniature pigs as model animal in the field of biomedical science. Therefore, miniature pig has broad application prospects in biomedical research, especially in human disease model, drug safety evaluation, xenotransplantation [2-4].

At present, though researches on the reliability of miniature pigs as an experimental model animal have reached the molecular level [5,6], the drug metabolic characteristics of Bama miniature pigs remains to be defined. In present study, so as to provide the basis for further clarifying the pharmacokinetic characteristics at the sub-cellular and molecular levels, Bama miniature pigs were used as experimental materials to clone the gene and then heterologously expressing the gene in HepG<sub>2</sub> cell lines by means of molecular technology. Materials and methods.

## EXPERIMENTAL SECTION

### Materials

The liver samples(n=2) were taken from Bama miniature pig(pig farm, Laboratory Animal Center, Third Military Medical University ),total RNAs of each species were individually extracted by means of Trizol kit.All studies were approved by theInstitutional Animal Care and Use Committee of Luzhou Vocational and Technical University.

### Reagents

pcDNA3.1 plasmid( Shanghai GeneCore BioTechnologies Co., Ltd.(Shanghai,China)); PMD-18-T subcloning vector, primescript TM 1st strand cDNA Synthesis kit(Takara Biotechnology (Dalian) Co., Ltd.Dalian,China); ECL chromogenic solution purchased from Pierce Company; DH5a competent cells, Anti-His Antibody, HRP-labeled goat anti-mouse secondary antibody, EndoFree Plasmid endotoxin-free plasmid extraction kit, agarose gel DNA extraction kit (TIANGEN Biotech(Beijing)Co.,Ltd.,Beijing,China));Trizol (total RNA extraction reagent), RIPA lysis buffer, Bradford Protein Assay kit,Coomassie Brilliant Blue G-250 (Beyotime Institute of Biotechnology,Shanghai,China);Lipofectamine2000, G418, High glucose DMEM,FBS(Invitrogen,USA);Nifedipine, Oxidized nifedipine, glucose-6 - phosphate (G-6-P), glucose-6 - phosphate dehydrogenase (G-6-P-DH), coenzyme II (NADP), bovine serum albumin, ketonazole and DMSO(Sigma-Aldrich,China);Trypan blue solution (0.4%), MTT(Amresco,USA) $\beta$ -actin antibody (Santa Cruz Biotechnology, Inc.USA); methanol(HPLC grade,Merck,Darmstadt,Germany ).

### Design and synthesis of primers

PCR primers based Sus.scrofa CY3A46 sequence were designed, the primes as follows:

The upstream primer:

CGCgatccGATGATGATGATGATGATG (BamHI, in lowercase letters),

The downstream primer:

CGGctcgagATGATGATGATGATGATGA AGTCAAGGTCTCTGATGCTCTG (XhoI,,in lowercase letters , histidine-tagged codon in Italics)

The online primer-designed tool (<http://frodo.wi.mit.edu/>)was utilized to evaluate the designed primers, which contains BamHI restriction sites in upstream primer, and XhoI restriction sites and histidine tag in downstream primer, the PCR products was a length of 1861bp for present primers.

The designed primer was synthesizd by Shanghai GeneCore BioTechnologies Co., Ltd.

### PCR cloning of target gene

Total RNA was extracted from Bama miniature pig liver according to Trizol (total RNA extraction reagent) reagent extraction kit manual;RT-PCR according to instructions of primescriptTM 1st strand cDNA Synthesis kit (Takara); Clones were identified by colony PCR, and the positive clones were sent to GeneCore Biotechnology Co., Ltd. for sequencing.

### Construction and identification expression vector

Pick the bacterial colonies containing correct target gene confirmed by sequencing to Proliferate culture,Extraction pMD-CYP46 by tiangen small plasmid extraction kit(Tiagen, China), and then the gene was PCRed after the purity and concentration of the extracted plasmid was detected. The reaction system as follows: forward primer, reverse primer 1 $\mu$ L, respectively Mg<sup>2+</sup> 4 $\mu$ L, template pMD-CYP3A461 $\mu$ L, Buffer (10  $\times$ ), dNTP 5 $\mu$ L, pfu DNA polymerase 1 $\mu$ L, deionized water 32 $\mu$ L, total reaction system volume was 50 $\mu$ L; reaction conditions: 95 $^{\circ}$ C 5min; 95 $^{\circ}$ C 4min; 68 $^{\circ}$ C 30sec;72 $^{\circ}$ C 1.5min, 72 $^{\circ}$ C 5min. The reaction product was purified using the PCR purification kit, After the reaction, the PCR product was purified using the PCR purification kit, and then BamHI/HindIII double digestion performed, and the restriction endonuclease product purified,and the ligation reaction carried out , the ligation product was transformed to DH5a, the positive clones were identified by colony PCR, the reaction system was as follows: forward primer, reverse primer, 1 $\mu$ L, Mg<sup>2+</sup> 2 $\mu$ L, Buffer (10  $\times$ ), dNTP2.5 $\mu$ L, pfu DNA polymerase 1 $\mu$ L, deionized water15 $\mu$ L; reaction conditions: 95 $^{\circ}$ C 5min; 95 $^{\circ}$ C4min, 68 $^{\circ}$ C 30sec, 72 $^{\circ}$ C 1.5min, 72 $^{\circ}$ C 5min. Colony PCR was utilized to identify the correct single colony,and the positive clones were sent Shanghai GeneCore BioTechnologies Co., Ltd. for sequencing.

**Screening for stably expressing Strain**

Cells in 24-well plate was inoculated  $3 \times 10^5$  cells/well, added anti-free DMEM (high glucose) 1ml/well containing 10% FBS, cultured to 85% confluence at 37°C, 5% CO<sub>2</sub>, saturated humidity condition. Referring to instructions offered by Lipofectamine 2000 Transfection Reagent kit, 1.6µg of plasmid was added to each well, 4µL liposomes in serum-free DMEM medium with a total volume of 500µL, which formulated mixture of plasmid - liposomes - serum-free DMEM medium, cultured 4h at 37.5% CO<sub>2</sub>, saturated humidity conditions, then cultured in DMEM (high glucose) containing 10% FBS in 24h before switched to the culture medium containing 400µgG418 until cell to death on Large-scale, replacing the culture containing 200µgG418 before the medium was maintained continuously for 10 generations, and then preparing a cell suspension, cell counts, the cells were diluted with medium to 1cell / 10µL. Medium was added 150µL / well in 96-well plates, cell suspension was added 10µL/well until increased their numbers gradually, and then transferred to 24-well plates using medium containing 200µgG418 for proliferation.

**Identification of recombinant expressing cells by RT-PCR**

All operations carried out by the methods 1.4.2 herein

**Western-blot detection of expressing products in HepG<sub>2</sub>-CYP3A46**

culturing and collecting the cultured stably expressing cell lines HepG<sub>2</sub>-CYP3A46、HepG<sub>2</sub>-pcDNA and HepG<sub>2</sub>, the protein extract solution offered by instructions of RIPA lysis kit, the protein concentration was determined by Bradford method, the protein solution was added to 5×SDS-PAGE sample buffer, and denatured by boiling 5 min, centrifuged 1min, 20µL supernatant sample was transferred to 12% polyacrylamide gel and then to nitrocellulose membrane with PBST (NaCl 8.5%, Na<sub>2</sub>HPO<sub>4</sub> 2.2%, NaH<sub>2</sub>PO<sub>4</sub> 0.4%, 2% (v / v) Tween 20, pH 7.2), and washing three times, 5 min each pass. 5% milk (PBST preparation) blocking antibody, shaking on shaker at room temperature for 1h. TBST washed three times, 5 min each pass, adding Anti-His Antibody antibody (1:1000, PBS formulation) or β-actin antibody (1:5 000), 37°C shook 2h, pBST washed three times, 5 min each pass, adding horseradish peroxidase-labeled secondary antibody (1:400, PBS formulation), shook at room temperature for 1h. PBST washed three times, 5 min each pass, plus ECL reagents, X-ray film exposure, washed the film. Analysis of protein expression by gel analysis system.

**Incubation recombinant cells with nifedipine**

Taking 24-well plate, inoculated cell suspension with  $1 \times 10^6$  HepG<sub>2</sub>-CYP3A46/ HepG<sub>2</sub>-pCDAN3.1/ HepG<sub>2</sub> cells, added 1200µg /mL nifedipine storage solution to 27.85µL (final concentration 200µM) each well, supplemented with 2% FBS containing antibiotic-free DMEM medium to 500µL, each well with three wells to repeat, mixed gently, incubated at 37 °C, 5% CO<sub>2</sub> for 60min, stopped the reaction in ice bath. The mixture in each well was transferred to Eppendorf microfuge tubes, the medium and methanol with a ratio of 1:2 was added to mixture to precipitate the protein in the incubation medium, and then centrifuged 10 min at 4 °C, 13000rpm, 50µL supernatant of each sample was drawn to detect the peak area of nifedipine (NF) - oxidized nifedipine (ONF) by HPLC, and calculated the amount of nifedipine and oxidized nifedipine by standard curves. All experiments concerned nifedipine operated in dark.

**Chromatographic conditions**

chromatographic column: Hypersil ODS C<sub>18</sub> (4.6mm×150mm, 5µm); mobile phase: methanol: water (64:36, v / v); Column temperature: 40 °C; Flow rate: 0.5 mL / min; detection wavelength: 237 nm; injection volume of 50µL.

**Data Analysis**

Statistics and comparison using excel and SPSS 17(Statistical Product and Service Solutions, USA)

**RESULTS AND DISCUSSION****1. Transformation of PMD-18-T-CYP3A46**

The transformation was successful by the fact that target gene CYP3A46 bands appear in Colony PCR electrophoresis and results showed in Fig 1.

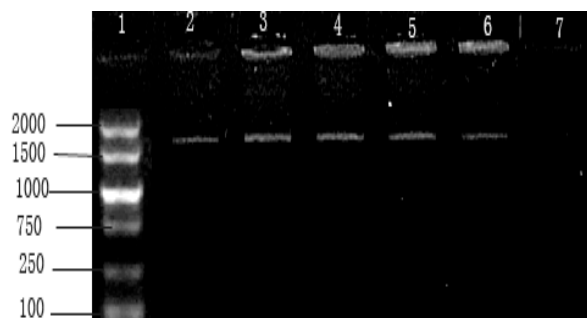


Fig 1. Screening for positive clone by clony PCR (Clony PCR products of CYP3A46(Lane1 DNA marker:DL2000;Lane2~6 positive Clone)

## 2. Sequencing results of comparative analysis

The cloned ORF in pMD-18-CYP 3A46 was sequenced, and the sequencing result was compared with the CYP3A46 ORF sequence deposited in GeneBank by NCBI Nucleotide Blast tool online ([http://blast.stva.ncbi.nlm.nih.gov/\\_10718](http://blast.stva.ncbi.nlm.nih.gov/_10718)), The blasting results as showed in fig 2 demonstrated that there was no difference between the two sequence in base composition.

Sus scrofa breed mixed unplaced genomic scaffold, Sscrofa10.2 ChrUScaf4074  
Sequence ID: [reflNWV\\_003540966\\_1](#) Length: 6630 Number of Matches: 2

Score	Expect	Identities	Gaps	Strand
375 bits(203)	3e-101	203/203(100%)	0/203(0%)	Plus/Minus
Query 1025	AGGCGCCTCCCACCTACGATGGTCTGGCGCAAATGGAGTATCTTGACATGGTGGTGAACG	1084		
Sbjct 4315	AGGCGCCTCCCACCTACGATGGTCTGGCGCAAATGGAGTATCTTGACATGGTGGTGAACG	4256		
Query 1085	AATCTCTCAGAATATTCCCGATTACTCCTAGACTTGAGAGGTCCTGTAAGAAGGATGTGG	1144		
Sbjct 4255	AATCTCTCAGAATATTCCCGATTACTCCTAGACTTGAGAGGTCCTGTAAGAAGGATGTGG	4196		
Query 1145	AAATCCATGGCGTGTTCGTTCCCAAAGGGACCCTGATGATGGTGCCAATCTTTGCGCTTC	1204		
Sbjct 4195	AAATCCATGGCGTGTTCGTTCCCAAAGGGACCCTGATGATGGTGCCAATCTTTGCGCTTC	4136		
Query 1205	ACAGAGCCCCGGAGCTCTGGCCA	1227		
Sbjct 4135	ACAGAGCCCCGGAGCTCTGGCCA	4113		

Fig2. the results of blast in NCBI

## 3. Identification of Recombinant Plasmid Transformants by Double Restriction Endonuclease Digestion

The results of the recombinant plasmid pcDNA- CYP3A46 digested by BamHI and XhoI demonstrated that the target gene CYP3A46 was integrated in vector pcDNA3.1 as showed in fig 3.

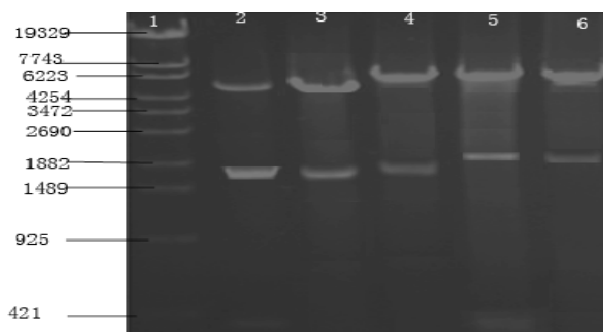


Fig3. Restriction anaLysis of recombinant plasmid pcDNA- CYP3A46 Lane 1λ-EcoT14I digest DNA Marker; Lane 2~6 Recombinant plasmid pcDNA-3A46 digested by BamHI and XhoI.

## 4. Eukaryotic expression transformants sequencing and analysis

The results of sequencing of CYP3A46 ORF in vector pCDNA3.1 as showed in fig 4 and fig 5 was blasted in NCBI by blast tool online showed that there was no difference between the present ORF and the one deposited in NCBI, indicating that the eukaryotic expression vector was constructed successfully.

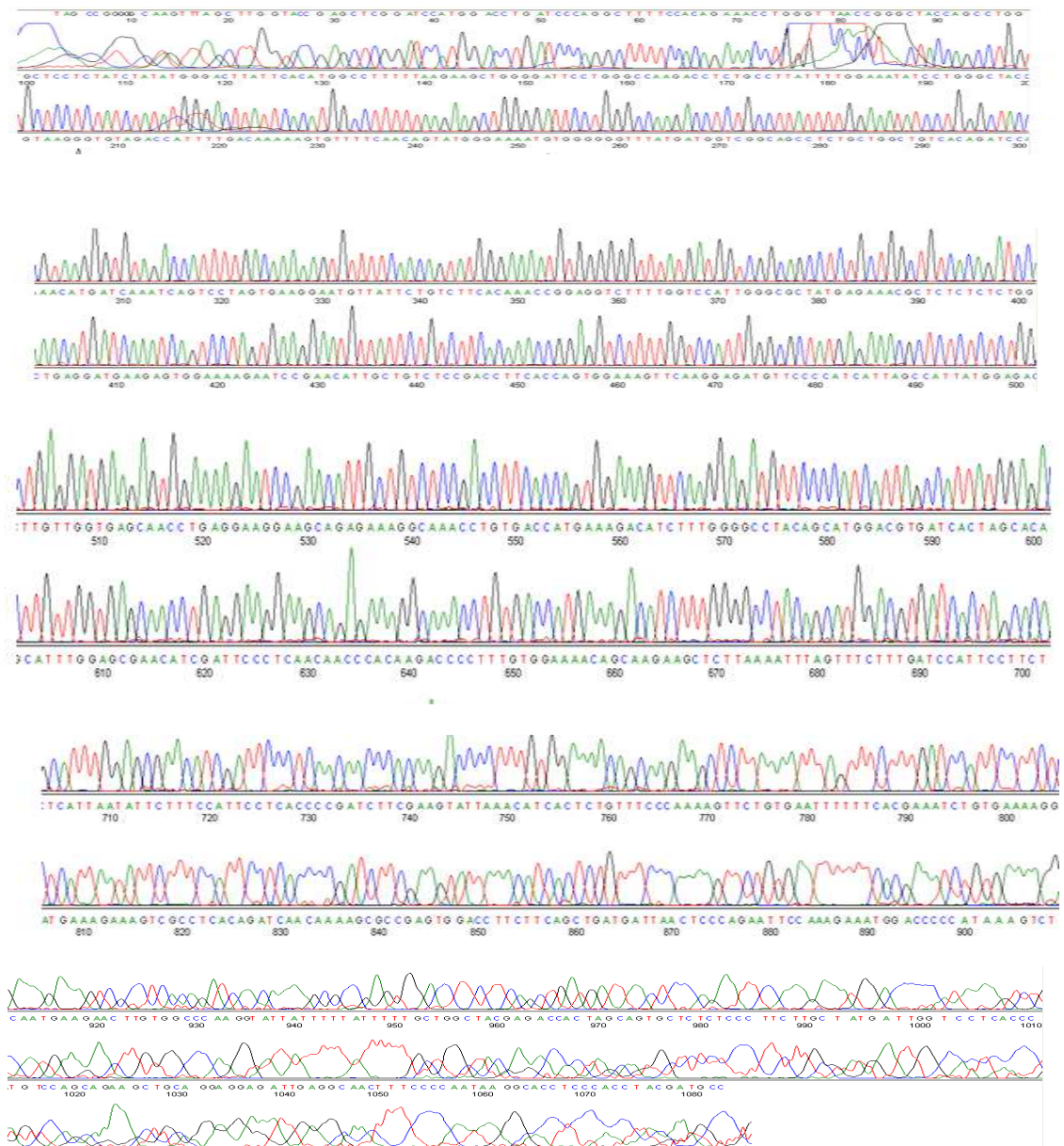
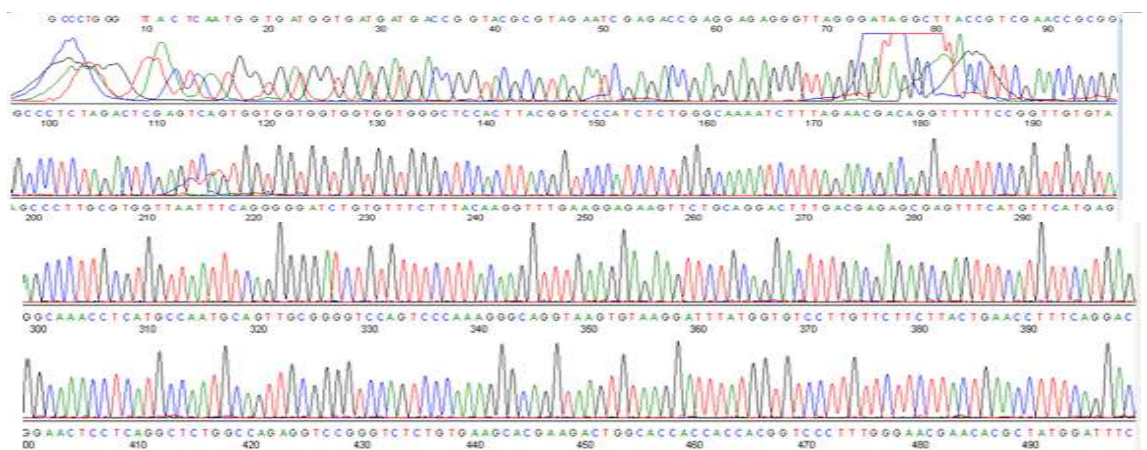


Fig4. Forward sequencing result of CYP3A46



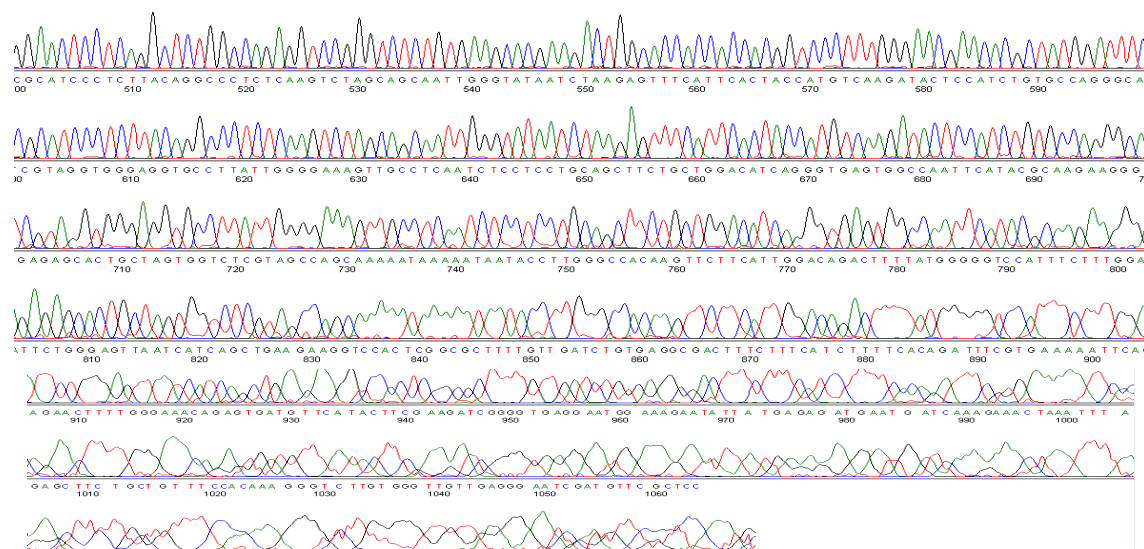


Fig 5. Reverse sequencing results of CYP3A46

### 5. The RT-PCR Identification of Recombinant HepG<sub>2</sub>-CYP3A46 Cell Line

The RT-PCR results as showed in Fig 6 indicated that a stable expression cell line of HepG<sub>2</sub>-CYP3A46 was established after the selection of recombinant cell line for 10 generations at the G418 concentration 200ng/mL.

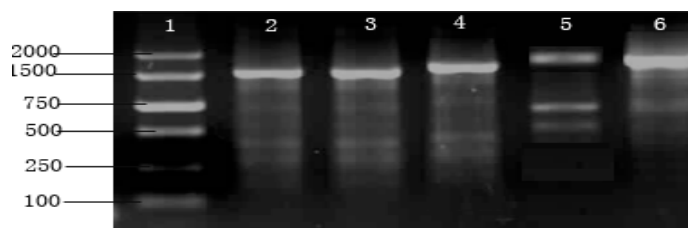


Fig6. Electrophoresis of HepG<sub>2</sub>-P4503A46 gene products derived from Recombinant cell lines of HepG<sub>2</sub>-CYP3A46by RT-PCR. Lane1 Marker: DL2000, Lane2~6 RT-PCR products of HepG<sub>2</sub>-CYP3A46

### 6. The West-blot results of recombinant cells HepG<sub>2</sub>-CYP3A46

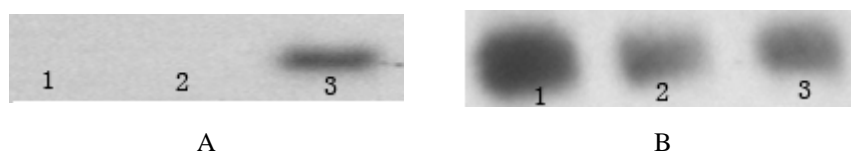
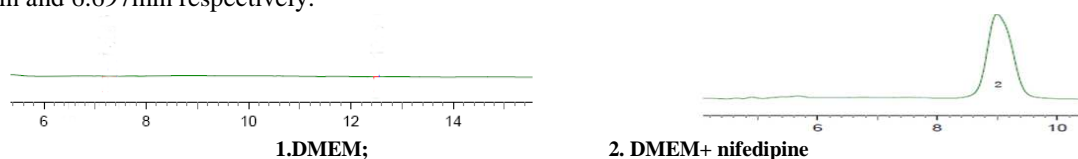


Fig 7.The results of West-blot. A CYP3A46 Ab test; B  $\beta$ -actin Ab test.1,HepG<sub>2</sub> cell line;2,HepG<sub>2</sub>-pcDNA3.1 cell line; 3,HepG<sub>2</sub>-CYP3A46 cell line

The West-blot results as showed in Fig7 indicated that the gene of CYP3A46 was stably expressed in recombinant cell line HepG<sub>2</sub>-CYP3A46.

### 7. Metabolic activity detection of recombinant cell line HepG<sub>2</sub>-CYP3A46 cell line by HPLC

Under the chromatographic conditions of this experiment set, the comparative chromatography of the blank chromatogram DMEM medium with DMEM + sample chromatogram, DMEM + sample + recombinant cell demonstrated that endogenous substances in the DMEM medium as showed in fig 8 does not interfere with the detection of nifedipine and oxidized nifedipine, and the retention time of nifedipine and oxidized nifedipine were 8.383min and 6.697min respectively.



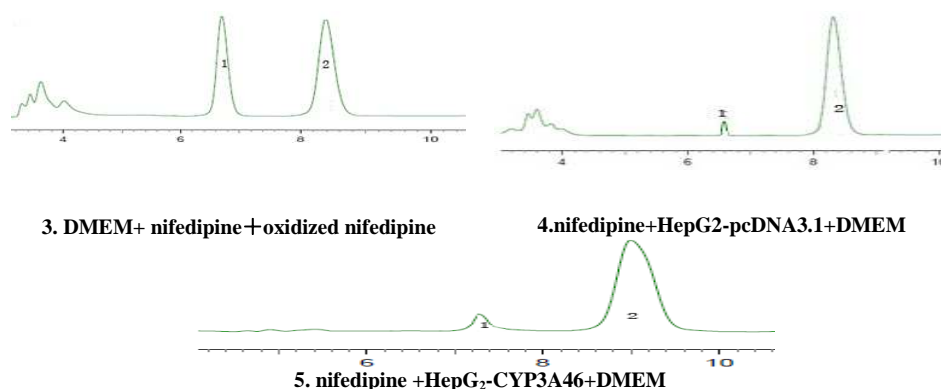


Fig8.HPLC chromatograms of nifedipine and oxidized nifedipine(1. oxidized nifedipine;2. nifedipine)

### 8. Nifedipine metabolic activity test of recombinant of HepG<sub>2</sub>-CYP3A46 cell line

Tab1. The significant test result of oxidized-nifedipine by one-Way ANOVA

Recombinant cell line	average Output of ONF (nmol)	HepG <sub>2</sub>	P HepG <sub>2</sub> -pcDNA3.1
HepG <sub>2</sub>	10.449±1.74		0.899>0.05
HepG <sub>2</sub> -pcDNA3.1	10.09±0.61	0.899>0.05	
HepG <sub>2</sub> -CYP3A46	163.31±5.44	0.00<0.01	0.00<0.01

\*ONF stands for oxidized-nifedipine

Comparing the products of oxidization nifedipine between HepG<sub>2</sub>-CYP3A46 cell line with the control group (HepG<sub>2</sub>-pcDNA3.1 and HepG<sub>2</sub> cell lines) by one-Way ANOVA, the results showed that the HepG<sub>2</sub>-CYP3A46 recombinant cell line produced very significantly greater metabolites of oxidized nifedipine than those of negative control HepG<sub>2</sub>-pcDNA3.1 and HepG<sub>2</sub> (P < 0.01), indicating that HepG<sub>2</sub>-CYP3A46 had a stronger nifedipine oxidative activity than HepG<sub>2</sub>-pcDNA3.1 and HepG<sub>2</sub>, the results further demonstrated that HepG<sub>2</sub>-CYP3A46 cell line expressed an CYP3A46 enzyme with nifedipine metabolized activity. (tab 1)

## DISCUSSION

At present, there are four subtypes of Cytochrome c oxidase in pig (miniature pig) CYP3A Subfamily, those are CYP3A22, CYP3A29, CYP3A39 and CYP3A46 (CYP3A88), CYP3A29 subtype was the most studied one in them, researches showed that it has the typical nifedipine oxidation activity (human CYP3A4 and CYP3A5 specific activity), which can be induced by phenobarbital, rifampin and dexamethasone [7-10].

Pig (miniature pigs) CYP3A46, also known as CYP3A88, is a cytochrome c oxidase composed of 503 amino acids in *Sus scrofa*, its full-length gene sequence was provided by Haito Shang (<http://www.ncbi.nlm.nih.gov/nuccore/EF625347>), the full-length sequence of size 1965bp, coding region of 1512bp. Shi-Chun Wang et al found that the amino acid sequence and senior structure of CYP3A46 are most similar to human CYP3A4 among the four CYP3A enzyme in the pig CYP3A subfamily by comparison [11], but its pharmacokinetic characteristics remains unknown.

Due to the presence of species differences among in CYP450s in different species experimental animal, in order to clarify the reliability of Bama miniature pig to be used in preclinical drug evaluation [12], the present study cloned the gene from Bama miniature pig liver tissue, and the gene was transfected into host cells HepG<sub>2</sub>, and stably expressing recombinant HepG<sub>2</sub>-CYP3A46 cell line was established by G418 screening. For the established cell line, RT-PCR analysis performed firstly, the results of which showed that monoclonal cell line of the HepG<sub>2</sub>-CYP3A46 was achieved through G418 selection, indicating that target gene expressed at the transcriptional level; for further characterized the expression of CYP3A46 at the translational level, the anti-His antibody was used for Western-blot test, the results of which showed that the HepG<sub>2</sub>-CYP3A46 cell line obtained in present research could express the CYP3A46 enzymes. In order to identify drug metabolizing activity of expressed CYP3A46 in

screened cell line HepG<sub>2</sub>-CYP3A46, CYP3A specific probe substrate nifedipine, which was recommended by FDA for CYP3A (<http://www.fda.gov/cder/drug/drugInteractions>), was utilized by means of incubation on whole cell level, the results of which showed that HepG<sub>2</sub>-CYP3A46 had extremely significantly high metabolic activity of nifedipine than that of control groups HepG<sub>2</sub>-pcDNA3.1 and HepG<sub>2</sub> (P < 0.001).

### CONCLUSION

In short, the results of present study demonstrated that CYP3A46 gene was cloned correct, the recombinant cell lines of HepG<sub>2</sub>-CYP3A46, which showed nifedipine metabolized activity significantly, was established successfully. However, in order to elucidate the drug metabolic characteristics, Further studies on the comparison of metabolic activities with human CYP3As, especially CYP3A4, should be carried out.

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