

CYP3A4 and CYP3A5 Expression is Regulated by CYP3A4*1G in CRISPR/Cas9-Edited HepG2 Cells[§]

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ABSTRACT

Functional CYP3A4*1G (G>A, rs2242480) in cytochrome P450 3A4 (CYP3A4) regulates the drug-metabolizing enzyme CYP3A4 expression. The objective of this study was to investigate whether CYP3A4*1G regulates both basal and rifampicin (RIF)-induced expression and enzyme activity of CYP3A4 and CYP3A5 in gene-edited human HepG2 cells. CYP3A4*1G GG and AA genotype HepG2 cells were established using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) single nucleotide polymorphism technology and homology-directed repair in the CYP3A4*1G GA HepG2 cell line. In CYP3A4*1G GG, GA, and AA HepG2 cells, CYP3A4*1G regulated expression of CYP3A4 and CYP3A5 mRNA and protein in an allele-dependent manner. Of note, significantly decreased expression level of CYP3A4 and CYP3A5 was observed in CYP3A4*1G AA HepG2 cells. Moreover, the results after RIF treatment showed that CYP3A4*1G decreased the induction level of CYP3A4 and CYP3A5 mRNA expression in

CYP3A4*1G AA HepG2 cells. At the same time, CYP3A4*1G decreased CYP3A4 enzyme activity and tacrolimus metabolism, especially in CYP3A4*1G GA HepG2 cells. In summary, we successfully constructed CYP3A4*1G GG and AA homozygous HepG2 cell models and found that CYP3A4*1G regulates both basal and RIF-induced expression and enzyme activity of CYP3A4 and CYP3A5 in CRISPR/Cas9 CYP3A4*1G HepG2 cells.

SIGNIFICANCE STATEMENT

Cytochrome P450 (CYP) 3A4*1G regulates both basal and rifampicin (RIF)-induced expression and enzyme activity of CYP3A4 and CYP3A5. This study successfully established CYP3A4*1G (G>A, rs2242480), GG, and AA HepG2 cell models using CRISPR/Cas9, thus providing a powerful tool for studying the mechanism by which CYP3A4*1G regulates the basal and RIF-induced expression of CYP3A4 and CYP3A5.

Introduction

In human adult livers, cytochrome P450 3A (CYP3A) and its major components, CYP3A4 and CYP3A5, play a crucial role in clinical drug metabolism, by metabolizing more than 60% of clinical prescription drugs. Genetic variation in CYP3A4, such as the single nucleotide polymorphism (SNP) CYP3A4*22 (rs35599367, G>A) in intron 6, which affects its transcript splicing, decreases CYP3A4 expression (Wang and Sadee, 2016), resulting in improved pharmacokinetics and clinically effective therapy (Wang et al., 2011; Mulder et al., 2021). The intronic genetic variation in CYP3A5, CYP3A5*3 (rs776746, A>G), leads to decreased normal CYP3A5 expression through alternative splicing (Kuehl et al., 2001) and is associated with tacrolimus dosage and pharmacokinetics (Dorr et al., 2017; Tang et al., 2019). Recently, these two

SNPs have become biomarkers for clinical individualized medications. Therefore, elucidating the mechanisms of individual differences in CYP3A enzyme activity from the perspective of intron SNP variation can provide new ideas for the implementation of precise and individualized treatments. Interestingly, another important CYP3A4 variation, CYP3A4*1G (rs2242480, G>A) (Fig. 1), is an intronic SNP enhancer (He et al., 2011; Yang et al., 2015) that is suspected to be a vital SNP in regulation of CYP3A4 expression and enzyme activity.

CYP3A4*1G is associated with the metabolism of numerous drugs (Gao et al., 2008; Zhang et al., 2010; Yuan et al., 2011; Dong et al., 2012; Ren et al., 2015; Yuan et al., 2015; Zhang et al., 2017; Lv et al., 2018; Zhang et al., 2018). CYP3A5 and CYP3A4 have highly substrate similarity, and CYP3A5 can metabolize most of substrates metabolized by CYP3A4. CYP3A5*3, which causes protein truncation, results in the absence of normal CYP3A5 expression, while CYP3A5*1 is related to normal CYP3A5 expression. Although CYP3A5*3 linkage with CYP3A4*1G might interfere with the metabolism of drugs metabolized by CYP3A4, the relation between CYP3A4*1G and tacrolimus in CYP3A5 expressers, or atorvastatin in CYP3A5 non-expressers, were further confirmed (Miura et al., 2011; He et al., 2014; Liu et al., 2017). Moreover, the allele frequency of CYP3A4*1G highly differs between ethnic lines, and its global allele frequency is about 0.15 (ranging between about 0.09 and 0.86 among 11 populations). Nine out of the eleven populations

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ABBREVIATIONS: CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; CYP3A4, cytochrome P450 3A4; EGFP, enhanced green fluorescent protein; ER6, everted repeat sequence separated by 6 bp; HDR, homology-directed repair; PCR, polymerase chain reaction; px330, PX330a-42230; PXR, pregnane X receptor; RIF, rifampicin; sgRNA, single guide RNA; SNP, single nucleotide polymorphism.

Examination of Basal Expression of *CYP3A4* and *CYP3A5* mRNA and Protein mRNA Analysis. Total RNA was extracted from *CYP3A4*1G* GG, GA, and AA HepG2 cells using TRizol Reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. cDNA was reverse transcribed from total RNA, and 5 ng of cDNA was amplified using an iQ5 Real-Time PCR Detection System with SYBR Green Master Mix from Thermo Fisher Scientific. Primers used are shown in Supplemental Table 2. The relative expression of *CYP3A4*, *CYP3A5*, and *PXR* was calculated by $2^{-\Delta\Delta C_t}$ method, and normalized to *glyceraldehyde-3-phosphate dehydrogenase*.

Western Blot Analysis. Total proteins of the above *CYP3A4*1G* GG, GA, and AA HepG2 cells were prepared using RIPA buffer. Protein concentrations were determined by BCA Protein Assay. Protein samples were separated by 12% SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried milk in 1xtris buffered saline, 0.1% Tween-20 and incubated overnight with primary antibodies for *CYP3A4* or *CYP3A5*-(Abcam) at 1/1000 dilution. The next day, the membranes were incubated in horseradish peroxidase-labeled secondary antibodies and visualized with an enhanced chemiluminescence method. Anti-glyceraldehyde-3-phosphate dehydrogenase (Abcam) at 1/5000 was used as a loading control.

Detection of *CYP3A4* and *CYP3A5* Induction Expression

*CYP3A4*1G* GG, GA, and AA HepG2 cells cultured in 12-well plates were treated with 10 μ M RIF (Sigma-Aldrich, St. Louis, MO) for 48 hours prior to harvesting. *CYP3A4*, *CYP3A5*, and *PXR* mRNA expression levels were examined by quantitative real-time polymerase chain reaction.

CYP3A4 Enzyme Activity and Tacrolimus Metabolism Study

P450-Glo *CYP3A4* Assay. *CYP3A4*1G* GG, GA, and AA HepG2 cells were washed with phosphate-buffered saline and luminescence was measured using the P450-Glo *CYP3A4* assay kit and Luciferin-IPA (Promega, Cat # V9001) with a plate reader as previous described (Pande et al., 2020).

Tacrolimus Metabolism Assays. *CYP3A4*1G* GG, GA, and AA HepG2 cells were treated with tacrolimus and incubated for 24 hours. Tacrolimus from Dalian Meilun Biotechnology Co., Ltd. (Shanghai, China) was diluted in methanol (1 mg/ml) so that tacrolimus final concentration in cell culture medium was 50 ng/ml. Media were collected and assayed for tacrolimus by ultra-high-performance liquid chromatography-mass spectrometry system according to previously described methods with minor modifications (Dorr et al., 2017; Liu et al., 2020; Shi et al., 2022). Collected media were centrifuged, and 40 μ l of supernatant with 160 μ l of glacial acetonitrile were vortexed for 30 seconds and placed on ice for 20 minutes before being centrifuged again. The supernatants were dried and resuspended in 320 μ l of methanol and mixed with 40 μ l of internal standard (ascomycin, 2000 ng/ml, dissolved in methanol) from Dalian Meilun Biotechnology Co., Ltd. (Shanghai, China). After vortexing and centrifugation at 14,000xg for 30 minutes at 4°C, the supernatants were filtered with a membrane and placed in a liquid injection flask. The residual amount of tacrolimus in the sample was determined using ultra-high-performance liquid chromatography-mass spectrometry including Shimadzu LC-30A system and 6500 QTRAP triple quadrupole mass spectrometer equipped with an ESI source) (ABSCIEX, USA). The mobile phase A was ammonium acetate aqueous solution, and mobile phase B was methanol. Loading volume was 3 μ l. An Atlantis T3 column (2.1 mm \times 100 mm, 3 μ m) was used, and the column temperature was 40°C. The transitions (precursor to product) monitored are m/z 821.7 to 768.5 for tacrolimus and m/z 809.7 to 729.6 for the internal standard, respectively. Ionization was performed in positive ion mode with a capillary voltage of 5.5 kV, a cone voltage of 61/74 (tacrolimus/internal standard), an ion source temperature of 550°C, and a collision gas pressure of 27.8/21 V (tacrolimus/internal standard).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions 21.0 software (IBM, Armonk, NY, USA). All comparisons were conducted using one-way or two-way ANOVA with Bonferroni post hoc test. Data were shown as mean \pm S.D. $P < 0.05$ was considered statistically significant.

Results

Establishment of *CYP3A4*1G* GG and AA HepG2 Cells by CRISPR/Cas9 Technology

Identification of px330 and pmCherry EGFP Reporter Recombination Plasmids. Sequencing results verified the correctness of the sequences and directions of positive recombination plasmids, px330-sgRNA, and pmCherry EGFP reporter with inserted target fragment sequences (Supplemental Fig. 1, A and B).

Identification of Gene-Edited *CYP3A4*1G* GG and AA HepG2 Cells. The efficiency of the sgRNA expression vector was screened in HEK293T cells (Supplemental Fig. 1C). To construct *CYP3A4*1G*, GG, and AA HepG2 cells, objective px330-sgRNA, pmCherry EGFP reporter recombinant vectors and the corresponding homologous recombination repair templates were co-transfected into *CYP3A4*1G* GA HepG2 cells. Monoclonal cells of the objective HepG2 cells were sorted and cultured. Identification of gene-edited *CYP3A4*1G* GG and AA HepG2 cells was performed by genomic DNA extraction, PCR amplification, and sequencing. Results of sequencing indicated that the genotypes of objective *CYP3A4*1G* GG and AA HepG2 cell strains were both homozygous compared with *CYP3A4*1G* GA HepG2 cells (Fig. 3). In *CYP3A4*1G* GG HepG2 cells the sequencing alignment (Supplemental Fig. 2A) unexpectedly revealed a deletion of two continuous bases (GA) at the ninth and tenth sites downstream of the *CYP3A4*1G* G allele in *CYP3A4* intron 10. For *CYP3A4*1G* AA HepG2 cells, there was one base deletion in 11th base (A) downstream of the *CYP3A4*1G* A allele. Above all, the *CYP3A4*1G* point mutation was correctly edited, and the *CYP3A4*1G* wild-type and mutant homozygote HepG2 cell strains were successfully constructed.

Off-Target Effects Validation of Monoclonal Cells. Genomic DNA of *CYP3A4*1G* GG, GA, and AA HepG2 cells was subjected to PCR and sequencing using off-target primers. Comparing the sequencing results with the predicted off-target sequences showed eight sequences without off-target (Supplemental Fig. 3, A and B), and two near sequences, 3M-2 and 2W-1, off-target in these ten sequences. The off-target sequence in *CYP3A4*1G* GG HepG2 was the second sequence, 3M-2 GAGTGGACAGTACATGGAGAA on chr7: -99710713 predicted by sgRNA-3M, where there was a base A insertion, highlighted by underline (Supplemental Fig. 2B). In *CYP3A4*1G* AA HepG2, the off-target sequence very near 3M-2 was the first sequence, 2W-1 GGACAGTACATGGAGAAGGA on chr7: -99710709 predicted by sgRNA-2W, where there was a 14 bp deletion, shown by underlined sequence, on chr7 (99710723-99710710) *CYP3A7* intron 10, as well as four SNP homozygous mutations in *CYP3A7* exon 10 (Supplemental Fig. 2B). The genotypes of these SNPs were rs754782383 (T>C) CC 90th, rs777212283 (C>T) new mutation C>G GG 127th, rs1336177699 (T>G) new mutation T>C CC 133rd, and rs756922005 (T>C) CC 145th in exon 10 of *CYP3A7*. *CYP3A4*1G* GG and AA HepG2 were used for further study.

*CYP3A4*1G* regulates basal expression of *CYP3A4* and *CYP3A5*

Basal expression levels of *CYP3A4* and *CYP3A5* mRNA and protein in *CYP3A4*1G* GG, GA and AA HepG2 cells were examined by quantitative real-time polymerase chain reaction and western blot. *CYP3A4* mRNA expression levels decreased in *CYP3A4*1G* AA HepG2 cells compared with *CYP3A4*1G* GG and GA HepG2 cells (both $P < 0.01$), and *CYP3A4* mRNA expression in *CYP3A4*1G* GA HepG2 cells was lower than that in *CYP3A4*1G* GG HepG2 cells ($P < 0.01$) (Fig. 4A). Moreover, *CYP3A4* protein expression level also decreased in *CYP3A4*1G* AA HepG2 cells compared with *CYP3A4*1G* GG ($P < 0.01$) and GA HepG2 cells, despite the fact that there was no significant difference between *CYP3A4*1G* AA and GA HepG2 cells (Fig. 4B). These results indicate that *CYP3A4*1G* regulates the mRNA and protein expression levels of *CYP3A4* in an allele-dependent manner. As *CYP3A5*3*

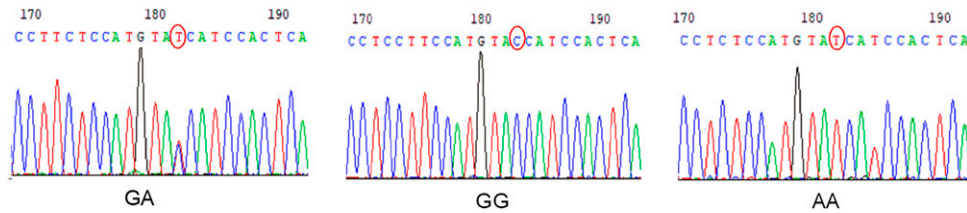


Fig. 3. Sequencing results of gene-edited *CYP3A4*1G* HepG2 cells. GA, GG, and AA indicate sequencing results of *CYP3A4*1G* GA, GG, and AA HepG2 cells, respectively. *CYP3A4*1G* GA HepG2 cells are the control. A base with a red circle in the sequence shows the *CYP3A4*1G* site and corresponding genotype.

showed linkage with *CYP3A4*1G*, *CYP3A5* mRNA and protein expression levels were also examined. Similarly, *CYP3A4*1G* also regulated *CYP3A5* mRNA and protein expression in an allele dependent-manner (Fig. 4, C and D). *CYP3A5* mRNA expression level in *CYP3A4*1G* AA HepG2 cells was lower than that in *CYP3A4*1G* GG HepG2 cells ($P < 0.01$), and there was a significant difference for *CYP3A5* mRNA expression level between *CYP3A4*1G* GA and GG HepG2 cells ($P < 0.05$) (Fig. 4C). The protein expression levels of *CYP3A5* were also lower in *CYP3A4*1G* AA HepG2 cells than that in *CYP3A4*1G* GG and GA HepG2 cells ($P < 0.01$ or $P < 0.05$), and there was also a significant difference for *CYP3A5* protein expression levels between *CYP3A4*1G* GA and GG HepG2 cells ($P < 0.05$) (Fig. 4D). In summary, *CYP3A4*1G* regulates basal expression of *CYP3A4* and *CYP3A5* at both mRNA and protein levels.

*CYP3A4*1G* Decreases RIF-Induced Expression of *CYP3A4* and *CYP3A5*

RIF, a strong inducer of *CYP3A4* enzyme, can induce *CYP3A4* expression by PXR; therefore, we investigated the impacts of *CYP3A4*1G* on the RIF-induced *CYP3A4* and *PXR* expression in different *CYP3A4*1G* HepG2 cells. As shown in Fig. 5A, RIF could induce the *CYP3A4* mRNA expression levels in *CYP3A4*1G* GG, GA, and AA

HepG2 cells, as compared with the control group ($P < 0.01$ or $P < 0.05$). However, diminished induction levels of *CYP3A4* by RIF were observed in the *CYP3A4*1G* AA and GA HepG2 cells as compared with the *CYP3A4*1G* GG HepG2 cells. RIF increased *CYP3A4* mRNA expression in the above three different *CYP3A4*1G* genotype HepG2 cells in an allele-dependent manner as compared with the DMSO alone control groups, with the induction of *CYP3A4*1G* AA and GG HepG2 cells being the weakest and the strongest (over 6-fold *CYP3A4* mRNA expression), respectively. The roles of induction of RIF in *CYP3A4*1G* GA HepG2 cells were between them. These results suggest that RIF induces *CYP3A4* mRNA expression in *CYP3A4*1G* allele-dependent manner.

*CYP3A4*1G* also affected *CYP3A5* mRNA expression; therefore, we analyzed whether RIF could also induce *CYP3A5* expression. Similar to *CYP3A4* induction by RIF, *CYP3A4*1G* decreased *CYP3A5* mRNA expression. Comparing the same *CYP3A4*1G* genotype HepG2 cells between RIF and DMSO groups, *CYP3A5* mRNA expression was also all increased after RIF treatment; however, the extent of induction of RIF in *CYP3A4*1G* AA HepG2 cells was much lower than that in *CYP3A4*1G* GG and GA HepG2 cells. There was significant difference in *CYP3A4*1G* GG or GA HepG2 cells after being induced ($P < 0.01$ or $P < 0.05$). RIF induction in *CYP3A4*1G* GG HepG2 cells was over

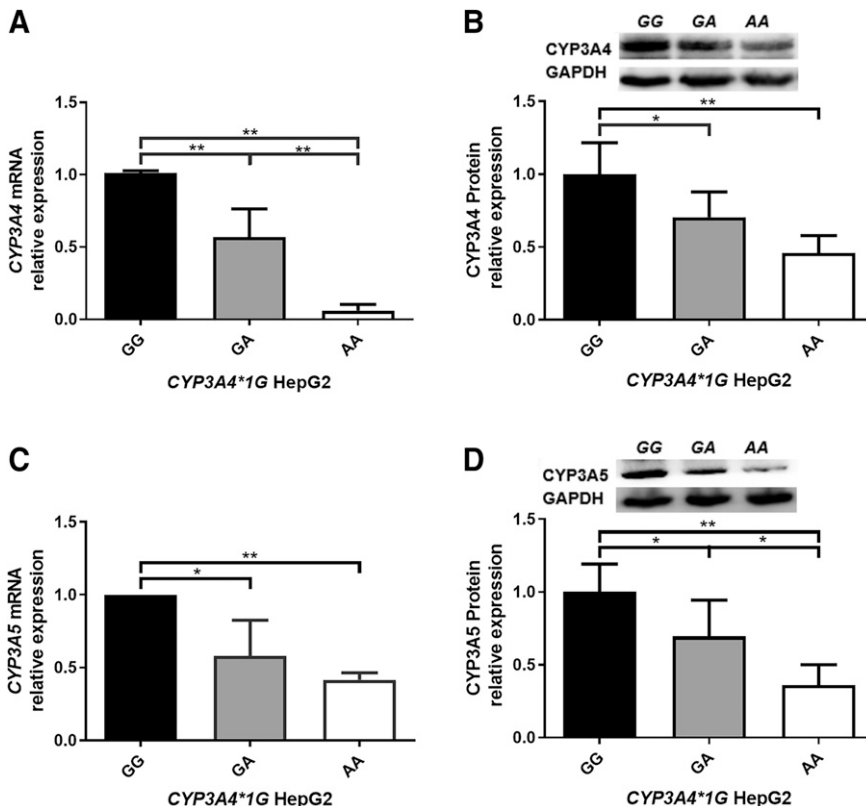


Fig. 4. *CYP3A* basal expression in *CYP3A4*1G* GG, GA, and AA HepG2 cells. *CYP3A4* mRNA (A) and protein (B) expression levels in *CYP3A4*1G* GG, GA, and AA HepG2 cells. *CYP3A5* mRNA (C) and protein (D) expression levels in *CYP3A4*1G* GG, GA, and AA HepG2 cells. The error bars represent S.D. ($n = 3$ per group for mRNA and protein). ($*P < 0.05$, $**P < 0.01$)

6-fold that of the *CYP3A5* mRNA expression of the same genotype HepG2 cells in the DMSO group (Fig. 5B). These results indicate that RIF also induces *CYP3A5* mRNA expression in a *CYP3A4*1G* allele-dependent manner, and its induction level in *CYP3A4*1G* AA HepG2 cells is much lower than that in *CYP3A4*1G* GG HepG2 cells.

Since RIF induced *CYP3A4* by PXR, PXR mRNA expression levels in different *CYP3A4*1G* genotype HepG2 cells were also determined. Comparing PXR expression between the DMSO and RIF group, we found that PXR mRNA expression level in *CYP3A4*1G* GG HepG2 cells was much higher in the RIF group than that in the DMSO group ($P < 0.05$); however, PXR mRNA expression levels in the other genotype HepG2 cells were not significantly increased (Fig. 5C). Particularly, diminished basal and RIF-induced mRNA expression levels of PXR were observed in the *CYP3A4*1G* AA (both $P < 0.05$) and GA HepG2 cells (both $P < 0.05$) as compared with the *CYP3A4*1G* GG HepG2 cells. These results reveal that *CYP3A4*1G*-decreases basal and RIF-induced mRNA expression levels of PXR.

*CYP3A4*1G* Affects *CYP3A4* and *CYP3A5* Activity in HepG2 Cells

To further study the effects of *CYP3A4*1G* on enzyme activity of *CYP3A4* and *CYP3A5*, *CYP3A4* enzyme activity and tacrolimus metabolism were also evaluated. *CYP3A4* enzyme activity decreased in *CYP3A4*1G* AA and GA HepG2 cells compared with *CYP3A4*1G* GG HepG2 cells, and *CYP3A4* enzyme activity in *CYP3A4*1G* GA HepG2 cells was the lowest of the three (both $P < 0.01$) (Fig. 6A). Similarly, for *CYP3A5* metabolism, the amount of tacrolimus metabolized in *CYP3A4*1G* GA HepG2 cells was lower than those in *CYP3A4*1G* GG ($P < 0.01$) and AA HepG2 cells ($P < 0.05$), while there was no significant difference between *CYP3A4*1G* AA and GG HepG2 cells (Fig. 6B).

Discussion

CYP3A plays a critical role in the metabolism of drugs and other exogenous and endogenous substances, and its function is modified by genetic factors (Zhai et al., 2022). *CYP3A4*1G* affects drug metabolisms by altering *CYP3A4* expression and enzyme activity in vivo and in human liver tissues (Miura et al., 2011; He et al., 2014; Yuan et al., 2015). Our previous study showed *CYP3A4*1G* as an enhancer, regulating *CYP3A4* expression indirectly in an allele-dependent manner (Yang et al., 2015). Until now, due mainly to the lack of validated *CYP3A4*1G* cell models, it was not clear whether the *CYP3A4*1G* variant could regulate *CYP3A4* expression and metabolism in gene-edited cells. Therefore, in this study, we constructed the homozygous point mutations *CYP3A4*1G* GG and AA in HepG2 cells using CRISPR/Cas9. These *CYP3A4*1G* HepG2 cells may provide cell models for studying the molecular mechanism by which *CYP3A4*1G* regulates *CYP3A4* and *CYP3A5* expression, and through further CRISPR/Cas9-induced SNPs, provide a means to research the mechanisms of *CYP3A5*3*, or its linkage with *CYP3A4*1G*, in regulating *CYP3A5*.

Our results showed that *CYP3A4*1G* regulates basal expression of *CYP3A4* mRNA and protein, in an allele-dependent manner (Fig. 4, A and B), and that *CYP3A4* expression in *CYP3A4*1G* AA HepG2 cells is lower than that in *CYP3A4*1G* GG HepG2 cells. These findings further support our previous in vitro luciferase enhancer reporter assays, which showed the *CYP3A4*1G* A allele has a lower enhancer activity than *CYP3A4*1G* G allele in a reverse orientation (Yang et al., 2015). Moreover, the results are consistent with studies in liver microsomes (Yuan et al., 2015) and pharmacokinetic studies among *CYP3A5* expressers (Miura et al., 2011; Liu et al., 2017). For example, Miura et al. suggested that *CYP3A4*1G* A allele carriers are related to the lower dose-adjusted area under the concentration-time curve (0–12) of tacrolimus than those with the *CYP3A4*1G* GG genotype among *CYP3A5*

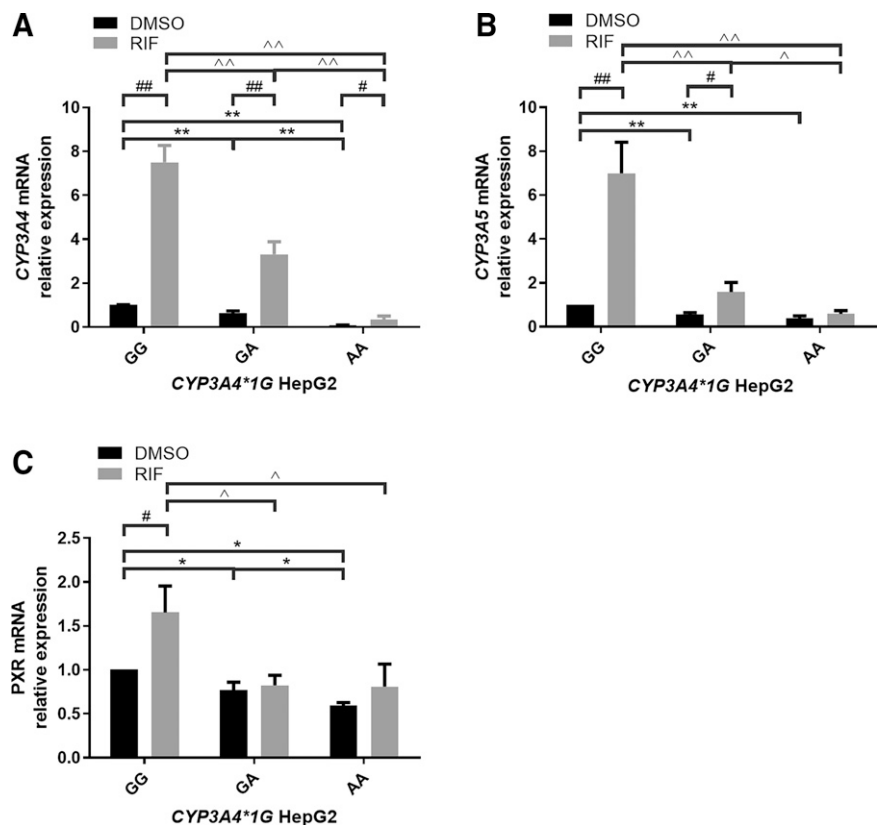


Fig. 5. *CYP3A* induction expression by RIF in *CYP3A4*1G* GG, GA, and AA HepG2 cells. *CYP3A* (A, B) and *PXR* (C) mRNA expression in *CYP3A4*1G* GG, GA, and AA HepG2 cells after 48 h induced by rifampicin (RIF). DMSO group for control. The error bars represent S.D. (n = 3 per group for mRNA). (* $P < 0.05$, ** $P < 0.01$ versus GG or GA control group; # $P < 0.05$, ## $P < 0.01$ versus the same genotype control group; ^ $P < 0.05$, ^^ $P < 0.01$ versus GG or GA RIF group).

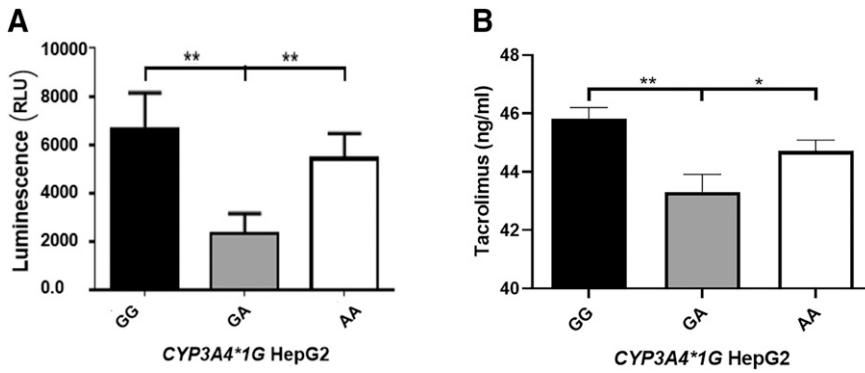


Fig. 6. *CYP3A4* enzyme activity and tacrolimus metabolism study in *CYP3A4*1G* GG, GA, and AA HepG2 cells. *CYP3A4* enzyme activity levels (A) and tacrolimus metabolism study (B) in *CYP3A4*1G* GG, GA, and AA HepG2 cells. The amount of tacrolimus metabolized in *CYP3A4*1G* GG, GA, and AA HepG2 cells is shown. (n = 3 per group for *CYP3A4* enzyme activity and tacrolimus metabolism). The error bars represent S.D. (* $P < 0.05$, ** $P < 0.01$).

expressers, but not *CYP3A5* non-expressers (Miura et al., 2011). Moreover, among *CYP3A5* non-expressers, He et al. also showed *CYP3A4*1G* AA and GA groups have lower area under the concentration-time curve (0– ∞) versus the *CYP3A4*1G* GG group (He et al., 2014). Importantly, to better understand the molecular mechanism of the *CYP3A4*1G* enhancer's regulation of *CYP3A4* through transcription factor binding, studies using these *CYP3A4*1G* HepG2 cell models should be conducted in the future.

Similar to *CYP3A4*, *CYP3A4*1G* also regulates basal expression of *CYP3A5* indifferent *CYP3A4*1G* genotype HepG2 cells (Fig. 4, C and D). It may be because of the strong linkage relation between the *CYP3A5*3* G variant allele and *CYP3A4*1G* G wildtype allele as well as *CYP3A5*3* A wildtype allele and *CYP3A4*1G* A variant allele (Uesugi et al., 2013; Qi et al., 2022). We believe the lower *CYP3A5* expression shown by *CYP3A4*1G* AA HepG2 cells, compared with the *CYP3A4*1G* GG and GA HepG2 cells, is a result of the *CYP3A5*3* G allele forming a new linkage with the *CYP3A4*1G* A allele, which replaces the primary G wild-type allele and *CYP3A4*1G* A allele, having lower enhancer activity than the *CYP3A4*1G* G allele. Conversely, in *CYP3A4*1G* GG HepG2 cells, as the *CYP3A5*3* A wild-type allele forms a new linkage with the *CYP3A4*1G* G wild-type allele (which replaces the primary A variant allele), and *CYP3A4*1G* G allele has a higher enhancer activity than *CYP3A4*1G* A allele, this results in *CYP3A4*1G* GG HepG2 cells having higher *CYP3A5* expression than the other *CYP3A4*1G* genotype HepG2 cells. Similar to our study, Dorr et al., used CRISPR/Cas9 to confirm the *CYP3A5*3* A allele increased *CYP3A5* expression, furthering the metabolism of midazolam and tacrolimus by editing *CYP3A5*3* GG into AA and AG genotype in Huh7 cells carrying *CYP3A4*1G* GA and *CYP3A4*22* CC genotypes (Dorr et al., 2017). Another study revealed that *CYP3A4*1G* A allele acts as a marker for the *CYP3A5*3* A allele, which increases tacrolimus clearance in Japanese liver transplant patients (Uesugi et al., 2013), also supporting the regulatory relationship of *CYP3A4*1G* and *CYP3A5*3* in our present study.

Accordingly, through *CYP3A4* and *CYP3A5* metabolic functional studies, we also found that *CYP3A4*1G* decreases *CYP3A4* enzyme activity and tacrolimus metabolism in *CYP3A4*1G* GA HepG2 cells. This finding is similar to previous results that dose-adjusted C_0 in *CYP3A4*1G* carriers is lower than *CYP3A4*1G* GG individuals in *CYP3A5* expressers (Miura et al., 2011). Enzyme activity may be affected by post-transcriptional regulation (protein expression) or post-translational modifications (phosphorylation, etc.). *CYP3A4*1G* affecting *CYP3A4* enzyme is partly different from its role for *CYP3A4* expression; it is suspected that multisite phosphorylation of *CYP3A4* is associated with enhancing its gp78- and CHIP-mediated ubiquitination (Wang et al., 2012; Kwon et al., 2019).

Taken together, our results show that *CYP3A4*1G* regulates basal expression and enzyme activity of *CYP3A4* and *CYP3A5* in different *CYP3A4*1G* genotype HepG2 cells with the *CYP3A5*3* AG genotype.

Lin et al. found that, in people with the *CYP3A5*3* AG genotype, there is a certain correlation between *CYP3A4* and *CYP3A5* protein and mRNA, and speculated there may be conserved 5'-flanking region elements involved in constitutive expression of *CYP3A4* and *CYP3A5* through sharing a common regulatory pathway (Lin et al., 2002). In agreement with this, in HepG2 cells with *CYP3A5*3* AG genotype, we found that *CYP3A4*1G*, as an enhancer, regulates expression of *CYP3A4* and *CYP3A5*. In addition, Collins and Wang proposed that *CYP3A4*1G* downregulates *CYP3A4* and *CYP3A5* expression through a lncRNA, AC069294.1 (Collins and Wang, 2022). Further validation will be done in these various *CYP3A4*1G* different HepG2 cells. *CYP3A4*1G* decreases *CYP3A4* enzyme activity and tacrolimus metabolism in *CYP3A4*1G* GA HepG2 cells, revealing that individuals with *CYP3A5*3* AG and *CYP3A4*1G* GA genotype, requires a lower dose of tacrolimus than those with *CYP3A5*3* AG and *CYP3A4*1G* GG or AA genotype.

We also found that *CYP3A4*1G* regulates the RIF-induced expression of *CYP3A4* and *CYP3A5* in a *CYP3A4*1G* allele-dependent manner in HepG2 cells (Fig. 5, A and B). It is well known that RIF increases expression of *CYP3A4* by regulating the transcription factor PXR. Meanwhile, we found that *CYP3A4*1G* may also affect PXR expression. Therefore, we believe that *CYP3A4*1G* and RIF act together on PXR to regulate *CYP3A4* and *CYP3A5* expression. As to the relation of *CYP3A5* expression and RIF, our results are consistent with the Burk group's suggestion that in liver tissue, RIF-induced *CYP3A5* expression may phenocopy the effects of the *CYP3A5*3* A, a high expression allele (Burk et al., 2004). Barwick et al. and Burk et al. found that RIF inductions of *CYP3A4* and *CYP3A5* share a similar mechanism in which the induction is mediated by the proximal everted repeat sequence separated by 6 bp (ER6) binding site for PXR in the promoter of *CYP3A4* or *CYP3A5* (Barwick et al., 1996; Burk et al., 2004). This still leads to two speculations. In combination with RIF, *CYP3A4*1G* enhances expression of *CYP3A4* or *CYP3A5* by PXR and its ER6-regulatory element in the *CYP3A4* or *CYP3A5* proximal 5'promoter. Furthermore, *CYP3A4*1G* alone can also enhance expression of *CYP3A4* or *CYP3A5* through PXR and its ER6 regulatory element in *CYP3A4* or *CYP3A5* proximal 5'promoter. Thus, further studies using *CYP3A4*1G* HepG2 cells, with or without RIF, are required to elucidate the molecular mechanisms by which *CYP3A4*1G*, as an enhancer, regulates *CYP3A4* or *CYP3A5* expression through PXR binding the ER6 regulatory element in the *CYP3A4* or *CYP3A5* proximal 5'promoter.

There are some limitations in this study. There is no data showing how *CYP3A4*1G* GG, GA, and AA genotypes affect the actual drug metabolism of *CYP3A4* substrates. Furthermore, similar to other CRISPR/Cas9 edited cells, in these point mutation *CYP3A4*1G* GG and AA HepG2 cells, there are also a few base deletions in *CYP3A4* intron 10 near *CYP3A4*1G* site. There are also a few base substitutions/insertions and deletions of 14 bases near the location of off-targets on

chr7 of *CYP3A7* gene far away from *CYP3A4*, although they have no obvious effects on the *CYP3A4*1G* regulated *CYP3A4* and *CYP3A5* expression, or combination with RIF treatment, in the present study.

In conclusion, we constructed *CYP3A4*1G* GG and AA point mutation HepG2 cell strains, and found *CYP3A4*1G* regulating both basal and RIF-induced expression and enzyme activity of *CYP3A4* and *CYP3A5*. These findings will provide a new theoretical basis for clinical precision therapy based on *CYP3A4*1G* as a biomarker. Considering the linkage relation of *CYP3A4*1G* and *CYP3A5*3*, and complex of *CYP3A* substrates, use of *CYP3A4*1G*, *CYP3A5*3*, and *CYP3A4*22* as biomarkers is recommended to guide *CYP3A* precision therapy.

Authorship Contributions

Participated in research design: Yang, Zhao, Z. Zhang, L.R. Zhang.

Conducted experiments: Yang, Zhao, Dou, Chang, Xu.

Performed data analysis: Yang, Zhao, Dou, Xu.

Wrote or contributed to the writing of the manuscript: Yang, Zhao, P. Wang, Chang, Qiao, X. Wang, L.R. Zhang.

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