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Regulation of CYP3A4 and CYP3A5 by a lncRNA: a potential underlying mechanism explaining the association between CYP3A4*1G and CYP3A metabolism

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Abstract

The cytochrome P450 3A4 (CYP3A4) enzyme is the most abundant drug-metabolizing enzyme in the liver, displaying large inter-person variability with unknown causes. In this study, we found that the expression of CYP3A4 is negatively correlated with AC069294.1 (ENSG00000273407, ENST00000608397.1), a lncRNA generated antisense to CYP3A4. Knockdown of AC069294.1 in Huh7 cells increased CYP3A4 mRNA ~3-fold, while overexpression of AC069294.1 decreased CYP3A4 mRNA by 89%. We also observed changes in CYP3A5 expression when AC069294.1 was knocked down or overexpressed, indicating dual effects of AC069294.1 on both CYP3A4 and CYP3A5 expression. Consistently, the expression level of CYP3A5 is also negatively correlated with AC069294.1. Previous studies have shown associations between an intronic SNP CYP3A4*1G (rs2242480) and CYP3A metabolism, but the results are inconsistent and the underlying mechanism is unclear. We show here that CYP3A4*1G (rs2242480) is associated with 1.26-fold increased expression of AC069294.1 ($p<0.0001$), and decreased expression of CYP3A4 by 31% ($p=0.008$) and CYP3A5 by 39% ($p=0.004$). CYP3A4*1G is located ~2.7kb upstream of AC069294.1 and has been previously reported to have increased transcriptional activity in reporter gene assays. Taken together, our results demonstrate the regulation of CYP3A4 and CYP3A5 by a novel lncRNA AC069294.1. Our results also indicate that the clinically observed CYP3A4*1G associations may be caused by its effect on the expression of AC069294.1, and thereby altered expression of both CYP3A4 and CYP3A5. Furthermore, since CYP3A4*1G is in high LD with CYP3A5*1, increased AC069294.1 expression caused by CYP3A4*1G may decrease expression of the normal-functioning CYP3A5*1, explaining additional inter-person variability of CYP3A5.

Keywords

cytochrome P450s; CYP3A4; CYP3A5; lncRNA; gene expression; polymorphisms

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Introduction

The cytochrome P450 3A4 (CYP3A4) enzyme is the most abundant drug-metabolizing enzyme in the liver [1] and is involved in the metabolism of more than fifty-percent of clinically prescribed medications [2]. Expression and enzymatic activity levels of CYP3A4 show large inter-person variability [1, 3], thereby influencing treatment outcomes of drugs metabolized by CYP3A4. Early studies showed that CYP3A4 expression/activity is highly heritable [4] [5], but these estimates are still under debate. To date, the cause of large variability in CYP3A4 expression and enzyme activity remains largely unknown.

While numerous coding region variants of CYP3A4 have been identified (https://www.pharmvar.org/gene/CYP3A4), they are all rare (<1%) and therefore cannot account for the observed variability in CYP3A4 activity. Several non-coding variants have also been implicated in regulating CYP3A4 expression. For example, we previously identified an intronic SNP in CYP3A4 (CYP3A4*22) that altered CYP3A4 expression and statin dosage [6] by causing alternative splicing of the CYP3A4 transcript [7]. So far, CYP3A4*22 has been associated with phenotypes related to CYP3A4 metabolism in numerous studies and has been used as biomarker predicting CYP3A4 activity [8]. Also, we recently identified an enhancer SNP rs62471956 that reduces CYP3A4 transcription [9], which was previously associated with higher plasma levels of the CYP3A4 metabolite of ticagrelor [10]. rs62471956 is in complete LD with CYP3A4*22, and likely contributes to the attenuated expression/activity of CYP3A4 in *22 carriers. Although CYP3A4*22 substantially reduces CYP3A4 enzyme activity in carriers, it can account for only part of the large population variability in CYP3A4 activity because of its low allele frequency (2-5%).

CYP3A4*1G (rs2242480), located within intron 10 of CYP3A4, has also been associated with CYP3A4 substrate drug pharmacokinetics and/or responses. However, reports documenting the effects of this allele are inconsistent. For example, CYP3A4*1G (rs2242480) has been associated with increased CYP3A activity, but this effect may instead be explained by its high linkage with the functional CYP3A5*1 allele. For instance, metabolism of tacrolimus (a CYP3A4/CYP3A5 substrate) is higher in CYP3A4*1G carriers, but the association is stronger with the CYP3A5*1 allele than the CYP3A4*1G allele [11–13]. In support of this, CYP3A4*1G is associated with higher CYP3A5 mRNA expression in the liver and intestine, but not with CYP3A4 expression, indicating that CYP3A4*1G is acting as a marker for CYP3A5*1 and thereby increased tacrolimus clearance in organ transplant patients [14]. Conversely, CYP3A4*1G has also been associated with lower CYP3A4 expression in the liver [15] and reduced metabolism of CYP3A4 substrate drugs, including fentanyl [15–20], sufentanil [21, 22], and atorvastatin [23]. These effects are contradictory to increased expression of CYP3A5*1 and likely to be unrelated to its linkage to CYP3A5*1.

CYP3A4*1G is located within a region enriched with epigenetic marks indicative of enhancers in several tissues, including the liver [24]. In reporter gene assays, both the wild-type and variant alleles of CYP3A4*1G enhance transcription, with the variant A allele having higher transcriptional activity [25, 26]. Therefore, if this enhancer regulates CYP3A4 expression, CYP3A4*1G carriers are expected to have higher CYP3A4 expression

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and enzymatic activity. However, this result is inconsistent with clinical association results showing reduced activity of CYP3A4*1G [15-23]. Moreover, our previous study showed no allelic expression differences between the A and G alleles in CYP3A4*1G heterozygous liver samples [6], arguing against a direct cis-acting regulatory effect of CYP3A4*1G on CYP3A4 expression in the liver.

The CYP3A locus is complex, harboring four functional CYP3A genes, two pseudogenes, and a lncRNA (AC069294.1-201, Ensembl gene ID: ENSG00000273407; transcript ID: ENST00000608397.1) (Figure 1). lncRNA-mediated regulation has been reported for many genes, including the CYP enzymes [see review by 27]. AC069294.1 is currently annotated as a 350 bp antisense lncRNA contained within the eighth intron of CYP3A4, but it is unclear whether this represents its full-length sequence. RNAseq data from The Genotype-Tissue Expression (GTEx) project show that AC069294.1 is expressed at a low level in a variety of tissues, including the liver and intestine [28]. Because of its location, we hypothesized that AC069294.1 may play a regulatory role in the expression of CYP3A4 or other CYP3A genes. In this study, we demonstrate the regulatory role of AC069294.1 on the expression of both CYP3A4 and CYP3A5 in human liver samples. In addition, we show a potential mechanism underlying the effects of CYP3A4*1G through increasing expression of AC069294.1 and thereby, decreasing the expression of both CYP3A4 and CYP3A5.

Materials and Methods

Liver tissue samples:

A total of 246 human liver samples (133 from European American and 113 from African American donors, see Supplemental Table 1 for demographic information of liver donors) were obtained from The Cooperative Human Tissue Network (CHTN). The University of Florida Institutional Review Board approved the human tissue study.

Quantitative analysis of mRNA or lncRNA:

Biopsy liver tissue samples were immediately frozen in liquid nitrogen, shipped in dry ice, and stored at −80°C. Total RNA was prepared from liver samples using direct-zol RNA miniprep plus kits (Zymo Research, CA, USA). RNA integrity and quality were estimated using the Qubit RNA IQ assay (Thermofisher, CA, USA). Samples with RNA IQ score <3 were excluded. To maximize the yield and minimize bias, cDNA was synthesized using two methods, each with 0.5 μg total RNA: qScript XLT cDNA SuperMix (VWR, PA, USA) and SuperScript IV Vilo Master mix (ThermoFisher, CA, USA). The cDNAs from the two reactions were combined, diluted 12-fold, aliquoted, and stored at −80°C. Expression levels of the CYP3A genes and AC069294.1 were measured with quantitative real-time PCR (qRTPCR) using the TaqMan assay (ThermoFisher Scientific) or SYBR Green with gene-specific primers (see Supplemental Table 2 for TaqMan probe ID or sequences of primers). Measurements were conducted on a Quantabio Q real-time PCR instrument (VWR, PA, USA), which showed minimal inter-run variability (coefficient of variation CV ranging from 0.007 to 0.01) and high reproducibility (correlation between two runs ranging from 0.987 to 0.998). Each sample was measured twice and Ct values were averaged. We also measured the expression levels of β-actin as an internal control. The relative expression of each gene

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was calculated using the following formula: expression level of tested gene = antilog2(mean Ct value of β -actin — mean Ct value of tested gene) *10^6. After Log10 transformation, the expression level of all genes followed a normal distribution.

AC06924.1 is located within an intron, and therefore heteronuclear CYP3A4 RNA (transcripts containing introns) that contains intron eight may also be detected by the AC069294.1 qPCR primers. To control for contamination by CYP3A4 heteronuclear RNA, AC069294.1 was further normalized by qPCR detecting expression of CYP3A4 heteronuclear RNA containing intron 11.

To validate this normalization approach, we re-synthesized cDNA using gene-specific primers targeting only AC069294.1, the CYP3A genes, and β-actin (see Supplemental Table 2 for primer sequence) in a subset of liver samples with either rs2242480 AA (n=20) or GG (n=24) genotypes. CYP3A cDNA was generated using a single primer set targeting a homologous region in exon 7 that is shared in all four CYP3A genes, resulting in CYP3A cDNA that only contains sequence upstream of exon 7 and therefore no CYP3A4 heteronuclear RNA containing intron eight (Figure 1). The relative expression levels of AC069294.1 and CYP3A genes were calculated using β-actin as an internal control as described above. The results from the two qPCR methods were similar.

Genotyping:

CYP3A4*1G (rs2242480) and CYP3A5*3 (rs776746) were genotyped using the OpenArray genotyping platform (probe# C_26201809, C_15746515, QuanStudio 12K Flex System) according to the manufacturer’s protocol (Life Technology, California, USA).

Antisense oligonucleotides and plasmid DNA:

Modified (2′-deoxy-2′-fluoro-arabinoguanosine, FANA) antisense oligonucleotides (FANA ASOs) targeting AC06294.1 (Supplemental Table 2) and the negative control ASO (no target in the human genome) were custom-made by AUM Biotech (Philadephia, PA, USA). DNA corresponding to AC069294.1 was synthesized and cloned into the pCDNA3.1 vector using the Biomatik (Wilmington, Delaware, USA) service.

Cell culture and transfection:

Huh7 and HepG2 cells were cultured at 37°C in a humidified incubator at 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Different concentrations of FANA ASOs were added directly to the cell culture medium and cells were harvested 48-72hrs post treatment. AC069294.1 expressing plasmid DNA was transfected into Huh7 cells using lipofectamine 2000 (Fisher Scientific, USA), and cells were harvested 72 hrs post transfection. FANA ASOs and transfection experiments were conducted in biological quadruplicates or triplicates. Total RNA was prepared from Huh7 cells using direct-zol RNA miniprep plus kits (Zymo Research, CA, USA). Total protein was prepared from the same cells using acetone precipitation of the flow-through Trizol lysate. Briefly, after passing the lysate through the column, the flow-through was collected, four volumes of acetone were added, the samples were incubated on ice for 30 min, and then centrifuged at 15,000 g for 10 min at 4°C. The pellets

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were washed once with 400 µl ethanol (95-100%), air-dried, and resuspended in sample loading buffer. Total protein concentrations were measured using the Bradford method (Thermofisher Scientific, CA, USA).

CYP3A protein quantification in Huh7 cells:

CYP3A protein expression in Huh7 cells was quantified using capillary western blot analysis with the ProteinSimple Jess system (ProteinSimple, CA, USA) as described previously [29]. CYP3A protein was detected using a mouse anti-CYP3A4 antibody (Santa Cruz, CA, USA) (1:3), followed by an HRP-conjugated anti-mouse secondary antibody (1:20) and chemiluminescent substrate (ProteinSimple, CA, USA). To adjust for the protein loading, we also measured total protein simultaneously in each capillary, as our previous study (in revision) and others [30, 31] showed total protein analysis is a more reliable loading control than using house-keeping genes for Western blots.

Data Analysis:

Data are shown as mean ± SD. Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA, USA). Pearson correlation was employed to determine the relationships between the mRNA levels of AC069294.1 and the CYP3A genes. The Student's t-test was used to determine differential expression of AC069294.1 in the rs2242480 AA and GG groups. A multiple linear regression model was used to test the association between the CYP3A4*1G genotypes and liver CYP3A gene expression using the Minitab software, adjusting for covariates (sex, age, race, and the CYP3A5*3 genotype for CYP3A5). Log10 transformed mRNA expression data of the AC069294.1 and CYP3A genes followed a normal distribution.

Results

The expression levels of AC069294.1 and CYP3A4/CYP3A5 are negatively correlated:

To determine if there is a relationship between expression of AC069294.1 and CYP3A4, we measured the expression levels of both transcripts in a 246-sample liver cohort (see Supplemental Table 1 for demographics). The expression levels of AC069294.1 and CYP3A4 are negatively correlated ($r=-0.53$, $p<0.0001$, Figure 2a). We also measured the expression of other CYP3A genes clustered in the same locus (CYP3A5, CYP3A7, and CYP3A43), to test whether a similar correlation exists. Like CYP3A4, the expression levels of CYP3A5 and AC069294.1 are negatively correlated ($r=-20.251$, $P<0.001$) (Supplemental Figure 1). Because CYP3A5*3 is the main factor affecting CYP3A5 expression [32], we excluded samples with CYP3A5*3/*3 genotypes. After only including samples with at least one copy of CYP3A5*1 allele (n=124), the negative correlation between CYP3A5 and AC069294.1 became stronger ($r=-0.52$, $p<0.001$, Figure 2b). In contrast, the expression level of AC069294.1 is not correlated with CYP3A7 ($r=-0.033$, $p=0.601$) and has a positive trend with CYP3A43 ($r=0.109$, $p=0.089$). Similar results were obtained when gene-specific primers were used in a subset of the samples (n=44). Namely, AC069294.1 is negatively correlated with CYP3A4 and CYP3A5 ($r=-0.506$ for CYP3A4, $P<0.0001$; $r=-0.555$ for CYP3A5 in CYP3A5*1 carriers, $p<0.0001$), but not with CYP3A7 ($r=0.202$, $P=0.189$) and slightly positively correlated with CYP3A43 ($r=0.358$, $P=0.017$) (Supplemental Figure

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2). Taken together, these results indicate that AC069294.1 may negatively regulate the expression of both CYP3A4 and CYP3A5.

The effect of knockdown or overexpression of AC069294.1 on expression of the CYP3As:

To test whether AC069294.1 directly regulates CYP3A expression, we conducted knockdown (KD) of AC069294.1 in the hepatocarcinoma cell line Huh7. We used modified (2′-deoxy-2′fluoro-arabinogunosine) antisense oligonucleotides (FANA ASOs) to silence AC069294.1. We tested two different FANA ASOs targeting AC069294.1 at two concentrations (1 μM and 10 μM) and two treatment periods, 48 and 72 hrs. The ASOs decreased the level of AC069294.1 by 20-60%, with ASO_1 at 10 μM (72 hr treatment) having the highest efficiency and therefore was selected for further experimentation. Compared to the negative control (ASO with no genomic target), ASO_1 decreased AC069294.1 expression ~57% (negative control 100 ± 22; ASO 43 ± 16, n=8, P<0.001) (Supplemental Figure 3a), increased expression of CYP3A4 3.5-fold and CYP3A5 2.5-fold, and had no effect on CYP3A7 and CYP3A43 (Figure 3a).

Using plasmid DNA transfection, we also tested the effects of overexpression of AC069294.1 on CYP3A expression. Transfection increased AC069294.1 expression nearly 40-fold (control: 100 ± 40.89; overexpressed: 3981 ± 1053, p<0.0001, n=3) (Supplemental Figure 3b). In agreement with the knockdown results, overexpression of AC069294.1 reduced the expression of CYP3A4 and CYP3A5 by 89% and 70%, respectively (Figure 3b). However, overexpression of AC069294.1 also reduced expression of CYP3A7 by 80%, while having no effect on CYP3A43 expression (Figure 3b).

We also tested the expression of CYP3A protein after AC069294.1 up- or down- regulation in Huh7 cells. We used an antibody that detects both CYP3A4 and CYP3A5 to determine the overall effect on CYP3A protein levels. As expected, knockdown of AC069294.1 increased CYP3A protein levels (Supplemental Figure 4a), while overexpression of AC069294.1 decreased CYP3A protein levels (Supplemental Figure 4b), consistent with our mRNA expression results.

Association between CYP3A4*1G and expression of AC069294.1, CYP3A4, and CYP3A5:

CYP3A4*1G is located within an enhancer region [24-26] in tenth intron of CYP3A4, ~2.7 kb upstream of AC069294.1, may potentially regulate expression of AC069294.1, and thereby alter the expression of CYP3A4 and CYP3A5. Indeed, in 246-sample liver cohort, CYP3A4*1G was associated with the expression level of AC069294.1 with each CYP3A4*1G allele (A allele) associating with 26% increase in the expression of AC069294.1 (P<0.0001, Table 1). Similarly, in the subset of samples with CYP3A4*1G AA or GG homozygous genotypes and cDNA synthesized using gene specific primers, the expression of AC069294.1 is 1.8-fold higher in homozygous CYP3A4*1G-AA samples than those with the CYP3A4*1G-GG genotype (P=0.042, Figure 4). Furthermore, CYP3A4*1G was also associated with decreased expression of both CYP3A4 (31%) and CYP3A5 (39%, after adjusting for CYP3A5*3) (Table 1), while there is no association between CYP3A4*1G and expression of either CYP3A7 or CYP3A43 (p>0.05, Table 1). Because expression of AC069294.1 is 40% higher in African American livers (p=0.001), we tested

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whether racial background was contributing to expression of AC069294.1. However, after adjusting for CYP3A4*1G, which is more prevalent in African Americans, ancestral background was not found to be a significant predictor for expression of either AC069294.1 or CYP3A4. However, ethnicity was significantly associated with expression of CYP3A5 (p=0.038), indicating that there are additional factors unrelated to AC069294.1 that are contributing to expression of CYP3A5 in different ethnic groups.

Discussion:

Using our liver cohort, we demonstrated that the expression level of the lncRNA AC069294.1 is negatively correlated with expression levels of both CYP3A4 and CYP3A5 (Figure 2). Furthermore, knockdown of AC069294.1 in Huh7 cells increased the expression of both CYP3A4 and CYP3A5 (Figure 3a), while overexpression of AC069294.1 decreased the expression of CYP3A4 and CYP3A5 (Figure 3b). These results suggest that AC069294.1 is a negative regulator for both CYP3A4 and CYP3A5, possibly acting through a *trans*-acting mechanism. Consistently, the CYP3A4*1G allele, which is located within an enhancer region upstream of AC069294 and has increased transcriptional activity in reporter gene assays [25, 26], is associated with increased expression of AC069294.1 (Table 1, Figure 4), and decreased expression of CYP3A4 and CYP3A5 (Table 1). Taken together, our results demonstrate a novel mechanism regulating the expression of both CYP3A4 and CYP3A5. In addition, our results suggest a potential explanation underlying observed CYP3A4*1G effects, namely through its up-regulation of AC069294.1, which, in turn acts to decrease expression of CYP3A4 and CYP3A5.

The role of CYP3A4*1G in regulating CYP3A4 is contentious. Our results agree with the previous study showing that *1G is associated with reduced expression of CYP3A4 [15]. Moreover, RNAseq data from GTEx (Genotype Tissue Expression project) [28] also showed that *1G is associated with decreased expression of CYP3A4 in the esophagus mucosa (normalized effect size = −0.29, p=0.000014, GTEx portal). However, our data suggests that the effect is indirect and is mediated by AC069294.1. This is consistent with our previous result showing CYP3A4*1G does not show allelic RNA expression imbalance of CYP3A4 in CYP3A4*1G heterozygous liver samples, indicating CYP3A4*1G does not have direct cis-acting regulatory effect on CYP3A4 expression [6]. The previously reported increased enhancer activity of CYP3A4*1G [25, 26] may alternatively promote transcription of AC069294.1, which then decreases expression of CYP3A4This effect appears to be *in trans*, as AC069294.1 also regulates the expression of CYP3A5 (see further discussion below). This additional layer of CYP3A4 regulation agrees with the numerous clinical association studies detailing the decreased activity of CYP3A4 in *1G carriers for those drugs mainly metabolized by CYP3A4 [15-23].

Conversely, the effect of CYP3A4*1G on drugs metabolized by CYP3A5 or both CYP3A4 and CYP3A5 is confounded by its high linkage with the functional CYP3A5*1 allele. Our results showed that CYP3A4*1G is also associated with decreased expression of CYP3A5. This is in contrast to clinical association studies showing increased CYP3A4*1G activity towards tacrolimus, a substrate for both CYP3A4 and CYP3A5 [11-14]. This discrepancy is likely caused by the relatively small effect of CYP3A4*1G on expression of CYP3A4

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and CYP3A5 (30-40% decrease) compared to the large effect of CYP3A5*1 on CYP3A5 expression, shown to increase CYP3A5 expression levels comparable to that of CYP3A4 [33]. Thus, the overall CYP3A metabolism toward tacrolimus in individuals carrying both CYP3A5*1 and CYP3A4*1G is still greater than individuals with the more common CYP3A5*3 allele. However, it should be noted that the coexistence of CYP3A4*1G on CYP3A5*1 allele may decrease the expression of CYP3A5 compared to those without CYP3A4*1G, and hence, may explain additional variability related to CYP3A5 metabolism.

Our results are inconsistent with a recent report by Fohner et al showing increased CYP3A4 activity associated with CYP3A4*1Gin American Indian and Alaska Native (AIAN) populations [34]. However, the discrepancy may be caused by the difficulty in differentiating CYP3A5 and CYP3A4 protein expression and activity due to their high sequence similarity and substrate affinities (including Luciferin-IPA used in Fohner et al. (2021)). This is especially true in CYP3A5*1 carriers, which express high levels of CYP3A5 [33], and that are likely to also carry CYP3A4*1G, due to the high linkage of these two SNPs. We also cannot exclude population-specific factors that may promote different CYP3A4*1G effects or cause alternate expression and/or regulation of AC069294.1 between our study and Fohner et al. (2021). Due to the complex interplay between CYP3A4 and CYP3A5, the clinical significance of CYP3A4*1G on CYP3A metabolism warrants further investigation.

The negative relationship between the expression levels of AC069294.1 and both CYP3A4 and CYP3A5 indicates that the effects of AC069294.1 may be in *trans* rather than in *cis*, as they are not restricted to the CYP3A4 locus. *trans*-lncRNAs can positively or negatively affect gene expression and can exert these effects both pre- and post-transcription [35]. AC069294.1 appears to negatively regulate CYP3A4 and CYP3A5 mRNA levels, but not CYP3A7 or CYP3A43. In addition to its complementarity with the CYP3A4 sequence, AC069294.1 also shows complementarity within intron eight of both CYP3A5 and CYP3A7. Despite this, knock-down of AC069294.1 did not alter expression of CYP3A7, suggesting that AC069294.1 may not regulate its targets through sequence-specificity alone, which is not uncommon for lncRNAs [35]. However, overexpression of AC069294.1 did decrease expression of CYP3A7, which indicates that this lncRNA has the potential to regulate CYP3A7, but with reduced efficiency compared to CYP3A4 and CYP3A5. Overall, these results indicate a common regulatory mechanism underlying CYP3A4 and CYP3A5 expression, one that under normal circumstances does not extend to CYP3A7, which is a fetal form of the CYP3A genes. Future experiments detailing the mechanism underlying the effect of AC069294.1 are warranted, as they will provide further insight into regulation of the CYP3A locus.

In summary, we demonstrated the regulation of CYP3A4 and CYP3A5 by a novel antisense lncRNA AC069294.1 located within CYP3A4 and provided potential mechanisms underlying the observed effects of CYP3A4*1G. These results explain some of the conflicting clinical associations of CYP3A4*1G and suggest including CYP3A4*1G as an additional factor regulating CYP3A5*1 expression in future clinical association studies.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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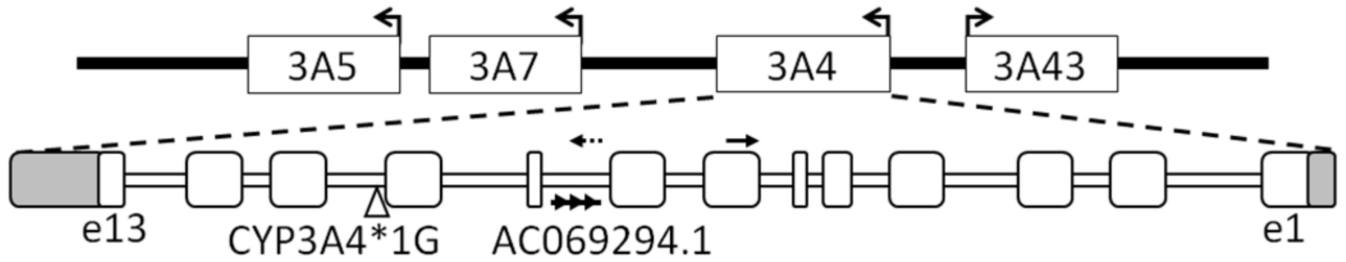

Figure 1. Diagram of the CYP3A locus.

The CYP3A locus contains four genes: CYP3A5, CYP3A7, CYP3A4, and CYP3A43. Gene orientation and the transcription start site are indicated. The expanded CYP3A4 diagram illustrates the location of its exons (right to left, e1 to e13) and the locations of CYP3A4*1G and AC069294.1. The location of the gene specific primers used for cDNA synthesis as shown in Figure 4 and Supplemental Figure 1 are indicated: dashed arrow, AC069294.1; solid arrow, CYP3A genes.

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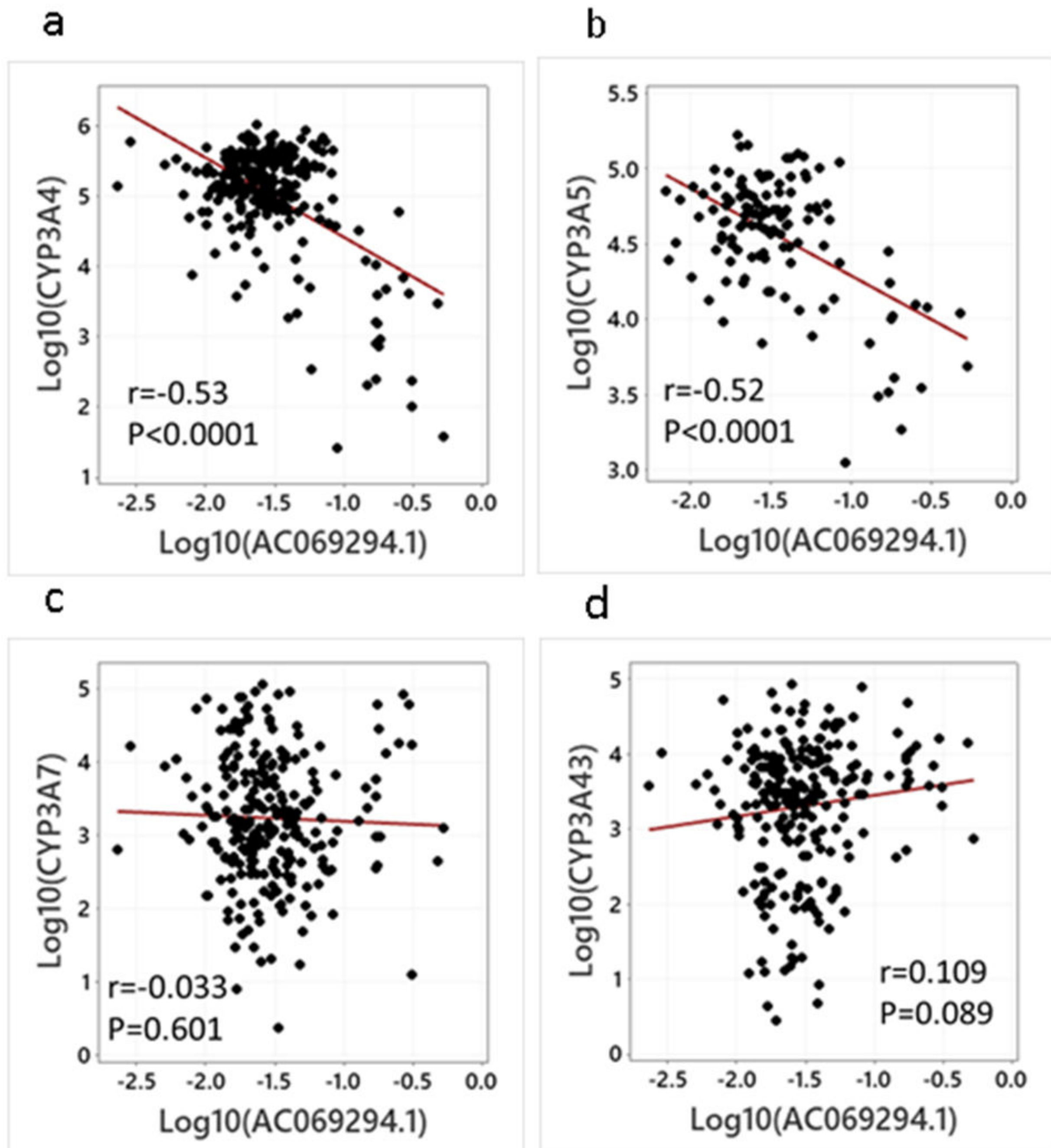

Figure 2. Correlation between expression of AC069294.1 and the CYP3A genes) in liver samples. (a) CYP3A4, (b) CYP3A5, (c) CYP3A7, and (d) CYP3A43.

Quantitative real-time PCR (qRTPCR) was used to measure gene expression of the CYP3A genes and AC069294.1. The level of AC069294.1 was normalized by the expression of CYP3A4 heteronuclear RNA measured with primers targeting intron 11. Pearson correlation results are inset on the graph. Note: Only CYP3A5*1 carriers (n=124) were included panel b.

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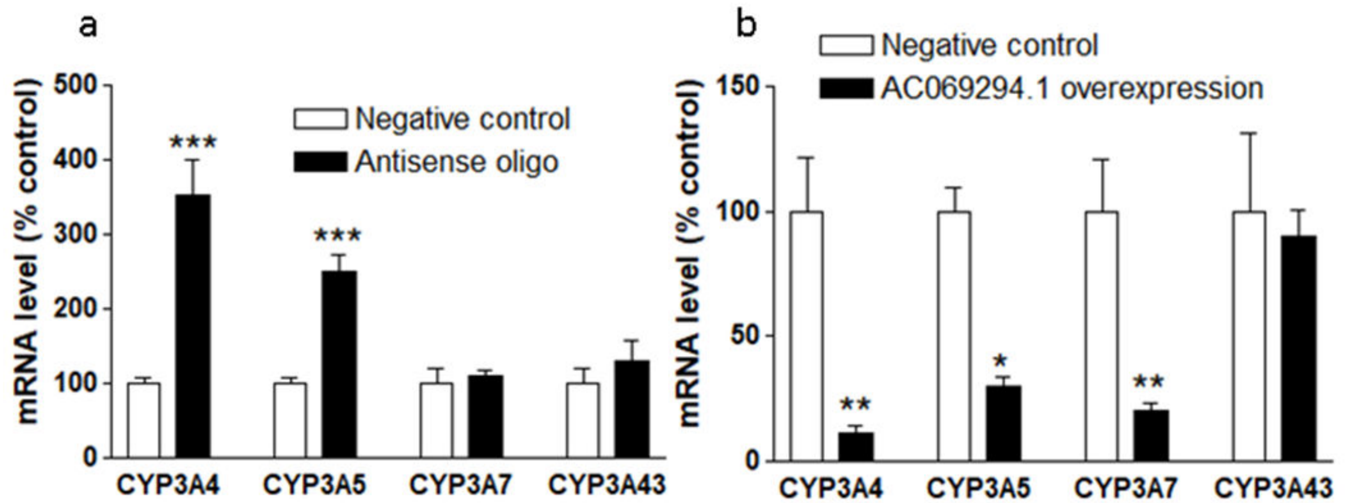

Figure 3. The effect of AC069294.1 knockdown (a) or overexpression (b) on expression of the CYP3As in Huh7 cells.

(a) AC069294.1 was knocked-down using 2′-deoxy-2′-fluoro-arabinoguanosine (FANA) antisense oligonucleotides (ASO), expression is shown as fold-change relative to the negative control ASO that does not have a target in the human genome. (b) AC069294.1 was overexpressed using plasmid DNA transfection, expression is shown as fold-change relative to the negative control, which was an empty vector. Bar graphs are the average of 3-4 biological replicates, each measured in duplicate, with standard deviation shown as error bars. *$P<0.05$; **$P<0.01$, *** $P<0.001$, ANOVA with Bonferroni post-test, compared to the negative control.

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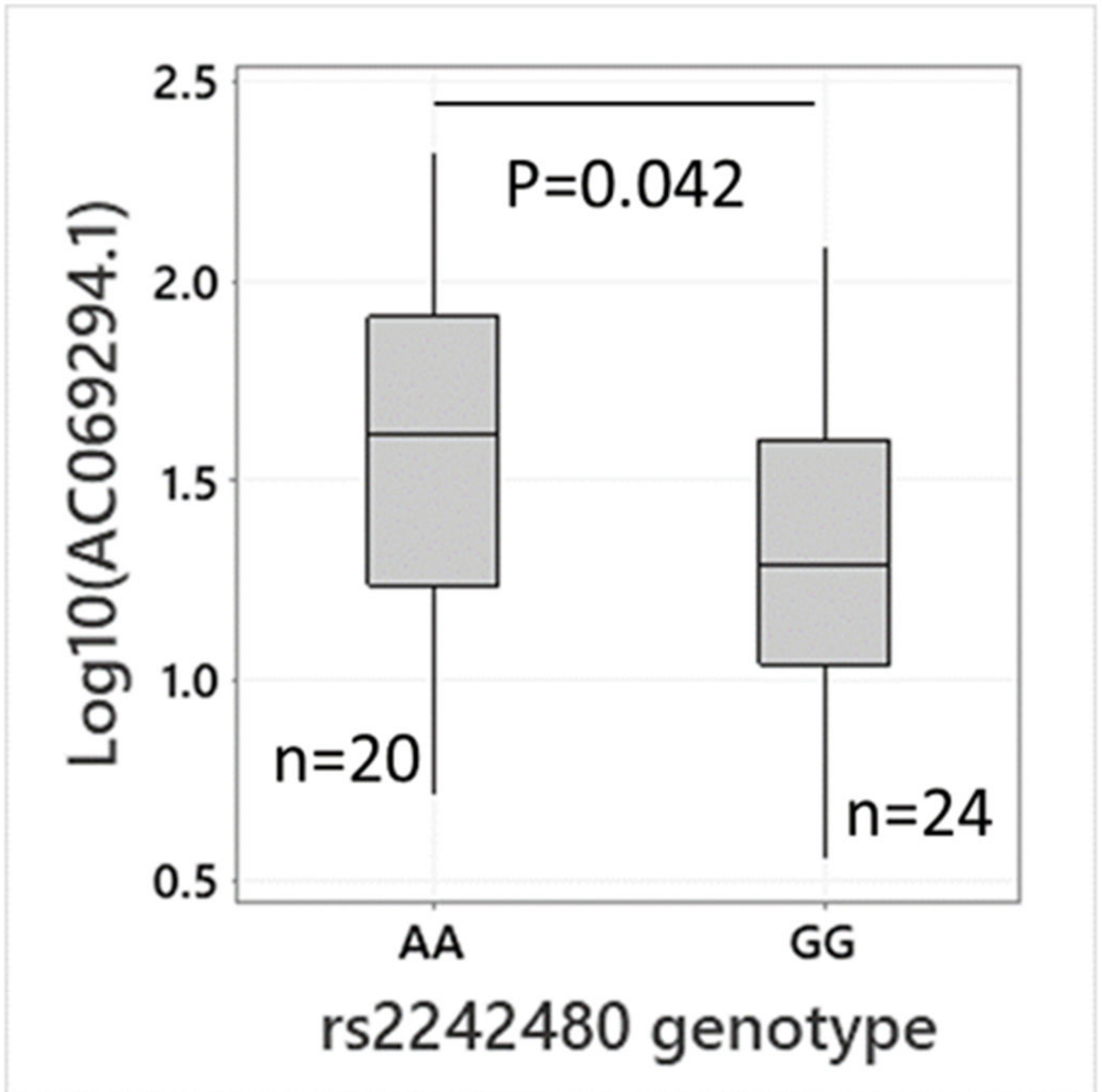

Figure 4. Expression level of AC069294.1 in liver samples grouped by rs2242480 genotype.
cDNA was synthesized using gene specific primers and expression level of AC069294.1 was normalized by β-actin.

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Table 1.

Association between CYP3A4*1G and the expression of AC069294.1 and the CYP3A genes in the 246-sample cohort.

Gene	beta	p-value	R^2
AC069294.1	0.101	<0.0001	5.34%
CYP3A4	−0.1609	0.008	2.86%
CYP3A5	−0.2111	0.004	2.19%
CYP3A7	0.0198	0.772	NA
CYP3A43	−0.0112	0.147	NA