Screening for MicroRNA Regulators of an Orphan Cytochrome P450 4V2 (CYP4V2)

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington

2012

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Program Authorized to Offer Degree:

Pharmaceutics

Introduction

CYP4 Family and CYP4V2 Disease Linkage

Cytochrome P450s are a superfamily of heme-containing monooxygenases that catalyze a number of oxidative metabolic reactions. Humans possess 57 functional P450 genes, many of which have well established roles in the metabolic clearance of drugs and xenobiotics, and in the anabolism and catabolism of endogenous sterols, bile acids, vitamins and fatty acids (Nelson 2009; Sim and Ingelman-Sundberg 2010). Human CYP enzymes are divided among 18 families, and isozymes are included within the same family if they contain greater than or equal to 40% protein sequence homology (Nelson 2009). A subset of thirteen human P450s have been termed 'orphans' because their functional roles have yet to be revealed (Stark and Guengerich 2007). Within this group of orphan P450s, almost half - CYP4A22, CYP4F11, CYP4F22, CYP4V2, CYP4X1 and CYP4Z1 – belong to the CYP4 family of enzymes.

In mammalian species, six CYP4 sub-families exist, comprising a total of 13 distinct CYP4 isoforms in the human (Figure 1). Three of these subfamilies, CYP4A, 4B, and 4F have been extensively studied. In contrast, the functions of four 'orphan' P450s, CYP4F22, CYP4V2, CYP4X1, and CYP4Z1 are just beginning to be explored. They are termed "orphans" because their substrate specificities and physiological roles are largely unknown. CYP4V2 is the most distantly related of the human CYP4 family, possessing only 35% sequence identity to the other family members. By classic definition, CYP4V2 is not a member of the CYP4 family because it does not meet the greater than or equal to 40% sequence homology criteria as defined by Nelson et al. (2009).

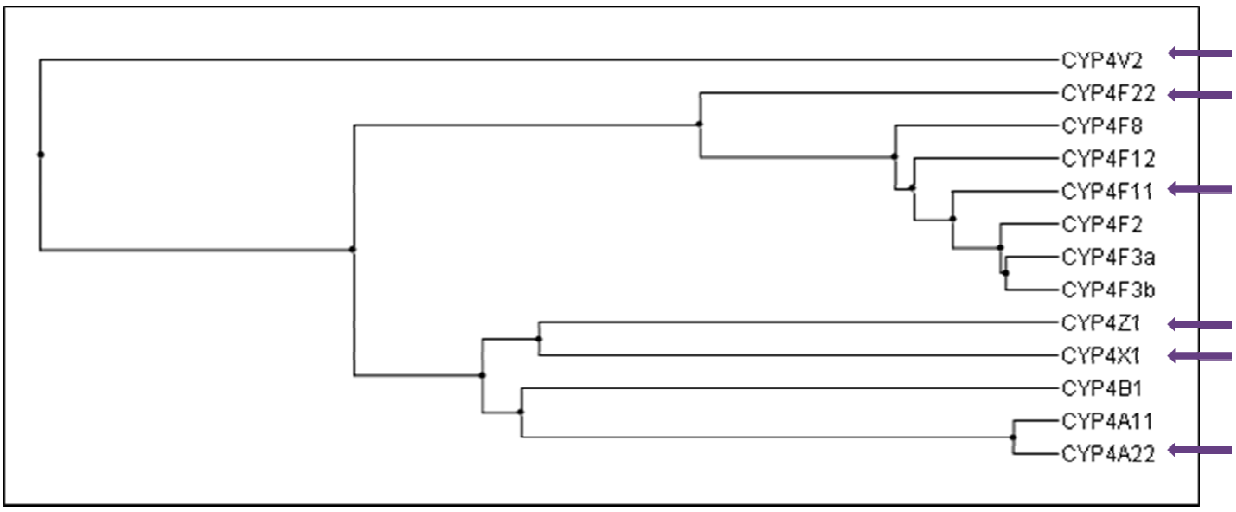

Figure 1. Phylogeny tree of CYP4 family enzymes, with arrows indicating orphan P450s (Rettie & Kelly, 2008).

Enzymes of the CYP4 family are traditionally associated with endogenous fatty acid metabolism (Hsu, Savas et al. 2007; Hardwick 2008; Rettie and Kelly 2008). For example, the leukocyte-specific isozyme CYP4F3A has an essential role in catalyzing the ω-hydroxylation of leukotriene B4, causing inactivation of its chemotactic properties. The polyunsaturated fatty acid (PUFA) arachidonic acid is metabolized by both CYP4A11 and CYP4F2 to 20-HETE, and these enzymes' relatively high expression in the kidney has stimulated much pharmacogenetically-based research investigating the potential roles of CYP4A11 and CYP4F2 in hypertension. One function of 20-HETE is to regulate vascular tone by inhibiting the Ca^{2+}-activated K^+ channel in vascular smooth muscle cells of the renal micro-circulation. Inhibition of the K^+ channel induces vasoconstriction of the kidney microvasculature which increases blood pressure. 20-HETE also functions in the kidney to regulate salt and water reabsorption by inhibiting

the Na^+/K^+ ATPase and Na^+/K^+ $2Cl^-$ co-transporter in the proximal tubule and thick ascending limb of the loop of Henle. Inhibition of the Na^+/K^+ ATPase and Na^+/K^+ $2Cl^-$ co-transporter prevents sodium reabsorption, thus lowering blood pressure (Kauser, Clark et al. 1991; Escalante, Erlij et al. 1994; Imig, Zou et al. 1994; Ribeiro, Dubay et al. 1994; Imig, Zou et al. 1996; Nowicki, Chen et al. 1997; Amlal, LeGoff et al. 1998; Alonso-Galicia, Falck et al. 1999). Gainer et al. reported a relatively common polymorphism in CYP4A11, a T-to-C transition at nucleotide 8590 which results in a phenylalanine-to-serine substitution at amino acid 434 and has an allele frequency of approximately 9% in Europeans and 35% in African Americans (Gainer, Bellamine et al. 2005). This single nucleotide (SNP) was associated with an increased risk of hypertension. The T8590C variant significantly reduced the catalytic activity of CYP4A11 and 20-HETE production to about 50% of the wild type enzyme, suggesting that the predominant role of CYP4A11 is to mediate sodium reabsorption.

In contrast to the association of CYP4A11 variants and hypertension, genetic mutations in CYP4V2 are linked to Bietti's Crystalline Dystrophy (BCD), an ocular disease (Li, Jiao et al. 2004). The *CYP4V2* gene comprises 11 exons, spanning 19 kB and encodes a protein consisting of 525 amino acids. Similar to other members of the CYP4 family, despite low sequence homology to other members, it functions as a fatty acid oxidase, with preferential activity for ω-hydroxylation of saturated, medium-chain fatty acids (Nakano, Kelly et al. 2009). Homology modeling predicts the CYP4V2 structure to contain a transmembrane segment located near the amino terminus with a globular structural domain typical of cytochrome P450 enzymes. The globular domain

of CYP4V2 comprises 18 α-helices and β-structural elements (Li, Jiao et al. 2004). Mutations in CYP4V2 is the only known cause of BCD.

Bietti's Crystalline Dystrophy is an inherited, chorioretinal degeneration, characterized by the presence of yellow-white crystals and/or complex lipid deposits in the retina (Figure 2). The typical age of onset is during the second to third decade of life, but it can range from the early teenage years to beyond the third decade. The progressive atrophy and degeneration of the retinal pigmented epithelium (RPE)/choroid lead to reduced visual acuity, poor night vision, abnormal retinal electrophysiology, and visual field constriction. Persons with BCD may also have impaired color vision (Kaiser-Kupfer, Chan et al. 1994; Lee 2005). A recent study involving 21 families with BCD showed visual acuities ranging from normal to lack of perception of hand motion (Xiao, Mai et al. 2011). This range of clinical phenotypes suggests that other factors (modifier genes, environment, lifestyle, etc.) may play a role in disease pathology.

While BCD is generally considered to be a rare disease, it is potentially underdiagnosed. For example, in a study done by Mataftsi et al., approximately 10% of persons with autosomal recessive retinitis pigmentosa were also diagnosed with BCD (Mataftsi, Zografos et al. 2004). According to Hartong et al., worldwide prevalence of retinitis pigmentosa was 1 in 4000, with autosomal recessive retinitis pigmentosa accounting for 50-60% of the cases (Hartong, Berson et al. 2006). This suggests prevalence of BCD can be up to 1:66,660 to 80,000. Prevalence of BCD appears to be higher in people of East Asian descent, particularly in Chinese and Japanese, but cases of BCD have also been reported in other populations including Lebanese, Mexicans,

(Lin, Nishiguchi et al. 2005; Lai, Ng et al. 2007; Zenteno, Ayala-Ramirez et al. 2008) and Europeans, presenting with retinitis pigmentosa (Mataftsi, Zografos et al. 2004).

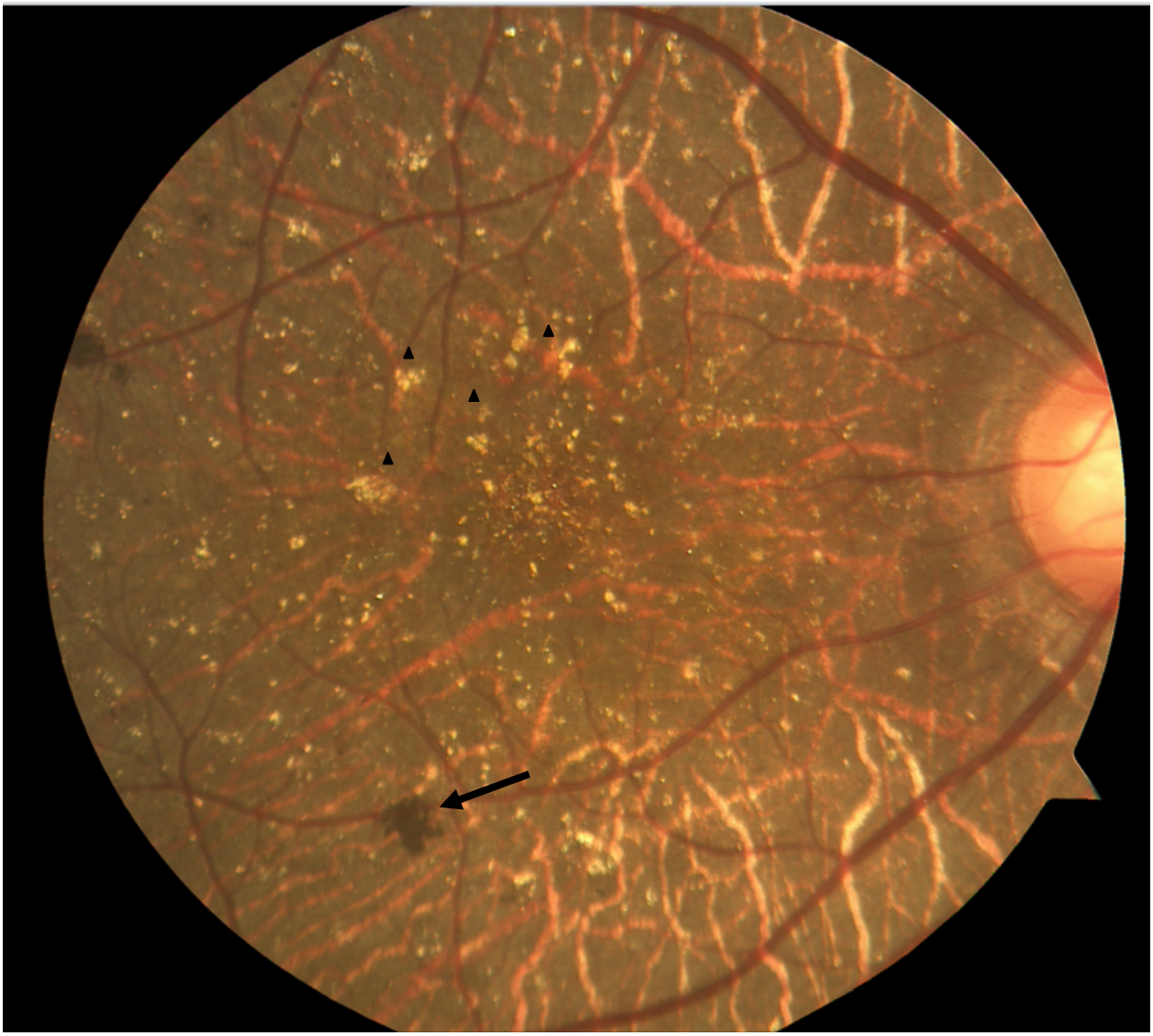

Figure 2. Fundus photograph of a Bietti's patient illustrating typical yellow crystalline deposits (arrowheads) and pigment clumping (arrow) (courtesy of Dr. Richard Weleber, OHSU).

In 2000, genetic linkage analysis identified the human chromosome locus 4q35 to be associated with BCD (Jiao, Munier et al. 2000). Fine-mapping of this locus revealed *CYP4V2* to be the likely causative gene as exonic and intronic mutations in *CYP4V2* were identified in 23 BCD patients (Li, Jiao et al. 2004). Subsequently, many investigators have corroborated this finding (Gekka, Hayashi et al. 2005; Lee, Koh et al. 2005; Lin, Nishiguchi et al. 2005; Shan, Dong et al. 2005; Wada, Itabashi et al. 2005; Jin, Ito et al. 2006; Lai, Ng et al. 2007; Zenteno, Ayala-Ramirez et al. 2008). To date, greater than twenty discrete *CYP4V2* mutations have been described in BCD. At least one mutation has been reported in each of the 11 exons (Figure 3).

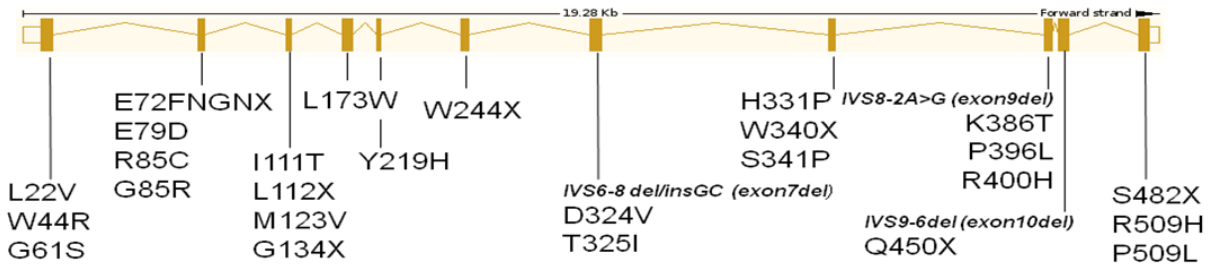

Figure 3. Mapping of CYP4V2 mutations found in Bietti's patients compiled from 10 separate studies (Kelly, Nakano et al. 2011).

The most frequent *CYP4V2* mutation found in affected individuals, c.800-1_8delTCATACAG_800_808delGTCATCGCGinsGC, results in the skipping of exon 7. Eight nucleotides of the 3' end of intron 6 and nine from the 5' end of exon 7 are deleted and GC is inserted (Kaiser-Kupfer, Chan et al. 1994; Lee, Jiao et al. 1998; Lee, Jiao et al. 2001; Lewis 2004; Shan, Dong et al. 2005; Baer and Rettie 2006; Hardwick 2008).

Two other deletions c.1091-1A>G and c.1226-6delTGACAG_1226_1235delCAGGTTACAG (a deletion spanning the splice acceptor site for exon 9) result in skipping of exons 9 and 10, respectively (Li et al. 2004; Shan et al. 2005). Six nonsense mutations and 23 missense mutations have also been described (Xiao, Mai et al. 2011; Li, Jiao et al. 2004; Lee, Koh et al. 2005; Lin, Nishiguchi et al. 2005; Shan, Dong et al. 2005; Wada, Itabashi et al. 2005; Lai, Ng et al. 2007; Hardwick 2008; Zenteno, Ayala-Ramirez et al. 2008).

There is currently no published data with direct evidence that abnormal gene products of *CYP4V2* cause disease through loss of function. However, expression of the H331P variant in SF9 cells resulted in protein that failed to incorporate heme and lacked fatty acid ω-hydroxylase activity (Nakano et al., manuscript submitted). Mutations resulting from exon 7, 9, and 10 skipping and exon 2 splicing are predicted to result in premature termination of the protein and are expected to cause gross structural changes in any translated protein with resulting loss of enzyme activity. Similarly, based on conservation across other CYP4 enzymes and homology modeling, several amino acid deletions and substitutions would be predicted to negatively affect CYP4V2 activity (Li, Jiao et al. 2004). Exon-skipping mutations are predicted to lead to more severe forms of the disease compared to missense mutations that lead to amino acid substitutions (Li, Jiao et al. 2004; Lai, Ng et al. 2007).

In an effort to gain a better understanding of how these coding-region variants may be affecting protein structure and function, structural modeling of human CYP4V2 was previously done in our group (Kelly, Nakano et al. 2011) using the Robetta server software (Kim, Chivian et al. 2004; Chivian and Baker 2006). We used a template of a

1.2 Å resolution x-ray structure of cytochrome P450 BM3 (pdb id: 2IJ2) from *Bacillus megaterium* (Girvan, Seward et al. 2007). This analysis revealed that the coding-region mutation sites in CYP4V2 associated with BCD are distributed throughout the enzyme's tertiary structure. Two amino acids, L22 and W44, are located in the predicted N-terminal membrane anchor, whereas others (I111, M123, L173, T325, H331, S341, and R400) are located inside the hydrophobic center of the enzyme towards the heme group, and the rest are located on the solvent-facing portion of the protein (Figure 4). This wide distribution suggests that coding-region mutations that impact CYP4V2 activity may do so through various mechanisms.

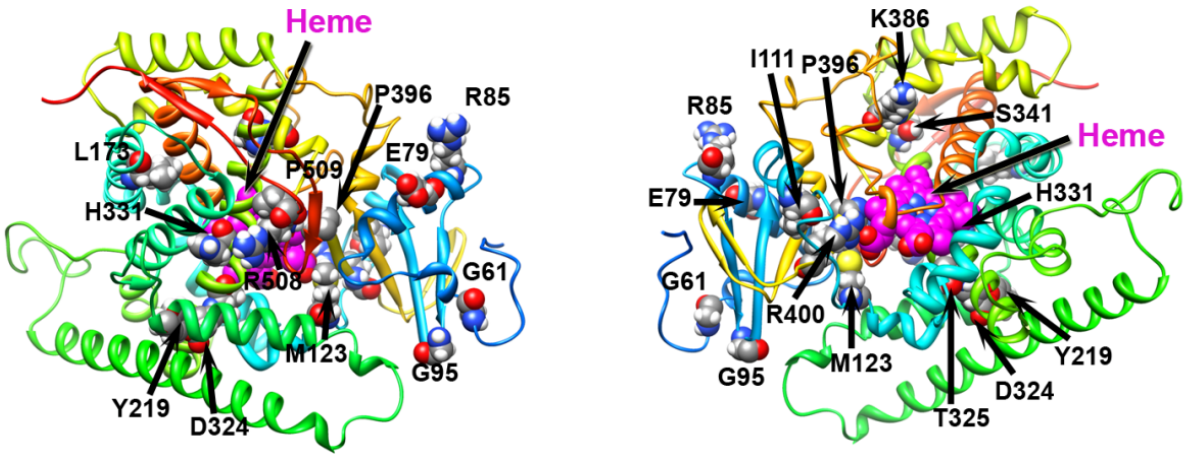

Figure 4. Homology Models of CYP4V2. The residues highlighted are those that are sites of mutation in CYP4V2-BCD (Kelly, Nakano et al. 2011).

In addition to its clear association with BCD, a common, non-synonymous polymorphism in CYP4V2 (rs13146272, K259Q), has been cited as a risk factor in deep

venous thromboembolism (Bezemer, Bare et al. 2008; Li, Bezemer et al. 2009; Tregouet, Heath et al. 2009). The association has been validated in a recent meta-analysis of five replication cohorts (Austin, De Staercke et al. 2011). K259Q is in linkage disequilibrium with SNPs in the Factor XI (F11) and pre-kallikrein genes (KLKB1) which are known to be involved in coagulation. Initially, this was thought to explain, at least in part, the observed association of rs13146272 with deep venous thromboembolism. However, more detailed genetic analysis of this locus indicated that subjects possessing the A-C-T haplotype corresponding to CYP4V2 rs13146272, F11 rs2036914, F11 rs2289252 were at highest risk of deep venous thromboembolism; OR of 1.55-1.57 (Li, Bezemer et al. 2009), indicating a modulating influence of the CYP4V2 SNP on the two more directly acting F11 polymorphisms.

CYP4V2 and Lipid Homeostasis

Though the biochemical basis of BCD remains unknown, findings suggest that the disease may result from a systemic abnormality in lipid metabolism. Studies by Lee et al. identified deficiency of a 32-kDa fatty-acid binding protein with a high affinity for the fatty acids docosahexaenoic acid (DHA; 22:6n-3), α-linolenic acid (18:3n-3), and palmitic acid (16:0) in the lymphocytes of patients compared to controls (Lee, Jiao et al. 1998). Fibroblasts and lymphocytes cultured from individuals with BCD exhibited altered lipid metabolism, with decreased synthesis of ω-3 polyunsaturated fatty acids (PUFAs) (e.g., eicosapentaenoic acid (EPA; 20:5n-3) and DHA) from α-linolenic acid compared to controls (Lee, Jiao et al. 2001).

More recently, free fatty acid profiling revealed significantly altered fatty acid concentrations in the serum of BCD patients compared to controls. Specifically, stearic acid (18:0) was elevated and oleic acid (18:1n-9) was decreased in persons with BCD compared to controls (Lai, Chu et al. 2009). Further support that BCD is a systemic dyslipidemia, and not just an ocular disease, is provided by studies using transmission electron microscopy to show the presence of crystalline material of unknown lipid composition in lymphoblasts and fibroblasts of afflicted patients (Welch 1977).

CYP4V2 and Polyunsaturated Fatty Acid, Docosahexaenoic Acid

Photoreceptor tips have the highest DHA content of any cell (Fliesler and Anderson 1983). Photoreceptor cells efficiently recycle lipids to regenerate disk membranes, with approximately ten percent of the photoreceptor cell outer segment shed daily in primates (Young 1967; Young 1971; Young 1976). DHA may be synthesized from the ω-3 fatty acid, α-linolenic acid (ALA). Fatty acid ALA is converted to DHA in the liver in a seven-step process, involving various desaturase and elongase activities with subsequent peroxisomal oxidation (Figure 5). However, this is a limited capacity process, with the majority of DHA coming from dietary sources, such as fish.

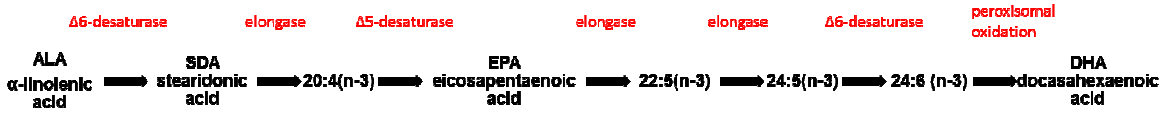

Figure 5. Metabolic scheme converting ALA to DHA.

After DHA enters the systemic circulation from the liver, it is shuttled by fatty acid binding proteins to retinal pigmented epithelial (RPE) cells (Scott and Bazan 1989). Lipids are transferred from RPE cells to rod inner segments for the biosynthesis of disk membranes (Figure 6). Immunohistochemical analysis revealed that CYP4V2 was highly expressed in the ocular tissues, with strong positive staining in RPE cells and moderate positive staining in the corneal epithelium (Nakano et al., manuscript submitted). In these same studies, CYP4V2 was also found to be highly expressed in the human liver, particularly the hepatocytes, as well as reproductive and endocrine organs. Additionally, recombinant CYP4V2 protein was found to metabolize DHA, forming ω and ω-1 hydroxy metabolites. Since RPE cells handle high concentration of PUFAs, they must have a rapid fatty acid-recycling mechanism, possibly involving CYP4V2. CYP4V2-dependent metabolism of PUFAs cycling through RPE cells may be an important mechanism to avoid excessive accumulation of PUFAs. Accumulation of DHA would be undesirable because the high oxygen tension and intense light environment in the eye would be ideal for lipid peroxidation reactions that could damage cells through oxidative stress and/or polymerization to insoluble aggregates that may more globally affect vision. Winkler hypothesized that the constant renewal of rod outer segments serves as a surrogate anti-oxidant because this cellular compartment lacks the key anti-oxidant glutathione (Winkler 2008).

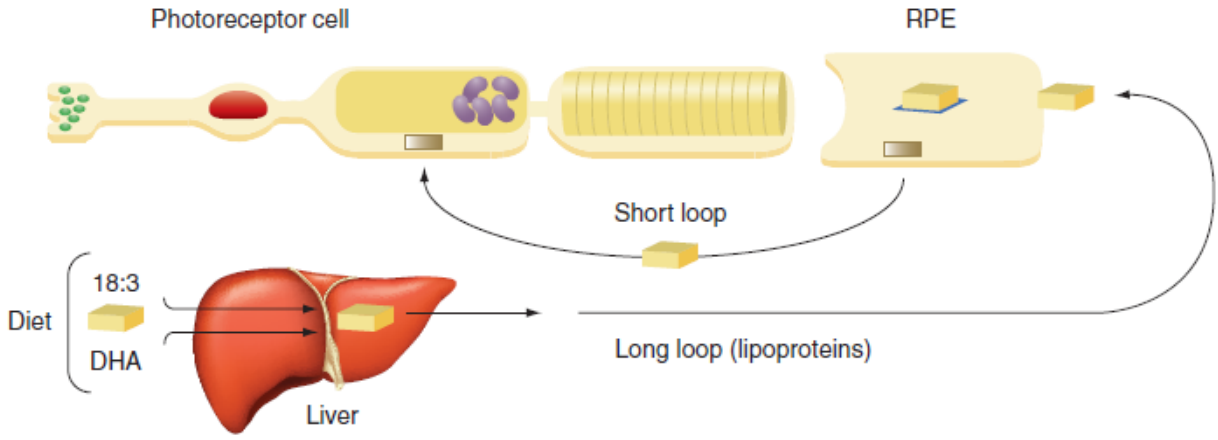

Figure 6. DHA conservation and uptake into photoreceptor cells. Highly efficient mechanisms for DHA utilization and recycling exist among RPE photoreceptor cells (short loop). These mechanisms complement the more significant uptake of dietary DHA from plasma lipoproteins (long loop) (Kelly, Nakano et al. 2011).

CYP4V2 Regulation

Bietti's Crystalline Dystrophy is thought to be an extreme condition resulting from loss of protein function due to CYP4V2 mutations, but there are other conditions of varying pathological severity in which CYP4V2 could be potentially involved. For example, as previously mentioned, rs13146272 (K259Q) has been linked with deep venous thromboembolism occurrence (Bezemer, Bare et al. 2008; Li, Bezemer et al. 2009; Tregouet, Heath et al. 2009; Austin, De Staercke et al. 2011). In addition, expression of CYP4V2 was inversely correlated with tumor grade in breast cancer (Murray, Patimalla et al. 2010). Thus, identifying mechanisms mediating inter-individual variability in CYP4V2 expression in normal populations warrants further exploration. While progress has been made in determining the activity and function of CYP4V2, regulation of its expression has not been examined to date. Many CYP enzymes are subject to transcriptional regulation by nuclear receptors, such as peroxisome

proliferator activated receptors (PPAR), retinoid X receptor (RXR), and others (Chawla, Repa et al. 2001), and these mechanisms have been extensively investigated. Transcriptional regulation of members of the CYP4 family is typically mediated by peroxisome proliferator-activated receptor alpha (PPARα) (Muerhoff, Griffin et al. 1992; Lee, Pineau et al. 1995), although CYP4V2 has not been shown to be regulated in this manner. Western blotting performed in our lab with CD1 mouse liver microsomes (Xenotech) from CD1 mice treated with prototypical inducers beta-napthoflavone and 3-methylcholanthrene (AHR agonists), pregnenolone-16-carbonitrile and dexamethasone (PXR agonists), and clofibrate (PPARα agonist), did not reveal an increase in Cyp4v3 expression, the mouse ortholog of CYP4V2 (Figure 7).

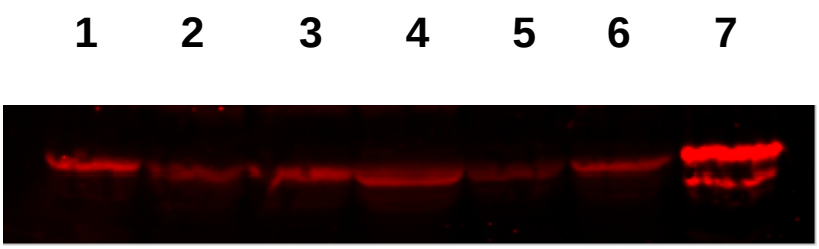

Figure 7. Western blot for Cyp4v3 in mouse liver microsomes (20 ug microsomal protein/lane). Lanes 1-6 are microsomes prepared from mice treated with corn oil, beta-napthoflavone, 3-methylcholanthrene, pregnenolone-16-carbonitrile, dexamethasone, and clofibrate, respectively. Lane 7 contains positive control, Cyp4v3 SF9 microsomes.

Other recent studies have focused on post-transcriptional regulation of P450 enzymes, and there is a growing body of research on the role of microRNAs (miRNAs) in the post-transcriptional regulation of various CYP isoforms (Yokoi and Nakajima 2011). MicroRNAs are a class of small, non-coding RNAs that play key roles in regulating gene expression. They are approximately 22-25 nucleotides long and

typically regulate their target gene by binding complementary regions on the 3'-untranslated region (UTR) of the messenger RNA (mRNA) transcript, causing mRNA degradation or translational repression (Bartel 2004). They have also been reported to interact at the 5' UTR end and within the coding region (Takagi, Nakajima et al. 2010). Currently, 1,424 different miRNAs have been identified in the human genome (miRBase Registry; version 17.0; Griffiths-Jones et al., 2011). MicroRNAs are expressed in a tissue- and/or cell-specific manner and are predicted to target approximately 60% of the human transcriptome (Friedman, Farh et al. 2009).

Processing of primary miRNA transcripts (pri-miRNA) into mature miRNAs requires a series of steps. Pri-miRNAs are 200-300 nucleotides in length and are transcribed from their respective genes by RNA polymerase III. Pri-miRNAs have a 5' cap and a 3' polyA tail (Gregory, Yan et al. 2004). The pri-miRNA transcripts are cleaved into 70- to 80-nucleotide precursor miRNA (pre-miRNA) hairpins by a protein complex in the cell nucleus containing RNase III enzyme (Drosha) and double stranded RNA-binding protein, Pasha (Gangaraju and Lin 2009; Kaikkonen, Lam et al. 2011). The pre-miRNA transcripts are then transported into the cytoplasm by exportin 5 and RanGTP where they are further processed by the RNase III enzyme Dicer into 19-25 nucleotide miRNA duplexes. One strand is degraded (the passenger strand, typically named miRNA*) while the other strand is used as the mature miRNA. The single strand mature miRNA is selectively incorporated into the RNA-induced silencing complex (RISC), which is comprised of RNase III Dicer, TAR RNA-binding protein and Argonaute protein Ago 2. RISC guides the miRNA in binding its complementary region on the 3'-UTR of its target mRNA with imperfect pairing, leading to mRNA decay or repression of

its translation (Ingelman-Sundberg and Rodriguez-Antona 2005; Gangaraju and Lin 2009).

As discussed, more than half of human mRNAs are predicted to be targets of microRNAs (Friedman, Farh et al. 2009). MicroRNAs are implicated in the regulation of a number of biological processes, such as development, cell differentiation, apoptosis, as well as contributing to the pathogenesis of human disease (Kloosterman and Plasterk 2006; Carthew and Sontheimer 2009; Krol, Loedige et al. 2010). MicroRNAs may also regulate drug metabolizing enzymes by acting directly on their transcripts or indirectly by targeting factors, such as nuclear receptors and transcription factors, that regulate drug metabolizing enzymes (Yokoi and Nakajima 2011). Research efforts on the role of microRNA regulation of genes involved in drug/xenobiotic absorption, distribution, metabolism, and excretion is rapidly expanding, including their impact on cytochrome P450 function.

In a study done in 2006, Tsuchiya et al. identified the first CYP to be subjected to regulation by microRNA (Tsuchiya 2006). This isozyme, human CYP1B1 is found primarily in the eye, ovary, uterus, and breast tissues (Sutter, Tang et al. 1994; Shimada, Hayes et al. 1996) and is responsible for the metabolic activation of many procarcinogens (Hayes, Spink et al. 1996; Spink, Spink et al. 1997; Lee, Cai et al. 2003). CYP1B1 is also highly expressed in malignant cancer tissues, such as breast cancer, relative to healthy tissues (Murray, Taylor et al. 1997). This difference between healthy and cancerous tissues was found with CYP1B1 protein levels but not CYP1B1 mRNA levels. This lack of correlation between protein and mRNA expression suggested potential post-transcriptional regulation of CYP1B1. Notably, CYP1B1

mRNA possesses an extremely long 3'-UTR, approximately 3 kb in length, suggesting the possibility of miRNA binding and regulation. Using the miRNA registry release 7.1 database, Tsuchiya et al. discovered a near-matching sequence between microRNA 27b (miR-27b) and the 3'-UTR of CYP1B1 in a region termed the miR-27b recognition element (MRE27b). *In vitro* studies using luciferase reporter assays demonstrated that the reporter activity of the plasmid containing the MRE27b was decreased in MCF-7 cells, a breast cancer cell line which are miR-27 positive but not in Jurkat cells, which are miR-27 negative. This evidence suggests that human CYP1B1 is post-transcriptionally regulated by miR-27b and that decreased levels of miR-27b may be the cause for higher CY1B1 content in cancerous tissues.

Interestingly, CYP1B1 is similar to CYP4V2 in that it is expressed in ocular tissues (Doshi, Marcus et al. 2006). Like CYP4V2, mutations of CYP1B1 are associated with a heritable ocular disease, primary congenital glaucoma (Stoilov, Akarsu et al. 1997; Bejjani, Stockton et al. 2000). Currently, no research has yet been published examining the effects of miR-27b on CYP1B1 protein in the eye.

Another P450 subject to miRNA epigenetic regulation is CYP2E1 (Mohri, Nakajima et al. 2010). CYP2E1 is a pharmacologically and toxicologically important CYP isoform, responsible for the metabolism of a number of xenobiotics, including acetaminophen and ethanol (Lu and Cederbaum 2008). CYP2E1 is highly expressed in the human liver as the most abundant P450 (56% of total P450) at the mRNA level (Bieche, Narjoz et al. 2007) and fourth highest (7% of total P450) at the protein level (Shimada, Yamazaki et al. 1994). *In silico* predictions identified complementary sequences between microRNA 378 (miR-378) and a region in the 3'-UTR of CYP2E1

mRNA, termed the recognition element of miR-378 (MRE378). Luciferase reporter assays in HEK293 cells demonstrated that MRE378 was recognized by miR-378 (Mohri, Nakajima et al. 2010). In HEK293 cells, transfection with precursor miR-78 caused a decrease in CYP2E1 protein level and activity in cells that contained CYP2E1 with the 3'-UTR, but not in cells that contained CYP2E1 without the 3'-UTR. Mohri et al. observed an inverse correlation between CYP2E1 protein and mRNA in 25 human livers ($r^2 = 0.55$, $p<0.05$), an inverse relationship between miR-378 levels and CYP2E1 protein levels ($r^2 = 0.47$, $p<0.05$) and an inverse relationship between miR-378 levels and the translational efficiency of CYP2E1 ($r^2 = 0.53$, $p<0.01$). These observations confirmed the involvement of miR-378 on CYP2E1 expression through post-transcriptional regulation.

Similar to CYP1B1, CYP4V2 stands out with regard to the length of the transcript 3' UTR, extending over 2.8 kb compared to 1.6 kb of coding sequence (Figure 8). This led to our hypothesis that CYP4V2 may also be subject to epigenetic regulation by microRNAs, which could account for the variability in CYP4V2 expression found among healthy individuals. The aim of our project was to identify microRNA regulators of CYP4V2 through microRNA microarray analysis of human liver samples.

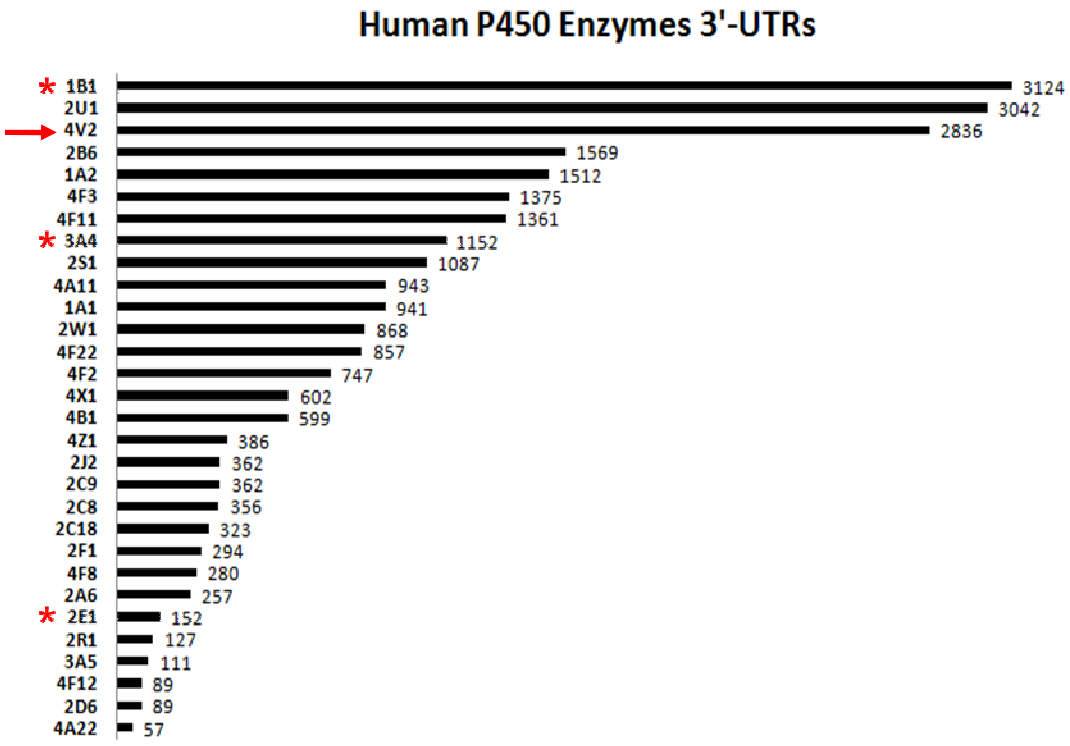

Figure 8. Length of 3'-UTRs of human P450 enzymes in families 1-4 (* indicates P450s known to be regulated by miRNAs).

Materials and Methods

Human Liver Samples:

Human liver samples (n = 55) were obtained from the University of Washington School of Pharmacy Human Liver Tissue Bank (Seattle, WA). Donor demographics are shown in Table 1. Human liver microsomes were prepared as described by Paine et al. (1997). Total mRNA from the human livers was extracted and normalized to 200 ng/uL. Whole genome expression measurement was carried out using the Illumina HumanRef08 v.2 Expression BeadChips according to the manufacturer's protocol. All samples were measured in duplicate and each sample and replicate was randomized between processed batches of 24 arrays analyzed on different days. Raw signal intensity measurements were processed using the Ilumina BeadStudio software v.2.3.41 using the 'average' normalization function and replicate data from each liver sample was averaged to obtain a final value. Use of the samples was classified as non-human subjects research by the University of Washington Human Subjects Division.

Table 1: Demographics of Human Liver Donors

Characteristic	
Age Range (years)	7 - 70
Sex	**N**
Male	29
Female	26
Ethnicity	
Caucasian	52
Asian	1
Black (non-Hispanic)	2

MicroRNA Microarray:

Total RNA (containing the miRNA fraction) was isolated using the miRNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's established protocol. The quality of the total RNA was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). Only samples with high integrity and adequate quantity were used for further analysis. Initial miRNA microarray analysis was performed on six livers with the highest CYP4V2 mRNA levels and six livers with the lowest CYP4V2 mRNA levels, to determine differential expression of miRNAs between the two groups. The entire liver bank was subsequently analyzed by miRNA microarray to validate potential miRNA regulators identified in initial study.

The Functional Genomics and Proteomic Core Laboratory used the GeneChip miRNA 2.0 Array (Affymetrix Inc., Santa Clara, CA) to measure levels of miRNA expression, following the manufacturer's established protocol to process the total RNA samples and arrays using the FlashTag Biotin HSR kit (Genisphere LLC., Hatfield, PA). Briefly, samples were processed with a poly(A) tailing reaction followed by ligation of the biotinylated signal molecule containing Genisphere's proprietary 3DNA dendrimer signal amplification technology to the target RNA sample. Samples were then hybridized to the Affymetrix array, followed by washing, staining and scanning of the arrays which were carried out using the manufacturer's established protocols.

Antibody production and assessment of cross-reactivity:

Anti-CYP4V2 antibody production was carried out by R&R Research (Stanwood, WA) using purified CYP4V2 as the antigen. Briefly, two rabbits were immunized with the purified CYP4V2 protein supplemented with complete adjuvant along with 3 boosts (1 mg total). Twelve weeks after initial immunization, the animals were sacrificed and sera isolated. Polyclonal IgG was isolated from the crude sera of pre- and post-immunized animals following fractionation with a saturated ammonium sulfate solution. The cross reactivity of the anti-CYP4V2 IgG towards other CYP4 enzymes (Supersomes® and purified CYP4B1 expressed in E.coli) was determined from Western blots of 0.2 pmol of purified CYP4V2, 1 pmol of Supersomes® (CYP4A11, CYP4F2, CYP4F3A, CYP4F3B, CYP4F12) and 1 pmol of purified rabbit CYP4B1.

SDS-PAGE and Western Blot Analysis:

The human liver microsomes (20 ug microsomal protein) were mixed with sample buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 20% BME, 0.004% bromophenol blue) and boiled for 5 minutes. Samples were separated on 15% SDS-polyacrylamide gels (100 V for 90 min at room temperature) using running gel buffer (250 mM Trizma base, 200 mM glycine, 3.5 mM SDS) and transferred to nitrocellulose membranes (90 V for 90 min) using cold transfer buffer (25 mM Trizma base, 200 mM glycine, 5 M methanol). Following the transfer, membranes were blocked overnight at 4^0C with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) containing 3% goat serum (Life Technologies, Grand Island, NY). Membranes were incubated with rabbit anti-human CYP4V2 in Odyssey Blocking Buffer (1:3,000) for 1 hour at room temperature

and washed with TPBS (0.3% Tween-20 and 1X phosphate buffered saline) and 50 mM sodium chloride (4X15 min). Following the washes, membranes were incubated with goat anti-rabbit IgG secondary antibody conjugated with IR680 dye (1:30,000; Fisher Scientific, Pittsburgh, PA). After washing with TPBS (4X15 min), the CYP4V2 bands were visualized and quantified using the Odyssey system.

Statistical Analysis:

Linear regression analysis was performed using GraphPad Prism 5.0 to correlate CYP4V2 mRNA and CYP4V2 protein levels and miR-146b-5p expression and CYP4V2 protein levels.

Results

Human Liver Samples:

Human liver tissue samples obtained from the UW Human Liver Bank were taken from anonymous donors ranging from seven to 70 years of age. There was equal representation of female and male donors (47% and 53%, respectively). The majority of the donors were Caucasian. The six liver samples with the lowest and highest CYP4V2 mRNA levels ranged in age from nine to 53 years (3 males and 3 females) and 21 to 59 years (5 males and 1 female), respectively. There were no noticeable trends in current medications, medical history, liver labs, and liver pathology between the two groups (Table 2).

MicroRNA Microarray:

The differential microRNA expression for all 4,560 measured human probe sets on the Agilent microRNA microarray is shown in Figure 9. Group 1 (n=6) consists of human liver samples with the highest CYP4V2 mRNA expression, and Group 2 (n=6) consists of human liver samples with the lowest CYP4V2 mRNA expression as determined by the Illumina mRNA microarray data. Plotted on the x-axis is the signal intensity which indicates the relative miRNA expression, while the y-axis represents the fold-difference in expression between Groups 1 and 2. The miRNAs plotted above the top line are more highly expressed by 1.5-fold in Group 1, the samples with higher CYP4V2 mRNA levels. The miRNAs located below the bottom line are more highly expressed by 1.5-fold in Group 2, the livers with lower CYP4V2 mRNA levels.

Highlighted in red are the four probes for miR-146b. Levels of both hsa-miR-146b-3p and hsa-miR-146b-5p were found to be statistically significantly higher in Group 2 (p-value < 0.05).

Table 2. Donor demographics of liver samples with the lowest and highest CYP4V2 mRNA levels, listed in order of increasing CYP4V2 mRNA.

Group	Liver	Donor Age	Donor Sex	Medications	Medical History	Liver Labs	Liver Pathology	CYP4V2 mRNA
2	138	9 yr	F	dopamine, nipride, RBC, cefazolin	normal	abnormal	acute injury, split liver	607
2	161	53 yr	M	alcohol (1/2 bottle day), dopamine, insulin, clindamycin, cefuroxime	alcoholic, palsy symptoms, psoriasis, sleep apnea	normal	mild fatty	1016
2	120	45 yr	F	prozac, Li, sinequin, codeine, promethazine, dilantin (1 day), dopamine, insulin, gentamycin, mannitol, doxacurium, thyroxine	depression, prior hx of alcohol (> 10 yr ago)	normal	fatty	1135
2	113	9 yr	F	none	normal	normal	normal	1215
2	154	26 yr	M	marijuana, alcohol, dopamine, mannitol, insulin, dilantin (x2 days), phenobarb, clindamycin, zinacef, synthroid, cleocin, zantac	alcoholism, testicular carcinoma	abnormal	mild fatty	1324
2	114	19 yr	M	none	normal	normal	normal	1427
1	163	59 yr	M	unknown	unknown	unknown	fatty	3185
1	158	55 yr	M	nitroprusside, dopamine, insulin, clindamycin, cefuroxime	untreated hypertension	normal	mild fatty	3290
1	145	38 yr	M	cimetidine, diazepam, baclofen, dopamine, epinepherine,bretylium, atropine, succ, lidocaine, cefuroxime, clindamycin	quadriplegia (12 yr), multiple pneumonias	abnormal	fibrotic	3529
1	167	44 yr	M	unknown	unknown	unknown	normal (excellent)	3556
1	133	45 yr	F	dopamine, vasopressin, insulin, clindamycin, ceftriaxone, mannitol, dopamine	normal	normal	fatty	3788
1	102	21 yr	M	none	normal	normal	normal	4239

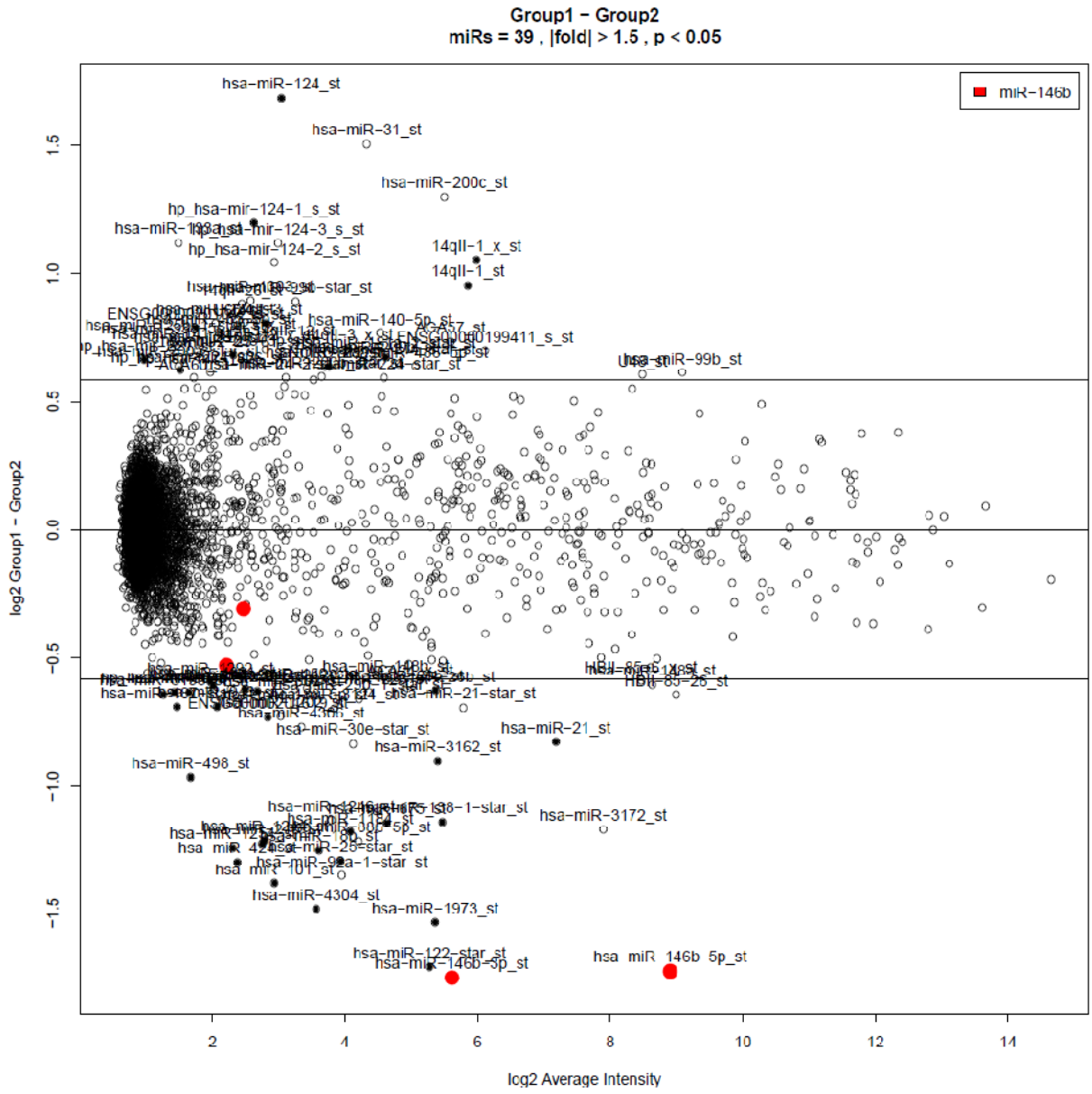

Figure 9. Differential microRNA expression for the livers samples in group 1 (high CYP4V2 mRNA expression, n=6) and group 2 (low CYP4V2 mRNA expression, n=6).

We examined the association of miR-146b-5p expression with CYP4V2 mRNA across the 55 human liver samples. The expression levels of miR-146b versus CYP4V2 mRNA, both expressed as the log2 absolute normalized levels, show a distinct separation between Group 1 and Group 2 and a significant trend for decreased CYP4V2 expression with increased miR-146b levels (Figure 10).

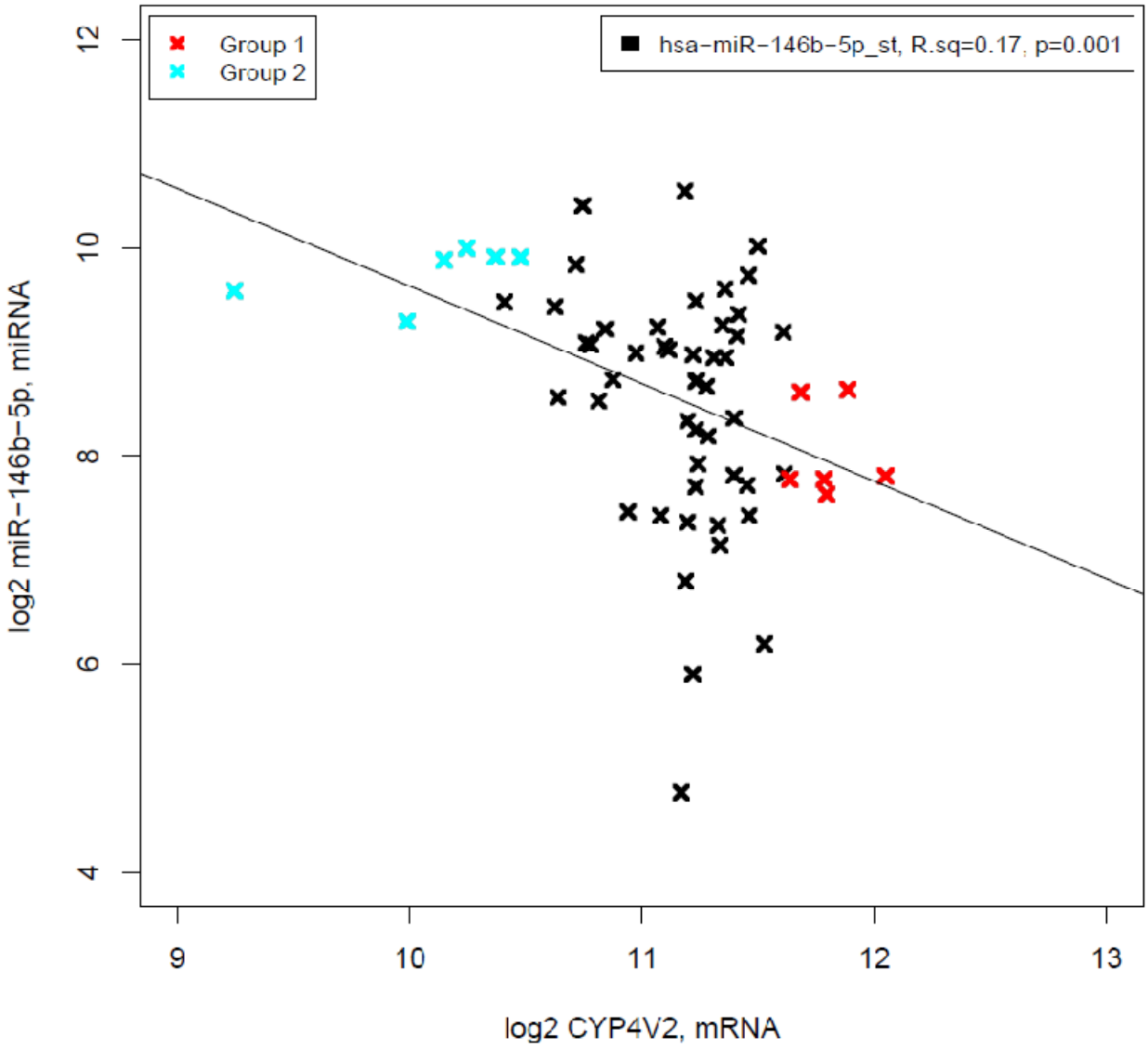

Figure 10. Association of CYP4V2 expression and miR-146b-5p microRNA levels for 55 livers from the UW Human Liver Bank. The vertical axis displays the microRNA levels in the samples for miR-146b-5p, while the horizontal axis displays the mRNA expression for CYP4V2.

Western Blot Analysis:

Western blotting of available human liver microsomes (n=10) from the two groups was done using purified CYP4V2 protein standards to quantify protein concentrations (Figure 11). CYP4V2 protein concentrations were normalized to amount of microsomal protein loaded (20 ug). Coomassie Blue staining was done to verify consistent microsomal protein levels loaded across the samples (Figure 12). CYP4V2 mRNA expression correlated with CYP4V2 protein expression ($R^2 = 0.472$, $p = 0.0282$; Figure 13). CYP4V2 protein expression was negatively correlated with miR-146b-5p expression ($R^2 = 0.4254$, $p = 0.041$; Figure 14).

For the other human liver microsomes (n=50) from the UW Human Liver Bank, Western blotting was performed to determine relative CYP4V2 protein expression using a single purified CYP4V2 standard to normalize each set of livers (Figure 15). In this larger set of samples (n=60), CYP4V2 mRNA levels were not correlated with CYP4V2 protein levels ($R^2 = 0.0143$, $p>0.05$; Figure 16). However, as CYP4V2 protein levels for these samples were quantified using the standard curve obtained from Group 1 and Group 2 western blots (Figure 11), more accurate CYP4V2 protein measurements could be made by running each set of samples with individual standard curves.

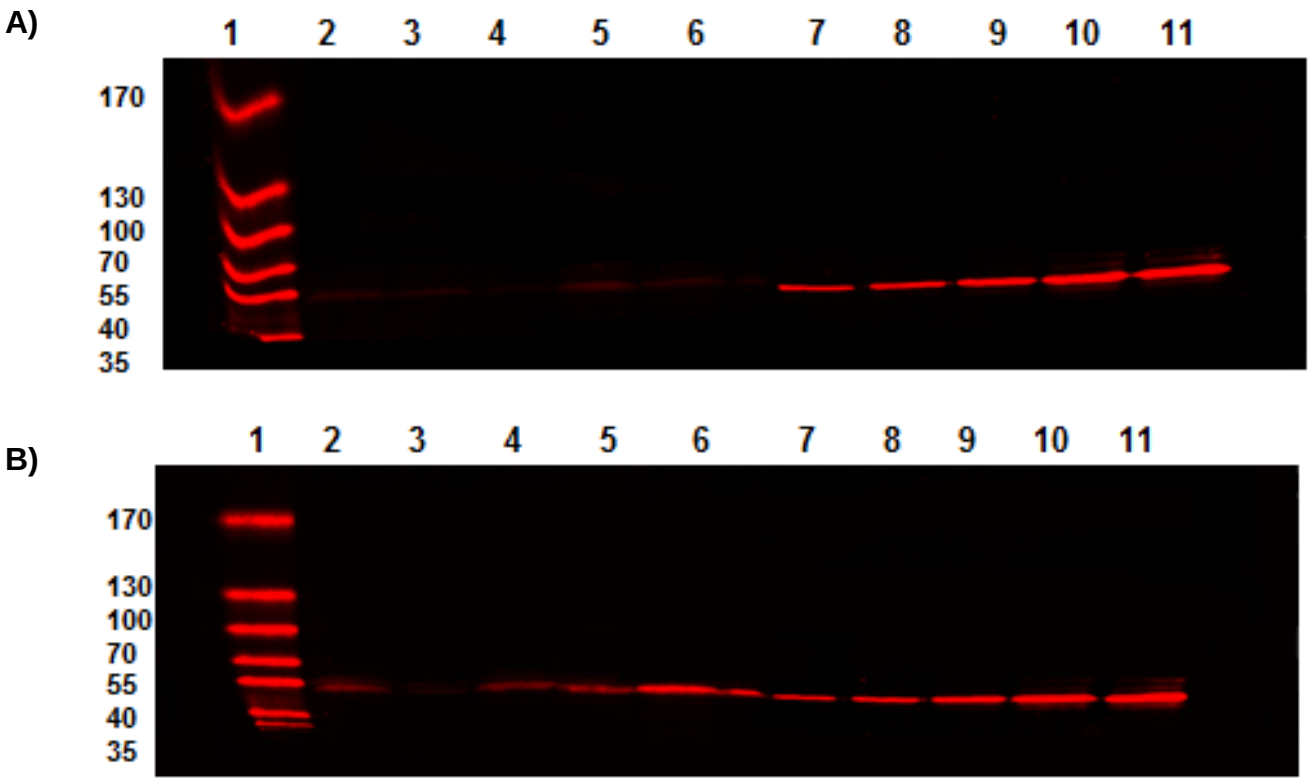

Figure 11. Western blot of human liver microsomes for CYP4V2 expression. Lane 1 contains the molecular weight marker, lanes 2 to 6 contain liver microsomes (20 ug microsomal protein), and lanes 7 to 11 contain purified CYP4V2 protein for quantification (0.0257 pmol, 0.1028 pmol, 0.4112 pmol, 0.257 pmol, 1.028 pmol). Livers with low CYP4V2 mRNA (Group 2) and high CYP4V2 mRNA (Group 1) are shown in Panels A and B, respectively.

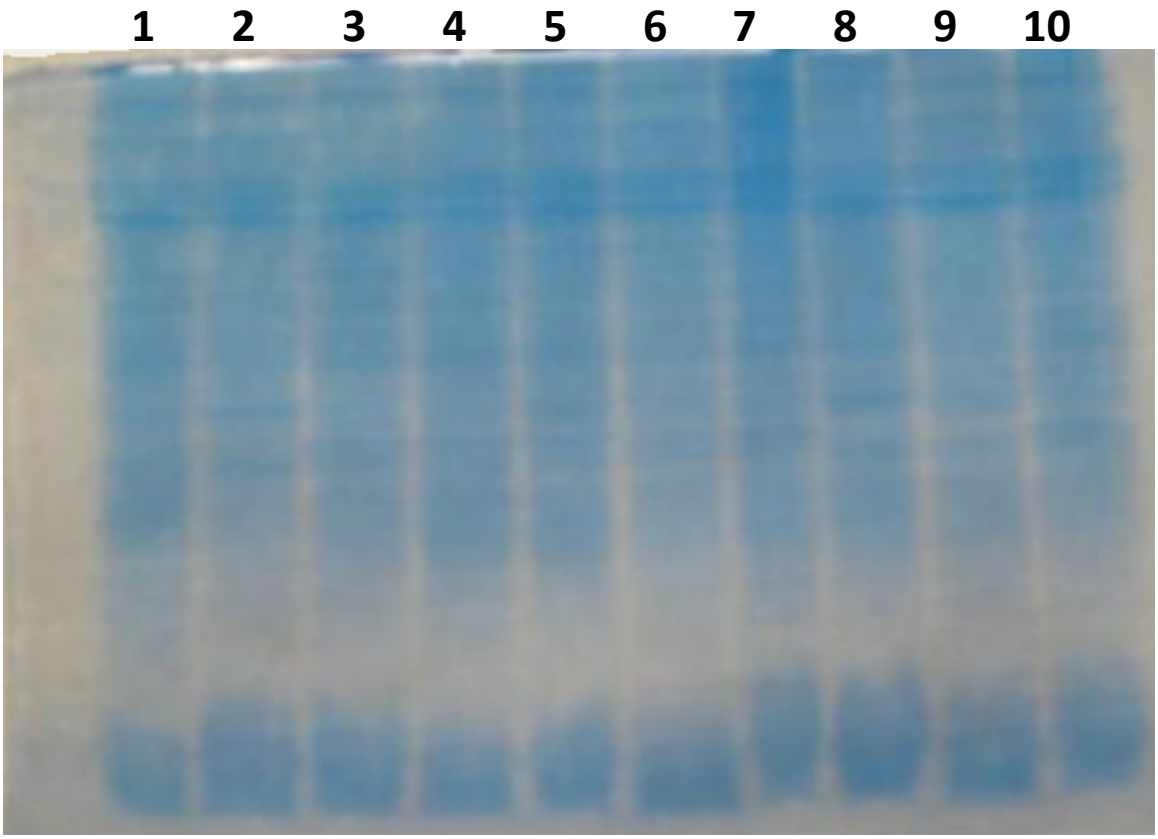

Figure 12. Coomassie Blue staining of human liver microsomes (20 ug microsomal protein). Lanes 1 to 5 correspond to livers with low CYP4V2 expression (Group 2), and lanes 6 to 10 correspond to livers with high CYP4V2 expression (Group 1).

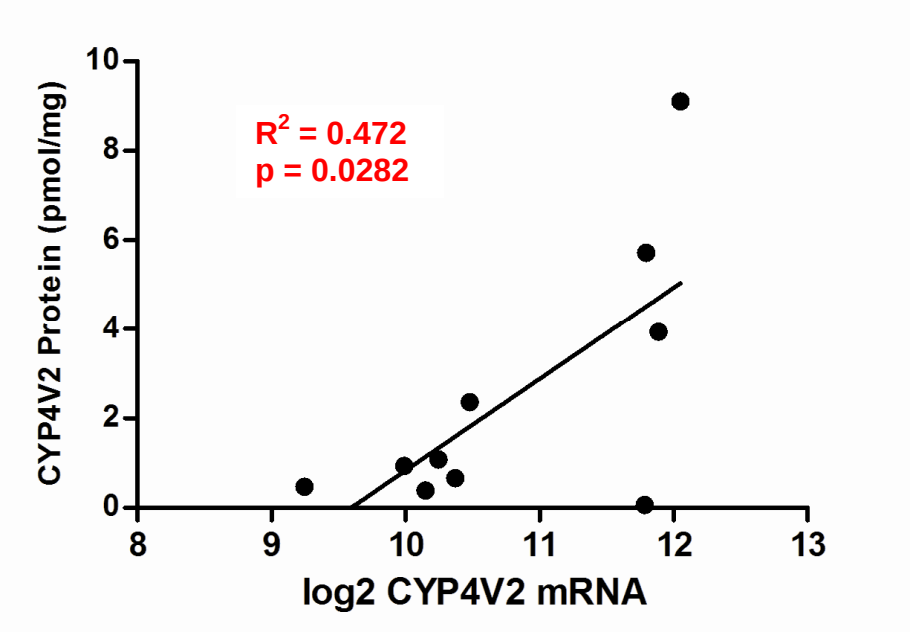

Figure 13. Association between CYP4V2 protein levels and mRNA levels.

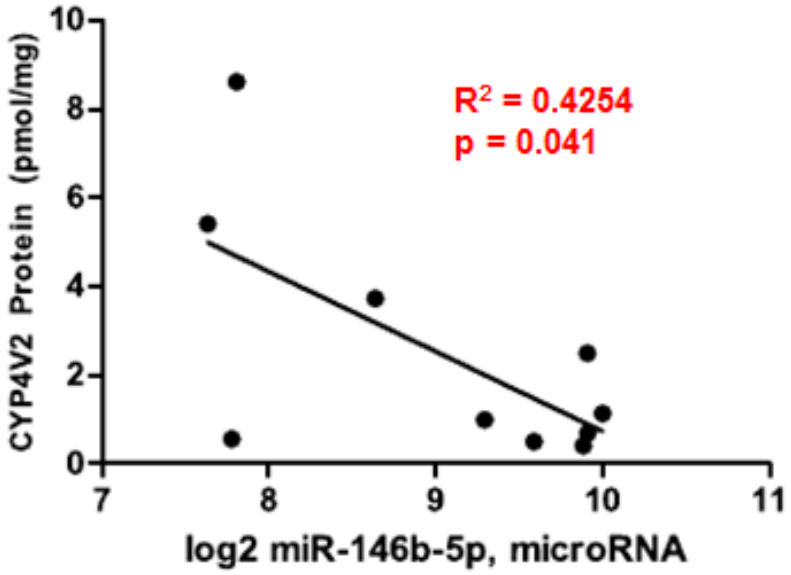

Figure 14. Association between CYP4V2 protein levels and miR-146b-5p expression.

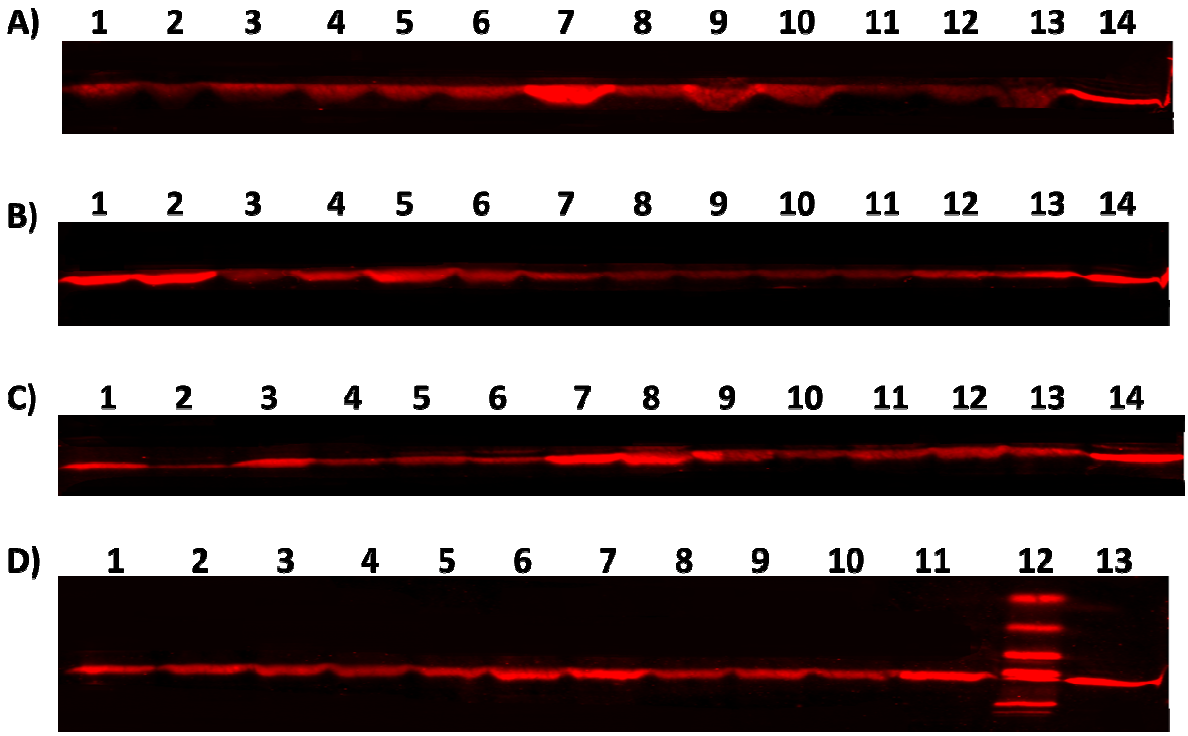

Figure 15. Western blot of human liver microsomes (20 ug microsomal protein). Lanes 1 to 13 (Panels A, B, C) and 1 to 11 (Panel D) correspond to samples from the UW Human Liver Bank, lane 12 (Panel D) is the molecular weight marker, and lanes 14 (Panels A, B, C) and 13 (Panel D) correspond to purified CYP4V2 protein (0.257 pmol).

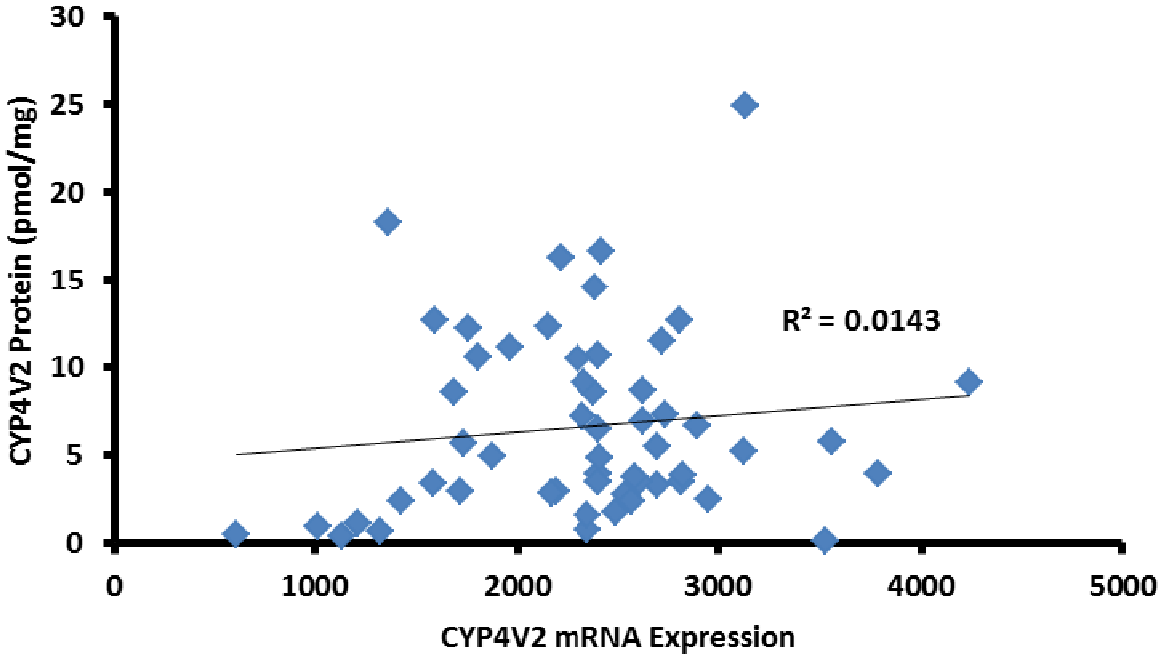

Figure 16. Scatter plot showing lack of correlation between CYP4V2 protein levels and mRNA levels (n=60).

Discussion

While attempting to ascertain possible mechanisms of regulation of CYP4V2 expression, we noted that the CYP4V2 mRNA transcript contains an interesting feature: an extremely long 3'-UTR of 2.8 kb in length, almost double the length of its coding region. This long 3'-UTR of CYP4V2 is similar in length to CYP1B1, a P450 known to be regulated by miR-27b (Tsuchiya 2006). Additionally, the 3'-UTR of CYP4V2 contains multiple miRNA candidate binding sites. MicroRNAs are emerging as key regulators in a number of physiological processes and have been shown to regulate multiple P450s, including CYP1B1, CYP2E1, CYP3A4, and CYP19 (aromatase) (Tsuchiya 2006; Ingelman-Sundberg, Sim et al. 2007; Mohri, Nakajima et al. 2010; Kaikkonen, Lam et al. 2011; Xu, Linher-Melville et al. 2011). In the case of CYP1B1, CYP2E1, and CYP19, the regulation is via direct interaction with the target mRNA, whereas CYP3A4 is regulated indirectly by miRNA modulation of PXR, a key transcriptional regulator of CYP3A4. We performed miRNA microarray analysis on human liver samples to determine if CYP4V2 may be subject to epigenetic regulation by miRNAs.

We observed a negative relationship between miR-146b-5p expression and CYP4V2 mRNA levels (Figure 11). Computational predictions of miRNA-mRNA binding (http://bioinfo.uni-plovdiv.bg/microinspector/) revealed that the 3'-UTR of CYP4V2 contains a binding site for miR-146b-5p, forming a hairpin structure at nucleotide 2115 with a binding energy of -21 kcal/mole (Figure 17). This may support our hypothesis that miR-146b-5p may be involved in the down-regulation of CYP4V2 expression at the mRNA level.

CYP4V2 mRNA and protein expression was correlated in a subset of samples (Figure 13). Consistent with the miR-146b-5p and CYP4V2 mRNA relationship, a negative correlation was found between miR-146b-5p and CYP4V2 protein levels (Figure 14). These data demonstrate that epigenetic regulation by miR-146b-5p affects both CYP4V2 mRNA and protein levels, most likely due to translational repression and/or increased mRNA degradation.

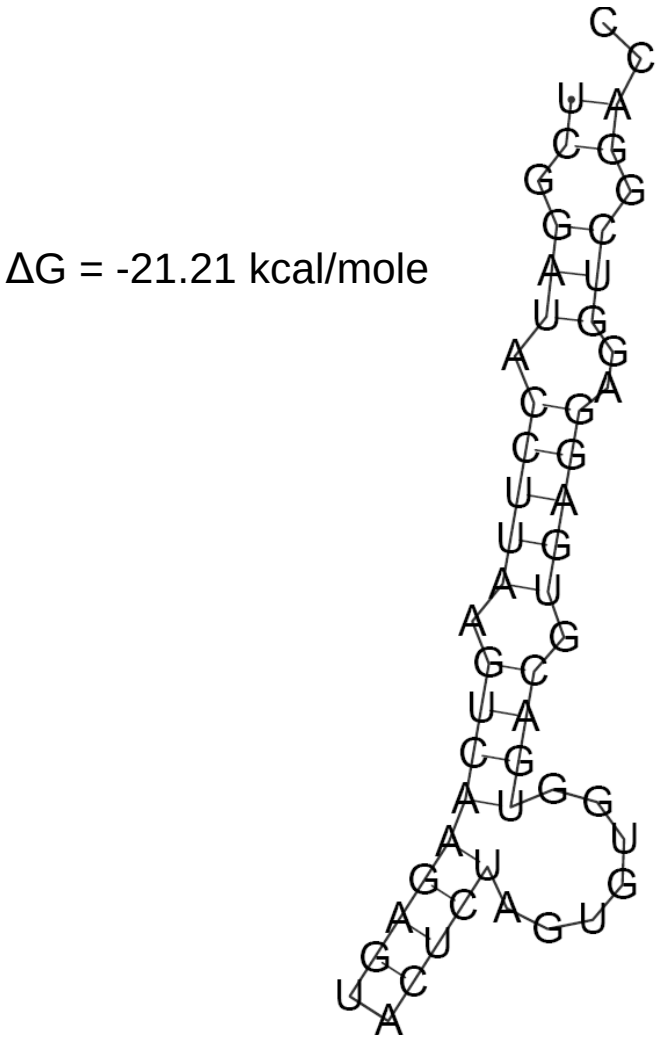

Figure 17. Hairpin formed by miR-146b-5p binding to nucleotide 2115 in CYP4V2 3'-UTR region (http://bioinfo.uni-plovdiv.bg/microinspector/).

Alterations in CYP4V2 expression may impact the viability of RPE cells. Retinal photoreceptor outer segments have the highest DHA content of any cell in vertebrates. Given that mammals have limited capacity to synthesize DHA, a key function of RPE cells is DHA uptake, recycling, and delivery to rod inner segments (Gordon, Rodriguez de Turco et al. 1992). While DHA is a critical structural component of disk outer membrane phospholipid bilayers, it is also an important signaling molecule. That is, DHA serves as a precursor to cytoprotective docosanoids, including the protectins and resolvins (Serhan, Hong et al. 2002; Bazan 2009). Resolvin D1 (RvD1) and protectin D1 (PD1)/Neuroprotectin D1 (NPD1) are oxygenated metabolites of DHA that possess anti-inflammatory and immuno-regulatory properties (Ariel and Serhan 2007). Formation of both docosanoids PD1 and RVD1 goes through a common lipoxygenase-generated intermediate, 17S-hydroperoxy-DHA. Resolvin D1 is the result of reduction of the hydroperoxide, subsequent 5-lipoxygenase (5-LO) action to form the 7,8-epoxide, followed by hydrolysis (Dangi, Obeng et al. 2009). Protectin D1/Neuroprotectin D1 are formed by hydrolysis of the 16,17-epoxide derived directly from 17S-hydroperoxy-DHA (Figure 18). These pathways are similar to leukotriene B4 formation from arachidonic acid (Figure 19). It is well established that inactivation of leukotriene B4 relies on initial ω-hydroxylation by CYP4F3A, a leukocyte-specific, splice variant of the CYP4F3 gene, followed by β-oxidation (Christmas, Ursino et al. 1999). Therefore, analogous pathways could be involved in the inactivation of the resolvins and protectins, and even DHA itself (Figure 19). In ARPE-19 cells, a human RPE cell line, the only CYP4 isozyme present is CYP4V2 (unpublished). Therefore, CYP4V2 may be the only option that RPE cells

have for mitigating DHA-related signaling and local PUFA metabolism via ω-hydroxylation.

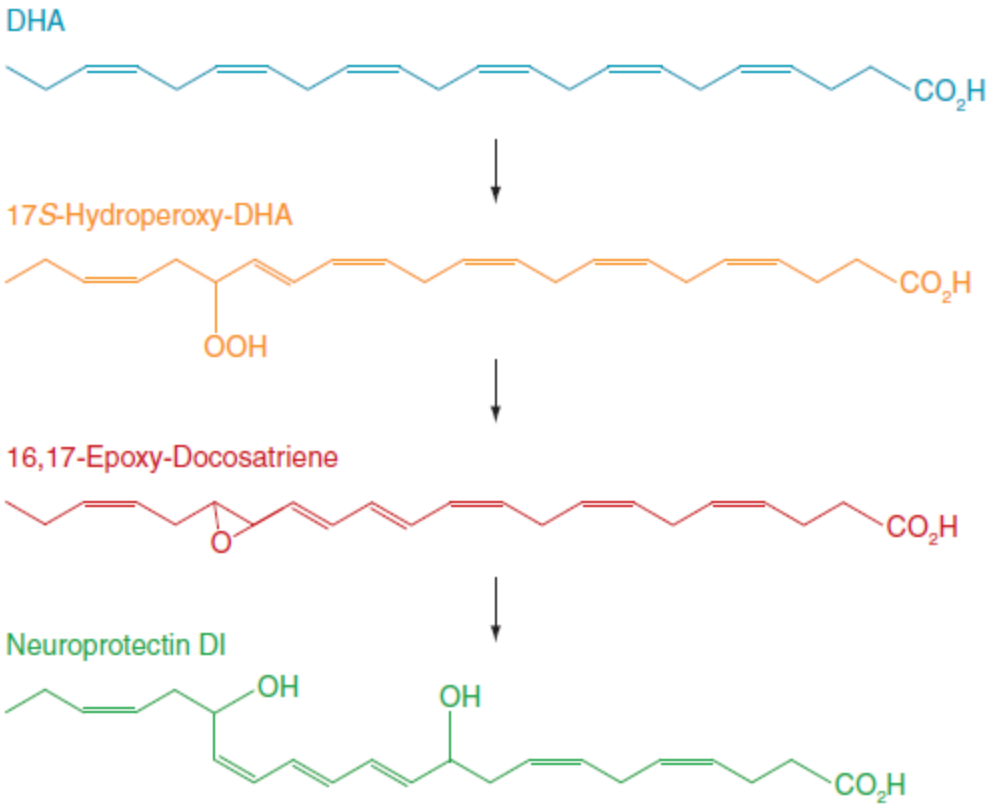

Figure 18. Formation of docosanoid NPD1 from DHA (Kelly, Nakano et al. 2011)

Interestingly, RvD1 was found to cause up-regulation of multiple miRNAs involved in the resolution of acute inflammation, including miR-146b (Recchiuti, Krishnamoorthy et al. 2010). This particular miRNA has been found to regulate a number of cytokines and signaling proteins involved in immune response, particularly the inhibition of NFκB signaling through the down-regulation of interleukin-1 receptor-associated kinase-1 and TNF receptor-associated factor 6 proteins, two key adapter molecules involved in the IRAK/ NFκB pathway causing inflammation (Taganov, Boldin

et al. 2006). Increased levels of miR-146b-5p were seen after 8 hr treatment with 100 ng/mL lipopolysaccharide (LPS) in transfected myeloid cell lines THP-1, U937, HL-60, and WEHI-3 (Taganov, Boldin et al. 2006). However, the opposite trend was seen *in vivo*. Schmidt et al. administered a 4-hour infusion of 2 ng/kg LPS to healthy volunteers and observed a decrease in miR-146b-5p in polymorphonuclear leukocytes (Schmidt, Spiel et al. 2009). This discrepancy with miR-146b-5p levels can be potentially explained by a couple reasons. First, measurement of miR-146b-5p was conducted in different cell types in the two studies, and it is possible that response to LPS administration may be different in leukocytes compared to monocytes, as proposed by Schmidt et al. But a more likely reason may be the disparity in dose between the *in vitro* studies and human studies. Using a 3 L blood volume, a dose of 2 ng/kg would yield concentrations >20,000-fold lower *in vivo* compared to the *in vitro* studies. This suggests that upon exposure to low doses of LPS, miR-146b-5p levels would decrease to facilitate the acute inflammatory response cascade. At higher doses of LPS, or continued exposure and inflammation, miR-146b-5p levels are increased to begin the resolution phase, thus preventing chronic inflammation. This hypothesis is supported by studies exploring the protective role of miR-146b-5p against inflammation.

Chronic, low-grade inflammation is associated with a number of disease states, including obesity, with studies showing that dysregulation of miRNA expression can be linked to this condition (Hulsmans, De Keyzer et al. 2011). Globular adiponectin, a known anti-diabetic adipokine solely expressed in monocytes/macrophages, possesses anti-inflammatory properties by causing induction of interleukin-1 receptor-associated kinase-3, a key inhibitor of the IRAK/ NFκB pathway (Hulsmans, Geeraert et al. 2012).

Globular adiponectin is associated with obesity-related inflammation and has been found to be present in lower levels in obese patients (Hulsmans, Geeraert et al. 2012). In addition, Hulsmans et al. observed decreased expression of miR146b-5p in circulating monocytes of obese patients and proposed that miR-146b-5p is a significant contributor to the anti-inflammatory action of globular adiponectin (Hulsmans, Van Dooren et al. 2012).

Additional studies have demonstrated the importance of miR-146b-5p in inflammation. For example, Takahashi et al. conducted microRNA expression profiling in cord blood cells to elucidate the molecular mechanisms underlying the decreased immune function in cord blood cells compared to adult peripheral blood. They observed differential expression of miR-146b-5p in cord blood cells versus adult peripheral blood cells, including decreased miR-146b-5p expression in CD14+ cord blood cells in response to treatment with the pro-inflammatory cytokine, interferon (IFN)-γ (Takahashi, Nakaoka et al. 2012). In addition, miR-146b decreased protein expression of the pro-inflammatory interleukin-8 (IL8) and Chemokine (C-C motif) ligand 5 (CCL5), potent chemoattractants driving leukocytes into sites of inflammation (Majno 1982; Rossi and Sawatzky 2008; Shimizu 2009).

Deregulation of miRNAs, including miR-146b-5p, has also been implicated in many types of cancers. *In vitro* studies have shown that overexpression of miR-146b-5p causes a decrease in cell migration and invasion in various cancer cell types and matrix metalloproteinase *MMP16* and epidermal growth factor receptor *EGFR* genes were identified as targets of miR-146b-5p (Hurst, Edmonds et al. 2009; Xia, Qi et al. 2009; Katakowski, Zheng et al. 2010; Lin, Wang et al. 2011). In contrast, miR-146b-5p

did not have a significant effect on the cellular proliferation, migration, or invasiveness of A549 lung cancer cells, despite being highly expressed in recurrent disease following surgical removal of non-small cell lung cancer tumors (Patnaik, Kannisto et al. 2010; Patnaik, Kannisto et al. 2011). Research by Garcia et al. suggest an increased expression of miR-146b-5p *in vitro* in basal-like mammary cancer cell lines and *in vivo* in triple negative breast tumor tissues, and this overexpression is associated with down-regulation of the *BRCA1* gene (Garcia, Buisson et al. 2011). Pallante et al. also observed higher levels of miR-146b-5p in papillary thyroid carcinoma cells (Pallante, Visone et al. 2010). This was followed by Geraldo et al. uncovered the potential oncogenic role of miR-146b-5p as a negative regulator of the transforming growth factor β pathway, via repression of the SMAD4 gene (SMAD family member 4) in thyroid cancer (Pallante, Visone et al. 2010; Geraldo, Yamashita et al. 2011).

The inflammatory response is important for protection and host defense but is a process that must be tightly regulated. Uncontrolled inflammation leads to a number of diseases: classic ones, such as psoriasis, periodontal disease and arthritis, as well as obesity and cancer (Calder 2006). Thus, resolution of inflammation is important to maintain homeostasis (Ryan and Majno 1977). Resolvins are key players in this resolution phase, exerting anti-inflammatory and immunoregulatory effects by decreasing neutrophil infiltration, regulating cytokines and reactive oxygen species (Serhan, Clish et al. 2000). These properties of resolvins are consistent with the anti-inflammatory effects of miR-146b-5p. Thus, RvD1 increases miR-146b expression, which would allow miR-146b to target genes involved in the immune system and control inflammation and resolution.

In our work, miR-146b-5p was associated with both CYP4V2 mRNA and protein levels. Thus, increased expression of miR-146b-5p may result in a down-regulation of CYP4V2 expression. These data provide preliminary evidence that epigenetic regulation of CYP4V2 by miRNA may explain the inter-individual variability in the human liver tissue bank. Whether miR-146b-5p is exerting its effects directly on CYP4V2 via the putative binding site in the 3'-UTR must still be elucidated. Such experiments could include, but are not limited to: 1. targeted over-expression of miR-146b-5p or siRNA directed towards miR-146b-5p via transfection into primary human hepatocytes; and 2. use of Luciferase reporter vectors containing the wild-type CYP4V2 3'-UTR compared to mutated miR-146b-5p target sequence in appropriate cell lines (e.g. primary hepatocytes or ARPE-19 cells).

The negative association between miR-146b-5p and CYP4V2 raises the question of what role CYP4V2 may have in docosanoid signaling pathways. It is plausible that this signaling circuit acts to decrease CYP4V2 mRNA and protein and attenuate ω-hydroxylation-mediated inactivation of RvD1 during the resolution phase of the inflammatory response (Figure 19). Investigating potential CYP4V2-catalyzed metabolism of RvD1 may help elucidate the role of CYP4V2 in the acute inflammatory process and provide more insight into the mechanistic causes of BCD.

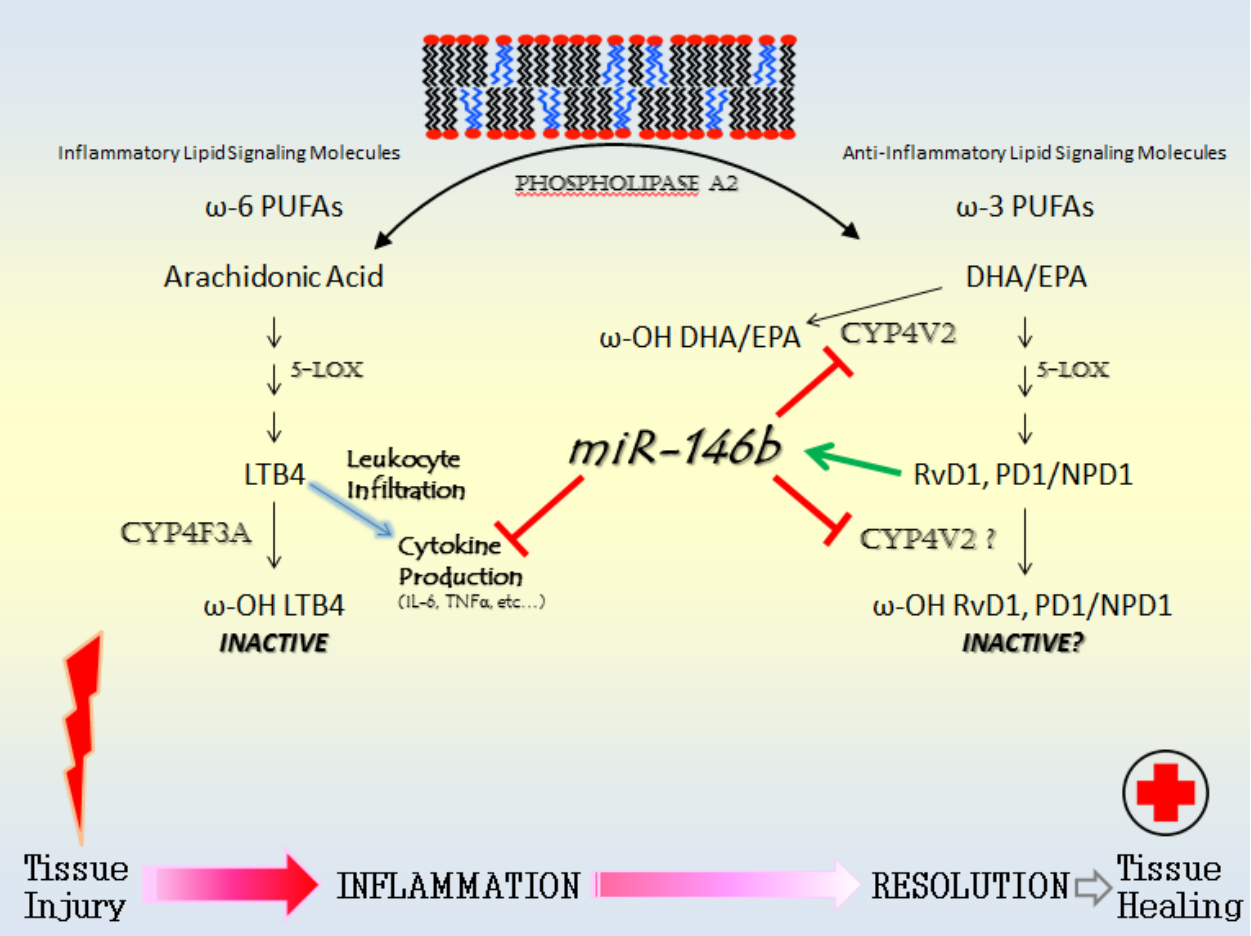

Figure 19. Formation and activation of LTB4 and RvD1 by 5-LO from PUFAs AA and DHA, respectively, during inflammation, and the potential involvement of miR-146b-5p in regulating this process.

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