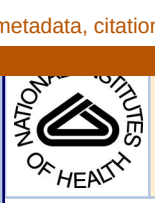

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In silico identification of microRNAs predicted to regulate the drug metabolizing cytochrome P450 genes

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Abstract

OBJECTIVE—Cytochrome P450 (CYP) enzymes exhibit high interindividual variability that is not completely explained by known environmental and genetic factors. To further understand this variability, we hypothesized that microRNAs (miRNAs) may regulate CYP expression.

METHODS—MiRNA identification algorithms were used to identify the miRNAs that are predicted to regulate twelve major drug metabolizing CYPs and to identify polymorphisms in CYP mRNA 3′-UTRs that are predicted to interfere with normal mRNA-miRNA interactions.

RESULTS—All twelve CYPs were predicted to be targets of miRNAs. Additionally, 38 SNPs in CYP mRNA 3′-UTRs were predicted to interfere with miRNA targeting of mRNAs. These predicted miRNAs and SNPs are candidates for future *in vitro* studies focused on understanding the molecular regulation of these CYP genes.

CONCLUSION—These *in silico* results provide strong support for a role of miRNA in the regulation and variability of CYP expression.

Keywords

Bioinformatic analysis; cytochrome P450s; microRNA; polymorphisms

INTRODUCTION

Cytochrome P450 (CYP) is a superfamily of heme-thioloate monooxygenase enzymes that are involved in the oxidative metabolism of a number of endogenous and exogenous compounds such as steroids, drugs, carcinogens and mutagens. Within the CYP superfamily, the drug metabolizing enzymes (DMEs) are involved in 70–80% of all phase I dependent drug metabolism [1]. The expression and activity of these enzymes are highly influenced by both genetic and environmental factors [2]. However, even after accounting for the known variability, there is still substantial unexplained interindividual variability in CYP enzyme activity. MicroRNAs (miRNAs) have been suggested to contribute to some of this unexplained variability [2]. Evidence for miRNA regulation of DMEs is starting to accumulate. Examples include cytochrome P450 1B1 (CYP1B1) [3], cytochrome P450 2E1

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(CYP2E1) [4], vitamin D receptor (VDR) [5], pregnane X receptor (PXR) [6] and ATP-binding cassette xenobiotic transporter ABCG2 [7]. However, a comprehensive analysis of miRNAs predicted to regulate the CYPs has not been published.

MicroRNAs are small (~22 nucleotides), non-coding RNAs that regulate gene expression post-transcriptionally. In animals, miRNAs typically bind to the 3′-untranslated region (3′-UTR) of the messenger RNAs (mRNAs) and negatively regulate gene expression either by blocking protein translation or by degrading the mRNA [8]. As more miRNAs are identified and studied, newer target sites and functions are being recognized. For example, it has now been shown that miRNAs can also bind to coding regions and repress gene expression [9]; this mechanism may explain some of the differential expression seen in mRNA splice variants. MiRNAs also appear to be involved in the induction of gene expression; this induction occurs through binding to complementary regions in the promoter [10] and the 5′-UTR [11]. In humans, 940 mature miRNAs have been reported so far (version 15 of microCosm release [12]). Bioinformatic predictions suggest that miRNAs can control 90% of human transcripts [13]. These miRNAs form a broad and complex regulatory network as each miRNA can regulate multiple genes and each gene can be regulated by multiple miRNAs. MicroRNAs are involved in a wide range of biological activities including cell differentiation, cell death, cancer and noncancerous human diseases [14].

Single nucleotide polymorphisms (SNPs) that occur either on the miRNA or on the mRNA (at or near the miRNA target site) can alter miRNA gene processing or affect the normal mRNA-miRNA interactions, respectively. These SNPs, referred to as miRSNPs [15], can create new miRNA target sites or destroy old target sites. Such loss or gain of miRNA targeting by miRSNPs can result in the development of drug resistance. Thus, miRSNPs represent another potential mechanism that may contribute to the inherited interindividual variability in CYP enzyme expression and activity.

In this study, we hypothesized that miRNAs regulate the expression of CYPs. In the first step in testing this overall hypothesis, we performed a comprehensive bioinformatic analysis to identify miRNAs that are predicted to target twelve of the major drug metabolizing CYPs. We also used bioinformatic algorithms to identify polymorphisms in the CYP 3′-UTR that are predicted to alter the normal mRNA-miRNA interactions. The results of the *in silico* analysis collectively suggest that miRNAs are likely to play an important role in the regulation of drug metabolism. These results provide a candidate list of miRNAs and SNPs that will be useful in testing and understanding the molecular regulation of the CYP genes.

METHODS

Bioinformatic analysis to predict microRNAs that target the CYPs

We used six different web-based bioinformatic algorithms to predict the miRNAs that target twelve of the major drug metabolizing CYPs. The programs are:

- **a.** miRanda [14] (http://www.microrna.org/microrna/getGeneForm.do),
- **b.** microCosm Targets [12] (http://www.ebi.ac.uk/enright-srv/microCosm/htdocs/targets/v5/; formerly referred to as miRBase Targets),

c. TargetScan [16] (http://www.targetscan.org/),

d. PicTar [17] (http://pictar.mdc-berlin.de/),

e. RNA22 [13] (http://cbcsrv.watson.ibm.com/rna22.html), and

f. PITA [18] (http://genie.weizmann.ac.il/pubs/mir07/index.html).

Analysis using these programs were performed using the default parameters. In brief, for miRanda and microCosm Targets, *homo sapiens* parameter was selected. For TargetScan, both conserved and non-conserved miRNAs were included in analysis For PITA, a minimum seed of 8 nucleotides, without any mismatches, a single G:U base pairing, and no flank was selected. For RNA22, which is a downloadable program with user defined mRNA and miRNA sequences, the CYP gene reference sequence identification numbers were identified from the Human Cytochrome P450 Allele Nomenclature Committee home page (www.cypalleles.ki.se/) when available, and then the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway) was used to identify the 3′-UTR sequence. The mature miRNA sequences (version 15.0) were downloaded from the microCosm database [12]. The parameters for analysis included, 0 unpaired bases in a 6 nucleotide seed, with a minimum of paired-up bases in heteroduplex, and a maximum folding energy of −25 Kcal/mol for the heteroduplex.

Identification of SNPs located in the CYP 3′-UTR

SNPs in the CYP mRNA 3′-UTR were identified using the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The minor allele frequencies (MAF) were obtained from the dbSNP database.

Bioinformatic analysis to predict the effect of CYP 3′-UTR SNPs on mRNA-miRNA interactions

Two programs, (a) Patrocles database [19] (http://www.patrocles.org/Patrocles_targets.htm), and (b) PolymiRTS database [20] (http://compbio.uthsc.edu/miRSNP/) were used to predict the effect of SNPs in the CYP mRNA 3′-UTR on the mRNA-miRNA interaction.

RESULTS

In silico analyses to predict miRNAs that target CYPs

Six bioinformatic algorithms were used to identify miRNAs that are predicted to target twelve of the major drug metabolizing CYP enzymes. These algorithms predicted that all the twelve genes were targets of miRNAs (Table 1 and Supplementary Table 1); while some genes were predicted to be targeted by many miRNAs, others were predicted to be targeted by relatively few miRNAs. The number of miRNAs predicted to target each gene appears to be correlated with the length of the 3′-UTR ($r^2 = 0.9$).

In silico analyses to predict the effect of CYP 3′-UTR SNPs in the miRNA target sites

Thirty eight SNPs were identified in the 3′-UTR of the CYPs that are predicted to alter miRNA targeting of these genes (Table 2). The algorithms predicted that 22 miRNA target

sites are destroyed by SNPs, 22 new miRNA target sites are created by SNPs, and 2 SNPs simultaneously created 2 new target sites and destroyed 2 target sites.

DISCUSSION

The drug metabolizing CYPs are involved in the metabolism of a number of clinically important drugs [1]. However, there is considerable interindividual variability in the activity of these enzymes and this consequently results in variability in both drug metabolism and response [2]. In this study, we performed bioinformatic analysis to investigate the role of microRNAs (miRNAs) in the regulation of CYP expression.

The *in silico* analyses indicated that all twelve of the drug metabolizing CYPs analyzed are likely to be regulated by miRNAs (Table 1). Some of these enzymes were predicted to be targeted by many miRNAs (e.g. CYP1A1, 1A2, 1B1, 2B6, 3A4); whereas others were predicted to be targeted by relatively few miRNAs (e.g. CYP2A6, 2D6, 2E1, 3A5). The intergene variability in the number of predicted miRNAs was largely explained by the length of the mRNA 3′UTR ($r^2 = 0.9$). These results may provide new insights as to why the expression of some of the CYPs is more regulatable than others. For example, the expression of CYP2D6 is not generally as regulatable as some of the other genes, such as CYP3A4 [21]. As expected from our analysis, CYP2D6 has the shortest 3′-UTR, whereas CYP3A4 has a relatively long 3′-UTR. Additional functional studies will be required to confirm these predictions. Furthermore, the vast number of miRNAs predicted to target the CYP genes indicates that it is likely that many miRNAs are involved in the regulation of those genes.

There was also substantial variability in the number of miRNAs predicted by the different programs (Table 1). The total number of miRNAs predicted by two or more programs (i.e., the overlap percentage in Table 1) ranged from 0–23%. This variability may be due to a number of factors including the inherent differences in the algorithms including differences in parameters, such as degree of complementarity and species conservation used. For example, three of the programs (miRanda, microCosm and PicTar) use evolutionary conservation parameter. Since the CYP isoforms are not highly conserved across species [22], this may contribute to the inter-algorithm variation. Part of the variability may be due to the different microCosm releases that are used by each algorithm; they ranged from versions 10.1 to 15. The total number of miRNAs predicted to target these CYP genes is likely to change as more miRNAs are being discovered and as new prediction algorithms arise and as the current algorithms evolve. Although we could have used additional algorithms that have recently come available (e.g. MiTarget, MirTarget2), the algorithms used in our analyses provided substantial evidence the CYP genes are very likely to be targeted by multiple miRNAs. As investigators initiate studies to prioritize and test the miRNA–mRNA interactions in laboratories, it would be advisable to use the most up to date versions of the algorithms and possibly to include the additional algorithms that are available at that time.

In vitro laboratory evidence from published studies confirms some of our predictions. For example, our bioinformatic analysis using miRanda, TargetScan, and RNA22 predicted that

miR-27b targets CYP1B1 mRNA. MiR-27b has been shown to regulate CYP1B1 mRNA [3]. Similarly, our bioinformatic predictions using MicroCosm Target algorithm suggested that miR-378* targets CYP2E1. MiR-378 (renamed as miR-378*) has been shown to regulate CYP2E1 mRNA [4]. Our bioinformatic analyses provide a focused list of miRNAs that are candidates for regulating additional CYPs that could be tested laboratory studies to verify the predicted CYP-miRNA interactions.

Polymorphisms that occur either on the miRNA or on the mRNA (miRSNPs) can alter normal mRNA-miRNA interactions [15]. These miRSNPs can either create new miRNA binding sites (resulting in down regulation of the target gene expression) or destroy miRNA target sites (resulting in a loss of targeting and elevated expression of the target gene expression) and thus affect enzyme activity [15, 23]. Using two bioinformatic programs, PolymiRTS [20] and Patrocles [19], we identified SNPs in eight of the CYP genes that are predicted to alter the mRNA-miRNA interactions (Table 2).

In the prioritization of SNPs for pharmacogenetics and functional studies, polymorphisms in the 3′-UTRs of genes have typically not been given high priority; however, based on our *in silico* analyses, these SNPs may have important functional consequences. Previous studies have shown that SNPs in the 3′-UTRs of CYP19A1 [24] and CYP2A6 [25] are associated with altered phenotypes. Although our bioinformatic analyses suggested that these SNPs do not directly target ‘seed’ regions (typically nucleotides 2–8 from the 5′ end of the miRNA) of predicted miRNAs, SNPs in ‘non-seed’ regions can also affect mRNA-miRNA interaction [15]. Since both PolymiRTS and Patrocles programs do not predict loss or gain of mRNA-miRNA interactions due to the presence of SNPs in the ‘non-seed’ regions, laboratory experiments will be required to determine if they affect miRNA targeting. It is likely that additional SNPs will be discovered in the 1000 Genomes Project and as that data matures, they should also be incorporated into this type of analysis. SNPs in the mature miRNAs and pre-miRNA may also affect the mRNA-miRNA interaction; however, not all miRNAs have not been resequenced in depth and hence, these are not included in our current analyses.

The studies presented here are the first steps in identifying miRNAs that target the enzymes involved in drug disposition. From this *in silico* analysis, miRNAs and SNPs can be prioritized for further *in vitro* functional studies (luciferase assay, western blotting, mRNA quantification, etc) to validate the bioinformatic predictions. Similar to miRNA regulation of drug metabolizing CYPs, recent studies also suggest that other genes involved in drug disposition, including Phase II enzymes, drug targets, and other drug transporters can also be regulated by miRNAs [3, 5–7]. As with any bioinformatics predictions, these studies will need to be confirmed with laboratory experiments. This would apply to both the identification of the targets and the effects of the SNPs. As more additional SNP data is generated (e.g. 1000 Genomes Project), those data will also need to be included in the miRSNP analyses.

CONCLUSION

In conclusion, the results of our *in silico* analyses indicate that miRNAs are likely to be an important mechanism that control CYP expression, and consequently drug metabolism. This would add at least two additional sources of variability that would affect drug metabolism. First, genetic variants that affect the CYP–miRNA interactions. The variants could be in either the CYP or the miRNA genes. Second, environmental factors that alter miRNA expression could have profound indirect effects on CYP expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

miRNAs predicted to target the UTRs of selected drug metabolizing CYPs.

CYP Gene	Reference ID [a]	3′-UTR length (bp)	Bioinformatic Programs [b, c]					Total no. of unique miRNAs [d]	Overlap [e] (%)
			Micro Cosm	miRanda	Target Scan	RNA22	PITA		
1A1	NC_000015.8 (NM_000499)	946	14	2	85	264	14	332	47 (12.4)
1A2	NC_000015.8 (NM_000761)	1512	7	g	70	341	11	386	43 (10)
1B1	NC_000002.10 (NM_000104)	3119	1	49	215	345	40	499	151 (23.2)
2A6	NC_000019.8 (NM_000762)	257	*g*	*g*	18	37	2	54	3 (5.3)
2B6	NC_000019.8 (NM_000767)	1569	5	*g*	125	347	27	416	88 (17.5)
2C8	NC_000010.9 (NM_000770)	355	35	*g*	31	65	2	114	19 (14.3)
2C9	NC_000010.9 (NM_000771)	362	46	*g*	34	77	9	129	37 (22.3)
2C19	L39102.1	*f*	57	*g*	*g*	*g*	*g*	57	0
2D6	NC_000022.9 (NM_000106)	74	33	*g*	*g*	4	4	40	1 (2.4)
2E1	NC_000010.9 (NM_000773)	152	24	*g*	10	5	*g*	35	4 (10.3)
3A4	NC_000007.12 (NM_017460)	1152	34	26	111	208	17	333	63 (15.9)
3A5	NC_000007.12 (NM_000777)	110	12	*g*	11	14	1	32	6 (15.8)

[a] Reference sequence and the corresponding RefSeq Gene id from UCSC Genome browser in parenthesis.

[b] PicTar predictions are not included in the table as the CYP genes do not appear to be a part of the program's database.

[c] Versions of the bioinformatic programs (retrieved on 07/12/2010): (A) MicroCosm Target uses miRNA Registry release 15.0, (B) for RNA22, we used miRNA Registry release 15.0, (C) MicroRNA.org and PITA use miRNA Registry release 11.0, and (D) TargetScan version 5.1 uses miRNA Registry release 10.1. Some of the predictions include multiple transcripts of the same gene.

[d] Total number of unique miRNAs predicted by all the programs.

[e] Total number of miRNAs predicted to target the genes by at least 2 programs.

[f] No 3′-UTR sequence available in UCSC genome browser.

[g] No results predicted for these CYPs by the corresponding bioinformatic program.

Table 2

CYP 3′-UTR SNPs predicted to destroy or create a miRNA target site.

CYP Gene	dbSNP rs#	MAF (%)	Seed sequence [Ancestral/Derived allele]	MicroRNA target destroyed	MicroRNA target created
1A1	4986880	0–10.8	T[C/T]TGCACA		−19a, −19b
1A2	34002060	NA [a]	TTT[T/-]GAGA [#]	−373*, −616*	−373*, −616*
1B1	1056876	0–1	GGAC[A/C]CC		−615-5p
	9309020	0–13.7	TGTTTA[T/C]A		−30a, −30b, −30c, −30d, −30e
	34169771	0–2.1	[T/C]GTACCAA [#]	−150*	
	9341262	0–2.4	A[T/C]TGAAAA		−30a*, −30d*, −30e*
			AATA[T/C]TGA	−16-2*, −195*	−16-1*
	34521017	0–6.2	AAT[A/C]TTGA [#]	−16-2*, −195*	
	2855658	0–91.7	CTTGT[A/G]TA	−300, −381	
			TTGT[A/G]TAA	let-7a*, let-7b*, let-7f-1*	
	1056843	0–6.9	GCAAA[G/A]AA [#]		−129-5p
	9341260	0–0.6	CAGA[G/A]ACA	−593	
	35007750	0–6.3	GGT[G/A]GGAA [#]	−126	
	2672	0 [b]	AC[T/A]ACTGA	−199a-3p, −199b-3p	
			C[T/A]ACTGAA	−222*	
2A6	8192733	NA	G[C/G]GGCTCA [#]		−1225-3p
	28399469	NA	[G/A]GGGCCAA [#]		−328
			C[G/A]GGGCCA [#]		−1291
2B6	3211376	0 [b]	CA[T/G]TGCAA	−106a*	
	28969419	NA	[C/G]ACCACCA [#]		−323-5p
			AAC[C/G]ACCA [#]	−876-3p	
	3211391	NA	GGT[G/T]GTGA [#]	−220b	
	3211392	21	TCCAC[C/A]CA [#]	−363*	
	3211393	NA	TCCACCC[A/G] [#]	−363*	

CYP Gene	dbSNP rs#	MAF (%)	Seed sequence [Ancestral/Derived allele]	MicroRNA target destroyed	MicroRNA target created
	3211398	NA	GAA[T/C]GCTA #	−1179	
	34031833	NA	TT[C/-]CCCCA #	−625	
	28399501	NA	AAAG[G/A]AT	−501	
	3211372	0–25	TCCCC[G/A]C	−491	
			CCCC[G/A]CC	−663	
	7246465	0–43.8	[C/T]GTTTTA		−570
	12979270	0–29.2	TTCCCC[A/C]		−625
	1042389	0–90	TGCCTC[T/C]		−650
	3211399	32	CTACTG[C/T]		−199a*
	3211403	NA	C[C/T]GCTGA		−214
			TC[C/T]GCTGA		−922
	3211404	NA	AATCT[G/A]C		−376a*
			CT[G/A]CTGA	−214	
2C9	9332240	0–4	TTATC[C/T]A		−577
	9332241	0–6	ATG[C/T]CTT		−641
	9332242	0–12.5	TCATCT[C/G]A	−143	
	9332243	0–4	A[C/T]GGAGA		−136
	28969379	NA	TAATTC[A/G]A #	−183*	
3A4	34469568	0–2.1	C[G/A/C]CCTGTA #		−552
	34141651	0–4.2	C[G/A]CCTGTA #		−552
	28988603	0–10	CAGAAC[T/G]A	−148b*	
3A5	17161788	0–6.2	T[A/G]CTTTG	−330	

NA - No frequency data available in dbSNP

Ancestral allele unknown

a Genotype information available from only one individual

b The minor allele frequency (MAF) appears to be 0% in over 90 individuals that have been genotyped for this SNPs.