Supplementary Information

The independent contribution of miRNAs to the missing heritability in

CYP3A4/5 functionality and the metabolism of atorvastatin

Ju-E Liu^{1,2,#}, Bin Ren^{1,#}, Lan Tang^{3,#}, Qian-Jie Tang^{2,4}, Xiao-Ying Liu², Xin Li^{2,5}, Xue Bai^{2,6}, Wan-Ping Zhong^{2,3}, Jin-Xiu Meng², Hao-Ming Lin⁷, Hong Wu⁷, Ji-Yan Chen^{2,8}, Shi-Long Zhong^{2,8,*}

[#]Ju-E Liu, Bin Ren and Lan Tang contribute equally to this paper

1. Department of Pharmacy, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510080, China

2. Medical Research Center, Guangdong General Hospital, Guangzhou, Guangdong 510080, China

3. Department of Pharmaceutics, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China.

4. Institute of Chinese medical science, Guangdong TCM key Laboratory for metabolism, Guangdong pharmaceutical university, Guangzhou 510006, China

5. Department of Pharmacology, School of Pharmaceutical Sciences, Guangzhou Medical University, Guangzhou 511436, China

6. School of pharmaceutical science, Sun Yat-Sen University, Guangzhou, Guangdong 510006, China

7. Department of Hepatobiliary Surgery, Sun Yat-Sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China

8. Guangdong Cardiovascular Institute, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080, China

*Correspondence to

Shi-Long Zhong, Ph.D.

Professor of Medical Research Center, Guangdong General Hospital,

Guangdong Cardiovascular Institute,

Guangdong Academy of Medical Sciences.

106 Zhongshan Road, Weilun Bldg.1112,

Guangzhou 510080, P. R. China

Tel: +8620-83827812 - 51157

Email: zhongsl@hotmail.com



Supplementary Figure S1. Correlation between CYP3A gene expression and the reduction rate of atorvastatin or the formation rate of two metabolites of atorvastatin. The expression of CYP3A4 was significantly associated with reduction rate of atorvastatin (a_1), formation rate of ortho-hydroxy atorvastatin (a_2), formation rate of para-hydroxy atorvastatin (a_3). Expression of CYP3A5 was associated with reduction of atorvastatin (b_1), formation of ortho-hydroxy atorvastatin (b_2), and formation of para-hydroxy atorvastatin (b_3).



Supplementary Figure S2. Luciferase reporter assay to evaluate the direct inhibition of miR-130a, miR-21, miR-27b and miR-142 on CYP3A4 or CYP3A5 expression. (a) miR-130a did not target the predicted binding sites in the CYP3A4 3'-UTR. (b) miR-21 did not target the predicted binding sites in the CYP3A4 3'-UTR. (c) miR-27b did not target the predicted binding sites in the CYP3A5 3'-UTR. (d) miR-142 did not target the predicted binding sites in the CYP3A5 3'-UTR.



Supplementary Figure S3. The chromatograms of testosterone and its metabolite. (a) blank microsomal; (b) reference standards; (c) inactive microsomal incubation mixtures; (d) active microsomal incubation mixtures. 1, 2 and 3 represent 6β -hydroxytestosterone, internal standard hydrocortisone and testosterone, respectively.



Supplementary Figure S4. Effect of testosterone incubation time and microsomal protein concentration on the formation of 6 β -OHT by human liver microsomes and enzyme kinetic parameters. (a) Time-dependent formation of 6 β -OHT in human liver microsome. (b) Microsomal protein concentration-dependent formation of 6 β -OHT. (c) Kinetic profiles of 6 β -OHT activity in HLM with obtained K_m= 83.06 \pm 14.93 μ M, V_{max}= 470.7 \pm 22.0 pmol/mg/min. Human liver microsomes from a pooled sample were incubated with testosterone (20 μ M) for different times (0-90 min with 0.5 mg/ml protein) or at different protein concentrations (0-2.0mg/ml for 20 min). Data shown are the average mean \pm S.D. of three independent experiments.



Supplementary Figure S5. Product ion spectra of atorvastatin (A), ortho-hydroxy atorvastatin (B), para-hydroxy atorvastatin (C), and carbamazepine (D).



Supplementary Figure S6. The chromatograms of atorvastatin and its metabolite. (a) atorvastatin; (b) ortho-hydroxy atorvastatin; (c) para-hydroxy atorvastatin; (d) internal standards carbamazepine; (e) mixtures. 1, atorvastatin; 2, ortho-hydroxy atorvastatin; 3, internal standard; 4, para-hydroxy atorvastatin.



Supplementary Figure S7. Enzyme kinetics and effect of atorvastatin incubation time and microsomal protein concentration on atorvastatin metabolism by human liver microsomes. (a) Time-dependent formation of ortho- and para-hydroxy atorvastatin in human liver microsomes. **(b)** Microsomal protein concentration-dependent formation of ortho- and para-hydroxy atorvastatin. Enzyme kinetics of the formation rates of (c) ortho- and (d) para-hydroxy atorvastatin from atorvastatin in pooled human liver microsomes. Human liver microsomes from a pooled sample were incubated with testosterone (1-150 μ M) with 0.3 mg/ml protein for 60 min at 37 $^{\circ}$ C. Data shown are the average mean ± S.D. of triplicate incubations.

а		Predicted consequential pairing of target region	Position
	hsa-miR-491	3' CAUCUUCCCUUAGAACGUAUUC 5'	93-124
	CYP3A4 MRE 491	5' GAAGATGGGCTTCATCCAATGGACTGCATAAA 3'	
	hsa-miR-27a	3' CGCCUUGAAU-CGGUGACACUU 5'	
		: * * ** *	593-614
	CYP3A4 MRE 27a	5' GTGAAAGTTAATCCACTGTGAC 3'	
	hsa-miR-107	3' ACUAUCGGGACAUG-UUACGACGA 5' ******* ****: *: : **	863-886
	CYP3A4 MRF 107	5' ACTTGAACCTGGGAGGCGGATGTTGAA 3'	
	hea-miR-103		
	lisa-milk-105	3. AGUAUCGGGACAUG-UUACGACGA 5.	
	CYP3A4 MRE 103		858-886
	bea miP 106a	31 CALIFICIAL CONCERNMENT ST	
	lisa-lilik-100a	**** ** ** *	991 002
	CYP3A4 MRF 106a	5' TGTTGAAGTGAGCTGAGATTGCAC 3'	001-905
	bee miD 27h		
	nsa-miK-27b	5. CG0C00GAA0-CGG0GACAC00 5.	593-614
	CVD2 AA MDE 27h	51 GTCAAACTTAATCCACTCTCAC 31	555 014
	CIPSA4 MKE 2/D	3' AGHIGHAGHCAG-ACHAHIGGAH 5'	
	hsa-miR-21	**•• •*** •***• • •	869-894
		5' GAGGCGGATGTTGAAGTGAGCTG 3'	000 004
	CYP3A4 MRE 21		
	nsa-mik-206	5. GGUGUGUGAAGGAAUGOAAGGU 5.	1073-1093
	CVD2 AA MDE 206		
	hsa-miR-130a	3' HAC-GGGAAAAHIIGHAACGUGAC 5'	
	nsa-mix-150a	** * : : * * * * * :	124 150
	CYP3A4 MRE 130a	5' GGGATTCTGTACA-TGCATTG 3'	134-150
	hsa-miR-130a	3' UACGGGAAAAUUGUAACGUGAC 5'	
		** :****: * **	887-905
	CYP3A4 MRE 130a	5' GAGCTGAGATTGCACCA 3'	
	hsa-miR-130a	3' UACGGGAAAAUUGUAACGUGAC 5 '	
		*** ****** : *	1067-1091
	CYP3A4 MRE 130a	5' TTTCCCTACACCTCTTGCATTC 3'	
h			
U		Predicted consequential pairing of target region	Position
		1 1 0 0 0	
	hsa-miR-491-3p	3' CAUCUUCCCUUAGAACGUAUUC 5'	105 000
		***********	185-203
	CYP3A5 MRE 491	5' TTTGTGTTAATATTT GCATAA G 3'	
	hsa-miR-27b	3' CGUCUUGAAUCGGUGACACUU 5'	
	provide and the second s	***********	338-361
	CYP3A5 MRE 27b	5' CCACTAATACCACACTGTGGT 3'	
	hsa-miR-27a	3' CGCCUUGAAUCGGUGACACUU 5'	
		***********	338-361
	CYP3A5 MRE 27a	5' CCACTAATACCACACTGTGGT 3'	
	hsa-miR-142	3' UCAUCACGAAAGAUGAAAUAC 5'	
		******	414-420
	CYP3A5 MRE 142	5' GGCAAUUCUUUCCACUUUAUU 3'	
	1 'D 271		
	nsa-miR-371	5' UUUCACGGCGGUA-GAAAACUCA 5'	
	CVD2 45 MDE 271		508-528
	CITSAS MRE 3/1	5 ACAMACCIOCCAIMAITTUATA 5	
	hsa-miR-126	3' GCGCAUGGUUUUCAUUAUUAC 5'	
		************	1065-1071
	CYP3A5 MRE 126	5' CCAUUAUUUCUCUCAAUAAUA 3'	

Supplementary Figure S8. MiRNA recognition element within CYP3A4 or CYP3A5 3'-UTR predictive by TargetScan, FINDTAR3 and miRanda. (a) predictive binding sites of miRNAs within CYP3A4 3'-UTR. (b) predictive binding sites of miRNAs within CYP3A5 3'-UTR

Supplementary Table 1. Baseline clinical characteristics and CYP3A4/5 genotypes impact on gene expression, CYP3A activity and metabolism of atorvastatin

	Aatorvastatin		Ortho-hydroxy atorvastatin		Para-hydroxy atory	CYP3A4 mR	RNA	CYP3A5 mR	NA	CYP3A activity			
Factors	B or Mean±SD P		B or Mean±SD P		B or Mean±SD	B or Mean±SD P		B or Mean±SD P		B or Mean±SD P		Р	
Sex(Male)	-10.49	0.017	-2.95	0.183	-5.45	0.149	-0.281	0.544	0.042	0.810	-86.73	0.137	
Age (year)	-0.13	0.437	-0.06	0.438	-0.14	0.290	-0.028	0.085	-0.007	0.292	-2.05	0.335	
Concomitant enzyme inducer	7.82	0.184	1.98	0.501	3.77	0.465	0.002	0.998	-0.283	0.208	29.75	0.703	
Liver cancer	-3.38	0.416	-0.74	0.722	-1.76	0.619	-0.775	0.066	-0.285	0.070	-37.45	0.492	
Hepatitis B	-2.69	0.600	-0.51	0.841	-1.26	0.773	-0.402	0.454	-0.038	0.853	-69.51	0.313	
CYP3A5													
*1/*1 or *1/*3	38.41±9.55	0.012	16.19±6.77	0.038	22.87±10.44	0.022	2.412±1.849	0.044	0.980±0.719	0.001	285.9±124.9	0.020	
*3/*3	32.52±17.78	0.012	12.94±7.86	0.058	17.49 ± 14.04	0.022	1.536±1.573	0.044	0.456±0.351	0.001	243.6±236.6	0.029	
CYP3A4													
*1/*1	34.19±19.43		13.81±8.76		18.86±15.45		1.530±1.217		0.484±0.353		266.2±249.6		
*1/*1G	36.00±9.85 0.500		15.37±6.17 0.194		21.35±9.81	21.35±9.81 0.234	2.118±1.700	0.222	0.764±0.619	0.033	258.5±130.4	0.654	
*1G/*1G	33.14±6.17		10.82±5.32		15.34±9.19		3.046±2.484		1.372±1.079		231.8±165.9		

mianoDNA	CYP3A4 mRNA		CVD2A5 mDNA		CVP3A activity		Antorvastatin			Ortho-hydroxy		Para-hydroxy						
Incrokiva			t			CII SA activity		Aatoi vastatiii			atorvastatin			atorvastatin				
	r	Р	FDR	r	Р	FDR	r	Р	FDR	r	Р	FDR	r	Р	FDR	r	Р	FDR
miR-27b	-0.37	0.006	0.020	-0.25	0.068	0.221	-0.50	0.000	0.007	-0.38	0.004	0.022	-0.43	0.001	0.013	-0.46	0.001	0.013
miR-206	-0.28	0.040	0.104	-0.21	0.129	0.335	-0.38	0.005	0.016	-0.42	0.001	0.013	-0.36	0.007	0.030	-0.39	0.003	0.013
miR-103	-0.02	0.908	0.910	0.04	0.791	0.861	0.00	0.990	0.990	-0.11	0.431	0.560	0.02	0.870	0.943	0.02	0.893	0.910
miR-107	-0.02	0.910	0.910	0.03	0.839	0.861	-0.01	0.951	0.990	-0.06	0.655	0.710	-0.01	0.949	0.949	-0.02	0.910	0.910
miR-371	-0.13	0.362	0.471	-0.06	0.664	0.861	-0.09	0.535	0.696	-0.07	0.624	0.710	-0.05	0.697	0.824	-0.09	0.515	0.670
miR-491	-0.11	0.417	0.493	-0.04	0.800	0.861	0.04	0.778	0.920	-0.03	0.819	0.819	0.07	0.604	0.785	0.03	0.813	0.910
miR-1260	-0.24	0.082	0.152	-0.14	0.297	0.499	-0.23	0.087	0.126	-0.13	0.362	0.523	-0.16	0.259	0.374	-0.19	0.170	0.246
miR-21	-0.46	0.000	0.003	-0.31	0.022	0.095	-0.35	0.009	0.023	-0.37	0.005	0.022	-0.32	0.018	0.059	-0.35	0.010	0.033
miR-27a	-0.27	0.049	0.106	-0.18	0.180	0.390	-0.38	0.005	0.016	-0.27	0.045	0.117	-0.31	0.024	0.062	-0.33	0.014	0.036
miR-106	-0.16	0.253	0.365	-0.02	0.861	0.861	-0.28	0.040	0.074	-0.23	0.094	0.204	-0.27	0.050	0.108	-0.28	0.040	0.087
miR-126	-0.22	0.114	0.185	-0.14	0.307	0.499	-0.24	0.073	0.119	-0.15	0.285	0.463	-0.20	0.149	0.242	-0.24	0.078	0.127
miR-130a	-0.45	0.001	0.004	-0.32	0.016	0.095	-0.40	0.002	0.013	-0.31	0.020	0.065	-0.36	0.007	0.030	-0.39	0.003	0.013
miR-142	-0.49	0.000	0.003	-0.32	0.005	0.065	-0.31	0.020	0.043	-0.22	0.116	0.215	-0.24	0.085	0.158	-0.25	0.066	0.123

Supplementary Table 2. Impact of miRNAs on CYP3A gene expression and activity, and atovastatin metabolism.

Dependent variable	Parameter	Estimate	Partial R ²	Model R ²	P value
CYP3A4 mRNA					·
	Intercept	2.459			
	miRNA142	-231.952	0.122	0.122	0.002
	liver cancer	-0.999	0.102	0.224	0.010
	CYP3A4*1G	0.649	0.069	0.293	0.032
CYP3A5 mRNA					
	Intercept	1.245			
	CYP3A5*3	-0.474	0.189	0.189	0.001
	miRNA142	-75.145	0.094	0.282	0.005
	liver cancer	-0.304	0.067	0.349	0.028
CYP3A activity					
	Intercept	5.401			
	miR-27b	-29.334	0.200	0.200	0.002
	CYP3A4 mRNA	0.158	0.095	0.295	0.009
	miR-206	131325.000	0.058	0.353	0.037
Atorvastatin					
	Intercept	19.621			
	CYP3A activity	0.059	0.600	0.600	<.0001
Ortho-hydroxy atorvasta	atin				
	Intercept	5.538			
	CYP4A activity	0.034	0.788	0.788	<.0001
Para-hydroxy atorvastat	in				
	Intercept	4.288			
	CYP5A activity	0.059	0.839	0.839	<.0001

Supplementary Table 3. Multiple regression analysis with forward selection for modeling metabolism of atorvastatin.