SUPPLEMENTAL DATA

Human Liver Methionine Cycle: *MAT1A* and *GNMT* Gene Resequencing, Functional Genomics and Hepatic Genotype-Phenotype Correlation

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Drug Metabolism and Disposition

METHODS

1. MAT1A mammalian expression

The open reading frame (ORF) of *MAT1A* was amplified from Origene clone SC119881 (Origene, Rockville, MD), and the amplicon was subcloned into pcDNA^{TM}3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA) in frame with the V5-His tag. The sequence of the insert was verified by DNA sequencing. Site-directed mutagenesis was used to create variant allozyme expression constructs. Sequences of the variant allozyme constructs were verified by DNA sequencing, and transient expression of the constructs in COS-1 cells was performed as described previously (1, 2).

2. MAT1A and S-COMT bacterial expression and purification

The *MAT1A* WT and variant constructs were cloned into the bacterial expression vector pET28a(+) (Novagen, Madison, WI) and transformed into BL21(DE3) *E. coli* to express MAT1A-His fusion proteins for use in substrate kinetic studies. The human catechol O-methyltransferase (COMT) gene open reading frame (ORF) for the soluble form of the enzyme (S-COMT) (3) was cloned into pET28a(+) and was also expressed in BL21 *E. coli*. After purification with a Ni-NTA column (Qiagen, Valencia, CA), the bacterially expressed S-COMT protein was used as a "reagent" in the MAT enzyme assay described subsequently.

The pET-28a-MAT1A and pET-28a-S-COMT vectors were transformed into *E. coli* BL21(DE3) cells that were grown to an OD_{600} of ~0.6 at 37°C before induction with 0.4 mM IPTG and incubation overnight at 18°C. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole) with the addition of Complete protease inhibitor cocktail (Roche, Indianapolis, IN) before being disrupted using a M-110Y microfluidizer (Microfluidics, Newton, MA). After centrifugation, the supernatant was loaded onto Ni-NTA resin and was washed with 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, and 5 mM imidazole. The expressed protein was eluted with 50 mM Tris-HCl, pH 8.0, that contained 0.5 M NaCl and, sequentially, with increasing concentrations of imidazole from 50-500 mM. The combined eluted fractions were dialyzed against 50 mM Tris-HCl, pH 8.0, with 150 mM NaCl and 2 mM DTT. The WT and the Lys238 allozymes were subjected to a final purification step performed by gel filtration on a Superdex 200 column (GE Biosciences, Piscataway, NJ) equilibrated with 50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 5% glycerol, and 2 mM DTT. The void volume of the column was ~8.0 mL, and the calibration standards used were β-amylase (200 kDa, eluting at 11.8 mL), albumin (67 kDa, eluting at 13.3 mL), and ovalbumin (43 kDa, eluting at 14.5 mL).

3. MAT enzyme activity assay

Recombinant allozymes were assayed for MAT1A activity using an *L*-[methyl-^{14}C]-methionine (specific activity 55 mCi/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO) radiochemical COMT coupled assay (4, 5). This assay was a modification of the method described by Wang et al. (5). Specifically, the [^{14}C]-labeled methyl group of AdoMet was transferred enzymatically to 3,4-dihydroxybenzoic acid (DBA) in a reaction catalyzed by COMT to form [^{14}C]-methyl labeled radioactive 3-methoxy-4-hydroxybenzoic acid, as described previously (6). The reaction mixture for this step contained 5 mM Tris-HCl buffer (pH 7.4), 1 mM DBA, 4 mM ATP, 15 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, 1 mM *L*-[methyl-^{14}C]-methionine (1 μCi/μmol), 20 μg purified COMT protein, and recombinant enzyme (in 5 mM potassium phosphate buffer, pH 7.4), in a final reaction volume of 248 μL. "Blanks" consisted of reactions that lacked ATP, DBA, or enzyme. The reaction mixture was incubated at 37°C for 30 min with shaking, and the reaction was stopped by the addition of 80 μL 1N HCl. 2.5 mL of toluene was added, and the mixture was vortexed for 10 sec. After centrifugation at 700xg for 10 min at room temperature, 1.5 mL of the organic layer was aspirated and added to 4 mL of Bio-Safe II liquid scintillation counting fluid. Radioactivity was then measured in a Beckman Coulter LS6500 liquid scintillation counter (Brea, CA). For substrate kinetic experiments, *L*-methionine concentrations varied from 8 to 1000 μM and concentrations of ATP varied from 8 to 5000 μM. Apparent K_m values were calculated using Prism 4 (GraphPad Software, La Jolla, CA). Average levels of allozyme activity and protein were compared using the two-sample *t*-test (Microsoft Excel, Redmond, WA).

4. β-Galactosidase enzyme activity assay

β-Galactosidase activity was measured spectrophotometrically using the Promega β-Galactosidase Assay System (Madison, WI), and levels of recombinant MAT enzyme activity and MAT1A protein for the COS-1 cell transfection experiments were corrected on the basis of the activity of the cotransfected β-galactosidase.

5. MAT1A structural analysis

A search of the Protein Data Bank identified several crystal structures for rat MAT1A, ranging in resolution from 2.7 to 3.5 Å, as well as one human MAT1A structure at a resolution of 2.1 Å (Structural Genomics Consortium, unpublished). Both rat MAT1A and human MAT1A crystallized as tetramers organized as dimers of dimers. Due to its higher resolution and direct relevance to this study, the 2.1 Å resolution crystal structure of human MAT1A bound to AdoMet, the reaction product (PDB accession code 2OBV), was used as a starting point for the structural analysis. The crystallized human MAT1A contained residues 16-395, together with 4 additional N-terminal residues that resulted from a subcloning artifact. The human MAT1A dimer was formed by extensive interactions between two monomers related by 2-fold (180°) rotational symmetry.

References

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3. Shield AJ, Thomae BA, Eckloff BW, Wieben ED, Weinshilboum RM. Human catechol O-methyltransferase genetic variation: gene resequencing and functional characterization of variant allozymes. Mol Psychiatry 2004;9(2):151-60.
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Forward or Reverse	Region amplified by the primer pair	PCR Primer Sequence
MAT1A		
F	Exon 1	CCTAACTTTTGCTTCCCACAGT
R		CTGAAGGGCTAGAAGAGGGAAT
F	Exons 2 & 3	TACGTGTCATCCAAAATTAGGC
R		CCTTATGCCAGAGTCTTTGACC
F	Exons 4 & 5	CTGGCTAGTTAGGGAACCCC
R		ACCATTCTTTGAATGCCCAG
F	Exon 6	TTTTTGAACGTAAGAATGTGTCAGA
R		TTCACCGACTTTACATTTAGTCCA
F	Exons 7, 8 & 9	GAGTTCTGGGCTAAGGGGTC
R		TGACAGGACAGGCTAAATGAGA
GNMT		
F	5'FR, Exons 1 & 2	CCCTGTTACATTTTGTGAGTTTAAATA
R		CTAGACCTGCATACCCCACTTGT
F	Exons 2, 3, 4 & 5	CGTGTGGCAGCTGTATATCG
R		AAGTGGTACCTCAAGCCAGGA
F	Exons 5, 6 & 3'UTR	CTATAAGGTGGGGCCCTCTG
R		TGGGAGACAAACCTAGTCCTG

Supplemental Table 1. Sequence of primers used in *MAT1A* and *GNMT* resequencing amplifications for the gene resequencing studies. The genomic region amplified by the primer pair is also listed.

SNP	Primer	Size of region amplified (bp)	Primer sequences
rs9471976	Forward	214	AGG ACA GGT ACC CCC TTT TTG GTT AGG CTG TC
	Reverse		AGG ACA ACG CGT TTT TAA ATG CTT GCG TGC AG
rs11752813	Forward	225	AGG ACA GGT ACC TGT CAC CAT GTC CCA GCT AA
	Reverse		AGG ACA ACG CGT CGA AGG AAG GCA TCA GCA TA

Supplemental Table 2. Sequences of oligonucleotides used to perform PCR reactions to amplify regions containing SNPs for luciferase reporter gene constructs. Sequences for restriction sites (Acc65I for forward primer and MIu1 for reverse primer) were added at the 5'-ends of each primer used in the reporter gene studies and are underlined in the table.

SNP rs no.	Spear man correlation	p-value (raw)	p-value (corrected)	R-sq (quality score)	Chr. location	Gene location	Distance to *GNMT*
rs9471976	-0.42	6.38E-12	3.89E-10	N/A	43028527	5'-	7951 bp
rs9471974	0.38	3.62E-10	2.21E-08	0.83	43026962	5'-	9516 bp
rs9462858	0.38	5.18E-10	3.16E-08	0.68	43054468	3'-	14872 bp
rs1129187	0.38	7.53E-10	4.59E-08	0.93	43040178	3'-	582 bp
rs9462859	0.37	9.56E-10	5.83E-08	0.68	43054921	3'-	15325 bp
rs9471983	0.37	1.03E-09	6.28E-08	0.72	43048343	3'-	8747 bp
rs9986447	0.37	1.31E-09	7.99E-08	0.69	43050757	3'-	11161 bp
rs9462857	0.37	1.39E-09	8.48E-08	0.70	43051076	3'-	11480 bp
rs6941212	0.36	4.02E-09	2.45E-07	0.74	43023898	5'-	12580 bp
rs11752813	-0.32	2.88E-07	1.76E-05	N/A	43035995	5'-	483 bp
rs11752813	0.32	2.88E-07	1.76E-05	1.00	43035995	5'-	483 bp
rs73432512	0.31	4.18E-07	2.55E-05	0.49	43030043	5'-	6435 bp
rs4714634	0.29	1.98E-06	1.21E-04	0.54	43009098	5'-	27380 bp
rs6907751	0.29	2.97E-06	1.81E-04	0.67	43052828	3'-	13232 bp
rs6920547	0.29	3.24E-06	1.98E-04	0.87	43032933	5'-	3545 bp
rs3763236	0.29	3.73E-06	2.28E-04	0.55	43010486	5'-	25992 bp
rs9462860	0.29	3.77E-06	2.30E-04	0.72	43054991	3'-	15395 bp
rs2274514	0.29	3.88E-06	2.37E-04	0.91	43042478	3'-	2882 bp
rs7770760	0.28	5.05E-06	3.08E-04	0.67	43023381	5'-	13097 bp
rs9471987	0.28	5.35E-06	3.26E-04	0.70	43052118	3'-	12522 bp
rs13216214	0.28	5.97E-06	3.64E-04	0.84	43032225	5'-	4253 bp
rs4714640	0.28	6.25E-06	3.81E-04	0.83	43032402	5'-	4076 bp
rs2104616	0.28	6.82E-06	4.16E-04	0.81	43027947	5'-	8531 bp
rs58497441	0.28	7.18E-06	4.38E-04	0.79	43029539	5'-	6939 bp
rs9471988	0.28	7.26E-06	4.43E-04	0.72	43052751	3'-	13155 bp
rs4714638	0.28	7.31E-06	4.46E-04	0.80	43024995	5'-	11483 bp
rs6940837	0.28	9.24E-06	5.64E-04	0.87	43032947	5'-	3531 bp
rs4469287	0.27	1.04E-05	6.34E-04	0.82	43028822	5'-	7656 bp
rs9296404	0.27	1.39E-05	8.48E-04	0.85	43033781	5'-	2697 bp
rs6458312	0.27	1.57E-05	9.58E-04	0.53	43012252	5'-	24226 bp
rs9462855	0.26	2.32E-05	1.42E-03	0.92	43033914	5'-	2564 bp
rs9462856	0.26	2.40E-05	1.46E-03	0.93	43034002	5'-	2476 bp
rs6458313	0.26	3.72E-05	2.27E-03	0.79	43029996	5'-	6482 bp
rs9471985	0.26	3.98E-05	2.43E-03	0.81	43049302	3'-	9706 bp
rs112538187	0.25	4.39E-05	2.68E-03	0.36	43034812	5'-	1666 bp
rs9296407	0.25	4.43E-05	2.70E-03	0.80	43050429	3'-	10833 bp
rs2234185	0.25	4.61E-05	2.81E-03	0.36	43005052	5'-	31426 bp
rs57295928	0.25	4.84E-05	2.95E-03	0.32	43031068	5'-	5410 bp
rs3293	0.25	7.25E-05	4.42E-03	0.81	43046355	3'-	6759 bp
rs7759302	0.24	9.61E-05	5.86E-03	0.37	43037230	Intron 1	
rs2395943	0.24	1.04E-04	6.34E-03	0.81	43048651	3'-	9055 bp
rs2296805	0.24	1.15E-04	7.02E-03	0.91	43036736	Intron 1	
rs1129186	0.24	1.35E-04	8.24E-03	0.90	43040180	3'-	584 bp
rs73432523	0.23	2.34E-04	1.43E-02	0.32	43032054	5'-	4424 bp

Supplemental Table 3. SNPs that were significant associated with hepatic GNMT protein level after multiple comparisons (corrected p-values > 0.05 or 5.00E-02) are listed. Chromosome (chr.) locations (NCBI/hg18, dbSNP 130), Spearman correlation coefficient values, imputation (R-sq) quality scores, as well as p-values for genotype vs. GNMT protein level are listed. Location and distance to *GNMT*, NM_018960.4 (43036478 – 43039596) of the SNPs are also listed. Only SNPs with a R-sq score for imputation > 0.3 are listed. Genotyped SNPs are highlighted with grey shading. Hepatic GNMT protein levels were adjusted for age.