

A Common 5′-UTR Variant in *MATE2-K* Is Associated With Poor Response to Metformin

JH Choi¹, SW Yee², AH Ramirez³, KM Morrissey², GH Jang¹, PJ Joski⁴, JA Mefford⁵, SE Hesselson⁶, A Schlessinger^{2,7,8}, G Jenkins², RA Castro², SJ Johns⁷, D Stryke⁷, A Sali^{2,7,8}, TE Ferrin⁷, JS Witte⁵, P-Y Kwok⁶, DM Roden⁹, RA Wilke³, CA McCarty¹⁰, RL Davis⁴ and KM Giacomini²

Multidrug and toxin extrusion 2 (*MATE2-K* (SLC47A2)), a polyspecific organic cation exporter, facilitates the renal elimination of the antidiabetes drug metformin. In this study, we characterized genetic variants of *MATE2-K*, determined their association with metformin response, and elucidated their impact by means of a comparative protein structure model. Four nonsynonymous variants and four variants in the *MATE2-K* basal promoter region were identified from ethnically diverse populations. Two nonsynonymous variants—c.485C>T and c.1177G>A—were shown to be associated with significantly lower metformin uptake and reduction in protein expression levels. *MATE2-K* basal promoter haplotypes containing the most common variant, g.–130G>A (>26% allele frequency), were associated with a significant increase in luciferase activities and reduced binding to the transcriptional repressor myeloid zinc finger 1 (MZF-1). Patients with diabetes who were homozygous for g.–130A had a significantly poorer response to metformin treatment, assessed as relative change in glycated hemoglobin (HbA_{1c}) (–0.027 (–0.076, 0.033)), as compared with carriers of the reference allele, g.–130G (–0.15 (–0.17, –0.13)) ($P = 0.002$). Our study showed that *MATE2-K* plays a role in the antidiabetes response to metformin.

The kidneys play an important role in the elimination of many endogenous compounds and drugs from the body.¹ Elimination processes in the kidney are driven, in part, by membrane transporters expressed on the basolateral and apical membranes of renal epithelial cells, working in sequential steps to mediate tubular secretion. In 2005, Moriyama *et al.* identified and functionally characterized the human H⁺/organic cation antiporter multidrug and toxin extrusion 1 (*MATE1*),² and in 2006, Inui *et al.* characterized a paralog, *MATE2*.³ *MATE1* is an H⁺/organic cation antiporter on the apical membrane of the kidney tubules and on bile canaliculi. It uses a countergradient of protons to drive the transmembrane flux of organic cations into urine and bile. Various drugs, including metformin, as well as endogenous substances such as guanidine, have been shown to be substrates of *MATE1*.⁴ The finding that the renal clearance of metformin and cephalixin

is lower in *Mate1* knockout mice suggested the importance of *MATE1* in the renal tubular secretion of drugs *in vivo*.^{5,6} Genetic variants in the basal promoter and exon regions of *MATE1* have been previously identified and characterized by our group^{7,8} as well as by others.^{9,10} Importantly, some clinical studies have found an association between *MATE1* genetic variants and response to metformin in patients with diabetes.^{11,12}

Much less is known about *MATE2*. Two isoforms of *MATE2* have been identified, one of which, *MATE2-K*, has been characterized as a membrane transporter in the kidney.³ *MATE2-K* is expressed specifically in the kidney and, like *MATE1*, it appears to transport an array of structurally diverse compounds, including many cationic drugs and endogenous compounds.³ Recently, Komatsu *et al.*¹³ characterized isoform 1 of *MATE2* (NP_690872). Their study showed that both human *MATE2*

The first two authors contributed equally to this work.

¹Department of Pharmacology, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, Seoul, Korea; ²Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, California, USA; ³Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA; ⁴Kaiser Permanente Georgia, Center for Health Research Southeast, Atlanta, Georgia, USA; ⁵Department of Epidemiology and Biostatistics, University of California, San Francisco, San Francisco, California, USA; ⁶Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California, USA; ⁷Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California, USA; ⁸California Institute for Quantitative Biosciences, University of California, San Francisco, San Francisco, California, USA; ⁹Department of Pharmacology and Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA; ¹⁰Center for Human Genetics, Marshfield Clinic Research Foundation, Marshfield, Wisconsin, USA. Correspondence: KM Giacomini (kathy.giacomini@ucsf.edu)

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(isoform 1) and MATE2-K (isoform 2) (i) operate in the kidney as electroneutral H⁺/organic cation exchangers; (ii) express and localize in the kidney, with MATE2-K being slightly more abundant than MATE2; (iii) transport tetraethyl ammonium (TEA); and (iv) have similar inhibitor specificities.¹³ Two nonsynonymous single-nucleotide polymorphisms (SNPs), Lys64Asn and Gly211Val, with allele frequencies of 0.6 and 1.7% respectively, identified in 89 healthy Japanese subjects, exhibit decreased transport activities.⁹ In fact, Gly211Val results in complete loss of transport activity due to loss of protein expression on the cell surface.

In the current study, we elucidated genetic variations in the basal promoter and exonic regions of *MATE2-K*, identified by sequencing 272 DNA samples from four major US ethnic groups. We discovered several genetic variants with significant functional effects (Pro162Leu (c.485C>T); Gly393Arg (c.1177G>A) and g.-130G>A). Consistent with its enhanced

basal promoter activity, g.-130G>A was associated with reduced clinical response to metformin in US diabetes patients who were of African or European origin. Pharmacogenomic studies of metformin disposition and response have focused on genetic variants in candidate transporters for metformin such as OCT1, OCT2, OCT3, and MATE1^{12,14–18} and in candidate genes associated with metformin targets such as AMPK and PRKAG2.¹² To our knowledge, this is the first study to examine the effect of variants in *MATE2-K* on response to metformin in ethnically diverse US populations. Our study suggests that MATE2-K plays an important role in the efficacy of metformin.

RESULTS

Genetic polymorphisms and linkage disequilibrium analysis of the coding and basal promoter region of *MATE2-K*

We sequenced the coding and the basal promoter region of *MATE2-K* in 272 DNA samples from ethnically diverse US

Table 1 Genetic variants of *MATE2-K* identified in 272 DNA samples (68 from each of the four major ethnic groups)

rs no. (dbSNP) or PMT ID	Genomic position	CDS position	Nucleotide substitution	Strand	Feature	Amino acid substitution	Minor allele frequency			
							African Americans	European Americans	Asian Americans	Mexican Americans
<i>Coding variants</i>										
PMT5599	19559039	207	G>A	–	Exon	Ser69Ser	0.000	0.000	0.037	0.000
rs115261507	19557846	327	C>T	–	Exon	Gly109Gly	0.025	0.000	0.000	0.017
rs4924792	19557828	345	C>A	–	Exon	Gly115Gly	0.458	0.306	0.492	0.364
PMT5634	19556609	485	C>T	–	Exon	Pro162Leu	0.056	0.000	0.000	0.000
PMT5640	19551676	702	G>A	–	Exon	Lys234Lys	0.000	0.008	0.000	0.000
rs4925042	19549365	885	C>T	–	Exon	Tyr295Tyr	0.538	0.336	0.750	0.545
rs34169093	19548024	969	G>A	–	Exon	Ala323Ala	0.200	0.023	0.000	0.038
rs34399035	19525563	1177	G>A	–	Exon	Gly393Arg	0.000	0.009	0.000	0.008
rs34416664	19525344	1320	C>G	–	Exon	Ala440Ala	0.000	0.009	0.000	0.008
rs113679066	19522778	1514	C>T	–	Exon	Thr505Ile	0.007	0.000	0.000	0.000
rs35526408	19522720	1572	C>T	–	Exon	His524His	0.000	0.000	0.000	0.007
rs113234335	19522719	1573	G>A	–	Exon	Ala525Thr	0.007	0.000	0.000	0.000
rs71369435	19522621	1671	C>T	–	Exon	Leu557Leu	0.000	0.007	0.000	0.000
<i>Basal promoter variants^a</i>										
PMT5598	19560626	–166	C>G	+	5'-UTR	None	0.015	0.000	0.000	0.000
rs12943590	19560590	–130	G>A	+	5'-UTR	None	0.277	0.262	0.485	0.341
PMT5596	19560506	–46	G>A	+	5'-UTR	None	0.008	0.000	0.000	0.000
PMT5595	19560505	–45	C>T	+	5'-UTR	None	0.000	0.000	0.007	0.000
<i>Noncoding variants</i>										
rs9900497	19557985	IVS2 (–107)	C>A	–	Intron	None	0.475	0.290	0.531	0.364
rs35263947	19550696	IVS7 (–30)	G>A	–	Intron	None	0.123	0.285	0.493	0.353
rs12942065	19550539	IVS9 (+14)	G>C	–	Intron	None	0.731	0.646	0.956	0.691
rs58045813	19549240	IVS10 (+101)	C>T	–	Intron	None	0.062	0.000	0.000	0.000
rs16960349	19546969	IVS12 (+28)	C>G	–	Intron	None	0.055	0.000	0.000	0.000
rs60425436	19523769	IVS16 (+88)	T>C	–	Intron	None	0.031	0.000	0.000	0.000

Nucleotide location of the variant is based on the translational start site according to the *MATE2-K* mRNA sequence NM_001099646.

CDS, coding sequence; PMT, pharmacogenomics of membrane transport; SNP, single-nucleotide polymorphism; UTR, untranslated region.

^aThe number for the basal promoter variants (–250 to +50 bp of the transcription start site) refers to the nucleotide position upstream from the MATE2-K start codon.

Table 2 Frequencies of the common haplotypes in the *MATE2-K* basal promoter region

Haplotype number	g.-166C>G	g.-130G>A	g.-46G>A	g.-45C>T	Minor allele frequency			
					African Americans	European Americans	Asian Americans	Mexican Americans
H1	C	G	G	C	0.708	0.738	0.507	0.659
H2	C	<u>A</u>	G	C	0.269	0.262	0.485	0.341
H3	<u>G</u>	G	G	C	0.016	0.000	0.000	0.000
H4	C	G	G	<u>T</u>	0.000	0.000	0.007	0.000
H5	C	<u>A</u>	<u>A</u>	C	0.008	0.000	0.000	0.000

The minor alleles are shown in bold with underlines. Data were obtained from DNA samples from 272 unrelated individuals: 68 from each of four major ethnic groups. Position of the variant is based on the translational start site.

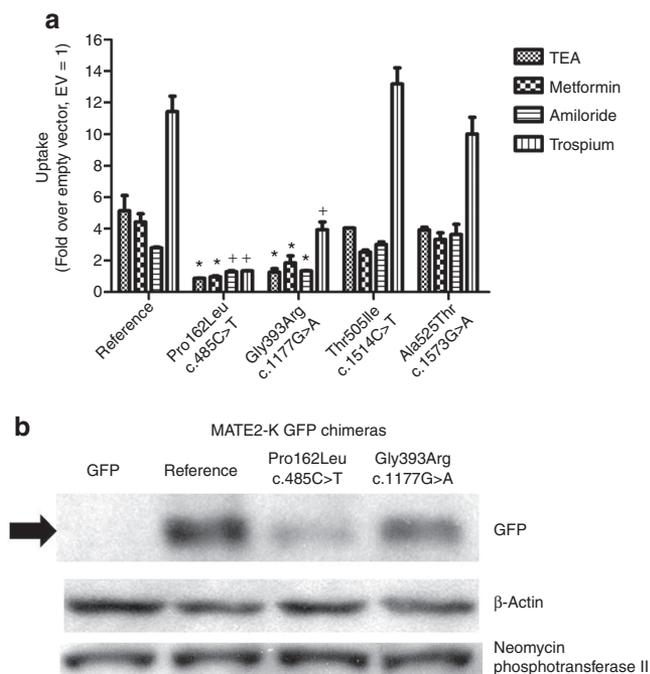


Figure 1 Effect of *MATE2-K* nonsynonymous variants on (a) transport of four compounds by HEK-293 cells expressing *MATE2-K* reference and (b) protein levels of *MATE2-K* green fluorescent protein (GFP) chimeras. (a) Functional activities were measured 48 h after transfection of plasmids containing *MATE2-K* reference or its variants into HEK-293 cells. The cells were incubated with various substrates including tetraethyl ammonium (TEA) (5 μmol/l), metformin (10 μmol/l), amiloride (10 μmol/l), and trospium (10 μmol/l) for 5 min each, and the uptake of each drug was determined. The functional activity of each construct was compared with that of the empty vector (pcDNA5-FRT). The data shown represent mean values ± SE from triplicate wells in a representative experiment. * $P < 0.05$, + $P < 0.01$ vs. the reference. (b) *MATE2-K* protein levels were determined by immunoblotting with an antibody against GFP. Cells were harvested 48 h after transfection of HEK-293 cells with *MATE2-K* reference or its variants—pcDNA3.1 vectors tagged with GFP. Proteins were blotted with antibodies against GFP, β-actin, and neomycin phosphotransferase II. β-actin was used as an internal control and neomycin phosphotransferase II was used as a *cis*-transfection marker. Expression levels were compared with that of the reference.

populations and identified genetic variants in these regions (Tables 1 and 2). Four nonsynonymous variants were identified, one of which, c.485C>T (Pro162Leu), was found to have an allele frequency >5% in African Americans. Four variants were identified in the basal promoter region. The variant g.-130G>A had the highest allele frequency in each of the four ethnic groups

(Tables 1 and 2). Table 2 shows the frequencies of the common haplotypes in the *MATE2-K* basal promoter region. Haplotype 1 (H1), which consists of all the major alleles, had the highest frequency, followed by haplotype 2 (H2), containing g.-130G>A. Among the genetic variants described in Tables 1 and 2, only two intronic SNPs (rs9900497 and rs35263947) and one synonymous SNP (rs4924792) were in linkage disequilibrium ($r^2 > 0.8$) with the 5'-untranslated region (UTR) variant g.-130G>A in European, Asian, and Mexican Americans. In African Americans, the two intronic SNPs, rs9900497 and rs3526397, and the synonymous SNP rs4924792 were in poor linkage disequilibrium ($r^2 = 0.38, 0.41, \text{ and } 0.17$, respectively) with the 5'-UTR variant.

Functional studies of *MATE2-K* variants in the coding region

Through uptake studies in human embryonic kidney (HEK)-293 cells transfected with *MATE2-K* reference or its variants, we investigated whether nonsynonymous variants of *MATE2-K* affect its transport activity. After the cells were incubated with various compounds such as TEA, metformin, amiloride, and trospium for 5 min, the uptake of each was determined. TEA and metformin have been previously identified as *MATE2-K* substrates,⁴ whereas amiloride and trospium were tested as *MATE2-K* substrates for the first time in this study. The uptake levels of TEA and metformin in *MATE2-K*-reference HEK cells were 5.1 ± 1.0 and 4.4 ± 0.5 times greater, respectively, than those of empty vector-transfected HEK-293 cells (Figure 1a). The uptake levels of TEA and metformin were significantly reduced in HEK-293 cells expressing two *MATE2-K* variants, c.485C>T and c.1177G>A (three- to fivefold reduction in uptake as compared to the reference). In addition, the uptake of amiloride and trospium in *MATE2-K*-reference HEK cells were 2.8 ± 0.1 and 11.4 ± 1.0 times greater, respectively, than those of empty vector-transfected HEK-293 cells, suggesting that both compounds are substrates of *MATE2-K*. The rates of transport of metformin, TEA, amiloride, and trospium by the variants c.1514C>T and c.1573G>A were comparable to those of the reference *MATE2-K* (Figure 1a).

Effect of genetic variants on expression levels of *MATE2-K* in HEK-293 cells

MATE2-K mRNA levels in HEK-293 cells harvested 30 h after transient transfection of the four nonsynonymous variants were comparable to the corresponding level in the reference cells (data not shown). We next assessed whether the nonsynonymous variants had any effect on *MATE2-K* protein expression levels.

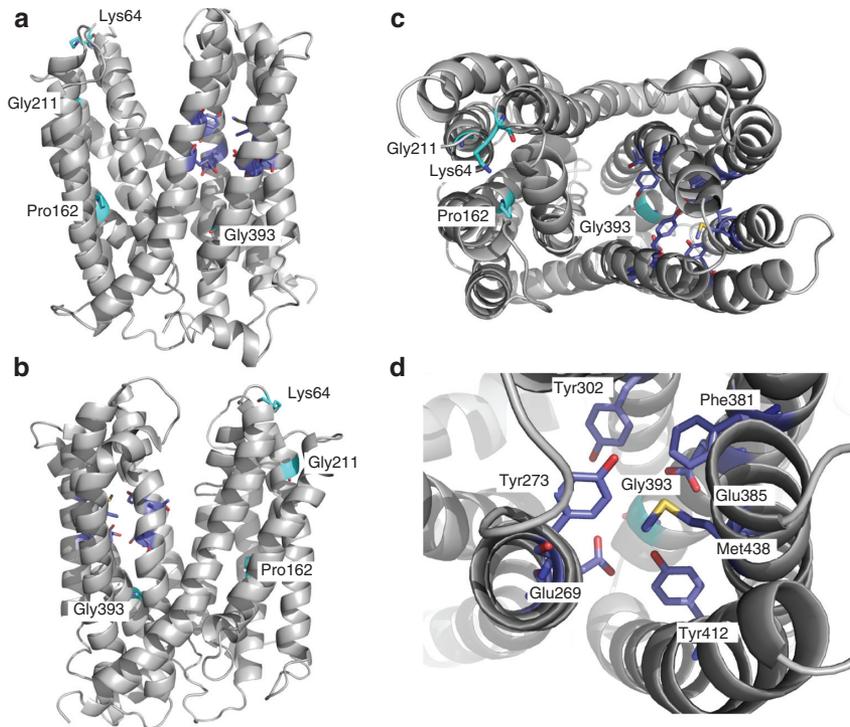


Figure 2 MATE2-K comparative model. **(a,b)** Side views and **(c)** top view of the predicted structure of MATE2-K. The backbone atoms of the model are depicted as a gray ribbon. Key residues are displayed as sticks; residues constituting the nonsynonymous variants are shown in cyan, and the putative proton binding site is shown in magenta. Oxygen, nitrogen, and hydrogen atoms are shown in red, blue, and white, respectively. **(d)** Top view of the putative proton binding site. In this model, two nonsynonymous variants, Pro162Leu (c.485C>T) and Gly393Arg (c.1177G>A), were discovered via Pharmacogenomics of Membrane Transporter (PMT) Projects (<http://www.pharmacogenetics.ucsf.edu>), and two nonsynonymous variants, Lys64Asn and Gly211Val, were previously reported by Japanese investigators.⁹ Also see **Supplementary Figure S1** online for the putative secondary structure of MATE2-K showing the positions of four nonsynonymous variants and nine synonymous variants.

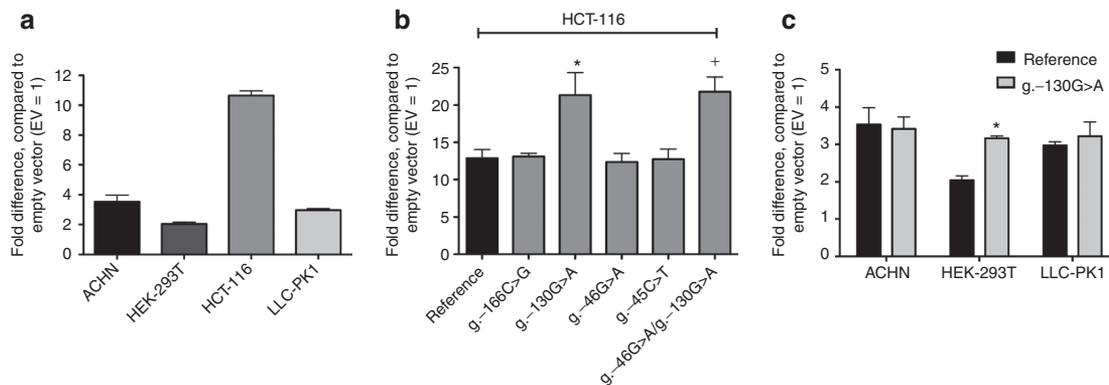


Figure 3 Reporter activities of MATE2-K reference basal promoter haplotype and variant haplotypes. Luciferase activities were measured 30 h after transfection with the reference plasmid in **(a)** four cell lines: ACHN (human kidney adenocarcinoma), HEK-293T (human embryonic kidney), HCT-116 (human colon carcinoma), and LLC-PK1 (porcine kidney epithelial); **(b)** the reference and variant plasmids in HCT-116 cell lines; and **(c)** the reference and g.-130G>A plasmids in ACHN, HEK-293T, and LLC-PK1. The reporter activity of each construct was compared with that of the empty vector (pGL4.11b(*luc2*)). The data shown represent mean values \pm SD from triplicate wells in a representative experiment. * $P < 0.05$, * $P < 0.01$ vs. the reference.

HEK-293 cells were transiently transfected with pcDNA3.1 plasmids containing the chimeras, green fluorescent protein (GFP)-MATE2-K reference and nonsynonymous variants (c.485C>T and c.1177G>A), or GFP (GFP vector only). We measured the GFP-tagged MATE2-K protein expression by means of immunoblotting, using an antibody against GFP. Consistent with their effect of reducing uptake, the MATE2-K variants c.1177G>A and c.485C>T resulted in expression levels that were lower by

31 and 66%, respectively, as compared with the reference cells (**Figure 1b**). The lower total protein levels of MATE2-K in the stable cell lines probably resulted in correspondingly lower levels on the plasma membrane; however, this was not determined.

Structure-based analysis of genetic variants of MATE2-K

The structure of MATE2-K was modeled on the basis of alignment with the X-ray structure of its recently crystallized

homolog from *Vibrio cholerae*, NorM.¹⁹ The model includes the channel residues and 12 of the 13 predicted transmembrane helices. Most of the residues constituting the putative proton-binding site in NorM are conserved in MATE2-K, reflecting the functional similarity between MATE2-K and NorM (Supplementary Figure S2 online). We mapped onto the model the genetic variants discovered in our cohort as well as the variants reported in healthy Japanese volunteers.⁹ In total, four of the six nonsynonymous variants could be mapped onto the comparative model (Lys64Asn, Pro162Leu, Gly211Val, and Gly393Arg) (Figure 2a-d). The model provided a rationale for the negative effects of these four variants on MATE2-K function. Gly393 is located in the channel in relatively close proximity to the putative proton-binding site. Therefore, mutation of Gly393 to the positively charged arginine is likely to prevent MATE2-K interaction with cationic ligands, including protons. Pro162 and Gly211 are located in transmembrane helices 4 and 6, respectively. Mutations of these residues to leucine and valine, respectively, are likely to impact MATE2-K structures through (i) a decrease in the main chain's flexibility (Gly) or rigidity (Pro), (ii) disruption of the helix-helix packing, and (iii) disruption of ligand transport, especially for Pro162, which is in close proximity to the channel. Lys64 is placed in the extracellular loop near the membrane-spanning residues; mutation to the uncharged asparagine residue can affect MATE2-K interaction with the membrane phospholipid head groups and other molecules as well as its posttranslational modifications.

Basal promoter activity of MATE2-K variants

To characterize the effects of the basal promoter variants, we performed luciferase gene reporter assays using MATE2-K-pGL4.11b. Of the four cell lines tested, the MATE2-K reference-HCT-116 cells showed the highest promoter activity (Figure 3a). HCT-116 cells were used in further reporter assays to examine the basal promoter activities of the variants (Figure 3b), whereas kidney cell lines (ACHN, HEK-293T, and LLC-PK1) were used to examine the basal promoter activities of the common haplotypes H1 (reference) and H2 (g.-130G>A) (Figure 3c, Table 2). The basal promoter activity levels of haplotypes 2 and 5, both of which contain the g.-130G>A variant, showed 60–65% higher reporter activity than the reference in HCT-116 and HEK-293T cells (Figure 3b,c).

Effect of the variant g.-130G>A on the binding affinity of MZF-1 within the MATE2-K basal promoter region

To determine the mechanism through which g.-130G>A enhances transcription rate, we used predictive algorithms to determine which of the transcription factors could bind to the MATE2-K, g.-130G>A 5'-UTR. The transcription factor myeloid zinc finger 1 (MZF-1) was identified using the Delta-Match query tool (David W. Williamson, personal communication). This query tool predicted that the g.-130G allele would bind with a higher affinity to MZF-1 than the g.-130A allele would. MZF-1 is known to regulate transcription during hematopoiesis, and it functions as an oncogene or tumor suppressor.^{20,21}

We used electrophoretic mobility shift assays (EMSAs) to determine whether MZF-1 could bind to the 5'-UTR containing the g.-130G>A site. Labeled oligonucleotides were incubated with nuclear protein extracts (25 µg) obtained from HCT-116 cells. There was a clear band when the MZF-1 probe was incubated

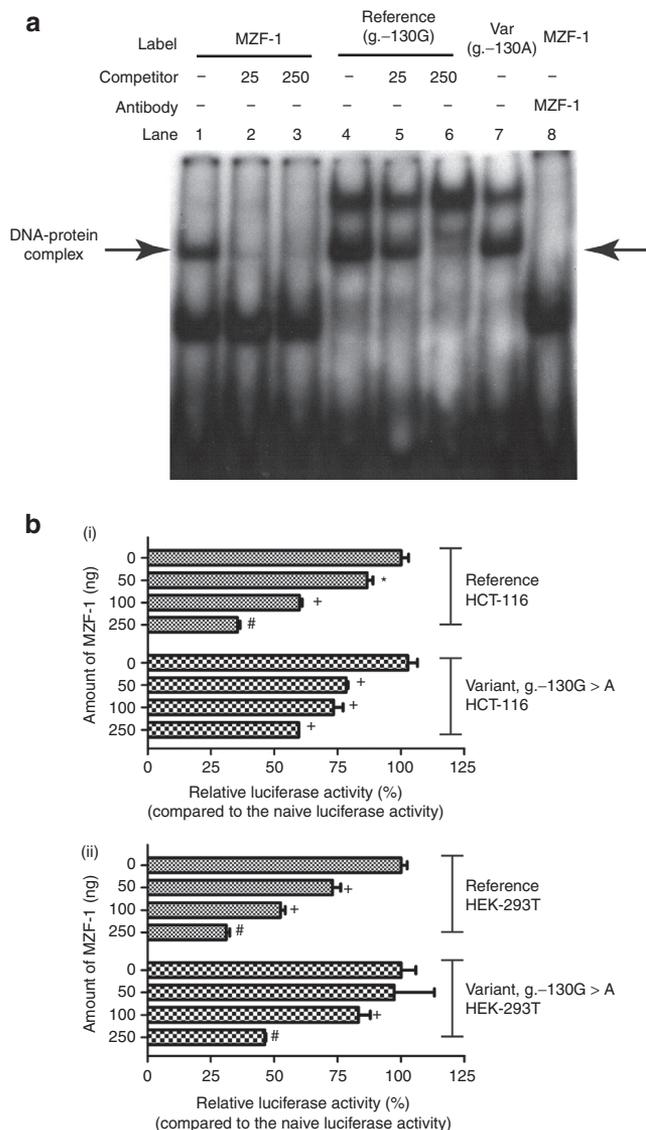


Figure 4 Effect of myeloid zinc finger 1 (MZF-1) on the binding and activity of the MATE2-K 5'-UTR variant, g.-130G>A. (a) Electrophoretic mobility shift analysis of MZF-1, MATE2-K reference, and MATE2-K variant g.-130G>A oligonucleotides. Oligonucleotides (MZF-1: lanes 1–3 and 8; reference g.-130G: lanes 4–6; variant g.-130A: lane 7) were incubated with nuclear protein extracts (25 µg) obtained from HCT-116 (human colon carcinoma) cells. The arrow indicates the position of the DNA–protein complex. Competition reactions and supershift assay were performed using different folds (25 (lanes 2, 5) and 250 (lanes 3, 6)) of molar excess of unlabeled consensus MZF-1 oligonucleotides and antibody against MZF-1 (lane 8). (b) Luciferase activities were measured 30 h after the cotransfection of the reference or variant reporters and various amounts of MZF-1 plasmids into (i) HCT-116 and (ii) HEK-293T (human embryonic kidney) cells. The reporter activity of each construct was compared with that of the empty vector (pGL4.11b(*luc2*)). The data shown represent mean values ± SD from triplicate wells in a representative experiment. **P* < 0.05, +*P* < 0.01, #*P* < 0.001 vs. the naive promoter activity.

with nuclear protein extract (lane 1, **Figure 4a**). The intensity of the band was reduced when a competition assay was performed using various concentrations of unlabeled MZF-1 consensus oligonucleotides (lanes 2 and 3, **Figure 4a**). These results suggest that MZF-1 is present in the complex. Both MATE2-K reference and variant probes formed DNA–protein complexes, but the intensity of the complex formed with the variant probe was lower by 13% as compared to the reference (lanes 4 and 7, **Figure 4a**). The intensities of the reference probe–protein complex bands were reduced with the competition assay using unlabeled MZF-1 consensus oligonucleotides, thereby indicating that these bands are complexes of DNA with MZF-1 (lanes 5 and 6, **Figure 4a**). A supershift assay was performed using an antibody against MZF-1 to demonstrate the specific binding of MZF-1 to this 5′-UTR of MATE2-K DNA (lane 8, **Figure 4a**). The addition of MZF-1 antibody resulted in the disappearance of the DNA–protein complex band (arrow), probably because the complex with the antibody shifted to a higher molecular weight. The supershifted band was not detectable with the labeled MZF-1 probe (lane 8, **Figure 4a**), probably because of the low intensity of the shifted bands. Other researchers have previously observed barely detectable bands in supershift assays.^{22,23}

Effect of MZF-1 on MATE2-K basal promoter activity

We examined the effect of MZF-1 on MATE2-K basal promoter activity by conducting a luciferase assay after overexpression of MZF-1 in HCT-116 and HEK-293T cells. Luciferase activity levels were measured 30 h after cotransfection of the MATE2-K reference or variant (g.–130G>A) reporter plasmids with increasing amounts of plasmids containing MZF-1. We observed that MZF-1 produced a dose-dependent repression of MATE2-K basal promoter activity, and that the repression was particularly

strong in the presence of the reference g.–130G in both HCT-116 and HEK-293T cells (**Figure 4b** (i) and (ii)). Using publicly available microarray expression data, we observed high expression levels of MZF-1 and MATE2-K in kidney tissues as compared with kidney carcinoma cell lines (**Supplementary Table S3** online).

Association between the MATE2-K 5′UTR variant and initial metformin response in type 2 diabetes mellitus patients

In a retrospective study, a total of 253 diabetes patients (189 self-reported Caucasians and 64 self-reported African Americans) met the study criteria and the quality-control standards for DNA and genotyping (**Supplementary Figure S4** online). **Table 3** shows the baseline characteristics of the patients with type 2 diabetes mellitus (T2DM) in whom metformin monotherapy was initiated, categorized by clinical site (**Table 3** (i)) and ethnic group (**Table 3** (ii)). Overall, there were no significant differences in the baseline characteristics of patients from the different clinical sites and ethnic groups. However, the body mass index of the patients from BioVU was significantly lower than that of patients from Marshfield Clinic and Kaiser South East ($P < 0.01$, Kruskal–Wallis test, followed by Dunn’s multiple comparison test). In addition, patients in Marshfield Clinic had significantly lower baseline glycated hemoglobin (HbA_{1c}) levels than did patients in Kaiser South East and BioVU ($P < 0.01$, Kruskal–Wallis test, followed by Dunn’s multiple comparison test). On average, the mean baseline HbA_{1c} level was $8.04 \pm 1.42\%$ and decreased to $6.81 \pm 0.89\%$. There was no statistically significant difference in the relative change in HbA_{1c} level among the three cohorts (Kruskal–Wallis test, $P = 0.09$) or between the two ethnic groups (Mann–Whitney test, $P = 0.56$). The average time between baseline HbA_{1c} measurement and

Table 3 Baseline characteristics of patients with type 2 diabetes on metformin monotherapy. The baseline characteristics are compared across (i) the three clinical sites and (ii) ethnicity

	(i) Clinical site			(ii) Ethnicity		Total
	Marshfield Clinic	Kaiser South East	BioVU	Caucasian	African American	
Number	105	73	75	189	64	253
Male	50 (47.6%)	23 (31.5%)	37 (49.3%)	94 (49.7%)	16 (25.0%)	110 (43.5%)
Female	55 (52.4%)	50 (68.5%)	38 (50.7%)	95 (50.3%)	48 (75.0%)	143 (56.5%)
Caucasian	105	20	64	—	—	189 (74.6%)
African American	0	53	11	—	—	64 (25.4%)
<i>Quantitative traits</i>						
Age (years)	57 ± 13	59 ± 11	57 ± 13	57 ± 13	58 ± 12	57 ± 12
BMI (kg/m ²)	37.5 ± 8.6 (35.7)	38 ± 8.6 (36.1) ^a	32.8 ± 6.0 (31.1) ^b	36.0 ± 8.5 (34.2) ^c	35.0 ± 6.0 (34.0) ^d	35.9 ± 8.1 (34.2)
Baseline HbA _{1c} (%)	7.72 ± 1.26 (7.50)	8.22 ± 1.39 (8.00)	8.32 ± 1.60 (7.90)	7.97 ± 1.42 (7.60)	8.25 ± 1.45 (8.00)	8.04 ± 1.43 (7.70)
Average metformin daily dose (mg)	901 ± 328	915 ± 454	1013 ± 472	963 ± 392	865 ± 467	938 ± 414
Creatinine clearance ^e (ml/min/1.73 m ²)	81.2 ± 21.1	82.0 ± 17.1	NA	80.2 ± 20.1 ^f	84.8 ± 17.7 ^g	81.6 ± 19.5

Data shown represent mean ± SD (median).

BMI, body mass index; GFR, glomerular filtration rate; HbA_{1c}, glycated hemoglobin; NA, not available; Scr, serum creatinine.

^aOnly 34 people with BMI information. ^bOnly 74 people with BMI information. ^cOnly 178 people with BMI information. ^dOnly 35 people with BMI information. ^eThe following equation was used to estimate creatinine clearance from serum creatinine levels: $\text{Scr. GFR (ml/min/1.73 m}^2) = 175 \times (\text{Scr})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if African American})$.⁴⁷ Weight was not required in this equation as the results are normalized to 1.73 m² body surface area, which is an accepted average adult surface area. ^fOnly 125 people with creatinine value. ^gOnly 53 people with creatinine value.

Table 4 Association analyses of MATE2-K variants with metformin response

(i) Marshfield Clinic (N = 105)		
Genotype group g.–130G>A	Number	Mean relative change (95% confidence interval)
GG	58	–0.13 (–0.16, –0.099)
GA	37	–0.13 (–0.18, –0.095)
AA	10	–0.0097 (–0.076, 0.068)
GG/GA	95	–0.13 (–0.16, –0.11)
<i>Statistical analyses</i>		
Kruskal–Wallis test (P value)		0.029
Mann–Whitney test (GG/GA vs. AA) (P value)		0.0091
Linear regression model (recessive): relative change = variant allele + dose		Coefficient = 0.13 (0.047, 0.21); P = 0.0025
(ii) Caucasians (N = 189)		
Genotype group g.–130G>A	Number	Mean relative change (95% confidence interval)
GG	98	–0.13 (–0.16, –0.11)
GA	77	–0.16 (–0.20, –0.13)
AA	14	–0.027 (–0.076, 0.033)
GG/GA	175	–0.15 (–0.17, –0.13)
<i>Statistical analyses</i>		
Kruskal–Wallis test (P value)		0.0035
Mann–Whitney test (GG/GA vs. AA) (P value)		0.0020
Linear regression model (recessive): relative change = variant allele + dose		Coefficient = 0.12 (0.052, 0.20); P = 0.00084
(iii) Caucasians and African Americans (n = 253)		
Genotype group g.–130G>A	Number	Mean relative change (95% confidence interval)
GG	139	–0.13 (–0.15, –0.11)
GA	96	–0.16 (–0.19, –0.13)
AA	18	–0.059 (–0.11, –0.0032)
GG/GA	235	–0.14 (–0.16, –0.12)
<i>Statistical analyses</i>		
Kruskal–Wallis test (P value)		0.047
Mann–Whitney test (GG/GA vs. AA) (P value)		0.031
Linear regression model (recessive): Relative change = variant allele + dose + ethnicity		Coefficient = 0.091 (0.025, 0.16); P = 0.0073
(iv) Caucasians and African Americans (n = 248): removed African-American carriers of the variant c.485C>T		
Genotype group g.–130G>A	Number	Mean relative change (95% confidence interval)
GG	139	–0.13 (–0.15, –0.11)
GA	93	–0.16 (–0.19, –0.13)
AA	16	–0.039 (–0.085, 0.017)
GG/GA	232	–0.14 (–0.16, –0.12)
<i>Statistical analyses</i>		
Kruskal–Wallis test (P value)		0.013
Mann–Whitney test (GG/GA vs. AA) (P value)		0.0078
Linear regression model (recessive): relative change = variant allele + dose + ethnicity		Coefficient = 0.11 (0.038, 0.18); P = 0.0026

Mean relative change of glycated hemoglobin (HbA_{1c}) levels were calculated for each MATE2-K g.–130G>A genotype group in the Marshfield Clinic (N = 105), Caucasians (N = 189), or the combined group of Caucasians and African Americans (N = 253). The 95% confidence intervals were calculated by nonparametric bootstrap estimates of the 95% confidence interval. Relative change = (treatment HbA_{1c} minus baseline HbA_{1c})/baseline HbA_{1c}. Relative change of –0.13 is interpreted as a decrease in HbA_{1c} level by 13% from baseline. In the linear regression model, the coefficient represents the increase (positive value) or decrease (negative value) in relative change in HbA_{1c} for those patients who carry the homozygous variant g.–130G>A allele. Boldface values signify P < 0.05.

the start of metformin therapy was 14 ± 23 days, whereas the average time between the start of metformin monotherapy and the first HbA_{1c} measurement after the initiation of treatment

was 160 ± 52 days. We evaluated the correlation between each of the following variables and relative change in HbA_{1c}: age, body mass index, gender, treatment gap (time between start of

treatment and HbA_{1c} measurement), average metformin dose, and creatinine clearance. Overall, only average metformin daily dose had a significant correlation with relative change in HbA_{1c} level ($r = -0.0015$ (95% confidence interval (CI): -0.00067 , -0.0023); $P = 0.00042$).

A total of 253 samples were successfully genotyped for the *MATE2-K* g.-130G>A variant, with 96% concordance in all the samples selected for validation by sequencing. The minor allele frequency for g.-130G>A was 28% in Caucasians and 22% in African Americans. All variants were in Hardy–Weinberg equilibrium ($P > 0.10$).

We evaluated the relative change in HbA_{1c} level for each genotype group in the (i) Marshfield Clinic alone ($N = 105$), (ii) the combined Caucasian group ($N = 189$), and (iii) the combined Caucasian and African-American group ($N = 253$) (Table 4). The results in Table 4 (i and ii) show that patients who were homozygous for the variant allele (g.130A/A) had an attenuated response to metformin. There were significant differences in relative change in HbA_{1c} between the homozygous variant group (AA) and the homozygous and heterozygous reference groups (GG and GA) in the Marshfield Clinic cohort and in the combined Caucasian group (all Mann–Whitney nonparametric P values < 0.01 ; Table 4 (i and ii)). The association was significant, but weaker, in the combined Caucasian and African-American group (Table 4 (iii)), whereas it was not statistically significant in the Kaiser South East, BioVU, or African-American cohorts (data not shown), probably because there were only a few patients who were homozygous for the variant allele (AA) in these cohorts (only 6, 2, and 4 patients, respectively). In the linear regression model, the average daily dose was added as a covariate. Based on the results for the mean relative change in HbA_{1c} in each genotype group (Table 4), linear regression analysis of reduction in HbA_{1c} levels was carried out under a recessive model for the genetic variant. The results showed that the *MATE2-K* homozygous variant allele was associated with a significantly smaller relative change in HbA_{1c} levels after initiation of metformin in the Marshfield Clinic (coefficient = 0.13 (95% CI: 0.047, 0.21); $P = 0.0025$, Table 4 (i)). The results were more significant in the combined Caucasian group (coefficient = 0.12 (95% CI: 0.052, 0.20); $P = 0.00084$, Table 4 (ii)) and remained significant in the combined Caucasian and African-American group (coefficient = 0.091 (95% CI: 0.025, 0.16); $P = 0.0073$, Table 4 (iii)).

Association between the *MATE2-K* nonsynonymous variant and initial metformin response in T2DM patients

We evaluated the effect of the common *MATE2-K* nonsynonymous variant c.485C>T on metformin response in African Americans from Kaiser South East. Of the 53 African Americans in the Kaiser South East cohort, 5 were heterozygous for c.485C>T. Although the changes were not statistically significant, there was a greater reduction in HbA_{1c} levels during treatment in the patients who were heterozygous for c.485C>T, (-0.16% (95% CI: -0.28 , -0.44) vs. -0.11% (95% CI: -0.16 , -0.070), Mann–Whitney nonparametric P values ~ 0.4). The linkage disequilibrium analysis showed that the

MATE2-K nonsynonymous variant c.485C>T was in complete linkage disequilibrium ($D' = 1.0$ (0.4–1.0), $\text{lod} = 2.62$) with the 5'-UTR variant g.-130G>A ($r^2 = 0.18$). These variants are $\sim 3,900$ bp apart. Given that the reduced-function coding variant, c.485C>T, would oppose the effect of the gain-of-function 5'-UTR variant, we removed the data for the five patients who were heterozygous for c.485C>T (two patients who were homozygous for the 5'-UTR variant g.-130G>A and three who were heterozygous for g.-130G>A) and determined the effect of the *MATE2-K* variant g.-130G>A on metformin response in the combined group of Caucasians and African Americans (Table 4, (iv)). Despite the small numbers, we saw a greater association of the homozygous variant g.-130G>A with reduced response to metformin (Mann–Whitney nonparametric P values < 0.05 in Table 4 (iii) vs. P values < 0.01 in Table 4 (iv)). Other functional variants in OCT1 (R61C, G401S, 420Del, and G465R),^{14,16–18} OCT2 (A270S),²⁴ and MATE1 (Intron 10, rs2289669)^{12,25} that have been shown to have an effect on metformin disposition or metformin response were selected to determine their associations with metformin response in this cohort. However, none of these functional variants was found to be significantly associated with relative change in HbA_{1c} (Supplementary Table S2 online).

DISCUSSION

Functional changes in the activity or expression level of *MATE2-K* caused by genetic variants in the coding or basal promoter regions of this transporter may result in changes in the expression and activity levels of drugs that are substrates for the transporter. This study was conducted to identify and functionally characterize genetic variants in the coding and basal promoter regions of *MATE2-K* in populations constituting four major ethnic groups. We identified four nonsynonymous variants in the coding region. The c.485C>T variant was found at a minor allele frequency of 5.6% in the African-American SOPHIE cohort ($N = 63$) and 4.7% in the African-American patients in the Kaiser South East cohort ($N = 53$). This novel variant has not been reported in any known SNP database, including the 1000 Human Genomes Project.²⁶

Recently, Kajiwaru *et al.*⁹ found that the nonsynonymous *MATE2-K* variant Gly211Val was associated with a complete loss of transport activity, mainly because of a decrease in *MATE2-K* protein expression. Although the allelic frequency of Gly211Val in the Japanese sample was 1.7%, it was not observed in our cohort of 68 individuals with Chinese ancestry. In our study, the two nonsynonymous *MATE2-K* variants, c.485C>T and c.1177G>A, were associated with significantly reduced transport activities in uptake assays using various cationic compounds (Figure 1). Our results from immunoblotting analysis suggest that the decreased activity levels of c.485C>T were due largely to a decrease in protein expression (Figure 1b). Consistent with the reduced function of Pro162Leu and Gly393Arg, we observed that Pro162 (located in the hydrophobic membrane domain (Figure 2)) and Gly393 (located in the hydrophilic cytoplasm site) are conserved across multiple species. By contrast, the variants that did not affect function occurred at sites that were not

evolutionarily conserved (**Supplementary Figure S3** online). The model showed that the Gly393Arg, which changes a glycine to positively charged arginine, would decrease the interaction of cationic ligands with MATE2-K (**Figure 2c,d**). This prediction is consistent with the dramatically reduced uptake of the cationic compounds in HEK-293 cells expressing the variant Gly393Arg, despite the observation that the total protein expression for Gly393Arg is comparable to that in MATE2-K reference cells. The model also predicted that two previously identified nonsynonymous variants⁹ would have deleterious effects on the transport function of MATE2-K.

The most common variant of the basal promoter region in this study, g.-130G>A, was associated with a significant increase in promoter activity in reporter assays (**Figure 3b**). Transcription factor binding site analysis suggested that MZF-1 could bind to the region encompassing g.-130G>A and that the binding affinity would be variant-dependent; MZF-1 would have a higher binding affinity for the g.-130G allele than for the minor allele. MZF-1 appears to be a repressor of the chloramphenicol acetyl transferase reporter gene in nonhematopoietic cell lines.^{27,28} MZF-1 contains 13 zinc fingers that interact with two different consensus sequences, AGTGGGGA and CGGGNGAGGGGAA, for fingers 1–4 and fingers 5–13, respectively.²¹ We observed that MATE2-K contains the sequence GGAGGAGGGGCTA, which is similar to the consensus sequence recognized by MZF-1. The variant g.-130G>A results in the sequence GGAGGAGGAGCTA, which is not as good a match as the reference is. Therefore, we predicted that MZF-1 would preferentially bind to the reference DNA sequence rather than to the variant sequence. The results of EMSA supported our hypothesis. The intensity of the DNA-MZF-1 complex was decreased in the presence of g.-130G>A (**Figure 4a**). We also demonstrated that MZF-1 represses MATE2-K transcription (**Figure 4b**), establishing that this transcription factor may be important in regulating the expression of MATE2-K. Data from microarray experiments suggest that there are higher expression levels of MZF-1 (and MATE2-K) in kidney tissue samples as compared with cancer cell lines (**Supplementary Table S3** online). These data suggest that MZF-1 could play a role in regulating MATE2-K expression levels in kidney tissue.

Previously, two separate studies have shown that the MATE1 intronic variants rs2289669 and rs8065082 have significant effects on metformin response,^{12,15} although the mechanism underlying the effects of these SNPs has not been determined. In our study group of 189 Caucasians, we observed an association of the intronic variant rs2289669 with relative change in HbA_{1c} level, almost at the level of statistical significance ($P = 0.055$) (**Supplementary Table S2** online). Unlike Becker *et al.*,¹⁵ we focused only on patients who had recently been diagnosed with T2DM and initiated on metformin monotherapy. Examination of the MATE1 intronic variant rs2289669 showed that it is not in linkage disequilibrium with the MATE2-K variants that we genotyped. Previously, we and others have shown that OCT1 reduced-function variants (R61C, 420Del, G401S, and G465R) and an OCT2 variant (A270S) are determinants of metformin

pharmacokinetics^{16,18,24,29,30} and pharmacodynamics in healthy subjects.¹⁷ However, our results (**Supplementary Table S2** online) also support findings from other studies showing that the variants may not have a significant effect on metformin response in patients with T2DM.^{12,14}

To our knowledge, there have been no studies investigating the effects of genetic variants of MATE2-K on metformin response. Although a small study in Japanese diabetes patients ($n = 48$) found no association between either of the MATE1 or MATE2-K coding region variants and metformin disposition,³¹ that study had only two patients who were carriers of the reduced-function MATE2-K variant Gly211Val. In our study, we focused on the common 5'-UTR variant g.-130G>A, which is associated with enhanced promoter activity. Our hypothesis was that individuals with this variant would eliminate metformin more quickly, and that the therapeutic effect in these patients would therefore be lower. Our clinical results were consistent with our hypothesis. That is, patients who were homozygous for the g.-130G>A variant had a markedly weaker response to metformin (only a 2.7% relative reduction in HbA_{1c} vs. 15% in all others) ($N = 189$ Caucasians, **Table 4** (ii); Mann-Whitney test P value = 0.002). With the available data, we were not able to determine whether a pharmacokinetic mechanism was responsible for the effect of the variant on metformin response and could not evaluate the contribution of this variant to metformin pharmacokinetics. The significance was weaker when we combined the Caucasian and African-American patients; however, when we removed the data for the five patients with the reduced-function nonsynonymous variant c.485C>T, the association became stronger (compare the results in **Table 4** (iii) and (iv)). We proposed that the reduced-function coding variant c.485C>T would oppose the effects of the gain-of-function 5'-UTR variant. After adjusting for the relative change in HbA_{1c} with average daily dose and ethnicity in a linear regression model, the significance of this association remained (**Table 4**).

Interestingly, the recent findings of the genome-wide association study of glycemic response to metformin in ~3,000 European T2DM patients did not identify any significant SNPs ($P < 0.0001$) in metformin transporter genes.³² However the platform used in that study did not include the MATE2-K g.-130G>A SNP, nor were any SNPs on the platform in strong linkage disequilibrium with the g.-130G>A SNP. Other studies of genetic factors associated with response to metformin have not included MATE2-K g.130G>A. Our study demonstrates that rare variants in MATE2-K, such as c.485C>T, and in other transporters that are functionally deleterious, if not considered in the analyses, may add complexity to the identification of significant common SNPs associated with response to metformin.

In conclusion, a common gain-of-function 5'-UTR variant of MATE2-K, g.-130G>A, is significantly associated with poor glycemic response to metformin in newly diagnosed T2DM patients. Future studies to evaluate its effects on metformin plasma levels and clearance will provide important information on the mechanism by which MATE2-K contributes to the anti-diabetes effects of metformin and on how the g.-130G>A

variant, in particular, modulates metformin response. The next key challenge in pharmacogenomics research is to demonstrate that the use of this genetic information will improve outcomes for patients and lead to personalized medicine in clinical practice.³³

METHODS

An overview of the methods used in this study is provided here; the details of these methods, statistical analyses, and associated references are available in the **Supplementary Data** online.

Genetic analysis of MATE2-K variants. Genomic DNA samples were collected from unrelated healthy individuals from four major ethnic groups (68 each from European Americans, African Americans, Chinese Americans, and Mexican Americans) as a part of the Study of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE).²⁹

Cellular assays. Cellular transport uptake studies and luciferase assays were performed as described previously.^{7,8,34,35} See **Supplementary Table S1** online for oligonucleotide primers used for different experiments related to molecular cloning, genotyping or EMSA.

Immunoblotting assay and real-time PCR. Both assays were performed as described^{36–38} on HEK-293 cells transfected with MATE2-K reference sequence or variants.

EMSA. EMSA was performed as previously described^{8,39} using nuclear protein extracts from HCT-116 cells.

Structure-based analysis and comparative model. The structure of MATE2-K was modeled using MODELLER-10v8,⁴⁰ based on the X-ray structure of the MATE homolog from *Vibrio cholerae*, NorM (Protein Data Bank identified 3MKT).¹⁹

Assessment of metformin response. We used large-scale DNA databanks linked to electronic medical records systems at Marshfield Clinic Research Foundation,⁴¹ Kaiser Permanente South East, Georgia,⁴² and BioVU (Vanderbilt University's DNA databank)^{43–46} to select T2DM patients who had been prescribed metformin monotherapy as their initial hypoglycemic medication. We used relative change in HbA_{1c} to assess metformin response in the first 90–270 days after the initiation of metformin monotherapy, where relative change = (treatment HbA_{1c} minus baseline HbA_{1c})/baseline HbA_{1c}.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/cpt>

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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