Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout

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Genome-wide association studies (GWAS) have successfully identified common single nucleotide polymorphisms (SNPs) associated with a wide variety of complex diseases, but do not address gene function or establish causality of disease-associated SNPs. We recently used GWAS to identify SNPs in a genomic region on chromosome 4 that associate with serum urate levels and gout, a consequence of elevated urate levels. Here we show using functional assays that human ATP-binding cassette, subfamily G, 2 (ABCG2), encoded by the ABCG2 gene contained in this region, is a hitherto unknown urate efflux transporter. We further show that native ABCG2 is located in the brush border membrane of kidney proximal tubule cells, where it mediates renal urate secretion. Introduction of the mutation Q141K encoded by the common SNP rs2231142 by site-directed mutagenesis resulted in 53% reduced urate transport rates compared to wild-type ABCG2 (P < 0.001).

Data from a population-based study of 14,783 individuals support rs2231142 as the causal variant in the region and show highly significant associations with urate levels [whites: P = 10−30, minor allele frequency (MAF) 0.11; blacks P = 10−4, MAF 0.03] and gout (adjusted odds ratio 1.68 per risk allele, both races). Our data indicate that at least 10% of all gout cases in whites are attributable to this causal variant. With approximately 3 million US individuals suffering from often insufficiently treated gout, ABCG2 represents an attractive drug target. Our study completes the chain of evidence from association to causation and supports the common disease-common variant hypothesis in the etiology of gout.

Urate is the end product of purine metabolism in humans. Humans and higher primates have much higher serum urate levels than other species because they lack the enzyme uricase, which converts urate into its breakdown product allantoin (1). Serum urate levels are highly heritable, suggesting a strong genetic component (2). Reduced excretion of urate by the kidney is the main cause for elevated urate levels (1), which can lead to gout, a painful condition affecting approximately 3 million individuals in the U.S. (3). Renal urate transport is complex and still poorly understood (1); although multiple renal urate transporters have been characterized in model systems, their role in human disease is mostly unclear. In a genome-wide association study (GWAS) of serum urate levels, we recently identified multiple SNPs in a genomic region on chromosome 4 containing the ATP-binding cassette subfamily G member 2 (ABCG2) gene as associated with urate levels and prevalence of gout (4). ABCG2 was first identified as a multidrug resistance protein (5) and has been shown to transport a wide range of structurally and functionally diverse substrates such as chemotherapeutics (6). Yet, the physiological substrate and the roles of ABCG2 in vivo have remained elusive.

Results and Discussion

ABCG2 Is a Urate Transporter. To investigate whether ABCG2 is a hitherto unknown urate transporter, we expressed human ABCG2 in Xenopus oocytes. Accumulation of radiolabeled urate in oocytes and urate efflux rates from oocytes were measured. Urate accumulation was significantly decreased by 75.5% in oocytes expressing ABCG2 compared with water-injected control oocytes (Fig. L4). In addition, ABCG2-expressing oocytes showed lower urate concentrations than oocytes expressing the known urate efflux transporter MRP4 (7). The reduced urate accumulation in ABCG2-expressing oocytes was absent in the presence of fumitremorgin C (FTC), a specific ABCG2 inhibitor, or after introduction of a mutation in ABCG2 (187T) that is known to disrupt transport of chemotherapeutic agents (5, 6) (Fig. LB). ABCG2-expressing oocytes showed significantly lower urate accumulation over a wide range of extracellular concentrations compared with control cells or cells expressing the loss-of-function mutation ABCG2 187T (Fig. LC). The reduced urate accumulation was due to ABCG2-mediated export of urate out of the cells as shown in experiments monitoring the decrease of the intracellular urate concentration over time in oocytes preloaded with radiolabeled urate (Fig. LD). Furthermore, ABCG2-mediated urate efflux was dependent on the intracellular urate concentration and significantly higher in ABCG2-expressing oocytes than in control oocytes (Fig. LE). Together, these data show that ABCG2 is a urate efflux transporter. ABCG2 in Renal Epithelial Cells. In mammals, the proximal tubule is the major site of renal urate handling (1). To study the urate transport capacity of endogenous ABCG2 in polarized renal epithelial cells, we measured urate accumulation in native LLC-PK1 cells. Inhibition of ABCG2 by FTC resulted in a significant impairment of urate export as shown by significantly higher intracellular urate accumulation compared with control cells (Fig. LF). To investigate whether ABCG2 mediates renal reabsorption or secretion of urate, we studied its subcellular localization. In polarized LLC-PK1 cells, ABCG2 was localized to the apical brush border membrane (Fig. LG), which is consistent with the recent demonstration of ABCG2 in the brush border of human proximal tubule cells (8). These data therefore establish that ABCG2 is a secretory urate transporter in the proximal tubule. Consequently, mutations in ABCG2 that increase serum urate concentrations must be loss-of-function mutations.

Impaired Urate Transport of ABCG2 Q141K. The most significant SNP in our recent GWAS of serum urate levels was the non-synonymous coding SNP rs2231142 in exon 5 of ABCG2 (4). The glutamine (Q) residue at ABCG2 position 141, encoded by


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urate concentration (H₂O [blue circles]; S187T [black squares]; WT [red triangles]; N = 10 samples each and n = 20 oocytes each for all accumulation experiments). (C) Urate accumulation is dependent on the extracellular urate concentration (H₂O [blue circles]; S187T [black squares]; WT [red triangles]; N = 10 and n = 20 each). (D) Urate efflux is dependent on the intracellular urate concentration (H₂O [blue circles]; S187T [black squares]; WT [red triangles]; N = 5 and n = 50 each). (E) Urate efflux depends on the extracellular urate concentration (H₂O [blue circles]; slope = 0.01, N = 55; ABCG2 [red triangles]; slope = 0.03, N = 55, P < 0.001). (F) FTC (5 μM) inhibits urate export in native LLC-PK₁ renal proximal tubule cells as shown by increased urate accumulation in FTC-treated cells compared to non-treated control cells (N = 6). (G) LLC-PK₁ cells express endogenous ABCG2 at the apical brush border membrane. Merged Z sections of LLC-PK₁ cells stained with BCRP1 antibody (green) and nuclear DAPI stain (blue; scale bar, 5 μm). Mean ± SEM; **, P < 0.001; *, P < 0.01.

Fig. 1. ABCG2 is a urate transporter. (A) C-14 urate accumulation data from Xenopus oocytes injected with either H₂O or mRNA coding for MRP4 or ABCG2. (B) Urate accumulation in oocytes injected with H₂O, ABCG2 WT mRNA (incubated with or without 5 μM FTC), or the nonfunctional ABCG2 mutant S187T (N = 10 samples each and n = 20 oocytes each for all accumulation experiments). (C) Urate accumulation is dependent on the extracellular urate concentration (H₂O [blue circles]; S187T [black squares]; WT [red triangles]; N = 10 and n = 20 each). (D) Urate efflux is dependent on the extracellular urate concentration (H₂O [blue circles]; S187T [black squares]; WT [red triangles]; N = 5 and n = 50 each). (E) Urate efflux depends on the extracellular urate concentration (H₂O [blue circles]; slope = 0.01, N = 55; ABCG2 [red triangles]; slope = 0.03, N = 55, P < 0.001). (F) FTC (5 μM) inhibits urate export in native LLC-PK₁ renal proximal tubule cells as shown by increased urate accumulation in FTC-treated cells compared to non-treated control cells (N = 6). (G) LLC-PK₁ cells express endogenous ABCG2 at the apical brush border membrane. Merged Z sections of LLC-PK₁ cells stained with BCRP1 antibody (green) and nuclear DAPI stain (blue; scale bar, 5 μm). Mean ± SEM; **, P < 0.001; *, P < 0.01.

the rs2231142 G allele, is highly conserved across species (Fig. 2A). Interestingly, Q141 is located in the nucleotide-binding domain of ABCG2 right next to the corresponding amino acid F508 in the nucleotide-binding domain of CFTR, a residue commonly mutated in patients with cystic fibrosis. To test whether this SNP is not only statistically associated but causally related to elevated urate levels, we introduced the mutation Q141K encoded by the rs2231142 T allele by site-directed mutagenesis. Wild-type and Q141K mutant ABCG2 showed 54% reduced urate transport rates compared with those expressing wild-type ABCG2 at similar levels (Fig. 2B and C), and decreased urate efflux across a range of intracellular urate concentrations (Fig. 2D). Our evidence for Q141K as a causal loss-of-function variant is supported by data showing reduced transport of chemotherapeutic agents by this mutation (6).

Relevance in the General Population. To investigate the genomic context and to quantify the effect of this common causal variant in the general population, we studied 10,902 self-reported white and 3,881 black participants of the Atherosclerosis Risk in Communities (ARIC) Study (9) who had serum urate measured and self-reported information on gout. Information on the SNP rs2231142, encoding the Q141K variant, was available as an imputed SNP from genome-wide Affymetrix 6.0 data along with 601 other SNPs in a 600-kb region containing ABCG2, and also genotyped directly using the TaqMan assay.

In a subset of 8,092 white participants with available GWAS data, the rs2231142 T allele showed highly significant association with higher urate levels in multivariable adjusted association analyses (P = 4 × 10⁻⁷; Fig. 3A). Other strongly associated SNPs were located in introns or downstream of ABCG2 and were in perfect or high linkage disequilibrium with rs2231142. After repeating the analyses conditional on genotype at rs2231142, no significant associations in the region remained (Fig. 3B), which supports the presence of a single common causal variant in the region.

In both white and black study participants, serum urate levels showed a graded and significant increase across genotypes at the directly genotyped rs2231142 variant (Fig. 3C). The association became more significant when adjusting for known correlates of serum urate levels: age, sex, BMI, alcohol consumption, and antihypertensive medication intake (P trend = 3 × 10⁻⁷⁰ for whites and P = 7 × 10⁻⁴² for blacks). Adjustment for sex explained the largest part of this difference, with the genetic effect in men significantly stronger than in women (4), which is in agreement with higher rates of renal ABCG2 expression in men compared with women (10). Similarly, the prevalence of gout was significantly different across genotypes in both whites and blacks (Fig. 3D). Of note, the prevalence of gout was 40% among black TT carriers, but genotypes GT and TT were pooled because of the low minor allele frequency of 3% in blacks. The multivariable-adjusted relative odds of gout per each copy of the T allele were 1.68 in both races (P = 2 × 10⁻⁷ in whites and P = 0.05 in blacks); the consistency of the effect size in groups of different ancestry further supports causality of rs2231142. In this context, it is of interest that the T allele frequency at rs2231142 among Asian populations (HapMap CHB, and JPT) is approximately 30% compared with 11% in white ARIC participants, and gout prevalence in U.S. individuals of Asian ancestry is about 3 times higher compared with individuals of European ancestry (11). Among the white study participants, population-attributable risk calculations yielded that a conservatively estimated 10% of gout cases could be attributed to the Q141K mutation.

Our findings are of substantial clinical interest because of the high prevalence of the Q141K mutation in individuals of European and Asian ancestry and the substantial population attributable risk. Gout has a high prevalence of approximately 3 million affected individuals in the U.S.; the treatment is often insufficient, with only 21% of gout patients who receive the most common urate-lowering treatment, reaching optimal serum urate levels in clinical trials (12, 13). ABCG2 therefore represents a potential drug target.

Fig. 4 summarizes and integrates our findings: based on the urate efflux capacity of ABCG2, its apical cellular localization, and the identification of a loss-of-function mutation that causes increased serum urate levels and gout, we postulate that ABCG2 is a secretory urate transporter in the proximal renal tubule. The physiological importance of ABCG2 in humans is illustrated by
the sizable differences in serum urate levels and the prevalence of gout caused by genetic variation in \textit{ABCG2}.

\textbf{Conclusion.} In summary, our study overcomes key limitations of GWAS, namely establishing gene function and determining causality of an associated genetic variant (14, 15). We show that \textit{ABCG2} is a urate efflux transporter and \textit{rs2231142} (Q141K) a loss-of-function mutation that causes hyperuricemia and gout. This completes the chain of evidence from association to causation and supports the common disease-common variant hypothesis in the etiology of gout.

\textbf{Materials and Methods}

\textbf{Functional Studies.} \textit{Xenopus laevis} oocytes. Oocytes were removed from female pigmented \textit{Xenopus laevis} (\textit{Xenopus} 1 Inc.) and defolliculated using collagenase A (Roche) in Ca\textsuperscript{2+}-free OR-2 ringer solution. Stage V-VI oocytes were selected and injected (Nanoinject II, Drummond Scientific) with 50 nL of either mRNA or H\textsubscript{2}O (as a control). All experiments were performed on the fourth day after injection. Oocytes after injection were cultured in a modified L-15 media on ice at room temperature in 50 \mu g/mL C-14 urate or overnight in 500 \mu M C-14 urate at 15 °C. After incubation the oocytes were washed in ice-cold ND96 solution 3–5 times to remove any superficial, residual C-14 urate from the oocytes. Oocytes were then pooled for scintillation counting. For the 1–2-h accumulation studies, after incubation in the C-14 urate solution, 2 oocytes were placed in each scintillation tube (N) with lysis buffer of 1 N NaOH; a total of 40–60 oocytes were lysed per tube. Total internal oocyte concentration was determined by liquid scintillation counting. Oocytes were then treated with alkaline phosphatase (Ambion) for 1 h at 37 °C and counted. Final values were corrected for the background levels determined by exposing the oocytes to carrier free C-14 urate.

\textbf{Oocyte transport experiments.} Accumulation studies were performed using the \textit{S. purpuratus} oocyte system. Oocytes were incubated with C-14 uric acid (American Radio-labeled Chemicals) in a modified seawater or H\textsubscript{2}O for 1 h at room temperature in 50 \mu g/mL C-14 urate or overnight in 500 \mu M C-14 urate at 15 °C. After incubation the oocytes were rinsed 3–5 times to remove any superficial, residual C-14 urate from the oocytes. Oocytes were then pooled for scintillation counting. For the 1–2-h accumulation studies, after incubation in the C-14 urate solution, 2 oocytes were placed in each scintillation tube (N) with lysis buffer of 1 N NaOH; a total of 60–90 oocytes were lysed per tube. Total internal oocyte concentration was determined by liquid scintillation counting. Oocytes were then treated with alkaline phosphatase (Ambion) for 1 h at 37 °C and counted. Final values were corrected for the background levels determined by exposing the oocytes to carrier free C-14 urate.

\textbf{Western blot of oocytes expressing ABCG2 WT or Q141K (monomers and dimers of ABCG2 are detected). Actin was used as a loading control. (C) Accumulation studies, after incubation in the C-14 urate solution, 2 oocytes were pooled for scintillation counting. For the 1–2-h accumulation studies, after incubation in the C-14 urate solution, 2 oocytes were placed in each scintillation tube (N) with lysis buffer of 1 N NaOH; a total of 60–90 oocytes were lysed per tube. Total internal oocyte concentration was determined by liquid scintillation counting. Oocytes were then treated with alkaline phosphatase (Ambion) for 1 h at 37 °C and counted. Final values were corrected for the background levels determined by exposing the oocytes to carrier free C-14 urate.}

\textbf{Fig. 2.} The \textit{ABCG2} Q141K mutation results in reduced urate transport. (A) Across-species comparison of the \textit{ABCG2} protein sequence. Note the close proximity of Q141 in \textit{ABCG2} to the mutational hot spot F508 in \textit{CFTR}. (B) Western blot of oocytes expressing \textit{ABCG2} WT or Q141K (monomers and dimers of \textit{ABCG2} are detected). Actin was used as a loading control. (C) Accumulation rates in oocytes expressing \textit{ABCG2} WT or Q141K normalized to \textit{ABCG2} expression level. (D) Efflux rates for \textit{ABCG2} WT and Q141K. (WT [red triangles]: slope = 0.01, N = 55; Q141K [black squares]: slope = 0.02, N = 55). Mean ± SEM; **, \(P < 0.001\).
of 20 oocytes (n) was used for each treatment. For the overnight accumulation studies, after incubation, 10 oocytes were pooled into 1 scintillation vial (N) with the lysis buffer; a total of 50 oocytes (n) was used for each treatment. For the efflux studies, 50 oocytes expressing each of the different proteins of interest were incubated overnight in 500 μM C-14 urate at 15–20 °C, washed 5 times in ice cold ND96 solution, and then, in groups of 10, placed into a well using 4% paraformaldehyde and permeablized using 0.1% Triton X 100. The oocytes were incubated in DMEM containing 500 μM urate, or without 5 μM urate, or with 10 μM urate, or with 10 μM urate and 10 μM auranofin (Sigma) secondary antibody was used (1:200) as well as a DAPI nuclear stain (Vector Labs). The cells were then blocked with 5% goat serum and the primary antibody was applied (anti-BCRP, Millipore Corp., clone BXP-21 at 1:100). An anti-mouse Cy3 (Sigma) secondary antibody was used (1:200) as well as a DAPI nuclear stain (Vector Labs). The cells were then incubated overnight in 500 μM urate, and then, in groups of 10, placed into a well using 4% paraformaldehyde and permeablized using 0.1% Triton X 100. The oocytes were then incubated in DMEM containing 500 μM urate, or without 5 μM urate, or with 10 μM urate, or with 10 μM urate and 10 μM auranofin (Sigma) secondary antibody was used (1:200) as well as a DAPI nuclear stain (Vector Labs).
data, 7,768 had data on rs2231142 by TaqMan available. Concordance of genotypes at rs2231142 obtained by TaqMan or imputed from the GWAS data was 98%.

**Genotype imputation.** For imputation, 602,642 high-quality SNPs with call rate greater than or equal to 95%, minor allele frequency greater than or equal to 1%, and conformation to Hardy-Weinberg expectations (P < 10−5) were used. Imputation was conducted using a Hidden Markov Model as implemented in MACH to a set of 2,557,232 high-quality polymorphic HapMap SNPs. For imputation, genotype data from each sample were combined with the HapMap CEPU samples. The software MACH10.16 was used to infer unobserved genotypes probabilistically based on identification of shared haplotype stretches between the study sample and the HapMap CEPU reference individuals [release 21 (build 35)]. Imputation results are then summarized for each genotypic as an “allele dosage,” a fractional value between 0 and 2, corresponding to the expected number of copies of the minor allele at that SNP.

**Outcome measures.** Uric acid was measured in serum at the baseline visit using an uricase method (19). Measurements of uric acid in 40 individuals were repeated greater than or equal to 1 week apart; the reliability coefficient was 0.91 and the coefficient of variation was 7.2% (20). Gout was self-reported at study visit 4 in answer to the question “were you ever told you had gout.” At a physiologic pH of 7.4, 98% of uric acid is present in its ionized form, urate. We therefore refer to serum urate levels rather than serum uric acid levels throughout the manuscript.

**Statistical analyses.** For the analyses of rs2231142 genotyped by TaqMan, differences in mean urate levels by genotype were evaluated using simple linear regression. Due to the low minor allele frequency of 3%, the genotype groups GT and TT were pooled among black participants. Adjusted differences in mean urate levels were tested using multivariable adjusted linear regression, assuming an additive genetic model (P trend). Prevalence of gout was graphed and crude and multivariable adjusted odds of gout were evaluated using logistic regression in both whites and blacks.

For analyses of GWAS data, mean serum urate levels were modeled using multivariable adjusted residuals in linear regression. Covariates in all regression models were age, sex, study site, BMI, antihypertensive treatment (yes/no), and alcohol consumption (g/week) at the time of uric acid measurement or ascertertainment of gout (4). All analyses were conducted separately by self-reported race and used an additive genetic model. The genomic control parameter for the GWAS analyses of serum urate levels across the entire genome was 1.02, indicating no substantial inflation of the test statistics.

Given the a priori hypothesis and prior evidence (4), the statistical significance threshold for the association analyses of rs2231142, and serum urate levels and gout was set at α = 0.05. In the analyses of the GWAS data, 601 additional SNPs at 88.9–89.5 Mb (Build 36) on chromosome 4 were analyzed, and the analyses were repeated conditional on genotype at rs2231142. The significance threshold for these analyses was set at α = 8 × 10−5, which corresponds to a Bonferroni correction for testing 601 independent markers.

Population attributable risk (PAR) was calculated using the formula PAR (%) = [2p(1−RR)]/([1 + p(1−RR)][p − 1]) × 100, where p corresponds to the proportion of individuals exposed to the risk allele (separate groups for heterozygotes and risk homozygotes) among those without gout, and RR is estimated by the respective multivariable adjusted odds ratio of gout (21). As gout is a common disease, analyses were repeated using the prevalence ratio rather than the odds ratio of gout to obtain a conservative estimate of PAR which was 10% as compared to 13% using the odds ratio.

Single SNP analyses were conducted using Stata, and GWAS analyses were performed using the ProbABEL package from the ABEL set of programs (http://mga.bionet.nsc.ru/yurii/ABEL/) (22) and the statistical software R.

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Supporting Information

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Fig. S1. Surface expression of wild type and mutant ABCG2 in Xenopus oocytes. Biotinylation experiments reveal that the protein amounts and surface expression of the Q141K mutant are similar to those of the wild-type ABCG2 transporter. Actin was used as a loading control. The blot is representative of results from oocytes taken from 2 different female frogs.
Fig. S2. Expression of *Xenopus laevis* uricase mRNA in female Xenopus tissues. (Upper) RT-PCR of RNA extracted from kidney, liver, and oocytes. The upper 458-bp bands show the uricase RT-PCR product. (Middle) No uricase PCR product was detected in reactions performed with inactivated reverse transcriptase. (Lower) The 157-bp actin RT-PCR product as a positive control. Tissues from 2 frogs produced the same results.