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# Functional variants of OCTN cation transporter genes are associated with Crohn disease

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**Crohn disease is a chronic, inflammatory disease of the gastrointestinal tract. A locus of ~250 kb at 5q31 (*IBD5*)[1,2] was previously associated with susceptibility to Crohn disease, as indicated by increased prevalence of a risk haplotype of 11 single-nucleotide polymorphisms[3] among individuals with Crohn disease, but the pathogenic lesion in the region has not yet been identified. We report here that two variants in the organic cation transporter cluster at 5q31 (a missense substitution in *SLC22A4* and a G→C transversion in the *SLC22A5* promoter) form a haplotype associated with susceptibility to Crohn disease. These variants alter transcription and transporter functions of the organic cation transporters and interact with variants in another gene associated with Crohn disease, *CARD15*, to increase risk of Crohn disease. These results suggest that *SLC22A4*, *SLC22A5* and *CARD15* act in a common pathogenic pathway to cause Crohn disease.**

By resequencing the five genes in the *IBD5* interval, we identified ten new single-nucleotide polymorphisms (SNPs; **Supplementary Table 1** online), including two in the organic cation transporter (OCTN) gene cluster (*SLC22A4* and *SLC22A5*, encoding OCTN1 and OCTN2, respectively) that are predicted to have functional effects. The first is a C→T substitution in *SLC22A4* exon 9 (1672C→T; numbered according to the cDNA sequence for *SLC22A4* in GenBank) that causes the amino acid substitution L503F. Leucine or isoleucine is conserved at this position in most OCTNs and other related transporters[4] (**Fig. 1a**), and its substitution with phenylalanine is predicted computationally (by PolyPhen[5], TMHMM2 (ref. 6) and PHAT transmembrane database[7]) to be nonconservative. The second SNP is a G→C transversion in the *SLC22A5* promoter (–207G→C), which disrupts a heat shock element (HSE) 207 bp upstream of the start codon (**Fig. 1b**).

1672C→T and –207G→C are in strong linkage disequilibrium and create a two-allele risk haplotype (TC) enriched in individuals with Crohn disease (frequency = 0.54 in affected individuals versus 0.42 in controls, $P = 0.0003$; **Table 1**). Odds ratios conferred by allele 1672T, allele –207C or the TC haplotype were similar. Population

**a**

```
        451                                                  500
hOCTN1  VFTAELYPTL VRNMAVGVTS TASRVGSIIA PYFVYLGAYN RMLPYIVMGS
mOCTN1  VFTAELYPTL VRNMAVGITS MASRVGSIIA PYFVYLGAYN RLLPYILMGS
hOCTN2  VYTAELYPTV VRNMGVGVSS TASRLGSILS PYFVYLGAYD RFLPYILMGS
mOCTN2  VYTAELYPTV VRNMGVGVSS TASRLGSILS PYFVYLGAYD RFLPYILMGS
mOCTN3  VYTAELYPTV VRNMGVGVSS TASRLGSILS PYFVYLGAYD RRLPYILMGS

                L503F
        501                                                  550
hOCTN1  LTVLIGILTL FFPESLGMTL PETLEQMQKV KWFRSGK... KTRDSMETEE
mOCTN1  LTVLIGIITL FFPESFGVTL PENLEQMQKV RGFRCGK... KSTVSVDREE
hOCTN2  LTILTAILTL FLPESFGTPL PDTIDQMLRV KGMKHRKTPS HTRMLKDGQE
mOCTN2  LTILTAILTL FFPESFGVPL PDTIDQMLRV KGIKQWQIQS QTRMQKDGEE
mOCTN3  LTILTAIITL FFPESSGVSL PETIDEMQKV KKLKQRQSLS KKGSPKESKG

        551        564
hOCTN1  NPKVLI.TAF ....
mOCTN1  SPKVLI.TAF ....
hOCTN2  RPTILKSTAF ....
mOCTN2  SPTVLKSTAF ....
mOCTN3  NVSRTSRTSE PKGF
```

**b**

```
HSE consensus   nGAAnnTTCn
HSP70           GGAATATTCC
ABCB1           AGAACATTCC
SLC22A5         GGAACCTTCC
                 –207G→C
```

**Figure 1** Sequence variants associated with Crohn disease. (**a**) Alignment of the C-terminal regions of OCTN proteins in humans (h) and mice (m). The L503F variant in the OCTN1 11th transmembrane domain (box) is indicated. (**b**) Heat shock elements (HSEs) from *HSP70*, *ABCB1* and *SLC22A5* and a consensus HSE. The –207G→C variant is indicated.

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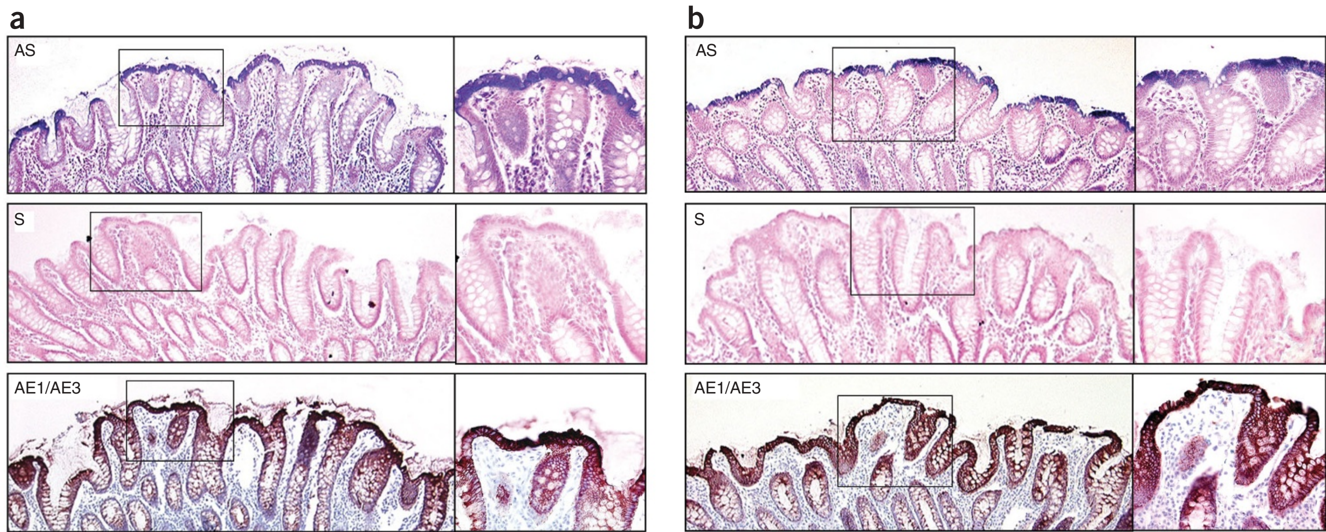


**Figure 2** Distribution of *SLC22A4* and *SLC22A5* transcripts in human colon. Photomicrographs ( 10; right boxes 40) of human colon tissue hybridized with *SLC22A4* (**a**) or *SLC22A5* (**b**) antisense probes (AS, blue stain; top panels) or sense probes (S; center panels) or subjected to immunohistochemical analysis with antibody to cytokeratin (AE1/AE3, brown stain; bottom panels).

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risk attributable to the TC haplotype was 19% for heterozygotes and 27% for homozygotes. A dose-effect test rejected recessive inheritance ($\chi^2 = 4.3$, $P = 0.04$) but supported both dominant ($\chi^2 = 1.19$, $P = 0.28$) and additive ($\chi^2 = 0.35$, $P = 0.56$) inheritance. Risk for disease in both a discovery and an independently collected replication cohort was similarly increased by presence of either the TC haplotype or at least one of the three main alleles of *CARD15* associated with Crohn disease[8]. Consistent with interaction between the *IBD5* locus and *CARD15* variants associated with Crohn disease[9], risk for disease was much greater in the presence of both the TC haplotype and the Crohn disease–associated *CARD15* alleles (**Table 1**). *IBD5* may also be associated with ulcerative colitis[10], but the TC haplotype did not influence disease risk in 216 individuals with ulcerative colitis that we studied ($P = 0.17$).

Among individuals lacking *IBD5* risk haplotypes (*i.e.*, homozygous with respect to the non-risk-associated allele of IGR2078a_1, a surrogate marker for the extended *IBD5* haplotype[3,9]), 53% of individuals with Crohn disease ($n = 99$) but only 23% of controls ($n = 120$) carried at least one 1672T or –207C allele ($P = 2 \quad 10^{-6}$). Haplotypes with at least one 1672T or –207C allele on the non-risk-associated *IBD5* background were also more frequent in individuals with Crohn disease (0.143, $n = 370$) than in controls (0.098, $n = 246$; $P = 3.5 \quad 10^{-9}$). By contrast, the haplotype containing the *IBD5* risk allele but neither of the 1672T or –207C alleles was almost as frequent among affected individuals as controls (0.012 versus 0.013). These data argue that the *SLC22A4* and *SLC22A5* 1672T and –207C variants *per se*, rather than other nearby alleles, confer risk for Crohn disease. Although the *SLC22A4–SLC22A5* interval has been hypothesized to contain an IBD-related human ortholog to the mouse gene *Slc22a9* (ref. 11), we and others were unable to identify a human *Slc22a9* counterpart or any other gene in this region.

*SLC22A4* and *SLC22A5* are widely expressed[11–14], but *in situ* hybridization data showed that they were specifically expressed in the principal intestinal cell types affected by Crohn disease, including epithelial cells (**Fig. 2**), CD68$^+$ macrophages and CD43$^+$ T cells, but not CD20+ B cells (**Supplementary Fig. 1** online). This expression pattern is consistent with the multisystem nature of Crohn disease and involvement of these genes in susceptibility to Crohn disease.

*SLC22A4* and *SLC22A5* encode the polytopic transmembrane sodium-dependent carnitine and sodium-independent organic cation transporters OCTN1 and OCTN2 (ref. 4). The L503F substitution in OCTN1 maps to a region (the 11th transmembrane domain) required for transport function[15,16] and may therefore alter this activity. We investigated OCTN1 function in fibroblasts and found carnitine uptake to be 2.7 times lower in cells expressing 503F than in those expressing 503L (**Fig. 3a**). Although both the

**Table 1 Linkage disequilibrium and Crohn disease association data**

| Data set | $n$ | $D'$ | $r^2$ | Odds ratios: Homozygous TC | Odds ratios: Heterozygous TC | Odds ratios: *CARD15* | Odds ratios: Joint TC-*CARD15* |
|---|---|---|---|---|---|---|---|
| Discovery cases | 203 | 0.95 | 0.62 | 3.43 (1.58–7.44), $P = 0.002$ | 2.10 (1.03–4.28), $P = 0.041$ | 2.10 (1.31–3.39), $P = 0.0011$ | 7.28 (2.63–24.79), $P < 0.0001$ |
| Discovery controls | 200 | 0.86 | 0.57 | – | – | – | – |
| Replication cases | 300 | 0.79 | 0.45 | 5.14 (2.52–10.45), $P < 0.001$ | 2.56 (1.42–4.60), $P = 0.002$ | 2.50 (1.54–4.04), $P < 0.001$ | 10.50 (3.31–36.27), $P < 0.0001$ |
| Replication controls | 190 | 0.99 | 0.70 | – | – | – | – |

$D'$ and $r^2$ between *SLC22A4* 1672C→T and *SLC22A5* –207G→C are indicated. Odds ratios for susceptibility to Crohn disease, 95% confidence intervals (in parentheses) and significance are shown for the TC haplotype, presence of a *CARD15* mutation and for the joint TC-*CARD15* effect.

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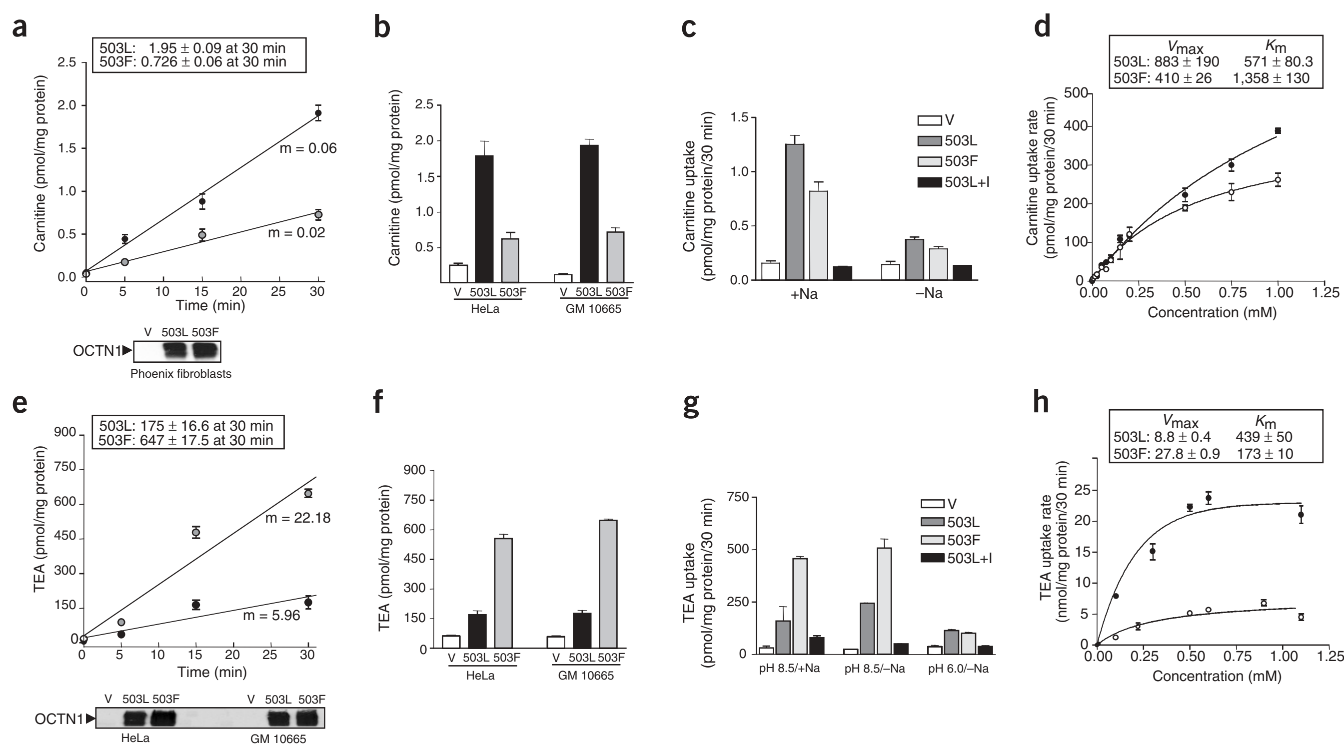


**Figure 3** Transport of carnitine and TEA by OCTN1. Transport by cells expressing the 503L variant (black circles or boxes), the 503F variant (shaded circles or boxes) or empty vector (V) over a time course (**a**,**e**) or 30 min after loading (**b**,**f**). Linear regression slopes (*m*) are indicated. Immunoblots (**a**,**e** bottom panels) show OCTN1 protein in transfected cells. Effects of sodium on carnitine uptake (**c**) and of sodium and pH on TEA uptake (**g**) are indicated. I, nonspecific inhibitor (5 mM quinidine). Concentration dependence, $V_{max}$ (carnitine, pmol per mg of protein per min; TEA, nmol per mg of protein per min) and $K_m$ ( M) of uptake of [$^3$H]-carnitine and [$^{14}$C]-TEA are shown (**d**,**h**).

503F and 503L variants transport carnitine in sodium- and concentration-dependent fashions (**Fig. 3c**,**d**), the 503F variant had a lower $V_{max}$ and higher $K_m$ for carnitine uptake (**Fig. 3d**). Although the 503F and 503L variants also transported tetraethyl ammonium (TEA) in a sodium-independent but pH-dependent manner (**Fig. 3g**), the 503F variant was associated with 3.7 times greater TEA uptake (**Fig. 3e**) and a higher $V_{max}$ and lower $K_m$ for TEA uptake (**Fig. 3h**). We observed similar effects in HeLa cells and OCTN2-deficient fibroblasts (**Fig. 3b**,**f**).

OCTN1 was previously characterized as a low-affinity carnitine transporter[17], but this conclusion was based on analysis of the 503F variant rather than the more prevalent wild-type 503L variant, shown here to be a better carnitine transporter. The 503F variant also had less affinity for carnitine and other endogenous substrates but greater affinity for TEA and various xenobiotics (**Table 2**) than the wild-type 503L variant. Collectively, these results confirm that the L503F substitution substantially alters OCTN1 function.

The –207G→C substitution in *SLC22A5* occurs within a heat-shock transcription factor (HSF)-binding element (HSE)[18,19]. We therefore used gel shift assays to evaluate inducible HSF binding to the *SLC22A5* HSE. We found that a radioactively labeled oligonucleotide corresponding to the –207G (wild-type) HSE formed high-molecular-weight complexes with nuclear extracts from heat-shocked or arachidonic acid–stimulated cells. We also found that mobilities of these complexes were supershifted by antibody to HSF1 but not by antibody to HSF2 (**Fig. 4**). In contrast, an oligonucleotide corresponding to the –207C (Crohn disease–associated) HSE formed no complexes. Like nonspecific oligonucleotides, unlabeled competitor –207C oligonucleotide had no effect on –207G binding activity (**Fig. 4a**).

We also examined effects of the –207C variant on HSF-induced *SLC22A5* transcriptional activation using a luciferase reporter driven by a *SLC22A5* promoter segment containing either –207C or –207G. Increases in promoter activity evoked by HSF were significantly greater in OCTN2-deficient fibroblasts expressing –207G than in

**Table 2 Effects of endogenous compounds and xenobiotics on TEA uptake in cells expressing OCTN1 503L and OCTN1 503F**

| | $K_i$ ( M) | |
|---|---|---|
| Compounds | 503L | 503F |
| Endogenous substrates | | |
| L-carnitine | 24.08 ± 3.01* | 3,642.0 ± 221.8 |
| Choline | 230.50 ± 47.05* | 864.9 ± 110.5 |
| N-methylnicotinamide | 76.66 ± 24.96* | 621.0 ± 59.95 |
| Competitive xenobiotics | | |
| TEA | 439.01 ± 32.31* | 172.93 ± 21.83 |
| Tetrabutyl ammonium | 535.00 ± 54.85* | 141.70 ± 25.53 |
| Tetrapentyl ammonium | 69.56 ± 0.05* | 10.67 ± 0.40 |
| Noncompetitive xenobiotics | | |
| Verapamil | 8.38 ± 0.20* | 22.31 ± 2.98 |
| Cimetidine | 434.80 ± 19.07* | 1,658.00 ± 142.0 |
| Lidocaine | 0.83 ± 0.02* | 21.06 ± 1.83 |
| Quinidine | 68.38 ± 8.89 | 60.99 ± 8.45 |

*$P < 0.05$, Student's *t*-test.

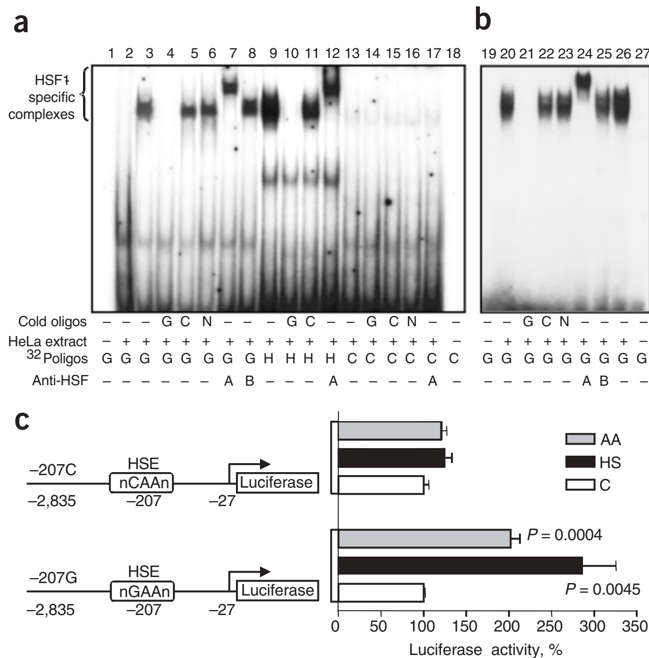


**Figure 4** Effects of *SLC22A5* –207G→C on heat shock–inducible HSE binding and promoter activity. (**a**) Gel-shift assay with radioactively labeled –207G (G), –207C (C) or *HSP70* (H) HSE and nuclear extracts from heat-shocked (lanes 1, 3–17) or untreated (lane 2) HeLa cells. Unlabeled (cold) –207G (G), –207C (C) or nonspecific (N) competitor oligonucleotides and antibodies to HSF-1 (A) or HSF-2 (B) are indicated. (**b**) Gel-shift assay in untreated cells (lane 26) or cells treated with arachidonic acid (lanes 19–25,27). (**c**) *SLC22A5* promoter assays. Luciferase activity from –207G or –207C variants left untreated (C), heat-shocked (HS) or treated with arachidonic acid (AA) and statistically significant differences from untreated cells are shown.

those expressing –207C (heat-shocked cells, 2.3 times higher; arachidonic acid–stimulated cells, 1.7 times higher; **Fig. 4c**). Increases in promoter activity evoked by HSF were also significantly greater in HeLa cells expressing –207G than in those expressing –207C (2.2 times higher, $P = 0.0001$; data not shown). These results suggest that the –207C variant disrupts a functional promoter element.

The data reported here identify variants of *SLC22A4* and *SLC22A5* that increase susceptibility to Crohn disease. Our data and a previous study of rheumatoid arthritis[20] suggest that these genes have a role in chronic inflammatory disorders. These variants may cause disease by impairing OCTN activity or expression, reducing carnitine transport in a cell-type and disease-specific manner. Defects in oxygen burst–mediated pathogen killing or impaired fatty acid β-oxidation in intestinal epithelium may ensue, the latter of which is exacerbated by bacterial metabolites and causes colitis in experimental models[21,22]. Alternatively, the effects of the 503F variant on OCTN1 transporter specificity may also diminish uptake of physiologic compounds while increasing uptake of potential toxins, such as putrescine, derived from bacterial catabolism. In either scenario, a role for OCTNs in handling enteric bacteria or their byproducts is consistent with the putative effects of *CARD15* mutations on cellular responses to bacterial products and provides a basis for the genetic interaction between these loci.

The L503F substitution probably has modest effects on OCTN1 function, perhaps substantial only under metabolic stress conditions. The *SLC22A5* promoter variant and the intronic variant in *SLC22A4* associated with rheumatoid arthritis[20] probably become important only after relevant transcription factor activation. Also, as observed for genes associated with other complex diseases[23,24], the OCTN variants are prevalent in the general population. These observations do not, however, mitigate their potential pathogenicity. The strong interaction between *SLC22A4*, *SLC22A5* and *CARD15* in Crohn disease and the suggested interaction between *SLC22A4* and *RUNX1* in rheumatoid arthritis suggest that OCTNs participate in multiple pathways underlying chronic inflammation. Our discovery provides a basis for diagnostic tests for Crohn disease risk and identifies the OCTNs as potential targets for therapeutic interventions.

## METHODS

We recruited unrelated individuals of European origin as described[1]. Replication cases were an independent cohort from the same center. We randomly selected healthy controls of European descent (average age 26 years) from 1,000 anonymous samples. We obtained ethics approval from the University of Toronto and informed consent from all subjects. Genotyping was done by single base extension (Beckman Coulter SNPstream) or allele-specific PCR[25]. Primer sequences are available on request. We determined haplotype frequencies through maximum likelihood estimation and the dose effect by a likelihood ratio test.

We transfected *SLC22A4* (1672C or 1672T) cDNAs into HeLa, GM 10665 or HEK293 fibroblasts using lipofectamine and evaluated them for carnitine or TEA uptake (in triplicate) in transport buffer (pH 8.5 or 6.0) in which NaCl was replaced isotonically with N-methyl-D-glucamine. We determined kinetics and $K_i$ values for *SLC22A4* transport and inhibition in stably transfected HEK293 by subtracting background uptake (5 mM quinidine).

We used digoxigenin-UTP-labeled sense or antisense riboprobes for whole-mount *in situ* hybridizations as described[26]. We used antibodies to digoxigenin conjugated with alkaline phosphatase to detect hybridized probes. We counter-stained sections with Fast Nuclear Red (Dako). We used monoclonal antibodies (Dako) against cytokeratin AE1/AE3 (1:100), CD20 (1:500), CD42 (1:400) and CD68 (1:100) for immunostaining.

For gel shift assays, we took nuclear extracts (10 g) from HeLa cells, left some untreated, heat-shocked some at 42 °C for 2 h and incubated some with 20 M arachidonic acid for 30 min. We incubated nuclear extracts with $^{32}$P-labeled or unlabeled (100 ) oligonucleotides (*SLC22A5* nucleotides –199 to –223 or *HSP70* HSEs[27]). For supershifts, we used antibodies to HSF1 or HSF2 (SantaCruz).

We transfected the *SLC22A5* promoter regions (–2.7 kb from ATG) carrying –207C or –207G alleles in the pRL-null reporter vector (Promega) into HeLa or GM10665 cells. After 24 h, we subjected cells heat shock at 42 °C for 2 h or incubated them with 20 M arachidonic acid for 30 min and assayed them for luciferase activity after 18 h.

**GenBank accession number.** *SLC22A4* cDNA, NM_003059.

*Note: Supplementary information is available on the Nature Genetics website.*

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COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Genetics* website for details).

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