Short Communication

Functional Analysis of Human Sodium-Phosphate Transporter 4 (NPT4/*SLC17A3*) Polymorphisms

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Abstract. We analyzed the functional properties of five nonsynonymous single nucleotide polymorphisms (SNPs) in the sodium-phosphate transporter NPT4 gene (*SLC17A3*) using the *Xenopus* oocyte expression system. NPT4 variants carrying SNP V257F, G279R, or P378L exhibited reduced transport of [¹⁴C]*para*-aminohippurate, [³H]bumetanide, [³H]estrone sulfate, and [¹⁴C]urate, when each variant clone was expressed in the plasma membrane of oocytes. This study suggests the possibility that the genetic variation of NPT4 contributes to inter-individual differences in disposition of anionic drugs such as diuretics as well as certain endogenous organic anions such as urate.

Keywords: single nucleotide polymorphism, diuretic, urate

Membrane transport proteins play an important role in the metabolism of endogenous substrates and the pharmacokinetics of drugs. Recently, it was reported that certain single nucleotide polymorphisms (SNPs) in transporters including ABC (ATP-binding cassette) transporters and SLC (solute carrier) transporters are related to their altered transport activities, which may have clinical implications (1). Thus, functional analysis of genetic polymorphism of transporters is clinically important.

Drug transporters of SLC22 and SLC17 families in the kidneys and liver mediate the transcellular transport of small organic cations and anions with different molecular structures (2). These organic ions include clinically used drugs (e.g., metformin, antibiotics, diuretics), endogenous compounds (e.g., catecholamine, prostaglandins, urate), as well as toxic substances (e.g., MPP⁺, mycotoxins). To date, several functionally relevant genetic variations in SLC22 transporters (3 – 6) were reported. In contrast, the functional characterization of genetic variations of SLC17 transporter has not been done except for sodium-phosphate transporter 1 (NPT1, *SLC17A1*) (7).

Human sodium-phosphate transporter NPT4 (hNPT4,

SLC17A3), identified recently as a novel voltage-driven organic anion transporter, expressed in the kidney and liver, participates in renal tubular efflux and elimination of various anionic drugs such as *para*-aminohippurate (PAH) and diuretics as well as in the secretion of endogenous substrates such as urate (8). In the public SNP database (NCBI dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>) five nonsynonymous SNPs of *SLC17A3* are reported, but functional analysis of these genetic variations has not been performed yet. In this report, we characterized the functional properties of the hNPT4 variants by using the *Xenopus* oocyte expression system.

Full-length cDNA of hNPT4 was obtained as described previously (8). The QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce point mutations into hNPT4 cDNA in the expression vector according to the instructions. Complementary oligonucleotides used for mutagenesis are described in Table 1. All the final sequences were confirmed by DNA sequencing.

cRNA synthesis and uptake measurements were performed as previously described (9, 10). Briefly, the cDNA was linearized with *Xba* I, and the cDNA insert transcribed in vitro with the T7 mMESSAGE mMACHINE cRNA synthesis kit (Ambion, Austin, TX, USA). Defolliculated *Xenopus* oocytes (stage IV and V) were injected with 20 ng of capped hNPT4 cRNA and

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Table 1. Summary of nonsynonymous polymorphisms of hNPT4 in NCBI database

refSNP ID	Protein residue	mRNA position	Heterozygosity	Oligonucleotide for mutagenesis	Genotype frequency	Allele frequency
rs1165165	A100T	408 (g to a)	0.340	For: 5'-gccccaagagctctctctacaaggctcctgtgtatg-3' Rev: 5'-catcacagaggcctttgttaggaagactcttggggc-3'	A/A: 0.065 A/G: 0.306 G/G: 0.629	A: 0.218 G: 0.782
rs34902660	G239V	826 (g to t)	0.025	For: 5'-gtcttctatatcttggagtggttgctgtgtctgctg-3' Rev: 5'-cagcagacacagccaactccaagatagatagaagac-3'	A/C: 0.026 C/C: 0.974	A: 0.013 C: 0.987
rs34376145	V257F	879 (g to t)	0.025	For: 5'-gttggtgatttatgatgcccttttctatccatggataagc-3' Rev: 5'-gcttatccatggataggaaggggtcatcataaatcacac-3'	A/C: 0.013 C/C: 0.987	A: 0.013 C: 0.987
rs56027330	G279R	945 (g to a)	0.500	For: 5'-ccttgaacaacaggtcaggtcttctaagcagcctc-3' Rev: 5'-gaggtgcttagaagacctgacctgttttcaagg-3'	No information	C: 0.875 T: 0.125
rs11966370	P378L	1243 (c to t)	N.D.	For: 5'-caattttaggaagtctctctcttcagcactcattgtg-3' Rev: 5'-cacaatgagtgtgaagagaggagacttctaaattg-3'	G/G: 1.000	G: 1.000

incubated at 18°C in a modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.4 mM CaCl₂, 0.8 mM MgSO₄, 2.4 mM NaHCO₃, and 10 mM HEPES] containing gentamicin (50 µg/ml). After incubation for 2–3 days, uptake experiments were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4) and high potassium solution (replaced NaCl in ND96 with equimolar KCl). The uptake experiment was initiated by replacing the ND96 solution with that containing radiolabeled 10 µM [¹⁴C]PAH, 0.05 µM [³H]bumetanide, 0.05 µM [³H]estrone sulfate (Perkin Elmer, Boston, MA, USA), and 30 µM [¹⁴C]urate (American Radiolabeled Chemicals, St. Louis, MO, USA) plus 270 µM cold urate and was terminated by adding ice-cold ND96 solution after 60 min of incubation. Oocytes were washed five times with ice-cold ND96 solution, solubilized with 5% SDS, and the radioactivity content was determined.

Two days after injection, *Xenopus laevis* oocytes, injected with cRNAs for wild-type NPT4 and mutants, were fixed with *para*-formaldehyde, and incubated with the anti-NPT4 antibody (1:50), followed by Alexa594-labeled goat anti-rabbit immunoglobulin-G (IgG) (Wako Pure Chemical Industries, Osaka; diluted 1:200). The sections were examined under an Olympus BX60 microscope equipped with a BX-FLA unit (Olympus, Tokyo).

For the uptake measurements, experiments were performed using three different batches of oocytes, and the results from the experiments were expressed as the mean ± S.E.M. Statistical significance was judged from Student's *t*-tests. Differences were considered significant at a level of *P* < 0.05.

In the public SNP database (NCBI dbSNP), we found five nonsynonymous nucleotide polymorphisms of hNPT4 (*SLC17A3*): A100T, G239V, V257F, G279R,

and P378L. As shown in Fig. 1A, A100 is located in the middle of the first extracellular loop between first and second transmembrane domains (TMDs): G239 and V257 are in the fifth TMD, G279 is in the middle of third extracellular loop between five and six TMDs, and P378 is located in the eighth TMD. Among these, A100 is conserved from hNPT1 to hNPT5, and G239 and V257 are conserved in hNPT4 and hNPT5, but G279 and P378 are found only for hNPT4 (11).

To examine whether hNPT4 polymorphisms found in the SNP database affect functional activities, we constructed site-directed mutants and expressed them in *Xenopus* oocytes. As shown in Fig. 1, B–E, V257F and G279R mutants showed the moderate reduction of uptake of all 4 substrates tested (10 µM [¹⁴C]PAH, 0.05 µM [³H]bumetanide, 0.05 µM [³H]estrone sulfate, and 300 µM [¹⁴C]urate in high potassium buffer). In contrast, the P378L mutant showed no uptake of these 4 tested substrates (no significant changes in the uptake from control oocytes). The uptake by the A100T mutant showed no significant changes from the wild-type clone for these 4 tested substrates, whereas the G239V mutant had statistically significant reduction of bumetanide uptake compared to that of the wild-type clone (Fig. 1C).

Plasma membrane localizations of NPT4 variants were confirmed by immunofluorescent analysis. Figure 2 (B–G) shows the staining of NPT4 proteins in oocytes injected with cRNAs for NPT4 variants. Wild-type (WT) and all mutant NPT4 proteins were largely localized in the plasma membrane. Loss-of-function in P378L is not due to the reduction of membrane expression, but due to the decreased functional activity of transporter per se.

In 2008, Dehghan et al. indicated that *SLC17A3*, encoding NPT4, was one of three gene loci associated with uric acid concentration and gout in their genome-wide association study (GWAS) (12). Although the locus of

Fig. 1. Functional characterization of NPT4 variants. A: Schematic representation of hNPT4 SNPs. Transmembrane topology is based on the previous report. Nonsynonymous nucleotide substitutions are indicated by arrows. B: The uptake of [¹⁴C]PAH (10 μ M) at 60 min in non-injected and NPT4 wild-type (WT) and mutant cRNA-injected oocytes was measured on day 2 after injection as described in the experimental procedures. C: The uptake of [³H]bumetanide (0.05 μ M) at 60 min in non-injected and NPT4 WT and mutant cRNA-injected oocytes was measured on day 2 after injection. D: The uptake of [³H]estrone sulfate (0.05 μ M) at 60 min in non-injected and NPT4 WT and mutant cRNA-injected oocytes was measured on day 2 after injection. E: The uptake of [¹⁴C]urate (30 μ M with cold urate 270 μ M) at 60 min in non-injected and NPT4 WT and mutant cRNA-injected oocytes was measured on day 3 after injection. The data represent the mean $\pm$ S.E.M. for 7 to 9 oocytes. * P < 0.05, ** P < 0.01, *** P < 0.001 relative to WT using Student's t -test.

SLC17A3 (rs1165205) was an intronic SNP and its association with uric acid concentration was weaker than that in the other loci in their study, this result prompted us to investigate the urate transport function of orphan transporter NPT4. Just recently, we have functionally

characterized NPT4 as a novel urate efflux transporter and have reported that two loss-of-function mutations of *SLC17A3* (not polymorphisms) were found in gout patients with reduced renal urate excretion (8). Based on its apical protein expression in renal tubules, NPT4 is pre-

Fig. 2. Surface expression of NPT4 wild-type and its variants at the plasma membrane of *Xenopus* oocytes. Immunodetection with a specific antibody raised against the N terminus of NPT4 showed that the wild-type (WT) (B) as well as five mutant proteins [A100T (C), G239V (D), V257F (E), G279R (F), P378L (G)] are expressed at the plasma membrane except for P378L whose expression was also found in the cytoplasm. This antibody did not exhibit specific staining in the control oocytes injected with water instead of cRNAs (A).

sumed to be an exit path for transtubular urate secretion. In the same way, functionally relevant hNPT4 variations shown in this study may explain some of the inter-individual variations in susceptibility to certain diseases like gout (13) and glycogen storage disease type Ic (14) observed in the individuals with altered hNPT4 function.

Since membrane transporters and metabolic enzymes are both involved in renal and hepatic clearance of drugs, alteration of drug transport activities in these tissues could have an important influence on pharmacokinetics of their substrate drugs (2). Since bumetanide is a transport substrate for hNPT4, its polymorphisms may affect the pharmacokinetics of loop diuretics (8). For example, it is known that about one third of the patients with chronic heart failure show the phenomenon called “diuretic resistance” (15). This term was generally defined as failure to decrease the extracellular fluid volume despite liberal use of diuretics. Decreased tubular delivery of loop diuretics is thought to be one of the causes for this phenomenon and is often ascribed to decreased renal perfusion in case of heart failure. In addition, aforementioned functionally relevant hNPT4 variations are likely to explain some of the inter-individual variations in diuretic disposition. Thus, it is interesting to know whether some cases of diuretic resistance can be explained by altered hNPT4 function.

From the point of the structure–function relationship,

the reduction of transport function in V257F of hNPT4 but no reduction of G239V seems interesting because both residues are in the same fifth TMD (Fig. 1A) and they are conserved in hNPT4 (*SLC17A3*) and $\text{Na}^+/\text{PO}_4^{2-}$ cotransporter homologue or hNPT5 (*SLC17A4*) (12). Functional analysis of the mutants that have the corresponding amino acid replacement in other isoforms of hNPTs may give us more information about this residue in the function of organic anion transport in SLC17 members.

P378 located in the 8th TMD may be important for the substrate binding of NPT4. P378L caused severe functional loss of transport function (Fig. 1), while no information about its allele frequency was reported in the NCBI database (Table 1). In addition, since P378L was not found from 175 gout patients (A. Taniguchi and W. Urano, unpublished observation), it seems to be a rare mutation, not a polymorphism.

In summary, we characterized the functional properties of the hNPT4 variants and found, for the first time, that three variants are associated with reduced transport activity. This study suggests that the hNPT4 variants contribute to inter-individual variations in anionic drugs and urate disposition.

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