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Functional analysis of NPHS1 mutations in Japanese patients

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Summary. Background: Many mutations in the *NPHS1* gene were detected among patients with congenital nephrotic syndrome. Functional analysis of those mutations was done with a stable-expression cell line. Nevertheless, establishing such a cell line is time-consuming. Methods and Results: We established an easier method using automatic counting software for functional analysis with transient-transfection cells rather than a stable-expression cell line. We demonstrated maltrafficking to the plasma membrane of abnormal nephrin for immunostaining on transient-expression cells by comparison without Triton X (detecting proteins on the cell membrane only) and with Triton X (detecting proteins both on the cell membrane and inside the cell cytoplasm). We obtained relevant results with data obtained previously using a stable-expression cell line. Furthermore, we conducted functional analysis of NPHS1 mutations in Japanese patients with congenital nephrotic syndrome using this simple method, which revealed that all pathogenic mutations impaired trafficking to the protein plasma membrane.

Conclusions: Functional analysis using transient-expression cells with automatic counting software was useful to demonstrate maltrafficking to the plasma membrane of a protein. All pathogenic mutations detected in Japanese patients impaired trafficking to the protein plasma membrane.

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Introduction

Nephrin, the main component protein of the podocyte slit diaphragm complex, plays a major role in preventing protein loss into urine (Johnstone and Holzman, 2006). Two well-known mutations cause Finnish-type congenital nephrotic syndrome (Kestila et al., 1998). The NPHS1 gene encodes nephrin protein. Earlier reports have described more than 100 mutations of this gene detected worldwide in patients with congenital nephritic syndrome (Lenkkeri et al., 1999; Aya et al., 2000; Koziell et al., 2002; Gigante et al., 2005; Sako et al., 2005; Heeringa et al., 2008; Schoeb et al., 2010). In ordinary clinical situations, a mutation found in a patient is believed to be causative when a substitution of amino acids causes a large change in the Grantham score (Grantham, 1974) and when a substituted amino acid is conserved among various species. Results of functional analyses support that speculation.

Liu et al. (2001) examined functional analysis with cells that stably express a nephrin mutant. However, establishing a stable-expressing cell line is time-consuming. This study examined whether functional analysis using transient-expressing cells is useful or not. Functional analysis of NPHS1 gene mutations found in Japanese patients was conducted using this method.

Materials and methods

Plasmid and mutagenesis

Plasmids were created with each mutation (Quick Change II XL Site-Directed Mutagenesis Kit; Agilent Technologies Inc., CO, USA) according to the manufacturer's protocol with pcDNA3-NPH1, which expresses human nephrin, as template. The primers used to create each plasmid are shown in Table 1. Each plasmid was cycle-sequenced using Big-Dye terminators (Applied Biosystems, CA, USA). The cycle sequence product was analyzed using an automated sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems).

Cell culture and DNA transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium high glucose (Life Technologies Inc., CA, USA) supplemented with 10% fetal bovine serum. PDN8, a stable HEK293 cell line expressing human podocin, was obtained as described previously (Nishibori et al., 2004). PDN8 cells were grown in culture medium as described above by adding 200 μg/ml Zeocin™ (Invitrogen Corp.). Plasmids expressing wild-type or mutated nephrin were transfected into HEK293 and PDN8 cells according to the manufacturer's protocol (with Lipofectamine™ 2000; Invitrogen Corp.).

Western blotting

HEK293 cells were cultured for 48 h after transient transfection with the plasmid cDNA of wild-type or mutants. Cells were washed with phosphate-buffered saline (PBS) before addition of hot sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 50 mM dithiothreitol, and proteinase inhibitor (Complete Mini EDTA-free; Roche Diagnostics Corp., Mannheim, Germany)]. The cell lysates were collected using a rubber scraper and were transferred into Eppendorf tubes. The lysates were sonicated, boiled, and centrifuged for 10 min before loading. After all samples had been subjected to SDS-polyacrylamide gel electrophoresis, the proteins were transferred onto nitrocellulose membranes, which were blocked with Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween-20. They were incubated with N20 anti-nephrin antibody (1:1000; Santa Cruz Biotechnology Inc., CA, USA) overnight at 4°C. The primary antibody was detected using horseradish peroxidase-conjugated anti-goat Donkey IgG (Santa Cruz Biotechnology Inc.) and a Western blot detection system (ECL plus; GE Healthcare UK Ltd., UK).

Two molecular standards were used for SDS-PAGE (Precision Plus Protein Standards, dual color prestained, cat. no. 161-0374; Bio-Rad Laboratories Inc., CA, USA and Santa Cruz Marker Molecular Weight Standard, cat. no. sc-2035; Santa Cruz Biotechnology Inc., CA, USA).

Immunostaining

HEK293 or PDN8 cells were cultured on glass cover slips and were transiently transfected with the plasmid cDNA of wild-type NPHS1 or mutants. Because podocin is an anchor protein for nephrin in the cell membrane, the PDN8 cells were used for experiments comparing nephrin on the cell membrane and inside cells (Roselli et al. 2002). The PDN8 cells were then cultured further for 48 h. The cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. They were then incubated with 0.1% Triton X-100 in PBS for 5 min for permeabilization to stain nephrin inside cells. Triton X-100 was not used for detection of nephrin on the cell membrane.

The cells were incubated with N20 anti-nephrin antibody after incubation with the blocking buffer (1% bovine serum albumin in PBS) for 10 min.

After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated donkey anti-goat IgG (1:200) for 1 h at room temperature. Hoechst33342 was used as the nuclear stain for the cells.

Immunofluorescence microscopy and array scan

All immunostained samples were examined using immunofluorescence microscopy (BZ-9000; Keyence Co., Osaka, Japan). Nine fields were recorded with every plasmid.

Then, three independent experiments were performed. The Hoechst33342 positive cells (showing all cells in that field) and Alexa 488 positive cells

Table 1. primer sets for mutagenesis.

Mutation		Primer
p.Cys160Ser	F	5'-ggtagctgcgtggtcaactctgtgtctgggg-3'
(c.479G>C)	R	5'-ccccagacacagagttgaccacgcagctacc-3'
p.Ile171Asn	F	5'-gccagcacctgacaacaccattctcc-3'
(c.512T>A)	R	5'-ggagaatggtgttgtcaggtgctggc-3'
p.Glu246*	F	5'-ggacccccctgtcatctagtggccaggcctgg-3'
(c.736G>T)	R	5'-ccaggcctggccactagatgacaggggtcc-3'
p.Leu376Val	F	5'-gctacgatggtgggtgggctggcggc-3'
(c.1126C>G)	R	5'-gccgccagcccacccaccatcgtagc-3'
p.Arg379Trp	F	5'-ggtggctgggctggtggcagctgctgccc-3'
(c.1135C>T)	R	5'-gggcagcagctgccaccagcccagccacc-3'
p.Glu447Lys	F	5'-gcccagaaactgtggattaagggtcccccag-3'
(c.1339G>A)	R	5'-ctgggggacccttaatccacagtttctgggc-3'
p.Gly601Arg	F	5'-ggagagccccattcaaacgctccgccgccgcc-3'
(c.1801G>C)	R	5'-ggcggcggcggagcgtttgaatggggctctcc-3'
p.Arg743Cys	F	5'-gctcccaccatctgtgccctccaggacccc-3'
(c.2227C>T)	R	5'-ggggtcctggagggcacagatggtgggagc-3'
p.Asp819Val	F	5'-ccagtgcattgtggtcaatggggtggcgc-3'
(c.2456A>T)	R	5'-gcgccaccccattgaccacaatgcactgg-3'
p.Arg827*	F	5'-ggggtggcgcctccagcatgacggctgctcc-3'
(c.2779C>T)	R	5'-ggagcagccgtcatgctggaggcgccacccc-3'
p.Gln839fs*8	F	5'-gtcagatttgccccccaggtggagcacccc-3'
(c.2515delC)	R	5'-ggggtgctccacctgggggcaaatctgac-3'
p.Arg1140Cys	F	5'-ggcagagccgtattactgctccctgaggg-3'
(c.3418C>T)	R	5'-ccctcagggagcagtaatacggctctgcc-3'

(showing cells expressing nephrin) in the immunofluorescence microscope images were counted using Cellomics BioApplications (Colocalization Ver.3.0; Thermo Fisher Scientific Inc., MA, USA).

Results

Confirmation of anti-nephrin antibody performance

To confirm the antibody performance, immunofluorescence staining was performed with anti-nephrin antibodies. The HEK293-cell transiently transfected plasmid expresses wild-type nephrin, p.Glu246*mutant, or no nephrin. The HEK293 was used for confirmation of antibody performance (Fig. 1). With N20 antibody, wild nephrin and p.Glu246*mutant were stained. N20 anti-nephrin antibody was used to detect wild-type nephrin and mutants.

Molecular size of nephrin mutants

Using cell lysates containing not only wild-type nephrin but also missense mutants, bands around 185 kD were detected using Western blot with anti-nephrin antibody, as reported previously for wild nephrin (data not shown). Truncated mutants p.Asp827* (c.2479C > T in exon18) and p.Gln839fs*8 (c.2515delC in exon 19) each had molecular weight of around 120 kD. The truncated mutant p.Glu246* (c.736G > T in exon 7) had molecular weight of around 30 kD.

Expression pattern of wild nephrin

Wild-type nephrin was expressed along the outline in many cells (Fig. 2). No difference was found in the ratio of Alexa488 positive cells to Hoechst 33342 cells between those with and without Triton X-100.

The ratio of cells expressing wild nephrin on the cell surface to cells expressing wild nephrin in and on a whole cell (i.e. the on-in ratio) was almost 1 (Fig. 3). This result indicates that wild-type nephrin was trafficking to cell membranes in cells that express nephrin inside once.

Comparison of stably and transiently expressing cells with the same mutants

Preliminarily, mutants already examined in earlier studies (Liu et al., 2001) using the stable-expression cell line below were used for certification of this transient-expression method: p.Ile171Asn mutant, with defect of trafficking to the cell surface; p.Leu376Val mutant, without defect of trafficking; p.Arg743Cys mutant, without a problem of trafficking, which shows a mild clinical course; and p.Arg1140Cys mutant, without defect of trafficking, which has an intracellular mutation site.

The ratio of the number of cells detected using anti-nephrin antibody without Triton X to those with Triton X (on-in ratio) was found using Cellomics BioApplications. Three experiments were performed independently, all showing similar on-in ratios. Herein, we present the average of three independently obtained experiment results (Fig. 3).

The on-in ratio of p.Leu376Val mutant was 0.84. The on-in ratio of p.Arg743Cys mutant was 0.92. The on-in ratio of p.Arg1140Cys mutant was 1.11. That of p.Ile171Asn mutant was 0.36. The trend of the on-in ratio of these four mutants in transient transfected cells is compatible with functional analysis using a stably

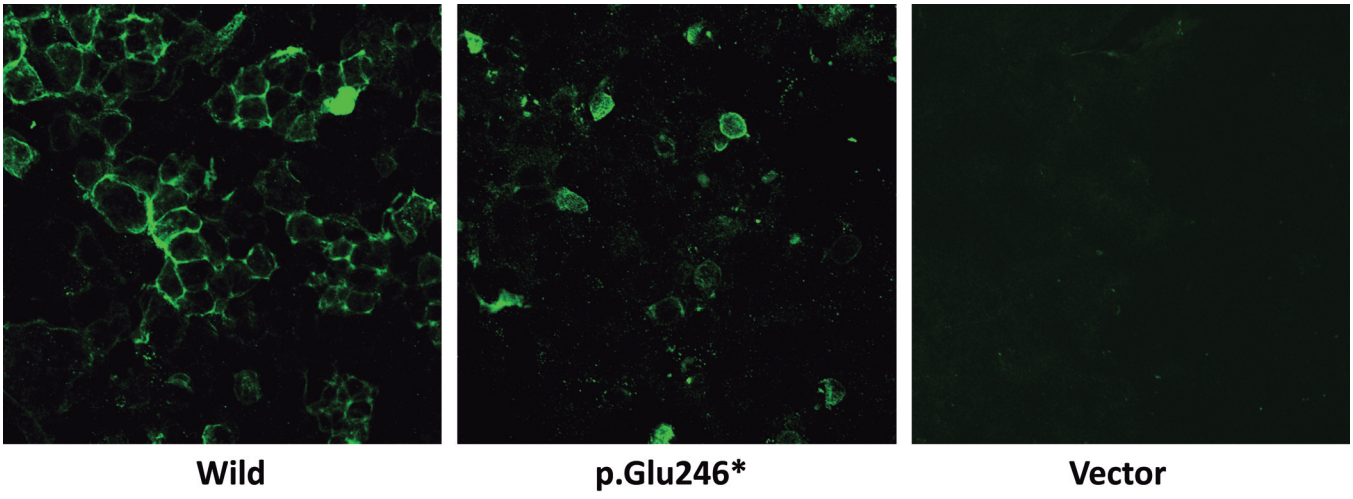


Fig. 1. Immunostaining for HEK293 cell transfected. HEK293 cells expressing wild-type nephrin and p.Glu246* mutant were immunostained with N20 antibody, with addition of Triton X-100. x 200

expressing cell line (Liu et al., 2001).

Functional analysis of NPHS1 mutations from Japanese patients

The on-in ratio with transient transfection method on each mutant from Japanese patients (Aya et al., 2000, 2009) is shown in Figure 3. The values are averaged results derived from those obtained from three independent experiments. First, p.Glu246*, p.Arg827*, and p.Gln839fs*8 (c.2515delC) are truncated mutants. The on-in ratios of the truncated mutants are small: around 0.40. The ratios of truncated mutants are nearly equal to the ratio of p.Ile171Asn.

These data indicate that these three truncated mutants have defects of trafficking to the cell surface. Aside from the on-in ratio, few fluorescence-positive cells with Triton X-100 have protein detected with anti-nephrin antibody both on and in.

Earlier reports (Aya et al., 2000, 2009) have described that four missense mutants are causative: p.Cys160Ser, p.Arg379Trp, p.Gly601Arg, and p.Val819Asp. The on-in ratios of these four missense mutants are also small. In contrast, the on-in ratio of p.Glu447Lys, which is not so causative, is nearly 1 (0.87). These data show that causative mutants detected in Japanese patients with congenital nephrotic syndrome have defects of trafficking to cell membranes.

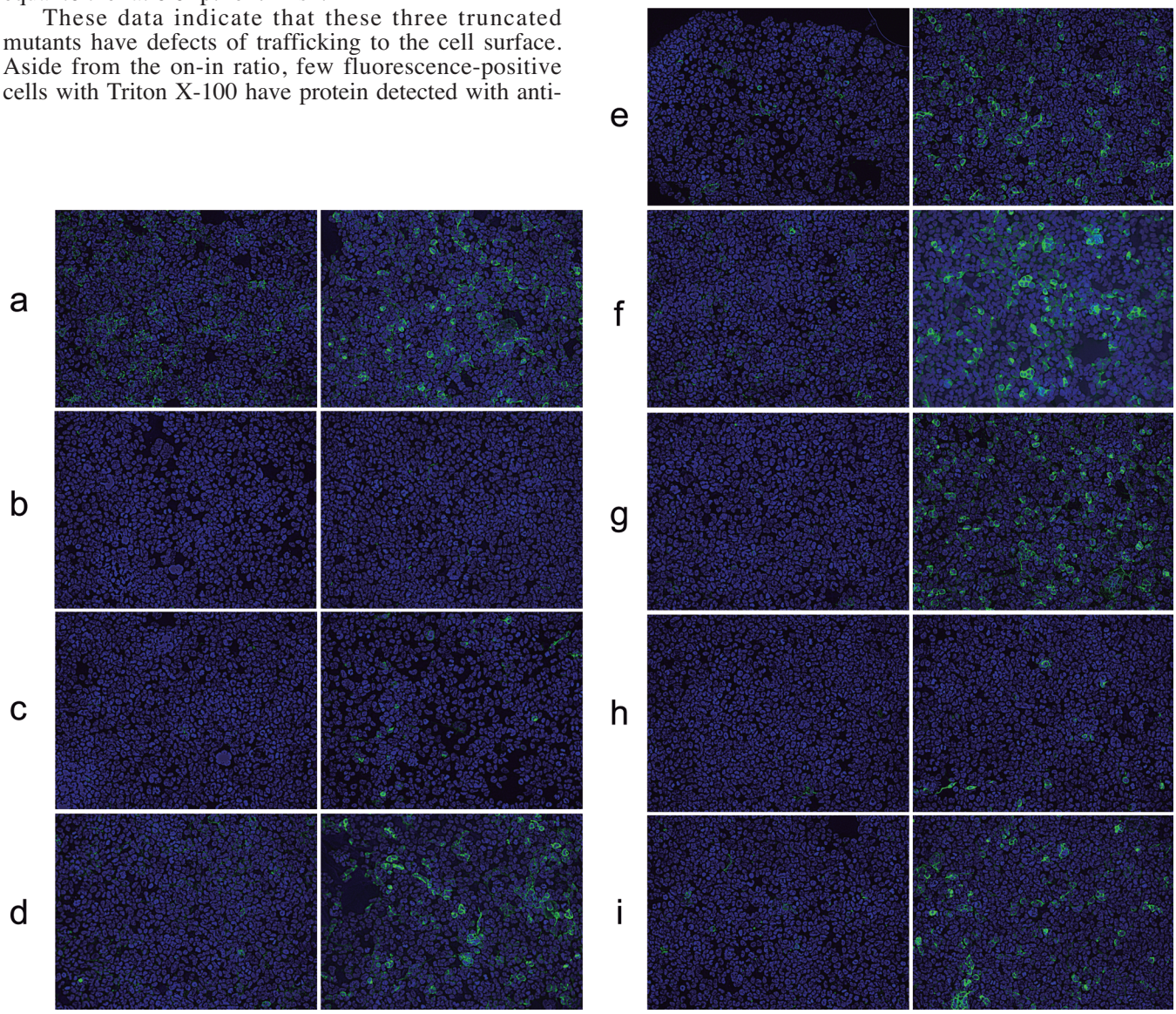


Fig. 2. Immunostaining for PDN8 transfected with each vector. Transfected cells were immunostained with Hoechst33342 and N20 anti-nephrin antibody (left panels) without Triton X-100 and (right panels) with Triton X-100: **a)** wild nephrin; **b)** empty vector; **c)** p.Ile171Asn mutant, maltrafficking, already demonstrated in Liu's study; **d)** p.Arg743Cys mutant, not maltrafficking, already demonstrated in Liu's study; **e)** p.Cys160Ser mutant, putatively pathogenic mutation; **f)** p.Glu447Lys mutant, putatively non-pathogenic mutation; **g)** p.Asp819Val mutant, putatively pathogenic mutation; **h)** p.Glu246*, non-sense mutation; and **i)** c.2515delC, frameshift mutant. x 200

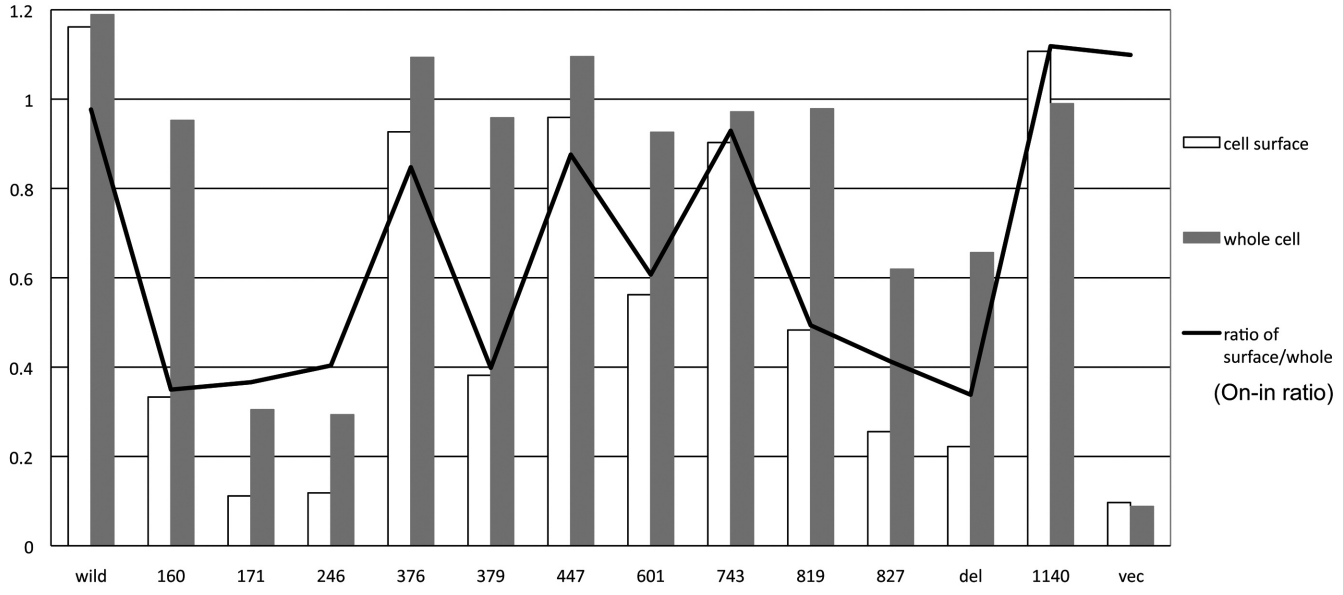
Discussion

Liu et al. (2001) reported that functional analysis of nephrin mutants in cultured cells was performed with a stable-expressing cell line. However, because establishment of a stable-expressing cell line is time-consuming, this mode of using a stable cell line is unsuitable for analysis of each mutant detected in patients. Our method of using software for automatic analysis of transient-expressing cells is not time-consuming.

Liu et al. (2001) reported the p.Ile171Asn mutant with the difficulty of trafficking to the cell surface. In the transient transfection experiment reported herein, p.Ile171Asn mutant was shown to have a low rate of expression on the cell surface, which is compatible with results described in Liu's report: p.Leu376Val, p.Arg743Cys, and p.Arg1140Cys mutants show no difficulty of trafficking to the cell surface (Fig. 2). In this experiment, these mutants have a high rate (higher than 0.8) of expression on the cell surface (Fig. 3).

Among seven missense mutations detected in Japanese patients, four mutations are inferred to be pathogenic: p.Cys160Ser, p.Arg379Trp, p.Gly601Arg, and p.Asp819Val. The four mutants have low rates of expression on the cell surface. Moreover, they have difficulty with trafficking to the cell surface (Fig. 3).

The p.Glu447Lys mutant, which is not regarded as pathogenic, has a high rate of expression on the cell surface. It presents no difficulty with trafficking to the



Nephrin	On-in ratio
Wild	0.97
p.Cys160Ser	0.34
p.Ile171Asn	0.36
p.Glu246*	0.40
p.Leu376Val	0.84
p.Arg379Trp	0.39
p.Glu447Lys	0.87
p.Gly601Arg	0.60
p.Arg743Cys	0.92
p.Asp819Val	0.49
p.Arg827*	0.41
c.2515delC	0.33
p.Arg1140Cys	1.11
Empty vector	1.09

Fig. 3. On-in ratios of nephrin. Ratios of Alexa488 positive cells to Hoechst 33342 cells with and without Triton X-100 (on-in ratio). Mutations showing low ratios were p.Cys160 Ser, p.Glu246*, p.Arg379Trp, p.Asp819Val, p.Asp827*, and c.2515delC, in addition to p.Ile171Asn.

cell surface (Fig. 3).

The strength of fluorescence itself is influenced by nephrin production capability, degradation with mutated protein, affinity of the anti-nephrin antibody to each mutant nephrin, and other factors. In the case of the three truncated mutants, few fluorescence-positive cells with Triton X-100 have protein detected with anti-nephrin antibody both on and in the cell. Therefore, it is difficult to ascertain whether the small number of fluorescence-positive cells without Triton X-100 on cells is attributable to the difficulty of trafficking to the cell surface or to other factors such as low nephrin production capability. However, calculating the ratio of the quantity of fluorescence for the cell surface and for the whole cell can resolve that issue.

The three truncating mutations detected in Japanese patients exhibit problems with trafficking to the cell surface, although some defects aside from trafficking to the cell surface might contribute to pathogenesis in these patients.

The on-in ratios of four missense mutants that are regarded as pathogenic and three truncating mutants from Japanese patients and p.Ile171Asn mutant were 0.4-0.6 (Fig. 3). The on-in ratios of p.Glu447Lys and three missense mutants without problems of trafficking to the cell surface described in Liu's report were higher than 0.8 (Fig. 3).

In conclusion, transient transfection is useful for the functional analysis of nephrin.

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Disclosure/Duality of Interest. None declared.

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