Complete Deletion of Slc52a2 Causes Embryonic Lethality in Mice

Congyun Jin, Yoshhiro Matsui, Atsushi Yonezawa, Satoshi Imai, Takashi Ogihara, Kotaro Itohara, Shunsaku Nakagawa, Takayuki Nakagawa, and Kazuo Matsubara

**Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital; Sakyo-ku, Kyoto 606–8507, Japan; and **Graduate School of Pharmaceutical Sciences, Kyoto University; Sakyo-ku, Kyoto 606–8507, Japan. Received September 17, 2020; accepted November 1, 2020

Riboflavin (vitamin B2) plays an important role in cellular growth and function. Riboflavin transporter 2 (RFVT2) is widely expressed in several tissues, especially in the brain and salivary glands, and plays an important role in the tissue disruption of riboflavin. During the last 10 years, mutations in SLC52A2 have been documented in patients with a rare neurological disorder known as Brown–Vialetto–Van Laere syndrome. However, no suitable animal model of this disease has been reported. Here, we aimed to clarify the physiological role of RFVT2 using Slc52a2-mutant mice. The appearance, body weight, and plasma riboflavin concentration of Slc52a2 heterozygous mutant (Slc52a2+/−) mice were similar to those of wild-type (WT) mice. However, intercrossing between Slc52a2+−/− mice failed to generate Slc52a2 homozygous mutant (Slc52a2−−/) mice. This suggested that Slc52a2 gene deficiency results in early embryonic lethality. Our findings suggested that RFVT2 is essential for growth and development, and its deletion may influence embryonic survival.

**Key words** riboflavin transporter 2; mouse model; embryonic lethality

**INTRODUCTION**

Riboflavin (vitamin B2) is an indispensable nutrient for cellular growth and function. The active coenzymes, flavin mononucleotide (FMN) and FAD, which are made from riboflavin. Riboflavin deficiency leads to growth impairment, which is causally related to the role of riboflavin in generation of energy from mitochondrial metabolism. Human riboflavin transporters RFVT1–3/SLC52A3 have been identified. RFVT2 predicted to have 10 membrane-spanning domains. The RFVT2-mediated uptake of riboflavin has been shown to be Na+-, Cl−, and pH-independent. RFVT2 mRNA is ubiquitously expressed. It has been suggested that RFVT2 is essential for tissue distribution of water-soluble riboflavin.

Since 2010, several mutations in the SLC52A3 and SLC52A2 genes have been shown to be linked to Brown–Vialetto–Van Laere syndrome (BVVLS). BVVLS patients with SLC52A3 mutations have a higher frequency of facial weakness and lower blood riboflavin levels. However, abnormal gait and/or ataxia and optic nerve atrophy appear to be more prevalent features of patients with SLC52A2 mutations. In addition, improvements in motor abilities, respiratory function and/or cranial nerve deficits upon riboflavin supplementation are observed in 70% patients, with the remaining patients showing stabilization of the current disease stage. The responses to riboflavin supplementation are similar in patients with SLC52A2 and SLC52A3 mutations. It has been suggested that immediate and continuous riboflavin administration may prevent neurological changes. In previous studies, we have shown that Slc52a3-knockout mice exhibit phenotypes similar to those seen in patients with SLC52A3 mutations, which are associated with riboflavin deficiency. An analysis of skin fibroblasts from patients with SLC52A2 mutations revealed a significant reduction in electron transport chain complex I and II activity. However, the pathophysiological mechanism of these symptoms is unclear.

In this study, we aimed to clarify the significance of Rfvt2 in vivo using Slc52a2-mutant mice. The appearance, body weight, and plasma riboflavin concentration of Slc52a2 heterozygous mutant (Slc52a2+/−) mice were not different from those of wild-type (WT) mice. However, intercrossing between Slc52a2+−/− mice failed to generate Slc52a2 homozygous mutant (Slc52a2−−/) mice. These results suggested that Rfvt2 deficiency causes embryonic lethality in mice.

**MATERIALS AND METHODS**

**Animals** All animal studies were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University. Embryos with an Slc52a2 mutation (C57BL/6-Slc52a2<sup>tm1(KOMP)Wtsi</sup>) were purchased from the Knockout Mouse Project (KOMP) Repository. The targeting vector is described in Fig. 1A. To determine mouse genotypes, genomic DNA was isolated from tail biopsies using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.), and PCR analysis was performed using the Takara Ex Taq® Hot Start Version reaction mix (Takara Bio, Shiga, Japan). The primer sets were as follows: a forward primer, 5′-CCAGGCCCTAAGGCCCATCAG-3′, and a reverse primer, 5′-CAGACGGCCATTGGTACAG-3′; for detecting the wild-type alleles and a forward primer, 5′-GGTTAAACCTGCTCGGATTAGG-3′, and a reverse primer, 5′-TTCAGTTGATGCCGCTGATTTG-3′, for detecting mutant alleles. PCR cycling conditions were as follows: 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Heterozygous mice (8 weeks old) were mated overnight and vaginal plugs were examined the following morning. Placenta was considered to correspond to day 0.5 of pregnancy. Embryos at E10.5, pups at postnatal day 0, and adult mice older than 8 weeks were used for subsequent experiments. The mice were housed...
under a 12-h light/dark cycle in a temperature-controlled environment, and were given water *ad libitum* and a standard chow diet (F-2; Funabashi Farm, Funabashi, Japan) before being used in experiments. All protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (Permission No. MedKyo20121).

**Real-Time PCR** Total RNA was isolated from brains dissected at 8 weeks of age, using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and was then reverse transcribed. TaqMan Gene Expression assays were obtained from Life Technologies (*Slc52a2*, Mm01205717_g1; Carlsbad, CA, U.S.A.). Real-time PCR was performed to determine the mRNA expression level of *Slc52a2* as described previously.4)

**Measurement of Riboflavin** We collected samples of blood and tissue from 16-week-old mice. The concentrations of riboflavin in blood and tissue samples were measured by HPLC (LC-10ADVP; Shimadzu, Kyoto, Japan) according to a previously reported method.11)

**Statistical Analysis** Statistics were performed using GraphPad Prism (version 7; GraphPad Software, Inc., La Jolla, CA, U.S.A.). All values are expressed as the mean ± standard error of the mean (S.E.M.), and the differences were analyzed for significance using an unpaired Welch’s *t*-test. Multiple comparisons were performed using Bonferroni’s two-tailed test, after a one-way ANOVA. The significance was shown based on the *p*-value (**** *p* < 0.0001).

**RESULTS**

**Targeted Disruption of the *Slc52a2* Gene** The mouse *Slc52a2* gene was deleted from exon 2 to 5, and was integrated with a trapping cassette. PCR analysis confirmed the targeted *Slc52a2* allele in genomic DNA isolated from tail biopsies of the offspring (Fig. 1A). Furthermore, real-time PCR analysis demonstrated that *Slc52a2* mRNA levels in the brain were significantly lower in *Slc52a2*+/− mice than in WT mice (Fig. 1B).

Genotyping of newborn pups from *Slc52a2*+/− parents revealed that intercrossing between heterozygotes only produced *Slc52a2*+/− and WT mice, and failed to generate *Slc52a2*−/− mutant mice (Fig. 2A). The same result was observed in embryos at E10.5 (Fig. 2B).
Riboflavin Homeostasis and Phenotypic Analysis in Slc52a2+/− Mice

Macroscopically, the appearance of Slc52a2+/− pups and adults were not different from WT mice (Fig. 3A), and the body weights of Slc52a2+/- and WT mice were similar within 3 weeks of birth (Fig. 3B).

We measured riboflavin concentration in plasma (Fig. 4A) and tissues, including the upper and lower small intestine, liver, kidney, lung, heart, muscle, and brain in 16-week-old WT and Slc52a2+/- mice (Fig. 4B). No differences in plasma or tissue riboflavin concentrations were observed between Slc52a2+/- and WT mice.

DISCUSSION

In this study, we attempted to produce Slc52a2-mutant mice as a pathological model of SLC52A2-mutant BVVLS. However, Slc52a2−/- mice were not observed among newborn pups or E10.5 embryos, including those that died due to maternal neglect. RFVT2 is widely expressed in tissues throughout the body. Therefore, the complete deletion of Slc52a2 expression resulted in embryonic lethality in the early stages of embryonic development.

In in vitro functional analyses, SLC52A2 mutations p.G306R and p.L312P show a moderate, but significant, decrease in function, while the mutations with a gray background showed almost complete loss of function.

Table 1. Gene Mutations in Patients with SLC52A2-Mutant BVVLS

| Gene mutation | G306R | G306R | L312P | G306R | G306R | L312P | G306R | L312P | G306R | L312P | L123P |
|---------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Number        | 17    | 1     | 1     | 1     | 4     | 2     | 2     | 1     | 1     | 1     |       |

Modified from Haack et al., Foley et al., and O’Callaghan et al. In in vitro functional analyses, the mutations with a white background showed a moderate decrease in function, while the mutations with a gray background showed almost complete loss of function.
transport activity. These mutations have been detected in 30 BVVLS patients (Table 1). Except for one patient, previous studies have shown that BVVLS patients with SLC52A2 mutations have one allele that encodes functional RFVT2. A previously described patient with mutations in p.L123P and p.L339P is thought to have survived due to the retention of a low level of RFVT2 activity. Taken together, these data suggested that RFVT2 is essential for embryonic cell survival in vivo, and complete deletion may lead to embryonic lethality.

Phenotypic analysis showed no difference between WT and Slc52a2+/− mice. In addition, the riboflavin concentrations in plasma and tissues were unchanged compared with those in WT mice. These results revealed that Slc52a2+/− mice show normal growth, which is consistent with the results reported for Slc52a3+/− mice. In clinical reports, parents or sibling with heterozygous mutation are healthy, suggesting an autosomal recessive mode of inheritance. Therefore, the Slc52a2+/− mouse phenotype may mimic the phenotype of parents of BVVLS patients.

When the Slc52a2 gene was completely deleted by homologous recombination with a long-chain sequence, Slc52a2+/− mice were not generated. Creating a single-nucleotide polymorphism animal model, in which some Rft2 function is retained, may be an alternative method for producing a pathomorphological model of RFVT2-mutant BVVLS.

In conclusion, RFVT2 is an essential transporter for growth and development, and its deletion may influence embryonic survival.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants-in-Aid for Scientific Research [C] to AY [24590190, 15K08095]).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1. Powers HJ. Riboflavin (vitamin B-2) and health. Am. J. Clin. Nutr., 77, 1352–1360 (2003).
2. Powers HJ, Corfe BM, Nakano E. Riboflavin in development and cell fate. Subcell. Biochem., 56, 229–245 (2012).
3. Yonezawa A, Inui K. Novel riboflavin transporter family RFVT/SLC52: identification, nomenclature, functional characterization and genetic diseases of RFVT/SLC52. Mol. Aspects Med., 34, 693–701 (2013).
4. Yao Y, Yonezawa A, Yoshimatsu H, Masuda S, Katsura T, Inui K. Identification and comparative functional characterization of a new human riboflavin transporter hRFT3 expressed in the brain. J. Nutr., 140, 1220–1226 (2010).
5. Foraker AB, Khantwal CM, Swaan PW. Current perspectives on the cellular uptake and trafficking of riboflavin. Adv. Drug Deliv. Rev., 55, 1467–1483 (2003).
6. Jaeger B, Bosch AM. Clinical presentation and outcome of riboflavin transporter deficiency: mini review after five years of experience. J. Inherit. Metab. Dis., 39, 559–564 (2016).
7. O’Callaghan B, Bosch AM, Houlden H. An update on the genetics, clinical presentation, and pathomechanisms of human riboflavin transporter deficiency. J. Inherit. Metab. Dis., 42, 598–607 (2019).
8. Yoshimatsu H, Yonezawa A, Yamanishi K, Yao Y, Sugano K, Nakagawa S, Imai S, Omura T, Nakagawa T, Yano I, Masuda S, Inui K, Matsubara K. Disruption of Slc52a2 gene causes neonatal lethality with riboflavin deficiency in mice. Sci. Rep., 6, 27557 (2016).
9. Manole A, Jaumarktane Z, Hargreaves I, et al. Clinical, pathological and functional characterization of riboflavin-responsive neuropathy. Brain, 140, 2820–2837 (2017).
10. Velocigene. Alleles produced for the KOMP project by Velocigene (Regeneron Pharmaceuticals). MGI Direct Data Submission (2008).
11. Yao Y, Yonezawa A, Yoshimatsu H, Omura T, Masuda S, Matsubara K. Involvement of riboflavin transporter RFVT2/Slc52a2 in hepatic homeostasis of riboflavin in mice. Eur. J. Pharmacol., 714, 281–287 (2013).
12. Haack TB, Makowski C, Yao Y, Graf E, Hempel M, Wieland T, Tauer U, Ahting U, Mayr JA, Freisinger P, Yoshimatsu H, Inui K, Strom TM, Mettiger T, Yonezawa A, Proksich H. Impaired riboflavin transport due to missense mutations in SLC52A2 causes Brown-Vialetto-Van Laere syndrome. J. Inherit. Metab. Dis., 35, 943–948 (2012).
13. Foley AR, Menezes MP, Pandraud A, et al. Treatable childhood neuropathy caused by mutations in riboflavin transporter RFVT2. Brain, 137, 44–56 (2014).
14. Green P, Wiseman M, Crow YJ, Houlden H, Ripphagen S, Lin JP, Raymond FL, Childs AM, Sheridan E, Edwards S, Josifova DJ. Brown–Vialetto–Van Laere syndrome, a ponto-bulbar palsy with deafness, is caused by mutations in c20orf73. Am. J. Hum. Genet., 86, 485–489 (2010).