

Novel *SLC39A4* Mutations in Acrodermatitis Enteropathica

Aoi Nakano, Hajime Nakano, Kazuo Nomura,* Yuka Toyomaki, and Katsumi Hanada

Department of Dermatology, Hirosaki University School of Medicine, Hirosaki, Japan; *Department of Dermatology, Aomori Prefectural Central Hospital, Aomori, Japan

Acrodermatitis enteropathica is an autosomal recessive disease characterized by skin involvement due to defective intestinal zinc absorption. Usually, the skin lesions include erythema, erosions, and small blisters in perioral, perianal regions, and hands and feet, which develop soon after weaning from the breast. The acrodermatitis enteropathica gene has been localized to chromosomal region 8q24.3 and subsequently the *SLC39A4* gene has been disclosed as the acrodermatitis enteropathica gene. *SLC39A4* mutations have been demonstrated in several acrodermatitis enteropathica families, and in this study

we have examined two Japanese acrodermatitis enteropathica families for *SLC39A4* mutations. The mutation detection strategy consisted of polymerase chain reaction amplification of all 12 exons and flanking intronic sequences, followed by direct nucleotide sequencing. It revealed three novel mutations, 1017ins53, which creates a premature termination codon, and two mis-sense mutations, R95C and Q303H. Key words: autosomal recessive disease/zinc deficiency/zinc and iron-regulated transporter-like protein. *J Invest Dermatol* 120:963–966, 2003

Acrodermatitis enteropathica (AE; OMIM 210100) is a rare autosomal recessive disease that manifests with acral dermatitis and low serum zinc levels. It was first named by Danbolt and Closs in 1943, and the pathogenesis of the disease had been unknown until Barnes and Moynahan (1973) observed that the disorder was caused by the inability to absorb sufficient zinc. The differential diagnosis of AE includes acquired zinc deficiency syndrome due to a low zinc level in mother's milk because both conditions are quite similar in terms of onset and clinical presentation. As soon as the low zinc breast-fed infants were weaned, they no longer require supplemental zinc, whereas AE patients experience recurrence of symptoms after discontinuation of oral zinc therapy.

Huang and Gitschier (1997) demonstrated that the lethal milk mouse, which was thought to be an animal counterpart of AE, is associated with a *ZNT4* gene mutation, strongly suggesting a relationship between AE and the human *ZNT4* gene; however, no pathogenetic *ZNT4* mutations were identified in AE patients in several studies (Bleck *et al*, 2001; Küry *et al*, 2001; Nakano *et al*, 2002).

Recently, linkage analysis revealed that the AE gene locus resides on chromosome 8q24.3 (Wang *et al*, 2001) and the *SLC39A4* gene (SLC, solute carrier) family 39, member 4, has been disclosed as a causative gene for AE (Küry *et al*, 2002; Wang *et al*, 2002). The *SLC39A4* gene encodes one member of a human zinc/iron-regulated transporter-like protein (hZIP) family, hZIP4 (Wang *et al*, 2002). This gene consists of a total of 12 exons and the corresponding 2.5 kb mRNA encodes a polypeptide of 647

amino acids, having eight transmembrane domains organized in two blocks of three and five. A histidine-rich region, which is a putative zinc-binding site, resides between the two blocks of transmembrane domains (Guerinot, 2000; Küry *et al*, 2002; Wang *et al*, 2002). Expression of hZIP4 mRNA was observed to be abundant in the small intestine, stomach, and colon, as well as in the kidney, and immunohistochemistry revealed that the presumed zinc-uptake protein was localized to the apical membrane of the enterocyte in mouse colon (Küry *et al*, 2002; Wang *et al*, 2002). Little is known about the function of hZIP4, although three other members of the ZIP family (hZIP1, hZIP2, and hZIP3) have been shown to transport zinc into the cytoplasm (Gaither and Eide, 2000a, 2000b).

In this study, we identified three novel mutations in the human *SLC39A4* gene in two Japanese AE families.

MATERIALS AND METHODS

Clinical details and diagnostic features Two Japanese families were studied, which were summarized in Nakano *et al* (2002). The use of human tissue was approved by the Ethics Committee of Hirosaki University School of Medicine.

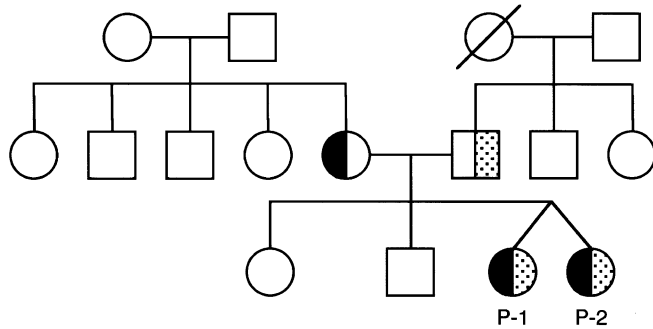
Family 1 Two 11 mo old female identical twins, now aged 8 y, were third and fourth children of healthy Japanese parents, who are not known to be related (**Fig 1**). Their older sister and brother had no history of acral dermatitis. The patients had blisters and erythema on their fingers and toes, and perioral and perianal regions at the age of 10 mo (**Fig 2A–C**). At the time of their first examination, the serum zinc levels of P=1 and P=2 in **Fig 1** were below the normal range of 59–135 µg per dl (20 µg per dl and 36 µg per dl, respectively). Hair zinc levels of the elder patient (P-1) was 68 µg per g, also below the normal range of 148 ± 41.5 µg per g. The serum zinc levels of their parents and siblings were normal. Histopathologic examination from the right dorsal foot of P-1 revealed pale staining of the corneal layer and an epidermal acantholytic blister (**Fig 2D**). After oral zinc sulfate treatment, blisters and erythema disappeared and their serum zinc level was within the normal range. At the age of 2.5 y, their mother discontinued zinc treatment, and they had a recurrence of skin involvement and

Manuscript received October 25, 2002; revised November 26, 2002; accepted for publication November 26, 2002

Reprint requests to: Hajime Nakano, MD, PhD, Department of Dermatology, Hirosaki University School of Medicine, Zaifu-cho 5, Hirosaki 036-8562, Japan. Email: hajime01@abelia.ocn.ne.jp

Abbreviations: AE, acrodermatitis enteropathica; *SLC39A4*, solute carrier family 39, member 4; hZIP, human zinc/iron-regulated transporter-like protein.

Family 1



Family 2

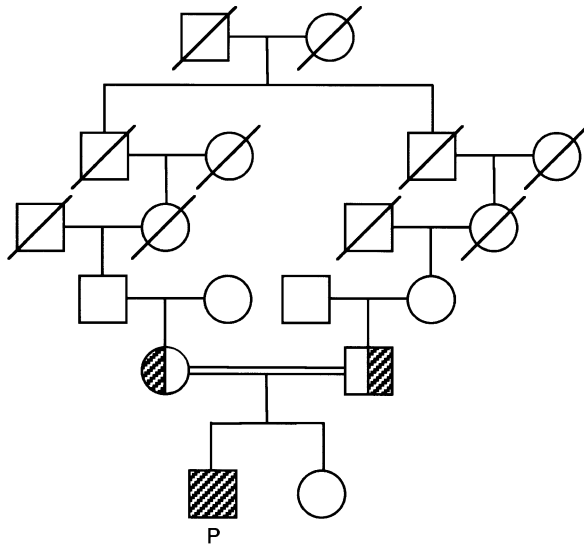


Figure 1. Pedigrees of family 1 and family 2 with AE. The solid symbols (●) indicate maternal mutations and the dotted symbols (◐) indicate paternal mutations in family 1. The diagonal stripe symbols (▨) refer to mutations in family 2. The open symbols refer to clinically unaffected individuals. The parents of family 2 are related.

remarkably low level of serum zinc. This clinical course suggested that their clinical diagnosis was congenital AE.

Family 2 A 9 mo old male infant, now aged 11 y, was the first child of healthy Japanese parents who are third cousins (**Fig 1**). There were no other affected individuals in this family, including his younger sister. He had acral erythematous plaques and small blisters at the age of 7 mo. His serum zinc level was undetectable, under 10 µg per dl, and his serum alkaline phosphatase level was 89 IU per liter, significantly below the normal range of 633 ± 192 IU per liter. The serum zinc and serum alkaline phosphatase levels of his parents and sister were normal. Oral zinc sulfate treatment was effective, however, discontinuation of the treatment resulted in recurrence of dermatitis and a low serum zinc level, which suggested AE. The details of these cases have been reported previously (Mikami *et al*, 1994).

Mutational analysis of human *SLC39A4* gene Informed consent was obtained from all participating family members and controls. DNA was extracted from peripheral blood samples from the probands, their parents, and close relatives using QIAamp Blood Maxi Kit (Qiagen, Valencia, CA). Control blood samples from 50 unrelated healthy individuals with no evidence of a skin disease were also obtained and DNA extracted.

Human *SLC39A4* cDNA and genomic DNA sequence (GenBank accession number NM 130849 and AF205589, respectively) were used to design primer pairs (**Table 1**) and polymerase chain reaction (PCR) amplification of the entire coding region was performed. PCR products were analyzed by direct nucleotide sequencing (ABI PRISM 310, Perkin-Elmer Cetus, Foster City, CA). The mutations were verified by restriction endonuclease digestion of PCR products from family members. As the

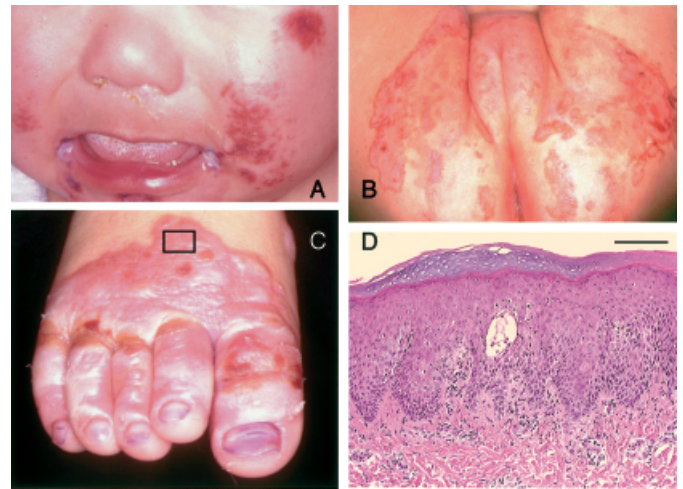


Figure 2. Clinical and histopathologic findings of patient 1 (P-1) in family 1 at the initial examination. Erythema, erosions, blisters, and crusts are shown in perioral and perianal regions and acral sites of the feet (A–C). The biopsy specimen from right dorsal foot, square box in C, showed pale staining of corneal layer, spongiosis, and epidermal acantholytic blister (D). Scale bar: 50 µm; Hematoxylin and eosin stain, original magnification × 100.

probands and their father in family 1 showed two distinct PCR products of exon 6, each of them was subcloned using pGEM-T and pGEM-T easy vector systems to isolate the coding PCR product (Promega, Madison, WI). Positive colonies were subjected directly to PCR amplification using the same primers, and the resulting PCR products were analyzed by direct nucleotide sequencing.

RESULTS AND DISCUSSION

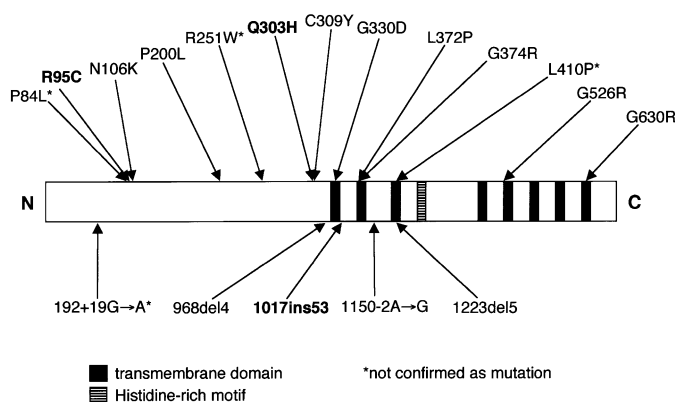
In this study, we identified a novel premature termination codon mutation and two mis-sense mutations in two Japanese AE families. The positions of these mutations are depicted in **Fig 3** together with *SLC39A4* mutations disclosed thus far in AE patients. During this screening, there was no evidence of coamplification of pseudogenes.

In family 1, screening of *SLC39A4* for pathogenetic mutations revealed that the two identical twins were heterozygous for a 53 bp insertion at nucleotide position 1017 and a C to T substitution at the nucleotide position 283. Agarose gel electrophoresis of the resulting PCR products demonstrated an additional longer band in the probands and their father, but not in the mother and the control, suggesting that the mutation was a small insertion in exon 6 of paternal origin (**Fig 4A**). Subcloning procedure confirmed that there was a 53 bp insertion at nucleotide position 1017, designated 1017ins53 (**Fig 4B**). This mutation causes a frame-shift and a premature termination codon immediately downstream from the insertion site. The production of a truncated protein missing the last seven of eight transmembrane domains as well as the zinc-binding site (**Fig 3**). Further screening revealed a nucleotide substitution 283C→T in exon 2, resulting in a change from arginine (CGC) to cysteine (TGC) at amino acid position 95, R95C (**Fig 4C**). This mutation abolishes a restriction enzyme site for *FauI*. Restriction digestion revealed that the R95C was of maternal origin (data not shown). Screening of 100 chromosomes in healthy, unaffected control subjects by *FauI* digestion was negative demonstrating that R95C is probably not a simple polymorphism but a disease-causing pathogenetic mutation. The implication of the R95C substitution is difficult to assess, as the sequence in this region is unique to hZIP4 (Wang *et al*,

Table I. Primers for amplification of the *SLC39A4* gene

Exon	5' primer	3' primer	Product size (bp)	AT (°C)
1	TGGACAACCCAGCAAAGCC -90 in cDNA ^a	GTGCCCAGGTACTGCCACTA + 128	410	62
2	CATCAAATTGGCAGTGGCTC -256	CCGAAGGCTTTGCAGCCAGG + 85	623	58
3	TGGATGGCAGAAGGTCACAC -53	CTGGCAGGGAGAAGAGTTGG + 116	362	62
4	GATTCCCCAGCCTCCACGTC -79	ATCACGTCCCTGGCACTCAG + 103	319	62
5	CTCATCAGCTCCAGCAACAG -116	ACTCTCTCCATCCCTCATCC + 94	382	60
6	CAGGAGAGTGGGGCTTTGAG -146	GAGGTGGGGTGGGTAAAGTC + 92	411	58
7	ACCTCCTGACCCCTCTCCCT -79	TACCTTCCCAAGAAGCCTGA + 21	238	60
8	CTCTTCAATCTCTGCTGCC -109	TGGGAGTGTGGGCGTGGGAA + 72	313	60
9	ACCGCGTTCCTCCTCCACTT -51	TTCGCGTGGCCTGTCGCCTA + 100	206	60
10	TAGGCGACAGGCCACGCGAA -43	TGTTCCAGGTCTCCCCGCCCA + 69	265	62
11	TAAGAGGGCGGGACCGAAAG -68	AGCTGAGGAGCAAGTGGGCA + 94	360	57
12	ACATGGTCAGGATGGCGAGG -97	TGGTTTCTGGGCTGTAGGTT 2012 in cDNA	294	57

AT, annealing temperature.

^aGenerated from the corresponding exon-intron border.**Figure 3. Schematic illustration of the *SLC39A4* polypeptide and positions of all *SLC39A4* mutations disclosed thus far in AE patients.** The mis-sense mutations are shown above the molecule, whereas the premature termination codon and the putative splice site mutation are shown below. The mutations disclosed in this study are shown in **bold type**. Four mutations with asterisk are not confirmed as a mutation.

2002) and functional data is lacking for hZIP4 as well as many of the other members of this gene family.

Screening of the *SLC39A4* gene in family 2 revealed that the parents were heterozygous for a G to C substitution at nucleotide position 909 in exon 5, whereas the affected individual was homozygous for this mutation (**Fig 4D**). The healthy sister was not a carrier. This nucleotide substitution resulted in a change from a glutamine (CAG) to histidine (CAC) at amino acid position 303, designated Q303H. This mutation creates a new restriction enzyme site for *Bst*XI. Restriction digestion of the exon 5 PCR product with the enzyme of 100 chromosomes in healthy,

unrelated controls was negative for Q303H mutation. This single-nucleotide mutation 909G→C leads to a substitution of highly conserved amino acid in the proteins of the ZIP family (see Wang *et al*, 2002). In fact, as the ZIP4 sequence has been identified only in humans and *Arabidopsis thaliana* (Guerinot, 2000), and other mammalian ZIP4 proteins have not yet been identified, we could not compare amino acid conservation between mammalian ZIP4 genes. The patient affected with AE in this study, however, was homozygous for this novel mis-sense mutation, whereas heterozygous carriers had no evidence of AE. Furthermore, this mutation was not present in any of the 50 unaffected and unrelated individuals we studied. Thus, it is not merely common polymorphism but more likely a pathogenetic mutation.

In a previous study, (Wang *et al*, 2002; Küry *et al*, 2002) three mis-sense mutations and a splicing mutation were not proven to be mutations (**Fig 3**). The P84L, which was identified in two affected individuals in the homozygous state and in one in the compound heterozygous state, was also detected in three of 120 control chromosomes (Wang *et al*, 2002). The R251W, probably resulting from 751C→T, is possibly a polymorphism because the cDNA sequence (NM 130849) shows a C at the nucleotide position 751, whereas the genomic DNA sequence (AF205589) shows a T. Whether 192 + 19G→A, originally reported as 193 - 19G→A by Küry *et al* (2002) is a pathogenetic mutation is also unclear. Because the +19 position is far from the exon-intron border, and evidence for altered transcripts resulting from mis-splicing is lacking, definite conclusions cannot be drawn.

The fact that simple administration of oral zinc can overcome zinc deficiency in AE patients is still enigmatic. One explanation is that there may be another zinc transporter in the intestine, which is less efficient in its ability to transport zinc compared with hZIP4, but can compensate for the defect in *SLC39A4* when excess zinc is supplied in the diet.

In conclusion, all three of the affected patients we investigated carry pathogenetic mutations on both *SLC39A4* alleles, supporting the hypothesis that *SLC39A4* is a candidate gene for AE.

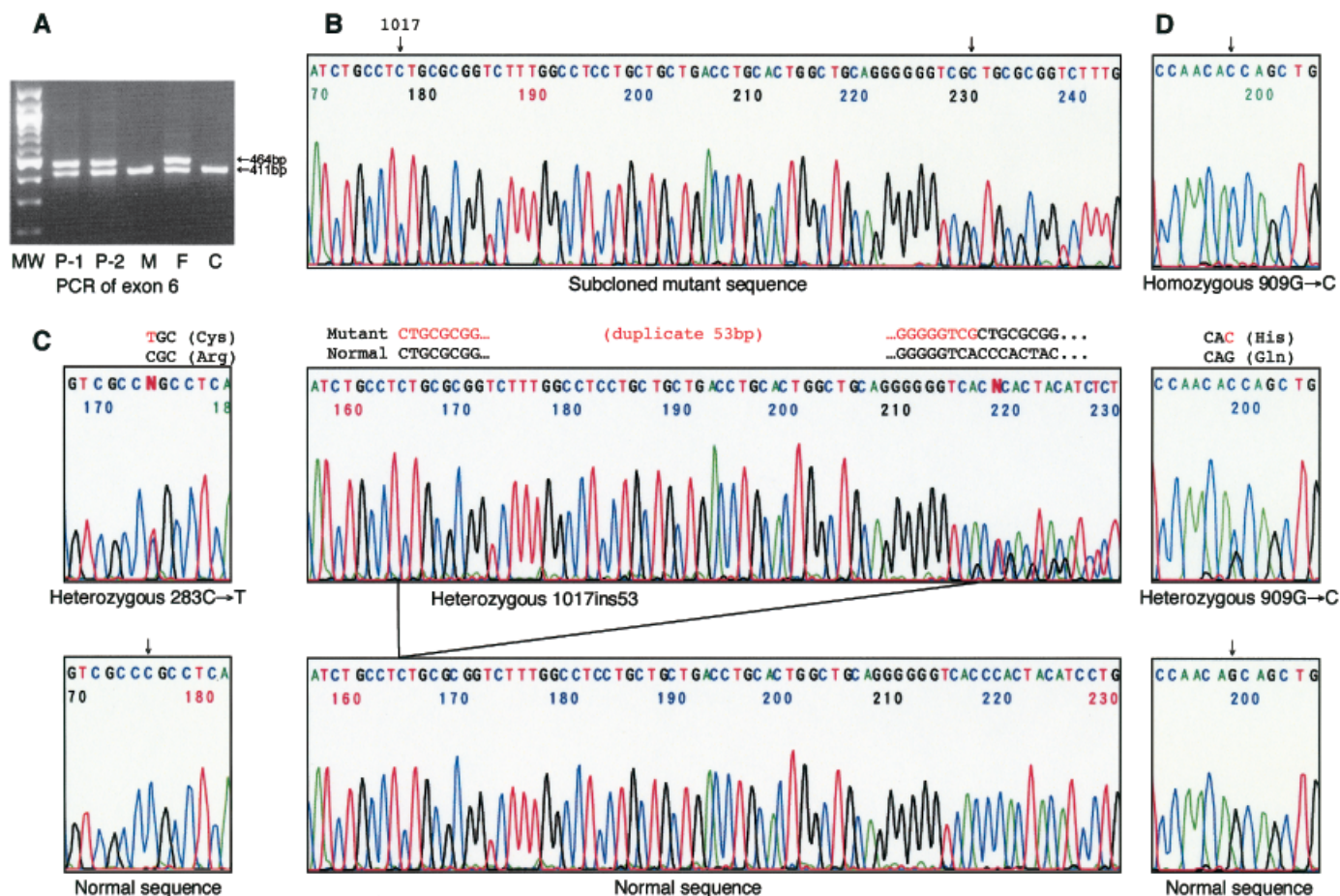


Figure 4. Identification of the *SLC39A4* mutations. Two percent agarose gel electrophoresis of the PCR products spanning exon 6 showed double bands in the affected individuals and father in family 1 (P-1, P-2, and F) (A). Direct nucleotide sequencing of the PCR products spanning exon 6 revealed a heterozygous 1017ins53 in these individuals (B, middle panel). The 1017ins53 mutation, which is the duplication of 53 bp from the nucleotide position 1017, was verified by sequencing of the subcloned mutant band (B, top panel). Sequencing of the PCR products spanning exon 2 revealed a heterozygous 283 C→T, designated R95C, in the affected individuals and mother (P-1, P-2, and M) (C, upper panel). In family 2, the patient was homozygous for 909 G→C, designated Q303H (D, upper panel), whereas the parents were heterozygous for this mutation (D, middle panel). MW, molecular weight markers 100 bp DNA Ladder; M, mother; F, father; C, control.

We thank Dr Ellen Pfendner, Thomas Jefferson University, PA, for critical reading of this manuscript. This study was supported by the JSID's International Fellowship Shiseido Award.

REFERENCES

- Barnes PM, Moynahan EJ: Zinc deficiency in acrodermatitis enteropathica: multiple dietary intolerance treated with synthetic diet. *Proc R Soc Med* 66:327–329, 1973
- Bleck O, Ashton GHS, Mallipeddi R, et al: Genomic localization, organization and amplification of the human zinc transporter protein gene, *ZNT4*, and exclusion as a candidate gene in different clinical variants of acrodermatitis enteropathica. *Arch Dermatol Res* 293:392–396, 2001
- Danbolt N, Closs K: Acrodermatitis enteropathica. *Acta Derm Venerol* 23:127–169, 1943
- Gaither LA, Eide DJ: Eukaryotic zinc transporters and their regulation. *Biomaterials* 14:251–270, 2001a
- Gaither LA, Eide DJ: Functional expression of the human hZIP2 zinc transporter. *J Biol Chem* 275:5560–5564, 2000
- Gaither LA, Eide DJ: The human ZIP1 transporter mediates zinc uptake in human K562 erythroleukemia cells. *J Biol Chem* 276:22258–22264, 2001b
- Guerinot ML: The ZIP family of metal transporters. *Biochim Biophys Acta* 1465: 190–198, 2000
- Huang L, Gitschier J: A novel gene involved in zinc transport is deficient in the lethal milk mouse. *Nature Genet* 17:292–297, 1997
- Küry S, Devilder M-C, Avet-Loiseau H, Dreno B, Moisan J-P: Expression pattern, genomic structure and evaluation of the human *SLC39A4* gene as a candidate for acrodermatitis enteropathica. *Hum Genet* 109:178–185, 2001
- Küry S, Dréno B, Béziau S, Giraudet S, Kharif M, Kamoun R, Moisan J-P: Identification of *SLC39A4*, a gene involved in acrodermatitis enteropathica. *Nat Genet* 31:239–240, 2002
- Mikami Y, Fukushi G, Shiraishi M, Hirayama Y: A case of hereditary acrodermatitis enteropathica. *Med J Aomori* 39:76–80, 1994 (in Japanese)
- Nakano A, Nakano H, Hanada K, Nomura K, Uitto J: *ZNT4* gene is not responsible for acrodermatitis enteropathica in Japanese families. *Hum Genet* 110:201–202, 2002
- Wang K, Pugh EW, Griffen S, et al: Homozygosity mapping places the acrodermatitis enteropathica gene on chromosomal region 8q24.3. *Am J Hum Genet* 68:1055–1060, 2000
- Wang K, Zhou B, Kuo Y-M, Zemansky J, Gitschier J: A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *Am J Hum Genet* 71:66–73, 2002