

Compound Heterozygous Mutations Including a *De Novo* Missense Mutation in *ABCA12* Led to a Case of Harlequin Ichthyosis with Moderate Clinical Severity

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Harlequin ichthyosis (HI) is one of the most devastating genodermatoses. Recently, *ABCA12* mutations were identified as the cause of HI. A newborn Japanese male demonstrated the typical features of HI. The patient was treated with oral etretinate and his general condition has been good (now aged 1.5 years). This patient with moderate clinical severity was compound heterozygous for a novel *de novo* missense mutation 1160G>A (S387N) in exon 10 and a maternal deletion mutation 4158_4160delTAC (T1387del) in exon 28 of *ABCA12*. T1387del was a deletion of a highly conserved threonine residue within the first adenosine 5' triphosphate-binding domain and is thought to seriously affect the function of the *ABCA12* protein. Conversely, the residue 387 is located outside the known active sites of *ABCA12* and S387N is predicted not to lead to a serious functional deficiency in *ABCA12*. Electron microscopy revealed abnormal lamellar granules in the granular layer cells and a moderate number of lipid vacuoles in the cornified cells. Disturbed glucosylceramide transport was confirmed in the cultured keratinocytes from the patient. No *de novo* mutation in *ABCA12* has yet been reported either in HI or lamellar ichthyosis. The present case suggested that a *de novo* *ABCA12* mutation might underlie HI.

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INTRODUCTION

Harlequin ichthyosis (HI) (MIM no. 242500) is a severe and often fatal congenital ichthyosis with an autosomal recessive inheritance pattern (Williams and Elias, 1987; Akiyama, 1999; Judge *et al.*, 2004).

In 2005, *ABCA12* mutations were identified in HI families (Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). The pathomechanisms of HI became known when functional defects in the lipid transporter *ABCA12* were shown to cause abnormal lipid transport via lamellar granules (LGs) in the keratinocytes, resulting in malformation of the patient's intercellular lipid layers of the stratum corneum (Akiyama *et al.*, 2005). However, genotype/phenotype correlations in HI cases with *ABCA12* mutations have yet to be fully elucidated (Akiyama, *in press a*).

In the study of this case, we have found a compound heterozygous *ABCA12* combination of mutations, a novel *de novo* missense mutation, S387N, in exon 10 and a maternal

deletion mutation T1387del in exon 28 in a newborn HI baby. In the majority of HI patients, deletion or truncation mutations seriously affecting *ABCA12* function were found. However, in the present case, one mutation was a missense mutation located in the cytoplasmic region of *ABCA12* polypeptide, not in the transmembrane domain or the adenosine 5' triphosphate (ATP)-binding cassettes. The nature and site of mutations might be the key to the relatively moderate phenotype of this HI patient.

In addition, the novel mutation S387N was the first reported *de novo* mutation in *ABCA12*. Our case suggested the possibility that *de novo* mutations can cause the occurrence of HI in non-consanguineous families.

RESULTS

Clinico-pathological features of the patient

The patient was a newborn Japanese male. He was born at 33 weeks and 3 days pregnancy by premature, vaginal delivery (body weight 1,876 × g). He was the first child of non-consanguineous healthy parents. There was no family history of congenital ichthyosis. At birth, the patient had presented with severe hyperkeratosis over his entire body, severe ectropion, eclabium, and malformed pinnae (Figure 1). The patient's clinical features at birth were characteristic of typical HI. He was admitted into a neonatal intensive care unit and had oral etretinate treatment (1 mg/kg/day). At this time, light microscopy of the lesional skin sample from the patient's trunk showed marked hyperkeratosis with only a

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Abbreviations: ATP, adenosine 5' triphosphate; HI, harlequin ichthyosis; LG, lamellar granule

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Figure 1. The patient showed a typical clinical phenotype of HI in the neonatal period. The entire body surface was covered with thick plate-like scales and fissures.

small number of parakeratotic cells. At the age of 1 year, the patient showed dark brownish, thick scales over the entire body surface including the face, palms, and the soles.

ABCA12 mutation analysis

Mutation analysis of the 53 exons including the intron-exon boundaries of the entire *ABCA12* gene revealed a 1160G>A transition in exon 10 and one previously reported deletion mutation 4158_4160delTAC in exon 28 in each allele in a compound heterozygous fashion in the patient (sequence according to Lefèvre *et al.* (2003)) (GenBank accession NM 173076) (Figure 2). The mutation 1160G>A transition was a novel missense mutation that changed a serine residue of codon 387 to an asparagine residue (S387N). This missense mutation S387N was not found in either in the parent's (father or mother), although the patient's mother was heterozygous for the deletion mutation 4158_4160delTAC (Figure 2). Thus, the missense mutation S387N was thought to be a *de novo* mutation and the deletion mutation was a maternal mutation. Paternity testing by microsatellite marker analysis proved the father was the patient's true genetic father (data not shown). These mutations were not found in 200 normal, unrelated Japanese alleles (100 normal unrelated Japanese individuals) by sequence analysis, and were unlikely to be a polymorphism (data not shown). The deletion mutation 4158_4160delTAC led to an in-frame deletion of a highly conserved threonine residue at codon 1387 (T1387del) within the first ATP-binding

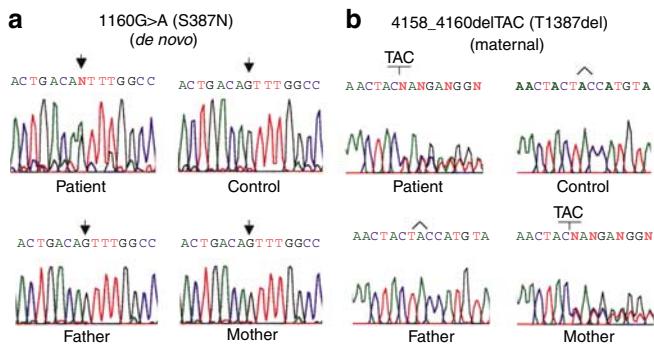


Figure 2. Compound heterozygous mutations of ABCA12 in the patient.
(a) Direct sequencing revealed a heterozygous 1160G>A transition (a missense mutation S387N) in exon 10 of *ABCA12* of the patient, but not in his parents or normal control samples. (b) A heterozygous deletion mutation 4158_4160delTAC (T1387del) was found in exon 28 of *ABCA12* of the patient and his mother, but not in his father or in normal controls.

domain of *ABCA12*. Thus, the deletion mutation is thought to seriously affect either the function or specific critical structures of the *ABCA12* protein (Akiyama *et al.*, 2005).

Ultrastructure of the patient's skin

Electron microscopy revealed that, in the keratinocyte cytoplasm of the granular layer, no normal LGs were apparent. Lipid droplets, vacuoles, and multivesiculated bodies were seen in keratinocytes in the keratinized and granular cell layers (Figure 3a and b). The number of abnormal lipid droplets in the keratinized stratum corneum cells was smaller than that in skin samples from HI patients harboring the homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12* (Akiyama *et al.*, 2005) (Figure 3c), although the number of lipid vacuoles was larger than those in lamellar ichthyosis patients with *ABCA12* mutations (data not shown). Cornified cell envelope formation was normal in the granular and cornified layer cells (Figure 3h).

Distribution of ABCA12 and glucosylceramide in patient's skin
Immunofluorescence studies revealed that *ABCA12* was positive in the upper epidermal layers, mainly in the granular layers, of normal human skin (Figure 4c). In the epidermis of the present patient, reduced *ABCA12* immunostaining was seen in the upper epidermis (Figure 4a), compared with seriously reduced immunolabeling in epidermal keratinocytes from the HI patient harboring a homozygous splice acceptor site mutation IVS23-2A>G (Figure 4b) (Akiyama *et al.*, 2005). These findings confirmed that the present patient expresses a significant amount of mutated *ABCA12* protein in his epidermis.

Immunofluorescent staining showed that glucosylceramide, a major lipid component of LGs (Vielhaber *et al.*, 2001; Ishida-Yamamoto *et al.*, 2004) and an essential component of the epidermal permeability barrier (Holleran *et al.*, 1993), was sparsely distributed in the patient's upper epidermis (Figure 4a), compared with a restricted, intense distribution in the granular layers of normal skin (Figure 4c). The condensed glucosylceramide staining was not seen in the patient's granular layers (Figure 4a).

Abnormal glucosylceramide transport in patient's cultured keratinocytes

Culture of the patient's keratinocytes under high-Ca²⁺ conditions (2.0 mM) induced a large number of cells to express condensed glucosylceramide staining around the

nuclei, and this glucosylceramide failed to become localized to the periphery of the keratinocyte cytoplasm (Figure 5a). Culture of normal human keratinocytes in high-Ca²⁺ conditions exhibited a more diffuse glucosylceramide staining throughout the cytoplasm (Figure 5b).

DISCUSSION

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families, encoding a highly conserved group of proteins involved in energy-dependent active transport (Higgins, 1992; Allikmets *et al.*, 1996; Dean *et al.*, 2001; Borst and Elferink, 2002) and, recently, this superfamily has been highlighted in the dermatology fields (Uitto, 2005). The ABCA subfamily is suggested to work in lipid transport (Weng *et al.*, 1999; Hayden *et al.*, 2000; Orso *et al.*, 2000; Schmitz and Langmann, 2001; Peelman *et al.*, 2003) and has received considerable attention (Klein *et al.*, 1999) because mutations in these genes have been implicated in several human genetic diseases (Allikmets *et al.*, 1997a,b; Brooks-Wilson *et al.*, 1999; Rust *et al.*, 1999; Oram, 2002). Keratinocyte LGs are known lipid-transporting organelles and LG contents are secreted into the intercellular space, forming an intercellular lipid layer between the granular layer cells and keratinized cells in the stratum corneum. Our previous study (Akiyama *et al.*, 2005) has clearly demonstrated that ABCA12 functions in the transport of endogenous lipid to the keratinocyte cell periphery via LGs.

The abnormal LGs in the granular layer keratinocytes and a lack of extracellular lipid lamellae reflect the defective lipid transport via LGs and the malformation of intercellular stratum corneum lipid layer in HI (Akiyama, *in press a*). In 2005, ABCA12 mutations that seriously affect its function were shown to cause a loss of the skin lipid barrier, leading to HI (Akiyama *et al.*, 2005). In addition to HI with defective lipid layers in the stratum corneum, ichthyosis syndromes are also thought to share similar pathomechanisms (Akiyama, *in press b*). For example, Dorfman-Chanarin syndrome (neutral lipid storage disease) showed malformation of LGs and

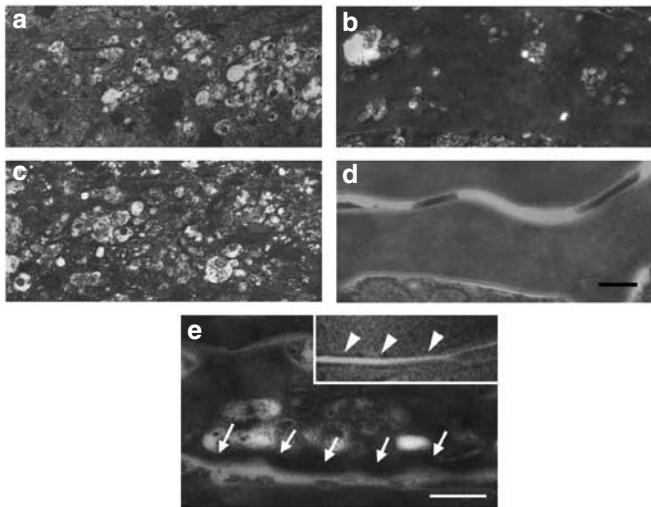


Figure 3. Abnormal LGs in the granular layer cells and accumulation of lipid droplets in the stratum corneum cells of the patient's epidermis.

(a) Abnormal LGs in the granular layer cells of the patient. (b) Abnormal lipid droplets accumulated in the cornified cells in the patient's epidermis, although the numbers and amounts of lipid droplets was smaller than that seen in an HI patient harboring a homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12* (c). (d) No lipid droplets were observed in control normal human stratum corneum cells. (a, b) the present patient; (c) control typical HI patient reported previously (Akiyama *et al.*, 2005); (d) normal control human skin. (e) Cornified cell envelope (arrows) with normal thickness was seen in the cornified layer cells in the present HI patient. Inset: the cornified layer cell of the control lamellar ichthyosis patient with transglutaminase 1 gene mutations showed malformed, thin cornified cell envelope (arrowheads). Bars = 200 nm.

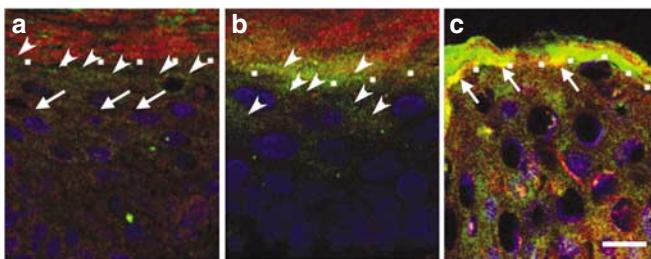


Figure 4. Abnormal ABCA12 immunostaining and disturbed distribution of glucosylceramide in the patient's epidermis. (a) In the patient's upper epidermis, weak ABCA12 immunostaining (red: arrows) was diffusely seen in the keratinocyte cytoplasm. Glucosylceramide labeling (green: arrowheads) was also diffusely observed in the keratinocytes of upper epidermis. (b) In the control HI patient carrying a homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12*, ABCA12 immunolabeling (red) was very weak, although diffuse glucosylceramide immunolabeling (green: arrowheads) was seen in the keratinocyte cytoplasm of upper epidermis. (c) In normal control human epidermis, both ABCA12 (red) and glucosylceramide (green) labelings overlapped, resulting in a yellow color in the granular layer (arrows). Dotted line: granular layer-cornified layer interface. ABCA12, tetramethylrhodamine isothiocyanate (red); glucosylceramide, FITC (green); nuclear staining, TO-PRO (blue). Bar = 10 μm.

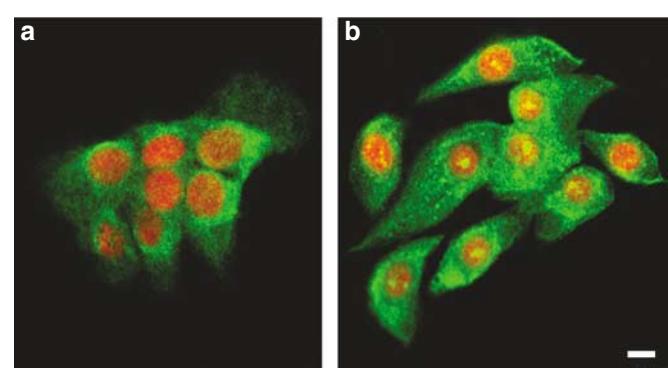


Figure 5. Altered glucosylceramide distribution in the patient's cultured keratinocytes. (a) In keratinocytes cultured from the patient, glucosylceramide labeling (green) was observed mainly in the perinuclear area of cytoplasm, suggesting disturbed glucosylceramide transport. (b) In normal cultured human keratinocytes, glucosylceramide (green) was seen diffusely in the cytoplasm to the cell periphery. Glucosylceramide, FITC (green); nuclear staining, propidium iodide (red). Bar = 10 μm.

defective lipid production in LGs caused by a deficiency in the CGI-58 protein that is thought to be involved in the pathogenesis of this form of ichthyosis (Akiyama *et al.*, 2003). In Sjögren-Larsson syndrome harboring fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) mutations, defective LG formation was reported as one sign of a putative pathogenetic mechanism (Shibaki *et al.*, 2004). Recently, mutations in a new gene, *FLJ39501*, encoding a cytochrome P450, family 4, subfamily F, polypeptide 2 homolog of the leukotriene B4- ω -hydroxylase (CYP4F2) were reported to underlie lamellar ichthyosis cases linked to chromosome 19p12-q12 (Lefèvre *et al.*, 2006). These facts further support the idea that abnormal LG lipid contents and defective intercellular lipid are prevailing concepts of pathogenetic mechanisms in the ichthyoses.

In the present case based on the reported amino-acid sequence (Annilo *et al.*, 2002), the deletion mutation 4158_4160delTAC led to an in-frame deletion of a threonine residue at codon 1387 (T1387del) within the first ATP-binding domain of ABCA12 protein (Figure 6). This threonine residue is a highly conserved residue between diverse species and is thought to be important in the function of ABCA12 (Akiyama *et al.*, 2005). Indeed, a compound heterozygous patient with this deletion mutation and a separate ABCA12 truncation mutation was previously reported as a typical HI newborn who died 15 days after birth (Akiyama *et al.*, 2005).

The novel *de novo* mutation S387N in the present patient is located outside all of the known ABCA12 active transporter sites, within the cytoplasmic domain at N-terminus of ABCA12 polypeptide (Figure 6). Both serine and asparagine are neutral amino acids with small side chains and this missense mutation would be expected to not significantly affect the conformation of ABCA12 molecule. Thus, one would predict the *de novo* missense mutation S387N not to lead to a serious ABCA12 functional loss. Considering the nature and site of the ABCA12 mutations in this case, we suggest that a combination of the deletion mutation and

the missense mutation leads to the current patient's HI phenotype with a moderate clinical severity.

In type 2 lamellar ichthyosis, a relatively mild form of congenital ichthyosis, all five reported ABCA12 mutations were missense mutations that resulted in only one amino-acid alteration (Lefèvre *et al.*, 2003). Conversely, in our previous study on HI families, no ABCA12 missense mutations were identified and most of the defects led to severe truncation of ABCA12 peptide, affecting important nucleotide-binding fold domains and/or transmembrane domains (Akiyama *et al.*, 2005). The other, non-truncation mutations in HI were deletion mutations affecting highly conserved ABCA12 sequences (Akiyama *et al.*, 2005). Thus, it was thought that only truncation or deletion mutations in conserved regions, which seriously affect the function of the ABCA12 transporter protein, can lead to the HI phenotype.

In an additional series of HI patients, most ABCA12 mutations were homozygous truncation mutations (Kelsell *et al.*, 2005). Only one mutation in one HI patient was a missense mutation and the patient was heterozygous for a truncation mutation and the missense mutation.

Recently, the prognosis of newborns affected with HI has improved, owing to better targeted oral retinoid treatment. More than half of HI newborns including cases with a serious functional loss of ABCA12 survive beyond the perinatal period (Akiyama *et al.*, 2005; Akiyama, in press a). Thus, it is difficult to discuss genotype/phenotype correlations and quality of life prognosis for ABCA12 mutations in HI.

Most families with HI or lamellar ichthyosis caused by ABCA12 mutations were consanguineous and, in those families, causative ABCA12 mutations were homozygous mutations inherited with an autosomal recessive trait. No *de novo* mutation has yet been reported in ABCA12, as far as we know (Lefèvre *et al.*, 2003; Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). The present novel missense mutation S387N in our case was the first *de novo* mutation reported in ABCA12. The genetic information of whether the causative mutations are inherited or *de novo* is very important for genetic counseling in severe genetic disorders such as HI. Indeed, in the present family, the parents had requested a prenatal diagnosis of HI for their subsequent pregnancy. However, we have demonstrated that the missense mutation was a *de novo* mutation and that the patient's father was not a carrier of any ABCA12 mutation. Thus, the family could be spared of the worry of an unnecessary HI prenatal diagnosis for the subsequent pregnancy almost, although we cannot completely exclude the possibility that the father has a mosaic mutation affecting his germ line. The present case suggested that, in non-consanguineous HI families, a *de novo* ABCA12 mutation might be one source of mutations and underlines that the detection of causative ABCA12 mutations is essential for the genetic counseling for HI.

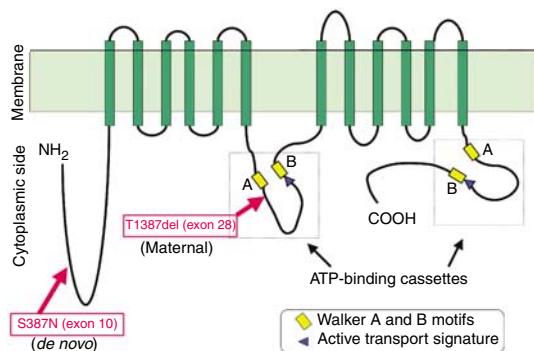


Figure 6. Schematic sequential arrangement of the domain structures of ABCA12 protein and the position of mutations in the present HI patient.

Mutations in the present HI patient are marked by red arrows. Note that the *de novo* missense mutation S387N is located in the intracytoplasmic region between the N-terminus and the first transmembrane domain, not in any active sites, and the other, deletion mutation T1387del is within the first ATP-binding cassette, which is thought to be important for ABCA12 lipid transporter activity.

MATERIALS AND METHODS

Mutation detection

Mutational analysis was performed in the affected baby and the parents. Briefly, genomic DNA isolated from peripheral blood was subjected to PCR amplification, followed by direct automated

sequencing using an ABI PRISM 3100 genetic analyzer (ABI Advanced Biotechnologies, Columbia, MD). Oligonucleotide primers and PCR conditions used for amplification of all exons 1–53 of *ABCA12* were originally derived from the report by Lefèvre *et al.* (2003) and were partially modified for the present study. The entire coding region including the exon/intron boundaries for both forward and reverse strands from the patient, family members, and 100 healthy Japanese controls were sequenced. No mutations were found in 200 normal alleles from the healthy Japanese population. Paternity testing was carried out using 16 microsatellite markers. The parents provided informed consent to the experiments. The experiments received institutional approval and the protocol adhered to The Declaration of Helsinki Principles.

Ultrastructural observations

Skin biopsy samples were fixed in 5% glutaraldehyde solution, post-fixed in 1% OsO₄, dehydrated, and embedded in Epon 812. The samples were sectioned at 1 μm thickness for light microscopy and thin sectioned for electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope. As controls, lesional skin samples from lamellar ichthyosis patients with transglutaminase 1 gene mutations (Akiyama *et al.*, 2001) or *ABCA12* mutations were also studied.

Cell culture

A skin sample from the patient was processed for primary keratinocyte culture, and cells were grown according to standard procedures in defined keratinocyte serum-free medium (Invitrogen Corp., Carlsbad, CA). After several passages in low-Ca²⁺ (0.09 mM) conditions, cultures were grown in high-Ca²⁺ (2.0 mM) conditions.

Antibodies

Polyclonal anti-*ABCA12* antiserum was raised in rabbits using a 14 amino-acid sequence synthetic peptide (residues 2567–2580) derived from the *ABCA12* sequence (NM 173076) as the immunogen (Akiyama *et al.*, 2005). The other primary antibody was mouse monoclonal anti-glucosylceramide antibody (Alexis Biochemicals, Lausanne, Switzerland).

Immunofluorescent labeling

Immunofluorescent labeling was performed as previously described (Akiyama *et al.*, 2000). Briefly, 6-μm-thick sections of fresh patient's skin was cut using a cryostat. The sections were incubated in primary antibody solution for 1 hour at 37°C. Antibody dilutions were as follows; 1/10 for anti-*ABCA12* antiserum and 1/10 for anti-glucosylceramide antibody. The sections were then incubated in FITC-conjugated to rabbit anti-mouse IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:100 (DAKO, Glostrup, Denmark) for 30 minutes at room temperature, followed by nuclear counterstain by TO-PRO-3 (Invitrogen, Carlsbad, CA). The sections were extensively washed with phosphate-buffered saline between incubations. The stained sections were then mounted with a cover slip and observed using a confocal laser scanning microscope.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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