DNA-Based Prenatal Diagnosis of Harlequin Ichthyosis and Characterization of *ABCA12* Mutation Consequences

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Until the identification of *ABCA12* as the causative gene, prenatal diagnosis (PD) for harlequin ichthyosis (HI) had been performed by electron microscopic observation of fetal skin biopsy samples. We report the first case of HI DNA-based PD. Direct sequence analysis of *ABCA12* revealed that the deceased proband was a compound heterozygote for two novel mutations. The maternal nonsense mutation p.Ser1249Term likely leads to nonsense-mediated messenger RNA decay. The paternal mutation c.7436G>A affects the last codon of exon 50 and was expected to be a splice site mutation. For their third pregnancy, the parents requested PD. Direct sequence analysis of fetal genomic DNA from amniotic fluid cells at 17 weeks gestation revealed the fetus was a compound heterozygote for both mutations. The parents requested the pregnancy to be terminated. Analysis of *ABCA12* transcripts of cultured keratinocytes from the abortus showed the presence of six abnormally spliced products from the allele carrying the splice site mutation. Four of them lead to premature termination codons whereas the two others produced shortened proteins missing 21 and 31 amino acids from the second ATP-binding cassette. This report provides evidence for residual ABCA12 expression in HI, and demonstrates the efficiency of early DNA-based PD of HI.

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INTRODUCTION

Harlequin ichthyosis (HI) is a severe and usually fatal congenital ichthyosis with an autosomal recessive inheritance pattern (Williams and Elias, 1987; Akiyama, 2006). The clinical features include thick, plate-like scales with ectropion, eclabium, and flattened ears. Infants affected with HI frequently die within the first few weeks of life. Skin development is altered *in utero*; hyperkeratosis of the hair canal occurs in the second trimester and characteristic ultrastructural abnormalities including abnormal lamellar granules, are present in the affected fetal epidermis (Dale *et al.*, 1990; Akiyama *et al.*, 1994, 1998). Before the gene underlying HI was identified in 2005, prenatal diagnosis (PD) of the disease relied on ultrastructural examination of fetal skin biopsy samples at 19–23 weeks estimated gestational age (EGA) (Blanchet-Bardon *et al.*, 1983; Suzumori and Kanzaki, 1991; Akiyama *et al.*, 1994, 1999).

ABCA12 is a transporter which belongs to the ATP-binding cassette (ABC) transporter superfamily (Annilo *et al.*, 2002; Uitto, 2005). In 2005, we identified serious loss of function mutations in *ABCA12* coding an ABC transporter, that leads to defective lipid transport in epidermal keratinocytes and results in an HI phenotype (Akiyama *et al.*, 2005). Another group independently demonstrated that mutations in ABCA12 underlie HI by linkage analysis (Kelsell *et al.*, 2005). Thus, HI PD using molecular *ABCA12* mutational analysis has become possible. We report here the first successful DNA-based PD for HI using fetal genomic DNA specimens from amniotic fluid cells.

RESULTS

Case history and the clinical features of the proband

The newborn proband was the first child from unrelated, healthy French parents. He was affected with HI and died soon after birth. He displayed severe hyperkeratosis with fissures over his entire body, severe ectropion, and eclabium (Figure 1a–c). There was no family history of genodermatoses in the family. The parents had a healthy son, their second child. For their third pregnancy, the parents requested HI PD.

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Abbreviations: ABC, ATP-binding cassette; cDNA, complementary DNA; EGA, estimated gestational age; HI, harlequin ichthyosis; HIK, HI keratinocytes; LG, lamellar granule; mRNA, messenger RNA; NHK, normal human keratinocytes; PD, prenatal diagnosis

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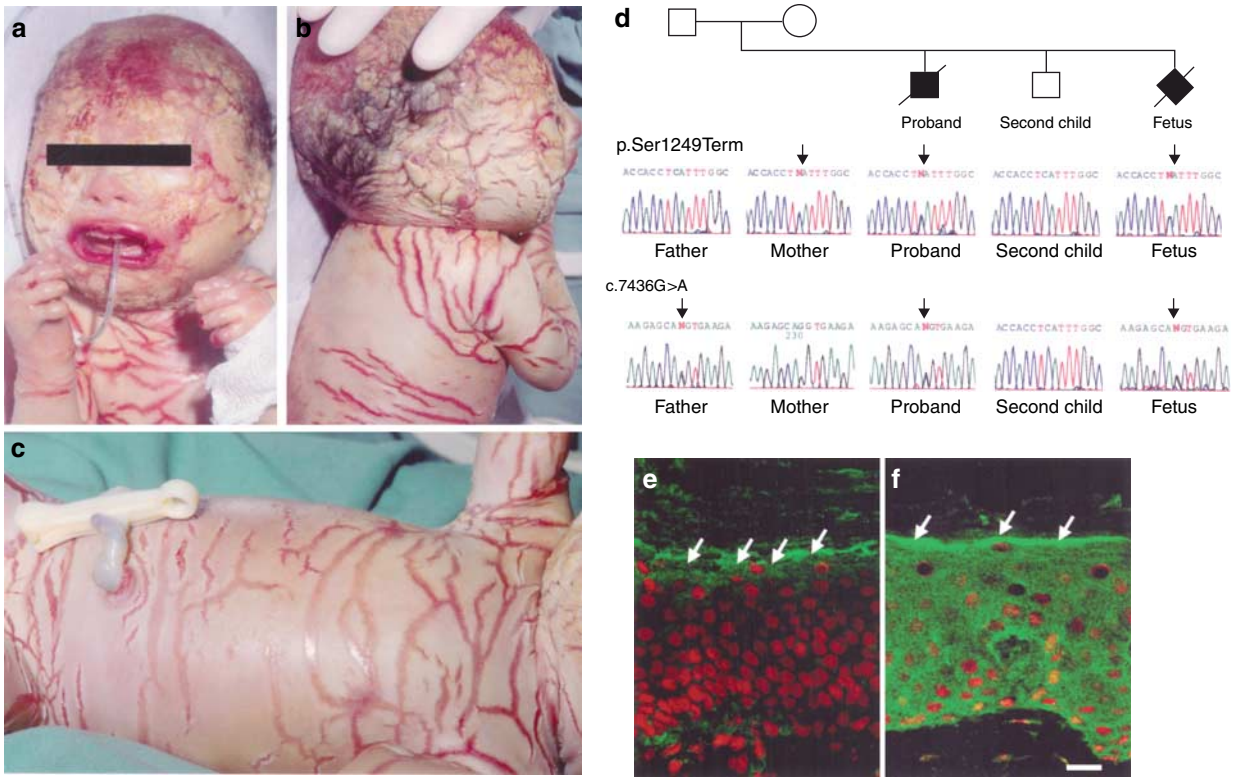

Figure 1. Clinical features of the proband, ABCA12 mutations in the family, and abnormal ABCA12 immunostaining in the patient's epidermis. (**a, b,** and **c**) Severe hyperkeratosis with fissures covering (**a**) the proband's face (**b**) scalp and back, (**c**) chest and abdomen. (**a**) Eclabium and (**b**) malformed pinna were apparent. (**d**) A novel nonsense mutation p.Ser1249Term was found in the proband, the mother, and the fetus (arrows). The novel splice site mutation c.7436G>A was detected in the proband, the father and the fetus (arrows). The fetus was prenatally diagnosed as affected. (**e**) In the patient's upper epidermis, weak ABCA12 immunostaining (green: arrows) was seen diffusely in the keratinocyte cytoplasm. (**f**) In normal control human epidermis, intense ABCA12 labeling (green) was noted in the granular layers (arrows). ABCA12, FITC (green); nuclear staining, propidium iodide (red). Bar = 10 μm.

ABCA12 mutation analysis

Mutation analysis of the 53 exons including the intron–exon boundaries of the entire *ABCA12* gene revealed that the proband was a compound heterozygote for two novel *ABCA12* mutations, c.3746C>A and c.7436G>A (sequence according to Lefèvre *et al.* (2003)) (GenBank accession NM 173076) (Figure 1d). c.3746C>A in exon 26 was a novel nonsense mutation that changed a serine residue at codon 1249 to a stop codon (p.Ser1249Term). This nonsense mutation p.Ser1249Term in exon 26 likely leads to nonsense-mediated messenger RNA (mRNA) decay rather than protein truncation, resulting in ABCA12 deficiency (see section *ABCA12* transcript analysis in cultured keratinocytes from the fetus). The mutation p.Ser1249Term was also found in the mother. The other mutation c.7436G>A in exon 50 affects the last amino acid of exon 50 and was expected to be a splice site mutation. Its potential effects on the splicing pattern of *ABCA12* pre-mRNA were investigated (see section *ABCA12* transcript analysis in cultured keratinocytes from the fetus). This splice site mutation was found in the father. Thus, the nonsense mutation p.Ser1249Term was of maternal origin and the splice site mutation c.7436G>A of paternal origin. These mutations were not found in 200 normal alleles (50 French and 50 Japanese healthy unrelated individuals) by sequence analysis, and were unlikely to be polymorphic variations (data not shown).

DNA-based PD of the fetus

Direct sequencing of PCR products including exon 26 or 50 of *ABCA12* from the fetal genomic DNA revealed the presence of the maternal nonsense mutation p.Ser1249Term in exon 26 and the paternal splice site mutation c.7436G>A in exon 50 (Figure 1d). Thus, the fetus harbored both *ABCA12* pathogenic mutations and was predicted to be affected. The pregnancy was terminated at 19 weeks gestation after the parents' request. The fetus showed characteristic changes including thin and fragile skin with petechia, eclabium, small, thickened and abnormally rimmed ears, rigid and swollen fingers and toes.

ABCA12 protein expression in the proband's skin

In the patient, ABCA12 immunostaining in the upper epidermis was reduced (Figure 1e), when compared with the intense ABCA12 immunostaining in the upper epidermal layers, mainly in the granular layers, of normal human skin (Figure 1f).

Ultrastructure of the skin from the abortus

Autopsy skin samples from the abortus showed abnormal, vacuolated lamellar granules in the upper intermediate cells and a large number of lipid droplets in the cytoplasm of incompletely keratinized keratinocytes.

ABCA12 transcript analysis in cultured keratinocytes from the fetus

Analysis of reverse transcription-PCR products from cultured keratinocytes of the fetus HI keratinocyte (HIK) and normal human keratinocytes (NHKs) on agarose gel electrophoresis revealed the presence of a single band in NHK PCR products and three bands in HIK PCR products (data not shown). PCR products were subsequently cloned and sequenced. A total of seven different mRNAs generated from the allele carrying the c.7436G>A mutation were identified (Figure 2a and b). Transcript p.Arg2479Lys corresponds to the full-length *ABCA12* transcript carrying a lysine residue in place of the arginine 2479. In addition, six of these transcripts are generated by splicing from several cryptic splice donor sites located in exon 50 of *ABCA12*. Transcript Δ4 carries a 4 bp deletion around the mutation (c.7433_7436del) leading to a frameshift and a PTC 11 amino acids downstream (p.Arg2479Leufs × 11). Transcript Δ31 displays a 31 bp deletion (c.7406_7436del) leading to a frameshift and a PTC 11 downstream (p.Ile2470_Arg2479>Leufs × 11). Transcript Δ43 presents a 43 bp deletion (c.7394_7436del) leading to a frameshift and a PTC 11 amino acids downstream (p.Lys2466_Arg2479>Leufs × 11). Transcript Δ50 shows a 50 bp deletion (c.7387_7436del) leading to a frameshift and a PTC 13 amino acids downstream (p.Asn2464_Arg2479>Trpfs × 13). Interestingly, transcript Δ63 predicts a truncated protein deleted from Leucine 2459 to Arginine 2479 (p.Leu2459_Arg2479del) owing to an in

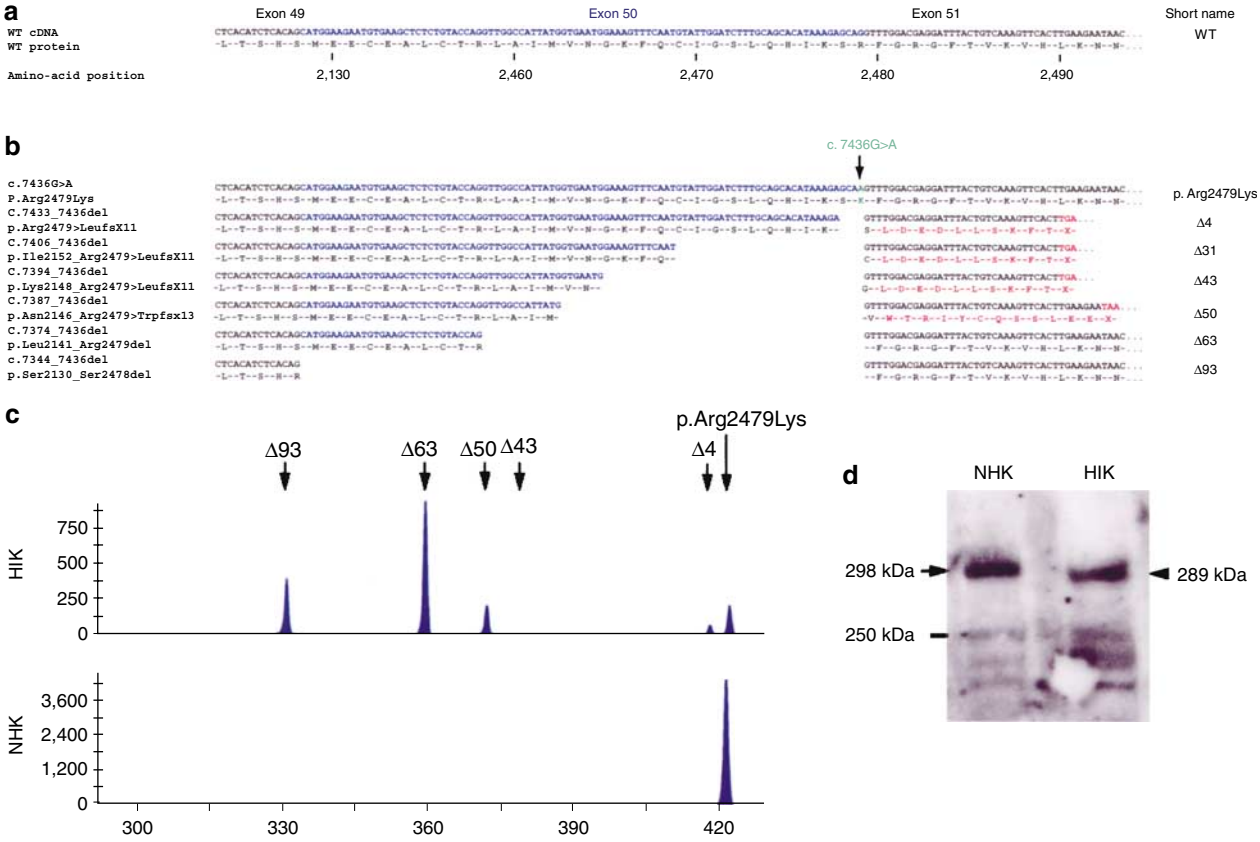

Figure 2. Expression analysis of ABCA12 transcripts and protein. (**a** and **b**) Sequence alignment of the several transcripts generated by the allele carrying the paternal splice site mutation c.7436G>A. (**a**) Wild-type cDNA (blue) and protein sequences of exon 50 of *ABCA12*. Amino-acid numbering is indicated. (**b**) Nucleotide and deduced protein sequences of splice variants of the allele carrying the c.7436G>A mutation (green). Premature stop codons and aberrant sequences owing to frameshifts are indicated in red. Splice variants include the correctly spliced product (p.Arg2479Lys) and six aberrantly spliced forms leading to out-of-frame (transcripts Δ4, Δ31, Δ43, and Δ50) and in-frame (Δ63 and Δ93) deletions. (**c**) Capillary electrophoresis analysis of *ABCA12* cDNA amplimers. Fragments extending over exons 49–53 were amplified by PCR using reverse-transcribed mRNA from primary cultures of HIK and NHKs. Although only one peak at 422 bp is present in NHK, several peaks are detected in HIK. The peak at 422 bp corresponds to the full-length transcript, arising either from the wild-type alleles (in NHK) or from the allele carrying the p.Ser1249Term null mutation and from the allele carrying the p.Arg2479Lys mutation (in HIK). The other peaks at 418, 379, 372, 359, and 329 bp, correspond respectively to transcripts Δ4, Δ43, Δ50, Δ63, and Δ93. The relative amount of each transcript, evaluated by the size of the peaks, shows that transcript Δ63 is predominant. Note that transcript Δ31 is missing, probably because its synthesis level is below the detection threshold of this technique. (**d**) Western blot analysis of ABCA12 protein in NHK and in HIK. A band at 298 kDa (arrow) is present in NHKs extract, however a slightly lower band (arrowhead, 289 kDa) is present in the HIK extracts. This lower band probably arises from the transcript Δ63 and to a less extent from the transcript Δ93.

frame deletion of 63 bp (c.7374_7436del), whereas transcript Δ93 presents an in frame skipping of the entire exon 50 predicting a truncated protein deleted from Serine 2448 to Serine 2478 (p.Ser2448_Ser2478del). The cryptic splice sites used to generate transcripts Δ4, Δ31, Δ50, and Δ63 were predicted using the automated splice site analysis software by Nalla and Rogan (2005).

Capillary electrophoresis analysis of the *ABCA12* complementary DNA (cDNA) amplimers showed one amplimer (422 bp) in NHKs corresponding to the normal sequence of exon 50, whereas five abnormal species were present in HIK (Figure 2c). All but one (Δ31) identified transcripts were found. The relative amount of transcript, indicated by the height of the peaks, showed that the Δ63 transcript is predominant, whereas the Δ31 transcript was not detectable. Interestingly, the height of the peak at 422 bp which arises from both the p.Arg2479Lys transcript and from the transcript synthesized from the other *ABCA12* allele carrying the p.Ser1249Term mutation is weak, indicating that the amount of both transcripts is low. This suggests that the allele carrying the null mutation is likely to be subjected to nonsense mediated mRNA decay and that only a small amount of ABCA12 protein arise from the natural splice site of the pre-mRNA carrying the c.7436G>A.

ABCA12 protein expression in fetal cultured keratinocytes

Western blot analysis revealed the presence of a band of an expected molecular weight of 298 kDa in protein extracts from NHKs, whereas a slightly smaller band of 289 kDa was seen in HI keratinocyte extracts (Figure 2d). This band probably corresponds to the proteins synthesized from both transcript Δ63 and transcript Δ93 which are predicted to encode proteins of calculated molecular weight of 291 and 289 kDa, respectively, and cannot be resolved on the SDS-PAGE. Smaller bands were seen in both extracts and probably correspond to degradation products. Western blot analysis failed to reveal the presence of other shortened proteins in the extract of HIK. However, the epitope recognized by the antibody is located at the end of the C-terminus domain of ABCA12 and thus is not present in the predicted proteins encoded by transcripts Δ4, Δ31, Δ43, and Δ50, and by the product of the allele carrying the p.Ser1249Term mutation.

DISCUSSION

HI is the most severe ichthyotic genodermatosis and has a very poor prognosis. Therefore, any parents' request for PD should be taken very seriously. However, until 2005 the causative gene had not been identified and previous prenatal diagnoses were performed using electron microscopic examination of fetal skin biopsies during the later stages of pregnancies (Blanchet-Bardon *et al.*, 1983; Suzumori and Kanzaki, 1991; Akiyama *et al.*, 1994, 1999). HI PD by fetal skin biopsy was usually performed at 21–22 weeks EGA (Blanchet-Bardon *et al.*, 1983; Suzumori and Kanzaki, 1991; Akiyama *et al.*, 1994, 1999). According to these reports, fetal skin biopsy specimens at that age showed characteristic abnormalities including a large number of lipid droplets in the keratinized cells and abnormal or absent lamellar granules, which were sufficient findings for the PD of the disorder.

However, PD of HI by fetal skin biopsy is technically difficult, requires excellent skin biopsy site selection, and is time-consuming. We need to gain a significant better understanding of fetal skin development and only a few experts are able to make this reliable diagnosis. Owing to the fact that the interfollicular epidermis at 19 weeks EGA or earlier is not sufficiently developed to exhibit the characteristic morphologic changes of keratinization (Holbrook and Odland, 1980), the observations of interfollicular keratinocytes are thought to sometime provide insufficient or unreliable information for PD or prenatal exclusion of HI. However, at 19 weeks EGA, we were able to see characteristic ultrastructural HI abnormalities in the keratinized cells in the hair canal or infundibulum of the developing hair follicle in the case of an affected fetus (Akiyama *et al.*, 1999). This is because keratinization in the hair cone and hair canal occurs at around 15 weeks EGA, approximately 8–9 weeks before the keratinization of interfollicular epidermis in human fetal skin development (Holbrook and Odland, 1978). However, fetal biopsy specimens at 19 weeks EGA may not always provide sufficient information for PD or exclusion of HI because the interfollicular epidermis has not yet keratinized at this stage of epidermal development (Shimizu *et al.*, 2005).

In 2005, *ABCA12* was identified as the underlying gene causing HI (Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). Additional HI cases harboring *ABCA12* mutations have now been reported (Akiyama *et al.*, 2006a, b). Owing to these discoveries, it has now become possible to undertake HI DNA-based PD by chorionic villus or amniotic fluid sampling from the earlier stages of pregnancy. These procedures are technically more reliable and have a reduced burden on the mothers, as in other severe genetic keratinization disorders (Tsuji-Abe *et al.*, 2004). We report here a successful PD of HI using fetal genomic DNA obtained at 17 weeks EGA. It is anticipated that in the future, even earlier prenatal diagnoses using completely non-invasive analysis of DNA from fetal cells in the maternal circulation will be possible (Uitto *et al.*, 2003), as well as pre-implantation genetic diagnosis for HI (Shimizu and Suzumori, 1999).

Expression analysis of *ABCA12* in cultured keratinoctyes from the abortus revealed that the allele carrying the c.7436G>A mutation produced seven different transcripts. The synthesis of six different truncated or deleted transcripts from this allele explains the loss of function of this allele. In particular, transcripts Δ63 and Δ93 predict the synthesis of deleted proteins (deletion of 21 and 31 amino-acid residues, respectively) with calculated molecular weights of 291 and 289 kDa, which cannot be separated on the SDS-PAGE. Thus, the protein detected at 289 kDa is likely to arise from both transcripts, but predominantly from transcript Δ63, which is present in higher amount. The two proteins lack part of the second ATP-binding cassette, which is predicted to dramatically affect ABCA12 function. Although, small amounts of a full-length ABCA12 protein carrying the p.Arg2479Lys mutation may theoretically be synthesized, they were not

detected. Thus, loss of ABCA12 function results from the combination of a null allele, and the synthesis of non-functional deleted proteins unable to restore a normal phenotype.

The terminated fetuses in the previous reports of positive PD of HI showed macroscopic changes consistent with the HI phenotype at 22 weeks or older EGA (Blanchet-Bardon *et al.*, 1983; Suzumori and Kanzaki, 1991; Akiyama *et al.*, 1994, 1999). In our previous report, we demonstrated that an affected fetus already showed a clinically apparent HI phenotype at 21 weeks EGA (Akiyama *et al.*, 1999). Characteristic HI changes of HI were seen both macroscopically and ultrastructurally at 19 weeks EGA and the present case suggests that the HI phenotype has started to emerge in the affected fetus at the late second trimester of pregnancy.

MATERIALS AND METHODS

Mutation detection

Mutational analysis was performed in the proband and both 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 as described previously (Akiyama *et al.*, 2005). The entire coding region including the exon/intron boundaries for both forward and reverse strands from the proband, the parents and 100 healthy control individuals (50 French and 50 Japanese) were sequenced.

DNA-based prenatal testing

Amniotic fluid cells were obtained under ultrasound guidance at 17 weeks gestation. Fetal DNA was extracted from fresh cells, and detection of *ABCA12* mutations targeting the mutations that were found in the proband was subsequently performed, as described above. There were no sonographic findings suggestive of an affected fetus. No complications arose from the procedure.

Cell culture

Primary human keratinocytes and fibroblasts were obtained from skin fragments of the abortus and a punch biopsy of a healthy control. Keratinocytes were cultured on a feeder layer of lethaly irradiated mouse 3T3-J2 fibroblasts as described previously (Barrandon and Green, 1987). Fibroblasts were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (Eurobio, Les Ulis, France).

RNA extraction and reverse transcription-PCR analysis

RNA from cultured keratinocytes and fibroblasts was extracted with the SV Total RNA Isolation System (Promega, Charbonnières, France), and first-strand cDNA synthesis was carried out using random hexamer primers and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Next, a segment of the *ABCA12* cDNA was PCR-amplified with primers 5′-TCCGTCATCCTCACATCTTCA-3′ (forward) and 5′-GAACCTTGGCTGCTGGTATC-3′ (reverse) using Go-Taq polymerase (Promega, Charbonnières, France). The cDNA amplimers were cloned into PGEM-T vector (Promega, Charbonnières, France) and sequenced using the SP6 primer (5′-ATTTAGGT GACACTATAGAATAC-3′) and the T7 primer (5′-GTAATACGACT CACTATAGGGC-3′). In parallel, the cDNA amplimers were analyzed on standard agarose gel and by capillary electrophoresis on an ABI 310 Genetic Analyzer running the GeneScan software (Applied Biosystems, Foster City, CA).

Immunohistological and immunoblot analysis of ABCA12 protein expression

Immunodetection of ABCA12 was performed using an affinity purified anti-ABCA12 serum raised in rabbits using a 14 amino-acid sequence synthetic peptide (residues 2,567–2,580) derived from the ABCA12 sequence (NM 173076) as the immunogen (Akiyama *et al.*, 2005). Immunofluorescent labeling was performed as described previously (Akiyama *et al.*, 2000). Briefly, 6-μm-thick sections of fresh patient's skin were cut using a cryostat. The sections were incubated in primary antibody solution, anti-ABCA12 antiserum diluted 1/10, for 1 hour at 37°C. The sections were then incubated in FITC-conjugated goat anti-rabbit Igs diluted 1:100 (DAKO, Glostrup, Denmark) for 30 minutes at room temperature, followed by nuclear counterstain by propidium iodide (Sigma Chemical Co., St Louis, MO). The sections were extensively washed with phosphate-buffered saline between incubations. The stained sections were then mounted with a coverslip and observed using a confocal laser scanning microscope. For Western blot analysis, proteins were extracted from cultured keratinocytes in the presence of protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche Diagnostics, Meylan, France), fractionated by SDS-PAGE using 30 μg/lane protein on a 4% gel, and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, Saclay, France). Membrane blocking and incubation with antibody (1:5,000) were carried out in phosphate-buffered saline with 5% skim milk. Secondary antibody was goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:5000, Cell Signalling Technology, Beverley). Signals were revealed with ECL + chemiluminescence reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Informed consent was obtained from the patients' parents. This study was approved by the medical ethical committees at the Hokkaido University, Sapporo, Japan, Purpan Hospital, Toulouse, France and Nantes University Hospital, Nantes, France. The study was conducted according to the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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