ABCA12 Is the Major Harlequin Ichthyosis Gene

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Harlequin ichthyosis (HI) is the most severe form of autosomal-recessive, congenital ichthyosis. Affected infants have markedly impaired barrier function and are more susceptible to infection. Abnormalities in the localization of epidermal lipids as well as abnormal lamellar granule formation are features of HI skin. Previously, we and others have shown that mutations in the *ABCA12* gene encoding an adenosine triphosphate-binding cassette (ABC) transporter underlie the skin disease HI. In this study, we have sequenced the *ABCA12* gene in an additional 14 patients and show that all contain mutations, with the majority being either nonsense substitution or frameshift mutations. Eleven HI patients had bi-allelic *ABCA12* mutations, whereas in the remaining three HI patients in this study, A*BCA12* mutations were detected on only one allele by sequencing. In addition, the one patient from the previous study where no sequence mutations were detected was screened for heterozygous deletions. A combination of oligonucleotide arrays, multiplex PCR analysis and single-nucleotide polymorphism genotyping revealed a heterozygous intragenic deletion in exon 8. These mutation data establish *ABCA1*2 as the major HI gene.

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Abbreviations: ABC, adenosine triphosphate-binding cassette; CGH, comparative genomic hybridization; HI, Harlequin ichthyosis; NBF, nucleotide-binding fold; SNP, single-nucleotide polymorphism

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INTRODUCTION

Harlequin ichthyosis (HI, OMIM 242500) is the most severe and often lethal form of recessive congenital ichthyosis (Hsu *et al.*, 1989; Moreau *et al.*, 1999; Sarkar *et al.*, 2000). Infants born with this condition have hard, thick skin covering most of their body. The skin forms large diamond-shaped plates separated by deep fissures that restrict movement (Hsu *et al.*, 1989; Dahlstrom *et al.*, 1995). These skin abnormalities also affect the shape of the eyelids, nose, lips, and ears. Owing to the impaired barrier function of the skin, neonates struggle to control water loss, regulate temperature, and are more susceptible to infection (Moskowitz *et al.*, 2004). In addition, the tightened skin can cause breathing difficulties leading to respiratory failure. In the past, an HI-affected neonate would often die within days of birth; however, with better intensive care and possibly the use of oral retinoids, patients now have an increased chance of survival (Hsu *et al.*, 1989; Moreau *et al.*, 1999; Elias *et al.*, 2000; Sarkar *et al.*, 2000). HI has been reported in both sexes and in a variety of ethnic backgrounds.

Our previous study utilized single-nucleotide polymorphism (SNP) array technology to map the HI locus to chromosome 2q35 and subsequently demonstrated that mutations in the *ABCA12* gene were a major cause of HI, with 11 out of 12 patients studied harboring bi-allelic *ABCA12* mutations (Kelsell *et al.*, 2005). Another study has independently associated *ABCA12* mutations with HI (Akiyama *et al.*, 2005). *ABCA12* is a member of the adenosine triphosphate-binding cassette (ABC) superfamily of active transporters.

Mutations in *ABC* genes underlie a variety of other diseases, including cystic fibrosis, Tangier disease, pseudoxanthoma elasticum, X-linked adrenoleukodystrophy and Dubin–Johnson syndrome (Hovnanian, 2005; Uitto, 2005). Interestingly, it has been shown that mutations in the first adenosine triphosphate-binding domain of ABCA12 underlie some cases of autosomal-recessive lamellar ichthyosis in families from Africa that showed linkage to chromosome 2q33–34 (Lamellar Ichthyosis type 2, (LI2), OMIM 601277) (Lefevre *et al.*, 2003). There is evidence to show that ABCA12 is a lipid-trafficking molecule involved in lamellar body formation and studies have reported abnormalities in the localization of epidermal lipids as well as abnormal lamellar granule formation in HI skin (Dale *et al.*, 1990; Akiyama *et al.*, 2005). In addition, the introduction of ABCA12 by gene transfer in HI keratinocytes increased lamellar granule lipid secretion (Akiyama *et al.*, 2005).

In this study, we have screened a further 14 unrelated patients affected with HI for disease-associated mutations in the *ABCA12* gene. In addition to sequence analysis, two different copy number analyses were performed to detect possible compound heterozygous exon or multiple exon deletions.

RESULTS

Sequence analysis of the *ABCA12* gene

Sequence analysis of all 53 coding exons of the *ABCA12* gene revealed likely disease-associated *ABCA12* mutations in all 14 patients described in this study. Examples are shown in Figure 1 and Table S1 gives the specific details of mutations found in each patient. Figure 2 shows a summary of HI-associated *ABCA12* mutations found in this and our previous study. Two patients were compound heterozygotes and nine were found to have homozygous mutations. In three patients, mutations were found on one allele only. The majority of mutations are nonsense substitutions arising from an inserted premature STOP codon, but four separate single base pair deletions and one single base pair insertion was detected, which are likely to result in frameshift mutations. Evidence for possible founder mutations was found within different ethnic groups. The UK patients of Caucasian origin, 66 and 96, had inherited the same mutation in exon 16 (2025delG). The mutations 7322delC and W1294* were previously identified in HI patients of Pakistani origin from our other studies (Kelsell *et al.*, 2005; Rajpar *et al.*, 2005). In addition, the Native American HI patient D938-01 was homozygous for the same *ABCA12* mutation (W1744* mutation in exon 34) as previously identified in an unrelated Native American HI patient from a previous study (Kelsell *et al.*, 2005). Although the vast majority of HI-associated *ABCA12* mutations identified to date (this study) (Akiyama *et al.*, 2005; Kelsell *et al.*, 2005) (Figure 2) would result in a truncated protein, patient 95 was found to contain a homozygous missense substitution (G1179R) in the first transmembrane domain of ABCA12. This residue is conserved in mouse, rat, and

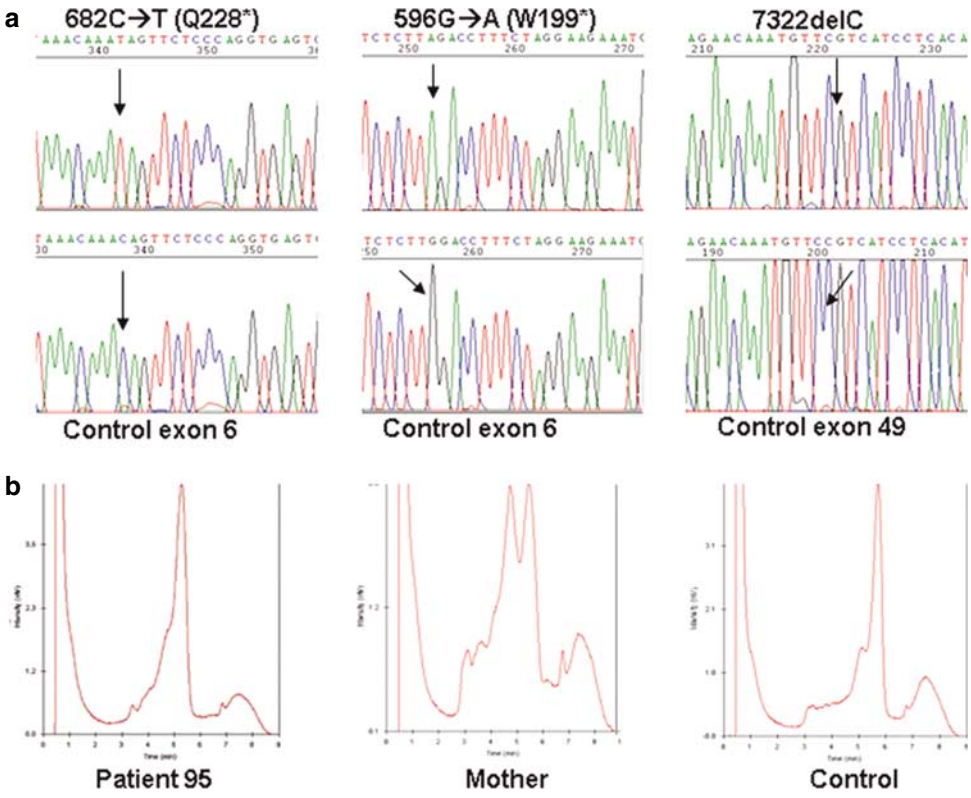

Figure 1. Sequencing and HPLC data from HI affected patients. (**a**) Sequences from three HI-affected patients along with normal controls. (**b**) Denaturing high-performance liquid chromatography traces of exon 24 showing patient 95 (homozygous G1179R), the mother of patient 95 (heterozygous), and a wild-type control.

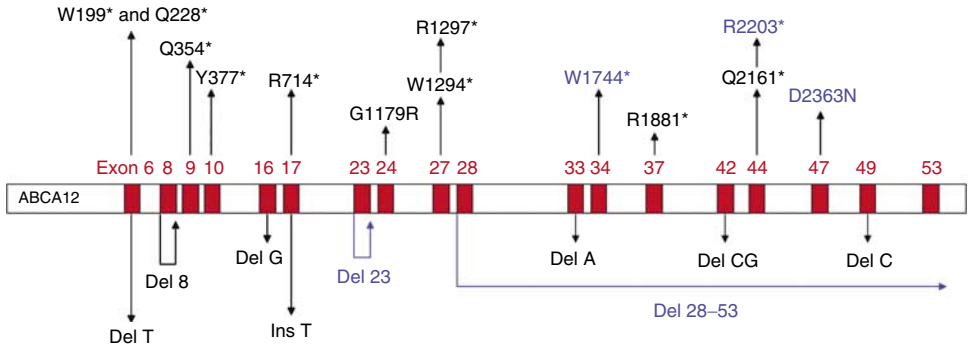

Figure 2. A schematic of the *ABCA12* gene showing the location of HI mutations found. Our previously published mutations are shown in blue, whereas novel mutations are shown in black. Deletions and insertions are shown below the gene, whereas nonsense and missense mutations are shown above. Exons are shown in red.

chicken orthologs of ABCA12. His unaffected mother was heterozygous for this amino-acid substitution. G1179R was not detected by denaturing high-performance liquid chromatography mutation screening in one hundred unrelated controls of mixed ethnicity. Functional studies are required to determine the proposed disease-causing nature of this mutation.

Multiplex and oligonucleotide array analysis

Two different methodologies were used to assess if single or multiple exon deletions in ABCA12 accounted for the ''missing'' mutation(s) in the predicted compound heterozygous HI patients (61, 131, and 96) and, from our previous study (Kelsell *et al.*, 2005), in patient 50 in whom no *ABCA12* mutations were identified. In a compound heterozygous form, these deletion mutations would be unidentifiable from a standard PCR reaction and sequencing. The first methodology was a multiplex PCR in which pools of primers used to amplify five different ABCA12 exons at a time were used to look for differences in amplification of each amplicon within the multiplex reaction. PCR products were resolved and quantified using Lab-on-a-Chip (Agilent Technologies, Germany) and also by running under size standard conditions on the WAVE system instrument (Transgenomic, France). The second method was a customized oligonucleotide array (Agilent Technologies, Germany) in which every exon of the *ABCA12* gene was represented. To validate both assays, two control samples were used: DNA from a patient homozygous for a deletion of exon 23 was mixed 1:1 with control DNA to generate an artificial exon 23 deletion compound heterozygote and a similar compound heterozygote control was generated for the homozygous deletion spanning exons 28–53. Both control heterozygous deletions were detected in the heterozygous form using the two deletion assays (data not shown). The assays were then performed on the patient DNA. Both methods clearly detected a previously undetectable heterozygous deletion of exon 8 of ABCA12 in patient 50 (Figure 3a and d). The exon 8 deletion was confirmed by genotyping the DNA of patient 50 and that of her parents with SNPs mapping in exon 8 and flanking intron (Figure 3f). The exon 8 deletion was inherited from her mother. Further molecular studies are required to define this putative deletion. Surprisingly, the sensitivity of the array methodology actually identified the single-nucleotide deletion within exon 16 of ABCA12 in patient 96 (Figure 3e). No other deletion mutations were identified.

DISCUSSION

The findings from this study firmly establish *ABCA12* as the major gene associated with HI. The increased number of patients with mutations in the *ABCA12* gene provides further evidence that *ABCA12* mutations are the main cause of HI and will allow prenatal diagnosis and/or pre-implantation genetic testing to be performed for most if not all HI carriers. A mutation on only one allele was discovered in three patients (90, 61, and 921–01). As the disease is recessive, we assume that there is another mutation on the other allele, perhaps of a more complex type, such as one in a regulatory region. We thought it possible that these patients may harbor heterozygous exon or multiple exon deletions that cannot be identified with standard sequencing techniques. These deletions are present in a homozygous form in HI patients from our previous study (Kelsell *et al.*, 2005). Unfortunately, skin biopsies were not available from these three patients to assay for ABCA12 protein levels. Large intragenic gene deletions have been reported in other *ABC* genes associated with disease. For example, in ABCC6 associated with pseudoxanthoma elasticum, a deletion of exons 23–29 was reported in 11% of patients screened (Miksch *et al.*, 2005). Indeed, other ABCC6 deletions have been described, which completely span the ABCC6 locus. To investigate for possible heterozygous exon deletions in ABCA12, we designed two laboratory investigations: multiplexing and peak height comparisons and the second was a comparative genomic hybridization (CGH) array approach. Both methods clearly detected a heterozygous deletion of exon 8 of ABCA12 from patient 50. Unexpectedly, the array methodology also identified the single-nucleotide deletion in exon 16 of ABCA12 from patient 96 previously found by sequencing. The 60mer oligonucleotide designed for exon 16 actually spans this G nucleotide deletion. This array-based mutation approach clearly will have applications for mutation analysis in other diseases in which heterozygous exon/intra–exon deletions are common.

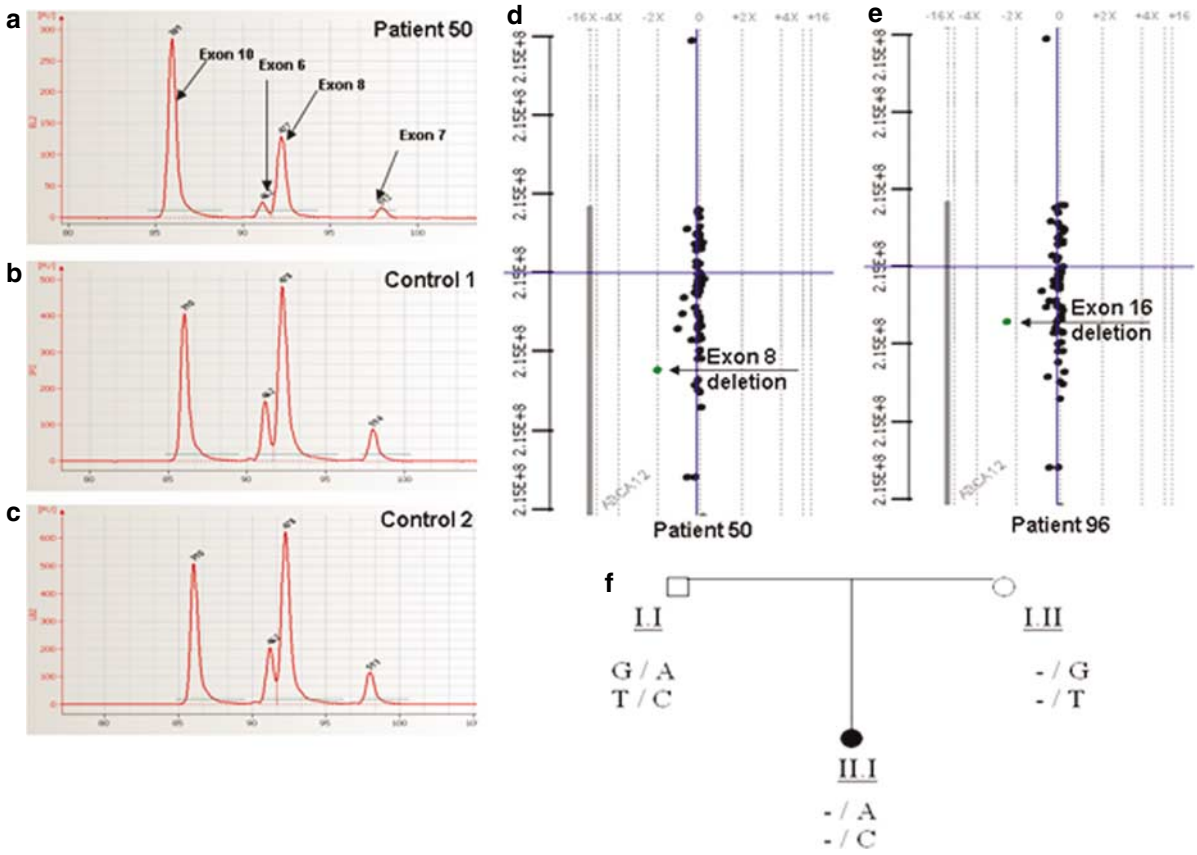

Figure 3. Details of heterozygous exon deletions. (**a–c**) Details of multiplex PCR from patient 50 and two controls along with (**d**) the Agilent oligo array data showing a reduction in copy number in exon 8 of patient 50, and (**e**) the presence of the known heterozygous G deletion in patient 96. (**f**) Deleted oligonucleotides are indicated by arrows. Pedigree of patient 50 with genotypes of exon 8 SNP (c.888 G>A (V296V)) and an intron 8 SNP marker (c.985+85 T>C). The genotypes clearly show non-maternal inheritance supportive of a maternal deletion spanning exon 8. Deleted SNPs are depicted as −.

Mutations in the *ABCA12* gene have also been identified in a subgroup of patients from Africa with LI2 (Lefevre *et al.*, 2003). The mutations in ABCA12 associated with LI2 have so far been shown to occur between exons 28 and 31, which correspond to the first nucleotide-binding fold (NBF1) located between amino acids 1370 and 1554. None of the mutations in this or the previous study have been found in the region of the NBF1. However, the vast majority of HI mutations found are truncations predicted to disrupt, at least, the C terminal end of the protein where the second NBF2 is located between amino acids 2282 and 2467. In our previous study, the only amino-acid substitution that was not a premature STOP codon was D2363N (Kelsell *et al.*, 2005). In contrast to ABCA12-associated LI2, this mutation is located directly in the second NBF region of the protein. It is therefore likely that missense mutations in these different regions of the gene determine the severity of the ichthyosis either LI2 if in NBF1 or the more severe HI if in NBF2. In this study, we have identified another missense substitution (G1179R) in exon 24 (homozygous in patient 95) that would substitute a glycine (uncharged polar residue) for an arginine (positively charged residue) in the first transmembrane domain of the protein. This variant was not seen in 200 control chromosomes, but the functional effect of this change requires further characterization.

It has been proposed that HI can be divided into three subtypes based on the expression patterns of keratin 16 and profilaggrin as well as the presence of keratohyalin granules (Dale *et al.*, 1990). In this study, we analyzed one patient (923-01) classified as type 1 under this system (all the rest were type IIs) and they were homozygous for a stop mutation in exon 6. This is a similar type of mutation seen in most HI patients that would lead to a severely truncated protein if expressed. This suggests that other factors unrelated to the site and type of mutation in ABCA12 account for this immunohistochemical classification

A key question raised is why is there reduced and immature lamellar granules in HI skin? Other ABC transporters including ABCA7 are also expressed in the skin and have been shown to be involved in the transportation of lipids in the epidermis. Over expression of ABCA7 in HeLa cells results in an increased expression of intracellular and cell surface ceramide as well as intracellular phosphatidylserine. It is suggested by the authors that ABCA7 may play a functional role in lamellar body lipid homeostasis in the epidermis (Kielar *et al.*, 2003). In addition, the transporters ABCC1, ABCC3, and ABCC4 translocate amphiphilic anions, such as conjugates of lipophilic compounds with glutathione, glucuronate, and sulfate. It has also been suggested that ABCB1 and ABCG1 are involved in phospholipid transport,

whereas ABCB1 and ABCG1 may be involved in the translocation of cholesterol (Kielar *et al.*, 2003). Therefore if these, and other, ABC transporters are expressed in the skin and are involved in lipid transport, why is it not possible for them to compensate for the lack of ABCA12 found in HI? Interestingly, the eletron microscopy features of HI lamellar granules grossly resemble those seen in the neurocutaneous syndrome (CEDNIK: cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma) caused by a mutation in a gene coding for a SNARE protein (Sprecher *et al.*, 2005), suggesting a possible link with abnormal lipid cargo loading of the vesicles.

In conclusion, we have screened a further 14 unrelated patients affected with HI and identified likely disease-associated mutations in the *ABCA12* gene. The majority of mutations are predicted to cause nonsense substitution or frameshift mutations. Patients without biallelic ABCA12 mutations following sequence analysis (this and our previous study: Kelsell *et al.* (2005)) were re-assessed using two copy number molecular strategies: a multiplex PCR, the other a CGH-based array platform. Indeed, the patient from our previous study (Kelsell *et al.*, 2005) in which no mutations were found by sequencing, was found by these methods to have exon 8 heterozygously deleted. From this study and our other studies, 22/26 unrelated HI patients of various ethnicities that we have analyzed by sequencing harbor bi-allelic mutations in the *ABCA12* gene. Four of twenty-six patients have mutations found by sequencing and copy number analysis on one allele only (Kelsell *et al.*, 2005; Rajpar *et al.*, 2005). Other plausible gene mutations that have not been assessed are inversions, promoter mutations and splice site mutations embedded deep in intronic sequence. In addition, disease-associated *ABCA12* mutations in four other HI cases have also been described (Akiyama *et al.*, 2005). These mutation data clearly emphasize that *ABCA12* mutations are the major genetic cause of HI. In addition, the identification of a heterozygous whole exon deletion in one patient highlights the requirement for such mutations to be taken into account when offering prenatal testing for HI.

MATERIALS AND METHODS

Clinical features and patient origins

Fourteen families with individuals affected with HI were used in this study: eight from the US, one from Ireland, five from Great Britain, and one from Israel. All patients and their families consented to take part in this study. Ethical approval was obtained from the relevant Local Research Ethics Committees. The clinical phenotype was verified as HI by at least two clinicians and relevant clinical information is shown in Table S2. Patient 50 was described in our previous study (Kelsell *et al.*, 2005). The study was conducted according to The Declaration of Helsinki Principles.

Sequence and denaturing high-performance liquid chromatography analysis of ABCA12

Primer design, PCR, and sequencing conditions used were the same as those described previously (Lefevre *et al.*, 2003; Kelsell *et al.*, 2005). The primers used for denaturing high-performance liquid chromatography analysis of G1179R in exon 24 are forward primer (5′-CGGACTACAGCTTCTCGGTTA-3′) and reverse primer (5′-AAT TTCCCACTCTGCCATTC-3′) to amplify a product of 205 bp. Briefly, PCR products covering each of the 53 exons and intron boundaries were purified using ExoSAP-IT (Amersham Pharmacia Biotech, UK). Each exon was then sequenced in both directions. Sequencing was carried out using ABI BigDye Terminator reagents (Applied Biosystems, CA) and an ABI 3700 sequencer. Sequencing reaction conditions were 5 μl of purified product, 1 μl of BigDye Terminator v.3.1, 3 μl of Better Buffer (Microzone, UK), and 1 μl water. Cycling conditions consisted of 25 cycles of 94°C for 30 seconds, 50°C for 15 seconds, and 60°C for 2 minutes. Reactions were precipitated on ice for 15 minutes following the addition of 2 μl of 3 M NaOAc (pH 5.2) and 50 μl ethanol. Sequence analysis was performed using Phred, Prap, and Consed. Variants were detected using reference sequence (cDNA reference sequence NM_173076 and genomic sequence for intronic variants) taken from the Ensembl Genome Browser (Ewing and Green, 1998). Denaturing high-performance liquid chromatography (Transgenomic WAVE system, France) was used to assess whether the exon 24 sequence variant G1179R found in patient 95 was likely to be a mutation or a polymorphism. The 205 bp fragment of exon 24 was analyzed in 100 control individuals plus patient and maternal samples, which are homozygous and heterozygous for G1179R, respectively. Five microliters of each PCR product was injected under the mutation detection program with an oven temperature of 59°C and a flow rate of 0.9. Site of mutation is based on the first nucleotide of the translation initiation codon ATG using cDNA reference sequence NM_173076.

Oligonucleotide microarray

The Human Genome CGH Microarray 44B (Agilent Technologies, Germany) contained ~43,000 *in situ* synthesized 60-mer oligonucleotide probes that span coding and non-coding sequences with an average spatial resolution of ~35 kb. The probes were designed *in silico* based on the National Center for Biological Information genome build 35 and were optimized for high-resolution array-based CGH. The catalog array was customized using the Agilent online design service eArray, by adding a further 73 probes (each replicated five times) designed specifically to span the *ABCA12* gene at chromosome 2q35 increasing the average spatial resolution in that region to ~1.7 kb. For each CGH hybridization, 3 μg of genomic DNA from the reference control (46, XY) and the corresponding experimental sample were digested with *Alu*I (25 U) and *Rsa*I (25 U) (Promega, UK) for 2 hours at 37°C. Individual reference and experimental samples were then purified using the QIAprep Spin Miniprep Kit (Qiagen, UK). Using the total amount of purified restricted DNA eluted, labeling reactions were performed with the BioPrime Array CGH Genomic Labeling Module (Invitrogen, UK) according to the manufacturer's instructions in a volume of 50 μl. Sixty micromolar Cy5-dUTP (for the experimental sample) or Cy3-dUTP (for the XY male reference) (Perkin-Elmer, Wellesley, MA) was added and *vice versa* for the corresponding dye-swap arrays. Experimental and reference targets for each hybridization were combined and purified using a Microcon YM-30 column (Millipore, Billerica, MA) to a final volume of 150 μl. The labeled targets were mixed with 50 μl of human Cot-1 DNA (Invitrogen, UK) and 50 μl of 10 × blocking agent (Agilent Technologies), and then mixed with an equal volume of Agilent 2 × Oligo aCGH Hybridization buffer. Before hybridization to the array, the 500 μl hybridization mixtures

were denatured at 95°C for 5 minutes and incubated at 37°C for 30 minutes. To remove any precipitate, the mixture was centrifuged at $\sim 17{,}000 \times g$ for 1 minutes and then 490 μl of the sample was applied to the array. Using an Agilent microarray hybridization chamber, the hybridization was carried out for 40 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA) at 20 r.p.m. The hybridization chambers were then disassembled in Agilent Oligo aCGH Wash Buffer 1 at room temperature and the arrays washed for 5 minutes at room temperature in fresh wash buffer 1, followed by 1 minute at 37°C in Agilent Oligo aCGH Wash Buffer 2 (prewarmed to 37°C overnight). The slides were removed from wash buffer 2 slowly (5–10 seconds) after which time they were completely dry and were then scanned using an Agilent G2565BA DNA microarray scanner. Microarray images were analyzed by using Feature Extraction software (version 8.1, Agilent Technologies) and the data visualized using CGH Analytics software (version 3.2, Agilent Technologies).

Multiplex PCR analysis by lab-on-a-chip electrophoresis

For the separation of multiplex PCR products, DNA 1000 LabChip Kits and the 2100 bioanalyzer (Agilent Technologies, Germany) were used according to the manufacturer's instructions. In brief, 9 μl gel–dye mix was added to the chip and then pressurized through the microchannels. Marker solution and DNA 1000 ladder were added then 1 μl of each PCR reaction was pipetted into each of the 12 sample wells of the prepared chip. After vortexing, the chip was placed in the Agilent 2100 bioanalyzer and run using the DNA 1000 assay. Electrophoresis of the 12 samples took 30 minutes including fragment analysis that was carried out using Agilent's 2100 Expert software. Electropherograms were overlaid to compare PCR patterns derived from patient and control DNA. Additional separation and peak height comparison was performed on the Transgenomic WAVE instrument under non-denaturing conditions.

SNP marker analysis

PCR products were sequenced using Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, High Wycombe, Buckinghamshire, UK) and run on the Beckman Coulter CEQ8000 Genetic Analysis System. The two relevant SNPs in the region shown to be heterozygously deleted in the affected member of the family (patient 50) are: exon 8, c.888 G>A (V296V) and intron 8, c.985 + 85 T>C.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Details of mutations found in *ABCA12*.

Table S2. Clinical details of HI patients.

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