

X-linked ichthyosis and Crigler-Najjar syndrome I: Coexistence in a male patient with two copy number variable regions of 2q37.1 and Xp22.3

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Abstract. X-linked ichthyosis (XLI) is an X-linked recessive skin disorder generally restricted to males, which arises from mutations in the steroid sulfatase (*STS*) gene located on Xp22.3. Crigler-Najjar syndrome (CN-I) is a rare autosomal recessive disease caused by the homozygous or compound heterozygous mutations in the UPD-glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*) gene on chromosome 2q37. A male patient was referred to the Department of Medical Genetics with of severe icterus and ichthyosis. The patient and his family members underwent genetic tests related to XLI and CN-I. Quantitative polymerase chain reaction on genomic DNA was performed to determine the gene copy number, while single nucleotide polymorphism array analysis was conducted to identify deletion mutations. Family pedigree analysis showed that the patient and his two cousins were all affected by ichthyosis, which was in accordance with the inheritance pattern of an X-linked recessive disease. In addition, the patient's serum bilirubin concentration (>340 mmol/l) was markedly greater than the normal level. The patient presented with kernicterus and phenobarbital treatment was ineffective. The clinical diagnosis of XLI was confirmed molecularly by laboratory evidence of a maternal 1.61 M deletion (including the *STS* gene) on ChrXp22.31. Coincidentally, the male patient was also confirmed to carry a rare maternal inherited microdeletion (374 Kb) comprising the entire *UGT1A1* gene combined with a paternal *UGT1A1* mutation (c.1253delT), a causative

event of CN-I. To the best of our knowledge, this study reported for the first time the comorbidity of XLI and CN-I in a male patient. The results suggested that co-occurrence of these two recessive diseases in a patient may be incidental.

Introduction

X-linked ichthyosis (XLI, OMIM#308100) is an X-linked recessive skin disorder that is caused by steroid sulfatase (*STS*) deficiency (1). The incidence rate of XLI is 1 in 6,000 males studied in various populations (1). It is characterized by desquamation of large brown scales on the limb and trunk (2). Extracutaneous manifestations, particularly corneal opacities and cryptorchidism, are frequently observed (3). The disease is caused by mutations in the *STS* gene that is located on the short arm of the X-chromosome (Xp22.3) (4). Approximately 90% of patients with XLI have large deletions involving the entire *STS* gene and flanking regions (5,6).

Crigler-Najjar syndrome type I (CN-I, MIM#218800) is a rare autosomal recessive disease caused by the genetic alterations in the UPD-glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*) gene (7). It is characterized by high levels of serum total bilirubin (342-684 μ mol/l) caused by the complete absence of the *UGT1A1* enzyme, with a frequency of less than one per million live births (8). The accumulation of unconjugated bilirubin in the serum may cause irreversible neurological damage (kernicterus). The brain damage can be prevented by intensive phototherapy that is effective at least until adolescence (9). However, CN-I is a lethal disease when not treated adequately and phenobarbital treatment is not effective for the treatment of symptoms. Liver transplantation is the only definitive treatment for CN-I. To date, the majority of *UGT1A1* mutations are single nucleotide substitutions, including missense mutations and nonsense mutations. Large deletions or insertions of *UGT1A1* gene are less reported.

Human genetic diseases are predominantly associated with DNA variations, ranging from single nucleotide changes to large DNA fragment variations, such as copy number variations (CNVs). XLI and CN-I are two genetic diseases with Mendelian inheritance patterns, however, they have distinct pathogenic mechanisms and clinical manifestations. XLI predominantly arises from CNVs involving the *STS* gene on the Xp22.3 region. By contrast, CN-I is usually the result of point mutations in the *UGT1A1* gene, and CNVs involving this

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Abbreviations: CN-I, Crigler-Najjar syndrome; *UGT1A1* gene, UPD-glucuronosyltransferase 1 family, polypeptide A1 gene; XLI, X-linked ichthyosis; *STS*, steroid sulfatase; CNVs, copy number variations; SNP, single nucleotide polymorphism

Key words: X-linked ichthyosis, Crigler-Najjar syndrome, 2q37.1, Xp22.31, copy number variation

gene have rarely been reported. Moreover, a coexistence of the two genetic alterations has not been reported.

The present study presents a case of a Chinese male patient affected by recessive XLI and CN-I, which were confirmed by DNA quantitative polymerase chain reaction (PCR) and single nucleotide polymorphism (SNP) array analysis. To the best of our knowledge, this is the first report this co-morbidity.

Materials and methods

DNA samples. Genomic DNA samples were extracted from the peripheral blood leukocytes of the patient and his parents, using the phenol-chloroform method (10). Informed consent was obtained from the patient's parents. This project was approved by the ethics committee of the Capital Institute of Pediatrics (Beijing, China).

Quantitative PCR for STS gene-copy number analysis. As 90% of the patients with XLI have large deletions involving the entire *STS* gene, the copy numbers of this gene were directly detected by quantitative PCR on genomic DNA. Primers were designed using the Primer Express 3.0 software (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The *STS* primers used were as follows: STS-E1F, 5'-GTT GCCCCTTCTGAAGAATCC-3'; STS-E1R, 5'-GAGGCG GAGACACTCTTTGC-3'; STS-E10F, 5'-TGGAGTGCAGTG GTGCAATC-3'; STS-E10R, 5'-GTGACTTGGGAGGCTGAA GTG-3'. The quantitative PCR was conducted using the ABI Prism 7500 sequence detection system (Applied Biosystems, Thermo Fisher Scientific Inc.) as described by Wilke *et al* (11). Data were analyzed using the ABI 7500 Prism sequence detection software (Applied Biosystems, Thermo Fisher Scientific, Inc.). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control gene for gene copy number analysis. The $2^{-\Delta\Delta C_q}$ method for relative quantification was used to evaluate *STS* gene copy numbers of the patient and his parents in comparison with those of healthy controls. DNA from healthy controls served as a calibrator that had a $2^{-\Delta\Delta C_q}$ of 1. The $2^{-\Delta\Delta C_q}$ value of the inspected sample is the ratio of the target gene copy number of inspected sample to the calibrator.

SNP array analysis. A genome-wide SNP array, Human CytoSNP-12 DNA Analysis BeadChip kit (Illumina, Inc., San Diego, CA, USA) was used to examine the deletion sizes of the two regions (2q37.1 and Xp22.31) containing *STS* and *UGT1A1* genes. The genome-wide SNP array was composed of 300,000 probe markers. Briefly, 4 μ l DNA sample (200 ng) was mixed with 4 μ l NaOH (0.1 mol/l) solution, followed by a 10-min incubation at room temperature for DNA denaturation. Next, 68 μ l MA2 solution and 75 μ l MSM solution (both from Illumina, Inc.) were added to the mixture to allow DNA renaturation. The reaction solution was put in the 37°C hybrid furnace for 20 h. The resulting DNA fragments were resuspended in RA1 solution (Illumina, Inc.) and the DNA solution was loaded onto the chip. The chip was kept in an airtight hybrid box (Illumina, Inc.) to permit hybridization for 16 h at 48°C. When the chip was washed to remove non-hybridized and nonspecific hybridized DNA fragments, single base extension and chip staining reactions were initiated. The iScan scanning system (Illumina, Inc.) was used to collect data, and

the data were further analyzed by Karyostudio software v1.4 (Illumina, Inc.).

Results

Clinical presentation. The male patient is the first offspring of healthy and nonconsanguineous parents. He was born at term by normal delivery; however, his mother had the premonitory symptoms of miscarriage two months into the pregnancy. The patient presented with yellowing of the skin 6 days after birth. He was admitted to the Capital Institute of Pediatrics due to yellowing of the skin that did not reduce at 4.5 months old. His total serum bilirubin level was 520.6 μ mol/l and serum unconjugated bilirubin level was 499.6 μ mol/l. The patient succumbed to kernicterus at 13 months of age, due to molecularly confirmed CN-I syndrome (12). However, upon reviewing the related medical records, other abnormal manifestations in the patient that could not be fully attributed to CN-I alone, were identified. Upon admission, the physical examination indicated that the patient was 7,000 g (20th) in weight and 66 cm in length (50-75th), with a head circumference of 41 cm (20th), which revealed that the patient had growth retardation. He could not raise his head at 4 months old of age, which suggested slight retardation of mental and motor development. A dispersed congestive rash were observed on his face and head, and rhombus-shaped brown pigmentation and furfuration were observed on his trunk and four limbs. A follow-up survey with the family revealed that the patient's two cousins also suffered from ichthyosis (Fig. 1); thus, XLI was suspected. Apart from ichthyosis, the patient had inspiratory stridor after birth and was diagnosed with congenital laryngeal cartilage softening disease. Abdominal computer tomography scanning indicated that the patient had left renal dysplasia and left renal artery stenosis. However, his renal function and routine urine test results were normal.

Gene copy number changes. For the *STS* gene, the patient's mother and father both carried only one copy number ($N=1$), although the male patient did not carry the gene ($N=0$). The results of real-time quantitative PCR are shown in Fig. 2, and the $2^{-\Delta\Delta C_q}$ value of the control (male or female) was considered to be 1.

The 'homozygous mutation' of c.1253delT in exon 4 of the *UGT1A1* gene was detected in the patient by DNA sequencing in our previous study (12). The same mutation was observed in his father, but not in his mother. It is possible that an allele lacking the entire *UGT1A1* gene may be transmitted to the patient from the mother who had a single *UGT1A1* gene copy. Therefore, for the patient with one point mutation (c.1253delT) on one allele and with an entire deletion of the *UGT1A1* gene on the other allele, the possibility existed of falling into the 'trap' of 'homozygous mutation' (c.1253delT) in the patient by direct DNA sequencing. Thus, quantitative PCR was performed to detect the copy number of *UGT1A1* gene in the core members. The results demonstrated that his father carried two copies of the *UGT1A1* gene, with c.1253delT detected on one of alleles. His mother only had one copy of the *UGT1A1* gene without base changes. The patient also carried a single copy of the *UGT1A1* gene that harbored the c.1253delT mutation.



Figure 1. (A) Pedigree analysis of CN-I and XLI. White square, normal male; white circle, normal female; circle right filled with vertical lines, female carrier with XLI; square filled with vertical lines: male patient with XLI; circle left filled with horizontal lines and right filled with vertical lines: female carrier with CN-I and XLI; square left filled with horizontal lines: male carrier with CN-I; square filled with vertical lines and horizontal lines: the male proband with XLI; diagonal line, death. (B) Rhombus-shaped brown pigmentation on the lower limbs of the patient's cousin with XLI. CN-I, Crigler-Najjar syndrome; XLI, X-linked ichthyosis.

Deletion range detection. SNP array results are shown in Fig. 3. Two CNVs were confirmed for this patient: One maternal microdeletion was located on ChrXp22.31 with an estimated size of 1.61 Mb; the other maternal microdeletion was found on Chr2q37.1 (Table I), with an estimated size of 374-kb (Fig. 3A and B). Moreover, SNP array results showed that his mother had the same microdeletion of 374 kb on Chr2q37.1, but a larger deletion (~1.67 Mb) on ChrXp22.31, including *STS* and additional five genes (for example, mental retardation related gene *VCX3A*) (Fig. 3C and D). Thus, these data suggested that the two CNVs in the patient may be inherited from his mother. One CNV contained an XLI-causing gene, *STS*, and the other CNV comprised a causative gene for CN-I, *UGT1A1*. These genetic abnormalities were well matched to the main clinical phenotypes of the patient, i.e., ichthyosis and icterus (Table I).

Discussion

In this study, a unique patient with XLI and CN-I, two recessive inherited diseases, was described. The two diagnoses were confirmed by molecular genetic evidence, as the patient exhibited a maternal 1.61 Mb deletion involving the *STS*

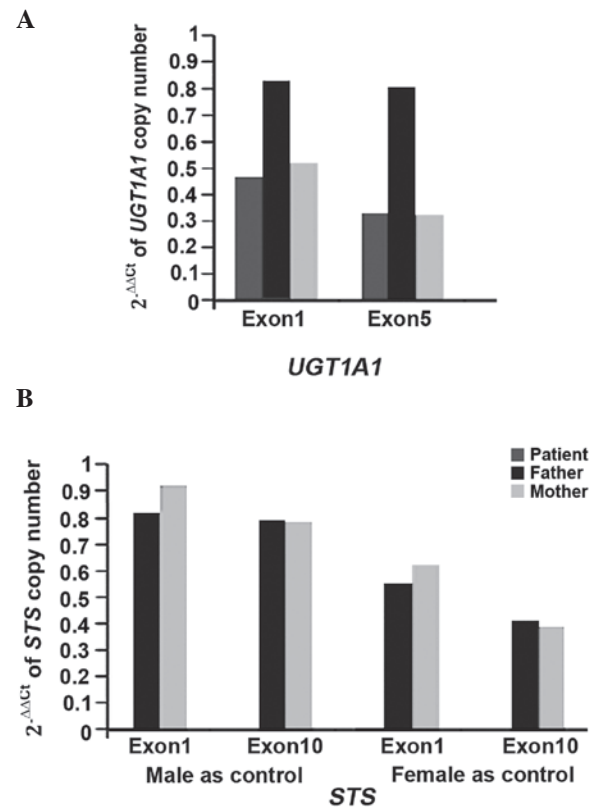


Figure 2. Quantitative polymerase chain reaction result of *UGT1A1* and *STS*. (A) *UGT1A1* gene in the patient and his parents; (B) *STS* gene in the patient and his parents. *UGT1A1*, UDP-glucuronosyltransferase 1 family, polypeptide A1; *STS*, steroid sulfatase.

gene, and a 374 Kb maternal deletion involving the *UGT1A1* gene combined with a paternal *UGT1A1* gene mutation (c.1253delT). Since these two recessive inherited diseases existed simultaneously in the same patient but independently with each other, it was speculated that the co-occurrence of two conditions was incidental. To the best of our knowledge, this is the first reported case with this comorbidity.

The patient's clinical symptoms of the skin were consistent with the diagnosis of ichthyosis. Pedigree analysis showed that there were two affected cousins in the extended family, which was consistent with the transmission characteristics of X-linked recessive diseases. Up to 90% of XLI patients have large gene fragment deletions involving the entire *STS* gene and its flanking regions, while the minorities of cases display partial deletions or point mutations in the gene (5,6,13). In this study, quantitative PCR and SNP microarray analysis confirmed the deletion of the entire *STS* gene in this patient. A previous study demonstrated that this aberration was the result of the unequal crossing-over in the two regions between the highly conserved CRI-S232 repeat regions flanking the *STS* gene (14). The deletion size of XLI in this patient was 1.61 Mb, which was close to the common 1.5-Mb deletion. Recent studies have shown that nervous system abnormalities, short stature, mental retardation, Kallmann syndrome or other clinical features may occur, when the deletion size on Xp22.3 is >1.5 Mb (15,16). Accordingly, the *VCX3A* gene located ~0.7 Mb telomeric to the *STS* gene has been proposed as the candidate mental retardation gene on Xp22.3 (17,18). In the present study, the *STS* and *VCX3A* genes were deleted on

Table I. Deletion result of single nucleotide polymorphism array analysis of the patient.

Region	From	To	Length (bp)	Gene	Related phenotype
Chr2p37.1	234604215	234978363	374148	<i>UGT1A8</i> ; <i>UGT1A10</i> ; <i>UGT1A9</i> ; <i>UGT1A7</i> ; <i>UGT1A6</i> ; <i>UGT1A5</i> ; <i>UGT1A4</i> ; <i>UGT1A3</i> ; <i>DNAJB3</i> ; <i>UGT1A1</i> ; <i>HJURP</i> ; <i>MSL3L2</i> ; <i>TRPM8</i>	Crigler-Najjar syndrome
ChrXp22.31	6516735	8131442	1614707	<i>HDHD1A</i> ; <i>STS</i> ; <i>VCX</i> ; <i>PNPLA4</i> ; <i>MIR651</i>	Ichthyosis

UGT1A1, UDP-glucuronosyltransferase 1 family, polypeptide A1; *STS*, steroid sulfatase.

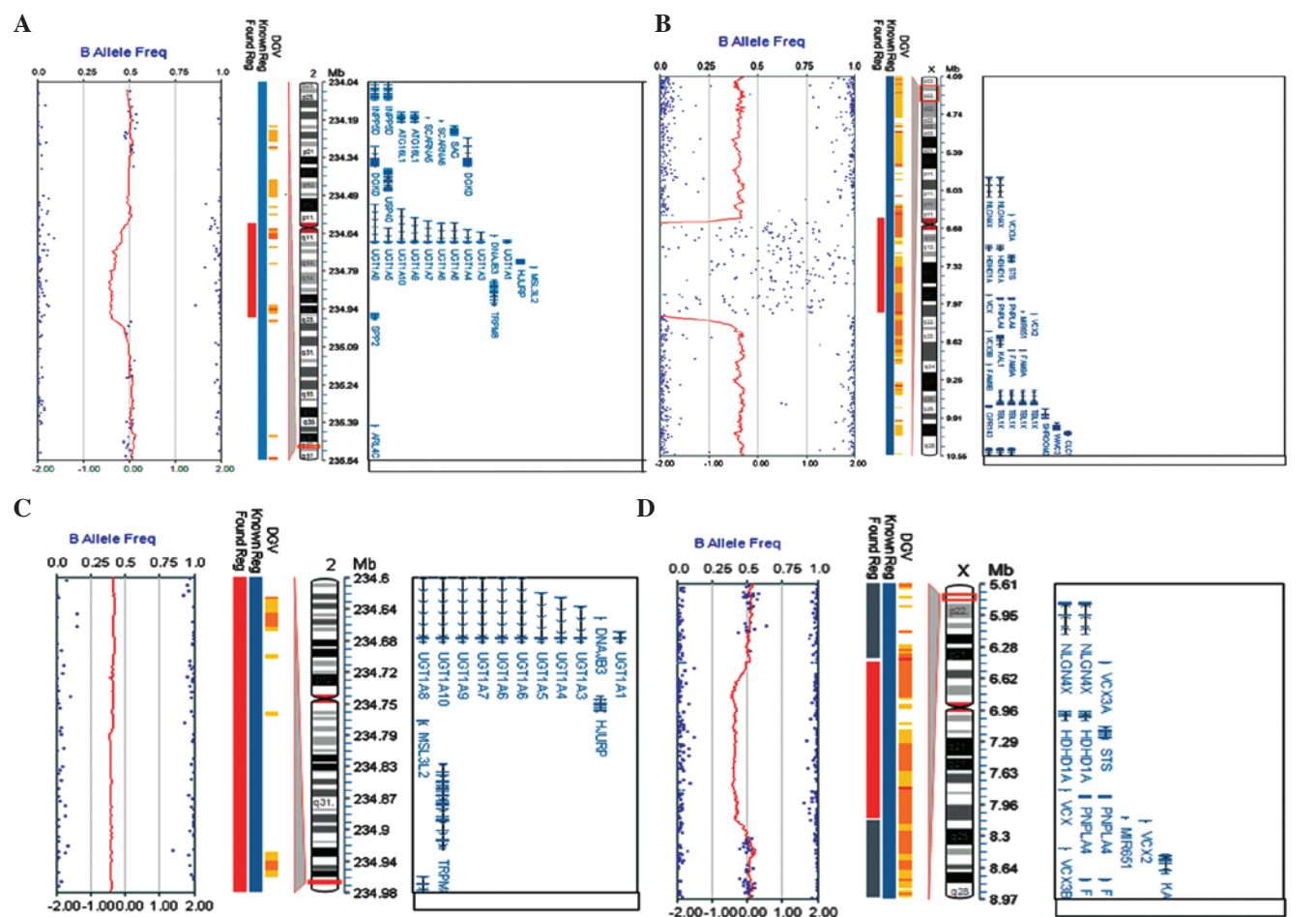


Figure 3. Single nucleotide polymorphism results of the patient and his mother. (A) Chromosome 2 of the patient; (B) chromosome X of the patient; (C) chromosome 2 of the patient's mother; (D) chromosome X of the patient's mother.

one of the X chromosomes of the patient's mother who had a normal phenotype. By contrast, the male patient presented with slight mental retardation, although he only lost the *STS* gene, but not the *VCX3A* gene. Such a disconnection of genotype and phenotype was considered to be due to the fact that number and location of the probes and hybridization signals of this region may limit the capacity to determine the accurate deletion size by SNP analysis. Unfortunately, we failed to repeat the SNP analysis or conduct another experiment to confirm the results, due to the lack of availability of the patient DNA samples. In addition, although the deletion range of the

patient did not include the Kallmann syndrome 1 protein, certain clinical manifestations of Kallmann syndrome were observed, such as left renal dysplasia. Conversely, he did not have the most common symptoms of XLI (corneal opacity or cryptorchidism). Moreover, the pathogenic mechanism for other complex manifestations, such as congenital laryngeal cartilage softening disease, require further elucidation.

To date, >130 *UGT1A1* gene mutations have been identified, including 91 single nucleotide changes (77 missense mutations and 14 nonsense mutations), 21 deletions, 10 insertions and 8 mutations located in the promoter and introns (19).

Complete loss of the *UGT1A1* gene is rarely reported. The present study initially performed direct DNA sequencing to screen the mutations, based on the feature of the *UGT1A1* gene mutation spectrum. This result revealed that the patient had a homozygous mutation-c.1253 Del T of the *UGT1A1* gene, which could not be fully explained by mutation transmission analysis of his parents as the same mutation was only observed in his father and not in his mother. Thus, this phenomenon prompted us to further careful analysis of DNA sequencing data for *UGT1A1*, the causative gene of the monogenic disease. Since the parents of the patient were unconsanguineous, it was presumed that the patient's mother only carried one copy of normal *UGT1A1* gene as indicated by DNA sequence analysis. Subsequently, PCR and SNP microarray analysis confirmed that the mother only had a single copy of *UGT1A1* gene. It is extremely rare for CN-I to be caused by a point mutation and a complete deletion of the *UGT1A1* gene. As the clinical phenotypes of this patient were more complex than what the known genotypes could explain, it was necessary to analyze the deletion range to understand the correlation between genotype and phenotype. Therefore, a Genome-wide SNP array analysis was conducted in this study.

CNV is a form of DNA structural variation (≥ 1 kb), which is present at a variable copy number, when compared with a reference genome. It occurs by recombination-based and replication-based mechanisms (18). Although CNVs can represent benign polymorphic variants widely distributed in the human genome, there are numerous CNVs that can cause Mendelian or sporadic traits, or be associated with complex diseases (20). To assess the pathogenicity of CNVs, the ISCA Consortium has published the criteria in their consensus statement of 2010 (21). For example, if the CNVs contain morbid MIM genes, they are probably pathogenic. By contrast, when the CNVs are inherited from a healthy parent, they are probably benign (22). In this study, the proband has been confirmed to carry two CNVs: One was a 374 Kb microdeletion located on Chr2q37.1 and the other was ~1.61 M deletion located on ChrXp22.31. The two CNVs contained two disease-causing genes, *UGT1A1* and *STS*, respectively. Family pedigree analysis showed that the two CNVs were inherited from the patient's mother. It was assumed that the 1.61 M deletion may be transmitted from the proband's grandmother as both cousins exhibited the phenotype of ichthyosis. Furthermore, a 374 kb microdeletion that resulted in CN-I was passed from the proband's mother. However, it could not be determined whether this microdeletion was inherited from the grandparents or was *de novo* in his mother. Cases with the two recessive genetic diseases have not been reported. The CN-I of the patient in the present study resulted from a rare 374 Kb maternal microdeletion combined with a paternal c.1253 delT mutation. Coincidentally, the proband was a male and also carried the maternal 1.61 M deletion located on ChrXp22.31, which caused him to lose the *STS* gene and consequentially develop XLI.

In conclusion, this study suggested that rare diseases may not simply be associated with the affected gene but may also be concurrent with genomic variations, such as CNVs. It also highlighted the importance of conducting detailed clinical examinations and unbiased genetic analyses, particularly for

the patients presenting with clinical phenotypes that could not be fully explained by general genetic testing results. Further studies using genome-wide scanning techniques (e.g., SNP array) may aid in determining disease-causing genetic alterations to understand the phenotypes in the affected individuals. Gaining a full understanding of the phenotype may assist in enabling a more accurate diagnosis to be made, and also in providing an improved basis for rational treatment strategies.

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