Clinical and genetic characterization of complete androgen insensitivity syndrome in a Chinese family

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ABSTRACT. We studied a family with two cousins who were diagnosed with complete androgen insensitivity syndrome, an X-linked disorder caused by mutations in the androgen receptor gene. A pedigree analysis and a molecular study using PCR and DNA sequencing clarified each female family member's androgen receptor status and revealed a mutation consisting of the deletion of exon 2 and surrounding introns of the androgen receptor gene. Based on the relative nucleotide positions, we concluded that the deletion mutation in exon 2 and its surrounding introns was approximately 6000 to 7000 bp. This mutation, never previously fully characterized using DNA sequencing, was responsible for complete androgen insensitivity syndrome in this family. Pedigree analysis with a molecular study of the androgen receptor gene in affected families facilitates genetic counseling provided to family members.

Key words: CAIS; Phenotype; Deletion mutation; AR gene

INTRODUCTION

Androgen insensitivity syndrome (AIS) is an X-linked disorder characterized by end-organ resistance to testosterone and dihydrotestosterone (DHT). It typically results from mutations in the androgen receptor (AR) gene in patients with typical male 46,XY karyotypes. A wide spectrum of presentations has been noted, ranging from complaints of male infertility or under-masculinization characteristic of mild AIS to complete AIS (CAIS) (formerly called testicular feminization syndrome) in phenotypic females. Patients with CAIS have normal appearing female external genitalia and breast development, but present a lack of pubic hair and developmentally immature testes present in the labia majora, inguinal ring or intrabdominally. Demonstration of a genetic mutation in the AR gene, located on Xq11-12, has become the gold standard for verifying a diagnosis of CAIS (Nitsche and Hiort, 2000; Brinkmann, 2001; Oakes et al., 2008).

The AR is an intracellular transcription factor that binds to androgen as a hormone receptor complex. It is comprised of 4 functional domains: an N-terminal domain, encoded by exon 1; a DNA-binding domain, encoded by exons 2 and 3; a "hinge" region, encoded by exon 4, and an androgen-binding domain, encoded by exons 4-8 (McPhaul et al., 1993; Quigley et al., 1995; Melo et al., 2003; Jaaskelainen et al., 2006). Over 400 mutations, mostly point mutations, of the AR gene have been reported (Gottlieb et al., 2004). These mutations include: a) single point mutations resulting in amino acid substitution or a premature stop codon; b) nucleotide insertions or deletions that most often lead to a frame shift and premature termination; c) complete or partial gene deletions (>10 nucleotides), and d) intronic mutations in either splice donor or acceptor sites, which affect the splicing of the AR RNA (McPhaul et al., 1993; Brinkmann, 2001).

We report our experience in treating two cousins with CAIS at Huashan Hospital of Fudan University. Diagnoses were made by physical examinations, B-ultrasounds and laboratory tests (including measurements of hormone levels and karyotype analysis). Polymerase chain reaction (PCR) and DNA sequencing were performed on leukocytes from the probands, obligate carriers, and female family members for the purpose of fully characterizing the AR gene mutation in this family and informing each female family member regarding her genetic status. The DNA mutation in the AR gene that was the cause of CAIS in this family, although previously reported in the AR mutation database (http://www.mcgill.ca/androgendb) (Gottlieb et al., 2004), has not yet been fully characterized. We also showed that our quick PCR exon analysis method can be easily adapted to genetic counseling for CAIS families.

MATERIAL AND METHODS

Clinical subjects

This study was approved by the institutional review board of Huashan Hospital, Fudan University. The present CAIS family first sought medical attention when a 24-year-old member with female social gender visited our hospital due to primary amenorrhea and who was later diagnosed with CAIS. The karyotype was 46,XY. Physical examination revealed normal breast development, vellus hair in the axilla and on the arms and legs, but absence of pubic hair, and a 4-cm deep blind-ending vagina. B-ultrasound showed a testis and hernia in

the right inguinal region. The uterus and Fallopian tubes could not be discerned by B-ultrasound. The patient expressed satisfaction with her sexual life. The karyotype analysis revealed 46,XY chromosome complement. With a clinical diagnosis of CAIS, the patient underwent bilateral orchidectomy without vaginal lengthening. Testicular histology showed developmental immaturity of seminiferous tubules containing monolayers of Sertoli cells without spermatogenic cells or spermatozoa. A surgical specimen also showed hyperplasia of mesenchymal cells and fibrous tissue. Hormone replacement was administered consisting of 0.3 mg/day diethylstilbestrol (DES) for 21 days/month; the use of DES has been approved in China (Tian et al., 2005).

A second proband diagnosed with CAIS was a 22-year-old cousin with female social gender who also visited our hospital due to primary amenorrhea. The presentation, diagnosis, and treatment were the same as that of the cousin, except that testes were found in both inguinal regions. The karyotype was 46,XY.

Pedigree analysis with molecular study

A pedigree analysis of the family was performed (Figure 1). The probands were subjects III-B and III-H. Subject I-B and subject II-C, both deceased, were reportedly tall, infertile with female phenotypes and were suspected to have CAIS.

Genomic DNA was extracted from leukocytes of the CAIS patients, an obligate carrier, and normal females in the family using a Qiagen Pure Gene Blood Core Kit C according to manufacturer instructions (QIAGEN, Shanghai, China). Genomic DNA samples were subjected to PCR for the AR gene. A Primer Express® software v5.0 kit (Applied Biosystems, Foster City, CA, USA) was used to design PCR primers and to assure their specificities. Based on the adjacent intron sequences of the human AR gene (GenBank ID NC000023.10), the exon-containing PCR primers were designed. AR coding regions and adjacent introns of exons 2-8 and non-coding regions of exon 1 were screened using 8 sets of primers, which are listed in Table 1. An additional set of primers was designed to locate the positions of deletions in exon 2; these are shown in Table 2.

Table 1. PCR primers for detecting exons of the AR gene.

Exon	Primer sequence	Location on chromosome X (start-end)	Length of amplicon (bp)
AR1-E1	Forward: 5'-ACCCGACTCGCAAACTGTTG	66763824-66763843	2879
	Reverse: 5'-TCTGAGTGTTCGCGCAGGTAG	66766682-66766702	
AR1-E2	Forward: 5'-CTGCCATTCAGTGACATGTGTTG	66863051-66863073	279
	Reverse: 5'-CTGGGCCCTGAAAGGTTAGTG	66863309-66863329	
AR1-E3	Forward: 5'-CTCATGGGCATTTCTGACTTTTG	66905530-66905552	586
	Reverse: 5'-TTGCCTATGAAAGGGTCAGCC	66906095-66906115	
AR1-E4	Forward: 5'-TCTGTGACCAGGGAGAATGGTG	66931127-66931148	528
	Reverse: 5'-GGGTTATGATGAAGACTGCCTGAG	66931631-66931654	
AR1-E5	Forward: 5'-CTTAATGGCCAGCCTGGATG	66937180-66937199	437
	Reverse: 5'-CGCCTCATACTGGATTGGCTG	66937596-66937616	
AR1-E6	Forward: 5'-GGCAATCAGAGACATTCCCTCTG	66941598-66941620	236
	Reverse: 5'-GGCATTCCCTGCACTTCTAGG	66941813-66941833	
AR1-E7	Forward: 5'-GTGCTTTGTCTAATGCTCCTTCG	66942603-66942625	314
	Reverse: 5'-AACAGGTGGTGCCAGACTCTAGAG	66942893-66942916	
AR1-E8	Forward: 5'-TCAGAGGTTGGGGAAGAGGCTAG	66943451-66943473	860
	Reverse: 5'-AATCAGTAACTCCCCATGGGCC.	66944289-66944310	

The primer design and the locations of exons on chromosome X refer to NC_000023.

Table 2. PCR primers for detecting the location of the exon 2 deletion mutation.

Primer	Primer sequence	Location on chromosome X (start-end)	Length of amplicon (bp)	Location of proposed amplicon	Amplification
I	F: 5'-ACCCGACTCGCAAACTGTTG R: 5'-TCTGAGTGTTCGCGCAGGTAG	66763824-66763843 66766682-66766702	2879	Within exon 1	Yes
II	F: 5'-ATCCATGTCCCTGCAAAGGACATG R: 5'-CCAATGGAACAGAACAGAGGTCTCAG	66857969-66857992 66858987-66859012	1044	Within intron 2	Yes
III	F: 5'-AAGTCAGGTAGCATGATGCCTCCAG R: 5'-ATACCCTCCCAAGCCTAAAGCAG	66859079-66859103 66860372-66860394	1316	Within intron 2	Yes
IV	F: 5'-CTCTGATGGTAATTTGTATTTCT R: 5'-CCCAACAACCTACCAGGTAGC	66860465-66860487 66861116-66861136	672	Within intron 2	No
V	F: 5'-ATGGCCTGGCATATAGAGTGC R: 5'-TTGGGTAAAAACTGTGCCCC	66861164-66861184 66861317-66861336	173	Within intron 2	No
VI	F: 5'-GGCAAGATCCAAAAGGCTC R: 5'-GTGCATGTGCAAGACCCTTTA	66862895-66862914 66863251-66863271	377	Within exon 1	No
VII	F: 5'-GCTCCTTCCATGTGGAACTTATGG R: 5'-GCAAGTGCATTTGGCCCTATG	66861402-66861426 66865407-66865427	4026	Exon 2-spaning (from intron 2 to intron 3)	No
VIII	F: 5'-GATGGTTTCCAGCTTCATCCATG R: 5'-GTTGGGGTGGTCTGAAGTCTCTG	66857306-66857328 66864819-66864841	7536	Exon 2-spaning (from intron 2 to intron 3)	No
IX	F: 5'-GATGGTTTCCAGCTTCATCCATG R: 5'-GCCTCCTGGGATCTACTTTCTATGAG	66857306-66857328 66868293-66868318	11013	Exon 2-spaning (from intron 2 to intron 3)	No
X	F: 5'-TTCAGATGGGGATAGAGCCAG R: 5'-GTTGAGGTGGGAAAGAATGGA	66866756-66866776 66867250-66867270	515	Within intron 3	No
XI	F: 5'-AGGATCTGCTTGCAGCTTCAACA R: 5'-GGGTATTGCAAGGAGGAGTGA	66867397-66867419 66868099-66868119	723	Within intron 3	Yes
XII	F: 5'-TGCTTATTTATAGCCTGTGC R: 5'-GCCATTTGACCCAGCCATC	66868544-66868563 66869240-66869258	715	Within intron 3	Yes
XIII	F: 5'-ATTTCCCTAATGACCAGTG R: 5'-TTGCCTTGAGCAGTGGTTTG	66869452-66869470 66869875-66869894	443	Within intron 3	Yes
XIV	F: 5'-AGGGAACAAGGTTTAGAAT R: 5'-GGGTGGAGTCGGAGGGTTT	66870615-66870633 66871133-66871151	537	Within intron 3	Yes
XV	F: 5'-CTCATGGGCATTTCTGACTTTTG R: 5'-TTGCCTATGAAAGGGTCAGCC	66905530-66905552 66906095-66906115	586	With exon 3	Yes

Polymerase chain reaction

According to the instructions for the HotStar Taq DNA PCR system (QIAGEN), PCRs were performed with a total volume of 50 μL that contained 1 μg genomic DNA, 200 μM of each dNTP, 0.25 μM of each primer, 2 mM $MgCl_2$, 1.25 U Hotstar Taq enzyme, and 5 μL 10X PCR buffer. Reactions were performed in a thermal cycler (PTC-200, Bio-Rad, Hercules, CA, USA) with the following conditions: 94°C denaturation for 15 min, 94°C denaturation for 30 s, 55°C annealing for 30 s, 72°C extension for 45 s, for a total of 32 cycles, followed by 72°C extension for 10 min. All PCRs were performed in duplicate. A negative control (no DNA) was included in the PCR analysis to rule out carryover contamination. An amplicon (10 μL) was subjected to gel electrophoresis. The remaining 40 μL was further purified using a DNA fragment purification kit B (BioDev Tech, China) pending DNA sequencing. The DNA sequencing was carried out using an ABI 3730 xl DNA analyzer (Applied Biosystems).

Quantitative real-time PCR (Q-PCR)

Genomic DNA (1 μg) extracted from a CAIS patient (III-B), an obligate carrier (II-B), and a normal female family member was subjected to amplification of the exon 2 containing the fragment using Q-PCR. Q-PCR was performed on an ABI 7500 fast real-time PCR system using the All-in-One™ Q-PCR Detection Kit (GeneCopoeia, USA) along with the AR1-E2 primers. For real-time PCR, the reaction was initiated at 95°C for 3 min for initial template denaturation, followed by a cycling protocol (95°C for 15 s, 60°C for 15 s, and 72°C for 20 s) for 30 cycles, and a final elongation (72°C for 1 min). After the final cycle, melt curve analysis was performed to confirm amplification specificity. PCR signals were analyzed using the delta-delta Ct method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control.

RESULTS

Probands' clinical characteristics

Figure 1 shows the pedigree analysis for this family with CAIS, including the two probands in this study (III-B and III-H). Table 3 shows the results of the hormone analyses for these probands (normal ranges are given for adult males). Other clinical presentations for the probands are given in Material and Methods (Clinical subjects).

PCR results

PCR amplification and sequencing of AR gene exons in the probands showed a deletion of exon 2 and its surrounding introns in the AR gene with a total of 6000-7000 bp missing. We used the human AR gene for comparisons (GenBank ID NC000023.10). Agarose gel electrophoresis imaging of PCR amplification of the AR gene exon 2 showed that PCR of genomic DNA from the CIAS patients produced no amplifications, indicat-

ing exon deletions. The result of an obligate carrier (II-B) showed amplification of the AR exon 2, but with less intensity than that of controls (Figure 2A). The PCR analysis indicated that exons 3 to 5 of the AR gene, but not exon 2, could be detected for the CAIS patient (III-B) (Figure 2B). AR gene deletions in the probands were located somewhere between 66860369 and 66867397 of chromosome X. Table 2 shows the primers used for detecting deletion mutations within exon 2. We used these primers to pinpoint the location of the exon 2 deletion mutation. The 5' end nucleotide of reverse primer III is 66860369; the 3' end nucleotide of forward primer XI is 66867397. 66867397 - 66860369 = 7028. Thus, the exon 2 deletion is about 7028 bp. We designed several primers to amplify DNA fragments between 66860369 and 66867397, but no PCR products were amplified for the CAIS patients.
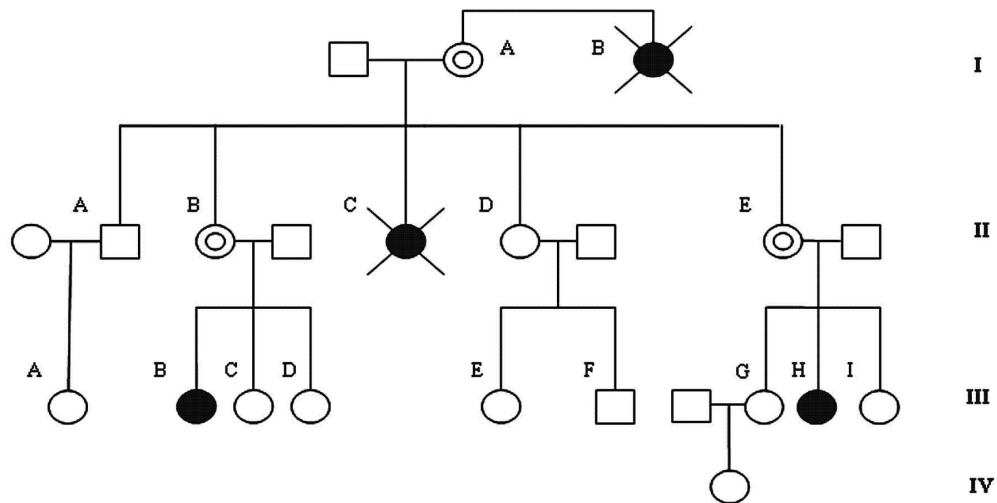
Figure 1. The pedigree of the family with complete androgen insensitivity syndrome (CAIS). Black circles = CAIS 46, XY individuals; open circles = normal females; open squares = normal males; double open circles = obligate carrier women; crossed black circles = deceased individuals suspected of having CAIS.

Table 3. Hormone analysis results.

	Normal male range	Patient III-B	Patient III-H
Testosterone	9.90-27.80 nM	23.5 nM	31.1 nM
Estradiol	28.00-156 pM	171.8 pM	143.3 pM
Luteinizing hormone	1.70-8.60 IU/L	45.81 IU/L	33.7 IU/L
Follicle-stimulating hormone	1.50-12.40 IU/L	2.35 IU/ L	13.8 IU/L
Dihydrotestosterone	55.10-386.5 ng/dL	51.0 ng/dL	49.1 ng/dL
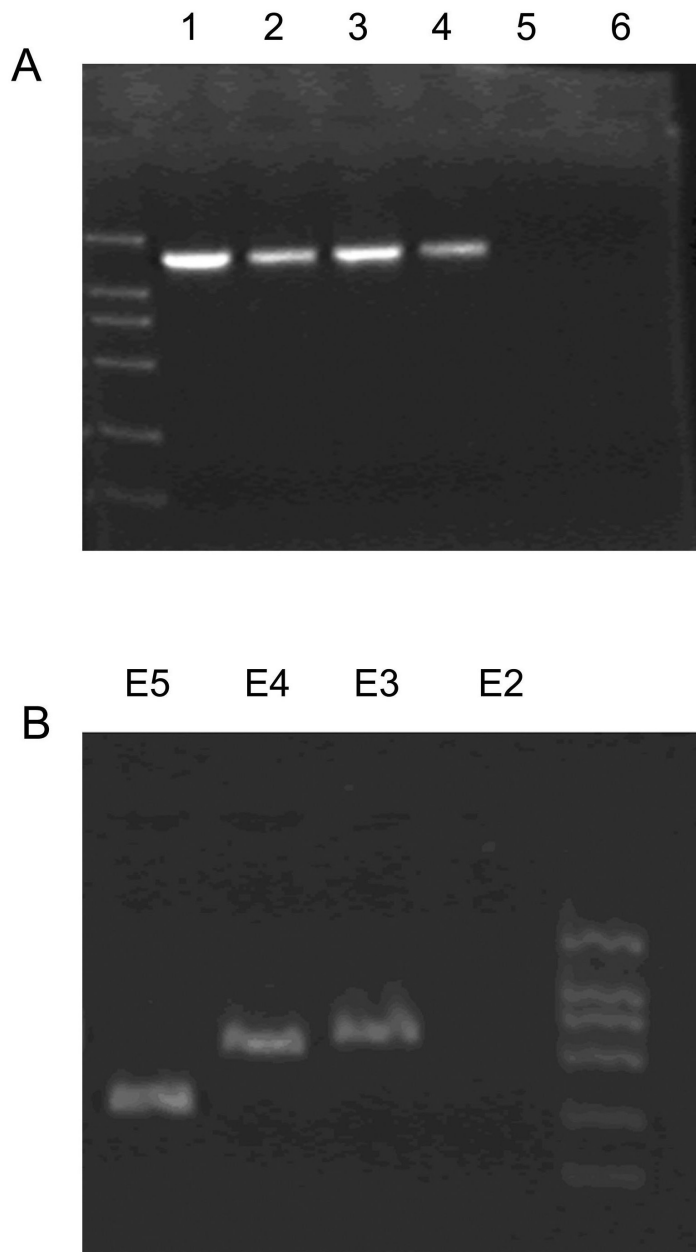
Figure 2. PCR amplification of the AR gene. **A.** Amplification of the AR gene exon 2 in family members. *Lanes 1* to *3* = Strong amplification in three normal family members. Notably, *lane 2* is a normal male family member. *Lane 4* = Intermediate amplification in an obligate carrier mother of a CAIS patient (II-B). *Lanes 5* and *6* = No amplification in the CAIS patients (III-B and III-H). A DL2000™ DNA Marker kit (TaKaRa, Japan) was used for the molecular weight markers, and the sizes of bands were 2000, 1000, 750, 500, 250, and 100 bp. **B.** Amplification of the AR gene exons 2 to 5 (E2-E5) in a CAIS patient (III-B).

DNA sequencing confirmed that there was no point mutation, except the deletion of exon 2, and showed that the remaining exons and introns were intact in the AR genes of the probands.

None of the females in the pedigree in generations III and IV, except for the probands, were found to have CAIS or to be carriers. I-A, II-B and I-E were confirmed to be carriers. The origin of the mutation in this family appeared to be from the predecessors of I-A.

Real-time PCR results

Compared to the normal female family member, the exon 2 fragment expression signal of the obligate carrier (II-B) was 0.55864. GAPDH, but not the AR exon 2, was detected in the CAIS patient (III-B). The Ct values of all GAPDH reactions ranged from 22.09 to 22.84, suggesting that equal amounts of genomic DNA had been applied. Consistent with the results of conventional PCR, the Q-PCR results suggested that II-B, the mother of the CAIS patient, was an obligate carrier.

DISCUSSION

Few studies of CAIS have shown clinical and hormonal data combined with pedigree analysis and direct DNA sequencing to identify an AR gene mutation, as we have presented in this report. In addition, very few previous reports of exon 2 deletions can be found in the AR mutation database (http://www.mcgill.ca/androgendb). Another important aspect of our study is that it shows that family studies can be easily undertaken to provide members with the information they need for family planning, which has become an increasing concern for families with AR gene mutations and members with CAIS (Fogu et al., 2003; Cheikhelard et al., 2008).

The molecular basis of CAIS in this family was a deletion of exon 2 of the AR gene, as shown in the CAIS patients by failure of PCR amplification of exon 2. Gel electrophoresis imaging of PCR amplification of the AR gene exon 2 showed that the mother of subject III-B was the obligate carrier. The deletion was not found in the normal subjects of this family.

To the best of our knowledge, only three previous reports have described androgen resistance involving deletions in exon 2 of the AR gene. Avila et al. (2002) screened 27 patients and found 2 involving exon 2 deletions, although it was unclear whether these 2 patients were related to one another. Beitel et al. (1994) and Ahmed et al. (2000) both reported cases in which only a few nucleotides were deleted within exon 2. These reports suggested the possibility of major structural changes in exon 2. Other reports have also suggested that major structural alterations in exons other than exon 2 might lead to androgen resistance (Ris-Stalpers et al., 1994; MacLean et al., 2006).

Normal male sexual development requires both the synthesis of testosterone and DHT by the testes and the ability to form an active complex between AR protein and circulating androgens (Wilson et al., 2002). AIS, with an incidence of about 1 in 20,000, is an X-linked disorder caused by mutations in the AR gene (Hashmi et al., 2008). It is thought to be related to approximately 6-10% of cases of primary amenorrhea (Ross, 1985). Individuals with AIS are androgen resistant and have disorders of male sexual differentiation ranging from CAIS, with a normal female phenotype in the presence of an XY genotype, to phenotypic males who present infertility problems. Partial AIS presents ambiguous genitalia in patients with an XY

karyotype (Alvarez et al., 2005). Individuals affected by CAIS may present a short, blind-ending vagina, the absence of Wolffian duct-derived structures, such as the epididymis, vas deferens and seminal vesicles, and the absence of a prostate gland. Breast development is observed at puberty, but pubic and axillary hairs are absent (Quigley et al., 1995). Müllerian structures are rarely seen in CAIS individuals (Ulloa-Aguirre et al., 1990). Gonadectomy may be indicated post-puberty because of the high incidence of testicular neoplasms in undescended testicles. However, as noted by Cools et al. (2009) the risk of germ cell tumors in CAIS patients is extremely low, and a more conservative approach to gonadectomy may be recommended.

Hormone replacement therapy is determined by serum hormone levels, which usually reveals an elevated testosterone in the setting of high luteinizing hormone (LH) (Oakes et al., 2008), as was observed in the probands in our case. As also noted, these probands had low DHT production, which is unusual for CAIS patients. This may be due to: 1) down-regulated LH receptors on Leydin cells in the testes resulting from chronic elevation of LH; 2) LH insensitivity syndrome, or 3) testicular atrophy.

The molecular basis of AIS is associated with a wide range of molecular defects that may or may not affect androgen binding. Genetic point mutations, which inhibit either DNA or hormone binding, are the most common genetic abnormalities (Brinkmann, 2001). According to the AR mutation database (http://www.mcgill.ca/androgendb), more than 400 different mutations, including deletions of exon 2, have so far been reported in the AR gene, and the number continues to increase. Most mutations observed in CAIS patients abolish either ligand or DNA binding activity, therefore AR exhibits no transactivation function (MacLean et al., 2004). The complexity of the phenotypic presentations of AIS, with genotype-phenotype variability, complicates both the diagnostic procedure and genetic counseling of affected families (Quigley et al., 2004).

Genetic testing empowered family members to realize that no one in generations III and IV of the family, other than the probands, was affected or was a carrier. Thus, our methods could be readily adapted to prenatal screening and genetic counseling for families harboring this rare disorder.

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