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Prenatal diagnosis and carrier detection for Athabascan severe combined immunodeficiency disease

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Objectives Severe combined immunodeficiency disease occurs at a high incidence among Athabascan-speaking Navajo and Apache children (SCIDA). We linked the SCIDA gene to chromosome 10p and recently identified a common nonsense mutation in Artemis/SCIDA. In this study we compared polymorphic markers linked to SCIDA and the point mutation which creates an NspI site on exon 8 for prenatal diagnosis and carrier detection.

Methods We tested five amniocentesis samples, two cord blood and two blood samples from eight at-risk families using polymorphic DNA markers tightly linked to SCIDA. We amplified the region of exon 8 of Artemis/SCIDA and evaluated the products for the NspI site in each sample plus samples from 30 unrelated healthy Navajos.

Results We correctly predicted that three were affected and six were unaffected. Two of the unaffected appear to be carriers based on our haplotype analysis. Retrospective analysis for the gene mutation confirmed the DNA analysis. Finally, 10% of the normal Navajo controls were carriers.

Conclusions We demonstrate the feasibility of prenatal diagnosis and carrier detection for SCIDA in the families at risk as well as the availability of a rapid screening test for the SCIDA founder mutation that can be used in all Navajo and Apache newborns and at-risk fetuses.

KEY WORDS: Athabascan SCID; prenatal diagnosis; carrier detection

INTRODUCTION

Athabascan severe combined immunodeficiency disease (SCIDA) is an autosomal recessive disorder with a high incidence (1:2000 live births) in Athabascan-speaking Navajo and Apache Native Americans (Murphy *et al*., 1980; Jones *et al*., 1991). Patients present early in life with serious infections, failure to thrive and oral and/or genital ulcers (Rotbart *et al*., 1986; Kwong *et al*., 1999). Diagnosis is usually made postnatally by combining laboratory immunophenotypes with clinical symptoms and a positive family history. The condition is fatal and affected children usually die from severe infection within 6 months of age without a successful bone marrow transplant (O'Marcaigh *et al*., 2001).

In a previous study, we established genetic linkage of the SCIDA gene to a region of 6.5cM on chromosome 10p in 12 Navajo families and we also presented data suggesting a strong founder effect for the SCIDA condition (Li *et al*., 1998). Subsequently, we evaluated several new SCIDA families, resulting in a total of 18 Navajo and 3 Apache families. Based on the results of our genetic analysis we developed a diagnostic test in affected families using analysis of the tightly linked DNA markers in the SCIDA candidate region. In the past four years, we evaluated five amniotic fluid and/or amniocyte culture samples, two cord blood specimens and two blood samples from eight Navajo and Apache at-risk families and the results are all in agreement with the clinical affected or non-affected outcomes.

Recently a novel gene, Artemis has been found to be mutated in RS-SCID patients (Moshous *et al*., 2001). In our evaluation of this gene, we identified a unique nonsense mutation that was homogeneously carried by all but one of the Navajo and Apache SCIDA patients. This finding is consistent with the founder haplotype that we previously defined (Li *et al*., 2002). This nonsense founder mutation creates a novel NspI cut (CCATGT → ACATGT) at the mutation site leading to a simple diagnostic assay. In a retrospective evaluation, we examined the SCIDA founder mutation in the previous nine prenatal and diagnostic cases and their at-risk families. We report here the complete results including previous diagnosis with polymorphic markers, clinical evaluation at birth and also the retrospective mutation detection for these nine cases, plus a screening result for 30 normal and unrelated adults from the Navajo population.

MATERIALS AND METHODS

Families

The study was approved by the Committee on Human Research at the University of California San Francisco, the Navajo Nation Health Board, and the Indian Health

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Service IRB. Informed consent was obtained from the participating family members and normal controls. Six of the seven families in the present report were of Navajo origin and one was Apache. Families 05 and 13 were described in our linkage study and families 25, 26 and 31 were related to family 15 in the previous report (Li *et al*., 1998). In fact, families 15, 25, 26 and 31 were all sub-families of one kindred. Families 25, 26 and 31 had no previously affected children but requested the diagnostic test because the parents were at risk to be carriers of SCIDA (Figure 1). The remaining three families (nos 27, 28 and 29) are not related to each other or any previously identified families. No consanguinity was found in their family histories. The 30 normal controls were from healthy, unrelated Navajo adults with no known relationship to any SCIDA families.

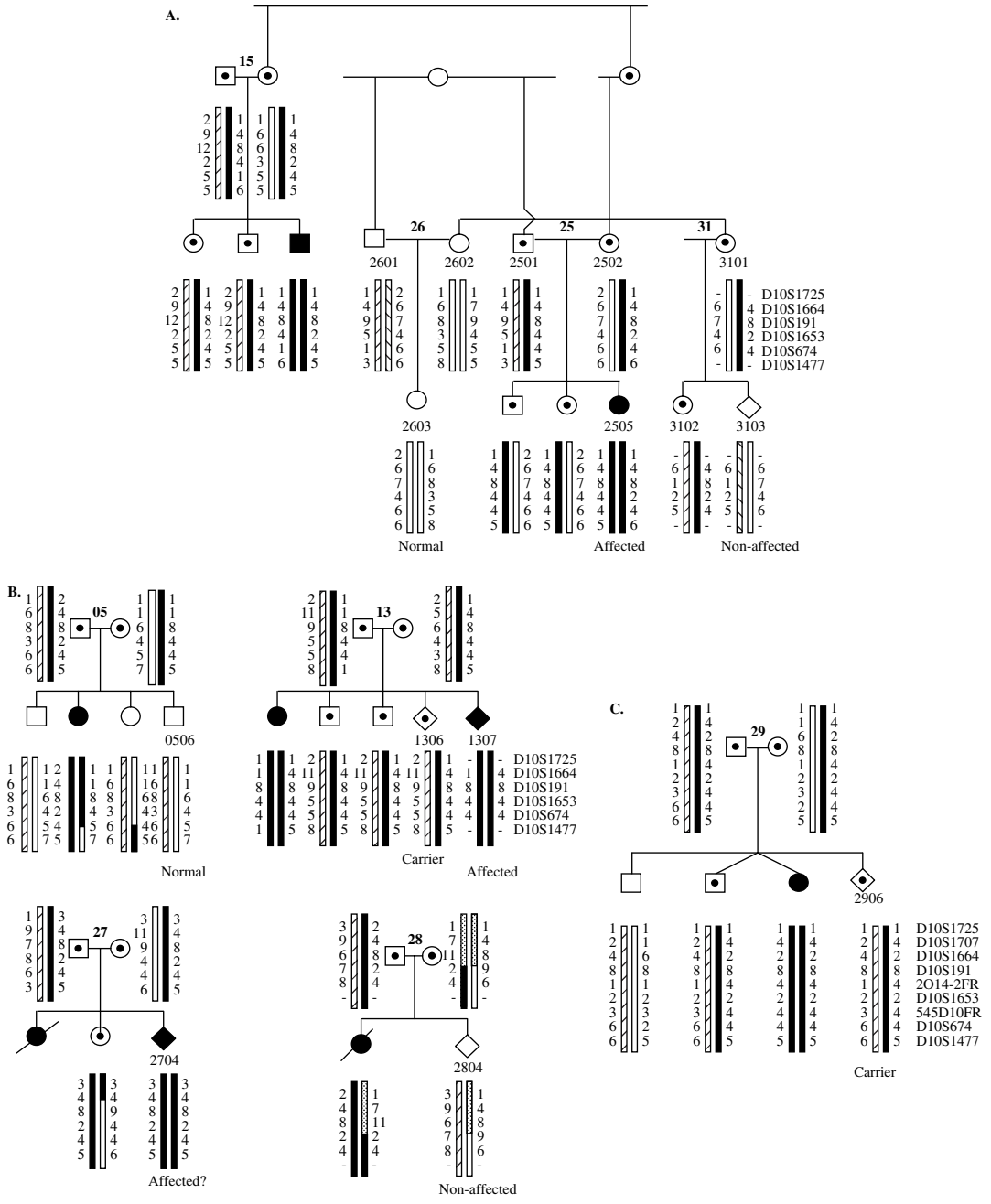

Figure 1—(A) The kindred consisting of families 15, 25, 26 and 31 showing haplotypes for markers D10S1725, D10S1664, D10S191, D10S1653, D10S674 and D10S1477 at the SCIDA locus. The order of the markers is from pter to centromere according to previous linkage maps (Hudson *et al*., 1995; Dib *et al*., 1996). Alleles are number coded. The blackened bars represent the disease haplotypes while unblackened and hatched bars stand for non-disease haplotypes and indicate parent of origin. (B) Pedigrees of families 05, 13, 27, and 28, showing haplotypes for the same six markers shown in (A). The dashed bars represent the undetermined haplotypes due to a possible historical recombination event. (C) Pedigree of family 29, showing haplotypes for markers D10S1725, D10S1707, D10S1664, D10S191, 2O14-2FR, D10S1653, 545D10FR, D10S674 and D10S1477 at the SCIDA locus. The order of the markers is from pter to centromere according to previous linkage maps and also physical mapping information from the human genome project and our own study

Amniotic fluid (AF) samples were obtained at 17–19 weeks of gestation, 10–15 ml of which was sent to us for direct DNA extraction and genotyping while the remaining was sent for amniocyte culture. The DNA from this volume of AF is usually sufficient to type 5∼10 markers. We requested amniocyte cultures for those showing positive results from AF typing and those in which more DNA was needed to complete the testing. The cord blood was obtained at birth. EDTA blood samples were obtained from patients, parents, siblings and normal controls. DNA was extracted using standard methods (Ford *et al*., 1989) from AF, amniocyte cultures, cord blood and EDTA blood samples.

Lymphocyte phenotyping and function assays

We conducted lymphocyte phenotyping and functional assays on newborn blood in six of the patients including two with negative genotyping results. T and B cell lymphocyte subsets were quantified by flow cytometric analysis using fluorescent conjugated monoclonal antibodies to human cell surface antigens (CD3, CD16, CD19, CD56) and a FACScan analyzer (Becton Dickenson, Mountainview, CA). Lymphocyte proliferative responses to phytohemagglutinin (PHA), and alloantigen in the mixed lymphocyte culture (MLC) were measured by the incorporation of [^{3}H]-thymidine as previously described (O'Marcaigh *et al*., 2001).

Genotyping analysis

All of the family members were genotyped using the four microsatellite DNA markers D10S1664, D10S191, D10S1653 and D10S674, which cover the 6.5cM SCIDA region. Two markers (D10S1725 and D10S1477) that flank the candidate region were also typed for most of the families. We included markers D10S1707, 2O14-2FR and 545D10FR for family 29 because of the uninformativeness of D10S1725, D10S1653 and D10S191 in this family. The order of the markers, from pter to centromere, is D10S1725, D10S1707, D10S1664, D10S191, 2O14-2FR, D10S1653, 545D10FR, D10S674 and D10S1477 according to previous genetic maps (Hudson *et al*. 1995; Dib *et al*., 1996). All markers were described in the linkage maps except 2O14-2FR and 545D10FR, which we developed during our physical mapping study. 2O14-2FR and 545D10FR are both CA-repeat markers: 2O14-2F, 5′-ACTTCCCATGTGACATCTTTGAG-3′; -2R, 5′-TATGAAGACCTGACTGTCTTA-3′; 545D10F, 5′-TGGATAAAGCCTCCTTCCATG; and 545D10R, 5′-GCCATTTCAGACAATTACACTC-3′. Each marker was typed by polymerase chain reaction (PCR) followed by separation on 6% polyacrylamide sequencing gel and autoradiography using the method that we previously described (Li *et al*., 1998).

SCIDA common mutation detection

The primer pair was designed from intron sequences to specifically amplify exon 8 of the SCIDA gene as follows, E8f: 5′-ATATCCTAACTGTCCCCGTAG-3′ & E8r: 5′-GGCCAACATGGTGAAATG-3′. We utilized the above primer pair to perform PCR on genomic DNA from patients and controls using a standard procedure. In brief, 100–300 ng DNA was added to a final volume of 25 µl PCR reaction containing 10 pmol of each primer, 200 µmol of each dNTP, 1 × PCR buffer with the $MgCl_2$ final concentration of 1.5 mM, and 2 units of Taq polymerase. We performed PCR with an initial denaturation at 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, and followed by a final extension at 72 °C for 10 min. For each PCR reaction, 5 µl was run on a 1% agrose checking gel to check for the amplification of the 488 bp PCR product, while the remaining reaction was purified using Qiagen PCR purification kit (Qiagen Inc. Valencia, CA) following the instructions. The purified PCR products were then digested with restriction enzyme NspI (New England Biolabs, Inc., Beverly, MA) and electrophoresed on 2% agrose gel or 12% polyacrylamide gel for a better resolution.

RESULTS

Evaluation results from using multiple polymorphic markers

The haplotypes were inferred so that recombinants are minimized in both previous and current studies. The results of genotyping and haplotype analysis for the kindred consisting of families 25, 26, 31 and 15 are shown in Figure 1A, the results for families 05, 13, 27 and 28 in Figure 1B and for family 29 in Figure 1C. The lymphocyte phenotyping and function assay for six of the newborns are summarized in Table 1.

Families 25, 26 and 31

Families 25, 26 and 31 were related to family 15 in our previous study and they all belonged to a big kindred. Family 25 requested the test in 1998 because of their risk of being carriers due to a positive family history in their close relatives (family 15) and the known high incidence of SCIDA in the population. We conducted the genotyping on cord blood for newborn 2505. The newborn baby girl carried a maternal haplotype that was similar to the one that appeared on 15005. She also had a paternal haplotype formed by SCIDA associated alleles on all four loci, which we had seen in a majority of SCIDA chromosomes but which is rare in the normal Navajo population (Li *et al*., 1998). The two healthy siblings carried the same paternal haplotype but also carried the normal maternal haplotype. The SCIDA diagnosis was highly suspected and SCID isolation precautions were taken at birth. The lymphocyte phenotyping and function assays on the newborn blood confirmed her SCIDA

Table 1—T cell immunity at birth

Id#	Sample	Results	ALC	CD3%	CD19%	CD16/56%	PHA	MLC
2505	Cord blood	Affected	612	<1	1	84	0	1488
2603	Blood	Normal	ND	ND	ND	ND	ND	ND
3103	AF	Non-affected	ND	ND	ND	ND	ND	ND
0506	Blood	Normal	ND	82	7	5	42 842	90 853
1306	AF	Carrier	2850	81	4	16	44 534	ND
1307	AF	Affected	340	<1	<1	100	298	ND
2705	AF	Affected	500	<1	<1	100	2113	8090
2804	Cord blood	Non-affected	5400	74	4	20	ND	ND
2906	AF	Carrier	3087	ND	ND	ND	ND	ND
Normal			>2900	>50	5–15	5–15	>27 000	>18 000

ALC, absolute lymphocyte count (per μl); CD3, T cells (% of lymphocytes); CD19, B cells (% of lymphocytes); CD16/56, natural killer (NK) cells (% of lymphocytes); MLC, mixed lymphocyte culture, cpm; PHA, phytohemagglutinin, cpm; ND, not done; AF, amniotic fluid.

status (Table 1). She underwent a successful BMT from a closely matched sibling at 5 weeks of age (O'Marcaigh *et al*., 2001).

Family 26 was closely linked to family 25 and requested carrier testing in December 1998. In this family, the mother (2602) was a sister of 2502 and the father (2601) was a half brother of 2501. We found that the mother 2602 carried different haplotypes from her sister and the father 2601 shared only the normal haplotype with his brother, suggesting their healthy status; their healthy daughter inherited normal haplotypes from the parents.

In family 31, the mother 3101 was another sister of 2502. The genotyping for the mother, a healthy sibling and the unborn fetus using the four markers linked to the SCIDA region were all informative in this family. The mother has the same haplotypes as her sister 2502 and is also a carrier of SCIDA. The healthy sibling received the maternal disease haplotype but what we suspect is a 'normal' paternal haplotype, although blood from the father is unavailable. Since the unborn fetus had the maternal normal chromosome and also the same 'normal' paternal haplotype as her healthy sister, we predicted that the fetus would be non-affected. The newborn was evaluated at birth and found to have a normal lymphocyte count and thymus shadow on chest X-ray (data not shown).

Family 05

This family was included in our linkage study and the disease haplotype was defined previously. Diagnostic testing for newborn 0506 was done in 1998. The newborn did not carry either of the disease haplotypes and carried the same haplotypes as his two healthy siblings. The child had normal immune studies (Table 1).

Family 13

This family was also included in our linkage study and the disease haplotype was established previously. We performed prenatal testing for fetuses 1306 in 1997 and 1307 in 1999. Fetus 1306 carried the maternal SCIDA haplotype but the normal paternal haplotype, suggesting a carrier status. The child was healthy at birth with normal immune studies (Table 1). Fetus 1307 had the same haplotypes to those seen in the affected child of this family and SCID isolation precautions were taken at birth. The lymphocyte phenotyping and function assays at birth confirmed the diagnosis of SCIDA and a matched sibling BMT was done at 5 weeks of age (O'Marcaigh *et al*., 2001).

Family 27

Family 27 was not included in the previous linkage study because their affected child died without a DNA sample being obtained. We tested the parents and the healthy sibling and defined the suspicious disease haplotypes by their formation from all SCIDA associated alleles on four markers that are tightly linked with SCIDA. The genotyping analysis was performed on AF and amniocyte DNA samples for fetus 2704 in 1998. The fetus had the same suspected SCIDA paternal haplotype and also the suspected SCIDA maternal haplotype. The healthy sibling carried the suspected SCIDA paternal haplotype but the suspected normal maternal haplotype, i.e. was a suspected carrier. The SCIDA diagnosis was highly suspected in the fetus and SCID isolation precautions were taken at birth. The diagnosis was confirmed at birth by lymphocyte phenotyping and function assays (Table 1). He received an HLA matched BMT from his healthy sibling at 4 weeks of age (O'Marcaigh *et al*., 2001).

Family 28

The disease haplotypes of this family were defined by the previously affected child. The mother carried the SCIDA-associated allele at all four loci, but the maternal haplotype transmitted to the affected child presented SCIDA-associated alleles only at adjacent loci D10S1653 and D10S674, but not at two distal adjacent loci D10S1664 and D10S191, which could have resulted from a possible historical recombination event. The fetus (2804) had the normal paternal haplotype and a different maternal haplotype from the affected child, suggesting a non-affected status. Because of the formation of the

maternal haplotype, we could not determine the fetus's carrier status. The child was healthy at birth (Table 1).

Family 29

Family 29 was also not among the families of our previous linkage study. Neither D10S1653 nor D10S191 were informative in this family. We included three more markers (D10S1707, 2O14-2FR and 545D10FR) that were in the SCIDA region and near the two uninformative markers. The disease haplotypes were defined by the previously affected child. Fetus 2906 was tested in 1998 and had a normal paternal haplotype and a maternal disease haplotype, suggesting a carrier status. The birth of a healthy child confirmed the prenatal test results (Table 1).

SCIDA common mutation evaluation

The retrospective mutation detection results for the previous diagnostic cases, as well as results for ten of the 30 normal controls, are shown in Figure 2. As expected, the normal control has the intact 488 bp PCR product while the positive control (from an affected SCIDA patient) shows two bands of 164 and 324 bp because of the homogenous mutation (CCATGT → ACATGT) resulting in the complete digestion of the PCR product by NspI. The carrier control (from a parent of the affected SCIDA patient) on the other hand shows one allele intact (488bp band) and the other one being digested (164 and 324 bp bands). The results for the nine cases are in complete agreement with the previous diagnoses with respect to the affected or non-affected status (Figure 2A). As previously diagnosed, cases 2505, 1307, 2704 are affected, 1306 and 2906 are carriers, 2603 and 0506 are normal and 3103 and 2804 are non-affected. Furthermore, the mutation analysis clarified the carrier or normal status for the two cases that were not determined previously (3103 and 2804). We have determined that 3103 is normal and 2804 is a carrier. Among the 30 healthy and unrelated Navajo controls, nos 6, 8 and 14 are carriers based on the assay (Figure 2B). This was further confirmed by sequencing analysis.

DISCUSSION

SCIDA is an inherited condition that is uniformly fatal early in life without a bone marrow transplant. Early diagnosis is crucial for identifying an optimal donor and performing a bone marrow transplant as early in life as possible before life-threatening infections occur. In our review of this patient population we found that virtually all affected children become symptomatic, usually with infections, within the first three months of life (O'Marcaigh *et al*., 2001). Because there is no other biochemical or pathological defect detectable in this condition, prior to the availability of DNA typing, the disease could only be diagnosed prenatally with fetal blood sampling or postnatally by immunologic testing. The risk of fetal blood sampling is significant and many families have elected to avoid undergoing that procedure. While amniotic fluid aspiration is not without risk, it is a relatively common procedure and readily obtainable.

DNA analysis using polymorphic markers linked to the SCIDA locus is a feasible diagnostic tool for prenatal diagnosis and carrier detection in the at-risk families when the direct mutation detection is not available. Based on our previous linkage and allelic association studies, the majority of the SCIDA chromosomes carry a common haplotype that is formed by certain rare alleles at the polymorphic marker loci in the SCIDA candidate region (Li *et al*., 1998 and data not shown). In the past four years, we have used these linked microsatellite markers for prenatal diagnosis and carrier detection. The combination of the four tightly linked markers was informative for eight of the nine families that we tested and the diagnosis of SCIDA could be confidently established. We added three more markers in the evaluation of family 29 because of the uninformativeness of two of the four linked markers. In the five prenatal evaluations, two cord blood examinations and two peripheral blood tests, we found complete agreement between the DNA analyses and clinical outcomes for the disease affection status. In addition, the carrier status was also determined for four of the six unaffected cases.

The recent identification of the SCIDA responsible gene and the common mutation now permits us to confidently and rapidly perform prenatal diagnosis and

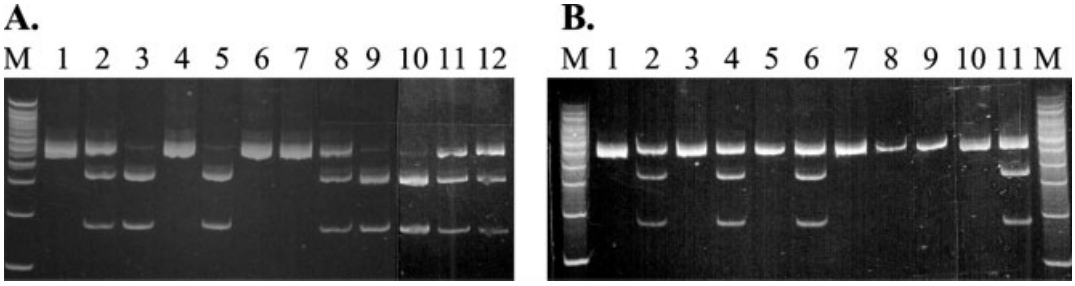

Figure 2—(A) Restriction analysis for the nine previous diagnostic cases. PCR was performed to specifically amplify exon 8 of the SCIDA gene and PCR products were digested by NspI as described in methods. Lane M: 100bp marker; Lane 1–3: normal, carrier and positive controls; Lane 4–12: results for nine previous cases 2603, 2505, 3103, 0506, 1306, 1307, 2704, 2804, 2906, respectively. (B) Restriction analysis for 9 of the 30 normal controls. Lane M: 100bp marker; Lane 1–2: normal and carrier controls; Lane 3–11: controls #5–12, and 14

carrier detection for SCIDA. Because almost all of the SCIDA cases identified to date are the result of a single founder mutation, and the mutation creates a new diagnostic restriction enzyme NspI site, we are able to use a direct, easy and accurate assay for pre- and postnatal diagnosis and/or carrier detection in not only the at-risk families but also the general Navajo and Apache populations. It is also feasible to establish a population-based screening program to identify unknown carriers, in addition to establishing a population-based newborn screening test considering its high incidence in these specific ethnic groups. In our screening for 30 unrelated Navajo adults with no known relationship to affected families, we surprisingly identified three carriers for the SCIDA founder mutation. This raises the possibility that the gene frequency of SCIDA may be higher than the previously estimated 2.1% (Jones *et al*., 1991). A population-wide survey and newborn screening program will be helpful to better define the prevalence of SCIDA in Navajo and Apache Native Americans.

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