CLINICAL REPORT

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Somatic Mosaicism Contributes to Phenotypic Variation in Timothy Syndrome

Susan P. Etheridge,[1]* Neil E. Bowles,[1] Cammon B. Arrington,[1] Thomas Pilcher,[1] Alan Rope,[2] Arthur A.M. Wilde,[3] Marielle Alders,[4] Elizabeth V. Saarel,[1] Rene Tavernier,[5] Katherine W. Timothy,[6] and Martin Tristani-Firouzi[1]

[1]Division of Pediatric Cardiology, University of Utah, Salt Lake City, Utah

[2]Division of Medical Genetics, University of Utah, Salt Lake City, Utah

[3]Department of Cardiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[4]Department of Clinical Genetics, Academic Hospital, University of Amsterdam, Amsterdam, The Netherlands

[5]Department of Cardiology, St. Jan Hospital Bruge, Bruge, Belgium

[6]Department of Pediatrics, University of Utah, Salt Lake City, Utah

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Timothy syndrome type 1 (TS-1) is a rare disorder that affects multiple organ systems and has a high incidence of sudden death due to profound QT prolongation and resultant ventricular arrhythmias. All previously described cases of TS-1 are the result of a missense mutation in exon 8A (p.G406R), an alternatively spliced variant of the L-type calcium channel gene (*$Ca_v1.2$*, *CACNA1C*). Most patients reported in the literature represent highly affected individuals who present early in life with severe cardiac and neurological manifestations. Here, we describe somatic mosaicism in TS-1 patients with less severe manifestations than the typical TS-1 patient. These findings suggest that the TS prognosis may not be as dismal as previously reported. Moreover, our findings have implications for genetic counseling in that previously described de novo TS mutations may represent cases of parental mosaicism and warrant careful genotyping of parental tissue other than peripheral blood lymphocytes.

Key words: Timothy syndrome; channelopathy; sudden death; arrhythmias

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INTRODUCTION

Timothy syndrome (TS) is a rare disorder that affects the development of multiple organ systems. Although originally described as severe Long QT Syndrome (LQTS) and syndactyly [Marks et al., 1995b], the ascertainment of additional subjects revealed a more complex clinical constellation, including congenital heart disease, autism spectrum disorder, abnormal dentition, and dysmorphic facial features [Splawski et al., 2004]. As a consequence of severe QTc prolongation, many TS patients present early in the newborn period with functional 2:1 atrioventricular (AV) block, T wave alternans, and frequent ventricular arrhythmias (*torsades de pointes*, TdP). The incidence of sudden cardiac death in TS subjects is higher than any other subtype of LQTS.

TS is a remarkably homogeneous disorder at the molecular level; all cases of TS-1 occur as the result of the identical, de novo missense mutation in exon 8A (p.G406R), an alternatively spliced variant of the L-type calcium channel gene (*$Ca_v1.2$*, *CACNA1C*) [Splawski et al., 2004]. The mutation is localized to the most terminal portion of the S6 transmembrane segment in Domain I, disrupting voltage-dependent channel inactivation to cause a gain-of-function effect. TS-2, characterized by the absence of syndactyly, is caused by mutations in the dominant splice variant exon 8 (p.G402S or p.G406R) [Splawski et al., 2004, 2005]. All previous TS-1 patients represent simplex cases, with the exception of a single family with two affected sibs [Splawski et al., 2004]. In that study, the mother's DNA samples were sequenced from the blood and oral mucosa. The blood DNA contained only wild-type sequences, but there was a minor peak for the missense mutation in DNA from her oral mucosa. Thus, the phenotypically unaffected mother was mosaic

*Correspondence to:
Susan P. Etheridge, M.D., Division of Pediatric Cardiology, University of Utah School of Medicine, 100 North Mario Capecchi Drive, Salt Lake City, UT 84113. E-mail: pcsether@ihc.com

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and transmitted this mutation to her two affected children [Splawski et al., 2004]. Mosaicism signifies the presence of genetically distinct populations in the somatic and germline tissues, with tissue-to-tissue variations that may not follow Mendelian rules of inheritance [Youssoufian and Pyeritz, 2002]. The designation of mosaicism is often based on the phenotypic appearance of the index patient and parents.

This reports details the clinical and genetic information of a mildly affected father and his severely affected infant and a third unrelated and moderately affected child, not identified until adolescence. While there has been a previous recognition of genetic mosaicism in TS [Splawski et al., 2004], our two more mildly affected patients are the first described with TS-1 due to mosaicism for the G406R *CACNA1C* mutation with manifest phenotypic features of the syndrome.

MATERIALS AND METHODS

Genotyping and Sequence Analysis

Genomic DNA was isolated from peripheral blood lymphocytes, buccal smears, or sperm using an Autopure LS (Qiagen, Valencia, CA) or QIAamp DNA Mini Kit (Qiagen). PCR amplification of exon 8A of *CACN1AC* from genomic DNA was performed using oligonucleotide primers spanning the exon and adjacent intron sequences: primer sequences and PCR conditions are available on request. PCR products were purified and sequenced using ABI3730 DNA Analyzers (Applied Biosystems, Carlsbad, CA).

In order to confirm the presence of the c.1216G>A substitution in mosaic patients the PCR product was cleaved using the restriction enzyme *Aci*I (New England Biolabs Inc, Ipswich, MA), since the mutation abolishes the recognition sequence. The digested PCR products were analyzed by agarose gel electrophoresis. In addition, the mutant allele was enriched for by performing a second PCR using the digested PCR product as a template and analyzed by DNA sequencing. In order to estimate the frequency of the mutated allele in the father's gametes, the PCR product obtained after amplification of sperm DNA was cloned into the plasmid vector pCR4 (Invitrogen, Carlsbad, CA) by TOPO cloning, according to the manufacturer's instructions. After bacterial transformation and selection, colonies were picked and grown overnight at 37°C in 200 µl of *E. coli* Fast-Media TB plus ampicillin (Invivogen, San Diego, CA). Three microliters of each culture was subject to PCR amplification, and, after purification using ExoSAP-IT (USB Corporation, Cleveland, OH), the product was analyzed by DNA sequencing. The percentage of colonies containing the WT or mutant allele was determined. This review conformed to the investigational review boards of the two participating institutions.

RESULTS

Clinical Presentation, Phenotype Ascertainment, and Genotyping

Patient 1. Index Patient 1 was brought to medical attention due to fetal bradycardia with functional 2:1 AV block. Preterm labor necessitated birth at 34 weeks gestation. Initial heart rates were 125–130 bpm but soon dropped to 60 bpm. A 12-lead ECG demonstrated functional 2:1 AV block (Fig. 1A) with marked QT prolongation and T wave alternans (Fig. 1B). The family history was not significant for sudden death or sudden infant death syndrome. The patient's father had complete cutaneous syndactyly of T1-3 of the right foot and of T1-2 of the left foot [Biesecker et al., 2009]. He was otherwise healthy, cognitively normal, and without a history of syncope. Examination of the index patient suggested features consistent with TS including the appearance of widely spaced eyes, a broad forehead, and wide nasal bridge with broad nasal tip [Allanson et al., 2009; Hall et al., 2009; Hennekam et al., 2009]. In addition, he had complete cutaneous syndactyly of F3-5 of the right hand and F4-5 of the left hand. An echocardiogram demonstrated a small anterior muscular ventricular septal defect and decreased biventricular systolic function.

Due to bradycardia a single chamber epicardial ventricular pacemaker was placed on the first day of life. The patient was not tried on beta-blockers prior to pacemaker implantation. He was managed in the neonatal intensive care unit for 2 months where 12-lead ECG

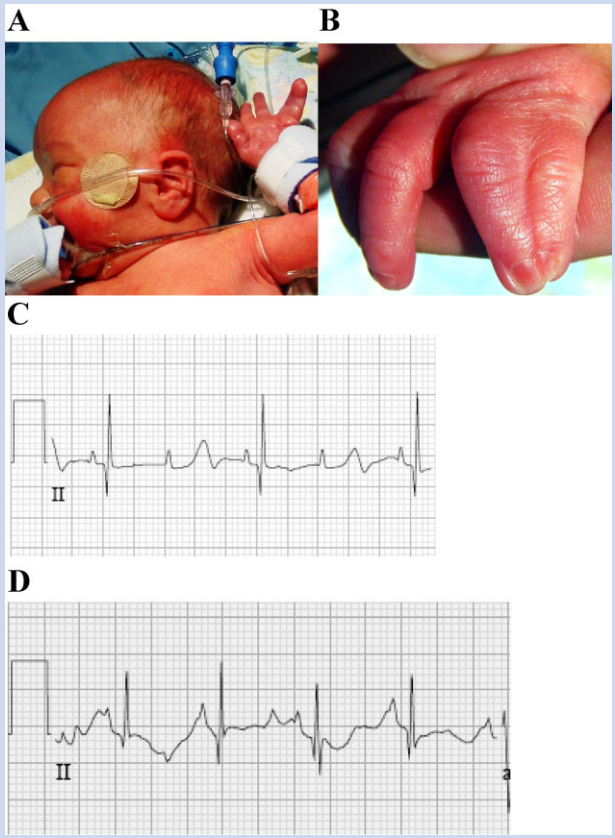

FIG. 1. A: Profile of index Patient 1 in the newborn period. The nasal root appears depressed, the nasal tip is prominent, the earlobe is large and the complete cutaneous syndactyly of F4-5 of the left hand can be appreciated. B: Dorsal aspect of the left hand of Index Patient 1: There is complete cutaneous syndactyly of F4-5 that includes fusion of the nails. C: Lead II from the initial ECG (index Patient 1) demonstrating QTc prolongation and functional second-degree AV block. D: ECG with QTc prolongation and T-wave alternans.

and bedside monitoring demonstrated QTc prolongation, T-wave alternans, significant ventricular ectopy, and frequent nonsustained TdP. He was initially managed with magnesium and potassium supplementation, spironolactone, and esmolol. He was converted to oral propranolol and mexiletene and this combination was effective in suppressing the frequent bursts of TdP. At this time, genetic testing identified a heterozygous c.1216G>A transition in *CACNA1C* resulting in the p.G406R missense mutation in exon 8A typical of TS-1 [Splawski et al., 2004]. Due to a previous report of successful medical management of TS-2 with verapamil, the mexiletene was discontinued and the patient was administered intravenous verapamil [Jacobs et al., 2006]. Verapamil administration was acutely associated with an increased frequency of TdP and the medication was discontinued. A few weeks later a second trial of verapamil was given, this time orally administered, and again there was a significant increase in TdP, necessitating discontinuation.

Implantation of a cardioverter-defibrillator (ICD) and left cervical sympathetic denervation (LCSD) were successfully undertaken once he attained a weight of 4 kg (birth weight 2.3 kg). The single chamber ICD was an epicardial device with dual epicardial ICD coils set VVI mode at 100 bpm and shock vector between the two coils. He tolerated the surgical procedure well without events of TdP. The arrhythmia detection of the ICD was purposely set with a long detect time allowing for spontaneous termination of TdP events. He received two appropriate ICD therapies while hospitalized. He was awake during the first therapy despite a long detection duration of 30/40 beats. He had syncope prior to the second appropriate ICD discharge. The detection interval was maximized to 120/160 beats.

Since hospital discharge he has had normalization of biventricular function but continues to have occasional nonsustained episodes of TdP and has had three appropriate ICD shocks for sustained tachycardia. On 2 occasions TdP resulted in loss of consciousness, while 1 event occurred during sleep (Fig. 2). He remains on spironolactone for potassium sparing effects, magnesium, and propranolol. At last visit, he was 1-year old, growing and developing normally. He has had syndactyly surgery and tolerated this without arrhythmias during anesthesia.

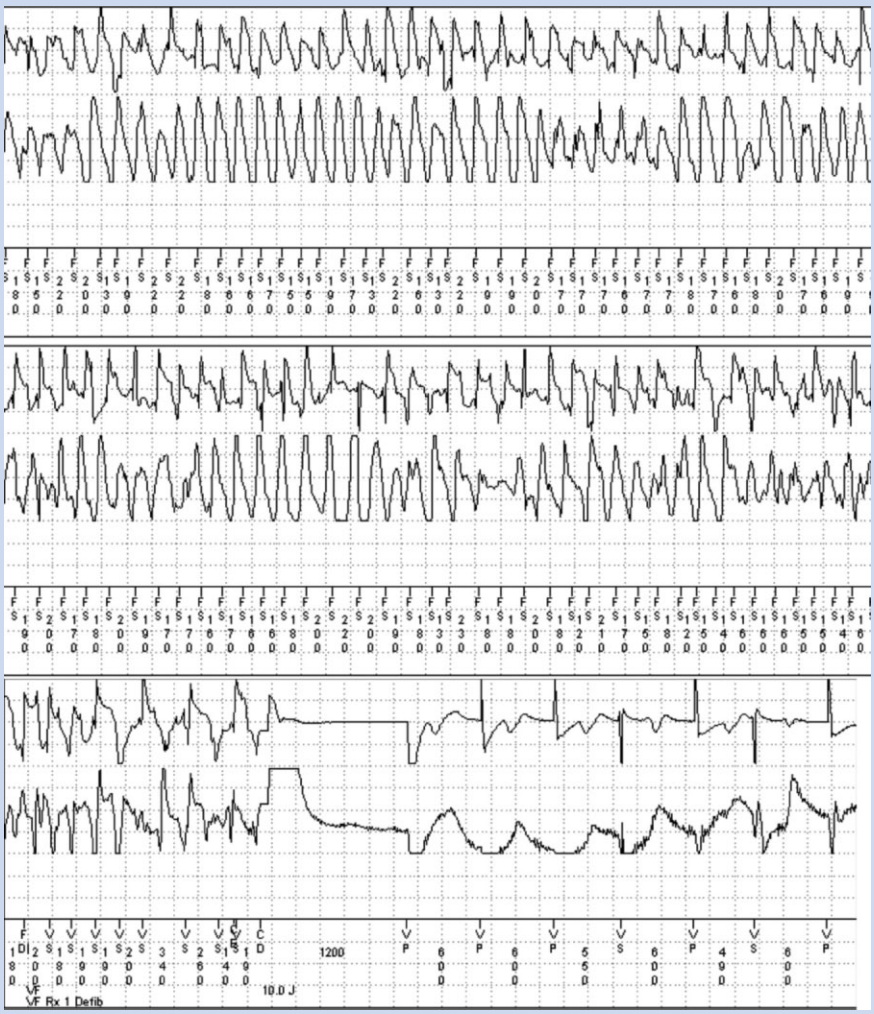

FIG. 2. Recordings from ICD (index Patient 1) an episode of ventricular tachycardia treated with an ICD shock.

The father of the index Patient 1 had complete cutaneous syndactyly of T1-3 of the right foot and T2-3 on the left, but no other dysmorphic features. He was cognitively normal and never experienced symptoms of syncope or seizure. A 12-lead ECG demonstrated prolonged QTc (480 ms) and he was started on prophylactic beta-blocker therapy. Analysis of the father's peripheral blood DNA (Fig. 3A) and buccal smear (data not shown) revealed the presence of a minor A peak, superimposed on a larger G signal at nucleotide 1216, that was absent in the control sample, suggestive of mosaicism in these tissues. The presence of this substitution was confirmed by digesting the PCR product with *Aci*I: a small proportion of the PCR product remained uncleaved (Fig. 3B). In addition, sequencing the PCR product after enrichment of the mutant allele identified a strong "A" peak (Fig. 3C). To further investigate the possibility of mosaicism, we analyzed DNA isolated from the father's gametes. The c.1216G>A substitution was still detected as the minor allele (Fig. 3A). Cloning of the PCR product, followed by PCR amplification and DNA sequencing of 32 of the resulting colonies identified "A" at position 1216 in five clones, indicating that approximately 16% of the sperm carried the mutant allele.

Patient 2. Index Patient 2 is a 14-year-old girl who came to medical attention after a cardiac arrest with documented ventricular fibrillation that occurred while making a phone call. Her past

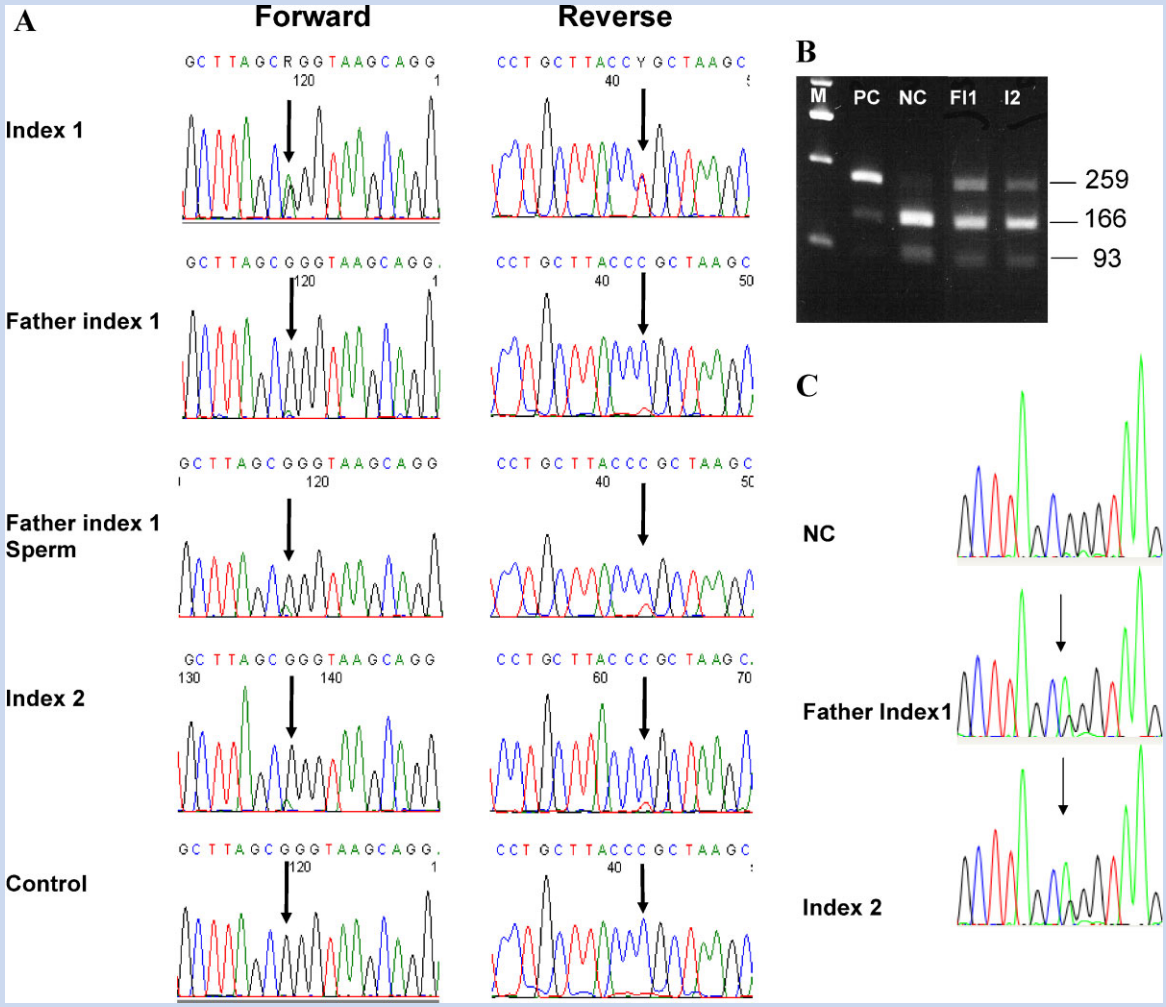

FIG. 3. Analysis of exon 8A of *CACNA1C*. Panel A: Electropherograms of DNA sequences derived from peripheral blood samples of index Patient 1, his father, index Patient 2 and a normal control (bottom). In addition, analysis of DNA purified from sperm of the father of index Patient 1 (sperm) is shown. Nucleotide 1216 is indicated by the arrow showing the presence of heterozygous G/A in index Patient 1 and a small A peak in the father. Panel B: Analysis of the CACNA1C exon 8A PCR products after digestion with *Aci*I by agarose gel electrophoresis. *Aci*I cuts the full-length PCR product (259 bp) into two (166 and 93 bp) fragments unless the c.1216G>A substitution is present which abolishes the recognition sequence. In the case of the father of the index Patient 1 and index Patient 2 a small proportion of the PCR product is not cut. M, Generuler Express DNA ladder (Fermentas); PC, positive control DNA (heterozygous mutation carrier); NC, normal control DNA; FI1, father index Patient 1; I2, index Patient 2. Panel C: Electropherograms of DNA sequences derived from *Aci*I cleaved PCR products shown in panel B. Note the major nucleotide at position 1216 is A (green), indicated by the arrow, although G (black) is still detected as a minor peak due to the incomplete digest seen in panel B. NC, normal control DNA; father of index Patient 1, index Patient 1 and index Patient 2.

medical history was unremarkable and she was described as an average student in high school with some behavior issues but no previous syncopal events. Her family history is unremarkable. Her clinical examination was significant for syndactyly involving the hands and the feet, bilaterally. Her 12-lead ECG was significant for a QTc interval of 560 ms at a heart rate of 60 bpm and tall and symmetric T waves. Her echocardiogram and cardiac MRI were both normal. She was started on beta-blocker therapy and underwent ICD implantation. She tolerated anesthesia well and has had no ICD discharges since hospital discharge 1 year ago. Analysis of DNA isolated from a blood sample identified a minor A peak at nucleotide 1216 suggesting she is mosaic for the c.1216G>A variant (Fig. 3A), which was confirmed by digestion with *AciI* (Fig. 3B) and sequencing the product after enrichment Fig. 3C).

In both of the mosaic cases, cardiac tissue was not available to determine the presence or quantity of the mutant allele, and, thus, confirm its contribution to the cardiac phenotype.

DISCUSSION

TS is a severe multisystem disorder primarily affecting the heart, brain, and limbs. The severe QT prolongation and consequent lethal nature of this disease are important factors in determining the rare inheritance pattern. Most TS-1 cases reported to date arise from an identical, de novo mutation in the alternatively spliced exon 8A *CACNA1C* variant. Most are simplex cases with the exception of a single family with two affected sibs and parents whose genotype and phenotype were apparently normal. The inheritance pattern in this family suggests parental somatic or germline mosaicism. Here, we report on two patients with TS-1 with molecular confirmation of somatic mosaicism. These observations have important consequences for genetic counseling as previously identified de novo mutations may actually represent parental mosaicism.

Mosaicism is defined as the presence of genetically distinct cell lines in a single organism derived from a single zygote. In somatic mosaicism, single or multiple tissues express more than one genotype and the degree of mosaicism can vary between tissues, while in germline mosaicism, the distinct genotypes are confined to the gametes. While somatic mosaicism has been implicated in over 30 monogenic disorders [Youssoufian and Pyeritz, 2002], mosaicism is rarely reported in LQTS. To the best of our knowledge, only a single report of parental mosaicism in typical LQTS has been published. Miller et al. [2004] reported a phenotypically normal mother with a low-level mosaic *SCN5A* missense mutation in lymphocytes, fibroblast, and buccal mucosal cells. The heterozygous *SCN5A* mutation was identified in two offspring with life-threatening TdP. In our study, the father of index Patient 1 manifested a mild TS phenotype (complete cutaneous syndactyly and asymptomatic LQTS) that escaped detection until the birth of his affected child. Mosaicism was suggested by the presence of a small peak, barely above background, at the 1216 position in exon 8A in peripheral blood lymphocytes and buccal mucosa. On further analysis, a subpopulation of the father's gametes carried the 1216G>A transition. The apparently normal cognition in this patient is consistent with the milder TS-1 phenotype. The somatic mosaicism identified in the father is the likely explanation for his mild TS phenotype. This observation raises the possibility that TS phenotype may not be as dismal as previously reported. In addition, some of the previously described de novo TS mutations may represent cases of parental mosaicism and warrant careful genotyping of tissue other than peripheral blood lymphocytes.

Because of the rarity of TS, precise management remains unclear and multicenter trials are unlikely to occur. A functional consequence of the TS mutation is disruption of voltage-dependent inactivation of L-type calcium channels with sustained inward, depolarizing current during the plateau phase and marked prolongation of the action potential duration [Splawski et al., 2004, 2005]. Thus, one would predict that L-type calcium channel blockers may be beneficial in TS patients. A previous report describes a reduction in TdP and ICD discharges in a TS-2 patient treated with verapamil [Jacobs et al., 2006]. However, in our TS-1 infant, verapamil resulted in increased TdP. Previous patients have been treated with beta-blockers but there have been deaths despite this therapy [Marks et al., 1995a]. In the case of the neonate, we chose to empirically start spironolactone to prevent hypokalemia and magnesium supplementation because of theoretical effects in patients with classic LQTS. Medical management and anti-bradycardia pacing did not fully control the tachycardia therefore a LCSD and ICD were undertaken. LCSD has been described as effective in the setting of drug refractory LQTS [Moss and McDonald, 1971; Schwartz et al., 2004] and has been attempted previously for TS but the patient died due to intractable arrhythmias that occurred at the moment of touching the stellate ganglion (A.A.M. Wilde, unpublished work). In our patient, LCSD was associated with a decrease in the arrhythmia burden and the ICD has proven effective at tachycardia recognition. A long tachycardia detect time has prevented frequent shocks for nonsustained TdP events yet has appropriately terminated three episodes of sustained tachycardia.

There are concerns about anesthesia-related complications and deaths in the TS population. There are other unpublished cases of death related to anesthesia including elective ICD (K.W. Timothy and A.A.M. Wilde, unpublished work) thus anesthesia and surgical interventions should be undertaken with caution in this population. Despite previous descriptions of anesthesia-related events [Splawski et al., 2004], anesthesia has been undertaken safely on three occasions in the first patient in this report and on one occasion in the second. The use of beta-blockers and attention to magnesium and potassium levels before and during the procedures may have been helpful.

A unifying and easy to recognize feature of TS-1, syndactyly was reported in 100% of the initial cohort [Splawski et al., 2004]. Complete cutaneous syndactyly as seen in our patients is a rare finding in the general population [McKiernan and McCann, 1993; Al-Qattan, 2006]. Performing ECG in neonates with this specific form of syndactyly and their similarly affected family members may help identify presymptomatic TS-1 patients and expand the phenotype to include less affected patients. Careful genotyping of parents of TS-affected offspring may identify parental mosaicism and improve genetic counseling in families where a de novo mutation was originally suspected. We suggest that laboratories screening CACNA1C for this variant consider analyzing the PCR product by *Aci*I digestion and agarose gel electrophoresis and/or

enrichment of the mutant allele-specific PCR product to reduce the chance that mosaicism will be missed.

REFERENCES

Allanson JE, Cunniff C, Hoyme HE, McGaughran J, Muenke M, Neri G. 2009. Elements of morphology: Standard terminology for the head and face. Am J Med Genet Part A 149A:6–28.

Al-Qattan MM. 2006. Expression of familial middle-ring-little finger syndactyly as either simple syndactyly or synpolydactyly. J Hand Surg Br 31:118–120.

Biesecker LG, Aase JM, Clericuzio C, Gurrieri F, Temple IK, Toriello H. 2009. Elements of morphology: Standard terminology for the hands and feet. Am J Med Genet Part A 149A:93–127.

Hall BD, Graham JM Jr, Cassidy SB, Opitz JM. 2009. Elements of morphology: Standard terminology for the periorbital region. Am J Med Genet Part A 149A:29–39.

Hennekam RC, Cormier-Daire V, Hall JG, Mehes K, Patton M, Stevenson RE. 2009. Elements of morphology: Standard terminology for the nose and philtrum. Am J Med Genet Part A 149A:61–76.

Jacobs A, Knight BP, McDonald KT, Burke MC. 2006. Verapamil decreases ventricular tachyarrhythmias in a patient with Timothy syndrome (LQT8). Heart Rhythm 3:967–970.

Marks ML, Trippel DL, Keating MT. 1995a. Long QT syndrome associated with syndactyly identified in females. Am J Cardiol 76:744–745.

Marks ML, Whisler SL, Clericuzio C, Keating M. 1995b. A new form of long QT syndrome associated with syndactyly. J Am Coll Cardiol 25:59–64.

McKiernan MV, McCann JJ. 1993. Familial syndactyly type III—Report of a large pedigree. Clin Genet 44:270–271.

Miller TE, Estrella E, Myerburg RJ, Garcia de Viera J, Moreno N, Rusconi P, Ahearn ME, Baumbach L, Kurlansky P, Wolff G, Bishopric NH. 2004. Recurrent third-trimester fetal loss and maternal mosaicism for long-QT syndrome. Circulation 109:3029–3034.

Moss AJ, McDonald J. 1971. Unilateral cervicothoracic sympathetic ganglionectomy for the treatment of long QT interval syndrome. N Engl J Med 285:903–904.

Schwartz PJ, Priori SG, Cerrone M, Spazzolini C, Odero A, Napolitano C, Bloise R, De Ferrari GM, Klersy C, Moss AJ, Zareba W, Robinson JL, Hall WJ, Brink PA, Toivonen L, Epstein AE, Li C, Hu D. 2004. Left cardiac sympathetic denervation in the management of high-risk patients affected by the long-QT syndrome. Circulation 109:1826–1833.

Splawski I, Timothy KW, Sharpe LM, Decher N, Kumar P, Bloise R, Napolitano C, Schwartz PJ, Joseph RM, Condouris K, Tager-Flusberg H, Priori SG, Sanguinetti MC, Keating MT. 2004. Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 119:19–31.

Splawski I, Timothy KW, Decher N, Kumar P, Sachse FB, Beggs AH, Sanguinetti MC, Keating MT. 2005. Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. Proc Natl Acad Sci USA 102:8089–8096,discussion 8086–8088.

Youssoufian H, Pyeritz RE. 2002. Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet 3:748–758.