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BRIEF REPORT

Timing of De Novo Mutagenesis — A Twin Study of Sodium-Channel Mutations

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SUMMARY

De novo mutations are a cause of sporadic disease, but little is known about the developmental timing of such mutations. We studied concordant and discordant monozygous twins with de novo mutations in the sodium channel $\alpha 1$ subunit gene (*SCN1A*) causing Dravet's syndrome, a severe epileptic encephalopathy. On the basis of our findings and the literature on mosaic cases, we conclude that de novo mutations in *SCN1A* may occur at any time, from the premorula stage of the embryo (causing disease in the subject) to adulthood (with mutations in the germ-line cells of parents causing disease in offspring).

DE NOVO MUTATIONS CAUSE SPORADIC FORMS OF A RANGE OF MENDELIAN disorders, including tuberous sclerosis, neurofibromatosis, achondroplasia,¹ and Dravet's syndrome. Recently, de novo copy-number variations have been identified as a cause of sporadic cases of some mendelian disorders and perhaps more commonly as susceptibility alleles for complex disorders. Thus, de novo mutagenesis is an important mechanism in human disease and probably explains an appreciable fraction of sporadic and apparently nongenetic disorders.¹

Twins represent a unique resource for studying the timing of de novo mutagenesis. There are numerous case reports of genetic disorders in which monozygous twin pairs are phenotypically discordant. Some of these disorders involve chromosomal syndromes (e.g., trisomies) and disorders known or postulated to be caused by conventional mutations or epigenetic mechanisms.² There are only two case reports of single-gene-point mutations found only in the affected monozygous twin, leading to phenotypic and genotypic discordance within the monozygous twin pair, but the timing of these de novo mutations has not been clearly determined.^{3,4}

Dravet's syndrome, or severe myoclonic epilepsy of infancy, is usually a sporadic disease yet is known to be a genetic disorder. The syndrome is characterized by an onset at approximately 6 months, with prolonged convulsions or hemic convulsions, often with fever. Other seizure types usually appear, and there is slowing in psychomotor development. The usual long-term outcome includes intellectual disability and intractable epilepsy.⁵

Heterozygous mutations in *SCN1A* are found in 70 to 80% of patients with Dravet's syndrome.^{6,7} Of these mutations, 95% are de novo, which explains why siblings or parents are usually unaffected.^{8,9} The absence of mutations in parental DNA, obtained from peripheral-blood lymphocytes, led to the inference that the

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usual mechanism involves a spontaneous mutation in *SCN1A* in parental gonadal tissue (i.e., testicular or ovarian cells). We sought to determine the timing of *SCN1A* mutation by analyzing different embryologic tissue lineages in a monozygous pair of twins who were discordant for Dravet's syndrome.

METHODS

CLINICAL STUDIES

The Australian epilepsy twin database was established in 1988; data were obtained from twin registries or referral, as described previously.¹⁰ The database includes 372 twin pairs in which one or both twins had seizures, with 169 monozygous and 203 dizygous pairs. Dravet's syndrome was identified in three monozygous twin pairs; two of the twin pairs were concordant and one twin pair was discordant for the syndrome. We have previously reported some of the clinical and molecular characteristics of these twins.^{7,11} The Human Research Ethics Committee of Austin Health approved the study, and written informed consent was obtained from patients or, when appropriate, their parents or legal guardians.

ZYGOSITY DETERMINATION

Lymphocyte-derived genomic DNA was examined with the use of nine independent polymorphic markers (D13S317, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, and vWA). In the discordant twin pair, these markers were also examined in genomic DNA from buccal cells (to search for evidence of chimerism), and 10 additional polymorphic markers (D2S2290, D4S2935, D5S422, D8S1799, D9S260, D10S580, D14S1065, D15S119, D16S3034, and D19S928) were tested in lymphocyte DNA; these additional 10 markers provide a stronger test of monozygosity.

ANALYSIS OF *SCN1A*

Mutations in *SCN1A* in genomic DNA from lymphocytes were sought in twin probands, as described previously.⁷ In the discordant twin pair, DNA was also extracted from cheek cells, hair-follicle cells, fibroblasts derived from skin-biopsy samples, and cell lines derived from olfactory neuroepithelium. Upper olfactory mucosa was extracted from a nasal-biopsy specimen obtained by an otolaryngologist after the administration of local anesthesia. This tissue was dissociated and grown in serum-free medium with epidermal

growth factor and basic fibroblast growth factor to produce neurospheres (phase-bright spheroids containing neural stem cells and neural progenitors), as described previously.¹² Neurospheres were dissociated, and these neurosphere-derived cells were grown as cell lines in 10% serum.¹² Genomic DNA was extracted from these neurosphere-derived cell lines.

GENETIC ANALYSES

To search for the presence of cell populations carrying an *SCN1A* mutation in apparently unaffected subjects, primer pairs specific for mutant and wild-type DNA sequences were designed for each twin-pair mutation so that one primer contained the mutant base change at the 3' end. Conditions for polymerase-chain-reaction (PCR) assay were optimized to allow selective amplification of mutant or wild-type sequence (for details, see the Supplementary Appendix, available with the full text of this article at NEJM.org).

To test for mosaicism, we performed allele-specific PCR and semiquantitative analysis. To determine the limit of sensitivity of the allele-specific PCR, we diluted mutant DNA from the patients in wild-type DNA and used the mutant primer for amplification of the mutant sequence while ensuring that no product was amplified in wild-type DNA from control subjects.

RESULTS

CONCORDANT MONOZYGOUS TWIN PAIRS 1 AND 2

Clinical characteristics of twin pairs 1 and 2 are shown in Table 1. On the basis of nine polymorphic DNA markers, the probability that these twin pairs could be dizygous is extremely low (8×10^{-5}).¹³ Twin pair 1 had two *SCN1A* gene variants, a protein truncation mutation (c.4573C→T, R1525X) and a missense rare variant (c.1811G→A, R604H); the functional significance of the latter is uncertain. Only their mother was available for testing, and sequencing showed that she carried neither the mutation nor the rare variant. Twin pair 2 had a de novo frameshift mutation (c.4949_4950insT, I1650fsX1672) that was predicted to cause protein truncation; both parents tested negative for the mutation.

DISCORDANT MONOZYGOUS TWIN PAIR 3

The twins in pair 3 were 25 years of age. They were born after an uncomplicated, spontaneous twin pregnancy, and the births were unremark-

able. The proband was delivered second and weighed 2880 g at birth, and her twin weighed 2520 g. They were third (unaffected twin) and fourth (proband) in a kindred of five siblings. One older sister had a history of two febrile seizures before the age of 4 years. Both parents were unaffected. There was no other family history of seizures.

Clinically, the twin pair was considered monozygous, although the affected twin was slightly heavier and had coarser facial features, findings that are consistent with long-term use of antiepileptic medication. Zygosity testing for 9 polymorphic DNA markers in lymphocyte and buccal cells and for an additional 10 markers in lymphocytes confirmed monozygosity. On the basis of tests for these 19 markers, the probability that these twins were dizygous was negligible (2×10^{-8}).¹³

The proband initially presented with prolonged, generalized febrile seizures at the age of 6 months. Multiple seizure types ensued. These included myoclonic seizures, tonic-clonic seizures, and partial seizures. Over the years, she had had recurrent hospital admissions for prolonged seizures (status epilepticus) and had received multiple medications for intractable seizures.

The proband had normal early milestones. After 15 months, developmental regression was noted, with delays in speech and walking. She attended a regular school and completed a modified 12th year with integration aids. At the time of evaluation, she was working in a restaurant. Neuropsychological testing showed a low average full-scale IQ of 89 on the Wechsler Adult Intelligence Scale-Revised. She was married and had an unaffected daughter. Magnetic resonance imaging (MRI) of the brain showed left hippocampal sclerosis with small hippocampal volume and an increased signal on T₂-weighted scanning.

Her twin had two simple febrile seizures, at 6 weeks and 4 years. Neuropsychological testing showed average intelligence. At the time of evaluation, she was working as a nurse. The findings on brain MRI were within normal limits.

The proband had a heterozygous base change c.664C→T in exon 5 of *SCN1A*, resulting in a premature stop-codon mutation (R222X) that was predicted to lead to a truncated protein. We detected this mutation in DNA extracted from the proband's lymphocytes, hair, buccal cells, skin fibroblasts, and cell lines derived from olfactory neuroepithelium and did not observe it in DNA

Table 1. Clinical Characteristics of Two Concordant Monozygous Twin Pairs with Dravet's Syndrome and *SCN1A* Sequencing Results for the Twins and Their Parents.*

Twin Pair No.	Sex	Age at Seizure Onset	Seizure Type	Intellectual Disability	Family History	Both Twins	SCN1A Sequencing	Parents
1	Female	8 mo for both twins	Febrile, tonic-clonic, absence, myoclonic	Moderate; some words and short sentences	None; 2 unaffected younger siblings	Protein truncation mutation (c.4573C→T, R1525X) and missense rare variant (c.1811G→A, R604H)	No evidence of mosaicism or mutation in mother; father deceased	
2	Female	4.5 mo for both twins	Hemidonic febrile, tonic-clonic, absence, partial, atonic	Severe; single words and regression in adulthood	None; 2 unaffected older siblings	Frameshift mutation (c.4949_4950insT, 11650fsX1672)	No evidence of mosaicism or mutation in either parent	

* *SCN1A* denotes sodium channel $\alpha 1$ subunit gene.

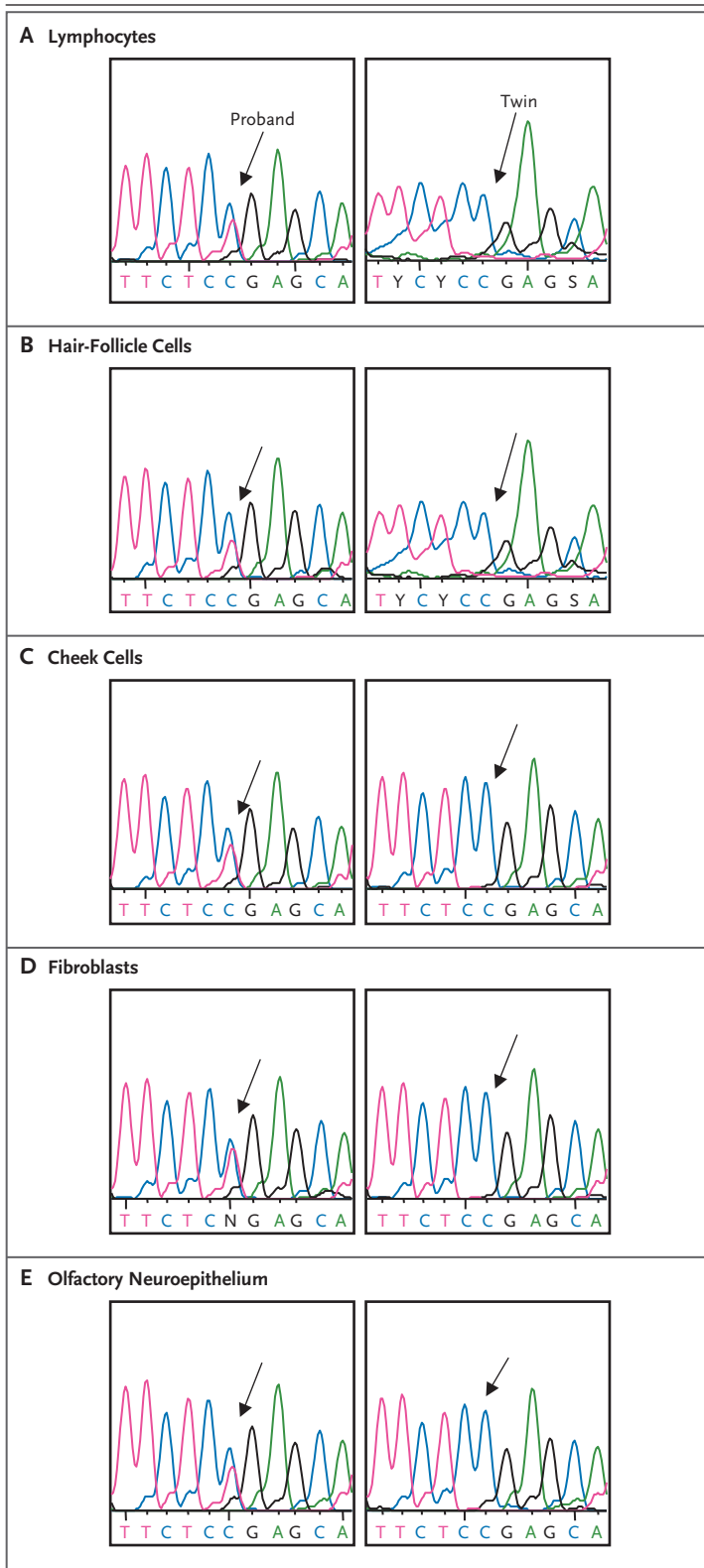


Figure 1. DNA Sequencing for a Monozygous Twin Pair Discordant for Dravet's Syndrome.

Shown is the sequence analysis of the c.664C→T mutation in exon 5 of the sodium channel $\alpha 1$ subunit gene (*SCN1A*) for DNA from lymphocytes (Panel A), hair-follicle cells (Panel B), cheek cells (Panel C), fibroblasts derived from skin-biopsy samples (Panel D), and cell lines derived from olfactory neuroepithelium (Panel E). In all tissue types, the proband has the heterozygous base change c.664C→T (two peaks, with the cytosine base in blue and the thymine base in pink), whereas the unaffected twin has a single peak (with the cytosine base in blue), which corresponds to the normal wild-type sequence (arrows).

extracted from tissues obtained from her twin (Fig. 1). We did not detect the mutation in DNA purified from lymphocytes from the parents and the older sibling with a history of febrile seizures.

DNA ANALYSIS FOR SOMATIC MOSAICISM

We analyzed each of the three *SCN1A* mutations by means of allele-specific PCR and semiquantitative analysis. The lowest percentage of mutant DNA in preparations in which mutant sequence was detected was 1.57% in twin pair 1, 3.13% in twin pair 2, and 1.57% in twin pair 3. We found no evidence of mosaicism in the mother of twin pair 1 or in either of the parents of twin pairs 2 and 3, although we cannot rule out the possibility that the parents have gonadal mosaicism. We also tested the unaffected twin in twin pair 3 and found no evidence of mosaicism for the R222X mutation.

DISCUSSION

We found mutations in all three monozygous twin pairs, which predict a truncated protein product (providing that the mutant RNA is not degraded), strongly suggesting pathogenicity. More than 200 similar truncation mutations of *SCN1A* that have been described in patients with Dravet's syndrome have not been reported in control subjects.¹⁴ Electrophysiological studies have also shown major functional effects of such *SCN1A* mutations.¹⁵ Moreover, the presence of the specific mutations R222X, in the discordant monozygous twin pair, and R1525X, in one of the concordant monozygous twin pairs, has been described previously in other patients with Dravet's syndrome.¹⁴

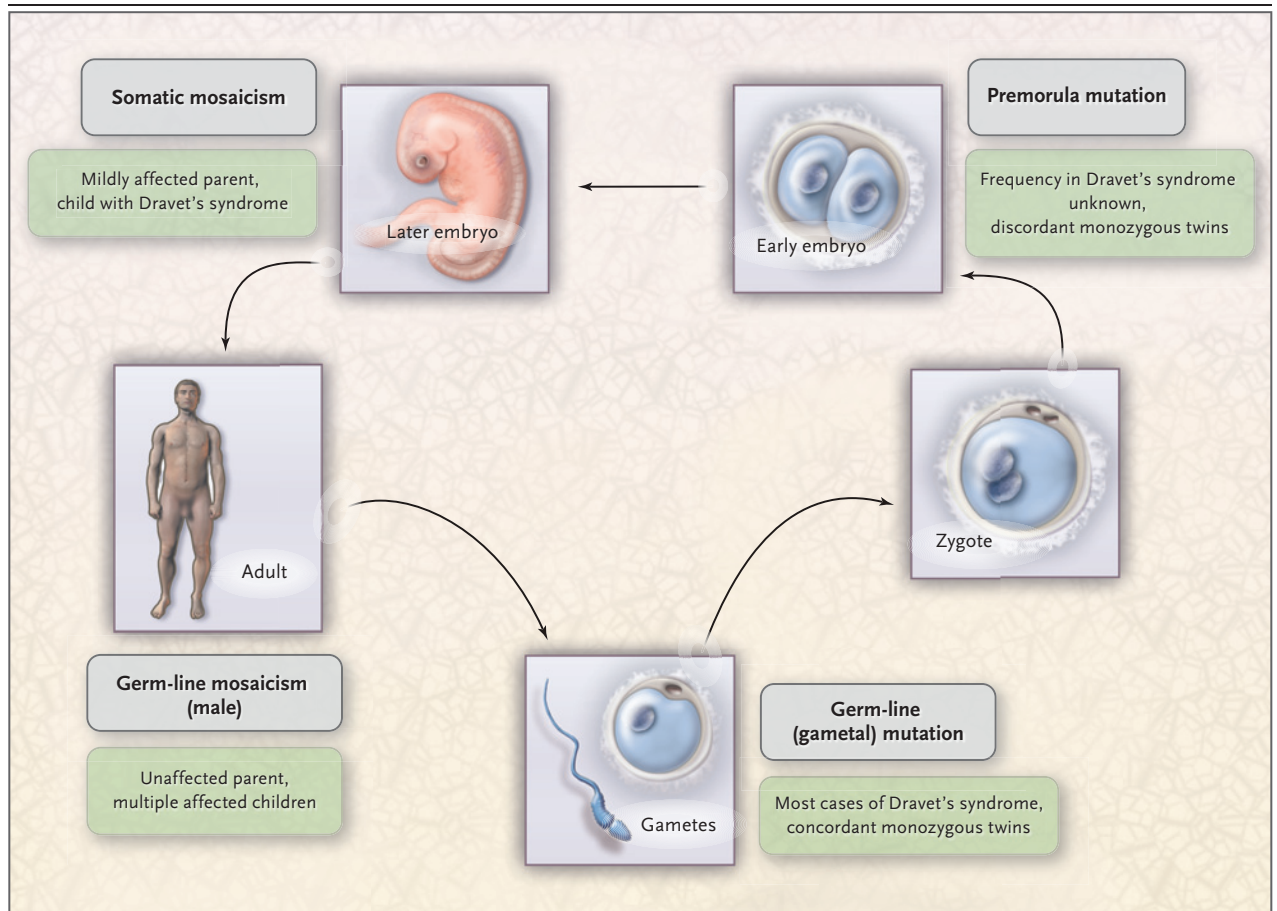


Figure 2. Timing of Mutations in Dravet's Syndrome.

Gray boxes show the timing of mutations, and green boxes describe evidence from twin and family studies. Most cases of this syndrome are believed to be due to de novo mutations in gametes (usually paternal¹⁹), although in nontwin patients with the syndrome, postzygotic mutation cannot be ruled out. The findings in twin pair 3 provide evidence for a premorula mutation, probably at the two-cell stage. Somatic mosaicism has been described in mildly affected parents (father or mother) with a child or children with Dravet's syndrome.²⁰⁻²² Germ-line mosaicism in fathers occurs in the adult testis, whereas germ-line mosaicism in mothers (not shown) occurs in early development, since oocytes are mature by 5 months of fetal life. Germ-line mosaicism is inferred when a healthy parent has multiple affected children.^{20,22-25}

Dravet's syndrome usually occurs sporadically, with *SCN1A* mutations in the affected patient but not in the parents or healthy siblings. In monozygous twins with this syndrome, both are typically affected (e.g., twin pairs 1 and 2 and those described previously^{16,17}). In such cases, the *SCN1A* mutation is most likely to develop in the parental germ line,¹⁸ giving rise to a mutated sperm or egg. Mutations in *SCN1A* occur more frequently in the male germ line.¹⁹

Mosaicism is the presence of two genetically different cell lines arising after fertilization, and

it informs the timing of postzygotic mutagenesis. Somatic mosaicism occurs in nonsex cells, whereas germ-line mosaicism occurs in the gamete-forming cells. In Dravet's syndrome, somatic mosaicism has been shown in cases in which mildly affected parents have one or more affected offspring.²⁰⁻²² Germ-line mosaicism is inferred in cases in which unaffected parents have multiple affected offspring.^{20,22-25}

The timing of mutagenesis is a critical factor in genetic counseling. Gametal mutations are associated with a negligible risk of recurrence,

whereas germ-line mosaicism (which may not be identified until after the birth of two affected children) is associated with a high risk of recurrence. The timing for germ-line and somatic mosaicisms is illustrated in Figure 2.

The findings in twin pair 3 are instructive with respect to the timing of mutagenesis. Since the mutation was found in all cell lines from different tissues in the proband but not in her twin, the de novo mutation probably occurred in the pre-morula embryo, most likely at the two-cell stage. The mutation may have occurred before or after the actual twinning process. If the de novo mutation occurred in the four-cell stage or later, the possibility of mosaicism would then arise. The implication for genetic counseling is that in a discordant monozygous twin pair without evi-

dence of mosaicism, the risk that the mutation will recur is negligible.

Similar timing could be postulated for the two other twin pairs in which the affected monozygous twin had a de novo mutation in a single gene, but the analysis of DNA, which was restricted to peripheral-blood leukocytes, precluded a more precise estimate of the timing.^{3,4} Our study highlights the value of studies in twins to determine the timing of de novo mutagenesis.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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