Novel G1481V and Q1491H SCN5A Mutations Linked to Long QT Syndrome Destabilize the Nav1.5 Inactivation State

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ABSTRACT

Background: Na\(_{1.5}\), which is encoded by the SCN5A gene, is the predominant voltage-gated Na\(^+\) channel in the heart. Several mutations of this gene have been identified and reported to be involved in several cardiac rhythm disorders, including type 3 long QT interval syndrome, that can cause sudden cardiac death. We analyzed the biophysical properties of 2 novel variants of the Na\(_{1.5}\) channel (Q1491H and G1481V) detected in 5- and 12-week-old infants diagnosed with a prolonged QT interval.

Voltage-gated Na\(^+\) channels are crucial for the amplitude and upstroke of cardiac action potentials (APs), which are important determinants for driving AP propagation and conduction velocity throughout the working myocardium.\(^4\) Mutations in SCN5A, the gene that encodes Na\(_{1.5}\), the predominant cardiac Na\(^+\) channel, have been implicated in rare familial forms of cardiac arrhythmias, such as type 3 long QT interval syndrome (LQT3), Brugada syndrome, progressive cardiac conduction disorder (PCCD), atrial fibrillation, and sudden infant death syndrome (SIDS). Another SCN5A mutation has been reported recently to be involved in dilated cardiomyopathy, a structural heart disease.\(^5\) In addition to their role in changing gating characteristics, there is growing recognition that such mutations may also be associated with alterations in channel protein trafficking and expression levels. The SCN5A gene is also the only gene for which there is definitive evidence for clinical validity of Brugada syndrome.\(^4\)

Long QT (interval) syndrome (LQTS) is an inherited cardiac channelopathy that may lead to syncope and even sudden cardiac death as a result of polymorphic ventricular tachycardia known as torsade de pointes. LQTS manifests as a
Methods: The Na\(_{1.5}\) wild-type and the Q1491H and G1481V mutant channels were reproduced in vitro. Wild-type or mutant channels were cotransfected in human embryonic kidney (HEK) 293 cells with the beta 1 regulatory subunit. Na\(^+\) currents were recorded using the whole-cell configuration of the patch-clamp technique.

Results: The Q1491H mutant channel exhibited a lower current density, a persistent Na\(^+\) current, an enhanced window current due to a +20-mV shift of steady-state inactivation, a +10-mV shift of steady-state activation, a faster onset of slow inactivation, and a recovery from fast inactivation with fast and slow time constants of recovery. The G1481V mutant channel exhibited an increase in current density and a +7-mV shift of steady-state inactivation. The observed defects are characteristic of gain-of-function mutations typical of type 3 long QT interval syndrome.

Conclusions: The 5- and 12-week-old infants displayed prolonged QT intervals. Our analyses of the Q1491H and G1481V mutations correlated with the clinical diagnosis. The observed biophysical dysfunctions associated with both mutations were most likely responsible for the sudden deaths of the 2 infants.

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We report here on 2 infants who presented with a prolonged QTc interval and who died suddenly, most likely after experiencing ventricular fibrillation. Although the 5- and 12-week-old infants were within the age range during which the incidence of SIDS peaks, their deaths were not attributed to SIDS. A sequencing analysis revealed a heterozygous G-to-T base substitution at position 4473 in exon 23 that resulted in a glycine (G)-to-valine (V) substitution (G1481V). The channel mutant G1481V exhibits an increase in the window current. All these characteristics point toward a gain-of-function due to the mutations.

Methods

Institutional Committee on Human Research

The study was conducted in accordance with the principles of the Declaration of Helsinki, and the protocol was approved by the local ethics committee. Parents provided written informed consent.

Cell cultures

Human embryonic kidney 293 (HEK 293) cells were used. The cells were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% streptomycin at 37°C in a 5% CO\(_2\) atmosphere. The human Na\(^+\) channel β1-subunit and enhanced Green Fluorescence Protein (eGFP) were inserted in the pIRES bicistronic vector in the form of β1-pIRES-eGFP. The cells were transfected with the plasmid cloning DNA3.1 vector containing either WT Na\(_{1.5}\) complementary DNA (1 μg) or...
Nav1.5/Q1491H and G1481V mutants with the pIRES2/EGFP vector containing β1 subunit complementary DNA (1 μg) in 10-cm cell culture dishes using the calcium phosphate method as previously reported. Whole-cell patch-clamp recordings

Na⁺ currents were recorded using low-resistance, fire-polished electrodes (≈1 MΩ) made from 8161 Corning borosilicate glass coated with HIPEC (Dow-Corning, Midland, MI) to minimize electrode capacitance. An Axopatch 200 amplifier and pClamp software (Molecular Devices, Sunnyvale, CA) were used to record Na⁺ currents. The series resistance was compensated to 80% to minimize voltage-clamp errors. The cells were allowed to stabilize for 5 minutes after the whole-cell configuration was established. The membrane potential was held at −140 mV before the currents were recorded. Sodium currents were filtered at 5 kHz and digitized at 83.33 kHz. The liquid junction potential was not corrected. All the experiments were performed at room temperature (22 °C).

Solutions

The intracellular solution was composed of 35 mM NaCl, 105 mM cesium fluoride, 10 mM ethylene glycol-bis(β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 10 mM 4-

Figure 1. (A) A standard electrocardiogram was recorded soon after birth. Paper speed was 25 mm/s; 10 mm/1 mV. The QT interval was 720 ms; the QRS was 60 ms; and 2:1 atrioventricular block developed due to an extremely prolonged QT interval. (B) Sequence analysis of the G1481V and Q1491H mutations. (C) The Q1491H mutation resulted from a G-to-T substitution at position 4473, leading to a glutamine (Q)-to-histidine (H) substitution at residue 1491. The G1481V mutation resulted from a G-to-T substitution at position 4442, leading to a glycine (G)-to-valine (V) substitution at residue 1481. (D) Schematic representation of the 4 domains of the α-subunit of the Nav1.5 channel showing the locations of the Q1491 and G1481V mutations. DEKA represents the selectivity filter of the channel. The Q1491H mutation caused a glutamine-to-histidine substitution 4 amino acids downstream from the isoleucine, phenylalanine, and methionine motif. The G1481V mutation caused a glycine-to-valine substitution 4 amino acids upstream from the isoleucine, phenylalanine, and methionine motif.
The pH was adjusted to 7.3 with 2 M CsOH. The first external solution (low Na\(^+\)) was composed of 35 mM NaCl, 115 N-methyl-d-glucamine, 2 mM KCl, 1.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 2 M HCl. Tetrodotoxin (TTX; LATOXAN, Portes-lès-Valence, France) was diluted to 75 μM in 2% acetic acid.

### Data analysis

The slope factor (K) and the midpoint (V\(_{1/2}\)) for activation and inactivation were calculated using standard Boltzmann functions: 1/[1 + exp ((V - V\(_{1/2\text{activation}}\))/K\(_{\text{activation}}\))] for activation and (1 - C)/(1 + exp ((V - V\(_{1/2\text{inactivation}}\))/K\(_{\text{inactivation}}\)))/C for inactivation. V is the voltage. The window current was obtained using equation 1: 1/[1 + exp ((V - V\(_{1/2\text{activation}}\))/K\(_{\text{activation}}\))] x ((1 - C)/(1 + exp ((V - V\(_{1/2\text{inactivation}}\))/K\(_{\text{inactivation}}\)))/C x 100, which is the probability of having the channel in the open state.

### Statistical analysis

Results are expressed as mean ± standard error of the mean. Statistical comparisons were performed using a 1-way analysis of variance in GraphPad Prism (La Jolla, CA) for statistical comparisons. Differences were considered significant at \(P < 0.05\).

### Results

**Available clinical data and identification of the SCN5A mutations**

Patient #1 was an infant with a QTc of 700 ms and a 2:1 atrioventricular block (AVB) at birth (Fig. 1A). The 2:1 AVB was detected in utero at a gestational age of 31 weeks of amenorrhea. This infant received 2 mg/kg of propranolol, and a pacemaker was implanted a few days after birth. He experienced salvos of torsades de pointes. Flecainide and mexiletine were unsuccessful in preventing torsades. The patient died at 3 months of age after experiencing ventricular fibrillation.

Patient #2 had a QTc of 600 ms and a 2:1 AVB block at birth. The 2:1 AVB was detected in utero at a gestational age of 25 WA. A pacemaker was implanted a few days after birth, and 2 mg/kg of propranolol was started. The infant experienced several episodes of torsades de pointes and died at 1 month of age after experiencing ventricular fibrillation.

The genomic DNAs of the 2 infants were screened using specific primers for variant changes in several genes associated with arrhythmia syndrome (see Supplemental Table S1), in particular in all 28 SCN5A exons. Molecular screening by automatic sequencing analysis of the genomic DNAs revealed 2 novel mutations in the SCN5A gene (G1481V and Q1491H), namely, c. 4473 G > T, which resulted in a Glu1491His substitution, and c. 4442 G > T, which resulted in a Gly1481Val substitution (Fig. 1B). No mutations were detected in other genes. Applying the American College of Medical Genetics and Genomics guidelines revealed unclear initial results. This is illustrated and confirmed by Varsome (https://varsome.com) and CardioClassifier (https://www.cardioclassifier.org), which respectively returned both variations as being likely pathogenic (class 4) or of uncertain significance (class 3). This initial variant classification consequently precluded their use in future presymptomatic diagnosis. This warranted further detailed biophysical characterization (see below). The 2 substitutions are highly conserved in the Na\(^+\) channels of many species and are located in the III-IV linker, which plays a role in channel inactivation in all voltage-gated Na\(^+\) channels. It was hypothesized that these 2 mutations were responsible for the
sudden death events. Family screening documented normal phenotypes in both parents of the 2 infants.

Biophysical characteristics of Na\(_{\text{v}1.5}\)/Q1491H and Na\(_{\text{v}1.5}\)/G1481V

Currents were elicited by sequential depolarizing steps of the cell membrane from \(-100\) mV to \(+30\) mV in 5-mV increments (Fig. 2A), with the low Na\(^+\) external solution (see Methods). The I–V curves were constructed by measuring the peak of each Na\(^+\) current and were normalized to the membrane capacitance to obtain current densities (pA/pF). The current density of the Q1491H mutant channel was lower than that of the WT channel, whereas the current density of the G1481V mutant channel was higher than that of the WT channel. These results suggest that the expression levels of the Q1491H and G1481V mutant channels were different than that of the WT channel. The potential of the maximum peak current amplitude of the Q1491H channel was shifted toward more positive potentials compared to the WT channel (Fig. 2B). To study the activation and inactivation parameters, we first calculated the G–V curves (steady-state activation), which were fitted with a standard Boltzmann function. The \(V_{1/2}\) of the Q1491H mutant channel was positively shifted by 9 mV, with significant differences in \(k\) values. There was no difference between the \(V_{1/2}\) values of the WT channel and the G1481V mutant channel (Fig. 3A; Table 1).

Voltage-dependent steady-state inactivation was assessed by applying 500-ms pre-pulses ranging from \(-140\) mV to \(-30\) mV to allow the channels to enter the inactivated state, followed by a test pulse at \(-30\) mV to assess the number of functional channels. The current amplitude of the test pulse was normalized to the maximum current recorded during the pre-pulse and was plotted vs the voltage of the pre-pulse to obtain the voltage-dependent inactivation curve, which was then fitted to a standard Boltzmann function. The \(V_{1/2}\) and \(k\) were generated by fitting each data set with a standard Boltzmann function (see values in Table 1). A \(+20\)-mV shift for Q1491H and a \(+7\)-mV shift for G1481V were observed, but \(k\) was not significantly affected (Fig. 3A).

Window currents were determined and were used to assess the open probability. The total areas were 1.4- and 11-fold larger, respectively, for G1481V and Q1491H than for the WT channel and were shifted to more-positive voltages (Fig. 3B).

Slow inactivation was measured using a 2-pulse protocol, with an initial conditioning pulse (pre-pulse) and a final test pulse. The current was normalized to the current amplitude during the pre-pulse. There was no difference between the G1481V and WT channels in terms of the kinetics of entry into slow inactivation. However, the Q1491H mutant channel entered into slow activation faster than the WT channel (Fig. 4A). Recovery from slow inactivation was measured using a 2-pulse protocol. The channels were first inactivated by a 500-ms pre-pulse and were then allowed to recover from inactivation. The peak currents were normalized to the maximum peak current. The Q1491H mutant exhibited a complex recovery with 2 (fast and slow) time constants (Table 1). The G1481V mutant also exhibited a 2-time constant recovery compared to the WT channel (Fig. 4B). The currents of the mutant channels (Q1491H and G1481V) did not decrease during closed-state inactivation. As the WT channel exhibited only a \(\tau_{\text{fast}}\) time constant, \(\tau_{\text{slow}}\) is not given in Table 1. Closed-state inactivation was assessed using a 2-pulse protocol. The currents were normalized to the first pulse. Neither mutated channel was affected, unlike the WT channel, which showed closed-state inactivation (Fig. 4C).
Steady-state activation
Closed state of activation
Recovery from slow inactivation
Peak current density (pA/pF)
and for their use in presymptomatic diagnosis.

We recorded the frequency dependence of the Na⁺ currents in order to determine the channels that enter the inactivated state by applying a series of 50 depolarizing pulses at −40 mV. To evaluate current inhibition during rapid pulsing, the channels were pulsed 50 times at 2 Hz, 5 Hz, and 10 Hz. There was a significant difference between the WT channel and the Q1491H mutant channel at 10 Hz, with the mutant channel displaying a reduced current during rapid pulsing. There was no difference between the G1481V mutant channel and the WT channel (Fig. 5).

Persistent Na⁺ currents in cells transfected with the Q1491H mutant channel

Persistent non-inactivating Na⁺ currents were recorded using a 400-ms depolarization pulse from −140 mV to −30 mV (see protocol inset) and were normalized to the peak. The current was inhibited using 25 μM tetrodotoxin (TTX). Only the Q1491H mutant channel displayed a significant decrease in current (2.66 ± 0.27 mV) and activation (+9.36 mV) that drove channel activity to more-positive potentials. We also showed that the Q1491H mutant channel displays a persistent current that is inhibited by 25 μM TTX (Fig. 6) and ranolazine (Fig. 7), which is characteristic of LQT3. This mutation also displayed pronounced frequency-dependent inactivation at 10 Hz, suggesting that the Na⁺ current in the Q1491H channel has an unstable inactivated state. These results provide a rationale for linking this mutation and the clinical phenotype, especially for the long QTc interval (700 ms), measured in the infant prior to his sudden death. Clearly, Q1491H resulted in several biophysical defects, which may contribute to the deleterious effect of the mutation. Obviously, some of the parameters are loss-of-function effects, but what is uniquely determinant is the persistent current.

The Q1491H mutation was located 4 amino acids downstream from the IFM motif. Glutamine has a polar uncharged side chain, whereas histidine has electrically charged side chains. A biophysical characterization revealed that this mutation has a marked effect on the normal function of the NaV1.5 channel. We observed a loss-of-function for peak current density that could be due to a consequence of a modulatory effect. We also observed a loss-of-function for recovery time (a slow time constant: 56.0 ± 8.73%; Table 1), which led to a decreased inward Na⁺ current and a longer recovery from inactivation time. An increase in the window current may lead to an increase in Na⁺ influx, which can increase the risk of fatal ventricular arrhythmias. This increase was correlated with the depolarized shift of steady-state inactivation (+20.27 mV) and activation (+9.36 mV) that drove channel activity to more-positive potentials. We also showed that the Q1491H mutant channel displays a persistent current that is inhibited by 25 μM TTX (Fig. 6) and ranolazine (Fig. 7), which is characteristic of LQT3. This mutation also displayed pronounced frequency-dependent inactivation at 10 Hz, suggesting that the Na⁺ current in the Q1491H channel has an unstable inactivated state. These results provide a rationale for linking this mutation and the clinical phenotype, especially for the long QTc interval (700 ms), measured in the infant prior to his sudden death. Clearly, Q1491H resulted in several biophysical defects, which may contribute to the deleterious effect of the mutation. Obviously, some of the parameters are loss-of-function effects, but what is uniquely determinant is the persistent current.

The G1481V mutation was located 4 amino acids upstream from the IFM motif. Given that valine and glycine are both uncharged, this may explain the more modest effect on the function of the Nav1.5/G1481V channel. The G1481V mutation resulted in an increase in the current density (Fig. 2) as well as a +7.03-mV depolarized shift in inactivation, which increased by 1.4-fold the window current (Fig. 3). Furthermore, the recovery from the inactivation was also faster, indicating that the sodium channel will recover faster from

| Table 1. Biophysical properties of the NaV1.5 WT and mutant channels |
|-------------------------|-------------------------|-------------------------|
|                        | NaV1.5/WT                | NaV1.5/Q1491H            | NaV1.5/G1481V            |
| Peak current density   | −235.8 ± 39.8 (n = 9)    | −49.2 ± 7.3 (n = 9)      | −391.2 ± 48.3 (n = 9)    |
| Steady-state inactivation | −89.6 ± 1.6 (n = 9)    | −69.4 ± 1.5* (n = 7)     | −82.6 ± 2.3* (n = 7)     |
| V1/2 (mV)              | 5.1 ± 0.1 (n = 9)        | −4.5 ± 0.3 (n = 7)       | −4.6 ± 0.2 (n = 7)       |
| K (mV)                 | −41.4 ± 2.5 (n = 9)      | −32.0 ± 1.5* (n = 9)     | −42.2 ± 1.2 (n = 9)      |
| Recovery from slow inactivation | 7.6 ± 1.0 (n = 9)    | 14.2 ± 1.3* (n = 9)      | 8.0 ± 0.6 (n = 9)        |
| Current plateau        | 0.9 ± 0.1 (n = 6)        | 0.7 ± 0.1* (n = 6)       | 0.8 ± 0.1 (n = 5)        |
| Closed state of activation | 0.8 ± 0.1 (n = 8)    | 1.3 ± 0.2* (n = 10)      | 1.0 ± 0.1* (n = 7)       |

A, fraction of recovery component; K, slope factor for activation or inactivation; t, time constant; V1/2, midpoint for activation or inactivation.

* P < 0.05.
† P < 0.01.
‡ P < 0.001.
§ P < 0.0001.

Discussion

Q1491H and G1481V are SCN5A heterozygous mutations identified in 2 infants who died from long QT (Q1491H, 700 ms AVB 2:1; G1481V, 600 ms AVB 2:1) at 5 (G1481V) and 12 (Q1491H) weeks of age, respectively. The mutations were located in the NaV1.5 intracellular loop between domains III and IV near the isoleucine, phenylalanine, and methionine (IFM) motif (Fig. 1), which is involved in fast inactivation.16,17 Such mutations in the inactivation gate are often associated with changes in inactivation of voltage-gated Na⁺ channels. Both mutations were identified in clinical practice. The SCN5A gene is known as a clinically actionable gene (https://search.clinicalgenome.org). The variants’ pathogenic status is thus critical to inform the family appropriately and for their use in presymptomatic diagnosis.
Figure 4. The gating properties of slow inactivation, recovery from slow inactivation, and closed-state inactivation. (A) Slow inactivation in wild type (●, n = 6), Q1491H (■, n = 6), and G1481V (▲, n = 6). The 2-pulse protocol described in the inset was used to generate the currents. (B) Time courses of recovery from slow inactivation in WT (●, n = 7), Q1491H (■, n = 8), and G1481V (▲, n = 7). (C) Closed-state inactivation in WT (●, n = 8), Q1491H (■, n = 10), and G1481V (▲, n = 7).
Figure 5. Frequency dependence of wild type (*, n = 8), Q1491H (■, n = 8), and G1481V (▲, n = 8). Currents were evaluated at (A) 2 Hz, (B) 5 Hz, and (C) 10 Hz. Fifty pulses were applied at −40 mV from a holding potential of −140 mV. Peak currents were normalized to the first peak current and were plotted vs the pulse number.
inactivation. All these changes represent a gain of function of G1481V mutant sodium channels.

The biophysical characterization of both Q1491H and G1481V SCN5A mutations thus revealed a functional defect. The manual patch-clamp is not validated as a well-established criterion in the American College of Medical Genetics and Genomics classification process.\textsuperscript{18,19} Interestingly, both variants were reclassified as either likely pathogenic (class 4) or pathogenic (class 5), indicating that with the support of the biophysical characterization, both SCN5A variants can now be used for presymptomatic diagnosis.

Interestingly, although the patient with the G1481V mutation presented a long QTc interval of 600 ms, the increase in the persistent current was not statistically significant. In addition, there was no significant difference in the time constants of inactivation for all conditions (data not shown).

The III–IV linker acts as a lid in which the inactivation particle IFM amino acid trio occludes the inner pore by binding to the docking site like a latch. We used the recently crystalized rat Nav1.5 to visualize the position of the 2 residues and their interacting sites.\textsuperscript{15} The IFM motif is highly conserved in all Na\textsuperscript{+} channels. The sites of the Q and G mutations investigated in the present study were located upstream and downstream, respectively, from the IFM. They are highly conserved in several Na\textsuperscript{+} channels, underlining their importance during the process of inactivation. The structure revealed that Q1493 residue interacts with N1661 (N1659 in hNav1.5) and is one of the key residues for optimum IFM docking. It has been shown previously that mutating N1661 to alanine abolishes fast inactivation.\textsuperscript{20} The recently published rat Nav1.5 structure showed that Q1493 at the C-terminal of the DIII–DIV linker (Q1491 in hNav1.5) forms a strong hydrogen bond with N1661 (Fig. 8). Q1491H mutation may lead to a weaker interaction with N1659 because of a histidine shorter imidazole side chain. This weakened interaction will impair fast inactivation and lead to a gain-of-function effect. This may explain the appearance of persistent current and the rightward shift of the steady-state inactivation curve of the Q1491H mutant. This could impede this interaction and contribute to change in pore closure and the slowing of slow inactivation. On the other hand, G1481, which is located at the tight turn of DIII S6 and the beginning DIII–DIV linker, is important for keeping the flexibility of movement of the IFM motif. A valine substitution, with its bulky side chain, at this position will lead to less flexibility of move-ment of the IFM.\textsuperscript{21} This structural insight explains the mild slow inactivation kinetics of the G1481V mutant compared to the wild-type channel. However, the effects of this mutation are expected to be less drastic compared to those of the Q1491H mutation, just as the effects of G1481V mutation on channel function are limited.

Many SCN5A mutations, especially in the III–IV linker and the voltage sensor domain, have been linked to BrS and LQT3. The difference between BrS and LQT3 is that BrS appears with age.\textsuperscript{21} The first mutation to be associated with LQT3 was a 3-amino-acid deletion (Lys1505-Pro1506-Gln1507, ΔKPQ). This mutation shares the same deletion (Q1507) as the delQKP deletion at position 1507-1509. Both are located in the III–IV linker region and are associated with a gain-of-function and a late persistent Na\textsuperscript{+} current.\textsuperscript{22,23} This region is a hotspot of mutations that induce inactivation disturbances, including a depolarized shift in inactivation that enhances the window current, as is the case for Q1491H and G1481V. This same effect was observed with F1486del, which involves a deletion of a phenylalanine on the IFM motif.\textsuperscript{24} This mutation alters lidocaine sensitivity, which is a local anesthetic that blocks late currents associated with LQT3 mutations.
Although IFM/QQQ (Q for Glutamine) mutants also display an alteration in lidocaine sensitivity,\textsuperscript{25} this effect is not seen if the phenylalanine (F) is replaced by a glutamine (Q).\textsuperscript{26} Other mutations have been shown to induce a long QTc interval, including N1774H, which is located in the C-terminal.\textsuperscript{27} Like the Q1491H mutations, N1774H results in a loss of function in peak current density and an increase in the late current. The N1774H mutation was detected in a 19-year-old woman. The S1333Y mutation on the S4-5 linker results in an enhanced window current and a persistent current. The S1333Y mutation was detected in a 25-day-old infant.\textsuperscript{28} Although all these mutations lead to LQT3, they are located in different parts of the Nav1.5 channel that all play a role in inactivation. The ineffectiveness of mexiletine and flecainide in case 1, and propranolol in case 2, is not very clear, especially given that these drugs all have some sodium channel-blocking properties. The possibility of interindividual pharmacokinetic and pharmacodynamic differences could explain the nonuniform clinical response to these drugs.

**Conclusion**

The Q1491H and G1481V mutations are both located near the IFM motif, which is involved in fast inactivation.

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Supplementary Material
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