Gain-of-Function Mutation in STIM1 (P.R304W) Is Associated with Stormorken Syndrome

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ABSTRACT: Stormorken syndrome is a rare autosomal dominant disorder characterized by a phenotype that includes miosis, thrombocytopenia/thrombocytopenia with bleeding time diathesis, intellectual disability, mild hypocalcemia, muscle fatigue, asplenia, and ichthyosis. Using targeted sequencing and whole-exome sequencing, we identified the c.910C > T transition in a STIM1 allele (p.R304W) only in patients and not in their unaffected family members. STIM1 encodes stromal interaction molecule 1 protein (STIM1), which is a finely tuned endoplasmic reticulum Ca²⁺ sensor. The effect of the mutation on the structure of STIM1 was investigated by molecular modeling, and its effect on function was explored by calcium imaging experiments. Results obtained from calcium imaging experiments using transfected cells together with fibroblasts from one patient are in agreement with impairment of calcium homeostasis. We show that the STIM1 p.R304W variant may affect the conformation of the inhibitory helix and unlock the inhibitory state of STIM1. The p.R304W mutation causes a gain of function effect associated with an increase in both resting Ca²⁺ levels and store-operated calcium entry. Our study provides evidence that Stormorken syndrome may result from a single-gene defect, which is consistent with Mendelian-dominant inheritance.

Introduction

Stormorken syndrome is a rare genetic disease (MIM# 185070) first reported in 1985 [Stormorken et al., 1985] with further details of the syndrome described subsequently [Sjaastad et al., 1992; Sjaastad, 1994; Stormorken, 2002]. Patients of Norwegian origin presented with moderate thrombocytopenia, thrombocytopenia, muscle fatigue, asplenia, miosis, migraine, dyslexia, and ichthyosis [Stormorken et al., 1985; Stormorken, 2002]. To date, only nine patients in seven different families have been reported [Misceo et al., 2014; Nesin et al., 2014], and the inheritance pattern is recognized as autosomal dominant. The moderate bleeding tendency has been referred to as “The inverse Scott syndrome” [Stormorken et al., 1995] because paradoxical to the phenotype, platelets from the patients were constitutively activated [Stormorken et al., 1995; Salles et al., 2008]. Moderate hypocalcemia has been identified in some of the patients [Stormorken, 2002] and probably responsible for the muscular fatigue reported in these patients since the symptoms were significantly improved upon administering calcitriol [Stormorken, 2002]. The muscle fatigue was also related to a tubular aggregate myopathy (TAM) as reported by Mizobuchi et al. (2000), in a Japanese family, where the mother and her daughter presented with a severe miosis and TAM.

Recently, stromal interaction molecule 1 (STIM1) (HGNC# 11386, MIM# 605921) has been identified as the genetic cause of dominantly inherited TAM [Bohm et al., 2013]. Furthermore, STIM1 has been described as a dynamic cellular calcium signal transducer [Soloboff et al., 2012; Fahrner et al., 2013] and has been...
found to be associated with thrombocytopenia and muscular hypotonia in a syndrome of immunodeficiency and autoimmunity [Picard et al., 2009], which suggested that STIM1 could be a candidate gene for Stormorken syndrome. Overlapping symptoms presented by patients with Stormorken syndrome and other disorders are still a challenge in clinical diagnosis.

Here, we describe two unrelated families with the syndrome. Family 1 is of French origin in which the father and a son are affected, and Family 2 is of German origin in which the mother and daughter are affected. Evidence for Stormorken syndrome in Family 1 included miosis, mild hypocalcemia, and thrombocytopenia in both the father and son, further supported by the presence of tubular aggregates in skeletal muscle fibers in a muscle biopsy from the father. This observation prompted us to sequence STIM1 in Family 1. In Family 2, following the clinical diagnosis of Stormorken syndrome whole-exome sequencing in the index case was undertaken as the molecular approach. Both the approaches led to the same result and allowed us to identify a STIM1 mutated allele associated with the disease in both families. The consequence of the mutation on the protein was further explored by structural modelling and by calcium homeostasis experiments. Based on our results, we suggest that the p.R304W mutation impairs the function of STIM1 and may be the cause for Stormorken syndrome in the two families.

Materials and Methods

Patients and Subjects

The study complied with the ethical principles set forth by the Declaration of Helsinki. Written informed consent was obtained from all family members to conduct the clinical and biological investigations and also to publish their photographs. Parents gave informed consent for children. The clinical investigations and experiments were conducted after obtaining approval from both the institutional review boards of the respective hospitals.

DNA Studies and Sequencing

Peripheral blood, buccal epithelial cells, and fibroblasts were obtained for isolation of genomic DNA from the probands and family members. Whenever kits were used, manufacturer’s instructions were followed unless otherwise stated. Genomic DNA was extracted from blood cells and fibroblasts using the DNeasy kit (QIAGEN GmbH, Hilden, Germany). DNA samples were calibrated to 50 ng μl⁻¹ by UV microvolume spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE) for further studies. Oligonucleotides and primers used for STIM1 sequencing and mutagenesis are listed in Supp. Table S1. PCR products were purified (ExoSAP-IT; VWR International SAS, Fontenay-sous-Bois, France) and sequenced on both strands using BigDye Terminator Cycle Sequencing V1.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequence alignments were performed using the T-Coffee software (http://tcoffee.vital-it.ch/apps/tcoffee/do/regular). Following DNA sequence analysis, genomic DNA (blood cells and epithelial cells) of all family members were screened for the mutation using Fas1 (New England Biolabs Inc. Ipswich, MA) restriction analysis of STIM1 exon 7 specific PCR product. Genomic DNA obtained from 100 white healthy controls (representing 200 chromosomes) was also analyzed for the presence of the STIM1 exon 7 mutation by Fas1 restriction analysis. The mutation numbering system used here is based on the cDNA sequence.

Whole-Exome Sequencing

Whole-exome sequencing was performed on DNA samples of the index case (mother) and child of Family 2. After enrichment with the SeqCap EZ Human Exome Library v3.0 (Roche NimbleGen; Madison, WI), the library was sequenced externally on an Illumina HiSeq 2000 Sequencer (Illumina, San Diego, CA). A coverage of 94% and 93%, respectively, >10× (mean coverage of 102× and 57×) for the two exomes was achieved. Sanger sequencing was performed as described above to study STIM1 in family members from Family 2.

Karyotyping and Array-CGH

Karyotyping was performed at a 400–550 GTG band level (ISCN 2009) on peripheral blood lymphocytes of all the patients using standard procedures. Genomic DNA used to perform array CGH (Comparative Genomic Hybridization) analysis was extracted from whole blood using the GenElute™ Blood Genomic DNA kit (Sigma–Aldrich, St. Louis, MO). Genome scan was performed using the Human Genome CGH Microarray Kit 244 K (Agilent Technologies, Santa Clara, CA), which consists of ~236,000 60-mer oligonucleotide probes covering the entire genome at an average spatial resolution of ~30 kb. Three micrograms of DNA from the test and normal individuals from both families underwent CGH analysis. Reference samples were processed following the manufacturer’s protocol. Dye emission was captured by means of a dual-laser scanner. The images were extracted using the Agilent Feature Extraction 9.1 software and analyzed by the DNA Analytics 4.0 software.

Muscle Biopsies

Muscle biopsies were obtained from the child and the father of Family 1 and samples processed within 10 min of excision. They were divided into three parts. The central part of the biopsy was embedded in TissueTek and snap frozen in liquid nitrogen cooled isopentane. A part of the biopsy was fixed in 4% buffered formalin and embedded in paraffin. Skin biopsy was obtained from the father of Family 1 and the fibroblasts were prepared as recommended by the protocol from EuroBiobank (INNCP, Italy). Normal human fibroblasts used in this study were purchased from Eurobiobank.

Histological Studies on Muscle Biopsy

Four micrometer sections were prepared from the paraffin block and stained with hematoxylin–phloxine–saffron. Six micrometer sections were prepared from frozen tissue and stained with hematoxylin-phloxine-saffron, PAS, and Gomori trichrome. The following enzymatic activities were explored: nicotinamide adenine dinucleotide-tetrazolium reductase, succinate dehydrogenase (SDH), Cox, phosphorylase, and ATPases at pH 3.4, 4.6, and 9.4.

Immunohistochemical Studies on Muscle Biopsy

Four micrometer sections of the paraffin-embedded tissue were mounted on SuperFrost® Plus slides, dried, dewaxed, rehydrated, and treated in a Benchmark Ultra instrument (Roche diagnostics, Meylan, France). CCI pretreated, incubated with primary antibody for 32 min. The primary antibodies used were Orai1 (rabbit polyclonal, dil. 1:100, GeneTex, Irvine, CA), Orai2 (rabbit polyclonal, dil. 1:100, GeneTex), STIM1 (rabbit monoclonal, clone EPR3414, dil. 1:100, GeneTex), and STIM2 (rabbit polyclonal, dil. 1:100, GeneTex). Six micrometer sections of frozen tissue were mounted on SuperFrost® Plus, dried for 20 min at room temperature, immersed
in acetone for 5 min, rinsed in PBS (Phosphate buffered saline), immersed in 3% hydrogen peroxide for 5 min, rinsed in PBS, and put in the Benchmark Ultra instrument without an antigen retrieval protocol. The primary antibodies used were the same as used on the paraffin-embedded tissues and incubated for 20 min.

**Site-Directed Mutagenesis and Transfection**

Mammalian expression vector pEYFP-N1 containing the STIM1 open reading frame was obtained as a gift from Murali Prakriya (Northwestern University, Chicago, IL) and designated as pEYFP-STIM1WT. The p.R304W mutation in STIM1 was created by site-directed mutagenesis using a Quick-change site directed mutagenesis kit (Agilent, Santa Clara, CA) using the primers STIM1R304WF as the forward primer and STIM1R304WR as the reverse primer (see Supp. Table S1 for sequence). The obtained plasmid was sequenced using the primer STIM1R304WSeq (Supp. Table S1) to confirm the presence of the mutation. The confirmed plasmid was designated as pEYFP-STIM1R304W. These vectors were transfected to human embryonic kidney cell line (HEK 293T) using a Fugene HD transfection reagent according to supplier’s instructions (Promega, Madison, WI) and maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS (Foetal calf serum), penicillin, and streptomycin.

**Preparation of Cell Lysate and Western Blotting**

HEK 293T cells expressing STIM1WT and STIM1R304W were obtained by trypsination and washed thoroughly with ice-cold PBS to remove the serum. The cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40 [v/v], 50 mM Tris–HCl, pH 8.0, 0.15 M NaCl) containing protease inhibitor cocktail and the lysate was clarified by high speed centrifugation. Protein content of the lysate was measured by the Bradford dye binding method, and 50 μg of total protein was resolved in a 12% SDS-PAGE. The resolved proteins were transferred to the membrane by electro blotting and expression of STIM1 was detected using Anti-STIM1 antibodies (rabbit monoclonal, dil. 1:1000, Cell signalling technology, Danvers, MA) and β-actin (goat polyclonal, dil. 1:500, Santa Cruz Biotechnology, Dallas, TX) used as a loading control.

**In Silico Prediction Tools and Molecular Modeling Studies**

In silico prediction of the consequence of the p.R304W mutation was performed using Polyphen-2 tool [Adzhubei et al., 2010] and Alamut (Interactive Biosoftware, Rouen, France). A comparison between the secondary structure of the variant versus the wild type was performed by using NETSurfP version 1.1 (http://www.cbs.dtu.dk/services/NetSurfP) [Petersen et al., 2009] and DisEMBL1™ (http://dis.embl.de). The coordinates file (PDB code: 4O9B) of the CC1-IH domain crystal structure [Cui et al., 2013] was used to study the molecular consequences of the mutation (http://spdbv.vital-it.ch). The mutation was introduced by the program COOT [Emsley and Cowtan, 2004]. The figure was drawn by the program PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC San Diego, CA). Multiple sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

**Ca²⁺ Homeostasis Studies**

Primary human fibroblasts and HEK 293T cell line were used to investigate the impact of the mutation on Ca²⁺ homeostasis. The cytosolic calcium concentration was measured using FURA-2-loaded cells. Cells plated onto cover slips were loaded for 30 min at 37°C with 3 μM of Fura-2-AM prepared in culture medium. Before recording, cells were washed twice with the extracellular dye free solution (NaCl 140 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 2 mM, d-glucose 5 mM, and HEPES 10 mM, pH 7.4). The glass coverslip was mounted in a chamber on a Zeiss microscope equipped for fluorescence. Ca²⁺ was measured as the ratio of the emitted fluorescence at 510 nm using excitation at 350 nm and 380 nm as described previously [Rybarczyk et al., 2012; Guilbert et al., 2013]. Metafluor software (version 7.1.7.0) was used for acquisition and analysis. All recordings were made at room temperature. The cells were continuously perfused with a saline solution and chemicals were added as indicated. The flow rate of the whole cell chamber perfusion system was set to 1 ml/min with the chamber volume of 500 μl.

**Statistical Analysis**

Data are presented as mean ± SEM and n refers to the number of cells or to the number of experiments. Data were compared using a t-test. Differences were considered as significant when \( P < 0.05 \) (\( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \)). The statistical analysis was performed using SigmaStat3.01A software (Systat Software, Inc., Chicago, IL).

**Results**

**Clinical Data of Family 1**

**Patient II-2 (son)**

A French male newborn (index case, patient II-2) was referred to the paediatrics department at 17 days of life for skin rash and thrombocytopenia (platelets: 48 × 10⁹/l; N = 140–410 × 10⁹/l). Inflammatory and bacteriological investigations were negative. On clinical examination, he had an urticaria-like eruption, mildly deep set eyes, retracted eyelids, and congenital miosis (1.5 mm). Abdominal ultrasound examination was normal, showing the presence of the spleen. The skin rash did not respond to antihistamines and corticosteroids, but progressively resolved in 6 weeks. During the first six years of life, the miosis became more prominent. The stature and the weight progressively declined to the bottom first percentile. Symptoms of muscular fatigue appeared, and the patient also had moderate learning difficulties.

Laboratory investigations showed a high creatine kinase (CK) level (1068 U/l; N = 28–300 U/l 0–90 days) and mild hypocalcemia (2.03 mmol/l; N = 2.1–2.7 mmol/l) with ionized calcium below the lower limit of the normal range (1.0 mmol/l; N = 1.10–1.30 mmol/l). Bleeding time was prolonged (Ivy method 15 min: N = 1–9 min). His platelets count varied from 28 × 10⁹ to 178 × 10⁹/l, suggesting an underlying thrombocytopenia. CK levels were consistently increased (371–1068 U/l). The numbers of CD⁴ and CD⁸, T cells, and B cells were normal.

**Patient I-3 (father)**

The father, a 32-year-old man, had a short stature (157 cm/0.5 percentile), and a chronic ichthyosiform skin eruption localized on his arms and shoulders but no relevant history of unusual infections. Ocular examination revealed a relative enophthalmos with hypertelorism, severe miosis (0.5 mm) without ocular malformation, and bilateral limitation of eye elevation. The miosis observed
Figure 1. Ophthalmological and facial features of the patients participated in this study. Family 1: (A) Father at the age of 26 years showing his full face and (B) close up photo of his eyes showing deep-set eyes and miosis. Index case at 3 weeks of age (C) presenting mildly deep-set eyes, retracted eyelids, and a moderate constriction of the pupil. At the age of 6 years (D), he presented with deep-set eyes and a miosis (E). Family 2: Index case at the age of 19 years showing full face (F). The patient presented with miosis, deep-set eyes, triangular face, and narrow forehead (G). Child at 2 weeks of age (H) and at 2.5 years of age (I). Note the deep-set eyes and moderate miosis in this patient (J). All the photographs shown here were taken without using flash.

was resistant to dilation with repeated application of topical tropicamide and 10% neosynephrine.

Laboratory screening revealed a high CK level (4412 U/l) and thrombocytopenia (97 × 10^9/l). The bleeding time determined by the Ivy method was within the normal range (7.5 min). An abdominal CT-scan revealed asplenia. Ophthalmological and facial features of the index case and the father are shown in Figure 1 A–E.

All other family members including the mother, two siblings, and two uncles had no clinical abnormalities, with normal ophthalmological examination, platelet count, ionized calcium, and CK levels. Information regarding the grandparents of the family was not available.

Clinical Data of Family 2

Patient II-2 (mother)

A diagnosis of Stromorken syndrome was made in the German woman (index case, patient II-2) at the age of 17 years, when she gave birth to an affected girl (patient III-1). Her height (160 cm) and weight (49 kg) were in the lower normal range. Miosis was the only striking facial feature observed. Ophthalmological investigations showed isochorus, narrow (diameter both sides: 1.25 mm), round, and centred pupils without any reaction (no dilatation) to either 1% or 5% phenylephrine. Other ophthalmological findings such as refraction, visual acuity, anterior, and posterior segments were normal. Repeated blood counts showed thrombocytopenia, with values ranging between 40 × 10^9 and 122 × 10^9/l. Peripheral blood smear showed anisocytosis of thrombocytes and erythrocytes with Howell–Jolly bodies indicating absence of the spleen, which was later confirmed by an ultrasound examination. There was no history of increased susceptibility to infections or increased bleeding tendency even during delivery. Further investigations on hemostasis revealed a prolonged in vitro bleeding time (Platelet function analyser test), reduced collagen-induced platelet ATP secretion (collagen concentration used was between 2 and 8 μM), and reduced thrombocyte aggregation with ADP (5 μM) and collagen (1 μg/ml) (Born method). Her CK level was elevated (919 U/l, N = 26–177 U/l) and she reported a mild muscle weakness without the occurrence of muscles cramps. Total calcium level was between low to normal (2.08–2.37 mmol/l), and ionized calcium was in the normal range.
(1.26 mmol/l). There was a history of severe migraine attacks approximately once per week and recurrent urticarial-like eruptions. Dyslexia has been known since childhood and psychological investigations confirmed impaired cognitive function (IQ in XY test 68) and attention deficit.

**Patient III-1 (daughter)**

Measurements at the time of birth were normal but height decreased at the third percentile (88 cm) at the age of 2.5 years, while head circumference (48 cm, 35th percentile) was normal. At birth there was no evidence of miosis but at the age of 2.5 years, a mild miosis appeared (diameter of pupils both sides: 2 mm) with marked reduced dilatation with maximum dose of phenylephrine in pupillometry. Other ophthalmological findings (refraction, visual acuity, orthoptic investigations, anterior, and posterior segments) were normal. Ultrasound showed the presence of the spleen and no Howell–Jolly bodies were detected in peripheral blood smear. She was thrombocytopenic at birth and received thrombocyte concentrates when thrombocyte count fell below 8 × 10^9/l. However, there was no history of bleeding complications or increased bleeding tendency. Her CK levels were slightly increased (260–491 U/l) and ionized calcium was within the reference level. Until now, there is no evidence of muscle weakness or suspicion of migraine. However, a mild speech delay was reported. Ophthalmological features of the index case and daughter are shown in Figure 1F–I.

The maternal grandmother had normal thrombocyte counts, normal platelet function, and hemostatic parameters with no miosis. No clinical information and blood sample of the grandfather were available. An overview on the clinical findings of these affected individuals from both the reported families is shown in Table 1 and compared to other reported cases.

**Muscle Biopsy**

Biopsy was obtained from the peroneus muscle of the index case in Family 1 at the age of 5 months. Gomori trichrome stain revealed some areas of red staining within cytoplasm of the muscle cells, possibly indicating the presence of early tubular aggregates (data not shown). Muscle biopsy of the father (Family 1) was obtained from the Vastus lateralis (quadriceps muscle) at 33 years of age. Standard light microscopy showed the presence of predominantly type II fibers atrophy. Tubular aggregates were seen as pink fields within the cytoplasm, or in the periphery of muscle fibers. In some areas, tubular aggregates occupied the entire muscle fiber. The aggregates were absent with SDH staining, indicating a reticular, but not mitochondrial, origin (Fig. 2A–D). Immunohistochemistry showed that the tubular aggregates observed were positively stained by anti-STIM1, anti-STIM2, anti-Orai1, and anti-Orai2 antibodies (Fig. 2E–H).

**Chromosomal and DNA Studies**

Conventional karyotyping in the index cases of the two families was normal. The array CGH profile was normal in the affected members from Family 1 and Family 2. Sanger sequencing of the STIM1 gene showed that the index case of Family 1 and his father were heterozygous for the c.910C>T (NM_001277961.1) transition, which would result in an inferred p.R304W (NP_003147.2) substitution in the protein. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. The observed variant has been submitted to dbSNP with accession number rs483352867. The mutation affected the cutting restriction site of *Fau1*. By using *Fau1* restriction enzyme analysis, we confirmed that the index case (blood cell and buccal epithelial cell DNA) and the father (blood cell and fibroblast DNA) were heterozygous for the c.910C>T substitution, whereas the mutation was absent in the unaffected family members (Fig. 3A). The c.910C>T substitution was not found in 100 unrelated controls tested in this study. This mutation was not annotated in human variation databases such as the 1000 Genomes Project (http://browser.1000genomes.org) [Siva, 2008] and dbSNP.

**Whole-Exome Sequencing (Family 2)**

In the German family (Family 2), exome sequencing was performed to identify the underlying gene defect in the patients. The index case II-2 and the affected child III-1 were included. Homozygous changes as well as silent variants, deep intronic changes, frequent polymorphisms (dbSNP 137), and changes not fulfilling internal quality parameters were excluded. After filtering 459 shared changes, mainly 428 missense mutations, 31 truncating mutations, six stop mutations, 10 frame shift mutations, eight mutations of the canonical splice site, and seven complex insertions/deletions remained as potential causative mutations (Supp. Table S2). An intensive analysis mainly focusing on truncating changes of the index patients did not lead to a promising candidate gene in the first place. Among the heterozygous missense variants, the change observed in the STIM1 gene was classified as relevant because of the known involvement in cases with myopathy with tubular aggregates, which also has been observed in cases with Stormorken syndrome. The missense mutation c.910C>T in the STIM1 gene was confirmed in heterozygous state by Sanger sequencing in the mother and daughter, and excluded in the unaffected grandmother (Fig. 3B).

**Impact of the p.R304W Substitution on Ca^{2+} Homeostasis**

To investigate whether STIM1<sub>R304W</sub> affects calcium homeostasis, we performed Ca^{2+} imaging using Fura-2 probe on primary fibroblasts isolated from patient I-3 (Family 1). Thapsigargin (TG) was used to empty the intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+} (0Ca), and store-operated calcium entry (SOCE) was then measured by the addition of 10 mM extracellular Ca^{2+} (10Ca). TG-induced Ca^{2+} response consists of two phases, a peak phase contributed by Ca^{2+} release from intracellular Ca^{2+} stores (endoplasmic reticulum [ER] Ca^{2+}) and a plateau phase contributed by SOCE. As shown in Figure 4, the basal F<sub>TG</sub>/F<sub>0</sub> Fura-2 fluorescence ratio was significantly elevated (33%) in fibroblasts from patient (1.45 ± 0.02, n = 90) versus control fibroblasts (1.09 ± 0.02, n = 90), P < 0.001, Fig. 4A), and the SOCE amplitude was increased by 26% in patient fibroblasts (0.93 ± 0.03) as compared with control fibroblasts (0.74 ± 0.06, n = 90, P < 0.01, Fig 4B and C). Furthermore, we show a drastically decreased or absence of the ER Ca^{2+} content in 50% of fibroblasts from patient I-3 (Fig. 4C, n = 90, P < 0.01). To further clarify the functionality of the STIM1<sub>R304W</sub>, we expressed both wild-type STIM1 (STIM1<sub>WT</sub>) and mutant STIM1 (STIM1<sub>R304W</sub>) in HEK 293T cells and evaluated their consequences on Ca^{2+} fluxes. Expression of STIM1<sub>WT</sub> and STIM1<sub>R304W</sub> was detected in transfected HEK 293T cells by immunoblotting experiments (Supp. Fig. S1). Sixty percent of STIM1<sub>R304W</sub> cells exhibited high Ca^{2+} levels as estimated by fluorescence intensity (basal fluorescence ratio of 1.18 ± 0.03 (n = 90) vs. 0.89 ± 0.01 (n = 90) for STIM1<sub>WT</sub>, P < 0.001 (Fig. 4), and displayed a higher (22%) TG-activated SOCE in cells expressing STIM1<sub>R304W</sub> when compared with cells expressing...
## Table 1. Clinical Characteristics of Patients Reported with Stormorken Syndrome

| Patients     | Family 1 (French) | Family 2 (German) | Misceo et al. (2014) | Nesin et al. (2014) | Total 13 Patients |
|--------------|-------------------|-------------------|----------------------|---------------------|------------------|
|              | This study        | This study        | Misceo et al.        | Nesin et al. (2014) |                  |
|              | Father [I-3]      | Son [II-2]        | Mother [II-2]        | Mother [II-2]       |                  |
| Sex          | Male              | Male              | Female               | Female              | Male             |
| Age at report (years) | 33 | 19 | 65 | 65 | ? | 52 |
| Miosis       | Marked            | Moderate          | Marked               | Marked              |                 |
| Short stature (cm) | 157 | 160 | 159 | 159 | ? | 3/7 |
| Low body weight (kg) | 57.6 | 49 | 53 | 53 | ? | 3/7 |
| Idiothyosis  | +                 | Urticaria         | +                    | Urticaria           |                 |
| Dyslexia     | ±                 | ±                 | ±                    | ±                   | ±                |
| Learning difficulties | ± | + | ? | ? | ? | 3/7 |
| Headache     | –                 | –                 | +                    | +                   | +                |
| Muscular weakness and cramps | ± | + | – | + | + | 3/7 |
| Asplenia     | +                 | –                 | +                    | Hypoplasia          | +                |
| Bleeding tendency | – | – | – | – | – | 3/7 |
| Thrombocytes 10^9/l | 97–146 | 28–207 | 75–90 | 70–95 | 18 | 13/13 |
| Bleeding time (s) (min) | 7.5 | 15 | 16–20 | 14–18 | ND | 13/13 |
| Increased creatine kinase level (U/L) | 4.412 | 769 | 919 | 491 | + | 13/13 |
| Muscle biopsy | TAM              | Mild TAM          | ND                   | TAM                 | ND               |
| Hypocalcemia | +                 | –                 | –                    | +                   | –                |
| Corrected calcium by protidemia (mmol/L) | 2.04–2.11 | 2.03–2.59 | 2.09–2.16 | 2.11–2.25 | ND | 1.95 |
| Ionized calcium (Ca^{2+}) (mmol/L) | 1.05 | 1.1 | 1.26 | 1.1 | 1.01–1.16 | 1.95 |

| Patients     | Family 1 Case 1 | Family 2 Case 2 | Lehmann\* | Total 13 Patients |
|--------------|-----------------|-----------------|-----------|------------------|
| Sex          | Male            | Male            | Male      | Male             |
| Age at report (years) | ? | ? | ? | ? |
| Miosis       | Marked          | Moderate        | Marked    | Marked           |
| Short stature (cm) | ? | ? | ? | ? |
| Low body weight (kg) | ? | ? | ? | ? |
| Idiothyosis  | ?                | ?                | ?         | ?                |
| Dyslexia     | ±                | ±                | ±         | ±                |
| Learning difficulties | ? | ? | ? | ? |
| Headache     | ?                | ?                | ?         | ?                |
| Muscular weakness and cramps | ± | ± | ± | ± |
| Asplenia     | ±                | ±                | ±         | ±                |
| Bleeding tendency | ± | ± | ± | ± |
| Thrombocytes 10^9/l | ? | ? | ? | ? |
| Bleeding time (s) (min) | ? | ? | ? | ? |
| Increased creatine kinase level (U/L) | ? | ? | ? | ? |
| Muscle biopsy | TAM             | Mild TAM        | ND        | ND               |
| Hypocalcemia | +               | –                | –         | –                |
| Corrected calcium by protidemia (mmol/L) | 2.04–2.11 | 2.03–2.59 | 2.09–2.16 | 2.11–2.25 | ND | 1.95 |
| Ionized calcium (Ca^{2+}) (mmol/L) | 1.05 | 1.1 | 1.26 | 1.1 | 1.01–1.16 | 1.95 |

\*Lehmann et al. (2010) Schweizerischen Gesellschaft für Innere Medizin 19–21 May 2010. Basel, Switzerland.
STIM1WT (n = 130, P < 0.01, Fig. 4E and F). To investigate whether SOC (Store Operated Calcium) channels contribute to the high basal Ca\textsuperscript{2+} entry, STIM1R304W cells were exposed to SOC inhibitors 2-APB or Gd\textsuperscript{3+} [Fernando and Barritt, 1994; Prakriya and Lewis, 2001] in the absence of TG and in the presence of 2 mM extracellular Ca\textsuperscript{2+}. As shown in Supp. Figure S2A and B, perfusion of 2-APB (70 μM) or Gd\textsuperscript{3+} (8 μM) decreased the resting Ca\textsuperscript{2+} level. In contrast, 70 μM 2-APB failed to affect basal fluorescence ratio in control cells (Supp. Fig. S2C). Moreover, basal F\textsubscript{350}/F\textsubscript{380} Fura-2 fluorescence ratio also showed a reduction of ER Ca\textsuperscript{2+} in 40% of STIM1R304W cells (Fig. 4F, n = 120, P < 0.01).

Structural Impact of p.R304W Mutation

The p.R304W mutation in STIM1 introduces a dramatic change in the variant compared to the wild type due to loss of a positive charge and a decrease in the access to the solvent (Grantham index of 101). The relative and absolute accessibility to the solvent are reduced to about 20% and 30%, respectively, when the R304W substitution occurs. The observed mutation was predicted to be “probably damaging” for the protein with a score of 0.998 by using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2). The Alamut program predicted a null result for a putative splicing defect. Sequence alignment
Figure 3. Pedigree of the two families reported in this study, sequencing data. A: Pedigree of Family 1 in which affected individuals are shown in black and the unaffected family members are shown in white with index case identified by a black arrow. The electrophoregram showing the c.910C > T variation in patients. B: Pedigree of Family 2 in which affected individuals are shown in black and the unaffected family members are shown in white with index case identified by a black arrow. The electrophoregram showing the c.910C > T variation. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence.

of CCI and IH region from various species shows that the amino acids are highly conserved including the R304 residue (Fig. 5A). The arginine 304 residue of STIM1 is localized at the C-terminal end of the CC1 domain. The lateral chain of the R304 residue creates a salt bridge with E318 and makes a hydrogen bond with the main chain oxygen atom of Q314 (Fig. 5B). When a tryptophan residue replaces arginine in position 304 both the salt bridge and the hydrogen bond are not formed (Fig. 5C). Thus, the conformation of the inhibitory helix may be severely affected, leading to the release of the active part of SOAR domain of STIM1 and resulting in the activation of the ORA1 channel [Shen and Demaurex, 2012; Yang et al., 2012].

Discussion

Stormorken syndrome is a rare but distinctive disorder characterized by miosis, thrombocytopenia/thrombopathia, mild hypocalcemia, and learning difficulties. Asplenia and tubular aggregates myopathy appear later in life [Stormorken et al., 1985]. Globally,
Figure 4. See figure legend on next page.

Figure 5. See figure legend on next page.
13 patients with classical Stormorken disease have been described including four patients reported in this study. Clinical presentation of all patients is summarized in Table 1. Taken together, the clinical observations suggest that Stormorken disease is a progressive disease characterized by a constellation of symptoms, which overlap with other disorders caused by mutations in STIM1, prompting us to examine this gene as a common underlying cause.

Initially, by targeted sequencing in patients from Family 1 affected with Stormorken syndrome, we found a heterozygous missense mutation in STIM1 (c.910C>T p.R304W). We identified the same mutation in Family 2 by using whole-exome sequencing and selecting variants consistent with the dominant inheritance of the disease. The observed mutation was not present in normal individuals from the two families and was also absent in 100 unrelated individuals tested in this study. Furthermore, this mutation is not listed in the EVS and 1000 genomes database. We could eliminate a major DNA rearrangement using standard chromosome analyses and array CGH in the affected members of both families. The father-son transmission also allowed us to eliminate mitochondrial and X-recessive inheritance. A mosaicism is very unlikely as the c.910C>T substitution was also found in the father’s fibroblasts and in the buccal epithelial cells of his son Family 1.

Mutations in STIM1 have already been studied but none of the reported patients presented with the classical phenotype of Stormorken syndrome. The c.380_381insA mutation that generates a premature stop codon (p.E136X in SAM domain of STIM1) has been found to be associated with immunodeficiency and autoimmunity syndrome [Picard et al., 2009]. Guanine to adenine substitution at the –1 position of 5’-exon 8 (1538-1G > A), which creates a new splice site, has been reported in Kaposi sarcoma [Byun et al., 2010]. Mutations in STIM1 have also been associated with TAM. These mutations are localized to EF1 (p.H72Q, p.D84G) and EF2 (p.H109N, p.H109R) domains of STIM1 [Bohm et al., 2013]. A recent report on granule secretion defect in platelet identified the p.R429P mutation in STIM1 located in the SOAR domain [Nakamura et al., 2013]. None of the previously reported mutations in STIM1 were found to be associated with the occurrence of miosis. It should also be noted that p.R304W mutation described in this study is localized to CCI-1H domain and is the first mutation to be identified in this domain.

Patients with homozygous nonsense or splicing mutations in STIM1 have immunodeficiency, autoimmunity, thrombocytopenia, and nonprogressive muscular hypotonia, whereas patients with heterozygous mutation in the STIM1 calcium sensing domain (EF-Hands) present with dominant TAM [Bohm et al., 2013]. The non-immunological disorders of SOCE deficiency already described in patients are dominated by generalized muscular hypotonia and hypohidrotic ectodermal dysplasia, the latter symptom being absent in all our patients. Until now, muscle tubular aggregates were reported in eight different genetic diseases (Table 2). This heterogeneity illustrates the multiple mechanisms that can lead to the formation of muscular tubular aggregates in genetic disease [Stiber et al., 2008] in addition to nongenetic acquired forms as in the case of prolonged anoxia, alcoholic myopathy, inflammatory muscle diseases, diabetes, endocrine disorders, and Whipple disease [Goebel, 2012].

Severe thrombocytopenia observed in patients with STIM1 mutations probably results from autoantibodies directed against platelet...
surface antigens since platelet activation and aggregation were severely impaired in Orai1<sup>−/−</sup> and Stim1<sup>−/−</sup> mice [Feske, 2010].

SOCE is an important Ca<sup>2+</sup> influx pathway in many nonexcitable and excitable cells. This influx is regulated by the sensing of intracellular Ca<sup>2+</sup> stores, particularly in the ER. Ca<sup>2+</sup> release activated Ca<sup>2+</sup> (CRAC) channel formed mainly by the interaction of two molecules (STIM1 and Orai1) has been demonstrated to be a major regulator of SOCE [Soboloff et al., 2012; Fahrner et al., 2013]. When the ER Ca<sup>2+</sup> stores are full, STIM1 molecules stay as dimers in a resting state and are localized in the ER. When the ER Ca<sup>2+</sup> stores are empty, STIM1 molecules oligomerize, move to the plasma membrane, bind to Orai1, and activate the CRAC channel, allowing the entry of Ca<sup>2+</sup> into the cells [Feske, 2010; Soboloff et al., 2012; Fahrner et al., 2013; Zhou et al., 2013].

The involvement of calcium homeostasis in smooth muscle cells could explain the pupillary miosis as a consistent feature of Stormorken syndrome. Both Ca<sup>2+</sup> influx through plasma membrane Ca<sup>2+</sup> channels and Ca<sup>2+</sup> release from the intracellular sarcoplasmic reticulum contribute to a rise in intracellular Ca<sup>2+</sup>. An increase in the intracellular free Ca<sup>2+</sup> concentration (Ca<sup>2+</sup><sup>++</sup>) could be an important determinant leading to miosis by acting on pupillary sphincter [Jernigan and Resta, 2013].

Recently Cui et al. used crystallization studies along with site-directed mutagenesis to investigate the structure of the CC1-IH domain (amino acids 237–340). They have shown that IH functions as a switch to inhibit and activate the C-terminus of STIM1 by interacting with the SOAR domain. They also demonstrate that CC1-IH domain exists as a long helix and that the oligomerization of CC1-IH depends on IH [Cui et al., 2013]. The arginine 304 residue of STIM1 is localized at the C-terminal part of the CC1 domain. Since tryptophan residue does not present with a positive charge and is much more hydrophobic than an arginine residue, the consequence is a loss of a salt bridge and a hydrogen bond affecting the conformation of STIM1 in the inactive state (Fig. 5B).

Recently, a link between STIM1<sup>R304W</sup> mutation and Stormorken syndrome has been reported by Misceo et al. (2014) and Nesin et al. (2014). Despite the identical mutations, the authors have found different results on Ca<sup>2+</sup> homeostasis. Misceo et al. (2014) found an elevation of resting Ca<sup>2+</sup> levels in platelets from patients compared to controls and a reduction of SOCE. They suggested a constitutive activity of STIM1 and ORAII. Nesin et al. (2014) found an increase in SOCE both in skin fibroblasts and lymphocytes from a patient with Stormorken syndrome without any variation in the resting Ca<sup>2+</sup> level or ER Ca<sup>2+</sup>. Heterologous expression of STIM1<sup>R304W</sup> with ORAII results in constitutive activation of the SOCE channel, and, by using patch clamp; they show that the fast Ca<sup>2+</sup>-dependent inactivation was suppressed in cells transfected with STIM1<sup>R304W</sup> and ORAII versus WT-STIM1 and ORAII. Therefore, they suggested that STIM1<sup>R304W</sup> acts as an activating mutation in terms of SOCE channel function. Here, our functional data including both confocal microscopy (Supp. Fig. S3) and calcium imaging demonstrated that STIM1<sup>R304W</sup> is in the active state. Our results obtained from calcium imaging experiments with the cells transfected with STIM1<sup>R304W</sup> show an increase in the resting cytosolic Ca<sup>2+</sup> levels and a modest increase in SOCE. In addition, we also observed a reduction of the resting intracellular Ca<sup>2+</sup> after the perfusion of a Ca<sup>2+</sup> free solution or SOCE channel blockers suggesting that an increase in basal calcium influx and resting cytosolic calcium is due to the activation of SOCE.

We also analyzed the punctate formation in HEK 293T cells transfected with STIM1<sup>WT</sup> and STIM1<sup>R304W</sup> in the presence and absence of 2 μM TG, and we show that TG induced the punctate formation in HEK 293T cells expressing STIM1<sup>WT</sup>. However, the cells transfected with STIM1<sup>R304W</sup> show the presence of punctates both in untreated and TG-treated cells (Supp. Fig. S3). Our results are in agreement with the results reported by Nesin et al. (2014). In addition, we also found a reduction of the ER Ca<sup>2+</sup> content in the STIM1<sup>R304W</sup> cells. Similar results were reported by Misceo et al. (2014) showing the depletion of ER in platelets from Stormorken patients. One explanation on this partial depletion of ER would be that basal SOCE activates RyR via a Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release mechanism, which, in turn, reduces the Ca<sup>2+</sup> levels into the ER, thus facilitating SOCE by reducing SOC channel inactivation [Thakur et al., 2012]. We also analyzed the surface localization of YFP-STIM1<sup>WT</sup> and YFP-STIM1<sup>R304W</sup> by indirect flow cytometry (Supp. Fig. S4). Interestingly, results showed the high level surface expression on YFP-STIM1<sup>R304W</sup> in comparison with YFP-STIM1<sup>WT</sup>. This phenomenon warrants detailed investigations on the structural implication of p.R304W mutation in STIM1.

The pleiotropic effect of mutations observed in the various channelopathies is probably due to the gene being expressed in a large set of tissues. This has already been reported in a large number of genetic diseases due to mutations in sodium channels. In most cases, mutations cause a gain-of-function at both the molecular and cellular levels [Feske, 2010] and are genetically dominant. The role of STIM1 in multiple biological processes is probably responsible for the pleiotropic effect and multisymptomatic disease observed with the p.R304W mutation. This report reinforces the observation that the phenotypic presentation of STIM1 mutation depends upon the location and the nature of the mutation.

The variable phenotypes observed with the different mutations in STIM1 could be compared to the highly variable phenotypes associated with the different types of mutations in LMNA, responsible for different diseases such as dilated cardiomyopathy, familial partial lipodystrophy of Dunnigan type, recessive form of Charcot–Mary–Tooth axonal neuropathy (CMT2B1), muscular dystrophies (Emery–Dreifuss muscular dystrophy, limb-girdle muscular dystrophy 1B), and premature aging syndromes (mandibuloacral dysplasia, Hutchinson–Gilford progeria, atypical Werner syndrome, restrictive dermopathy) [Bertrand et al., 2011].

Patients with Stormorken syndrome display a wide spectrum of signs and present a challenge to a clinician. The identification of p.R304W mutation in the patients with Stormorken syndrome could aid in the timely diagnosis of the disease.

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