Homozygous mutation in *murine retrovirus integration site 1* gene associated with a non-syndromic form of isolated familial achalasia

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**Abstract**

**Background:** Achalasia is a condition characterized by impaired function of esophageal motility and incomplete relaxation of the lower esophagus sphincter, causing dysphagia and regurgitation. Rare cases of early-onset achalasia appear often in combination with further symptoms in a syndromic form as an inherited disease.

**Methods:** Whole genome sequencing was used to investigate the genetic basis of isolated achalasia in a family of Tunisian origin. We analyzed the function of the affected protein with immunofluorescence and affinity chromatography study.

**Key Results:** A homozygous nonsense mutation was detected in *murine retrovirus integration site 1 (MRVI1)* gene (Human Genome Organisation Gene Nomenclature Committee (HGNC) approved gene symbol: IRAG1) encoding the inositol 1,4,5-trisphosphate receptor 1 (IP\(_3\)-R1)-associated cyclic guanosine monophosphate (cGMP) kinase substrate (IRAG). Sanger sequencing confirmed co-segregation of the mutation with the disease. Sequencing of the entire *MRVI1* gene in 35 additional patients with a syndromic form of achalasia did not uncover further cases with *MRVI1* mutations. Immunofluorescence analysis of transfected COS7 cells revealed GFP-IRAG with the truncating mutation p.Arg112* (transcript variant 1) or p.Arg121* (transcript variant 2) to be mislocalized in the cytoplasm and the nucleus. Co-transfection with cGMP-dependent protein kinase 1 isofrom \(\beta\) (cGK1\(\beta\)) depicted a partial mislocalization of cGK1\(\beta\) due to mislocalized truncated IRAG. Isolation of protein complexes revealed that the truncation of this protein causes the loss of the interaction domain of IRAG with cGK1\(\beta\).

**Conclusions & Inferences:** In individuals with an early onset of achalasia without further accompanying symptoms, *MRVI1* mutations should be considered as the disease-causing defect.

**KEYWORDS**
cGMP-pathway, isolated achalasia, next-generation sequencing, smooth muscle relaxation
1 | INTRODUCTION

Achalasia is a rare disorder of esophageal motility characterized by esophageal aperistalsis and impaired relaxation of the lower esophageal sphincter (LES) during deglutition. The annual incidence of achalasia is approximately 1 in 100,000 people worldwide, with an overall prevalence of 9-10 per 100,000.\(^1\) Up to now, several proteins involved in this process have been identified. However, there are still a number of unresolved cases with suggested genetic background.

The first described syndrome with achalasia was the triple A syndrome (MIM 231550) with the further main symptoms alacrima, adrenal insufficiency, and neurological impairment.\(^2\) In this syndrome, the achalasia-addisonianism-alacrima syndrome gene (AAAS) is mutated.\(^3\) This gene encodes a protein of the nuclear pore complex named ALADIN (alacrima achalasia adrenal insufficiency neurologic disorder).\(^4\) Achalasia due to ALADIN dysfunction is predicted to originate by degradation of myenteric neurons of the LES due to increased oxidative stress.\(^5,6\) In recent years, mutations in several new genes have been described in humans with a monogenic form of achalasia. These are genes encoding for proteins of the cGMP-pathway such as nitric oxide synthase 1 (NOS1) and guanylate cyclase 1 soluble subunit alpha 1 (GUCY1A1: previous name: soluble guanylate cyclase 1 alpha 3; GUCY1A3) as well as genes with other cellular protein functions like cytokine receptor-like factor 1 (CRLF1), mitochondrial isoleucyl-tRNA synthetase (IARS2), stem cell factor receptor gene (KIT), trafficking protein particle complex subunit 11 (TRAPPC11), and guanosine diphosphate mannose pyrophosphorylase A (GMPPA).\(^7,13\) Association studies led to the identification of an eight-amino-acid insertion in the cytoplasmic tail of HLA-DQ\(\beta\)1 as a major risk factor for disease onset of idiopathic achalasia suggesting autoimmune processes to be involved in the pathogenesis of achalasia.\(^14,15\) Genes mediating esophageal motor function and gastrointestinal motility defects in mice such as the sprouty2 gene (SPRY2), myosin phosphatase target subunit 1 (MYPT1), and MRVI1 are suspected as candidate genes for achalasia in humans.\(^16-18\) A possible involvement of microRNAs regulating MRVI1 in esophageal muscle tissue of achalasia patients was recently postulated.\(^19\)

MRVI1 encodes the inositol trisphosphate receptor-associated cGMP kinase substrate IRAG. There are different transcript variants (V1-V6) encoding several IRAG isoforms (a-d) with distinct protein length. In its phosphorylated form, IRAG blocks calcium release from the endoplasmic reticulum (ER) into the cytoplasm and mediates the relaxation of smooth muscle cells.\(^20\) This process is regulated by nitric oxide (NO) that stimulates soluble guanylyl cyclases and increases cGMP levels.\(^21\) The interaction of cGK1\(\beta\), IRAG, and IP\(_3\)R1 is essential for this regulation.\(^20,22\) Decreasing the expression of IRAG in human smooth muscle cells has been shown to result in the abrogation of the inhibitory effect on calcium release from IP\(_3\)-sensitive stores, which points to a physiologic role of IRAG in human gastrointestinal smooth muscle relaxation.\(^23\)

Here, we report a homozygous nonsense mutation in human MRVI1 associated with familial early-onset isolated achalasia in a Tunisian family with three affected siblings. The mutation causes loss of the interaction domain of IRAG with cGK1\(\beta\) and therefore contributes to the loss of its central role in calcium regulation supporting its pathogenic role.

2 | METHODS

2.1 | Whole genome sequencing

Blood samples from patients, parents, and healthy siblings were collected after informed consent for whole genome sequencing (WGS). DNA preparation was performed according to standard protocols using QIAamp DNA Blood Mini Kit (Qiagen). WGS was performed in two (II:1 and II:8) of the three patients of this family using the BGISEQ-500 pipeline, which generated 1252 Mio and 1403 Mio 100 bp paired-end FASTQ-encoded reads that were aligned to the human GRCh37.p11 (hg19) genome build using BWA-MEM v0.7.1 (http://arxiv.org/pdf/1303.3997.pdf).\(^24\) The clean reads of the samples had high Q20 (96.26% and 97.09%) and Q30 (87.65% and 89.34%), which showed high sequencing quality. A variant file for variants located in coding and flanking intronic regions was generated using the GATK v3.8 software package and sent to MutationTaster2 for assessment of potential pathogenicity (http://www.mutationtaster.org).\(^25,26\) Variants were filtered using allele frequencies from gnomAD removing all variants with a minor allele frequency of >5E-5 for the dominant disease model and a homozygote frequency of 1E-4 for the recessive disease model.\(^27\) All relevant variants were visually inspected using the IGV-software (http://www.broadinstitute.org/igv) and checked...
for entry in the gnomAD database (http://gnomad.broadinstitute.org/), the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org), the 1000 genome (1000G) database (http://www.1000genomes.org) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP).

2.2 | Sanger sequencing

For verification and co-segregation analysis of the MRVI1 mutation with the disorder in the family, we used the oligonucleotide primers FW 5′-AAG TTT GAG ATT GAT TTT CTG AAC-3′ and REV 3′-CTG ATC TAC CCT GTC CTG TTG-5′ flanking exon 4.

For sequencing of the 21 coding exons of MRVI1 in 19 further achalasia patients, primer pairs were designed using OLIIGO software (Table S1). 40 ng genomic DNA was amplified in 20 µL of PCR mix containing 15 mmol/L Tris-HCl pH 8.0, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs, 0.3 µmol of forward primer, and 0.3 µmol of reverse primer and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: Initial denaturation at 94°C for 10 minutes, 35 cycles with 94°C denaturation for 30 s, 60°C annealing for 30 seconds, and 72°C extension for 60 seconds, and 72°C extension for 10 minutes. PCR products were cleaned-up by RApid Alkaline Phosphatase (Sigma-Aldrich Chemie GmbH) and Exonuclease I (New England Biolabs GmbH ) digestion at 37°C for one hour and sequenced on an ABI 3130 XL genetic analyzer using BigDye Terminator Cycle Sequencing Kit 1.1 (Applied Biosystems).

2.3 | Plasmids

Full-length MRVI1 cDNA of transcript variants 1 and 2 was cloned into pEGFP-C1 (BD Biosciences Clontech) in frame at the carboxy terminus of the enhanced green fluorescence protein (EGFP). For cloning of MRVI1 and mutagenesis of patient’s mutation, we used the In-Fusion HD Cloning Plus kit (TaKaRa Bio Europe SAS) according to the manufacturer’s instructions. Plasmid DNA was prepared using the QIAfilter™ Plasmid Maxi Kit (Qiagen GmbH) and verified by sequencing. cGK1β-dsRed plasmid used was from previous study.22

2.4 | Cell culture and transfection

COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Biochrom AG) supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics, at 37°C and 5% CO2. The day before transfection, cells were plated in 24 well plates on cover slips at 5 × 10⁴ cells per well or in 10 cm petri dish at a density of 2 × 10⁶ cells in DMEM without antibiotics. After cultivation for 24 hours, cells were transiently transfected with 100 ng or 7.5 µg of each plasmid DNA using Fugene HD (Promega) or Polyethyleneimine (Sigma-Aldrich) respectively according to the manufacturer’s instructions.

2.5 | Immunofluorescence analysis

Forty-eight hours after transfection, cells were fixed with 2% formaldehyde and permeabilized with 0.5% Triton X-100 for 5 minutes. Blocking was performed with 3% BSA in PBS for one hour at room temperature (RT). Anti-Calnexin antibody (AF18, sc-23954; Santa Cruz Biotechnology Inc.) was diluted 1:50 in blocking solution and incubated at 4°C overnight in a humidified chamber. Secondary antibody Alexa Fluor 405 goat anti-mouse IgG (1:500) (Molecular Probes, Life Technologies) was incubated one hour at RT in the dark. Excess antibodies after primary and secondary antibody staining were removed by five washing steps using PBS. Cells on cover slips were attached on object slides with Vectashield® mounting medium (Vector Laboratories). Confocal laser scanning was performed on an inverted Zeiss LSM 780 microscope (Zeiss) of the CMCB light microscopy facility. Images were acquired by setting the gain just below the threshold of signal saturation in blue, green, and red channels, and values for brightness, gamma, and contrast and brightness of individual color were adjusted equally by ZEN software.

2.6 | Affinity purification

Isolation of protein complexes consisting of cGK1β, IRAG, and IP3R1 from transfected COS7 cells by affinity chromatography was performed as described previously.20,28 In brief, the cGK1β and IRAG co-transfected COS7 cells were solubilized in RIPA lysis buffer (Santa Cruz Biotechnology) for 30 minutes at 4°C and then centrifuged (15 000 g 10 minutes, 4°C). The supernatant was added to 8-AET-cGMP-agarose beads (Biolog Life Science Institute GmbH & Co. KG) and incubated over night at 4°C. After washing the beads with Lubrol wash buffer (0.1% Lubrol-PX in 20 mmol/L Tris pH 8.0, 80 mmol/L NaCl, protease inhibitor cocktail), proteins were eluted with Laemmli buffer and analyzed by SDS-PAGE and Western blot.

2.7 | Western blot

Affinity-purified protein complexes (AP) or a total of 10 µg cell lysate (L) were run on an SDS-polyacrylamide gel. The fractionated proteins were transferred to a nitrocellulose membrane, blocked for 1 hour with 5% skim milk powder in 1 x PBS-T (PBS with 0.1% Tween 20) and then incubated overnight at 4°C with an anti IRAG (1:200; amino acid 53-449) or anti cGK1α/β (1:100; Sc-271766; Santa Cruz Biotechnology) in 5% skim milk powder in 1 x PBS-T. Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000 in 5% skim milk powder in 1 x PBS-T; Cell Signaling Technology Europe BV) was incubated one hour at room temperature. After washing the membrane, the signal was developed using ECL Prime Western Blotting detection system (GE Healthcare) according to the protocol of the manufacturers and visualized on an Azure c300 Chemiluminescent Western Blot Imaging System (Azure Biosystems).
TABLE 1 Clinical presentation of the affected individuals

| Patient | II:1 | II:5 | II:8 |
|---------|------|------|------|
| Ethnicity       | Tunisian | Tunisian | Tunisian |
| Consanguinity   | Not known | Not known | Not known |
| Sex            | Female | Female | Female |
| Current age (years) | 34 | 28 | 13 |
| Skeletal height (cm) | 159 | 159 | 149 |
| Bodyweight (kg)  | 59 | 56 | 39 |
| Head circumference (cm) | 58 | 57 | 53 |

Cardinal symptoms of triple A syndrome

| Symptom          | II:1 | II:5 | II:8 |
|------------------|------|------|------|
| Age of onset     | From birth | 9 mo | From birth |
| Balloon dilatation at age (years) | 3 | 1 | 3 |
| Treatment with PPIs | Yes | No | Unknown |
| Alacrima age of onset | 6 mo | Absent | Absent |
| Adrenal insufficiency | Absent | Absent | Absent |

Other symptoms of triple A syndrome and achalasia-related syndrome

| Symptom                | II:1 | II:5 | II:8 |
|------------------------|------|------|------|
| Development            | Normal | Normal | Normal |
| Skin                   | Normal | Normal | Normal |
| Neurological symptoms  | Absent | Absent | Absent |
| Scoliosis              | Absent | Absent | Absent |
| Gastrointestinal symptoms | Present | Absent | Absent |
| Globus abdominalis     | Heartburn | Present | Absent |
| Cardiovascular function | Absent | Normal | Normal |
| Arterial hypertension  | Absent | Present | Absent |
| Raynaud’s phenomenon   | Absent | Absent | Absent |
| Bleeding time          | Normal | Not tested | Normal |

Abbreviation: PPIs, proton-pump inhibitors.

3 | RESULTS

To investigate genetic basis of a familial form of isolated achalasia, we performed WGS in two patients from a large Tunisian family. A full clinical information of all three patients is provided in Table 1. WGS in patient II:1 and II:8 revealed a homozygous variant [c.361C>T, NM_130385 | p.(Arg121*)] in MRVI1 (Figure 1). Other potential candidate variants were only present in one of the two patients (data not shown). Compound heterozygous predicted disease-causing variants in myosin heavy chain 8 (MYH8: rs200412862 and rs145863180) and phospholipase C gamma-2 (PLCG2: rs187956469 and rs75472618) were detected but are unrelated to the achalasia phenotype. Co-segregation of the MRVI1 variant with the disease phenotype was confirmed by Sanger sequencing of the parents, 3 patients, and 4 unaffected siblings (Figure 1B). As expected, both parents are heterozygous carriers of the mutation. The healthy siblings are all heterozygous carriers as well, which confirms the co-segregation of homozygosity for the mutation with the disease. In order to determine the allele frequency for this variant in the general population, we searched population databases and found the variant in heterozygous form twice in gnomAD v3 (allele frequency 0.000007) and once in ExAC (allele frequency 0.000008). Based on the rounded allele frequency of $q = 0.00001$, the calculation of the number of homozygous mutation carriers according to the Hardy-Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) for two alleles yield $q^2 = 1 \times 10^{-10}$ for non-consanguineous pairing. This indicates that the MRVI1 c.361C>T variant is a potential candidate for familial form of isolated achalasia.

Additionally, we screened 35 patients affected by achalasia selected from a cohort of triple A syndrome-like patients without mutations in AAAS by Sanger sequencing ($n = 19$) or WGS ($n = 16$). None of these patients had a pathogenic MRVI1 mutation in any of the 21 coding exons and exon-intron boundaries. This indicates that a mutation in MRVI1 is a very rare condition in achalasia and that syndromic forms of achalasia with triple A syndrome-like conditions have other causes than the isolated form of achalasia. Interestingly, the polymorphism p.Pro186Ser (rs35857561) in MRVI1 related to moyamoya-complicated neurofibromatosis type 1 was detected in a heterozygous form in 2 out of the above 35 tested achalasia patients and in one patient in a homozygous form. In the latter patient, we additionally identified the previously described splice mutation in TRAPPC11 (c.1893 + 3A>G).12

To explore whether the c.361C>T mutation in MRVI1 interrupts proper protein-protein interaction and subcellular localization of the protein, we used an in vitro test system because no patient material was available for functional analysis. It is known that the membrane protein IRAG which interacts with the soluble enzyme cGK1β is assembled in a ternary complex with IP$_3$R1.29 For visual analysis (immunofluorescence and biochemical isolation of protein complexes (affinity chromatography experiments), we used COS7 cells, naturally expressing IP$_3$R1, but not cGK1β and IRAG.20 We cloned full-length MRVI1 cDNA of transcript variant 1 and 2 in frame to the green fluorescent GFP-protein and included the mutation by mutagenesis to express N-terminally GFP-tagged wild-type and mutant IRAG protein. Immunofluorescence analysis of transfected COS7 cells revealed that wild-type IRAG (isoform a and d from transcript variant 1 and 2) is located at ER and co-localizes with cGK1β. GFP-IRAG with the truncating mutation p.(Arg112*) or p.(Arg121*) mislocalizes into cytoplasm and nucleus. Co-transfection with cGK1β revealed a partial cytoplasmic mislocalization of cGK1β due to the mislocalization of truncated IRAG (Figure 2).

Next, we functionally analyzed the protein-protein interaction between cGK1β and IRAG by isolating protein complexes from

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co-transfected COS7 cells via cGMP-agarose affinity chromatography. By this method, a cyclic nucleotide responsive protein is separated out of a cell lysate together with its binding partners. A loss of protein-protein interaction corresponds to the loss of protein detection of binding partners in the affinity-purified protein fraction in Western blot. The truncating p.(Arg112*) or p.(Arg121*) mutation interrupted the interaction between IRAG and cGK1β so that, in contrast to the wild-type GFP-coupled proteins at 147 kDa, the mutated GFP-IRAG protein at about 40 kDa was undetectable after cGMP-agarose precipitation (Figure 3). This is in agreement with the proposed interaction of cGK1β with the N-terminal interaction domain of IRAG in the trimeric protein complex. The results confirm loss of interaction by the truncating mutation.

4 | DISCUSSION

This is the first description of a nonsense mutation in MRVI1 causing early onset of achalasia in humans. In this study, we investigate the cause of achalasia in three affected siblings from healthy parents. Not having found pathogenic AAAS or GMPPA variants that are known to cause syndromes with achalasia, WGS was performed on two of the patients. A homozygous nonsense mutation in the MRVI1 gene was identified, while variants in other genes could be excluded as being disease-causing. Based on WGS, we were able to exclude further genes responsible for achalasia such as NOS1, GUCY1A1, CRLF1, IARS2, KIT, and TRAPPC11 as well as two genes responsible for gastrointestinal motility defects in mice SPRY2 and MYPT1. Our findings complement the results from Geiselhöringer and colleagues who created a mouse line with a targeted in-frame deletion of Mrvi1 exon 12 coding for the N-terminus of the coiled-coil domain of IRAG. These IRAGD12/D12 animals expressed a dilated gastrointestinal tract and disturbed gastrointestinal motility. Accordingly, one of the patients exhibit additional gastrointestinal symptoms such as globus abdominalis and heartburn. IRAG contains an amino-terminal domain that specifically interacts with the amino terminus of cGK1β and a coiled-coil domain in the carboxy-terminal
part of the protein. \(^{20,28,30,31}\) The coiled-coil domain is responsible for the interaction of IP\(_3\)R1 with IRAG and is essential for assembly of the IP\(_3\)R1 within the cGK1–IRAG macro-complex. \(^{18}\) The deletion of the IP\(_3\)R1–IRAG interaction domain in IRAG\(^{D12/D12}\) mice is involved in the inability of cGMP to relax receptor-triggered phasic and tonic smooth muscle contractions. \(^{18}\) In a similar manner, the MRVI1 mutation of the described family causes the loss of the interaction domain of IRAG with cGK1β, thereby disrupting the IP\(_3\)R1–IRAG–cGK1 complex and presumably contributing to the loss of cGMP-regulated smooth muscle relaxation (Figure 4). The interplay of smooth muscle cells (SMC) and interstitial cells of Cajal (ICC) in nitrergic neurotransmission in the LES highlights disturbances in novel signaling pathways responsible for primary achalasia. It has been shown that basal LES tone is dependent on NO-sensitive guanylyl cyclase in both ICC and SMCs whereas nitrergic LES relaxation is predominantly mediated via ICC. \(^{32}\) The extent of the described MRVI1 mutation in ICC function remains to be determined.

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**FIGURE 2** Immunofluorescence analysis of co-transfected COS7 cells (GFP-IRAG green, cGK1β-dsRed red) reveal that wild-type IRAG (isoform a and d) is located at endoplasmic reticulum (ER-marker Calnexin; blue) and co-localizes with cGK1β (yellow arrows); co-localization on the right column (merged images). GFP-IRAG\(^{aArg112*}\) and GFP-IRAG\(^{dArg121*}\) are mislocalized in the cytoplasm (white arrow heads) and the nucleus (white arrows) and induce a partly mislocalization of co-transfected cGK1β (yellow arrow heads)
IRAG is a 125-kDa membrane protein that resides in the ER membrane via a COOH-terminal transmembrane anchor. Two NH2-terminal variants of IRAG, a 912-amino acid IRAG (isof orm d; transcript variant 2; NM_130385) and a 904-amino acid IRAG (isof orm a; transcript variant 1; NM_001098579), derived from alternative splicing of the same gene, are known as the main IRAG isoforms. A functional difference of these splice variants has not been shown, which is in agreement with our transfection results. 32 Both wild-type isoforms are localized at the ER and both mutated isoforms IRAGaArg112* and IRAGdArg121* are mislocalized in the same way to the cytoplasm and nucleus.

We anticipate that the transcript variants 4 (NM_001100167) and 6 (NM_001206881) of IRAG encoding for a 597 amino acid protein (isof orm c) of 66 kDa (NP_001093679) with a start codon in exon 9 of MRVI1 are not affected by the mutation in exon 4. It is conceivable that the identified mutation in exon 4 of MRVI1 is only relevant for specific tissues and therefore results in an isolated form of achalasia. Werder and colleagues described a tissue-specific alternative splicing with exon skipping and alternative splice donor and acceptor site usage. 33 They showed that COOH-terminally truncated IRAG variants lacking both the cGK1 phosphorylation and the IP3;R1 interaction site counteract cGMP-mediated inhibition of calcium transients and relaxation of human colon smooth muscle cells. 34 Recently, the polymorphism p.Pro186Ser (rs35857561) in MRVI1 was hypothesized to be a genetic susceptibility factor for moyamoya in European patients with neurofibromatosis type 1. 29 We detected it twice in a heterozygous and once in a homozygous form out of the 35 tested achalasia patients, which corresponds to the expected minor allele frequency in the Caucasian population (0.06 vs. 0.07 in ExAc browser).

Interestingly, our patients did not show any signs of cardiovascular involvement. This is surprising in the context of the affected NO-cGMP signaling pathway and the phenotype of patients with mutations in GUCY1A1 and NOS1. 7,8 Five genes encoding proteins in this pathway have thus so far been identified by genome-wide association studies to convey an increased risk for coronary artery disease; these are GUCY1A1, endothelial nitric oxide synthase gene (NOS3), phosphodiesterase 3A gene (PDE3A), phosphodiesterase 5A (PDE5A), and MRVI1. 25 Although we thoroughly investigated our patients concerning cardiovascular or hemostasiologic symptoms neither altered blood pressure or bleeding time nor any other symptom could be detected. However, it has to be emphasized that the IRAG-mutant mice did reveal only a mild cardiovascular phenotype with a slight hypotension and showed unaltered basal platelet aggregation at injured artery. 22,36 One further reason could be the availability of IRAG isoform c in our patients. Furthermore, we did not determine other manifestations seen in achalasia-related syndromes.

In conclusion, although MRVI1 mutations seem to be a rare cause of achalasia, testing a panel of genes associated with achalasia including MRVI1 should be considered in individuals with an early onset of the disorder.

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FIGURE 3 Immunoblot of cGMP-agarose affinity-purified proteins (AP) vs. whole cell lysate (L) of cGK1β and different IRAG forms co-transfected COS7 cells. The truncated GFP-IRAGaArg112* or GFP-IRAGdArg121* proteins with the molecular size of 38.5 and 39.5 kDa were undetectable after cGMP-agarose precipitation (white arrows) whereas wild-type (WT) IRAG (GFP-IRAG 147 kDa) co-precipitates with cGK1β (cGK1β-dsRed 111 kDa) on cGMP-agarose. PS, protein standard. Plus sign under the blot indicates the corresponding transfected plasmids in COS7 cells.
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DISCLOSURES
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
KK, NR, and AH conceived and designed the experiments. KK analyzed and interpreted the data. DH recruited and clinically characterized patients. JS provided protocols and experimental expertise. DL performed experiments. MS performed the bioinformatics analysis of sequencing data. KK and DH wrote the paper. All authors reviewed and approved the final version of manuscript.

REFERENCES
1. Patel DA, Lappas BM, Vaezi MF. An overview of achalasia and its subtypes. Gastroenterol Hepatol. 2017;13:411-421.
2. Allgrove J, Clayden GS, Grant DB, et al. Familial glucocorticoid deficiency with achalasia of the cardia and deficient tear production. Lancet. 1978;1:1284-1286.
3. Handschug K, Sperling S, Yoon SJ, et al. Triple A syndrome is caused by mutations in AASS, a new WD-repeat protein gene. Hum Mol Genet. 2001;10:283-290.
4. Cronshaw JM, Krutchinsky AN, Zhang W, et al. Proteomic analysis of the mammalian nuclear pore complex. J Cell Biol. 2002;158:915-927.
5. Khelif K, De Laet MH, Chaouachi B, et al. Achalasia of the cardia in Allgrove’s (triple A) syndrome: histopathologic study of 10 cases. Am J Surg Pathol. 2003;27:667-672.
6. Kind B, Koehler K, Krumholz M, et al. Intracellular ROS level is increased in fibroblasts of triple A syndrome patients. J Mol Med Berl. 2010;88:1233-1242.
7. Shteyer E, Edvardson S, Wynia-Smith SL, et al. Truncating mutation in the nitric oxide synthase 1 gene is associated with infantile achalasia. Gastroenterology, 2015;148:533-536.e4
8. Hervé D, Philippi A, Belbouab R, et al. Loss of α1β1 soluble guanylate cyclase, the major nitric oxide receptor, leads to moyamoya and achalasia. Am J Hum Genet. 2014;94:385-394.
9. Busch A, Žarković M, Lowe C, et al. Mutations in CRLF1 cause familial achalasia. Clin Genet. 2017;92:104-108.
10. Schwartzentuber J, Buhas D, Majewski J, et al. Mutation in the nuclear-encoded mitochondrial isoleucyl-tRNA synthetase IARS2 in patients with cataracts, growth hormone deficiency with short stature, partial sensorineural deafness, and peripheral neuropathy or with Leigh syndrome. Hum Mutat. 2014;35:1285-1289.
11. Hirota S, Nishida T, Isozaki K, et al. Familial gastrointestinal stromal tumors associated with dysphagia and novel type germline mutation of KIT gene. Gastroenterology. 2002;122:1493-1499.
12. Koehler K, Milev MP, Prematilake K, et al. A novel TRAPPc11 mutation in two Turkish families associated with cerebral atrophy, global retardation, scoliosis, achalasia and alacrima. J Med Genet. 2017;54:176-185.
13. Koehler K, Malik M, Mahmood S, et al. Mutations in GMPPA cause a glycosylation disorder characterized by intellectual disability and autonomic dysfunction. Am J Hum Genet. 2013;93:727-734.
14. Gockel I, Becker J, Wouters MM, et al. Common variants in the HLA-DQ region confer susceptibility to idiopathic achalasia. Nat Genet. 2014;46:901-904.
15. Becker J, Haas SL, Mokrowiecka A, et al. The HLA-DQβ1 insertion is a strong achalasia risk factor and displays a geospatial north-south gradient among Europeans. Eur J Hum Genet EJHG. 2016;24:1228-1231.
16. Taketomi T, Yoshida D, Taniguchi K, et al. Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and esophageal achalasia. Nat Neurosci. 2005;8:855-857.
17. He W-Q, Qiao Y-N, Peng Y-J, et al. Altered contractile phenotypes of intestinal smooth muscle in mice deficient in myosin phosphatase target subunit 1. Gastroenterology. 2013;144:1456-1465, 1465. e1-5.
18. Geiselhöringer A, Werner M, Sigl K, et al. IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase. EMBO J. 2004;23:4222-4231.
19. Palmieri O, Mazza T, Bassotti G, et al. microRNA-mRNA network model in patients with achalasia. Neurogastroenterol Motil. 2020;32(3):e13764.
20. Schlossmann J, Ammendola A, Ashman K, et al. Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. Nature. 2000;404:197-201.
21. Friebe A, Koelsing D. Regulation of nitric oxide-sensitive guanylyl cyclase. Circ Res. 2003;93:96-105.
22. Geiselhöringer A, Gaisa M, Hofmann F, et al. Distribution of IRAG and cGKI-isosforms in murine tissues. FEBS Lett. 2004;579:19-22.
23. Fritsch RM, Saur D, Kurjak A, et al. InsP3R-associated cGMP kinase substrate (IRAG) is essential for nitric oxide-induced inhibition of calcium signaling in human colonic smooth muscle. J Biol Chem. 2004;279:12551-12559.
24. Huang J, Liang X, Xuan Y, et al. A reference human genome dataset of the BGISEQ-500 sequencer. GigaScience. 2017;6:1-9.
25. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20:1297-1303.
26. Schwarz JM, Cooper DN, Schuelke M, et al. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods. 2014;11:361-362.
27. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016;536:285-291.
28. Ammendola A, Geiselhöringer A, Hofmann F, et al. Molecular determinants of the interaction between the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and cGMP kinase Ibeta. J Biol Chem. 2001;276:24153-24159.
29. Santoro C, Giugliano T, Kraemer M, et al. Whole exome sequencing identifies MRV11 as a susceptibility gene for moyamoya syndrome in neurofibromatosis type 1. PloS One. 2018;13:e0200446.
30. Desch M, Sigl K, Hieke B, et al. IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation. Cardiovasc Res. 2010;86:496-505.
31. Casteel DE, Boss GR, Pilz RB. Identification of the interface between cGMP-dependent protein kinase Ibeta and its interaction partners TFII-I and IRAG reveals a common interaction motif. J Biol Chem. 2005;280:38211-38218.
32. Groneberg D, Zizer E, Lies B, et al. Dominant role of interstitial cells of Cajal in nitrogentic relaxation of murine lower oesophageal sphincter. J Physiol. 2015;593:403-414.
33. Schlossmann J, Desch M. IRAG and novel PKG targeting in the cardiovascular system. Am J Physiol Heart Circ Physiol. 2011;301:H672-682.
34. von Werder A, Mayr M, Schneider G, et al. Truncated IRAG variants modulate cGMP-mediated inhibition of human colonic smooth muscle cell contraction. Am J Physiol Cell Physiol. 2011;301:C1445-C1457.
35. Wobst J, Schunkert H, Kessler T. Genetic alterations in the NO-cGMP pathway and cardiovascular risk. Nitric Oxide Biol Chem. 2018;76:105-112.
36. Antl M, von Brühl M-L, Eiglsperger C, et al. IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. Blood. 2007;109:552-559.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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