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REGULAR ARTICLE

A novel *RNASEH2B* splice site mutation responsible for Aicardi–Goutieres syndrome in the Faroe Islands

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ABSTRACT

Aim: The aim of the study was to identify the genetic background for Aicardi–Goutieres syndrome (AGS) in the Faroe Islands.

Methods: Four patients with AGS were identified. The patients had a variable phenotype, from a severe prenatal form with intrauterine foetal death to a milder phenotype, albeit still with an early onset, within the first 2–3 months.

Results: A genome-wide search for homozygosity revealed one single 15.6 Mb region of homozygosity on chromosome 13, which included *RNASEH2B*, where a splice site mutation c.322-3C>G was identified. Screening of 170 anonymous Faroese controls revealed a carrier frequency of approximately 1.8%, corresponding to an incidence of AGS in the Faroe Islands of around 1 in 12 300.

Conclusion: The previously identified *RNASEH2B* mutations comprise altogether 20 mutations (missense, nonsense and splice site) with all patients harbouring at least one missense mutation. The severe phenotype of the Faroese patients compared with the previously reported patients with *RNASEH2B* mutations may be caused by the presence of two null alleles (although some residual normal splicing cannot be ruled out), whereas patients with one or two missense mutations may have some, albeit abnormal, RNASEH2B proteins, and hence some residual activity of RNASEH2B, explaining their milder phenotype.

INTRODUCTION

The Faroe Islands consist of 18 small islands situated in the North Atlantic Sea, 300 km north-west of Scotland between Iceland and Norway. The population of around 48 000 inhabitants (http://www.faroeislands.com) has been very isolated, and therefore several hereditary disorders have a high incidence owing to a founder effect, whereas other disorders are rarely or never seen (1–3). We have recently identified Aicardi–Goutieres syndrome (AGS (MIM #225750)) as an additional disorder with a high prevalence in the Faroe Islands.

AGS is inherited as an autosomal recessive trait, although autosomal dominant inheritance has been described in a few cases (4,5). The disorder is characterized by a combination of basal ganglia calcification, leukodystrophy and cerebrospinal fluid (CSF) lymphocytosis. Pregnancy, birth and the neonatal period are usually unremarkable, although onset within the first days is seen in around 20%. The remaining cases present after a period of normal development with a severe, subacute encephalopathy with feeding problems, irritability and psychomotor regression or delay. Associated symptoms include epilepsy, chilblain skin lesions on the extremities and episodes of aseptic febrile illness. The disorder progresses over several months, and microcephaly, pyramidal signs and psychomotor retardation develop. After this period, the disease course stabilizes. Prenatal and neonatal presentations of the disorder have been reported. Treatment is symptomatic, for example, anticonvulsants to treat epilepsy. Most patients with the severe form die within the first 10 years of life, but longer survival has been reported in the milder forms.

Neuroimaging typically shows bilateral calcification of the basal ganglia, a periventricular leukodystrophy that may

Key notes

- A novel splice site mutation in *RNASHE2B*, c.322-3C>G, was identified in four patients with Aicardi–Goutieres syndrome from the Faroe Islands.
- cDNA analysis showed that the mutation leads to a predominant skipping of exon 5.
- The patients had a variable phenotype, from a severe prenatal form with intrauterine foetal death to a milder phenotype, albeit still with an early onset, within the first 2–3 months.

show cystic degeneration, and cerebral atrophy. In the CSF, elevated interferon (IFN)-alpha and lymphocytosis are found in the initial phase of the disorder, but the levels usually normalize within a few years. In accordance with this, a study of gene expression in CSF showed up-regulation of IFN-related and lymphocyte-activating genes in AGS cases compared with controls, whereas genes related to cell cycle suppression, pro-apoptosis and RNAse activity were down-regulated (6). There was a trend towards decreased expression of IFN-alpha-related genes over time, with an increase in expression of angiogenesis-related genes. AGS may be mistaken for a congenital infection (TORCH: toxoplasma, rubella, CMV, HSV1 or HSV2), which should be ruled out before diagnosing AGS.

In 2006, mutations in four different genes responsible for AGS were reported: *TREX1*, encoding a 3′->5′ exonuclease, and *RNASEH2A*, *RNASEH2B* and *RNASEH2C* encoding subunits of the RNase H2 endonuclease complex (7,8). Mutations in *SAMHD1*, a SAM domain- and HD domain-containing protein, were recently reported in 13 families (9). Mutations in *TREX1* and *RNASEH2B* are found in around 65% of AGS patients.

The lymphocytosis and elevated levels of IFN-alpha in AGS are similar to what is found in a viral CNS infection and indicate activation of both innate and adaptive immunity; but the lack of infectious reagents in AGS and the autosomal recessive inheritance suggested an endogenous cellular defect leading to an inflammatory response, likely autoimmune, as the cause of the disorder. *TREX1* mutations have also been implicated in systemic lupus erythematosus and in autosomal dominant familial chilblain lupus (5,10), autoimmune disorders where a disturbance of IFN-alpha is considered central, and the overlap between these disorders and AGS is emphasized by the fact that children with AGS may also develop SLE or chilblain lesions (11,12).

TREX1 encodes the homodimeric $3' \rightarrow 5'$ deoxyribonuclease that preferentially degrades single-stranded DNA, whereas *SAMHD1* encodes a deoxynucleoside triphosphate triphosphohydrolase that inhibits an early step of the viral life cycle (13,14). RNase H2 endonucleolytically cleaves ribonucleotides from RNA/DNA duplexes (8). It may function in the removal of lagging-strand Okazaki fragment RNA primers during DNA replication and in the excision of single ribonucleotides from DNA/DNA duplexes.

A possible mechanism behind the autoimmune reaction in TREX1 and RNASEH2 deficiency is that accumulation of endogenous nucleic acids, which are sensed as viral or nonself, leads to induction of an IFN-alpha-mediated immune response via, for example, Toll-like receptor (TLR)-dependent pathways and cytoplasmic receptors.

The aim of the present study was to identify the genetic background for AGS in the Faroe Islands.

METHODS

Human subjects

Four patients of Faroese origin with AGS were identified, among them two siblings. There was no known relationship between the three families. We have not identified additional Faroese patients with AGS, although reportedly two deceased persons in the family of patients 1 and 2 had a diagnosis of 'congenital CMV infection'. The families gave informed consent for the study.

DNA and RNA extraction and cDNA synthesis

DNA was extracted from peripheral blood using standard procedures. RNA was extracted from cultured skin fibroblasts from patient 1 with the RNAeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) and reverse transcribed to cDNA with the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA).

Microarray analysis and homozygosity mapping

DNA from two siblings (patients 1 and 2) was used for a genome-wide search for homozygosity with the Affymetrix Genomewide Human SNP Array 6.0 (Affymetrix Inc., Santa Clara, CA). In brief, DNA was digested with the restriction enzymes StyI and NspI, mixed with StyI and NspI adapters and ligated with the T4 DNA ligase. The ligated DNA was PCR-amplified, pooled and purified. The purified PCR product was fragmented with DNase I and end-labelled with biotin. The samples were hybridized to an array for 18 h in a hybridization oven. The array was washed, stained and scanned with an Affymetrix GeneChip scanner 3000. Affymetrix software was used to analyse the data, and homozygositymapper (http://www.homozygositymapper.org) was used to identify the homozygous regions.

Mutation analysis

PCR of genomic DNA or cDNA was performed with the Promega GoTaq PCR system and the following conditions: 0.2 mmol/L dNTPs, 1× buffer, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L of each primer, 10 ng template and 1.5 U polymerase in a total volume of 50 μL. The PCR program was 94°C for 2 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension step of 72°C for 7 min.

Sequencing was performed with the ABI Big Dye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were vacuum-purified with the Montage Seq96 Sequencing Reaction Cleanup Kit (Millipore SA, Saint-Quentin, France) and analysed on an ABI 3130xl Gene Analyzer (Applied Biosystems).

The data were analysed with Sequencing Analysis Software Version 5.2 from Applied Biosystems, and mutation screening was performed using Mutation Surveyor v.3.1 Software (SoftGenetics, State College, PA, USA). Primer sequences and PCR conditions are available on request.

RESULTS

Clinical findings

Patient 1 was the first child of healthy parents who were related five generations back. He was born by Caesarean section in gestation week 34 owing to severe intra-uterine

growth retardation (IUGR). Microcephaly was noted at birth. A percutaneous endoscopic gastrostomy was implanted at age 9 years owing to eating difficulties. At that time, he was 105 cm tall and weighed 17 kg.

At age 10 years, he is severely psychomotor retarded and has no expressive language. He has spasticity of the extremities, microcephaly and chilblain lesions of the hands and feet.

Cerebral CT showed periventricular calcifications, similar to what is seen in congenital CMV infection. Cerebral MRI showed severe atrophy with hypoplasia of corpus callosum, periventricular hyperintensities, high signal changes in the basal ganglia and dysmyelination.

Patient 2 was the younger sister of patient 1. In gestation week 27, an ultrasound showed foetal hydrops, enlarged heart and ascites, in addition to subcutaneous oedema. Severe hyperperfusion of the brain was seen, and thus foetal anaemia was suspected. Cordocentesis showed a normal haemoglobin level but reticulocytosis and thrombocytopenia. A foetal infection was excluded. Karyotype and multiplex ligation-dependent probe amplification of subtelomeric regions and for a panel of syndromes were normal. In the following weeks, the condition worsened with a severely abnormal foetal cord blood flow. The head was small with ventriculomegaly. In gestation week 34, an ultrasound showed heart failure, ascites, subcutaneous oedema and hyperperfusion of the brain (V_{max} > 130 cm/s). In addition, oligohydramnios and decreased foetal movements were found. Foetal biometries showed biparietal diameter and head circumference below the 5th centile. The pregnancy ended with intrauterine foetal death in gestation week 35. Neuropathological examination showed multiple calcifications, micro- and macrogliosis and tissue decay.

Patient 3 was the second child of healthy parents; her older sister was healthy. The pregnancy was normal and her birth weight was 3000 g. During her early development, she showed lack of eye contact and hypotonia. She was admitted to hospital 1 month old owing to an episode of apnoea, and from 3 months of age, she had severe irritability and nystagmus. A cerebral CT scan showed multiple periventricular calcifications in the basal ganglia, central pons, medulla oblongata and cerebellum, and cerebral atrophy. At age 6 months, she was microcephalic (−2 to −3 SD), hypertonic and had inverted mammary papillas and an enlarged liver.

Analysis of CSF at age 6 months showed lymphocytosis, 24 million/L (ref, <3 million/L), IFN-alpha was elevated at 5 U/mL (ref. <2 U/mL) and neopterin was elevated at 2008 nmol/L (ref. 12–30 nmol/L). She died 8 months old.

Patient 4 had normal pregnancy and birth at term. His birth weight was 2870 g and birth length 50 cm. He developed normally the first month and was admitted to hospital 4 months old owing to failure to thrive and decreased visual contact. He had developmental regression with feeding problems from age 3 months. He was found to have decreased spontaneous movement with episodes of hypertonia. Seizures were suspected, but an EEG was normal. The ophthalmological examination showed unspecific decreased vision. Cerebral MRI at age 4 months showed leukodystrophy, decreased amounts of grey matter, hypoplasia of corpus callosum and general atrophy.

At age 2 years, he is severely psychomotor retarded and uses a wheelchair. He is hypertonic and has dystonias that are treated with baclofen. He has decreased vision and nystagmus. His height is −2 to −3 SD, and he is moderately microcephalic. He has a gastric tube owing to feeding difficulties and has frequent, almost daily, episodes of vomiting.

Genetic analyses

A genome-wide search for homozygosity in the two siblings revealed one single 15.6 Mb region of homozygosity on chromosome 13, which included *RNASEH2B* (Fig. 1A). Sequence analysis in patient 1 revealed a novel homozygous *RNASEH2B* intronic mutation, c.322-3C>G (Fig. 1B). Parental analysis confirmed the homozygous nature of the mutation. The other three patients were also homozygous for the mutation. Evaluation of the intronic variant *in silico* predicted the destruction of the acceptor splice site at exon 5 (http://www.fruitfly.org/seq_tools/splice.html). In accordance with the prediction, RT-PCR analysis of cDNA from fibroblasts showed predominantly skipping of exon 5 (Fig. 1C,D), leading to an out-of-frame transcript. Several other, less-abundant abnormal splice variants were observed; however, none corresponded to wild-type transcript. The c.322-3C>G mutation is thus not expected to give rise to any functional protein.

Screening of 170 anonymous Faroese controls for the mutation revealed three heterozygous carriers, and thus a carrier frequency of approximately 1.8%. None of the controls were homozygous for the mutation. Assuming Hardy–Weinberg equilibrium, the carrier frequency corresponds to an incidence of AGS in the Faroe Islands of around 1 in 12 300.

The four patients were born during a 10-year period, which results in an observed incidence of around 1 in 2500.

DISCUSSION

A high incidence of AGS of around 1 in 1700 has been reported in the Cree population in North America, where the disorder is caused by a *TREX1* founder mutation (8). The incidence of AGS in the Faroe Islands (1 in 2500 to 12 300) is the second-highest hitherto reported. This is in accordance with the incidence of other diseases caused by founder mutations in the Faroe Islands, for example, Bardet–Biedl syndrome (15). The difference between the observed and calculated incidence may be owing to the relatively small number of controls analysed, and disturbance of Hardy–Weinberg equilibrium by consanguineous mating.

In general, patients reported with *RNASEH2B* mutations have a milder phenotype compared with patients with mutations in *TREX1*, *RNASEH2A* and *RNASEH2C*; in a study of 89 families with AGS, patients with *RNASEH2B* mutations had a significantly later onset of disease, and in five patients the onset was delayed to age 12 months or later (16). Six children had relatively preserved intellectual function with some speech, and one of the patients even had

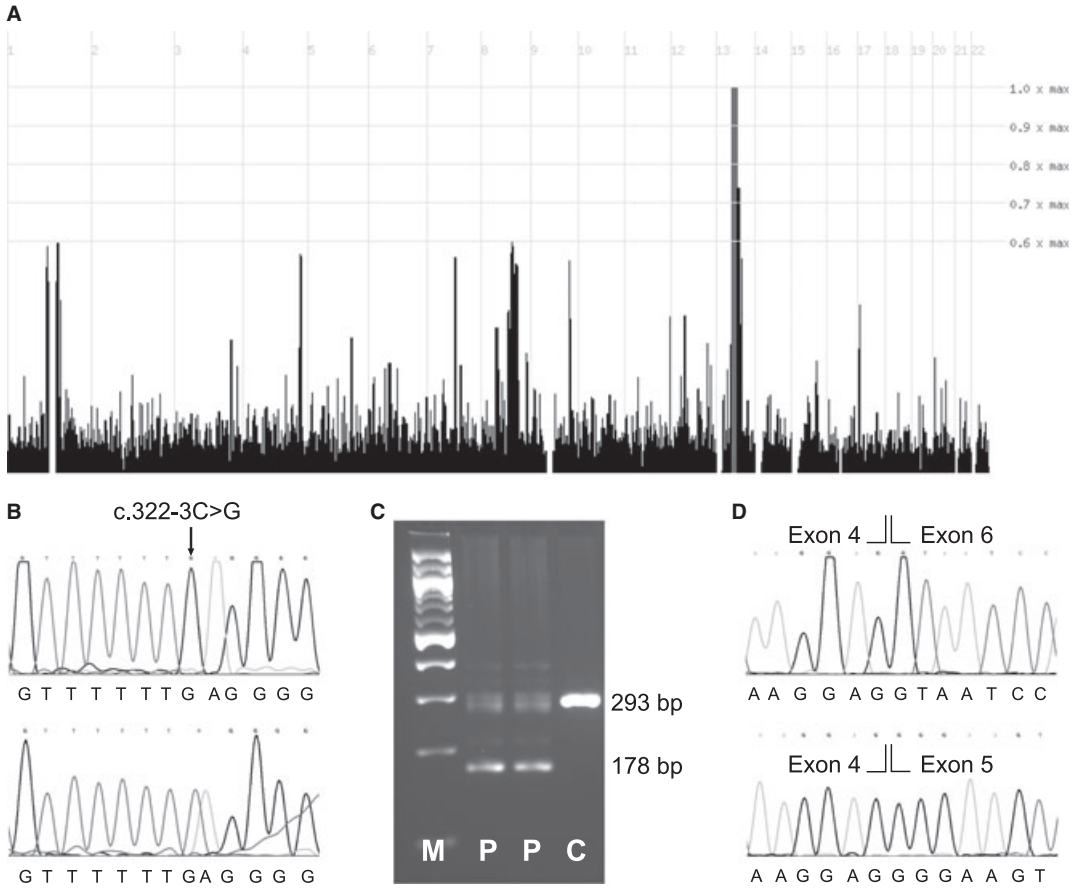

Figure 1 Identification of an RNASEH2B mutation in Faroese patients. (A) Homozygosity mapping in two siblings showing a single 15.6 Mb homozygous region on chromosome 13. (B) Sequencing analysis showing the homozygous c.322-3C>G splice site mutation in patient 1 (top) and the wildtype sequence (bottom). (C) Gel image of cDNA showing a band of 293 bp representing the wild-type (C) and a band of 178 representing skipping of exon 5 (P). M: molecular weight marker. (D) Sequence analysis of cDNA (band of 178 bp cut from gel) showing skipping of exon 5 in the patient (top) and the wild-type sequence (bottom).

normal intelligence and head circumference at age 19 years. In another study, the mean age at onset in patients with *RNASHE2B* mutations was 2–3 months (17). Congenital microcephaly was not reported, but most patients later developed microcephaly and seven of eight patients had skin lesions.

The Faroese AGS patients had a variable phenotype, from a severe prenatal form with intrauterine foetal death to a milder phenotype, albeit still with an early onset, within the first 2–3 months. One patient had skin lesions. Two patients had prenatal onset of disease (IUGR and microcephaly), and two patients died before age 1 year, whereas the other two are alive at 2 and 11 years, respectively. The two patients that are alive have severe psychomotor retardation, growth retardation and microcephaly. Patient 2 had intrauterine onset with unspecific signs of severe nonimmune hydrops, and extreme hyperperfusion and enlarged heart, which are sonographic findings not found in other intrauterine infections. Hydrops is lethal in most cases, and owing to the high carrier frequency of AGS in the Faroe Islands, AGS should be suspected in cases of intrauterine death in the Faroe Islands.

In this study, we identified the same novel *RNASEH2B* splice site mutation, c.322-3C>G, in all four Faroese patients. The previously identified *RNASEH2B* mutations comprise altogether 20 mutations (missense, nonsense and splice site) (7) with all patients harbouring at least one missense mutation. The severe phenotype of the Faroese patients compared with the previously reported patients with *RNASEH2B* mutations may be caused by the presence of two null alleles (although some residual normal splicing cannot be ruled out), whereas patients with one or two missense mutations may have some, albeit abnormal, RNASEH2B proteins, and hence some residual activity of RNASEH2B, explaining their milder phenotype.

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COMPETING INTERESTS

None.

PATIENT CONSENT

Obtained.

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