Phenotypic variability including Behçet's disease-like manifestations in DADA2 patients due to a homozygous c.973-2A>G splice site mutation

G.T.J. van Well[1], B. Kant[2], A. van Nistelrooij[3], S. Sirma Ekmekci[4], S.S.V. Henriet[5], E. Hoppenreijs[6], M. van Deuren[7], J. van Montfrans[8], S. Nierkens[9], A. Gül[10], M.E. van Gijn[11]

Affiliations: see page S-145.

*Gijs T.J. van Well, MD, PhD**
*Benjamin Kant**
Annabel van Nistelrooij, MD
Sema Sirma Ekmekci, PhD
Stefanie S.V. Henriet, MD, PhD
Esther Hoppenreijs, MD
Marcel van Deuren, MD, PhD
Joris van Montfrans, MD, PhD
Stefan Nierkens, PhD
*Ahmet Gül, MD***
*Mariëlle E. van Gijn, PhD***

**These authors contributed equally to this work and should be considered as shared first authors.*
***These authors contributed equally to this work and should be considered as shared last authors.*

Please address correspondence to:
Dr Ahmet Gül, Dept. of Internal Medicine, Istanbul Faculty of Medicine, Istanbul University, 34390 Istanbul, Turkey.
E-mail: agul@istanbul.edu.tr
and
Dr Mariëlle E. van Gijn, Dept. of Genetics, University Medical Centre Groningen, 9700 Groningen, The Netherlands.
E-mail: m.e.van.gijn@umcg.nl

Received on August 4, 2019; accepted in revised form on October 28, 2019.

Clin Exp Rheumatol 2019; 37 (Suppl. 121): S142-S146.

Key words: ADA2, DADA2, systemic inflammation, vasculitis, polyarteritis nodosa, early-onset stroke, Behçet's disease-like manifestations, splice site mutation, genotype, phenotype

Funding. This work was partially supported by Scientific Research Projects Coordination Unit of Istanbul University, Turkey (grant no. 55150 to S.S. Ekmekci, and A. Gül).

Competing interests: see page S-145.

ABSTRACT

Objective. *To describe phenotypic and functional characteristics of patients with the homozygous c.973-2A>G splice site mutation in the adenosine deaminase 2 (*ADA2*) gene (rs139750129), resulting in deficiency of ADA2 (DADA2).*

Methods. *We present case synopses of six patients from three unrelated families. Clinical data were analysed and next-generation sequencing (NGS) was performed. We also tested for aberrant RNA splicing and measured ADA2 enzyme activity.*

Results. *One family had common DADA2 symptoms, whereas Behçet's disease-like manifestations were observed in the other two families. We detected the homozygous c.973-2A>G splice site mutation in* ADA2 *in all patients tested.* ADA2 *enzyme activity was significantly lower in patients than in healthy controls, but no correlation between* ADA2 *activity levels and disease severity was observed. Aberrant splicing was detected in a minority of mRNA transcripts, but the formation of other, undetected, aberrant splicing products could not be excluded. Patients were treated with TNF-α inhibitors to prevent recurrence of inflammatory findings including cerebral vasculitis-associated stroke.*

Conclusion. *We describe three families with the same homozygous splice site mutation in* ADA2 *and observed a novel combination of manifestations resembling Behçet's disease. This further expands the range of phenotypes caused by* ADA2 *mutations, although no complete genotype-phenotype association could be determined. Even without active disease, the risk of stroke should be addressed in making decisions regarding treatment of DADA2 patients.*

Introduction

Deficiency of adenosine deaminase 2 (DADA2) is a monogenic vasculitic syndrome associated with systemic inflammation. The most prevalent manifestations of DADA2 are early-onset strokes and polyarteritis nodosa-like findings, along with fever, livedoid rash and splenomegaly. Immunodeficiencies and haematological manifestations have also been described along with the significant expansion of phenotypes since its first definition (1-6).

DADA2 is an autosomal recessively inherited disease caused by loss-of-function mutations in *ADA2* gene, previously known as *CECR1*, encoding for adenosine deaminase 2 (ADA2) enzyme (7), which is produced by activated myeloid cells and involved in growth and development of mononuclear cells (8). It may also be involved in regulation of extracellular adenosine, which has an important function in tissue damage response (3, 9). The described *ADA2* mutations have been associated with reduced enzymatic activity, leading to defective inflammatory cell regulation as the main cause of manifestations (1).

Over 50 *ADA2* mutations have been described so far (3), but no clear genotype-phenotype correlations have yet been established. Phenotypic variability was reported in siblings carrying the same mutations (2, 10, 11), implying that epigenetic and environmental factors are also involved.

We hereby present six patients from three families who ascertained by investigations for potential hereditary autoinflammatory conditions and showed an identical homozygous c.973-2A>G splice site mutation in *ADA2*. We describe their characteristics with an em-

phasis on Behçet's disease-like manifestations as an expansion of the clinical spectrum.

Methods

For each patient, written informed consent was obtained for genetic testing and publication according to the principles of the Declaration of Helsinki.

NGS sequencing

From genomic DNA, coding sequences of the relevant genes (Supplementary Table S1) were enriched using a custom Agilent SureSelectXT kit (Agilent, Santa Clara, CA, USA). DNA sequencing was performed using the SOLiD 5500XL Genetic Analyser (ThermoFisher, Waltham, MA, USA). Analysed 50 bp DNA fragment reads were mapped and compared to the human reference genome (hg19) to detect sequence variation. Variant interpretation was performed using Alissa Interpret (Agilent) and Alamut Visual (Interactive Biosoftware, Rouen, France). Genotyping of patient 3.1 was performed similarly, using the Fever and Autoinflammatory Diseases NGS panel, an Illumina sequencer (San Diego, CA, USA) and Sophia Genetics software (Saint-Sulpice, Switzerland). Sanger sequencing was performed to confirm the results and for testing variants in family members. Primer sequences are available upon request.

RNA splicing

mRNA was isolated from blood samples using PAXgene kit (Qiagen, Hilden, Germany) and subjected to hexamer primed reverse transcriptase PCR using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA products were amplified by PCR using primers for exons 6 and 8. Primer sequences are available upon request. PCR products were separated according to size using 2% agarose gel and also Sanger sequenced.

ADA2 activity

ADA2 enzyme activity measurement was performed in serum/plasma as described elsewhere (11). In brief, we modified the procedure of an ADA measurement kit (Diazyme) by adding Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; Sigma-Aldrich) for inhibition of ADA1. Measurement was performed in a Spectramax M2 plate reader at 37°C and 550 nm after 15 minutes. Ten anonymised adult volunteers were used as healthy controls.

Table I. Clinical manifestations and laboratory results of included DADA2 patients. In each cell it is stated whether a patient has or has had a certain manifestation. Values in the last column represent the percentage of DADA2 patients with this manifestation known in literature [3]. Blank cells represent missing data.

	1.1	1.2	2.1	2.2	3.1	3.2	DADA2 patients (%)
neurologic involvement	no	no	no	no	yes	no	51
Fever	yes	yes	yes	yes	yes	no	50
Livedo	no	no	yes	yes	no	yes	50
Arthralgia/Arthritis	yes	no	yes	no	yes	yes	36
Polyarteritis Nodosa(-like)	no	no	yes	yes	no	no	34
Gastrointestinal involvement	yes	yes	no	no	no	no	33
splenomegaly	yes	no	no	no	yes	no	29
Digital necrosis	no	no	no	no	no	no	22
hypogammaglobulinaemia	no	no	yes	no	no	no	22
Non-specific rash	yes	yes	no	no	no	no	20
(recurrent) infections	no	yes	no	no	no	no	20
hepatomegaly	yes	no	no	no	no	no	19
Erythema nodosum	yes	no	yes	no	yes	yes	14
lymphadenopathy	no	no	no	no	no	no	9
oral ulcers	yes	no	no	no	no	yes	8
genital ulcers	no	no	no	no	yes	no	
ophthalmologic involvement	no	no	no	no	yes	no	
low i_gM			yes	no			18
low i_gA			yes	no			12
low i_gG			yes	no			
low b-cells (CD20+/Memory)			yes	yes			11
low Nk cells			no	no			2
low $CD4^+$ cells			no	no			
low $CD8^+$ cells			no	no			
anaemia	yes		no				13
leukopenia	no	no	yes		yes	yes	5
high crp/esr	yes		yes	no	yes	no	
high ace							
high asat/alat/af			yes	no	no	no	
low albumin			no		no	no	
lac presence					no	no	
ana/ena positive	no		no		no	no	
anca positive			no	no	no	no	
high hla*B51	no		no		no	no	
ADA2 homozygous mutation	yes	yes	yes	yes	yes	yes	
low ada2 activity	yes	yes	yes	yes			

Results

Clinical synopsis

Clinical and laboratory features of six patients from three unrelated families are summarised in Table I. These investigations included routine immunological evaluations, ADA2 enzyme activity measeurements, and further genetic evaluations of the ADA2 gene variants conducted in DADA2 patients.

Family 1 consists of two brothers of Dutch ancestry. The oldest boy (1.1) presented with recurrent mild vasculitic features since the age of 1. At 5, his disease progressed with high spiking fevers, erythema nodosum, arthralgia, severe aphthous stomatitis and ulcerative lesions in his gut, for which he was successfully treated with azathioprine. At 9, he was diagnosed as DADA2. His youngest brother (1.2) had the same mutation but described only mild exanthema.

Family 2 consists of a brother and sister of Dutch ancestry, unrelated to family 1. The younger brother (2.1) presented with recurrent fevers accompanied by livedo reticularis, erythema nodosum, and arthralgia at the age of 6. Histol-

ogy revealed necrotising vasculitis of a small arteria with granulomatous reaction. He was treated for polyarteritis nodosa (PAN) with corticosteroids. He developed hypogammaglobulinaemia with low numbers of B-cells. Due to recurrent disease activity, methotrexate (MTX) was started, followed by high dose immunoglobulin, mycophenolate mofetil (MMF) and infliximab. He had no history of stroke. After documentation of DADA2, immunoglobulin and infliximab was continued with good response. His older sister (2.2) presented with an isolated cutaneous PAN and was treated with steroids. After she was diagnosed with DADA2, despite her mild symptoms, anti-TNF treatment was initiated due to the risk of stroke.

Family 3 consists of a female patient of Turkish origin (3.1). She presented with painful legs and acute onset upper eyelid ptosis due to left oculomotor nerve palsy at age of 3. At 13, she developed recurrent genital ulcers, arthralgias and fever. Colchicine was initiated for suspected Behçet's disease (BD), and her ulcers responded. At the age of 21, she started to experience erythema-nodosum and vertigo attacks that responded to short courses of corticosteroids. At age of 23, she was given canakinumab 150 mg monthly and azathioprine to control fever and neurologic manifestations and reduce corticosteroid requirement. After diagnosing DADA2, her treatment was successfully switched to adalimumab.

Her sister (3.2) presented with erythema nodosum at the age of 10 and recurrent oral aphthous ulcers at 12, for which she was diagnosed as possible BD and treated with colchicine. She was evaluated at age 23 for progression of oral ulcers and arthralgia. She also had folliculitis on her trunk and livedoid rash in hands. After DADA2 diagnosis, anti-TNF treatment was planned.

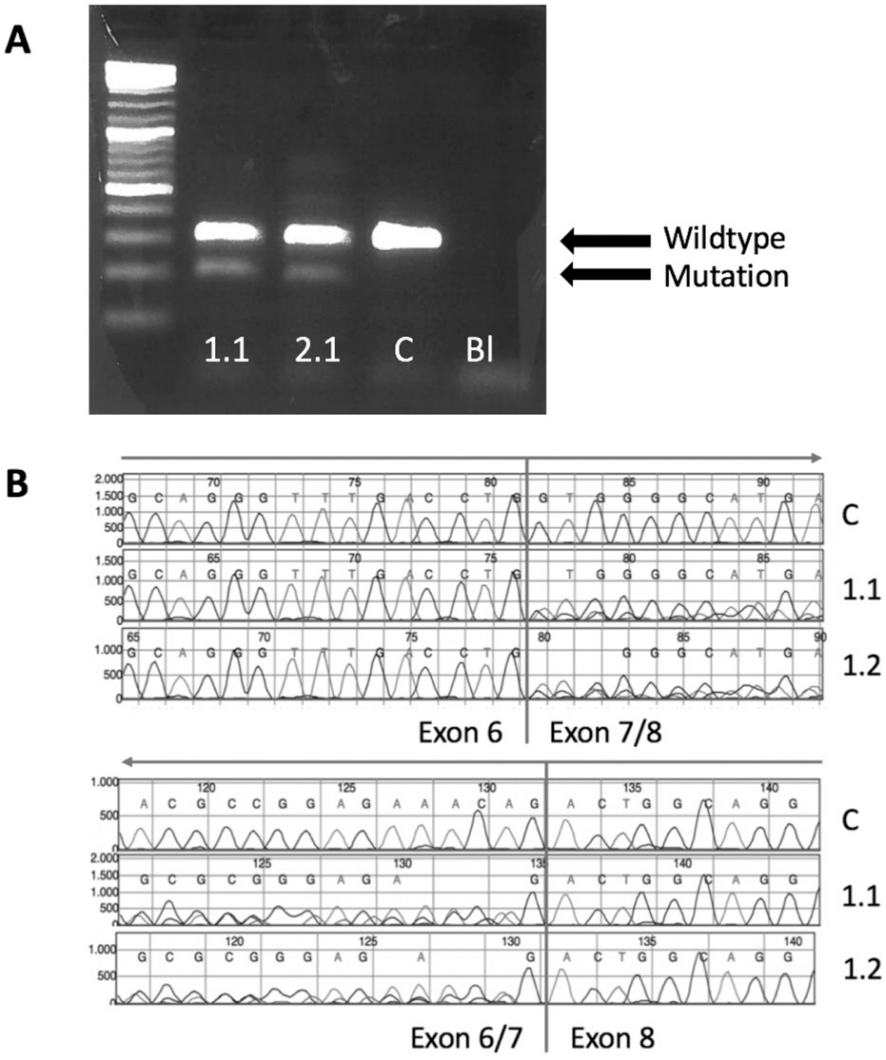

Fig. 1. mRNA transcripts of patients with a homozygous c.973-2A>G p.(?) splice site mutation.
A: Gel image of the amplified cDNA PCR products of patients 1.1, patient 2.1, healthy control sample (C), and a water control sample (Bl). In patients 1.1 and 2.1 two bands were detected, which included both full-length (wild type) and a shorter exon-skipped bands. Image analysis by Image Lab software (Bio-Rad) revealed that the intensity of the shorter band was approximately 25% of the wildtype band. Healthy control (C) had only wildtype band.
B: cDNA sequences in forward (top) and reverse (bottom) direction for patients 1.1 and 1.2, a healthy control sample (C). Sequence of the healthy control sample shows continuation of exon 6 with exon 8 in forward direction (upper panel) and continuation of exon 8 with exon 7 in the reverse direction. However, in patients 1.1 and 1.2, exon 6 sequence continues with both exon 7 and exon 8 overlapping sequences in the forward direction, and exon 8 continues with both exon 6 and exon 7 sequences in the reverse direction due to presence of both wildtype and exon 7 skipped mRNA transcripts.

Genetic screening

NGS gene panel testing revealed homozygosity for the rare c.973-2A>G p.(?) splice site mutation in the *ADA2* gene (NM_001282225.1, rs139750129), in patients 1.1, 2.1 and 3.1, which is present in 0,02% of alleles in the European population (ExAC database). No other relevant autoinflammatory variant was detected. Patients 1.2, 2.2 and 3.2, all symptomatic, were found to have the same homozygous mutation. All parents were found to be heterozygous carriers, as were three siblings of patients 3.1 and 3.2.

RNA splicing

The c.973-2A>G mutation was predicted to result in total disruption of the acceptor splice site of intron 6 by MaxEntScan (10) (Suppl. Fig. 1). Although the mutation was present on both alleles in all patients, only ±25% of the mRNA transcripts of patients 1.1 and 1.2 show skipping of exon 7 (Fig. 1), and the majority of the cDNA products were wildtype. These findings were confirmed in patients 3.1 and 3.2 (data not shown).

ADA2 activity

ADA2 enzyme activities were measured for patients 1.1, 1.2, 2.1 and 2.2 at least three different times over one

year. The mean ADA2 activity level for our patients was 0.3 U/L (range 0.0–1.4 U/L) compared to 5.5 U/L (range 2.8–8.3 U/L) for healthy controls. In all patients, amount of residual ADA2 activity could not be linked to disease severity.

Discussion

We describe six patients from three unrelated families sharing the same homozygous splice site mutation in *ADA2* with varying disease manifestations and severity. Although compound heterozygosity for the c.973-2A>G and other *ADA2* mutations has previously been reported, homozygosity for this mutation was not described before (13-16). Next to typical DADA2 symptoms, some of our patients developed recurrent oral and/or genital aphthosis as well as erythema nodosum, folliculitis and arthralgias as well as neurologic findings (13, 14). Mucocutaneous lesions and less frequently recurrent uveitis mimicking BD have been described in other monogenic autoinflammatory disorders, including mevalonate kinase deficiency and haplotype insufficiency for A20. Similarly, erythema nodosum and oral aphthous ulcers were previously reported in DADA2 patients without splice site mutations (15), but none of them described in c.973-2A>G mutation carriers (13, 15, 16). Also, homozygosity has previously been described for the c.973-1G>A splice site mutation in *ADA2* (15), however this patient was reported to have different phenotype suggesting functional variations between splice variants. Therefore, relatively higher frequency of Behçet disease-like symptoms including neurologic findings in currently described families may be important, but no clear genotype-phenotype association could be determined.

A correlation between ADA2 activity and disease severity has been mentioned before (3, 11). However, our patients had low to absent ADA2 activity levels, regardless of the patient's phenotypes. This suggests existence of additional mechanisms in disease pathogenesis as potential triggers of manifestations.

Despite very low ADA2 levels, we detected exon 7 skipping in only ±25% of transcripts using the primers for exon 6 and 8 in both members of family 1 and 3, not in all as predicted. However, this limited analysis does not rule out other potential mechanisms leading to mis-spliced variants and reduced translation.

TNF-α inhibitors have been reported to be effective in inducing and maintaining remission from various typical and atypical DADA2 symptoms and are currently regarded as treatment of choice for all symptomatic patients (2, 11, 17). Although the patients from the first two families were free from neurological manifestations and MRIs showed no abnormalities, treatment with TNF-α inhibitors was initiated because of the risk of stroke in untreated patients. For patient 3.1, after the identification of the *ADA2* mutation, canakinumab treatment was switched to anti-TNF-α treatment because of the favourable observations in other patients.

In conclusion, we describe a homozygous *ADA2* splice site mutation in 3 unrelated families with novel combination of common DADA2 and BD-like phenotypic expressions in two of them. Disease severity greatly varied between families and siblings due to not yet discovered mechanisms. It is important to consider *ADA2* mutation screening for a broad range of phenotypes including BD-like symptoms with or without stroke or other immunologic manifestations, especially in patients with juvenile disease onset.

Acknowledgements

We gratefully thank the UMC Utrecht NGS team for performing NGS and Rumo Jansen for performing functional testing of mRNA splicing. Lysette Ebskamp is kindly acknowledged for performing the ADA2 activity measurements.

Affiliations

[1]Department of Paediatrics and School for Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands; [2]Department of Genetics, University Medical Centre Utrecht, The Netherlands; [3]Department of Paediatrics, Maastricht University Medical Centre, Maastricht, The Netherlands; [4]Department of Genetics, Institute for Experimental Medicine, Istanbul University, Turkey; [5]Department of Paediatric Infectious Diseases and Immunology, Amalia Children's Hospital, Radboud University Medical Centre, Nijmegen, The Netherlands; [6]Department of Paediatric Rheumatology, Radboud University Medical Centre, Nijmegen, The Netherlands; [7]Department of Internal Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands; [8]Department of Paediatric Immunology, University Medical Centre Utrecht, The Netherlands; [9]Centre for Translational Immunology, University Medical Centre Utrecht, The Netherlands; [10]Department of Internal Medicine, Istanbul Faculty of Medicine, Istanbul University, Turkey; [11]Department of Genetics, University Medical Centre Groningen, The Netherlands.

Competing interests

J. van Montfrans has served on the Advisory Board for Takeda. A. Gül has received consultancy payments or honoraria from Abbvie and Novartis. The other co-authors have declared no competing interests.

References

1. ZHOU Q, YANG D, OMBRELLO AK *et al*.: Early-onset stroke and vasculopathy associated with mutations in ADA2. *N Engl J Med* 2014; 370: 911-20.
2. NAVON ELKAN P, PIERCE SB, SEGEL R *et al*.: Mutant Adenosine Deaminase 2 in a polyarteritis nodosa vasculopathy. *N Engl J Med* 2014; 370: 921-31.
3. MEYTS I, AKSENTIJEVICH I: Deficiency of Adenosine Deaminase 2 (DADA2): Updates on the phenotype, genetics, pathogenesis, and treatment. *J Clin Immunol* 2018; 38: 569-78.
4. LEE PY: Vasculopathy, immunodeficiency, and bone marrow failure: The intriguing syndrome caused by deficiency of Adenosine Deaminase 2. *Front Pediatr* 2018; 6: 282.
5. RIAZI MA, BRINKMAN-MILLS P, NGUYEN T *et al*.: The human homolog of insect-derived growth factor, CECR1, is a candidate gene for features of cat eye syndrome. *Genomics* 2000; 64: 277-85.
6. ZAVIALOV AV, GRACIA E, GLAICHENHAUS N, FRANCO R, ZAVIALOV AV, LAUVAU G: Human adenosine deaminase 2 induces differentiation of monocytes into macrophages and stimulates proliferation of T helper cells and macrophages. *J Leukoc Biol* 2010; 88: 279-90.
7. KARMOUTY-QUINTANA H, XIA Y, BLACKBURN MR: Adenosine signaling during acute

and chronic disease states. *J Mol Med* 2013; 91: 173-81.
8. UETTWILLER F, SARRABAY G, RODERO MP *et al*.: ADA2 deficiency: case report of a new phenotype and novel mutation in two sisters. *RMD Open* 2016; 2: e000236.
9. MONTFRANS JM VAN, HARTMAN EAR, BRAUN KPJ *et al*.: Phenotypic variability in patients with ADA2 deficiency due to identical homozygous R169Q mutations. *Rheumatology* 2016; 55: 902-10.
10. YEO G, BURGE CB: Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J Comput Biol* 2004; 11: 377-94.
11. SPRINGER JM, GIERER SA, JIANG H *et al*.: Deficiency of adenosine deaminase 2 in adult siblings: Many years of a misdiagnosed disease with severe consequences. *Front Immunol* 2018; 9: 1361.
12. RAMA M, DUFLOS C, MELKI I *et al*.: A decision tree for the genetic diagnosis of deficiency of adenosine deaminase 2 (DADA2): a French reference centres experience. *Eur J Hum Genet* 2018; 26: 960-71.
13. SCHEPP J, PROIETTI M, FREDE N *et al*.: Screening of 181 patients with antibody deficiency for deficiency of adenosine deaminase 2 sheds new light on the disease in adulthood. *Arthritis Rheum* 2017; 69: 1689-700.
14. TROTTA L, MARTELIUS T, SIITONEN T *et al*.: ADA2 deficiency: Clonal lymphoproliferation in a subset of patients. *J Allergy Clin Immunol* 2018; 141: 1534-7.
15. ALSULTAN A, BASHER E, ALQANATISH J, MOHAMMED R, ALFADHEL M: Deficiency of ADA2 mimicking autoimmune lymphoproliferative syndrome in the absence of livedo reticularis and vasculitis. *Pediatr Blood Cancer* 2018; 65: e26912.
16. MACCORA I, FRONGIA I, AZZARI C, RICCI S, CIMAZ R, SIMONINI G: A misleading case of deficiency of adenosine deaminase 2 (DADA2): the magnifying glass of the scientific knowledge drives the tailored medicine in real life. *Clin Exp Rheumatol* 2018; 36 (Suppl. 115): S146.
17. LAMPRECHT P, HUMRICH JY, DIEBOLD I, RIEMEKASTEN G: Diagnosis of deficiency of adenosine deaminase 2 with early onset polyarteritis nodosa in an adult patient with a novel compound heterozygous CECR1 mutation. *Clin Exp Rheumatol* 2018; 36 (Suppl. 111): S177.