Functional characterisation of a novel homozygous p.Y227C *ADA2* variant in a child with deficiency of adenosine deaminase type 2

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Abstract

Objective

Deficiency of adenosine deaminase 2 (DADA2) is a rare inflammatory disorder caused by biallelic loss-of-function mutations in ADA2. We sought to functionally characterise a novel homozygous ADA2 variant, p.Y227C (c.680A>G), identified in a six-year-old patient presenting with recurrent fevers, erythema nodosum, and tumor necrosis factor (TNF) inhibitor-responsive myositis

Method

Monocyte-derived macrophages from the patient were analysed for ADA2 protein expression, enzymatic activity, and TNFα secretion. To model the inflammatory phenotype in vitro, THP-1 cells were engineered to express the p.Y227C variant. Lentiviral gene correction with wild-type ADA2 was performed to assess rescue of enzymatic function and inflammatory responses.

Results

Patient-derived macrophages exhibited markedly reduced ADA2 protein levels and enzymatic activity, accompanied by increased TNFα secretion. THP-1 cells expressing the p.Y227C variant recapitulated this proinflammatory phenotype. Lentiviral reconstitution with wild-type ADA2 restored protein expression and enzymatic activity and normalised TNFα release.

Conclusion

The p.Y227C ADA2 variant is pathogenic and promotes inflammation through loss of ADA2 function. Functional rescue following gene correction confirms the causal role of this mutation and underscores the therapeutic potential of restoring ADA2 activity in DADA2.

Key words

adenosine deaminase 2, deficiency of adenosine deaminase 2, p.Y227C variant, autoinflammatory disease, TNF-alpha

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Introduction

Deficiency of adenosine deaminase 2 (DADA2) is a rare autosomal recessive autoinflammatory disorder caused by mutations in the *ADA2* gene, leading to reduced or absent enzymatic activity of ADA2 (1-6). This condition is characterised by a spectrum of clinical manifestations, including vasculitis, recurrent strokes, immune dysregulation and bone marrow failure (7-9). Although many *ADA2* variants have been associated with disease, the functional consequences of several others remain unconfirmed (9). In this study, we characterised the functional impact of a novel homozygous p.Y227C (c.A680G) variant in *ADA2* identified in a 6-year-old male patient presenting with recurrent fevers, myositis, erythema nodosum and elevated inflammatory markers.

Methods

This study was approved by the Bloomsbury Ethics Committee (no. 08H071382). Written informed consent and age-appropriate assent were obtained. Genetic sequencing was performed using the routine NHS diagnostic gene panel for autoinflammatory disorders (R413.1), available in the national genomic test directory for rare and inherited diseases (https://www.england.nhs.uk/publication/national-genomic-test-directories/).

Monocyte-derived macrophages (MDMs) were generated from PBMCs via Ficoll separation. PBMCs (4×10^9) were plated in RPMI-1640 medium supplemented with glutamine (Sigma-Aldrich, St. Louis, MO) and 10% foetal calf serum (FCS). After 2 h, non-adherent cells were removed to enrich for monocytes. Adherent cells were cultured with macrophage colony-stimulating factor (M-CSF, 50 ng/mL; PeproTech, Rocky Hill, NJ) to induce differentiation into macrophages. On day 7, cells were harvested, stained for the macrophage-specific marker CD68, and analysed by flow cytometry using a CytoFLEX instrument (Beckman Coulter) (7). ADA2 knockout (KO) THP-1 cells were generated via CRISPR/Cas9 and transfected using Lipofectamine 2000 (7). Site-directed mutagenesis (New England Biolabs) was used to introduce the *ADA2* c.A680G variant (corresponding to p.Y227C) into the ADA2 construct.

THP-1 cells were differentiated into macrophages following incubation with PMA and subsequently polarised into M1 macrophages using LPS/IFN-γ stimulation.

MDMs and THP-1 cells were also transduced using a third-generation lentiviral vector with a pCCL backbone containing codon-optimized wild type human ADA2 cDNA under the EFS promoter tagged with GFP (LV-ADA2-eGFP) for gene correction or mock transduced with an empty vector (LV-eGFP) as a control (7). Lentiviral particles were produced via transient transfection of HEK293T cells as previously described (6).

Genomic DNA was extracted from cells on day 14 post-transduction (DNeasy Blood and Tissue Kit, Qiagen). Viral copy number (VCN) was determined by TaqMan qPCR targeting the PSi vector sequence and the human albumin reference gene following standard protocols (10).

ADA2 expression was assessed by Western Blotting and flow cytometry (7). ADA2 activity in cell culture supernatants and serum was quantified with selective ADA1 inhibition with EHNA distinguished ADA2 activity (Diazyme). TNF-α in cell culture supernatants was quantified using a Meso Scale Discovery multiplex kit or ELISA (Thermo Fisher).

Identification of a novel homozygous ADA2 Variant (p.Y227C): clinical presentation and genetic sequencing results for index case

A six-year-old Nigerian boy of non-consanguineous parents was referred to our rheumatology clinic at Great Ormond Street Hospital for Children NHS Foundation Trust with a 12-month history of myalgia, arthralgia, headaches, erythema nodosum and recurrent fevers. Laboratory investigations revealed elevated C reactive protein (CRP; median 48 mg/L, reference range <10 mg/L) and erythrocyte sedimentation rate (ESR; median 41 mm/h, reference range <15 mm/h)) on

Table I. ADA2 expression, enzyme activity and TNF-α secretion in monocyte derived macrophages (MDM) obtained from a patient with DADA2 homozygous for the p.Y227C *ADA2* variant. MDM from healthy controls and MDM derived from this patient (uncorrected or gene corrected post transduction with the LV-ADA2-EFS-eGFP vector) were examined. ADA2 expression (mean fluorescent intensity) in the MDM was measured by flow cytometry. Results are presented as mean± SEM when more than one replicate was available.

Cells	ADA2 expression	ADA2 activity (U/L)	TNF-α (pg/ml)
Healthy controls MDM	10.21 ± 0.68	9.42 ± 1.34	1.97 ± 0.52
DADA2 (p.Y227C/p. Y227C) patient derived MDM- uncorrected	6.37	0	9.75
DADA2 (p.Y227C/p. Y227C) patient derived MDM-gene corrected	10.96	11.56	3.68

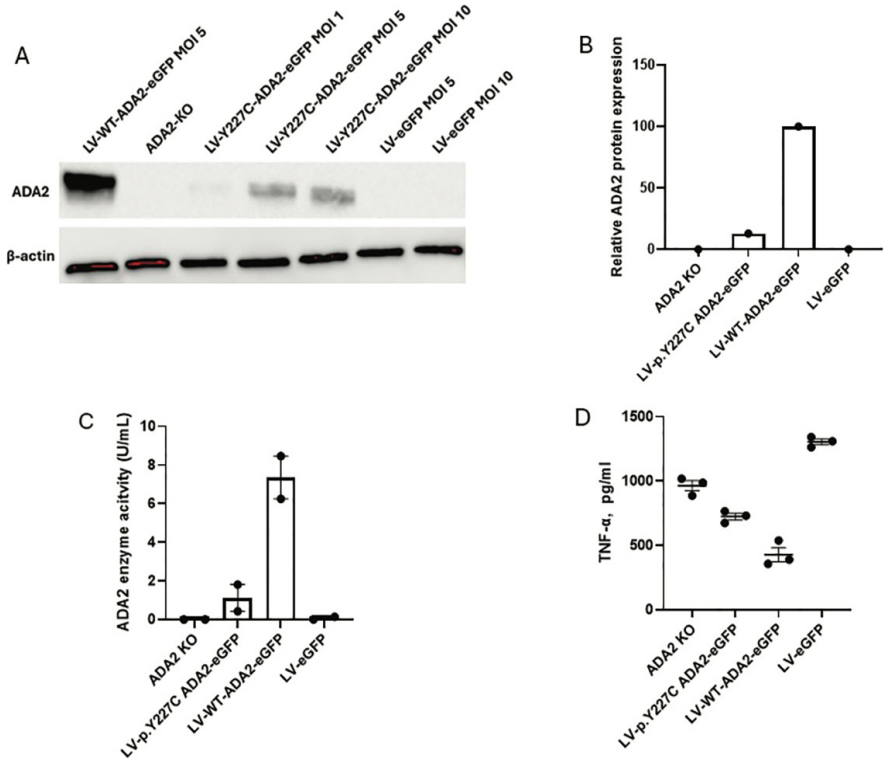

Fig. 1. Functional analysis of p.Y227C ADA2 variant in THP-1 Cells. **A, B**: Western blot showing the relative ADA2 protein expression in THP-1 cells transduced with lentiviral vectors (LV) expressing wild-type ADA2 (WT-ADA2-eGFP), the p.Y227C *ADA2* variant (p.Y227C-ADA2-eGFP), or empty vector (LV-eGFP). ADA2-knockout (KO) cells served as controls. Western blot results (**A**) are shown for different MOI (1-10). Protein quantification plot (**B**) is shown for MOI 5. The p.Y227C variant resulted in significantly reduced ADA2 protein expression (<20% of wild type levels). (**C**) ADA2 enzymatic activity in cell culture supernatants under the same conditions. Enzymatic activity was markedly reduced in cells expressing the p.Y227C variant. (**D**) Cells expressing the p.Y227C variant secreted elevated TNF-α, consistent with a pro-inflammatory phenotype. Results are shown as median and range when replicates were available.

several occasions; negative antinuclear antibodies (ANA) and anti-neutrophil cytoplasmic antibodies (ANCA), normal C3, C4 complement levels, normal complement function and immunoglobulin levels, negative myositis associated antibodies. Chest radiograph showed no lung changes; abdominal ultrasound revealed mild hepatomegaly and whole-body magnetic resonance scan (MRI) showed diffuse myositis. Brain MRI and echocardiography were normal. He had a muscle biopsy that showed non-specific inflammation. Skin biopsy revealed mild perivascular lymphocytic infiltrate in the superficial and deep dermis with associated focal mild panniculitis. Bone marrow aspiration showed myeloid hyperplasia likely secondary to systemic inflammation. Potential differential diagnoses included juvenile dermatomyositis, polyarteritis nodosa, systemic autoinflammatory diseases including monogenic interferonopathies.

He was started on oral prednisolone 2 mg/kg/day with a plan to taper over 3-6 months and methotrexate sc 15 mg/m2 weekly and genetic sequencing was undertaken as part of the diagnostic work up. This revealed a novel homozygous variant in the *ADA2* gene: c.A608G (p.Y227C), based on transcript NM_001282225.1. ADA2 enzyme activity was undetectable 0 U/L (healthy control range 5-18 U/L). With a possible diagnosis of DADA2, he was started on the TNF inhibitor adalimumab (given subcutaneously at 40 mg every fortnight). His symptoms have subsequently completely resolved, and he currently remains well 4 years since the initial diagnosis.

Markedly reduced ADA2 expression and enzymatic activity in patient monocyte derived macrophages (MDM)

Monocytes from the patient were differentiated into MDM and compared with those from healthy donors. Flow cytometry revealed reduced ADA2 protein expression in patient MDM (mean fluorescence intensity: 6.37) versus healthy controls (10.21±0.61) (Table I). Correspondingly, ADA2 enzymatic activity was undetectable in the patient's MDM culture supernatants. These findings confirmed that the homozygous p.Y227C variant is associated with severe loss of ADA2 protein and function in primary immune cells, consistent with a pathogenic loss-of-function effect.

ADA2-Y227C variant confers loss of function in ADA2-knockout THP-1 macrophages

To directly assess the functional consequence of the p.Y227C variant, we transduced ADA2-knockout (KO) THP-1 cells with lentiviral vectors encoding either wild-type ADA2, ADA2-Y227C, or a control empty vector. In macrophages derived from these cells:

ADA2-Y227C protein expression was <20% of wild-type ADA2 levels, as measured by western blotting and flow cytometry (Fig. 1A-B). Enzymatic activity of ADA2-Y227C was <15% of wild-type, consistent with a severe loss-of-function effect (Fig. 1C). This validated the deleterious impact of the p.Y227C substitution in a controlled cellular model.

Lentiviral gene correction restores ADA2 expression and function

To assess the reversibility of the defect, patient-derived MDM were transduced with the LV-ADA2-EFS-eGFP vector encoding wild-type ADA2. Following transduction, ADA2 protein expression was fully restored to levels comparable to healthy controls. ADA2 enzymatic activity returned to within the normal range (Table I). These rescue experiments provide strong functional evidence that the p.Y227C variant is causative of the observed loss of ADA2 function.

Increased TNF-α production in ADA2-Y227C expressing macrophages

Macrophages expressing the ADA2-Y227C variant exhibited a pro-inflammatory phenotype, with significantly elevated TNF-α secretion: THP-1 MDM expressing ADA2-Y227C released increased levels of TNF-α, mean of 723.9 pg/mL (range: 674.6–776.1), compared to 427.9 pg/mL in wild-type ADA2 MDM (range: 356.9–537.8, p=0.03) (Fig. 1D). Patient-derived MDMs released 9.75 pg/mL of TNF-α, markedly higher than healthy controls (1.97±0.52 pg/mL). TNF-α release was suppressed upon gene correction with wild-type ADA2 in both models. These results confirm that the ADA2-Y227C variant not only disrupts ADA2 function but also drives a pro-inflammatory macrophage response, providing a mechanistic link to the clinical phenotype and therapeutic response.

Discussion

We report the identification and functional characterisation of a novel homozygous *ADA2* variant, p.Y227C, in a child with systemic autoinflammatory disease. Functional assays in both patient-derived cells and an engineered THP-1 model demonstrated severely impaired ADA2 expression and enzymatic activity. This loss of function was accompanied by heightened TNF-α production, indicating a shift toward a pro-inflammatory macrophage phenotype.

Importantly, these effects were reversed upon gene correction with lentiviral-mediated wild-type ADA2 expression, establishing the pathogenicity of the p.Y227C variant. Clinically, the patient's sustained remission with TNF inhibition further supports a causal relationship between ADA2 dysfunction and inflammation.

Recent work characterising undifferentiated recurrent fever syndromes, including the comprehensive review and survey by Batu *et al*., further emphasises the clinical heterogeneity within autoinflammatory diseases and the importance of distinguishing monogenic entities such as DADA2 from broader inflammatory phenotypes (11). Our findings add to the expanding evidence base that DADA2 arises from ADA2 loss-of-function mutations leading to dysregulated macrophage activation and cytokine overproduction. This study highlights the utility of combining patient-derived and *in vitro* models for functional validation of novel variants and underscores the importance of early genetic diagnosis for targeted treatment.

In conclusion the p.Y227C *ADA2* variant is a pathogenic loss-of-function mutation associated with severe reduction in ADA2 expression and enzymatic activity, and with increased pro-inflammatory cytokine production. Functional correction via lentiviral gene transduction and clinical response to TNF blockade confirm the relevance of this variant in DADA2 pathogenesis and support its inclusion in diagnostic variant panels.

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