

## ORIGINAL ARTICLE

# Novel loss-of-function mutations in TNFAIP3 gene in patients with lupus nephritis

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## ABSTRACT

**Background.** Heterozygous loss-of-function mutations in the tumour necrosis factor alpha induced protein 3 (TNFAIP3) gene cause an early-onset auto-inflammatory disease named haploinsufficiency of A20 (HA20). Here we describe three unrelated patients with autoimmune lupus nephritis (LN) phenotypes carrying three novel mutations in the TNFAIP3 gene.

**Methods.** Whole-exome sequencing (WES) was used to identify the causative mutations in three biopsy-proven LN patients. Sanger sequencing and quantitative polymerase chain reaction (qPCR) were used to validate the mutations identified by WES. RNA sequencing, qPCR and cytometric bead array was used to detect inflammatory signatures in the patients.

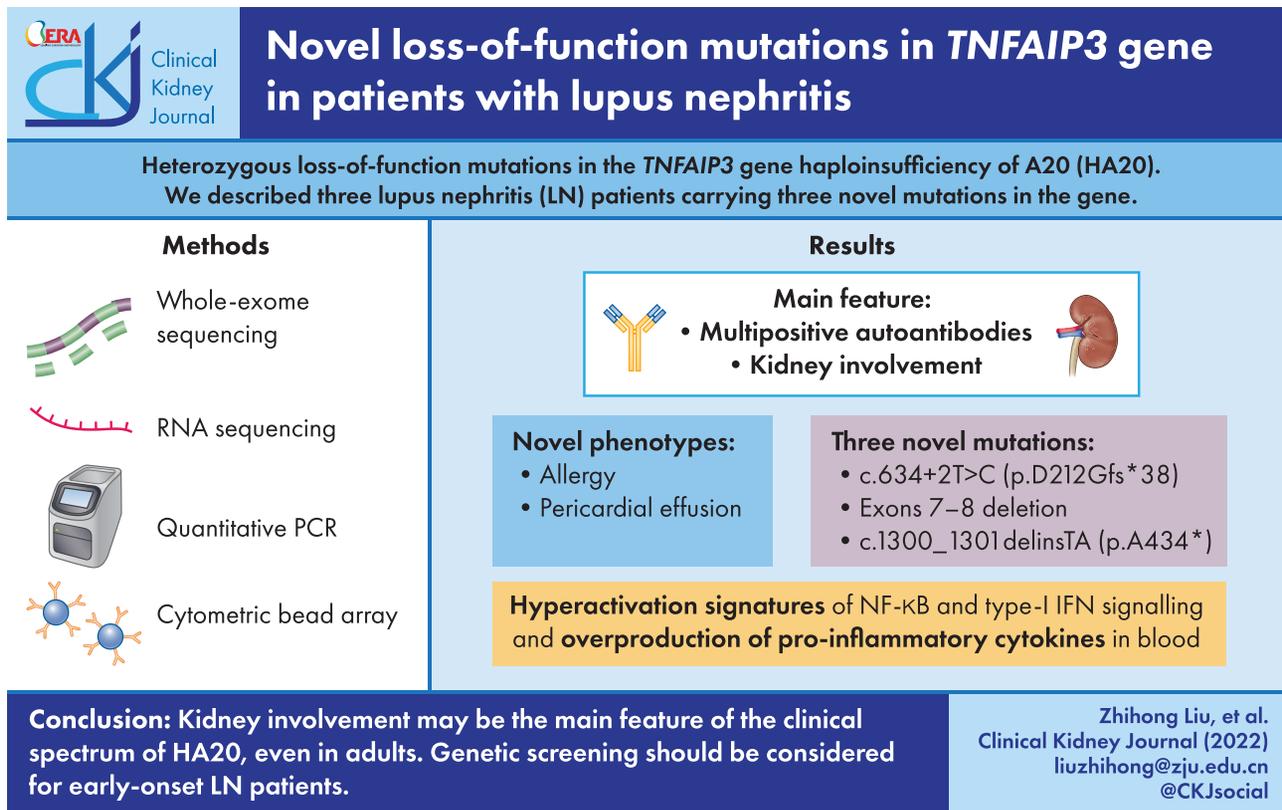
**Results.** The patients predominantly presented with an autoimmune phenotype, including autoimmune haemolytic anaemia, multipositive autoantibodies and LN. Additionally, novel phenotypes of allergy and pericardial effusion were first reported. WES identified three novel heterozygous mutations in the TNFAIP3 gene, including a novel splicing mutation located in the canonical splicing site (c.634+2T>C) resulting in an intron 4 insertion containing a premature stop codon, a *de novo* novel copy number variation (exon 7–8 deletion) and a novel nonsense mutation c.1300\_1301delinsTA causing a premature stop codon. We further identified hyperactivation signatures of nuclear factor-kappa B and type I IFN signalling and overproduction of pro-inflammatory cytokines in the blood. This report expanded the phenotype to a later age, as two girls were diagnosed at age 3 years and one man at age 29 years.

**Conclusions.** Kidney involvement may be the main feature of the clinical spectrum of HA20, even in adults. Genetic screening should be considered for early-onset LN patients.

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## GRAPHICAL ABSTRACT



**Keywords:** activation of NF-κB and type I IFN pathways, haploinsufficiency of A20, lupus nephritis, novel *TNFAIP3* mutations

## INTRODUCTION

The A20 protein, encoded by the tumour necrosis factor alpha induced protein 3 (*TNFAIP3*) gene, is a negative regulator of the nuclear factor kappa B (NF-κB) signalling pathway. Heterozygous loss-of-function mutations in the *TNFAIP3* gene cause haploinsufficiency of A20 [HA20; Online Mendelian Inheritance in Man (OMIM) 616744], characterized by an early-onset auto-inflammatory disease [1]. The major phenotype of HA20 is Behçet-like symptoms, including recurrent aphthous stomatitis, genital ulcers and intestinal symptoms [2]. However, HA20 was also identified in patients with childhood-onset autoimmune diseases and autoimmune lymphoproliferative syndrome [1, 3]. To date, the predominant autoimmune phenotype associated with HA20 may vary in family members carrying the same mutation.

Systemic lupus erythematosus (SLE; OMIM 152700) is one of the most heterogeneous systemic autoimmune diseases and presents great challenges in diagnosis and treatment. To develop innovative therapies, a better understanding of SLE pathogenesis is needed. Multiple genetic factors are presumed to be involved in SLE. For example, genome-wide association studies have identified a number of common alleles, including *TNFAIP3* gene polymorphisms, which increase the risk of developing lupus [4–7]. In addition, >30 causative genes for monogenic SLE and lupus-like syndrome have been described, which are associated with complement components, type I interferon, NF-κB,

self-tolerance and other pathways that have yet to be characterized [8–13]. Although the proportion of patients with monogenic SLE is small, they provide considerable insight into signalling pathways contributing to the SLE phenotype. Discovery of shared autoimmunity pathways in SLE can provide clues for better understanding of this complex disorder, its prognosis and new therapeutic approaches.

In this study we identified by whole-exome sequencing (WES), three novel mutations in the *TNFAIP3* gene leading to a predominantly lupus nephritis (LN) phenotype. We further confirmed the hyperactivation signatures of NF-κB and type I interferon (IFN) signalling and overproduction of pro-inflammatory cytokines. The results expand the genotypic and phenotypic spectrums of *TNFAIP3*-associated disease. They provide evidence to highlight the need to consider genetic screening in early-onset SLE patients as well as in sporadic adult patients.

## MATERIALS AND METHODS

## Patients

Three biopsy-proven LN patients with *TNFAIP3* loss-of-function mutations were studied. Six adult controls were healthy colleagues in our laboratory, including three males and three females. The age range was 24–34 years. Three male children as controls (0–3 years old) underwent routine physical examination in the clinic. All subjects or their guardians provided written

informed consent in accordance with the Helsinki Declaration for enrolment in research protocols approved by Jinling Hospital, Children's Hospital of Fudan University or Jilin University.

## WES

Genomic DNA was extracted from peripheral blood using the Chemagic DNA Blood 3K Kit Special H24 (CMG-1073; PerkinElmer, Waltham, MA, USA). One microgram of DNA was used for WES at BGI, Shenzhen, China. All genes across the whole-exome regions were explored. For patient 1 (P1), variants were annotated by Annotate Variation (ANNOVAR) software. Candidate variants were filtered to remove those presenting in the gnomAD, Kaviar, dbSNP and an in-house database and further filtered by dominant inheritance. For patient 2 (P2) and patient 3 (P3), WES was performed and analysed concurrently for the proband and both parents. Other family members were tested by Sanger sequencing for the presence or absence of the variants identified in the probands.

## Assessment of the splicing mutation in P1

The PAXgene Blood RNA Kit (762174; Qiagen, Venlo, The Netherlands) was used for the purification of intracellular RNA from whole blood of P1 collected in the PAXgene Blood RNA tube. The cDNA was synthesized using the PrimeScript Reverse Transcriptase kit with gDNA Eraser (RR047A; Takara Bio, Shiga, Japan). Then a set of specific primers for exon 3 and exon 7 was used to assess the effect of the splice-site variant in intron 4.

## Quantitative polymerase chain reaction (qPCR) assay

One microgram of RNA was used for cDNA synthesis. qPCR was performed using TB Green Premix Ex Taq II (RR820A; Takara). The reactions were run on a LightCycler 480 Instrument II (Roche, Indianapolis, IN, USA). Relative mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and analysed by the  $2^{-\Delta\Delta Ct}$  method.

## Copy number variant analysis by qPCR

Copy number variant (CNV) of the *TNFAIP3* gene identified in P2 by WES was validated by qPCR, which was performed as described earlier with specific primers for exons 5–9.

## RNA sequencing

One microgram of RNA was used for library preparation using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations. Index codes were added to attribute sequences to each sample. The libraries were sequenced on a Novaseq (Illumina, San Diego, CA, USA) and 150-bp paired-end reads were generated. Sequenced reads were mapped against the human reference genome (GRCh38) using HISAT2. The tool featureCounts was used to count the read numbers mapped to each gene. Differential expression analysis was performed using the DESeq2 R package.

## Cytokine detection in serum

The serum concentrations of cytokines were measured by cytometric bead array. All data were analysed by FCAPArray version 3 software (BD Biosciences, Franklin Lakes, NJ, USA).

## RESULTS

### Clinical features of the patients

The clinical features of the enrolled patients are summarized in Table 1 and renal involvement in Table 2.

P1 was a 29-year-old male diagnosed with SLE because of alopecia, shoulder pain, proteinuria, mild thrombocytopenia, positive autoantibodies (Table 3) and decreased serum complement. In addition, he suffered from dry mouth, dry eyes and multiple patch rash in the chest and upper limbs. He was allergic to mould and pollen and his immunoglobulin E (IgE) level was high (>1000 IU/mL). P1's mother had recurrent oral ulcers, while P1's son (6 years old) had oral ulcers; skin rashes; duodenal ulcers; anaemia; allergies to milk, egg, fish and shrimp; and had been diagnosed as Henoch–Schönlein purpura.

P2, a 3-year-old girl, suffered from recurrent fever with elevated leucocytes, lymphadenopathy, massive pericardial effusion, persistent hepatosplenomegaly and abnormal liver function since she was 10 months old (Fig. 1A), without any autoantibodies. About 1 year later, P2 developed weak positive anti-nuclear antibodies (ANA) and persistent pulmonary hypertension. Computed tomography scan showed interstitial lung disease (Fig. 1B). Then she developed vasculitic rashes, leukopenia, haemolytic anaemia, strongly positive ANA and anti-double-stranded DNA (dsDNA) antibodies and decreased C3 and C4. Urinalysis revealed proteinuria and microscopic haematuria. Based on these findings, SLE was diagnosed.

P3 was a 3-year-old girl born at term with low birthweight. She was diagnosed with SLE because of intermittent fever, frequent oral ulcers, hyperaemic rash on both palms, haemolytic anaemia, proteinuria, haematuria, decreased C3 and C4 and multiple positive autoantibodies (Table 3). Additionally, she had hepatosplenomegaly and multiple swollen lymph nodes. Her mother was asymptomatic.

### Kidney involvement

The renal involvement of the patients manifested as proteinuria and haematuria without renal insufficiency (Table 2). P1 presented with nephrotic syndrome at onset. P2 and P3 had low to moderate proteinuria. Serum creatinine levels were normal. Renal biopsy findings are summarized in Table 2.

For P1, global sclerotic glomeruli and crescents were identified (Fig. 1C). Endocapillary hypercellularity, adhesion and fibrinoid necrosis were occasionally seen. Periodic acid–silver methenamine (PASM)–Masson staining showed mesangial, subendothelial and subepithelial immune deposits (Fig. 1D and E). Immunofluorescence (IF) staining revealed that IgG<sup>+</sup>, IgA<sup>++</sup>, IgM<sup>++</sup>, C3<sup>++</sup> and C1q<sup>+</sup> was diffusely and granularly deposited in the mesangium and capillary walls (Fig. 1F–J). Extraglomerular deposits of IgG, IgA and C1q were seen in tubular basement membrane (TBM) and blood vessels (Fig. 1K and L).

For P2, global sclerotic glomeruli and crescents were identified without endocapillary hypercellularity, adhesion or fibrinoid necrosis. Phenophilin was deposited in the subepithelial area. IF staining revealed that IgG, IgA, C3, C4 and C1q was diffusely and granularly deposited along the capillary walls, without extraglomerular deposits. Numerous subepithelial electron-dense deposits were seen by transmission electron microscopy.

For P3, mild endocapillary hypercellularity and cellular crescents were seen in ~20% of glomeruli, without adhesions or fibrinoid necrosis. Karyorrhexis was occasionally present.

Table 1. Clinical characteristics of patients with TNFAIP3 mutations

Characteristics	Patient 1	Patient 2	Patient 3
Genotype	c.634+2T>C p.D212Gfs*38	Exon 7–8 deletion	c.1300_1301delinsTA p.A434*
Mutation origin	From mother	<i>De novo</i>	From mother
Sex	Male	Female	Female
Age at onset (years)	29	10 month	3
Age at LN diagnosis (years)	29	3	3
Age at genetic diagnosis (years)	35	3.5	3
Recurrent fever	No	Yes	Yes
Haematological	Thrombocytopenia	Leukopenia, AIHA	AIHA
Cutaneous	Alopecia	Acute cutaneous lupus	Oral ulcers
Serositis	No	Pericardial effusion	No
Musculoskeletal	Arthralgia	No	Bilateral knee effusion
Renal	LN-IV	LN-V	LN-IV
Ocular	Dry eyes, increased secretion	No	No
Cardiovascular	No	Pulmonary hypertension, tricuspid valve prolapse	No
Other features	Dry mouth, allergy	Hepatosplenomegaly, multiple swollen lymph nodes, ILD, mild growth retardation	Multiple swollen lymph nodes, hepatosplenomegaly

AIHA, autoimmune haemolytic anaemia; ILD, interstitial lung disease.

Table 2. Clinical and renal biopsy findings in patients with TNFAIP3 mutations

Findings	Patient 1	Patient 2	Patient 3
24 h-Uprot(g)	7.29	0.67	1.11
Haematuria	160/μL	15/HP	646.40/μL
Scr (μmol/L)	60	22	29
GSG	1/42	1/50	0/34
Crescents	3/41	2/49	7/34
Endocapillary hypercellularity	Present	Absent	Present
Adhesion	Present	Absent	Absent
Karyorrhexis	Absent	Absent	Present
Fibrinoid necrosis	Present	Absent	Absent
Phenophilin deposition	Mesangial, subendothelial, subepithelial	Subepithelial	Mesangial, subendothelial
Tubulointerstitial lesions	Mild	Mild	Interstitial inflammation
Immunofluorescence staining	IgG, IgA, IgM, C3, C1q	IgG, IgA, C3, C4, C1q, C9	IgG, IgA, IgM, C3, C1q
EGD's location	TBM, blood vessels	Absent	TBM
EDD's location	No EM	Subepithelial	No EM
Diagnosis based on ISN/RPS classification of LN	LN-IV	LN-V	LN-IV

Uprot: proteinuria; Scr: serum creatinine; GSG, globally sclerotic glomeruli; crescents, open glomeruli with cellular or fibrocellular crescents; EGD, extraglomerular deposit; TBM, tubular basement membrane; EDD, electron-dense deposit.

Mesangial and subendothelial deposits were seen. Tubulointerstitial inflammation occurred in the absence of interstitial fibrosis. IF staining showed IgG<sup>++</sup>, IgA<sup>+</sup>, IgM<sup>+++</sup>, C3<sup>+++</sup> and C1q<sup>+++</sup> deposits in the mesangium, capillary walls and TBM.

According to the International Society of Nephrology (ISN)/Renal Pathology Society (RPS) classification of LN, the histopathological findings were categorized as LN-IV in P1, LN-V in P2 and LN-IV in P3.

### Genetic variants in the TNFAIP3 gene

**A novel splicing variant in the TNFAIP3 gene causes intron 4 insertion in P1.** In P1, a novel heterozygous variant (c.634+2T>C) was identified in the TNFAIP3 gene, which alters the canoni-

cal donor splice site downstream of exon 4. The mutation cosegregated with disease phenotype in the family (Fig. 2A and B). *In silico* bioinformatics tools, including regsnp-splicing (<http://regsnps-splicing.cccb.iupui.edu/>) and HSF (<http://www.umd.be/HSF3/>), predicted that the variant was 'disease-causing'. To assess mRNA splicing, we used cDNA synthesized from the proband's blood RNA and compared it with a healthy control. Sanger sequencing of P1's cDNA identified a mutant allele with an intron 4 insertion containing a premature stop codon resulting in p.D212Gfs\*38 (Fig. 2C).

**A novel CNV in the TNFAIP3 gene in P2.** A trio WES revealed no candidate single-nucleotide variant or small indels in any gene associated with inflammatory diseases in P2. However, WES-based CNV analysis identified a heterozygous deletion of exons 7

Table 3. Immunologic characteristics of patients with TNFAIP3 mutations

Characteristics	Patient 1	Patient 2	Patient 3
ANA	1:128	1:1280	1:3200
dsDNA		1:1000	1:32
C3 (g/L)	0.167	0.4	0.14
C4 (g/L)	0.0327	0.08	0.01
CRP (mg/L)	ND	19	6.7
ESR (mm/h)	ND	31	54
IgG (g/L)	10.3	24.2	37.29
IgA (g/L)	2.35	0.91	1.72
IgM (g/L)	1.49	1.98	1.97
IgE (IU/mL)	>1000.0	134.62	ND
RF (IU/mL)	<20.0	10.9	19.87
CD4:CD8 ratio	0.37	2.24	1.6
Other autoantibodies	Anti-C1q <sup>+</sup> , SSA-Ro60 <sup>+++</sup> , SSA-Ro52 <sup>+++</sup> , aAnti-RPA <sup>+++</sup> , TRAb 37.4 IU/mL	Negative	aCL <sup>+</sup> , anti-β2GPI <sup>+</sup> , SSA-Ro60 <sup>++</sup> , anti-AnuA <sup>+++</sup> , SSA-Ro52 <sup>+++</sup> , antihistone <sup>++</sup>

ND, no data.

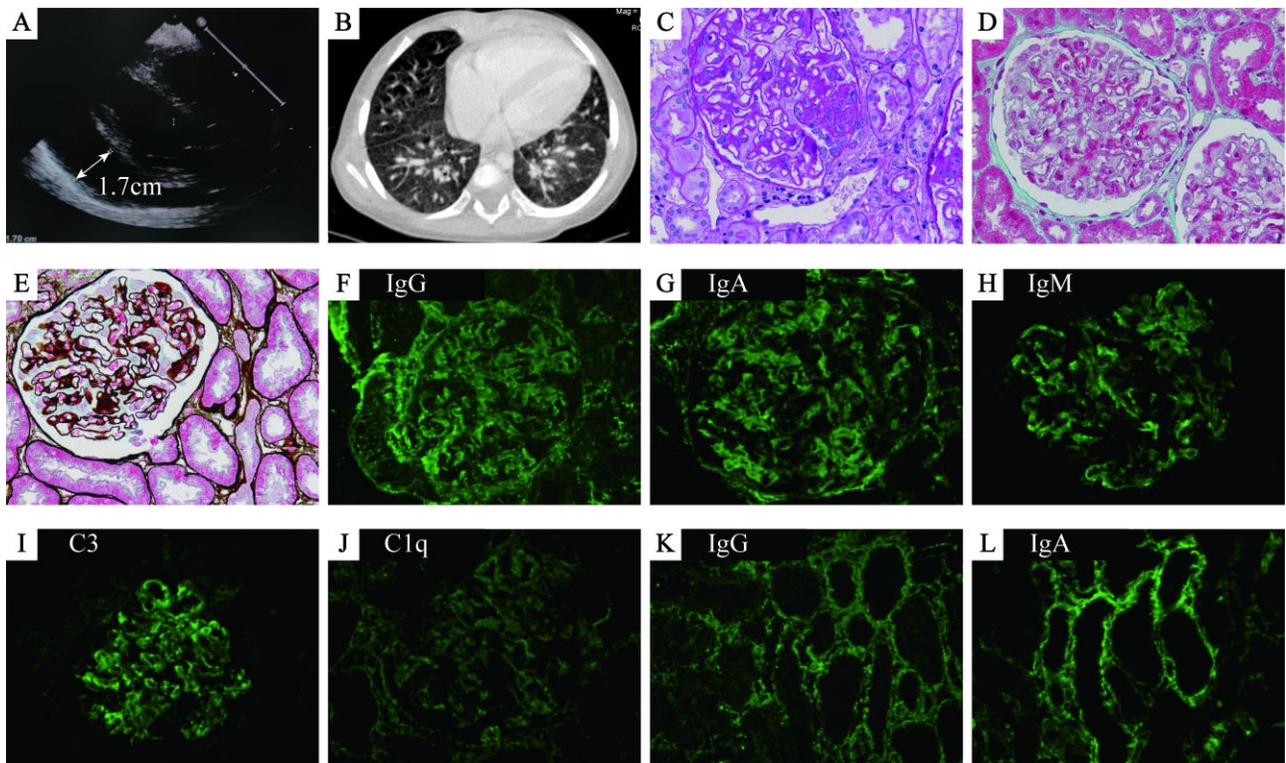


FIGURE 1: Clinical manifestations and renal biopsy findings. (A) Massive pericardial effusion and (B) interstitial lung disease of P2. Renal biopsy of P1. (C) Mesangial hypercellularity and crescents (periodic acid-Schiff,  $\times 400$ ). (D, E) Subendothelial, mesangial and subepithelial fuchsinophilic deposits (PASM–Masson,  $\times 400$ ). (F–J) Diffuse and granular deposition of IgG, IgA, IgM, C3 and C1q in mesangial areas and vascular loops (immunofluorescence,  $\times 400$ ). (K, L) Extraglomerular deposits of IgG and IgA in tubular basement membrane and blood vessels.

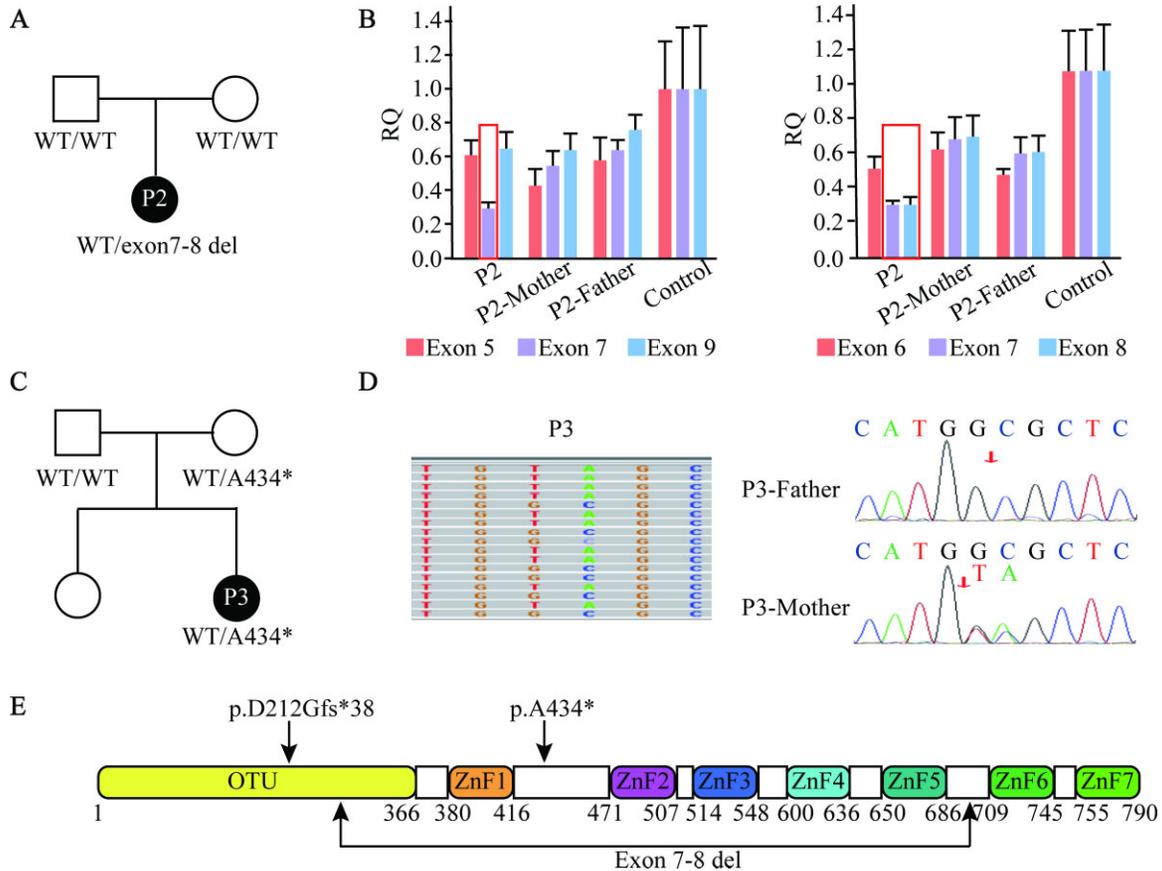
and 8 of *TNFAIP3*. qPCR confirmed the deletion in the proband, which is *de novo*, as neither parent harboured the variant (Fig. 3A and B).

**A novel nonsense variant in the *TNFAIP3* gene in P3.** A novel nonsense heterozygous variant [c.1300\_1301delinsTA (p.A434\*)] in the *TNFAIP3* gene was identified in P3. This mutation caused a premature stop codon directly and was presumed to result in A20 haploinsufficiency. Her asymptomatic mother

had the same mutation confirmed by Sanger sequencing (Fig. 3C and D).

All novel mutations were absent from gnomAD, ChinaMAP [14] Chinese Millionome database, in-house controls and Infervers (an online database for auto-inflammatory mutations; <https://infervers.umai-montpellier.fr/web>). The A20 protein structure and location of the novel mutations is marked in Fig. 3E.





**FIGURE 3:** Pedigree and validation of the mutations of P2 and P3. (A) Pedigree and mutation of the *TNFAIP3* gene of P2. (B) qPCR of P2 and family members confirmed the deletion of exons 7–8 in P2. Left image shows the relative *TNFAIP3* expression using primers targeting exons 5, 7 and exon 9, respectively. Right image shows the relative *TNFAIP3* expression using primers targeting exons 6–8, respectively. The red box in the two images indicates the decreased *TNFAIP3* expression in P2 using primers targeting exons 7 and 8. (C) Pedigree and mutation of the *TNFAIP3* gene of P3. (D) Sanger sequencing of P3 and family members. (E) A20 structure and the location of three novel mutations of *TNFAIP3*.

### Activation of inflammatory signalling

Table 3 summarizes the immunologic characteristics of the patients. All were strongly positive for ANA and/or anti-dsDNA and had decreased C3 and C4 levels. Additionally, P2 and P3 had increased CRP and erythrocyte sedimentation rate (ESR) without infection. IgE levels of P1 and P2 were increased.

To investigate their inflammatory signature, we performed RNA sequencing using RNA extracted from whole blood cells of P1. The gene set enrichment analysis (GSEA) showed activation of the inflammatory response, such as NF- $\kappa$ B, Toll-like receptor and tumour necrosis factor (TNF) signalling pathways (Fig. 4A–D). A20 is a potent inhibitor of the NF- $\kappa$ B pathway. As expected, we observed significant up-regulation of NF- $\kappa$ B signalling compared to three controls (Fig. 4E). A20 also can inhibit the retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA-5) pathway and restrict the activation of type I IFN. A strong type I IFN signature is observed in whole blood cells of P1 compared with controls (Fig. 4F). The 28-gene IFN score confirmed the activation of type I IFN signalling (Fig. 4G). qPCR confirmed the up-regulation of IFN-stimulated genes in P1's whole blood cells, such as *IFIT1*, *IFI44L*, *RSAD2*, *ISG15*, *OAS1*, and *MX2* (Fig. 4H). The serum cytokine interleukin-6 (IL-6) was also significantly elevated compared with controls (Fig. 4I). Together, our results indicated that

both NF- $\kappa$ B and type I IFN pathways were activated when A20 was deficient.

### Treatments and outcomes

All patients received steroids and two patients also received mycophenolate mofetil (MMF) and/or tacrolimus or cyclosporine A. Belimumab, a human monoclonal antibody targeting B-cell activating factor and the Janus kinase (JAK) inhibitor tofacitinib were also used (Table 4). P1 initially received a combined therapy with prednisone, MMF and tacrolimus. His condition gradually improved, as autoantibodies turned negative, complement levels returned to normal and proteinuria decreased. Proteinuria relapsed twice during prednisone and immunosuppressant reduction. His renal function, autoantibodies and complement levels remained normal during follow-up of 6.5 years. Methylprednisolone pulse therapy and methylprednisolone with cyclosporine were successively prescribed to P2. Later, belimumab was added because of the high titres of autoantibodies. Her proteinuria, complement levels and autoantibodies quickly returned to normal. P3 received four rounds of methylprednisolone pulse therapy then prednisone, hydroxychloroquine, and the JAK inhibitor tofacitinib were used. During follow-up, she had no fever or proteinuria. Her haemoglobin

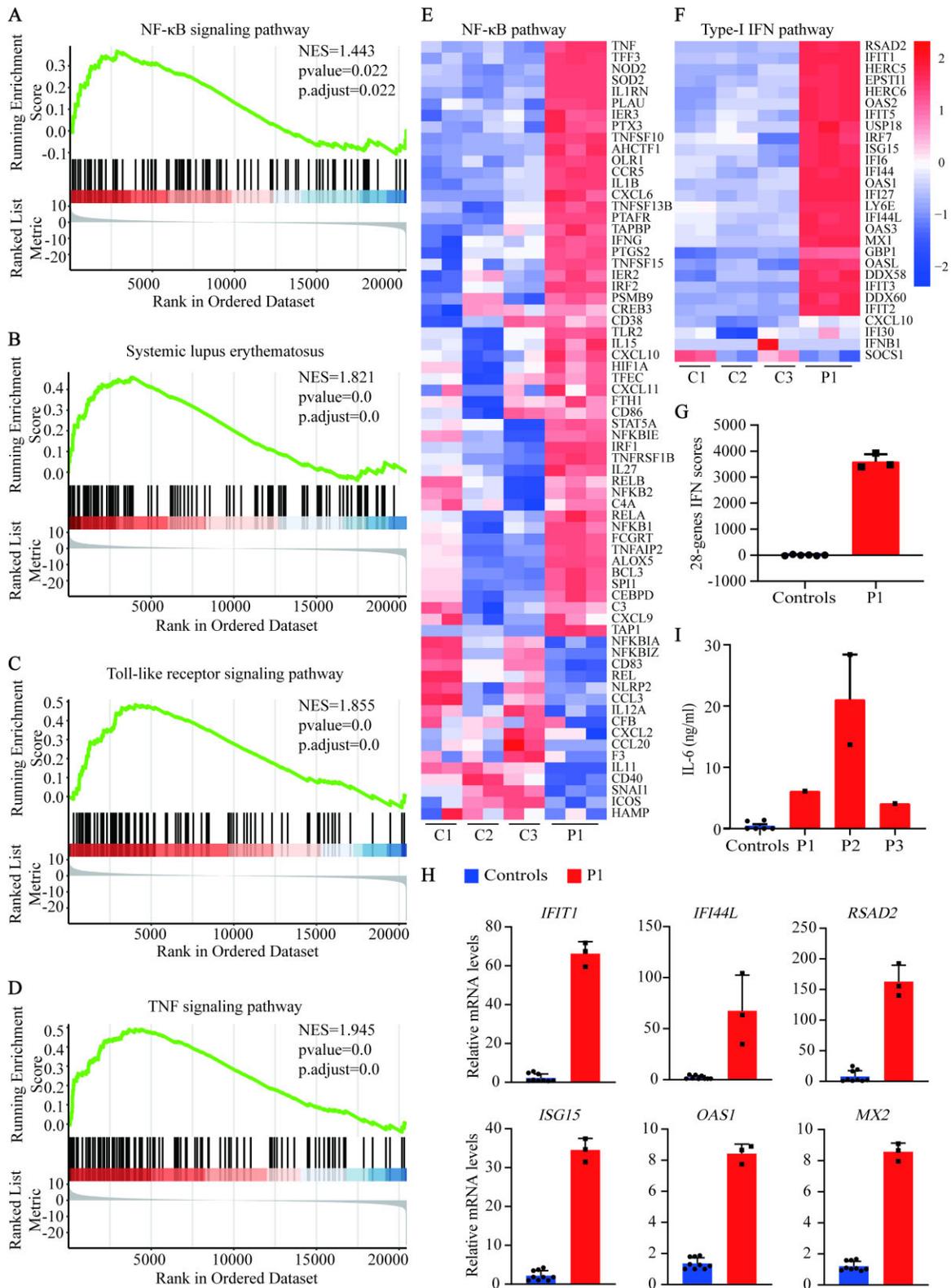


FIGURE 4: Activation of inflammatory signalling. (A–D) GSEA of RNA sequencing of whole blood cells of P1. Activation of (E) NF-κB and (F) type I IFN signalling in whole blood cells of P1. (G) The 28-gene IFN score in whole blood cells of P1. (H) qPCR results of IFN-stimulated genes in whole blood cells of P1 (controls = 3). RNA of whole blood cells per subject was divided into three parts equally and then qPCR was performed in parallel. (I) Elevated serum cytokine IL-6 levels in the three patients (controls = 6). Serum IL-6 levels in P2 were tested twice at different time points.

Table 4. Treatments and outcomes of patients with TNFAIP3 mutations

Patient	Treatment	Outcome	Time of follow-up (years)
Patient 1	P + MMF + tacrolimus P + TW + tacrolimus P + tacrolimus P + TW	Proteinuria relapsed, normal renal function, negative autoantibodies, normal complement	6.5
Patient 2	Methylprednisolone pulse therapy P + cyclosporine A P + belimumab	Complete remission	2
Patient 3	Methylprednisolone pulse therapy P + hydroxychloroquine + tofacitinib	Complete remission	1.5

P, prednisone; TW, *Tripterygium wilfordii*.

returned to normal and hepatosplenomegaly and lymphadenopathy improved.

### Common SLE risk allele

To explore additional factors modifying the disease phenotype, we screened the common SLE risk allele using the WES data and found that in addition to the deleterious TNFAIP3 mutation, several other common SLE risk alleles were identified in all three patients (Table 5).

## DISCUSSION

In the current study we described the clinical and pathological features of three patients carrying novel mutations in the TNFAIP3 gene leading to HA20. HA20 classically presents with auto-inflammatory features. In contrast, our patients were predominantly characterized by autoimmune features with LN.

The first mutation, c.634+2T>C, affects the splicing of mRNA and creates a frameshift mutation that removes both the ovarian tumour (OTU) domain and all zinc finger domains. The second mutation, exon 7–8 deletion, results in the absence of the OTU domain proximal to the zinc finger domains and zinc finger domains 1–5. The third mutation, c.1300\_1301delinsTA (p. A434\*), results in a premature stop codon that removes zinc finger domains 2–7. They all lead to the truncating of the A20 protein. Previous molecular and biochemical studies showed that truncating mutations of A20 protein, even only the seventh (last) zinc finger domain, correlated with HA20 clinical disease as well as diminished A20 expression and suppressive activity, leading to increased TNF- $\alpha$ -associated NF- $\kappa$ B activity [1, 16, 17]. Similar findings of obvious activation of the NF- $\kappa$ B pathway were observed in our patients.

In addition to expanding of genotypic spectrum, we reported for the first time an allergy history in P1 and massive pericardial effusion in P2 associated with TNFAIP3 mutation. Additionally, P1 was adult onset, different from previous reports. These are novel phenotypes. All three patients met the classification criteria for SLE [18] and were biopsy-proven LN. They shared some clinical hallmarks with auto-inflammatory phenotype of HA20 [1, 2, 15], including recurrent oral ulcers, intermittent fever, hepatosplenomegaly, skin lesions, arthralgia or arthritis and elevated pro-inflammatory markers. They also presented with distinct autoimmune features, including autoimmune haemolytic anaemia, multiple positive autoantibodies, decreased complement levels and immune-complex deposits in

the kidneys. Therefore the study expands both the genotypic and phenotypic spectrum of TNFAIP3-associated disease.

While >36 unique TNFAIP3 mutations that are associated with auto-inflammatory and autoimmune disease have been collected in the Infervers database, only 2 mutations were associated with renal involvement, including a p.Q187\* mutation in a proliferative glomerulonephritis (class III) patient and a p.F224Sfs\*4 mutation in a membranous lupus (class V) patient [2, 19]. The three particular novel mutations in our patients were associated with LN. Whether the particular mutations are associated with organ-specific manifestations is currently unknown. More cases are needed in further studies.

HA20 is commonly reported in early-onset patients [15, 20]. However, P1 in our study was adult onset. Manifestations and disease courses were quite different among the three patients, suggesting other possible genetic and environmental factors. Additionally, P3's mother had a corresponding mutation but is still asymptomatic, suggesting a significant degree of variable expressivity and reduced penetrance in HA20 patients.

For P2, an auto-inflammatory phenotype was predominant in the first year of her illness, without any autoantibodies. Then weak autoantibodies appeared and progressed to a strong autoimmune phenotype. The transition from auto-inflammatory to autoimmune disorder suggested the dysregulation of immune homeostasis. A20 is an anti-inflammatory molecule that restricts multiple intracellular signalling cascades in multiple cell types including myeloid cells, dendritic cells (DCs), B cells and intestinal epithelial cells [21]. Mice with B-cell-specific ablation of A20 spontaneously developed an autoimmune condition similar to human SLE, whereas intestinal cell-specific A20 deficiency resulted in intestinal inflammation [22, 23]. Further, two independent DC A20-deficient mouse strains showed remarkably different phenotypes. One strain developed SLE-like symptoms, including the presence of anti-dsDNA autoantibody, glomerulonephritis, antiphospholipid syndrome and arthritis [24]. In contrast, the other strain spontaneously developed colitis, seronegative ankylosing arthritis and enthesitis [25]. Therefore A20 may regulate crucial steps in immune cell homeostasis and additional factors such as major affected cell types, genetic backgrounds and the environment may play a determinative role in the disease phenotype.

Autoimmune features were reported in aging TNFAIP3 knockout mice [26]. Autoimmunity is considered a complication of HA20 with aging and/or the accumulation of other genetic factors [27]. However, two patients (P2 and P3) showed marked autoimmunity at a very early age (<3 year) and P3 showed obvious autoimmune characteristics from the onset. Therefore the

Table 5. Common SLE risk variants identified in patients by WES

Patients	Chromosome	Position	Ref	Alt	Type	Gene	Consequence	dbID	ExAC-freq	GT	[Ref]
P1	chr1	1243545	G	A	Exonic; splicing	C1QTNF12	NM_001014980:exon5:c.C539T:p.A180V	rs12093154	0.1283	0/1	[28]
	chr10	71356185	G	A	Exonic	SLC29A3	NM_001174098:exon5:c.G715A:p.V239I	rs2252996	0.8217	1 1	[29]
	chr12	118244946	C	T	Exonic	TAOK3	NM_001346487:exon4:c.G140A:p.S47N	rs428073	0.7142	0/1	[30]
	chr17	4809322	C	T	Exonic	PLD2	NM_001243108:exon6:c.C514T:p.R172C	rs2286672	0.1472	0/1	[6]
	chr19	32615715	C	T	Exonic	ANKRD27	NM_032139:exon22:c.G2118A:p.A706A	rs405858	0.589	0/1	[28]
	chr6	34856859	A	G	Exonic	UHRF1BP1	NM_017754:exon11:c.A1361G:p.Q454R	rs11755393	0.3681	0/1	[30]
	chrX	30559729	A	C	Exonic	CXorf21	NM_025159:exon3:c.T627G:p.V209V	rs887369	0.8242	1/1	[6]
P2	chr1	116533925	G	T	Intronic	CD58	.	rs1016140	.	1/1	[28]
	chr10	71356185	G	A	Exonic	SLC29A3	NM_001174098:exon5:c.G715A:p.V239I	rs2252996	0.8217	0 1	[29]
	chr12	118244946	C	T	Exonic	TAOK3	NM_001346487:exon4:c.G140A:p.S47N	rs428073	0.7142	1/1	[30]
	chr14	102797451	A	G	Intronic	TRAF3	.	rs12148050	.	1/1	[28]
	chr17	4809322	C	T	Exonic	PLD2	NM_001243108:exon6:c.C514T:p.R172C	rs2286672	0.1472	0/1	[6]
	chr19	32615715	C	T	Exonic	ANKRD27	NM_032139:exon22:c.G2118A:p.A706A	rs405858	0.589	0 1	[28]
	chr6	34856859	A	G	Exonic	UHRF1BP1	NM_017754:exon11:c.A1361G:p.Q454R	rs11755393	0.3681	1/1	[30]
	chrX	30559729	A	C	Exonic	CXorf21	NM_025159:exon3:c.T627G:p.V209V	rs887369	0.8242	1/1	[6]
P3	chr1	183563302	G	A	Exonic; splicing	NCF2	NM_001190789:exon11:c.C940T:p.R314W	rs13306575	0.0151	0/1	[31]
	chr10	71356185	G	A	Exonic	SLC29A3	NM_001174098:exon5:c.G715A:p.V239I	rs2252996	0.8217	1 1	[29]
	chr12	12717761	T	C	UTR5	CDKN1B	.	rs34330	.	1/1	[29]
	chr2	162267541	C	T	Exonic	IFIH1	NM_022168:exon15:c.G2836A:p.A946T	rs1990760	0.5047	0/1	[30]

trigger factor of auto-inflammatory or autoimmune cannot be simply attributed to aging. By screening the common SLE risk alleles in the WES data, we found that several common SLE risk alleles may have contributed to disease expressivity [6, 28–31]. Analysing the combined effect of rare and common gene variants in LN may shed light on interactions between signalling pathways and may further reveal potential therapeutic targets.

LN pathogenesis is associated with immune complex deposition within the kidney as well as activation of resident cell types, infiltration of immune cells and expression of inflammatory cytokines [32]. Many of these inflammatory mediators are the result of activation of the NF- $\kappa$ B pathway, which is a key transcription factor for maturation of DCs, activation and differentiation of T-cells, survival and recruitment of neutrophils and inflammatory signalling and polarization within macrophages [33]. Blocking classical NF- $\kappa$ B signalling markedly abrogated disease, with an improvement in proteinuria, kidney function and histopathology [34]. In addition, type I IFN may drive the pathogenesis of LN and is associated with disease flares [35]. Single-cell studies also demonstrate high type I IFN signatures in LN patients [36]. A20 not only inhibits NF- $\kappa$ B signalling by modifying ubiquitylated protein substrates in multiple ways, but also blocks type I IFN signalling by removing K63-linked ubiquitin chains from TBK1/IKKi, which is activated by the RIG-I/MDA5-mitochondrial antiviral signaling protein axis [21]. In this study we observed that loss of function of A20 resulted in significant activation of both the NF- $\kappa$ B and type I IFN pathways. This might be the core of LN pathogenesis.

Biologics targeting cytokines are commonly used as novel treatments because of excessive production of pro-inflammatory cytokines in HA20 patients [1, 27, 37]. For example, anti-TNF, anti-IL-1 $\beta$  and anti-IL-6 agents can effectively inhibit systemic inflammatory responses in these patients [2, 38]. Other immunotherapies, such as anti-CD20 monoclonal antibodies and JAK inhibitors, have also been reported as useful [39–41]. Hematopoietic stem cell transplantation can be considered

for patients with refractory disease [39]. More cases should be accumulated to elucidate the pathophysiology and novel treatment strategies for LN patients with pathogenic TNFAIP3 mutations.

Our probands with LN are likely to remain under the direct care of a nephrologist. The key message of our report is to alert nephrologists to recognize a kidney-predominant presentation of systemic inherited diseases to avoid missing any extrarenal manifestations of HA20. Genetic screening of patients based on clinical characteristics alone is unlikely to be sensitive enough to detect all relevant cases. Next-generation sequencing, like WES/whole-genomic sequencing, combined with RNA sequencing is a powerful tool to identify pathogenic TNFAIP3 mutations and potential novel pathogenic genes in patients with LN [42–44].

In summary, we report the clinical manifestations and immunological characteristics of three biopsy-proven LN patients with novel pathogenic mutations in the TNFAIP3 gene. Continuous improvement and widespread application of genetic testing technology provide new diagnostic and therapeutic options for autoimmune diseases. Monogenic diseases, such as HA20, may help advance our understanding of disease pathogenesis and develop targeted therapies for more common, multifactorial disorders with lupus-like manifestations.

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## AUTHORS' CONTRIBUTIONS

C.Z. performed the experiments and analysed the data. X.H. performed the bioinformatics analysis. J.P. performed the experiments. C.Z., L.S., S.Y., Y.J., Y.C. and F.X. enrolled the patients and collected and interpreted clinical and pathological information. Q.Z. and Z.L. designed the study and directed and supervised the research. C.Z. wrote the manuscript. Q.Z. and Z.L. edited the manuscript. All authors contributed to the review and approval of the manuscript.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no relevant financial interests.

## DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article.

(See related article by Villalvazo et al. Gain-of-function TLR7 and loss-of-function A20 gene variants identify a novel pathway for Mendelian lupus and lupus nephritis. *Clin Kidney J* (2022) 15: 1973–1980.)

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