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FULL LENGTH ARTICLE

Clinical heterogeneity of NLRP12-associated autoinflammatory diseases

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Nuclear factor-Kappa B (NF-κB)

Abstract Nod-like receptor family pyrin domain-containing protein 12 (NLRP12) is one of the critical pattern recognition receptors which participates in the regulation of multiple inflammatory responses. Mutations in *NLRP12* cause exceptionally rare NLRP12-associated autoinflammatory disease (NLRP12-AID). So far, very few patients with NLRP12-AID have been identified worldwide; therefore, data on the clinical phenotype and genetic profile are limited. In this study, we reported 10 patients who presented mainly with periodic fever syndrome or arthritis. Next-generation sequencing (NGS) identified 6 heterozygous mutations of *NLRP12*, including 2 novel null mutations. Of the patients, some with same mutations showed different clinical features. Compared to healthy controls, the increased levels of cytokines were revealed in the patients' plasmas, as well as in the supernatants of patients' cells stimulated with lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). The missense mutations did not change the protein expression; but decreased level of NLRP12 protein was shown in the null mutations. And *in vitro* expression assay demonstrated a truncating protein induced by the frameshift mutation. Further functional studies revealed the deleterious effect of mutations on nuclear factor-kappa B (NF-κB) signaling. Both the null and missense mutations impaired their inhibition of NF-κB activation induced by p65. Collectively, this study reported a relatively large NLRP12-AID case series. Our findings expand the clinical spectrum, and reinforce the diversity of genetic mutations and clinical phenotypes. The NLRP12-associated

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disorder should be considered when autoinflammatory diseases are encountered in the clinical practice, especially for patients presenting with periodic fever but no other genetic cause identified.

Introduction

Nod-like receptor family pyrin domain-containing protein 12 (NLRP12) is a member of the NOD-like receptors (NLRs) family and encoded by *NLRP12* gene on the chromosome 19. It is expressed mainly in immune cells and down-regulated in response to pathogen products or inflammatory cytokines, so it is believed to regulate the inflammation and immune response. NLRP12 is able to down-regulate the canonical and non-canonical nuclear factor-kappa B (NF-κB) signaling pathways by inducing proteasome degradation of NF-κB related enzymes.[1–5] It also interacts with apoptosis-related dot-like protein (ASC) and pro-caspase-1 to form inflammasome which drives caspase-1 activation and the release of pro-inflammatory cytokines. Then, it regulates the production of interleukin-18 (IL-18) and IL-1β production.[1,6–8] As an important pattern recognition receptor, NLRP12 can recognize multiple pathogens and initiate downstream immune responses to eliminate invading pathogens. It plays an important role in maintaining the migration state of neutrophils and peripheral dendritic cells.[9,10] In addition, it also negatively regulates T cell responses in adaptive immunity to control dominant activation and maintain intracellular environmental stability.[11]

NLRP12-associated autoinflammatory disease (NLRP12-AID) is an extremely rare autosomal dominant genetic disorder caused by *NLRP12* mutations. In 2008, heterozygous *NLRP12* mutations were first reported in patients with hereditary periodic fever (HPF).[12] Studies have shown that *NLRP12* mutations are well known to cause Familial Cold Autoinflammatory Syndrome Type 2 (FCAS2, OMIM #611762), which is characterized by periodic fever, recurrent rash, arthralgia and myalgia. Most patients reported cold exposure as a trigger for the episodes.[13,14] The disease onset often occurs in the childhood, but also has been reported in the adult. In addition to autoinflammation disease, increased susceptibility to infection due to the related immunodeficiency was also described in the patients with *NLRP12* mutations. So far, very few NLRP12-AID patients have been discovered[15,16]; therefore, limited data on the clinical spectrum and genetic profile are available. In addition, scarce studies have been performed to clarify the mechanism underlying the enhanced inflammatory response, which thus remains still unclear.

In this study, we reported 10 patients carrying different *NLRP12* mutations. They presented mainly with periodic fever syndrome or arthritis. The clinical and genetic features were analyzed. Functional experiments were performed to evaluate the effects of the mutations on NLRP12 protein expression, cytokine production and NF-κB activation. Our findings expand the clinical spectrum of NLRP12-AID, and reinforce the diversity of genetic and clinical profiles. The impaired suppression of NF-κB signaling by mutant NLRP12 may contribute, at least in part, to the hyperinflammation of the disease.

Materials and methods

Patients

A total of 7 probands and their families were enrolled in this study. They were diagnosed with periodic fever syndrome or arthritis. Next-generation sequencing identified *NLRP12* mutations in the patients. Clinical data and family history were collected from all probands, and blood samples were available from some patients. Informed consent was obtained from all of the participants. This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Children's Hospital of Chongqing Medical University (approval number: 030/2013).

Genetic study and bioinformatic analysis

Peripheral blood samples were sent to MyGenostics (Beijing, China) and subjected to exome sequencing. *NLRP12* mutations in all patients were confirmed by NGS and Sanger sequencing (NM_144687.4). Bioinformatic analysis was performed for all the mutations. The conservation of mutations was analyzed on the weblogo.berkeley.edu. The pathogenicity of mutations was evaluated by four algorithms: PROVEAN (http://provean.jcvi.org/index.php), SIFT (http://sift.jcvi.org/), Mutation Taster (http://www.mutationtaster.org/), and MutPred2 (http://mutpred2.mutdb.org/index.html). Minor allele frequencies (MAF) for novel mutations (p.F303Sfs*25 and p.C680X) were obtained from publicly available databases (1000 genomes, ESP6500, ExAC and ExAC-EAS). At last, the two novel mutations were evaluated by the ACMG classification for variant pathogenicity.

Structure analysis of NLRP12 mutants

The NLRP12 front structure was modeled on the crystal structure of NLRP12 (4XHS from the Protein Data Bank). Due to no crystal structure of the posterior segment of NLRP12 available, NLRP3 crystal structure (6NPY from the Protein Data Bank) was used as the template for the modeling of the posterior part of mutant NLPR12 as described elsewhere,[17,18] since both of them are NLRs family proteins

with similar primary structure and functions. The three-dimensional structures were predicted by Swiss-model, and the potential impact of the mutations were analyzed by PyMOL2.5 (PyMOL Software, Inc., CA, USA.)

Cell preparation and plasma isolation

Plasma was separated from peripheral blood by centrifugation at room temperature. Peripheral blood mononuclear cells (PBMCs) were obtained from the patients and healthy controls (HCs) by gradient centrifugation of heparinized blood over Ficoll—Paque PREMIUM (GE Healthcare, Sweden) as described before.[19]

Cytokine quantitation

PBMCs were cultured in RPMI 1640 media (Gibco, USA) at a density of 0.8×10^6 cells/mL containing 10% FBS (Gibco, USA), and penicillin-streptomycin (100 U/mL each; Sigma—Aldrich, USA). Cell supernatants were collected 24 h after TNF-α (20 ng/mL) or LPS (100 ng/mL) stimulation. The production of TNF-α stimulated by LPS, and IL-1β and IL-6 by TNF-α stimulation were examined, respectively. Cytokine levels in the plasmas and cell culture supernatants were determined by Multi-Analyte Flow Assay Kit (Human Inflammation Panel, Biolegend, USA). Data were collected with a FACS Canto II flow cytometer (BD Bioscience) and analyzed using LEGENDplexv8.0 software (Biolegend, USA).

Western blot analysis

PBMCs were immediately lysed in radioimmunoprecipitation assay (RIPA) buffer with complete protease inhibitor (Sigma—Aldrich, USA) after centrifugation. The concentration of protein lysate was measured with BCA kit (Byotime, China), and boiled at 100 °C for 10 min with 5 × SDS-PAGE buffer. Protein lysates in protein loading buffer were separated in 8% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany), PVDF membranes was blocked with QuickBlock™ Blocking Buffer (Byotime, China), and then probed overnight at 4 °C with primary antibodies, including anti-NLRP12 (Abcam, UK) and anti-GAPDH (Bioss, China). Then, PVDF membranes were rinsed three times with 1 × TBST for 15 min. After incubation with horseradish peroxidase (HRP) conjugated secondary antibody (Bioss, China) for 1 h, the blots were washed with 1 × TBST and imaged using an enhanced chemiluminescence system (Thermo Scientific, USA). Same procedure was performed for transfected cells, and the membrane was incubated overnight at 4 °C with antibodies against the FLAG (Proteintech, USA) and GAPDH (Bioss, China).

Plasmids transfection

Wild-type (WT) NLRP12 complementary DNA (cDNA) sequences were obtained from NLRP12 clone plasmid by polymerase chain reaction (PCR), fused with a FLAG tag and inserted into pLVX-IRES-ZsGreen1 vector. Mutant NLRP12 constructs were generated by site-directed mutagenesis to introduce the p.F303Sfs*25 and p.G729R substitutions according to the manufacturer's instruction. Human embryonic kidney 293T (HEK293T) (2×10^5) in a 12-well plate were transfected by using Lipofectamine™ 3000 (Invitrogen, USA) with 1 μg expression plasmid. The expression of NLRP12 was examined by Western blot with an antibody against FLAG (Proteintech, USA).

Dual-luciferase reporter assay

HEK293 cells (1×10^5) in 24-well plate were transfected with 200 ng of pNF-κB-LUC luciferase reporter (Byotime, China), 50 ng of Renilla luciferase reporter (Byotime, China), and 250 ng of NLRP12 expression plasmids or empty vector as indicated. The NF-κB signaling pathway was induced by transfection of 200 ng of p65. After 24 h of transfection, cells were treated with 20 ng/mL TNF-α or not for 16 h. Luciferase activities were determined on cell lysates (Dual-Luciferase® Reporter Assay System, Promega, USA). Using the same cell lysates, the NLRP12 protein tagged with FLAG were quantitated by Western blot with an antibody against FLAG (Proteintech, USA).

Statistical analysis

All statistical analyses were conducted with GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA). The statistics of plasma cytokines and dual-luciferase reporter assay were analyzed by two sample *t* test. If the variance was not homogeneous, it was assessed with Welch's correction. The statistics of cytokine production levels by PBMCs were analyzed by two sample *t* test or Mann—Whitney *U* test. Differences were considered statistically significant at *P* values less than 0.05.

Results

Clinical features of patients

There were seven pedigrees enrolled in this study (Fig. 1A). And 10 patients, including 7 probands and 3 family members (P1, P2, P2 father(P2F), P3, P4, P4 mother(P4M), P5, P6, P7, P7M) were identified. Seven probands, two females and five males, were diagnosed at a median age of 2 years (2 months—5.5 years). Except of P5 who presented with Raynaud phenomenon of fingers post cold exposure, other probands were diagnosed with periodic fever syndrome or arthritis. The clinical features of the patients were shown in Table 1. P2, P4 and P7 had related family history. P2 presented with recurrent fever and rash. Her father had similar clinical phenotype when he was a child, but the symptoms gradually improved at the adulthood. P4 and P7 had arthritis and periodic fever respectively; while both of their mothers presented with arthralgia upon cold exposure. The paternal uncle of P5 had similar Raynaud phenomenon like P5, but was unavailable for genetic test.

The most common clinical manifestations of patients were recurrent fever (5/10). The duration of fever was 2—5 days in the majority of patients. Fever intervals ranged from half a month to several months. The fever of all patients recovered spontaneously or with medication. Joint

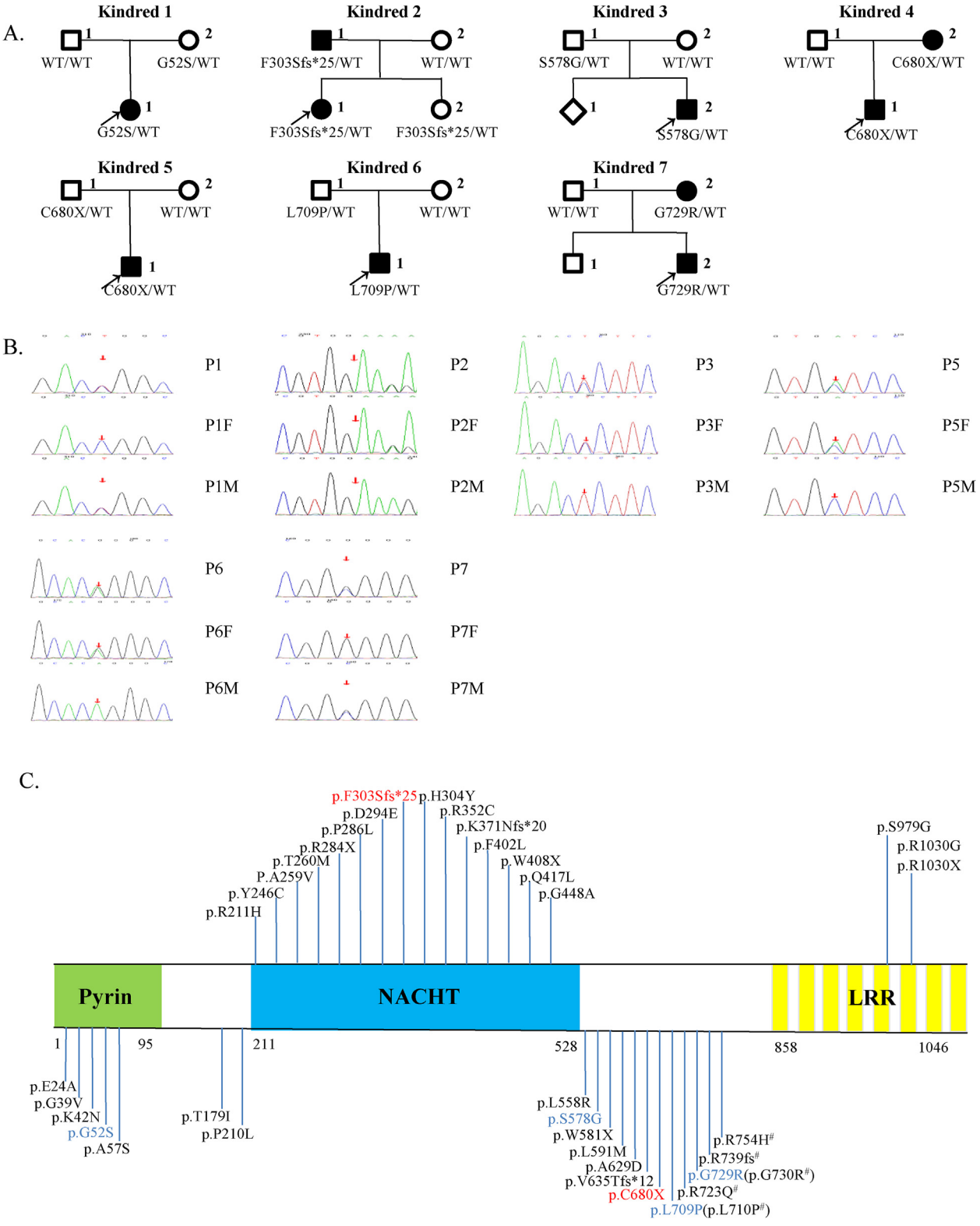

Figure 1 Genetic characterization of the patients. **(A)** Family pedigrees of the patients. Male subjects, squares; female subjects, circles; unknown gender, rhombus; patient, black squares/circles; proband, arrows. **(B)** Sequence analysis of the *NLRP12* gene. The mutations are indicated by the arrow. **(C)** Schematic structure of the NLRP12 protein with the individual domains labeled and distribution of reported variants. c.2126T > C, p.L709P(NM_144687.4) and c.2129T > C, p. L710P(NM_001277126.2) are the same mutation on different transcript versions, so are 2185G > C, p.G729R(NM_144687.4) and 2188G > C, p. G730R(NM_001277126.2). Variants reported before are labeled in black. Variants reported before and also identified in this study are shown in blue. The novel mutations are shown in red. #: the transcript version is NM_001277126.2. LRR, leucine rich repeat; NACHT, nucleotide-binding oligomerization domain; PYD, pyrin-like domain.

Table 1 Clinical presentation of the 10 patients with NLRP12 mutations.

Phenotype	P1	P2	P2F	P3	P4	P4M	P5	P6	P7	P7M
Sex	F	F	M	M	M	F	M	M	M	F
Mutation	c.154G > A (p.G52S)	c.908delT (p. F303Sfs*25)	c.908delT (p. F303Sfs*25)	c.1732A > G (p.S578G)	c.2040C > A (p.C680X)	c.2040C > A (p.C680X)	c.2040C > A (p.C680X)	c.2126T > C (p.L709P)	c.2185G > C (p.G729R)	c.2185G > C (p.G729R)
Age	2.5y	9y	30+y	3y	3y	30y	5.5y	9y	5y	39y
Age of onset	7m	3y	8+y	2m	1y	In adulthood	2y	5.5y	3y	In adulthood
Family history	−	+	+	−	+	+	−	−	+	+
Fever	−	+	+	+	−	−	−	+	+	−
Duration of fever, days	−	2−3	2−3	3−4	−	−	−	4−5	2−5	−
Rash	−	+	−	+	−	−	Raynaud phenomenon post cold exposure	+	−	+
Oral ulceration	−	+	−	−	−	−	−	+	−	−
Sensorineural hearing loss	−	−	−	−	−	−	−	−	−	−
Abdominal pain	−	−	−	−	−	−	−	+	−	−
Joint involvement	Arthritis	−	−	−	Arthritis	Arthralgia	−	Arthralgia	−	Arthralgia
Myalgia	−	−	−	−	−	−	−	+	−	−
Lymphadenopathy	−	+	−	−	−	−	−	+	−	−
Hepatosplenomegaly	−	−	−	−	−	−	−	−	+	−
CRP/ESR level	−	↑	−	ND	↑	−	−	↑	↑	↑
RF	+	−	−	−	+	−	−	−	−	−

Note : F : female; M : male; y: years; m: months; ND: not determined; RF: rheumatoid factor.

symptom was also one of main complaints (5/10). Two patients (P1 and P4) had multiple joints swelling, pain and limited mobility. Juvenile idiopathic arthritis (JIA) was diagnosed for them. And intermittent arthralgia was reported in other three patients (P4M, P6 and P7M). Besides, skin rash, oral ulceration, abdominal pain, lymphadenopathy and hepatosplenomegaly were also noted in a couple of them. The level of acute-phase reactant, erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP), was increased in half of the patients on disease attack. Positive rheumatoid factor was revealed in two patients with arthritis. As for the therapy, besides symptomatic support given to all patients, P1 is now on tocilizumab and P4 on etanercept treatment. All of them are stable so far.

NLRP12 mutation identified by next-generation sequencing and bioinformatic analysis

Next-generation sequencing (NGS) was performed and six *NLRP12* mutations were found in the patients. Of them, four (c.154G > A, p.G25S; c.2126T > C, p.L709P; 2185G > C, p.G729R and c.1732A > G, p.S578G) had been reported before in patients with periodic fever syndrome or arthritis.[20–23] Mutation c.2126T > C, p.L709P was labeled as c.2129T > C, p. L710P based on a different transcript version (NM_001277126.2). So was 2185G > C, p.G729R, labeled as 2188G > C, p.G730R (NM_001277126.2). Other two null mutations (c.908delT, p.F303Sfs∗25 and c.2040C > A, p.C680X) were novel ones. Three mutations (p.F303Sfs∗25, p.S578G and p.C680X) were located in exon 3(amino acid residue 124 − amino acid residue 691) (Fig. 1A–C). Bioinformatic analysis showed that most of the mutations were highly conserved in multiple species (Fig. 2A), and predicted to be functional damaging by a couple of algorithms including PROVEAN, SIFT, Mutation Taster and MutPred2. The MAF value of p.F303Sfs∗25 is 0.0005, while p.C680X does not presented in the general population database. The ACMG classification for variant pathogenicity of them are pathogenic or likely pathogenic.

The effects of mutation on protein structure were further analyzed. NLRP12 has the common characteristic structures of NLR family proteins, and contains N-terminal pyrin-like domain (PYD), nucleotide-binding oligomerization domain (NBD) and C-terminal leucine-rich repeat (LRR). So far, the crystal structure of human NLRP12 is only available for the PYD domain, which was used as the template for the modeling of N-terminal part of mutant NLPR12.[17] Both NLRP3 and NLRP12 belong to the NLRs protein family, and their sequence identity is over 40%, which indicates that NLRP3 and NLRP12 are homologous proteins.[18,24] Therefore, the major three-dimensional structure of NLRP12 can be predicted by using NLRP3 as a template.[18] Then the structure of the posterior part of mutant NLRP12 was modeled with NLRP3 as a reference. It was found that the missense mutations led to alteration of potential hydrogen bonds and spatial structure, and the frameshift mutations (p.F303Sfs∗25) and nonsense mutation (p.C680X) could lead to premature termination of protein translation and shortened protein structure (Fig. 2B). These data suggested that these mutations may impair protein function by altering its structure.

The null mutations changed NLRP12 protein expression

Functional studies were performed to examine the biological effects of *NLRP12* mutations. We first checked the protein expression by Western blot. Several patients were available for peripheral blood collection, and PBMCs were prepared and examined for NLRP12 protein expression. It was found that the missense mutations had no significant effects on protein expression compared with the healthy controls. However, both the samples from patients with frameshift mutation (p.F303Sfs∗25) and nonsense mutation (p.C680X) showed decreased expression of NLRP12 protein (Fig. 3A).

The effect of mutation on protein expression was further validated by *in vitro* expression assay. The wild-type *NLRP12* cDNA was fused with a FLAG tag and cloned into an expression plasmid. Mutant *NLRP12* expression constructs were generated by site-directed mutagenesis to introduce the p. F303Sfs∗25 and p.G729R alterations. The expression plasmids were then transfected into HEK293T cells for protein expression. As shown in Figure 3B, the missense mutation did not alter NLPR12 protein expression significantly. However, no protein of normal size was expressed by the plasmid containing the frameshift mutation, which, instead, produced a truncated protein.

Increased cytokine expression and impaired inhibition of NF-κB activation by the mutations

It has been reported that NLRP12 protein can modulate the inflammatory response by negatively regulating NF-κB signaling pathway. Then the cytokine response and NF-κB activation were further examined for the effects of *NLRP12* mutations. The plasmas were collected from the patients (P1, P2, P4, P7) and tested for the expressions of IL-1β, IL-6 and TNF-α. The levels of IL-6, IL-1β or TNF-α was significantly higher in the plasma of some patients than in healthy controls (Fig. 4A); but the levels of such cytokines in P4 were normal. Next, we examined the cytokine production of patient cells induced by TNF-α or LPS stimulation. The PMBCs were available from the patients (P2, P4, P4M, P6, P7, P7M) and stimulated with TNF-α or LPS. As shown in Figure 4B, post TNF-α stimulation, the productions of IL-1β and IL-6 were significant higher in the patients' PBMCs than those in normal controls. Similarly, LPS-triggered TNF-α production was also markedly higher in cells from patients than those from normal controls.

Next, we investigated the effect of *NLRP12* mutation on NF-κB signaling by *in vitro* luciferase reporting assay. As shown in Figure 4C, the wild-type NLRP12 significantly inhibited the activation of NF-κB induced by p65; whereas the inhibitory effect on NF-κB activation was almost abrogated by the frameshift mutation (p. F303Sfs∗25) (Fig. 4C). The NLRP12 with missense mutation (p.G729R) still showed

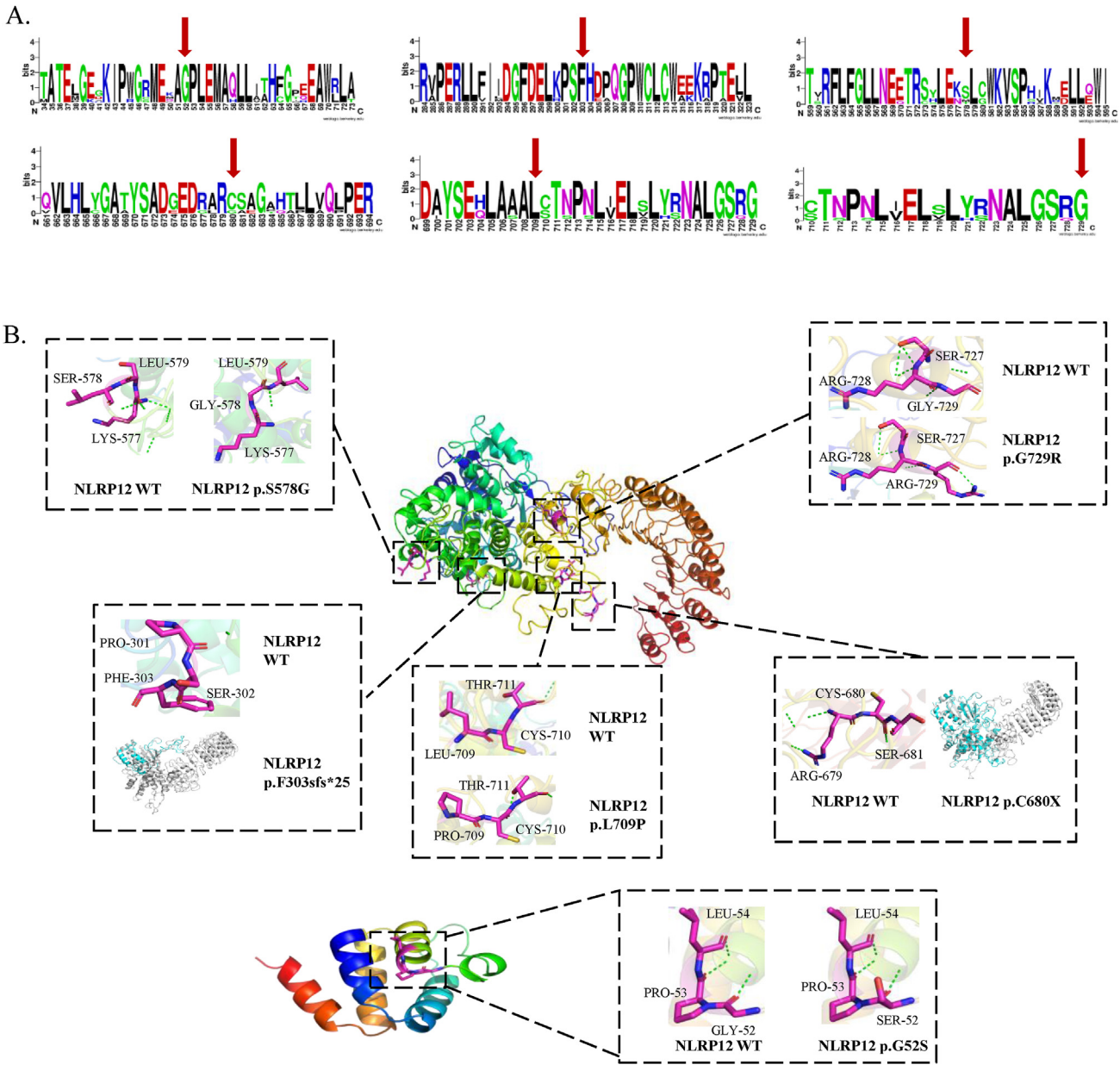

Figure 2 The effects of mutations on NLRP12 protein structure. **(A)** Amino acid conservation in the variation sites was analyzed and highlighted by a red arrow. Amino acid sequence of mutation was aligned on the weblogo.berkeley.edu across various species. **(B)** The structural impact of mutants and nearby residues were modeled on the basis of the template of 4XHS and 6NPY from PDB by Swiss-model and PyMOL2.5. Hydrogen bonds are shown as green dotted lines. Truncated proteins resulting from mutations were shown in blue, while structures of wild-type were shown in grey.

some inhibition of NF-κB activation; which, however was impaired when compared to the wild-type protein.

Discussion

In this study, we reported 10 Chinese patients mainly presenting with periodic fever, rash and arthritis. One patient had Raynaud phenomenon upon cold exposure. Exome sequencing identified *NLRP12* mutations including novel ones. Patients with same mutation could have different clinical phenotypes. The mutations induced increased cytokine expression, and impaired the inhibition of NLRP12 on NF-κB activation. These data expanded the clinical spectrum and genetic profile of NLRP12-AID.

As a member of the NLR family, NLRP12 plays a vital role in the inflammatory response and host immunity.[25–27] Its mutations were known to cause autoinflammatory diseases, such as periodic fever syndrome and familial cold autoinflammatory syndrome type 2.[13,14,28,29] So far, there are less than 90 cases of NLRP12-associated AIDs in the literature.[15,16,22,23,30–32] The clinical manifestations were variable. Recurrent fever and rash were commonest reported symptoms, followed by arthralgia, myalgia,

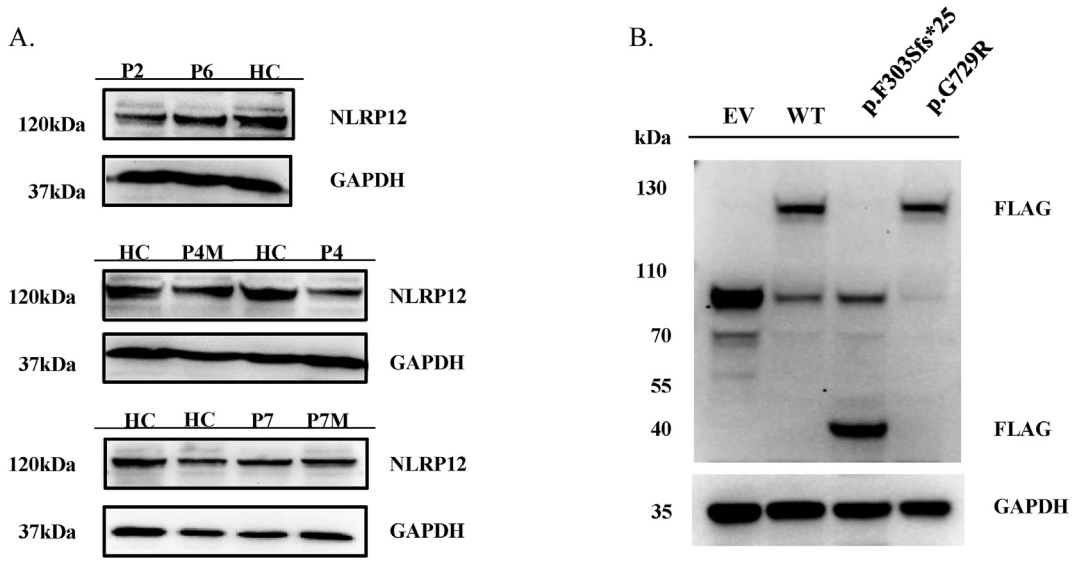

Figure 3 Impact of the mutations in NLRP12 protein expression. **(A)** The protein level of NLRP12 in whole-cell lysate was examined by using western blotting of PBMCs. **(B)** The constructed expression plasmids were transfected into HEK293T cells. The NLRP12 protein tagged with FLAG were quantitated by Western blot. The GAPDH was used as internal control. The representative result from three independent experiments is shown. EV, empty vector; WT, wild type.

lymphadenopathy and neurological condition. In addition, increased susceptibility to infection was also described.[20,33] In this study, we reported 10 patients with NLRP12 mutations. Periodic fever syndrome and arthritis were the two main clinical phenotypes. One patient showed Raynaud phenomenon triggered by cold exposure, which has not been reported before. While no infection or neurological symptom was observed in our patients. In addition, in previous reports, cold exposure was often described as a trigger for disease attack.[13,14] But in this study, it was seldom reported by our patients.

On the other hand, it seems that the patients with same NLRP12 mutation could have different clinical phenotypes. Herein the P1 patient presented with arthritis; however, another patient with the same mutation reported before had periodic fever syndrome.[20] Moreover, both the P4 and P5 had same nonsense mutation of NLRP12, but one presented with arthritis, while another with skin rash. In the pedigree 7, also distinct clinical features were reported by the P7 and his mother. The former had recurrent fever, and the latter just showed arthralgia triggered by cold exposure. Indeed, this phenomenon has also been reported in NLRP3, where a same NLRP3 mutation was identified in different cryopyrin-associated periodic syndromes.[34–36]

In addition to distinct clinical manifestations, incomplete penetrance was also noted for NLRP12-AID before. And both the missense and null mutations have been reported with it.[13,16,37,38] Consistently with this, incomplete penetrance was also demonstrated in our patients herein. Indeed, this phenomenon has also been described in the mutations of NLRP3 and TNFRSF1A, where they caused cryopyrin-associated periodic syndrome and periodic fever syndrome respectively.[39,40] Taken all together, these findings clearly revealed the highly clinical heterogeneity of NLRP12-AID. Other factors such as modifier gene, epigenetic modification and environment could contribute, at least in part, to the diversity of clinical phenotype of the disease. It has been shown that the environment could cause the epigenetic modification of genes and regulate gene expression.[41,42]

So far, the majority of *NLRP12* mutations reported before were missense mutations.[15,16,20,30] In this study, five patients had missense mutations and other half carried null mutations. Among all known mutations, most of them were located on the third exon (amino acid residue 124 — amino acid residue 691) of *NLRP12* gene. The third exon of *NLRP12* has the nucleotide binding oligomerization domain (NACHT). NACHT is located in the center of the NLR molecule and is present in all members of the NLR family, which is essential for the oligomerization and activation of NLR proteins. Therefore, mutations in this region are highly likely to affect immunomodulatory function of NLRP12, and thus are pathogenic.

It has been reported that NLRP12 can regulate the inflammatory response. NLRP12 negatively regulates canonical and non-canonical NF-κB signaling by inducing proteasome-mediated NF-κB related enzyme degradation, and therefore suppresses inflammatory response.[43] Thus, it would be expected that NLRP12 dysfunction could lead to hyperinflammatory response. Herein we showed that *NLRP12* mutations caused increased cytokine expression in the patient cells post stimulation, compared to the health controls. In addition, *NLRP12* mutations also remarkedly impaired their inhibition of NF-κB activation. These could contribute to the pathogenic mechanism underlying the hyperinflammation associated with NLRP12-AID.

In conclusion, in this study, we reported a relatively large case series of patients with NLRP12-AID. Our findings expanded the clinical spectrum and genetic profile of the disease. NLRP12-AID has a high heterogeneity of clinical phenotypes. The impaired suppression of NF-κB signaling by mutant NLRP12 may contribute to the hyperinflammation of

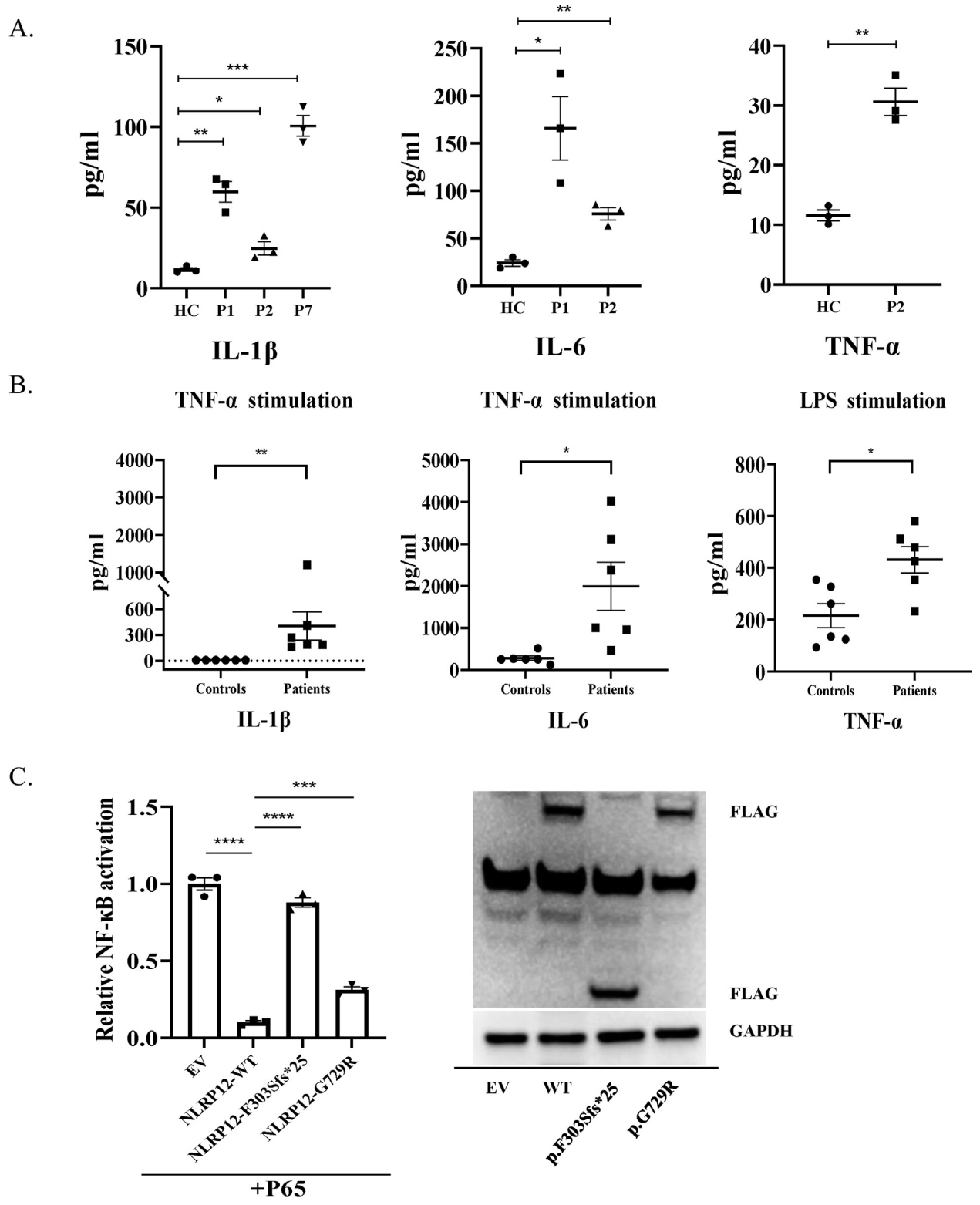

Figure 4 Increased cytokine expression and impaired inhibition of NF-κB activation by the mutations. **(A)** Elevated plasma levels of IL-1β, IL-6 and TNF-α were found in proband patients compared to healthy controls. The data were pooled from 3 independent experiments. **(B)** PBMCs were stimulated with LPS (100 ng/mL) or TNF-α (20 ng/mL) for 24 h. Then the supernatants were collected and tested for the cytokine expression. The representative result from three independent experiments is shown. **(C)** HEK293 cells were transfected with pNF-κB-LUC luciferase reporter, renilla luciferase reporter, and constructed expression plasmids as indicated. The NF-κB signaling pathway was induced by transfection of p65. After 24 h of transfection, cells were continued to culture for 16 h. Luciferase activities were determined on cell lysates. Protein levels were quantitated by Western blot analysis on the same lysates. The representative result from three independent experiments is shown. Values are represented as mean ± SEM. EV, empty vector; WT, wild type. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

the disease. Of course, large scale studies are warranted in order to dissect the full picture of NLRP12-AID. In clinical practice, NLRP12-AID should be considered in face of patients with autoinflammatory disease, especially those with genetically unknown periodic fever.

Ethics declaration

The studies involving human participants were reviewed and approved by Institutional Review Board of Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the parents.

Author contributions

H. Mao conceived, designed and guided the study, and revised the manuscript critically. Y. Li, M. Deng, Y. Li, X. Mao and S. Yan performed the experiments. Y. Li and M. Deng collected, and analyzed the data. Y. Li wrote the manuscript. X. Tang participated in the clinical assessment and management of patients. All authors contributed to and approved the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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