Abnormal processing of IL-1β in NLRP7-mutated monocytes in hydatidiform mole patients

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Abstract

Background NOD-like receptor pyrin 7 (*NLRP7*) has been identified as the major gene responsible for the recurrent hydatidiform mole (RHM). The immunological role of *NLRP7* mutation in HM patients has not been conclusively demonstrated. Hence, we aim to demonstrate this role in our study.

Methods We followed 12 new patients with *NLRP7* nonsynonymous variations (NSVs) from date to date. Peripheral blood mononuclear cells (PBMCs) were collected from patients with and without *NLRP7* mutation, separately. Supernatant IL-1β secretion, intracellular pro-IL-1β and mature-IL-1β expressions were measured after 24h lipopolysaccharide (LPS) stimulation. Plasmids with corresponding NSVs were generated to evaluate the ability of processing pro-IL-1β into mature-IL-1β *in vitro*.

Results Homozygous or compound heterozygous *NLRP7* mutation secreted less IL-1β in root of abnormal intracellular pro-IL-1β or mature-IL-1β according to different domain defective. Plasmids with NSVs could also affect processing or/and trafficking together with caspase-1 and apoptosis-associated speck-like protein (ASC).

Conclusion Inflammasome related *NLRP7* mutation is a potential mechanism of RHM.

Introduction

Hydatidiform moles (HM) is one of the most common abnormal pregnant outcomes, with an incidence of sporadic HM higher than 1 in 600-1000 in developing countries [1]. Patients with third HM are likely to carry a rare maternal-effect autosomal recessive condition. *NLRP7* and *KHDC3L*, have been identified from familial recurrent HMs (RHM) as two main pathogenic genes. *NLRP7* (NACHT, leucine rich repeating and PYD containing 7) has been identified in 48%-80% RHM patients among different populations [2-4] and *KHDC3L* (also known as c6orf221) is mutated in 10-14% RHM patients with no *NLRP7* mutation [5, 6].

The theory of abnormal methylation and abnormal maternal inflammation are two etiologies of HM. *NLRP7*, as the first identified maternal effect gene [7], has been found associated with multilocus imprinting disturbance in offsprings [8, 9]. Still, the underlying mechanism of abnormal methylation is unknown.

For another, oligomerized NLRP7 was believed to function as a multiprotein recruiting the adaptor protein, apoptosis-associated speck-like protein (ASC) through PYD-PYD interaction. Besides the PYD domain on the N terminus, ASC contains a CARD domain on the C terminus, which can active caspase-1 through CARD-CARD interaction. The complex of NLRP7, ASC and caspase-1 is believed to processing and trafficking interleukin-1β (IL-1β) [10]. Messaed *et al* point out that peripheral blood mononuclear cells (PBMCs) from patients with NSVs secrete lower IL-1β [11, 12]. However, it is difficult to obtain fresh blood cells. In this study, only 11 patients with NLRP7 mutations and rare variants were accessed to evaluate IL-1β secretion and intracellular IL-1β expression.

Considering the abundant *NLRP7* NSVs, the abnormal maternal inflammation hypothesis needs to be further confirmed. Therefore, we enrolled *NLRP7*-associated HM patients to analyze IL-1β expression of PBMCs to estimate the immunological response to LPS stimulation. Further, we generated corresponding plasmids for *in vitro* study to understand the pathogeneticity of *NLRP7* mutations with HM.

Materials and Methods

Subjects The RHM outpatients were clinically evaluated in the First Affiliated Hospital, Zhejiang University School of Medicine. The study was approved by the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine. All the participants in this study provided a written consent for collecting the blood samples, and were followed up with the telephone interview to track their reproductive outcomes. The enrolled HMs are based on clinical features, ultrasound and measurement of β-human chorionic gonadotropin (β-hCG) levels (β-hCG>100,000 U/L). Consensus diagnosis was based on H&E stained slides reviewed by two gynecologic pathologist independently. The controls did not have family histories of inflammatory condition and recurrent fetal loss, we screened them to exclude any *NLRP* variants.

Genotyping DNA was extracted from formalin-fixed, paraffin-embedded molar tissues section of patient 691 and 791 (2017) for genotyping separately, these two patients were ≥2HM without any *NLRP7* mutation or rare variants. PCR assays that amplify DNA at 21 different short tandem repeat loci. The genotypes of the molar tissues were comparing with those of the patients and their partners in order to determine the parental origin of the alleles.

Immunohistochemistry Patient 772, 815, 823, 843 and patient 691, 791 provided 4-μm formalin-fixed paraffin-embedded tissue sections for immunohistochemistry with IL-1β antibody (2022, Cell Signaling Technology).

Cytokine Assay Blood (with K_3EDTA) from patients 838, 783, 843, 815, 806, 639, 772, 734, 776, 823, 737 and 293 who carried *NLRP7* mutation and rare variants were analyzed in parallel with blood from controls of unrelated outpatient subjects (between 20 and 40 years) within 24 h after withdrawal. All of the controls had no family history of immunological, inflammatory condition or fetal losses. PBMCs were isolated using Ficoll-Paque PLUS, 1.5×10^6 cells were counted, plated in 24-well plates and stimulated with lipopolysaccharide (LPS) (1000 ng/mL) (Sigma, L6529, from Escherichia coli 055:B5) for 24 hours.

Cell Culture and Transfection One day prior to the transfection, HEK293T cells were seeded at a density of 1×10^5 cells per well using 24-well plates. The human FLAG-pro-IL-1β vector, FLAG-caspase-1 vector and FLAG-ASC vector were co-transfected with pcDNA-3.1(-)-FLAG-NLRP7 for 24 h.

Western Blotting Monoclonal antibody against FLAG (1:1000) (F3165, Sigma), monoclonal antibodies directed against human IL-1β (1:1000) (2022, Cell Signaling Technology), human NLRP7 (1:1000) (ab126979, abcam) and β-actin (1:1000) (4970S, Cell Signaling Technology) were used to detect the immunoblots. Protein bands were revealed using the NIH ImageJ software.

Site-directed Mutagenesis of Human NLRP7 Plasmid Human wtNLRP7 cDNA was cloned into PCR-Blunt-II-TOPO vector (IMAGE ID 40036028, accession no. BC109125; Open Biosystems). The *NLRP7* vector was verified following the instructions from Rima Slim *et al* . [11] FLAG-wtNLRP7 was inserted into a pcDNA-3.1(-) vector (Invitrogen) using restriction enzyme AflII and KpnI. Missense mutations in the *NLRP7* gene were produced by site-directed mutagenesis with PfuUltra High-fidelity DNA polymerase AD (Agilent Technologies) and the QuikChange™ site-directed mutagenesis (Stratagene).

Statistical Analysis The data were analyzed by SPSS17.0 software (SPSS, Inc., Chicago, IL, USA). ELISA measurements were performed using Student's *t* test. *P* values<0.05 were considered as statistically significant.

Results

Characteristics of NLRP7 mutation—Totally 81 RHM patients were diagnosed in our team between 2007 and 2018, among which 20 *NLRP7* NSVs were detected and patient 838, although with only one HM history, contained the previous reported mutation [13] (Table 1). The new 12 patients carry 6 novel variants and all the missense mutations clustered in the leucine-repeat region (LRR). Considering c.1137G>C and c.1976G>T were also found in a 300 subject control people (Hu *et al*., under review), these two were recognized as rare variants. It is reported that *NLRP7* is mutated in 48%-80% of sporadic and familial RHM

patients [3, 4]. However, the ratio depended on our study was 27.2% (22/81), much lower than previous study, which offer new evidence for theory that *NLRP7*-associated RHM varies among different ethnic groups and the genetic background underlying Chinese Han people is complex.

Table 1. Patients with defective *NLRP7* alleles

ID	**DNA**	**Protein**	**Reproductive history**	Reference
783	c.1137G>C	p.Lys379Asn *	3CHM	This study
815	c.3062A>T	p.Asp1021Val	SA,PHM,CHM	This study
838	c.251G>A	p.Cys84Tyr*	HM	This study
843	c.2155G>A	p.Thr718Ala	CHM PHM	This study
639	c.2078G>A+c:2078G>A	p.Arg693Gln*+p.Arg693Gln*	SA,PHM,CHM,PHM	This study
734	c.1137G>C+ c.1976G>T	p.Lys379Asn*+p.Arg659Leu	2CHM	This study
737	c.2165A>G +c.2471+1G>A	p.Asp722Gly*+p.Leu825X*	2CHM,3SA,PHM_GTT	This study
772	c.2161C>T+c.2161C>T	p.Arg721Trp+p.Arg721Trp	CHM,CHM_GTT	This study
776	c.2165A>G + c.2760G>A	p.Asp722Gly*+p.Trp920Ter	3CHM	This study
806	c.1294C>T+c.1294C>T	p.Arg432X *+ p.Arg432X *	1DA,2CHM	This study
823	c.1294C>T+c.2111G>A	p.Arg432X*+p. Cys704Tyr	2HM	This study
522	c.1719_1720insT + c.2165A>G	p.Asp722Gly*	2PHM,1HM_GTT	This study
293	c. 1294C>T +c.2156C>T +c.27T>C	p.Arg432X*+p.Ala719Val+p.Ile 858Thr	2CHM, CHM_GTT	[14]
492	c. 251G>A	p.Cys84Tyr	CHM,SA,failed ART	[13]
501	c.1137G>C	p.Lys379Asn *	1SA/HM,CHM,1SA	[13]
765	c. 2468T>A	p.Leu823X	2ET,2CHM	[14]
29	c.2165A>G+	p.Asp722Gly*+p.Asp722Gly*	2SA,2PHM,	[13]

	c.2165A>G			
77	c. 1294C>T + c.2471+1G>A	p.Arg432X*+ p.Leu825X*	SA,3CHM	[13]
78	c. 1294C>T + c.2471+1G>A	p.Arg432X*+ p.Leu825X*	3SA,4CHM	[13]
101	c. 2101C>T+ 2078G>A	p.Arg693Gln*+p.Cys701Ser	2HM,SB,SA,CHM	[13]
517	c.295G>T+ c.1970A>Ta	p.Glu99X+p.Asp657Val	2CHM,1failed ART	[15]
519	c.295G>T+ c.1970A>T	p.Glu99X+p.Asp657Val	3CHM,PHM	[15]
781	c.2130-312_2300+737del1218+c.2130-312_2300+737del1218		2CHM	[14]
791	c.1622_1698del76+C.2471+1G>A	p.Arg541RfsX1+ p.Leu825X*	3CHM	[14]

New variants are underlined. Asterisk indicates mutations reported in at least two unrelated patients of Chinese origin. HM, hydatidiform mole; CHM, complete HM; PHM, partial HM; SA, spontaneous abortion; DA, voluntary termination using drug; GTT, gestational trophoblastic tumor.

Expression of IL-1β in NLRP7-associated RHM patients-The genotypic results showed that patient 691 and 791 were biparental HM (Table 2).

H&E and immunohistochemistry of IL-1β of patient 772, 815, 823,843 and patient 691,791 were showed in Fig. 1. It is diagnosed that all the 6 POC (product of conception) were HM. Meanwhile, the expression of IL-1β of patient 691 and 791 were negative whitle the other four *NLRP7*-mutated patients showed IL-1β positive only between decidua.

Table 2. Microsatelite DNA genotyping of patient 691 and 791

Loci	Patient 691	POC	Partner
D1S1677	14/16	14	13/14
D1S1627	13/14	12/13	12/14

D19S433	13/16.2	15.2/16.2	14/15.2
D1GGATA113	7/12	7	7/12
D10S1435	11/13	12/13	12/14

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Loci	Patient 791	POC	Partner
D18S51	13	13/16	15/16
D7S820	11	11/12	12
Penta D	13	11/13	9/11
vWA	16/17	14/16	14
Penta E	9/15	5/9	5/11

168 POC, product of conception.

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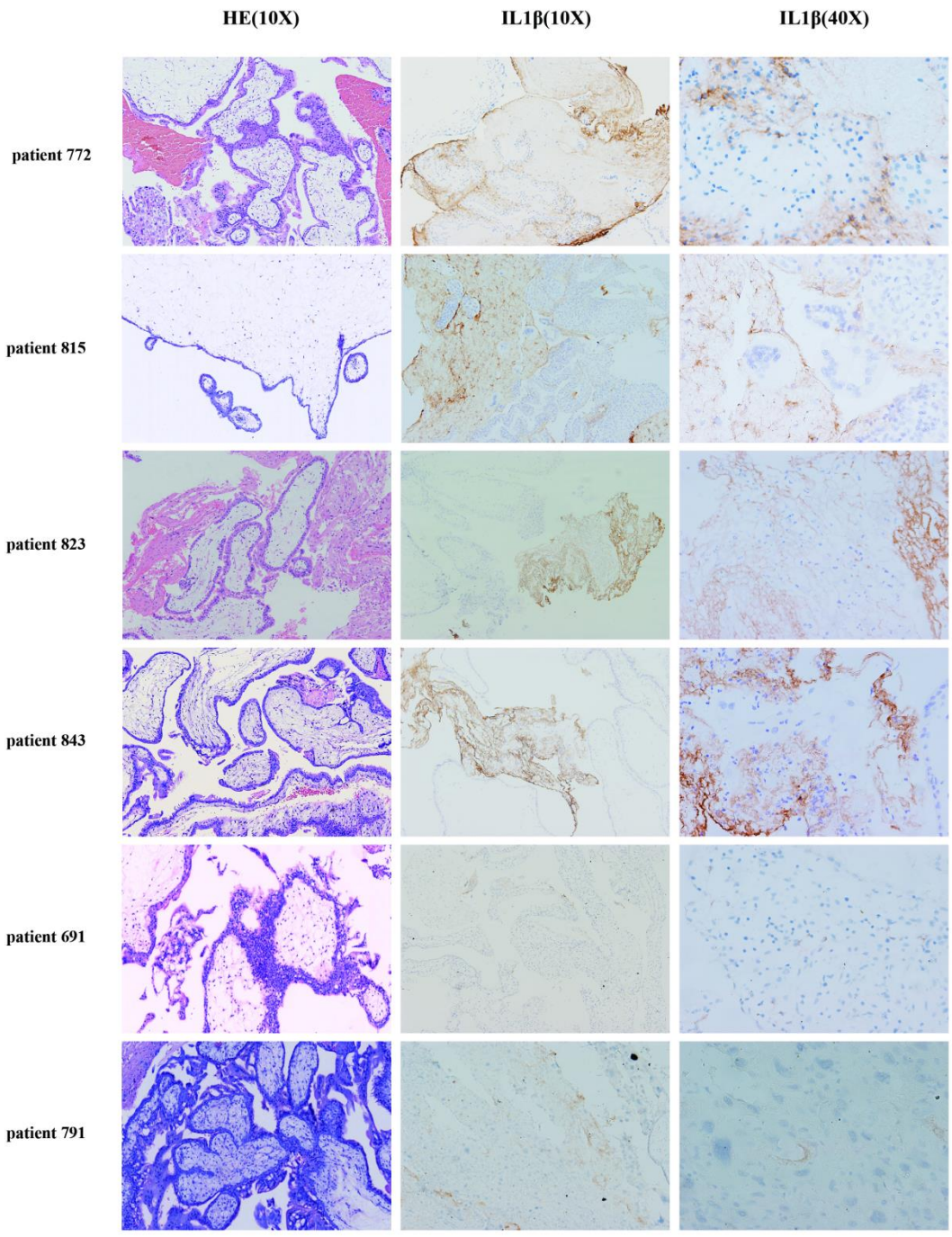

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172 **Fig. 1. Expression of IL-1β in NLRP7-associated RHM patients.** H&E staining (100×),

173 IL-1β staining (100×, 400×) of patient 772, 815, 823, 843, 691 and 791.

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Low IL-1β and TNF-α secretion by PBMCs from patients with homozygous and compound heterozygous mutation-- 12 patients were analyzed for the first time and PBMCs were assessed.

Our data demonstrates that *NLRP7*-mutated patients tended to secrete less TNF-α except patients 737 and 293 who were diagnosed later with gestational trophoblastic tumor (GTT) (Fig. 2B). However, 4 patients with only one defective allele did not secrete less IL-1β (Fig. 2A). Meanwhile, patients with one homozygous *NLRP7* mutation or compound heterozygous defective alleles, except patient 734 containing two NSVs, tended to secrete lower levels of IL-1β than controls after 24 h LPS stimulation. Additionally, the 2 RHM patients without *NLRP7* NSVs, patient 691 and 791were proved to secrete no less IL-1β or TNF-α and patient 691 was diagnosed later with GTT.

Together, TNF-α of culture supernatant can be affected by *NLRP7* mutation while only homozygous and compound heterozygous mutations secreted less IL-1β compared with controls after 24 h LPS stimulation.

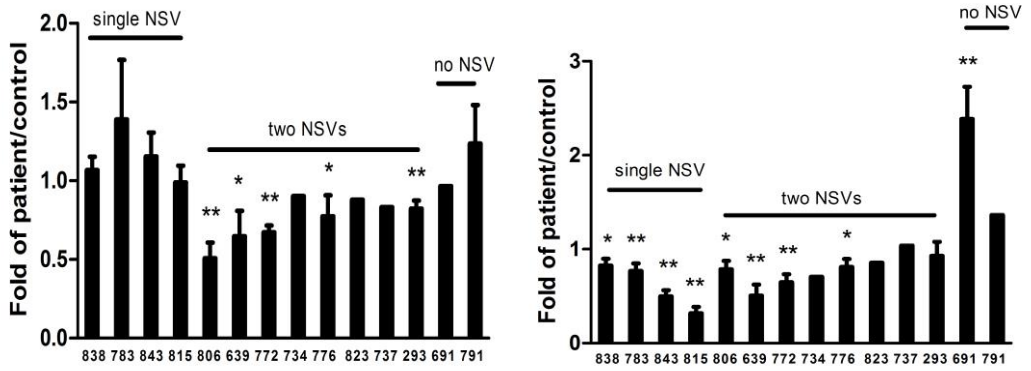

Fig. 2. **Low IL-1β and TNF-α secretion of PBMCs from patients with homozygous and compound heterozygous mutations.** Relative amounts of each cytokine refer to the secreted amounts by patients cells divided by those secreted by control cells (Δpatient/Δcontrol). The averages and SD were calculated on two to three different ELISA assays on supernatants from the same LPS stimulation.*, $p<0.05$;**, $p<0.01$. A for IL-1β. B for TNF-α.

Pro-IL-1β and mature-IL-1β expression in vitro-stimulated patient PBMCs--

The ratios of patients (patient 838, 843, 815, 806, 639, 772, 734, 776, 823 and 293) intracellular pro-IL-1β and mature-IL-1β change with controls after and before LPS stimulation were measured in 10 patients (patient 737 could not offer enough blood).

The results from patients showed that the processing of pro-IL-1β and the trafficking of mature-IL-1β are affected by the NSVs.

Patient 806, with one homozygous NACHT protein-truncating mutation, expressed less pro-IL-1β and more mature-IL-1β. Patients 639 and 772, with one homozygous LRR mutation separately, expressed more pro-IL-1β and less mature-IL-1β (Fig. 3B). These data implied that different domain of NLRP7 may play different role to affect the supernant IL-1β.

Meanwhile, except patient 734 containing two rare variants, showed no significant change of both pro-IL-1β and mature-IL-1β (Fig. 3C and 3D).

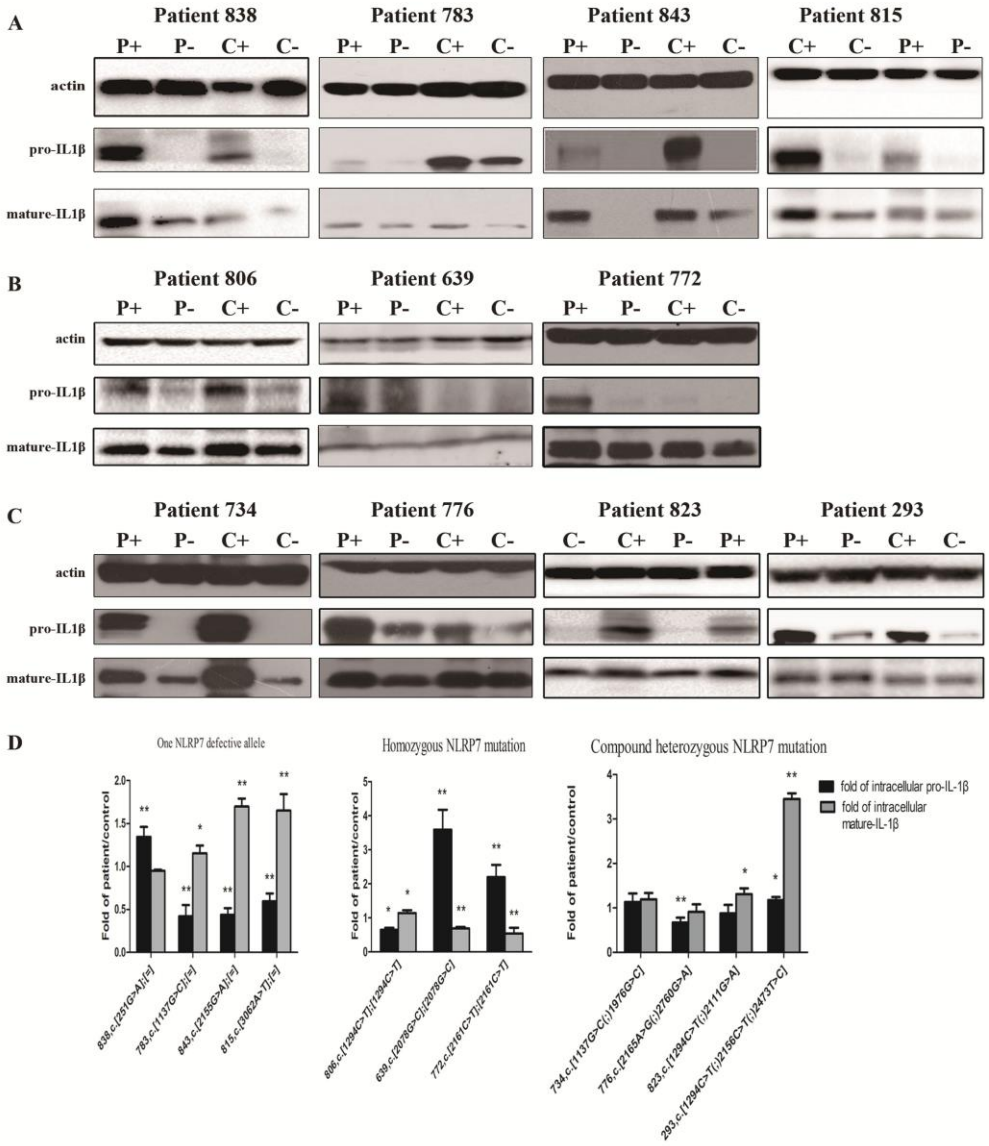

Fig. 3. Immunoblots of whole cell lysates show expressed intracellular pro-IL-1β and mature-IL-1β in patients with NLRP7 mutations in ratio to controls. The ratios of pro-IL-1β and mature-IL-1β from the cells of patients divided by control cells (Δpatient/Δcontrol) were presented after signal quantification using Image J software. A for one defective alleles patients. B for one homozygous mutation patients. C for compound heterozygous mutation patients. *, $p<0.05$; **, $p<0.01$.

NLRP7 NSV affect process and trafficking of IL-1β--Up to now, 260 mutations of NLRP7 are listed in *Infevers*, whether these mutations are HM-linked

are unclear.

Our data demonstrated that NLRP7 together with caspase-1 and ASC could process the pro-IL-1β into mature-IL-1β *in vitro* (Fig. 4).

Added with site-directed mutated plasmids, the intracellular pro-IL-1β expression and intracellular mature-IL-1β were affected according to the location of NSVs (Fig. 5). *In vitro*, from the plasmid 2078 and 2161, the LRR NSVs could affect both pro-IL-1β and mature-IL-1β. Comprehensively, plasmid 1137 together with plasmid 1137+1976 as well as plasmid 1294 together with 1294+2111, the NACHT NSVs may play a different role from LRR NSVs.

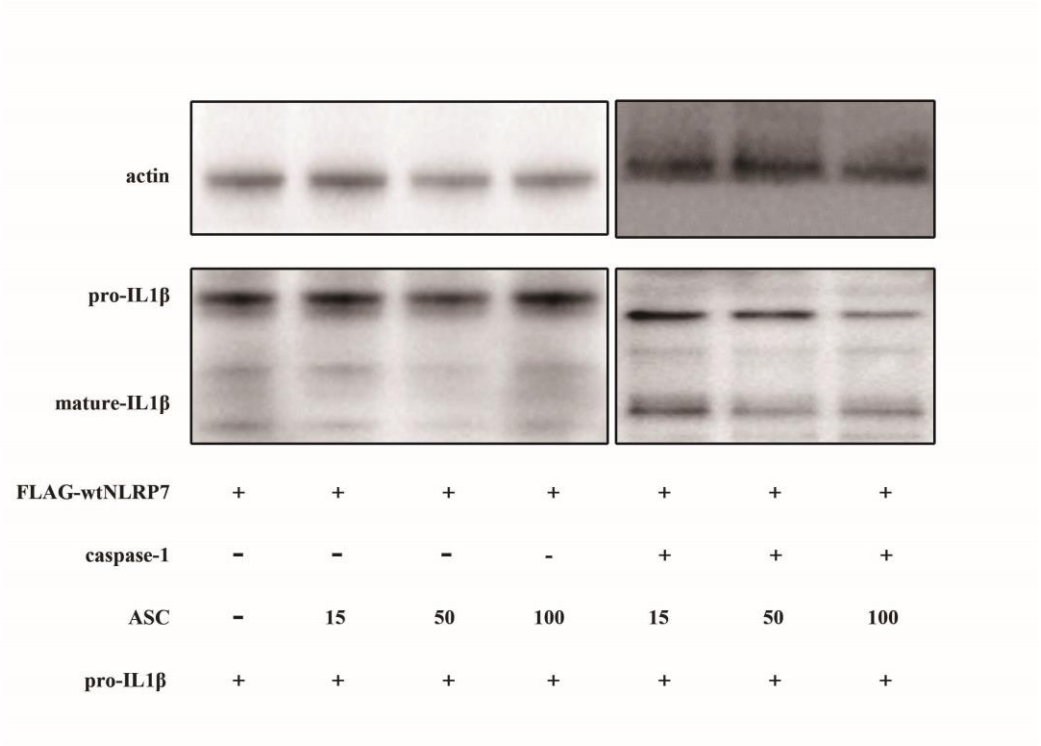

Fig. 4. ASC processed pro-IL-1β into mature-IL-1β together with flag-pro-IL-1β, flag-caspase-1 and FLAG-wtNLRP7 *in vitro.* Immunoblot of whole cell lysates of HEK293 cells that were transfected simultaneously with expression vectors flag-wt-nlrp7 (100 ng), flag-pro-IL-1β (150 ng) and different amount (0, 15,248 50,100 ng) of flag-ASC with and without flag-aspase-1 (15 ng).

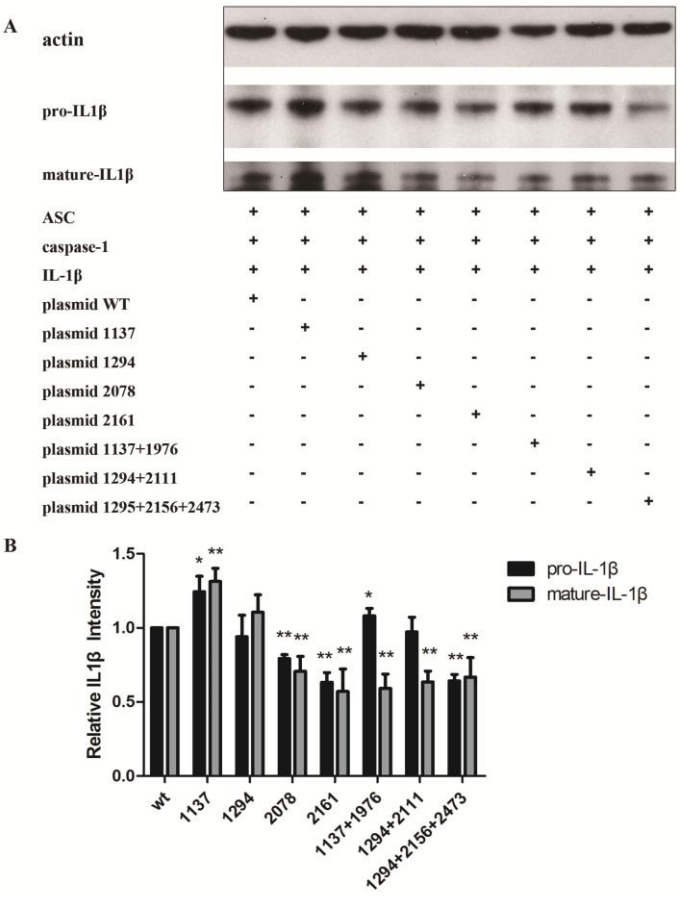

Fig. 5. Missense mutations in NLRP7 affected the IL-1β expression. A, immunoblots of HEK293 cells that were transfected simultaneously with expression vectors encoding FLAG-pro-IL-1β (150 ng), FLAG-caspase-1 (15 ng), FLAG-ASC (100 ng) and FLAG-wtNLRP7 (100 ng) or mutant NLRP7 (100 ng) expression vectors. B, representations of the quantification of pro-IL-1β and respectively, using ImageJ software. The averages and S.D. were calculated on three different Western blotting on cellular lysates from different transfection experiments. *, $p<0.05$;**, $p<0.01$.

Discussion

In this study, we reported 12 new patients with 6 new mutations. The data showed that one defective allele patients only occupy a small portion (5/22) and the LRR domain was more frequently involved in other domains (16/23). RHM can also occur in the absence of *NLRP7* mutations, which underlines the multifactorial

nature of HM. Obviously, IL-1β expressed in the decidua of *NLRP*-associated RHM, while the no *NLRP7*-mutated BiCHM barely expressed IL-1β. Additionally, PBMCs from HM patients with *NLRP7* NSVs were hyporesponsive to LPS stimulation, which rooted in either processing with/or trafficking of IL-1β. Furthermore, both the PBMCs and plasmids ascertained the NACHT domain and the LRR domain may work differently.

Long before the epigenetics in the pathology of moles, immunology has been recognized as a reason for various forms of pregnancy loss including HMs. Presently, the evidence of abnormal maternal inflammation is limiting. It is verified that NLRP7 downregulates intracellular inflammation and impairs IL-1β secretion in various monocytes, which is consistent with the fact that PBMCs from *NLRP7*-defective alleles patients secrete less IL-1β [10-12].

Many immune cells have been identified in the endometrium include uterine NK (uNK) cells, macrophages, mast cells, dendritic cells (DC) and T cells. These endometrial lymphocytes together constitute the maternal immune microenvironment. Single-cell transcriptome profiles from early human maternal-fetal interface showed that placental extravillous trophoblast cells were adjacent to macrophages [16]. AnCHM is able to introduce maternal immune responses which leading to fetal rejection and recruitment of immune cells to the decidual tissue[17] . Compared with AnCHM, although containing more maternal genetic materials, the BiCHM is also charaterized by proliferative trophoblast cells as well. Reduced levels of NLRP7 accelerate trophoblast differentiation of human embryonic stem cells [18], however, little is known about the exact function of leukocytes involved in BiCHM.

According to Singer et al. that domains of *NLRP7* play certain roles in inflammasome activity [19]. In this study, mutation located in LRR domain is different from protein-truncating mutation in NACHT domain, which suggests that each domain of *NLRP7* plays a different role in either activating or polymerizing the inflammasomes and the lower IL-1β was caused by less processing or/with trafficking of IL-1β. Interestingly, the IL-1β of patient , whose NSVs were

considered as rare variants did not show differences in pro-IL-1β and mature-IL-1β expression or IL-1β secretion.

The exact mechanism underlying the *NLRP7*mutations and RHM is unclear, whether the NSV is a missense mutation or rare varients may lead to different therapeuties. Presently we are still far from offering a comprehensive view of the relationship between mutation and pathogenicity due to the incomplete data from the patients. Whether HM-linked *NLRP7* mutants is gain-or loss-of-function defects impact inflammasome activity, we provided more data, especially by adding the essential inflammasome adaptor ASC *in vitro*.

In conclusion, our results directly support the hypothesis of lowering the level of immunity in cases of *NLRP7* mutation due to the decreased levels of IL-1, and hence the decreased immunologic ability to repel HMs. On the other hand, the plasmids 734, taken from a patient with AnCHM, did not show such a decrease in the *in vitro* processing *of* pro-IL-1β. This observation further supports that *NLRP7*mutation helps the formation of BiCHM. In the future, larger trials are needed to better understand the association by assessing other types of interleukins and interferons for being potential cofounders.

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