Associations with Single Rare Heterozygous NLRP7 Variants for Chinese Patients with Sporadic Gestational Trophoblastic Diseases

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Research article

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Abstract

Background Gestational trophoblastic diseases (GTDs) encompass a group of clinically heterogeneous tumors of trophoblastic cell origin. In some populations and studies, single *NLRP7* heterozygous mutations and some non-synonymous variants (NSVs) have been shown to confer genetic susceptibility to sporadic GTDs. Our study aims to investigate whether single rare heterozygous NSVs in *NLRP7* predispose patients to GTDs in Chinese.

Methods We performed Sanger sequencing of all *NLRP7* exons and exon-intron boundaries in a cohort of Chinese patients with non-recurrent sporadic GTDs and normal childbearing women. Using *in-silico* analysis and several prediction programs, we investigated the effects of identified NSVs on the function of the protein. Then we examine the function in inflammatory pathway to assessing the functional consequences of the identified rare NSVs in *in-vitro* studies.

Results We found that the number of patients with rare single heterozygous NSVs that are predicted to have functional effects on the protein is significantly higher in the first cohort of 292 patients than in the 300 controls ($p=0.028$). Analysis of the validation cohort of 130 patients confirmed the significant higher number of patients with rare NSVs than controls ($p=0.0014$). We show that most of the rare variants that are identified only in the patients are predicted to have functional effects on the protein by various algorithms and in-vitro experiments.

Conclusion Our results demonstrate that Chinese patients with GTDs have a higher burden of single heterozygous *NLRP7* variants which were predicted to have effects on the protein than controls. We suggest that some of these single NSVs may contribute to the genetic susceptibility for GTDs in China.

Background

Gestational trophoblastic diseases (GTDs) are defined by the abnormal proliferation of trophoblastic cells, including hydatidiform mole (HM), invasive mole, choriocarcinoma, placental site trophoblastic tumor (PSTT) and some other rare tumors. Gestational trophoblastic neoplasia (GTNs) has been applied collectively to the latter four conditions, that have potential for invasion and metastasis and require chemotherapy and/or additional surgical procedures. GTNs may manifest in the form of high HCG levels several weeks or months after molar evacuation or as tumors demonstrated by histopathological evaluation in the form of invasive mole (IM), choriocarcinoma (CC), PSTT, and epithelioid trophoblastic tumor (ETT) [1]. GTNs occur mostly after HM, and rarely after other types of pregnancies. HM occurs once in every 600 to 1500 pregnancies in western countries, but at higher frequencies in several underdeveloped and developing countries [2–4]. In China, the reported incidence of HMs varies from 1 to 8.83 in every 1000 pregnancies with the highest incidence being in the province of Zhejiang where it reaches 1 in every 700 pregnancies [3, 5].

Familial recurrent HMs (RHMs) are rarer [6–9] and the analysis of such cases has led to the identification of two causative genes, *NLRP7* and *KHDC3L*, responsible for this condition [10, 11]. *NLRP7* is a major gene for RHMs and is mutated in 48–80% of patients with RHMs depending on populations and patients ascertainment criteria [12–15]. To date, approximately 59 mutations in *NLRP7* have been reported in patients with two defective alleles and are listed on INFEVERS [16]. In addition, 18 rare single heterozygous non-synonymous variants (NSVs), mostly missenses, in *NLRP7* have been seen in patients with sporadic HMs and recurrent spontaneous abortions [17–21]. Some of these NSVs seemed to underlie the genetic susceptibility for sporadic HMs [13, 18, 19]. However, these data were not replicated in other populations and studies [21–23]. Similar studies on patients with GTNs have been limited due to their lower frequencies in western societies. To date, only one study looked at the presence of *NLRP7* NSVs in patients with post-molar choriocarcinomas in Senegalese patients and showed the presence of three novel NSVs that were not found in controls from the general Senegalese population [14]. Therefore additional studies are needed to reach a clear conclusion.

NLRP7 belongs to the CATERPILLER family and contains four conserved and functional domains, including the N-terminal pyridine domain PYRIN, the C-terminal leucine rich repeat, 9 to 10 leucine-rich repeats (LRRs), the NACHT-related domain (NAD) (physical medium of oligomeric assembly) and the NACHT region in the middle of the protein (this domain contains the Walker A / P-loop motif, which is the binding site for ATP) [24, 25]. The LRR and PYRIN domains on *NLRP7* are involved in protein-protein interactions, and the NACHT domain is involved in apoptosis and MHC class II transactivation, and PYRIN also interacts with a variety of inflammatory components to negatively regulate inflammatory body reactions [26]. Members of the CATERPILLER protein family are widely expressed in various tissues of different species, from *C. elegans*, *Drosophila melanogaster*, rats, mice to humans [27]. They act as components of inflammation and play a role in innate immunity, inflammation and apoptosis. Patients with *NLRP7* mutations have significantly lower levels of IL-1 β and TNF- α produced by LPS stimulation and are therefore considered to function as part of the immune system [24]. Some studies suggest that *NLRP7* mutations in patients can result in impaired cytokine secretion and subsequent inflammatory responses, allowing them to tolerate the growth of abnormal hydatidiform moles without embryos, thereby demonstrating the phenotype of the mole [28]. Inflammasomes regulate the innate immune response by promoting the maturation of inflammatory cytokines, interleukins IL-1 β and IL-18. There is evidence that *NLRP7* protein is also a component of Inflammasome, and several proteins which interact with *NLRP7* have been identified, including PRO-CASPASE-1, PRO-IL-1 β , apoptosis-associated spot-like protein (ASC). CASPASE-1 mediates proteolytic cleavage of PRO-IL-1 β and PRO-IL-18, affecting the biological activity, secretory form of these cytokines, to initiate and extend inflammatory host responses. CASPASE-1 recruits activated PRRs via a central inflammatory adapter, ASC, which contains the CASPASE recruitment domain (CARD) [24]. Subsequently, CASPASE-1 clustering caused its automatic activation by inducing proximity. ASC is critical for all inflammasomes that are activated by members of the NLR family that contain the PYRIN domain (NLRP) and LRR.

To address whether single heterozygous *NLRP7* NSVs predispose the patients to GTDs, we sequenced *NLRP7* in a first cohort of 292 Chinese patients with GTDs (155 with GTNs and 137 without GTNs), and 300 controls. As opposed to previous studies, in which controls were screened for specific NSVs found in patients [13, 18, 19, 21], we sequenced the 11 *NLRP7* exons in all patients and controls and found that the number of subjects with rare single heterozygous NSVs is higher in patients than in controls. We then used a validation cohort of 130 Chinese patients with GTDs (88 with GTNs and 42 without GTNs). Our data on the two independent cohorts demonstrate a higher burden of rare NSVs in *NLRP7* on Chinese patients with GTDs as compared to normal bearing women. However, no significant differences in the number of rare NSVs were observed between patients with GTNs and without GTNs. Meanwhile, there are 14 rare NSVs identified only in the patients, most of them are predicted to have functional effects on the protein by various algorithms. And we tested 13 of

these 14 rare NSVs *in vitro*, as Glu488dup was not a single rare heterozygous *NLRP7* variants, the results show that some variants have effects on the inflammatory pathway which have a positive effect predicted by various algorithms. And the variants predicted to be benign show the negative symptom.

Methods

Patients

The first cohort of patients contained a total of 292 unrelated Chinese patients with sporadic GTDs ascertained from various clinics and hospitals in the province of Zhejiang. We did not include any patient with RHMs in this study. Patients from cohort 1 were recruited between 2003 and 2013. The size of the initial patient cohort was based on the number of cases that were available at the time of the initiation of the study. A total of 300 controls were ascertained from the same population and ethnic group between 2006 and 2014. A second cohort of 130 patients was ascertained from the same hospitals and clinics between 2013 and 2015. Informed consents were obtained from all individuals. Questionnaires recapitulating medical and family histories was completed for every patient.

The diagnosis of sporadic HM was based on histopathological reports according to standard criteria [29]. HM can be classified into CHM and PHM. CHM were diagnosed based on the presence of abnormal circumferential trophoblastic proliferation and absence of embryonic tissues while PHMs were diagnosed based on the presence of mild trophoblastic proliferation. The diagnosis of GTNs, their staging and scoring was carried out according to the revised criteria of the International Federation of Gynecology and Obstetrics and the World Health Organization (WHO) scores [30]. These criteria are: 1) a plateau in the HCG level of four values (plus or minus 10%) recorded over a 3-week period on days 1, 7, 14, and 21; 2) an increase in HCG level of more than 10% in three tests recorded over a 2-week period on days 1, 7, and 14; 3) a persistent detectable HCG for more than 6 months after molar evacuation; 4) a histological diagnosis of choriocarcinoma or invasive mole on uterine curettage or the identification of clinical or radiographic evidence of metastases. According to these criteria, patients from cohorts 1 and 2 were divided into two groups, those with GTNs and those without GTN (Table 1). The antecedent pregnancies of patients with GTNs are provided in Supplementary Table 1, 2. Control group consisted of 300 control women of Chinese origin who had one child and no reproductive loss. All patients and controls are of Han ethnic origin.

NLRP7 mutation analysis

DNA was isolated from whole blood cells using Flexi-gene DNA Kit (Qiagen Inc. Shanghai, China). Sequence analysis was performed by PCR amplification of genomic DNA of the 11 *NLRP7* exons followed by sequencing in both directions as previously described (Qian *et al.*, 2011) at the Shanghai Biosune Biotechnology Co. Ltd (Shanghai, China). Identified NSVs in patients and controls were confirmed in a repeating PCR reaction and in available parents. Variants were annotated according to GenBank accession numbers NM_001127255.1 and Q8WX94 as recommended by the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/recs.html>) [31]. Protein numbering starts from the initiation codon (codon 1). We refer to NSVs for DNA changes leading to missense amino acids substitutions. We refer to common NSVs for missense variants with MAF >5%, low frequency NSVs for missense variants with $0.5\% \leq \text{MAF} \leq 5\%$, and rare NSVs for missense variants with $\text{MAF} < 0.5\%$ in the 300 Chinese controls.

Statistical Analysis

For each cohort of patients, statistical analyses were performed on patients with GTNs and patients without GTNs, combined or separately, as compared to the same cohort of 300 controls. The mutation burden test was performed for a single gene, *NLRP7*, as previously described using the collapsing method [32]. One patient (525) had a variant in a homozygous state and this patient was counted only once. Patients with more than one NSV were also counted only once and are listed in Table 1 with the least frequent variant. Chi-square analysis was performed using two-by-two contingency table at the Simple Interactive Statistical Analysis (SISA) (<http://www.quantitativeskills.com/sisa/distributions/binomial.htm>), using Fisher exact test. P values less than 0.05 were considered significant.

Prediction of pathogenic impacts

The potential pathogenic effects of the rare NSVs on the protein function were assessed by four different protein prediction software programs: PolyPhen-2 (Polymorphism Phenotyping v2) (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (sorts intolerant from tolerant) (<http://sift.jcvi.org/>), AGVGD (Align GVGD) (<http://agvgd.iarc.fr/>), and SNAP. PolyPhen-2 prediction is based on sequence similarity, phylogenetic conservation and structural information. Polyphen-2 score ranges from 0 to 1 where 0 is neutral and 1 is the highest damaging. SIFT is a sequence homology based prediction tool that sorts intolerant from tolerant using multiple alignment files. SIFT score ranges from 0 to 1, where 0 is predicted not to be tolerated and 1 is predicted to be tolerated. AGVGD uses both biophysical characteristics of amino acids and multiple sequence alignment of proteins. AGVGD scores are classified in to 7 classes C0, C15, C25, C35, C45, C55 and C65, where C65 is predicted most likely damaging and C0 most likely neutral. In this study, C15 was not considered to have an effect on the protein since the AGVGD algorithm considers it as less likely to be damaging. SNAP (screening for non-acceptable polymorphisms) is a neural network based prediction tool that uses secondary structure, conservation and solvent accessibility of a protein to predict the functionality of the mutated proteins. SNAP scores range from 1 to 100 and -1 to -100. Variants with positive scores have no effects on the protein function while the ones with negative scores are functionally damaging.

Protein stability predictions were performed using four programs, I-Mutant 2.0, I-Mutant 3.0, iPTREE-STAB and Mupro. I-Mutant 2.0 and 3.0 show the free energy change ($\Delta\Delta G$) upon mutation. A $\Delta\Delta G$ of < -0.5 Kcal/mol is predicted to be largely destabilizing, $\Delta\Delta G$ of > 0.5 Kcal/mol is predicted to be largely

stabilizing and values between the two are predicted to have a weak effect on the stability of the protein. Mupro prediction scores are shown as $\Delta\Delta G$ and the negative scores are predicted as destabilizing and positive as stabilizing. iPTREE-STAB uses mainly residue base information for its prediction. Again, scores are shown as $\Delta\Delta G$ in Kcal/mol with negative scores predicted to be destabilizing and positive stabilizing.

Experiment on functional effects of 13 rare NSVs

From two cohort of patients, there are 14 rare NSVs identified only in the patients, with one variant, Glu488dup was not a single rare heterozygous *NLRP7* variants, We test the remain 13 variants of these 14 rare NSVs *in vitro*.

Site-directed Mutagenesis of Human NLRP7

A standard PCR was conducted with the primers in a total volume of 50 μ l by the following procedure: 94°C 2min, then 98 °C 10s and 68 °C 3.5min for 10 cycles, and 4 °C for one hour. Then add of DpnI (2 μ l, 10 U/ μ l) to it and incubate at 37°C for one hr. The Ki nation /ligation reactions was performed in a total volume of 20 μ l (DpnI-treated PCR product 2 μ l, PCR grade water 7 μ l, Ligation high 5 μ l, T4 Polynucleotide Kinase 1 μ l, at 16°C for 1hr) and then transformed the into E.coli (DH5 α).All the plasmids were extracted by SanPrep Column Plasmid Mini-Prep Kit (Shanghai Sangon Biotech CO., Ltd) and the sequences were confirmation by Sangon Biotech (Shanghai) .

Cell Culture and Transfection

HEK293 cells were cultured using 12-well plates in 2ml of DMEM (Invitrogen) supplemented with 10% of FBS, when it reaches a density of 80% to 100% per well, we began the transfection. In co-transfection experiments, the pcDNA 3.1(+)-pro-IL-1 β (200ng) vector encoding the human IL-1 β protein, the pcDNA 3.1(+)-ASC(200ng) vector encoding the human ASC protein, the pcDNA 3.1(+)-FLAG-caspase-1(30ng) vector encoding the human caspase-1 protein (NM_033292.21) were co-transfected with pcDNA 3.1(+)-FLAG-*NLRP7* (200ng). and diluted in Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Use the indicated volume of DNA and PLUS™ Reagent with each of the four volumes of Lipofectamine® LTX.

Western blot

Incubate cells for 1–3 days at 37°C, extract Proteins from cells culture in, separated proteins with 10% SDS-PAGE, then transferred electro-phoretically onto a polyvinylidene fluoride blotting membrane. Blocked with 5% Gibco for 2 h at room temperature, and immunodetected using a monoclonal antibody directed against Flag (Cell Signaling Technology) (1:2000), and human IL-1 β (2022, Cell Signaling Technology) (1:1000), and caspase-1 (2144, Cell Signaling Technology) (1:1000), and β -actin (MAB1501, Chemicon Intl.) (1:1000) overnight at 4°C. Protein bands were revealed using the Hyperfilm ECL Western blotting detection reagents (GE Healthcare) and quantified by NIH ImageJ software.

Results

Patients with GTDs have several rare novel NSVs

The screening of *NLRP7* gene for NSVs and mutations in two independent cohorts (cohort 1, n = 292 and cohort 2, n = 130) of Chinese patients with GTDs (with GTNs and without GTNs) revealed one common NSV, V319I, with a minor allele frequency of 14.3%; three low frequency NSVs, S126,K379N and V699I, with MAF of 4.7%–1% and 1.8%, respectively; and 21 rare NSVs with MAF of < 0.5% (Table 1). All rare NSVs found in the patients were in heterozygous state with the exception of one variant in patient 525 (Supplementary Table 1). This patient was found homozygous for p.Arg721Trp, a previously reported disease-causing mutation found in a homozygous state in three unrelated patients with RHMs, one of Armenian origin [33] and two of Indian origin (unpublished data). Another patient, 370 (Supplementary Table 1), was found heterozygous carrier of a previously reported disease-causing mutation, p.Asp722Gly, observed in a homozygous state in a Chinese patient [18]. In this study, only one patient was found to have a novel duplication of AGG leading to an in-frame insertion of a Glutamic acid at position 488 and the remaining had NSVs. This variant was not used to compute the p-values because it is not possible to predict its impact on the protein function with four software. Of the 21 rare NSVs, 14 were found only in patients and not in controls. Three of these variants are listed on the 1000 Genome database but do not have frequencies because of their scarcity and the remaining are novel.

Table 1

The screening of *NLRP7* gene for NSVs and mutations in two independent cohorts (cohort 1, n = 292 and cohort 2, n = 130) of Chinese patients with GTDs (with or without GTNs). And the result of NSVs predicted by four computational analysis programs, Polyphen, SIFT, AGVGD, and SNAP, that use various structural, evolutionary, and comparative considerations to predict the functional impact of all identified NSVs.

cDNA variant	Protein variant	RS number	Patients with GTN (n = 155 + 88 = 244)	Patients without GTN (n = 137 + 42 = 179)	Controls (n = 300)	MAF in patients (n = 422)	MAF in controls (n = 300)	MAF in 1000Genome database in Han Chinese populations (CHS, CHB, CDX)	Polyphen	SIFT	AGVGD
NSVs found in patients but not in controls											
c.198G > C	Trp66Cys		1/155			0.0011	not found	not found	Pro D	T 0.07	C35
c.728G > C	Ala243Val			1/137		0.0011	not found	not found	Pos D	T 0.10	C0
c.1071 C > G	Phe357Leu		1/155			0.0011	not found	not found	Pro D	A 0.00	C0
c.1538C > T	Pro513Leu		1/155			0.0011	not found	not found	Pro D	T 0.09	C0
c.2165A > G	Asp722Gly	rs104885540	1/155			0.0011	not found	n.a.	Pos D	T 0.12	C0
c.2201C > T	Thr734Met	rs150220721	1/155			0.0011	not found	0.0000	Pro D	T 0.09	C0
c.2161C > T	Arg721Trp *	rs104885525	1/88			0.0011	not found	n.a.	B	A 0.02	C65
c.1090G > A	Ala364Thr			1/42		0.0011	not found	not found	Pro D	T 0.35	C0
c.1148C > T	Pro383Leu	rs199475826		1/42		0.0011	not found	n.a.	Pos D	T 1.00	C35
c.2573T > C	Iso858Thr			1/42		0.0011	not found	not found	B	A 0.04	C65
c.1463AGGdup	<i>Glu488dup~</i>		1/88			0.0011	not found	0.0000	n.a.	n.a.	n.a.
c.2444 G > A	Arg815His	rs150034626	2/88			0.0023	not found	0.0000	B	T 0.78	C0
c.1757T > C	Val586Ala		1/155			0.0011	not found	not found	B	T 0.69	C0
c.924C > A	Asp308Glu		1/155			0.0011	not found	not found	B	T 0.36	C0
NSVs found in patients and controls											
c.251G > A	Cys84Tyr	rs104885509		2/137, 1/42	1/300	0.0035	0.0017	0.022	Pos D	A 0.05	C0
c.2809C > T	Arg937Cys	rs145163808		1/137	1/300	0.0011	0.0017	0.0000	Pro D	T 0.12	C15
c.376T > G	Ser126Ala	rs187063197	4/155		2/300	0.047	0.0033	0.001	B	T 0.74	C15
c.1879G > C	Gly627Arg	rs201120887	1/155, 1/88			0.0023	0.0000	0.01 in CHS	B	T 0.47	C15
c.1137G > C	Lys379Asn		3/88	5/137	6/300	0.0106	0.0100	not found	B	T 0.06	C0
c.316G > A	Gly106Arg	rs201987540	1/155	2/137, 1/42	2/300	0.0047	0.0033	0.0000	B	T 0.23	C0
c.1460G > A	Gly487Glu	rs775881	1/155, 1/88	1/137		0.0047	0.0000	0.01 in CHS	B	T 1.00	C0
c.1441G > A	Ala481Thr	rs61747414	1/155		1/300	0.0035	0.0017	0.0000	B	T 0.44	C0
c.271G > A	Met91Val	rs201748256		1/137	1/300	0.0011	0.0017	0.0026	B	T 0.96	C0
c.2095G > A	Val699Ile	rs77072552	7/155, 2/88	8/137	15/300	0.0184	0.0250	0.04	B	T 0.93	C0

cDNA variant	Protein variant	RS number	Patients with GTN (n = 155 + 88 = 244)	Patients without GTN (n = 137 + 42 = 179)	Controls (n = 300)	MAF in patients (n = 422)	MAF in controls (n = 300)	MAF in 1000Genome database in Han Chinese populations (CHS, CHB, CDX)	Polyphen	SIFT	AGV
c.955G > A	Val319Iso	rs775882	44/155, 13/88	32/137,9/42	40/300	0.1436	0.152	0.2889	B	T 0.45	C0
			9/292 (3.08%)		2/300 (0.66%)	Cohort 1: all patients vs controls p = 0.028					
			5/155 (3.2%)			Cohort 1: patients with GTN vs controls, p = 0.048					
				4/137 (2.9%)		Cohort 1: patients without GTN vs controls, p = 0.08					
			8/130 (6.15%)			Cohort 2: all patients vs controls p = 0.0014					
			4/88 (4.5%)			Cohort 2: patients with GTN vs controls, p = 0.0256					
				4/42 (9.5%)		Cohort 2: patients without GTN vs controls, p = 0.0024					

Prediction of the pathogenic effects of the rare NSVs

To evaluate the potential functional consequences of the NSVs found in the patients and/or the controls, we used four computational analysis programs, Polyphen, SIFT, AGVGD, and SNAP, that use various structural, evolutionary, and comparative considerations to predict the functional impact of all identified NSVs (Table 1 and Fig. 1). The results of this analysis showed that 76% of the rare NSVs that were found only in the patients are predicted to be damaging by any of the four programs while 25% of rare NSVs observed in both patients and controls are predicted to be damaging (Figure. 1A). This difference between the predicted pathogenicity of NSVs seen only in patients versus those seen in patients and controls was significant (p = 0.029). In addition, NSVs observed only in patients were predicted to have functional effects by more programs than those observed in patients and controls (Figure. 1B). Therefore, our analysis showed that rare NSVs observed only in patients have, in general, a higher probability to be predicted damaging than those observed in both patients and controls. We also investigated the predicted impact of three variants, p.Trp66Cys, p.Cys84Tyr, and p.Met91Val, located in the pyrin domain on its recently described three-dimensional structure [28]. We found that p.Trp66Cys is highly predicted to affect the stability of the protein (Figure. 1C); the two others, p.Cys84Tyr and p.Met91Val, were also predicted to reduce the stability, but had milder effects. This was only possible for variants located in the pyrin domain for which a three-dimensional structure is available.

Patients with GTDs have a higher burden of rare variants in NLRP7

We next performed a rare variant burden test by comparing the number of subjects with rare NSVs that are predicted to have a functional effect on the protein, by any of the four programs, between patients and controls. This analysis revealed a significant higher number of patients with rare NSVs that are predicted to be functional than of controls (p = 0.028). We next divided the patients from cohort 1 according to the malignant degeneration of their GTDs between patients with GTNs and patients without GTNs. The analysis of mutation burden on these two categories of patients as compared to controls revealed a borderline difference between patients with GTNs and controls (p = 0.048) but no significance was observed between patients without GTNs and controls. Also, no significant difference was detected between patients with GTNs and patients without GTNs in cohort 1.

To validate the results obtained on the first cohort, we performed the mutation burden test on a second cohort of 130 patients, 88 patients with GTNs and 42 patients without GTNs, as compared to the same 300 controls. This analysis confirmed the finding obtained on the first cohort and demonstrated a significant higher number of patients with rare NSVs predicted to have functional effects on the protein than controls (p value = 0.0014). In the second cohort, a significant difference was detected between the number of patients with (p = 0.0256) and without GTNs (p = 0.0024) and rare NSVs predicted to be functional as compared to controls even after splitting them into the two categories. Again, in cohort 2, no significant difference was detected between patients with GTNs and patients without GTNs.

Some NLRP7 mutants effect the synthesis of IL-1β

It has reported that, *NLRP7* have an negative effect on synthesis of Pro-IL-1β, and mutation and variants in *NLRP7* may disrupt this effect [13]. There are fourteen mutation variants were probably associated with GTDs, because they were found in patients and not in control subjects (Table 1). Glu488dup couldn't be built in plasmid. As shown in Fig. 2A, other thirteen mutations located in different regions of *NLRP7*, only one variant in PYRIN domain, five in NACHT domain, one in NAD domain, and five of thirteen mutants belong to LRR. To investigate the rare NSVs roles *in vitro*, we co-transfected the FLAG-tagged-NACHT domain, and *NLRP7* mutants/wild type (we treated wild type as control), caspase-1, ASC, and IL-1β to deduce the process of the synthesis and the maturation of IL-1β. We had used western blotting to observe the difference in the protein expression. And the result indicated that patients 283, 324, respectively with

T66C and V586A variants, have an augment in Pro-IL-1 β ($p = 0.04857$, $p = 0.02853$), thus, there is an increased mature-IL-1 β in 283 ($p = 0.02116$). Also, Pro-IL-1 β tended to be increased in V586A, A721T, I858T, but these variants did not reach statistical significance (Fig. 2B).

Discussion

In these study, we identified a total of 21 rare NSVs only in patients which were more frequently predicted to have pathogenic effects and tended to be predicted harmful by more programs. However, no significant differences were detected between patients with GTNs and patients without GTNs in the two cohorts. Our results demonstrate that Chinese patients with GTDs have a higher burden of single heterozygous *NLRP7* variants predicted to have effects on the protein than controls. We suggest that some of these single NSVs may contribute to the genetic susceptibility for GTDs in China.

Among the 422 analyzed patients, we found two patients with previously described pathogenic mutations, one in a heterozygous state seen as a single variant and another in a homozygous state. The latter indicates that the frequency of homozygous *NLRP7* mutation among Chinese women with sporadic non-recurrent GTDs in China is approximately 1 in 422 and is in agreement with a recent finding from England where one patient with recessive mutations in *NLRP7* was found among 650 patients with a prior CHM[9].

Previous research has established an association between homozygous or compound-heterozygous maternal effect variants in the *NLRP7* gene and hydatidiform moles/reproductive wastage[34]. As we know, normal *NLRP7*, in addition to its role in cytokine secretion, has another role in down-regulating the intracellular level of pro-IL-1 β . Some mutations and variants in this gene can impair this function and consequently lead to higher amounts of pro-IL-1 β production[13]. This is the pathway many studies adopted to research on RHM and reproductive loss. It is currently believed that the *NLRP7* gene may cause hydatidiform moles and other types of failed pregnancies by impaired cytokine secretion and consequent inflammatory response[13]. Nevertheless, explicit mechanism has not been clarified. So, as for GTDS, we try to investigate whether this rare NSVs of *NLRP7* also have roles on this pathway. As is mentioned above, there are 13 *NLRP7* mutants founded in patients and absent in control groups. And we examined them *in vitro* to explore any possible influence in inflammatory pathway. The result indicated that patients 283, 324, respectively with rare variants T66C and V586A, have a higher secretion of pro-IL-1 β . There was also an elevation in mature-IL-1 β in patient 283. It's worth noting that, among the two variants we identify as meaningful, three bioinformatic prediction tools generated damaging results for variant T66C, which is consistent with our results above. It showed that comprehensive evaluation of pathogenicity of *NLRP7* mutation through these tools has certain instructing value. As for the lack of effect on IL-1 β in other 8 variants identified damaging by at least one of the softwares, *NLRP7* may exert its pathogenicity in GTDs through other mechanisms, like affecting DNA methylation as maternal imprinted gene. Also, it is possible that our *in vitro* system is not sensitive enough to detect milder defects caused by these variants. There are still many features that haven't been yet elucidated, and further research is needed to reveal specific role of *NLRP7* mutations in the reproductive system.

Because of the One-Child Policy in China, our control subjects consisted of women who had only one child and no reproductive loss, which may not be a sufficient criterion to exclude the potential genetic susceptibility of the control women to sporadic HMs or reproductive loss. It is, possible that some of our control women with rare NSVs may develop in the future molar pregnancies. Consequently, we believe that this limitation may have attenuated the observed differences between patients and controls. Other limitations to our study are the lack of access to another cohort of controls to validate our findings in the controls.

Epidemiological studies have always shown that GTDs are more frequent in Asian populations[2]. The absence of differences between patients and controls in the frequencies of the common NSV, V319I, and the low frequency NSVs, V699I and K379N, suggests that these NSVs do not underlie the higher frequency of GTDs in China, which is in agreement with our previous observations on a different population[13]. On the contrary, our study suggests that rare NSVs that are predicted to be functional may underlie the genetic susceptibility for the higher incidence of GTDs in China and is in agreement with previous observations in several other diseases[35–38]. Similar analyses of large cohorts of patients with GTDs and controls from other countries and from China will be important to replicate our data and identify whether this higher burden of rare variants is specific to some ethnic groups and populations known to have higher frequencies of GTDs than western countries.

Conclusions

Our results show that the rate of *NLRP7* NSVs in Chinese GTN patients is relatively high, and the prediction of the effect of mutation on the protein is meaningful.

Abbreviations

GTDs gestational trophoblastic diseases

GTNs gestational trophoblastic neoplasia

HM hydatidiform mole

PSTT placental site trophoblastic tumor

IM invasive mole

CC choriocarcinoma

ETT epithelioid trophoblastic tumor

LRRs leucine-rich repeats

NAD NACHT-related domain

ASC apoptosis-associated spot-like protein

NSVs non-synonymous variants

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving patient were in accordance with the ethical standards of Institute Research Ethics Committee of The First Affiliated Hospital, ZheJiang University School of Medicine . The consent was written and the reference number was 2013-270.

Consent for publication

The author confirms that the work described has not been published before and its publication has been approved by all co-authors.

Availability of data and material

Competing Interest

The authors have no conflicts of interest to declare.

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Informed consent

Informed consent was obtained from all individual participants included in the study.

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Author Contributions

YJ, DY, SJ, Zhou W and Zhao W collected biological samples and isolated DNA; CQ and XX performed PCR on all the samples; SY and HJ performed mutational analysis; RR verified all the variants, performed statistical analysis and functional predictions; RS supervised variants verification and performed statistical analysis and; YX, SY, RS, PW and QJ conceived the study and wrote the manuscript.a

All authors have read and approved the manuscript.

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Figures

Figure 1

A. Comparison between the predicted functional effects of rare NSVs identified only in patients and those identified in patients and controls using various prediction programs. B. Comparison between the number of programs that predicted functional effects for rare NSVs observed only in patients and rare NSVs observed in patients and controls. The nuclear magnetic resonance structure of the pyrin domain was used as template to predict the protein stability of three NSVs. The four programs calculate energy changes ($\Delta\Delta G$) between the mutant and native proteins. Positive values indicate increased stability, negative values decreased stability. The four programs predicted that W66C has the most destabilising effect while the two others had milder effects.

A

B

Figure 2

A. Schematic of NLRP7 protein structure with representative mutations identified in patients but not found in control. PYD=pyrin domain; NACHT=found in NAIP, CIITA, HET-E, and TP1 protein family; NAD=NACHT-associated domain; LRR=leucine rich region; B. Expression of protein in Wide-type group transfected with IL-1 β (150 ng), FLAG-caspase-1 (15 ng), FLAG-Asc(100 ng), FLAG-tagged NLRP7 wild-type (200 ng), and control group; Western blotting results of HEK293 cells that were transfected simultaneously with expression vectors encoding IL-1 β (150 ng), FLAG-caspase-1 (15 ng), FLAG-Asc(100 ng), FLAG-tagged NLRP7 mutants (200 ng), and FLAG-tagged NLRP7 wild-type (200 ng); Relative expression of pro-IL-1 β and mature-IL-1 β in wt-group and the impact of mutants-group.

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