The Function Changes of the Ala^{348}to Thr Polymorphism in the P2X7R Closely Related to the Onset of Gout

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Research Article

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Abstract

Gout is a common disease of inflammatory arthritis caused by hyperuricemia and the deposition of MSU crystals. Our previous study has shown that ATP action on P2X7R could be the second signal to induce the onset of gouty arthritis. However, the biological function changes of SNPs in the P2X7R, which affect the ATP-P2X7R-IL-1β signaling pathway with high uric acid, remained unknown. To further research P2RX7 gene variant (encoded by the SNP as rs1718119) polymorphism association between the gout susceptibility and the functional effect, 270 patients with gout and 70 hyperuricemia patients without history of gout flare (over 5-years course) were recruited in this study. The current study first time analyzed the genotyping study in genomic DNA samples from gout and hypeluricemia patients, then flow cytometry was used to test the transfected $Ala^{348}$to Thr mutant in HEK-293T cells. Expression levels of IL-1β, NLRP3, ASC, caspase-1 mRNA in THP-1 cells were analyzed by quantitative real-time polymerase chain reaction. Expression levels of IL-1β were measured by enzyme-linked immunosorbent assay. The gout-sensitivity allele at rs1718119 was A. The AA and AG genotypes exhibited a higher risk of gout and the gout-sensitivity allele at rs1718119 was A. Moreover, $Ala^{348}$to Thr increased P2X7-dependent ethidium^{+} bromide uptake. What's more, Ala^{348} to Thr significantly up-regulated the serum and mRNA levels of IL-1β compared with wild type (P=0.0007;P=0.0334,respectively). Expression levels of NLRP3 mRNA in cells with Ala^{348} to Thr also showed a higher level than wild type(p=0.0003). However, no statistical significance were found in the mRNA expression of ASC and caspase-1 between $Ala^{348}$to Thr and wild type in all three groups(P 0.05). Our study revealed that the biological function changes of one SNP (rs1718119) mutation affected the ATP-P2X7R-IL-IL-1β signaling pathway with high uric acid. And, both rs1718119 was dominant gene. The genetic variability of the P2X7R rs1718119 gene might, in part, be associated with susceptibility for gout onset.

Introduction

Gout is a inflammatory arthritis caused by hyperuricemia. It is a type of inflammatory diseases with the Monosodium Urate (MSU) crystals deposit in the tissues surrounding the joint resulting in joint swelling and pain[1]. Current studies[1] have shown that MSU crystals induced gout, by means of the activation of toll-like receptors (TLRs), the oligomerization domain (NOD)-like receptor family and pyrin domain containing 3 (NLRP3) ,the signal transduction produced interleukin-1beta (IL-1β). In addition, the changes in extracellular adenosine triphosphate(ATP) can also stimulate the purinergic receptor P2X ligand-gated ion channel 7 (P2X7R) signaling pathway, synergistically MSU crystal stimulation to produce NLRP3 inflammatory bodies, leading to gout attack. Therefore, the functional status and the genetic polymorphisms of P2X7R might be an important factor in determining the incidence of acute gouty arthritis.

The P2X7R is an ATP-gated ion channel supporting $Na^{+}$and Ca^{2+} influx into and K^{+}efflux out of the cell cytoplasm[2]. P2X7R stimulation with high (0.5 to 1 mM and above) concentrations of ATP causes in as yet poorly understood fashion the opening of a non-selective pore permeable to hydrophylic solutes of

MW up to 900 Da, such as ethidium^{+} bromide, YO-Pro, or Lucifer yellow [3–5].The P2X7R is activated by extracellular ATP to induce NLRP3 inflammasome assembly, which is one of the most potent activators of the NLRP3 inflammasome[6].Constitutive expression of NLRP3 inflammasome has predominantly detected in macrophages[7]. Imani[8] analysed four common SNPs of NLRP3 (rs10754558, rs35829419, rs3806265, rs4612666) with relapsing-remitting MS (RRMS). The results showed that the SNPs of NLRP3 gene (rs3806265) locus C allele and C/C genotype were more frequent in the RRMS patients than the control group, and the expression of NLRP3 in patients with relapse was significantly reduced. Therefore, it is speculated that NLRP3 inflammatory bodies play an important role in the development of MS. The NLRP3 inflammasome is a multi-protein complex and is composed of NLRP3, Apoptosis-associated speck-like protein containing a caspase-1 recruitment domain (ASC) and pro-caspase-1[9]. The NLR family contains two inflammatory bodies, NLRP3 and absent in melanoma 2 (AIM2). NLRP3 recruits caspase-1 in an ASC-dependent manner[10].After recognition of specific agonists, NLRP3 and AIM2 form inflammatory bodies. Therefore ASC is an inflammasome adapter protein that is required for the formation of the AIM2 and NLRP3 inflammasomes[11].In addition, caspase-1 is initially expressed as an inactive precursor(pro-caspase-1) and plays a biological role that needs to be activated[12].It also plays a critical role as a mediator that activates the subsequent maturation of the pro-inflammatory cytokines IL-1β[13].

Single nucleotide polymorphisms (SNPs) have been used to study human P2RX7 gene, such as rs2230911, rs3751143, rs208294, et al[14–17], which can affect the K^{+} outflow velocity and change the functional status of P2X7R by affecting the pore formation ability[15].According to Tao[15] and Ying[16] et al. studies, both gout and hyperuricemia patients, whom were carrying the susceptibility genotype AA or AT of rs1653624, had a significantly higher level of IL-1β than patients carrying the non-susceptibility genotype TT. Beyond that, there were no obviously differences in allele or genotype frequencies between gout and hyperuricemia patients at a different SNP (rs1718119)[15].The P2X7R containing the Ala^{348} to Thr polymorphism (rs1718119) at position 1068 of the P2RX7 gene from single base substitutions have been identified in exon 11 located in transmembrane domain 2. The Ala^{348}to Thr polymorphism exhibits increased P2X7R functional responses, and stimulation of human monocytes from individuals carrying gain-of-function haplotypes induces increased secretion of the proinflammatory cytokine IL-1β when compared to wild type P2X7R in monocytes[17].

In conclusion, overall the data presented in this study demonstrate that the variant(rs1718119) affected the function of P2X7R by changing the structure of P2X7R ,which may influence the susceptibility to gout. The allele and genotype frequencies of rs1718119 was found to be associated with gout susceptibility. A allele and GG/(AA + AG) genotypes in rs1718119 were at a higher risk of gout.We further constructed the models of wild type and Ala^{348}to Thr mutation SNP in cells, found that the Ala^{348}to Thr polymorphism has the major effect in conferring increased receptor function that ATP induced with high uric acid.

Materials And Methods

2.1 Subjects and collection of clinical information

This study was designed as a case-control study and a total of 270 gout patients were recruited in current study. All gout patients were diagnosed according with the American College of Rheumatology classification criteria[18]. In present study, we only selected male cases because male individuals were used as more susceptible than females to develop gout. And beyond that, there is research showing that urate levels were associated with gout and the development of gout within five years[19]. But not all individuals with hyperuricemia develop the clinical features of gout. Based on the above, a total of 70 hyperuricemia patients over 5-years course of disease with serum uric acid levels > 480 μmol/L (8 mg/dl) having no history of gout flare were recruited from the physical examination center or a related department of the first affiliated hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China in this study. After the informed consent had been obtained, two groups of genomic DNA samples were respectively extracted from 270 gout patients and 70 hypeluricemia patients peripheral venous blood using a Qiagen DNA Kit (Qiagen, Germany) following the standard DNA isolation instructions.

2.2 Selection of SNP loci of P2X7R

In the process of searching for suitable point mutation sequences from the human P2X7R cDNA sequence(Genebank accession number: NM_002562.5), rs1718119 (Ala^{348}to Thr) was selected, which were believed to impact the secretion of IL-1β and thus play a vital role in the pathogenesis of gout[20], were examined in our present study.This SNP was genotyped using an EP1™ high-throughput gene analysis system (Fluidigm, U.S.). The linkage disequilibrium (LD) between selected SNP was caculated based on HapMap genotype data using PLINK software version 1.07[21]. Moreover, construction of THP-1 cell model of transfected Ala^{348}to Thr mutation and stimulation of THP-1 cells from P2X7 MUT subjects with PMA, MSU and ATP induced a strong rise in levels of IL-1β.

2.3 Reagents

RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, bovine serum albumin were purchased from Gibco and Hyclone. ATP, MSU and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St Louis, MO, USA).

2.4 Cell culture

The attachment-dependent, human embryonic kidney cell line, HEK-293 T cell (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco's Modified Eagle's Media containing high glucose (4.5 g/L), GlutMAXTM I (3.97 mM) (DMEM, Gibco), with added 5% fetal bovine serum (Hyclone). Monocytic THP-1 cells, human monocyte line, obtained as a gift of Department of Immunology (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China), were grown in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco)

at 37°C and 5% CO_2. THP-1 and HEK-293 T cells were plated at the density of 1.0–1.5×10^6/mL in 6-well plates Cells were cultured in 75 cm^2-tissue culture flasks (Costar) and subcultured every 2–3 days as the population approached 80–90% confluency in a humidified 5% CO_2 atmosphere at 37°C.

2.5 Lentivirus production and transduction

Lentivirus was generated for overexpression of P2X7R in HEK-293 T cells and THP-1 cells. Lentivirus vectors were generated by inserting the P2X7R sequences encoding human P2X7R into the multicloning site of the lentivirus backbone plasmid of pHBLV-CMV-MCS-3FLAG-EF1-ZsGreen-T2A-PURO. The constructs were co-transfected with packaging vectors into HEK-293 T cells for packaging followed by purification (Hanbio Biotechnology Co.,LTD, Shanghai, CHINA). Then, infect the HEK-297 T cells and THP-1 cells with concentrated virus solution. Cells were used for further assays 3 d after transfection. HEK-293 T cells was transduced at MOI 10, THP-1 cell was transduced at MOI 100. Cells transduced with lentiviral overexpression vector containing P2X7R lentiviral particles in the presence of 6 μg/ml polybrene. After 24 h, culture medium was removed and fresh medium was added. After transfected, stable transfected HEK-293 T or THP-1 cells were obtained for one day or three days, respectively.

2.6 Ethidium influx measurement

By observing the uptake ability of HEK-293 T cells to ethidium$^+$ bromide by flow cytometry (BD Biosciences, San Jose, USA), it was observed that the effects of different mutations on P2X7R were observed on the P2X7 channel. Cells (1×10^5) prelabeled with GFP-conjugated LVs were washed once and resuspended in 1.0 ml of HEPES-buffered KCl medium(150 mM KCl, 5 mM D-glucose, 0.1% bovine serum albumin, 10mM HEPES, pH 7.5) at 37°C. All samples were stirred and temperature-controlled at 37°C using a Time Zero module. Ethidium$^+$ (25 uM) was added followed 40 s later by addition of 1.0 mM ATP. Cells were analyzed at 1,000 events/s on a flow cytometry. The linear mean channel of fluorescence intensity (0–255 channel) for each gated subpopulation over successive 5-s intervals was analyzed by flwojo software and plotted against time.

2.7 Functional experiment

THP-1 cells were plated at the density of 2.5 × 10^4mL in 24-well plates. THP-1 cells were stimulated for 3 h with 100 ng/mL PMA the day before stimulation[22]. This treatment enhances the phagocytic properties of the cells and prompts a constitutive production of pro-IL-1[23, 24], Then dissolve the purchased MSU crystals with sodium hydroxide to prepare an MSU chylosus suspension with a concentration of 100 ug/mL. And use this suspension to stimulate THP-1 cells into cell culture medium. Finally, THP-1 cells were randomized into three groups: wild type or mutant types were added MSU (labeled group M), MSU + ATP (labeled group MA), unstimulated control group (labeled group C), respectively. Group M and MA were added MSU for 24h, then ATP was added to the group MA for 60 minutes.

2.8 Enzyme-linked immunosorbent assay

Serum samples were obtained from the the serum in THP-1 cell. The presence of IL-1β in serum was measured with commercial enzyme-linked immunosorbent assay (ELISA) kits(R&D Systems, Minnesota, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm using a microplate reader.

2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from treated splenocytes using Trizol reagent (Sigma) and the concentration of RNA was determined using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNAs were reverse-transcribed with cDNA Reverse Transcription Kit (QIAGEN, Duesseldorf, Germany), after which quantitative real-time PCR amplification was performed with SYBR® Green PCR Kit (Qiagen) according to the manufacturer's instructions. Primers for human IL-1β, NLRP3, ASC and caspase-1 were synthesized by Sangon Biotech (Shanghai, China). The sequences of the primers were as follows in Table 1. All cDNA samples were amplified in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA). Data were analyzed using the $^{\Delta\Delta}$Ct comparative quantification method following normalization to β-actin.

Table 1 PCR primers

IL-1β	Forward primer	5'-TCATTGTGGCTGTGGAGAAG-3'
	Reverse primer	5'-AGGCCACAGGTATTTTGTCG-3'
NLRP3	Forward primer	5'-GATCTTCGCTGCGATCAACAG-3'
	Reverse primer	5'-CGTGCATTATCTGAACCCCAC-3'
ASC	Forward primer	5'-TGGGCCTGCAGGAGATG-3'
	Reverse primer	5'-ATTTGGTGGGATTGCCAG-3'
caspase-1	Forward primer	5'-AATAAATGGCTTGCTGGATGAG-3'
	Reverse primer	5'-CCTCCTGGTCCTGAAGATGC-3'
β-actin	Forward primer	5' -CCTTCCTGGGCATGGAGTCCTG-3'
	Reverse primer	5' -GGAGCAATGATCTTGATCTTC-3'

2.10 Statistical analysis

Data were determined using the SPSS 23.0 software (SPSS, Chicago, IL, USA). and the graphics were drawn by GraphPad Prism 6. Data are presented as mean ± SEM. To determine statistical significance among multiple comparisons, we used a one-way ANOVA test followed by a post hoc analysis. Differences between two individual experimental groups were compared by a two-tailed t test. In addition,we compared SNP genotype and allele frequencies between gout and hyperuricemia group for

each SNP. Odds ratios (ORs) and 95% confidence interval (CIs) were calculated using non-conditional logistic regression analyses. Quantitative data are presented as the means ± standard deviation. Hardy-Weinberg equilibrium (HWE) in gout patients and normal controls was determined using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php)[25]. All statistical analysis was performed using Stata version 14.2 (Stata Corp, College Station, TX, USA). A two-sided P value of < 0.05 was considered as statistically significant.

Results

3.1 Demographic characteristics

To study the Ala^{348}to Thr in patients, 270 gout patients and 70 hypeluricemia patients were enrolled. All of them are males. The mean ages of the patients in the hypeluricemia and gout groups were 49.4 ± 13.4 and 52.7 ± 16.2 years, respectively. There was no significant difference in average age at presentation between the two groups (P = 0.29).

3.2 Hardy-Weinberg equilibrium test

H-W balance testing for all genotyping results revealed that rs1718119 genotype frequency was consistent with HWE equilibrium in Table 2(P > 0.05).

Table 2
Hardy-Weinberg equilibrium test of the genotype frequencies distribution in gout and hyperuriceamia patients

SNPs	group	genotype			HWE	
						P-value
		AA	AG	GG		
rs1718119	GOUT	16	94	160	0.20	0.658
	HUA	2	10	58	2.98	0.084

3.3 Differences in the distribution of SNP genotype in P2X7R between gout and hyperuricemia patients

Differences in the prevalence of rs1718119 were observed between gout and hyperuricemia patients. The GG, GA and AA genotype frequencies of rs1718119 were 82.9%, 14.3% and 2.9%, respectively, in hyperuricemia group, and 59.3%, 34.8% and 5.9%, respectively, in gout group. There was statistically significant difference between the two groups in rs1718119 genotype distribution ($\chi 2 = 13.48$, P = 0.001). In the hyperuricemia group, the frequency of the A and G alleles were 10.0% and 90.0%, respectively, which was 23.3% and 76.7%, respectively, in gout group. The gout-sensitivity allele at rs1718119 was A (OR = 2.74, 95%CI: 1.50–5.33). The AA and AG genotypes exhibited a higher risk of gout (AG vs. GG, OR = 3.41(95%CI: 1.62–7.82); (AA + AG) vs. GG, OR = 3.33(95%CI: 1.66–7.10)) (Table 2). Although the

comparison between gout and hyperuricemia patients carrying the recessive genetic model (AG + GG) showed a slightly increased odds ratio [OR = 2.14, 95% CI (0.48–19.62)], the difference was not significant in Table 3(P = 0.307) .

Table 3
Allele and genotype frequencies and genetic model of SNP rs1718119 in gout and hyperuricemia patients

Gennetic model	GOUTs	HUAs	χ2	P-value	OR	95%CI
A vs. G	126/414	14/126	12.09	P < 0.001	2.74	1.50–5.33
AA vs. GG	16/160	2/58	2.11	0.147	2.90	0.65–26.68
AG vs. GG	94/160	10/58	12.20	P < 0.001	3.41	1.62–7.82
(AA + AG) vs. GG	110/160	12/58	13.45	P < 0.001	3.33	1.66–7.10
AA vs. (AG + GG)	16/254	2/68	1.04	0.307	2.14	0.48–19.62

3.4 Association between the number of susceptible genotypes and the risk of gout.

Rs1718119 was dominant gene. Homozygous and heterozygous hyperuricemia patients with this allele exhibit a higher risk of gout than patients without this allele. Patients with hyperuricemia may have a relatively low risk of gout if they carry a single SNP-susceptible genotype, but if they carry more than one susceptible SNPs, the risk of gout may be increased. Based on this hypothesis, we further analyzed the risk of gout in patients with hyperuricemia who carry two susceptible SNP genotypes. The results demonstrated that more susceptible genotypes increased gout risk, and the OR reached 4.91(95%CI:2.34–11.23) in patients with susceptible genotype in Table 4.

Table 4
Association between the number of susceptible genotypes and the risk of gout

Contains all gout susceptibility genotypes?	Gout (n)	Hyperuricemia (n)	P-value	OR	95%CI
Yes	108	10	P < 0.001	4.91	2.34–11.23
No	132	60			

3.5 Dominant positive effect of Ala^{348} to Thr on P2X7 pore formation function in HEK-293 T cells

HEK-293 T cells were transfected with virus carrying Ala^{348} to Thr mutation. Non-transfected cells (empty virus) were set as a blank control, and the transfected cells (wild type) were set as a experimental control. We examined the functional effect of Ala^{348} to Thr on P2X7 pore formation using ATP-induced ethidium^{+} bromide uptake assay. Cell were stimulated with ATP for 260s. Wild type showed brisk uptake of

ethidium^{+} bromide dye via the P2X7R.The Ala^{348} to Thr mutation showed a P2X7-mediated ethidium^{+} bromide uptake which was more than the wild type value. In Fig. 1A.

The Ala^{348} to Thr mutation has a gain-of-function effect on the uptake function of ATP-induced ethidium^{+} bromide. The Ala^{348} to Thr increased P2X7-dependent ethidium^{+} bromide uptake in Fig. 1B (145% of wild type P2X7 response, $P < 0.001$).

3.6 Establishment of a THP-1 cell line stably expressing P2X7R with Wild Type and Ala^{348} to Thr

As shown in Fig. 2, lentivirus carrying Ala^{348} to Thr mutation□wild type or empty virus was stably transferred into THP-1 cells.The mRNA expressions of P2X7R in cells with Ala^{348} to Thr mutation showed a higher level than the wild type ($p = 0.035$).

3.7 The Ala^{348} to Thr mutation enhanced the expressions of IL-1β and NLRP3

THP-1 cells were transfected with virus carrying Ala^{348} to Thr mutation□wild type or empty virus. Cells then were stimulated with or without MSU or MSU + ATP. Supernate and cells was collected to detect the level of IL-1β and NLRP3. In MA group, the Ala^{348} to Thr mutation significantly up-regulated the levels of IL-1β compared with the wild type and empty virus ($P = 0.0007$; $P = 0.013$, respectively). The wild type had higher levels of IL-1β than the empty virus, but with no statistical significance ($P>0.05$) in Fig. 3A.Moreover, the mRNA expressions of IL-1β in cells with Ala^{348} to Thr mutation showed a higher level than the wild type and empty virus ($P = 0.0334$; $P = 0.0307$, respectively)(Figure.3B).The mRNA expressions of NLRP3 in cells with Ala^{348} to Thr mutation also showed a higher level than the wild type and empty virus($p = 0.0003$; $P = 0.0001$, respectively)(Figure.3C).However, the level of IL-1β and NLRP3 among Ala^{348} to Thr, wild type and empty virus in group C and M have no statistical significances in Fig. 3, respectively ($P>0.05$).

3.8 ASC and Caspase-1 were not affected by the Ala^{348} to Thr mutation

As shown in Fig. 4, Unlike IL-1β and NLRP3 gene expression, no statistical significance were found in the mRNA expression of ASC and caspase-1 among Ala^{348}to Thr, wild type and empty virus in all the three groups($P>0.05$).

Discussion

Gout is defined by arthritic inflammation in the joints, and the cause of the disease is due to an excess of circulating uric acid, a byproduct of purine degradation[26]. Previous study has found that the key factor for the generation of acute gouty arthritis are the dramatic changes of extracellular ATP, which was followed by activation of P2X7R signaling pathway as well as MSU crystals-induced activation of NLRP3 inflammasome signaling pathway[1]. These mechanisms provided a new avenue for understanding acute gouty arthritis and new methods for treatment. However, it is not everyone with hyperuricemia to develop

gout. Some patients have hyperuricemia, but never developed gout. The reason could be related to genetic factors, especially variants of P2RX7 gene[15].

P2X7R is expressed highly in almost all tissues and organs, especially in the immune cells of monocyte–macrophage origin[27]. The expression and function of the P2X7R are affected by the concentration of extracellular ATP. A study[28] has shown that dilatation of the P2X7 channel to form a “pore” stimulated by extracellular ATP, that is recognized as a unique feature of the P2X7R. However, opening of the ionic channel and formation of the “pore” are two distinct processes. At this moment, short stimulation of P2X7R with extracellular ATP activates K^+ efflux. Prolonged or repeated exposure to the P2X7 ligand opens a non-selective cation channel, then the formation of a cytolytic pore permeable to allows NLRP3 agonists to enter the cytoplasm to activate NLRP3 inflammasome [29, 30]. It has found that $Ala^{348}$to Thr affected bipolar disorder included the gain-of-function haplotypes[17]. The search for all SNPs in the P2RX7 gene was performed within the Chinese population in the HapMap project database and reported in the dbSNP database [National Center for Biotechnology Information (NCBI), Bethesda, MD, USA]. One major identified non-synonymous coding SNPs in P2RX7 were screened out: rs1718119 ($Ala^{348}$to Thr). Several studies[31, 32] that investigate the functional characterization of P2X7R polymorphism in autoimmunity diseases are limited, and there are only two studies showed that the rs1718119 polymorphism of P2X7R were associated with Systemic lupus erythematosus(SLE) and Rheumatoid arthritis (RA). Thus, the rs1718119 polymorphism of P2X7R were associated with gout is still unknown.

Therefore, the current study first time analyzed the genotyping study in genomic DNA samples from gout and hypeluricemia patients, then analyzed the transfected $Ala^{348}$to Thr mutant in HEK-293T cells using fow cytometric and in THP-1 cells using ELISA and qRT-PCR. We excluded age and gender and selected hyperuricemia patients over 5-years course of disease with serum uric acid levels > 480 μmol/L (8 mg/dl) having no history of gout flar.The results of the present genotyping study demonstrated that mutation in the P2X7R gene at the rs1718119 loci were related to gout onset. A allele and GG/(AA + AG) genotypes in rs1718119 may become dangerous genes if mutated and activate gout. A hyperuricemia patient who carries these dangerous genes at these loci may be more susceptible to a gout attack. What’s more, rs1718119 was dominant gene. Homozygous and heterozygous hyperuricemia patients with these alleles exhibit a higher risk of gout than patients without these alleles. Patients with hyperuricemia may have a relatively low risk of gout if they carry a single SNP-susceptible genotype, but the risk of gout may be increased if they both carry one or more susceptible locus.

Furthermore, the function of ATP-stimulated P2X7R detected by the amount of ethidium$^+$ bromide. Stokes [17] has shown a significant association that massive K^+ loss from the cell occurs on P2X7 pore dilatation, due largely to the presence of the $Ala^{348}$to Thr mutation. As shown in our results, our fndings were consisted with previous studies.In our research,the transfected $Ala^{348}$to Thr mutant in HEK-293T cells increased ATP-induced P2X7-dependent ethidium$^+$ bromide uptake to values of 145% of wild type. Under the agitation of ATP, it suggested that P2X7R was activated in $Ala^{348}$to Thr mutant and wild-type, and we observed the strength of P2X7R function by detecting the amount of ethidium$^+$ bromide uptake

by HEK-293 T cells. We further founded that THP-1 monocytic cells with the P2X7R carrying Ala^{348}to Thr increased ATP-induced secretion of proinflammatory cytokines IL-1β gene. It suggested that the Ala^{348}to Thr polymorphism has the major effect in conferring increased P2X7 receptor function and could up-regulate inflammation via ATP-stimulated P2X7R with high uric acid.Besides,the NLRP3 inflammasomes play an important role in exogenous (pathogen-associated molecular patterns) PAMPs or endogenous (damage associated molecular patterns) DAMPs in this process. Among DAMPs, extracellular ATP and other nucleotides play an undisputed role[9]. P2X7R is activated by extracellular ATP to activates K^+ efflux. NLRP3 inflammasome assembly and caspase-1 recruitment occurs spontaneously at K^+ concentrations below 90 mM, but is prevented at higher concentrations. Thus, low intracellular K^+ may be the least common trigger of NLRP3 inflammasome activation. Activated P2X7R promotes formation of a cytolytic pore permeable to allows NLRP3 agonists to enter the cytoplasm to activate NLRP3 inflammasome[30]. In our research, The mRNA expression level of NLRP3 gene can directly reflect the involvement of NLRP3 inflammatory bodies in the ATP-P2X7R-IL-1β signaling pathway. The expression level of NLRP3 gene in THP-1 cells expressing the Ala^{348}to Thr mutant via ATP-stimulated was increased and was significantly stronger than the wild type and empty virus. It shows that the Ala^{348}to Thr mutant has increased the function of P2X7R by changing the structure of P2X7R,and the mRNA expression of NLRP3 involved the down-stream signaling of inflammasomes with this variant could regulate the development of inflammation via ATP-stimulated P2X7R with high uric acid.

Another point to note was that in our study ASC plays an important role in gout as a adaptor protein[33]. The NLRP3 inflammasome was a molecular platform activated upon extracellular stimulator to trigger inflammation through the maturation of pro-inflammatory cytokines such as IL-1β[34]. This study has showed that the expression of ASC has not changed significantly in wild type compared to the Ala^{348}to Thr mutation.These findings indicate that ASC as an inflammasome adapter protein also formulates the ASC speck, a supramolecular aggregate of ASC dimers, which serves as another platform for the activation of caspase-1 after inflammasome activation[35, 36]. It also implies that since the activation of inflammasomes stimulated by the specific agonists,such as MSU and ATP, results in pyroptosis[37] and thereby the release of most of the ASC specks in the extracellular space. It is conceivable that extracellular ASC specks were ingested by macrophages, which resulted in partial ASC loss on THP-1 cells from wild type and the variant[38]. More worth mentioning is that inflammasome complexes are required for activation of caspase-1 have been identified[39]. Here we have shown that the expression of caspase-1 has unchanged significantly between wild type and the Ala^{348}to Thr mutation, suggesting that caspase-1 may be a protein that usually undergo proteolytic processing or caspase-1 signal pathway may not have important role in development of gout by ATP-stimulated P2X7R mediated biological process[40].

Gout attacks are the result of the interaction of MSU and ATP, and the SNPs present in the P2RX7 gene may lead to the occurrence of gout. These findings provide a new SNP to improve the pathogenesis of acute gouty arthritis and may explain why some hyperuricemia patients never develop acute gouty

arthritis. In our study, we first found this SNP, rs1718119 altering $Ala^{348}$to Thr, changed the functions of $P2X_7R$ in P2RX7 gene with high uric acid. In addition, the genetic variability in P2RX7 gene with this variant was involved in the process of NLRP3 inflammasome activation. It might influence susceptibility for the development of gout. These findings may provide a new therapeutic strategy for the prevention and treatment of gouty arthritis.

Declarations

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Author Contributions

Conceptualization, J.-H.T.; Formal Analysis, M.-Y.L. and X.F.; Investigation, M.-Y.L ,X.F. , X.-Y.P. and Y.M.; Writing – Original Draft, M.-Y.L. ;Writing–Review & Editing, Y.-P.W., J.-H.T. and X.-P.L.; Funding Acquisition, X.-P.L.; Supervision, X.-P.L. and X.-M.L.

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Data Availability Statement

all data included in this study are available upon request by contact with the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Consent for publication

All authors read and approved the manuscript.

Ethics approval

This study conformed to the principles expressed in the Declaration of Helsinki. All subjects gave their written informed consent as approved by the respective ethical committees to participate in the study, which was approved by the Medical Ethics Committee of the first affiliated hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China□2021 KY London Examination No. 155□.

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Figures

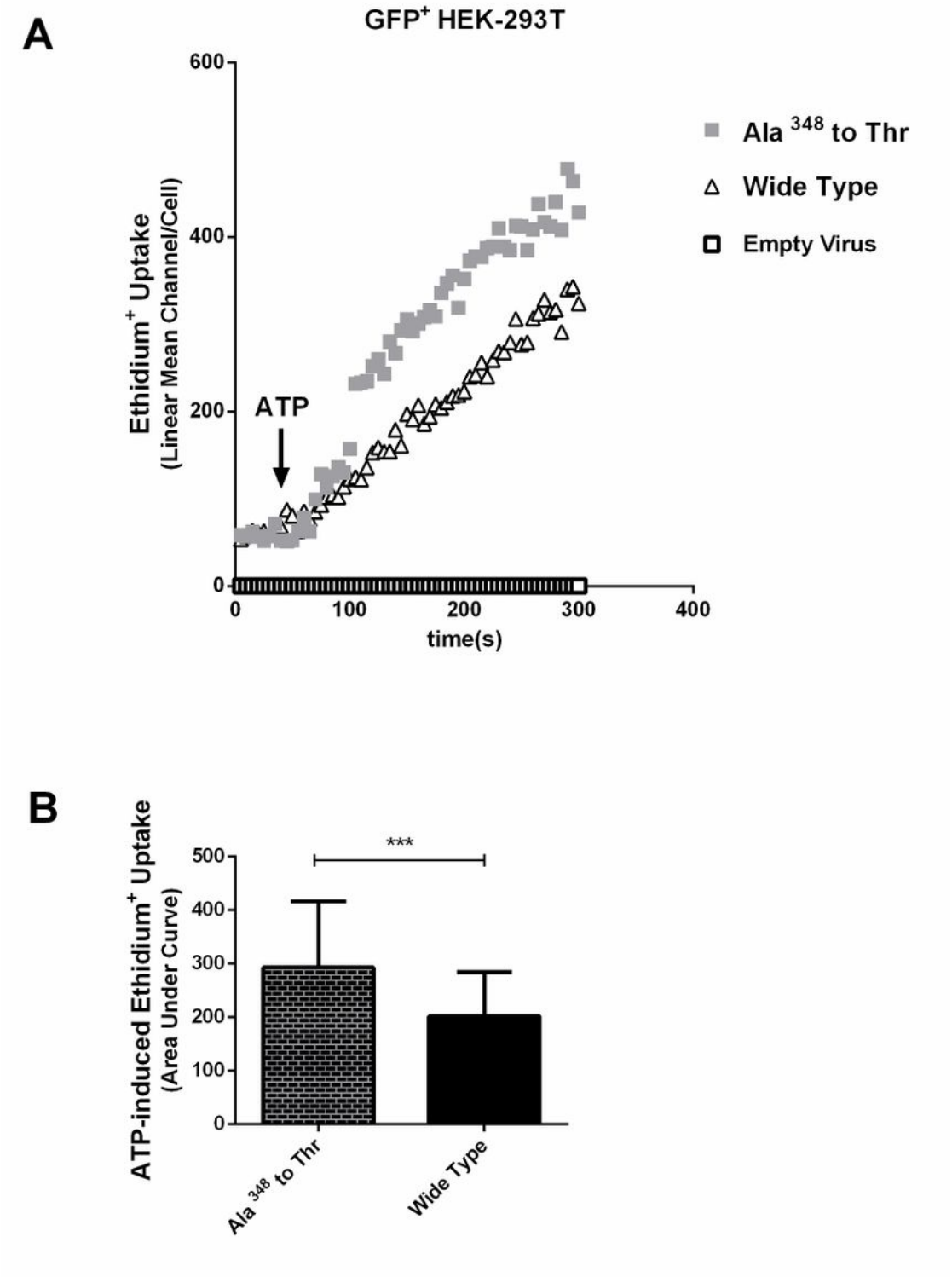

Figure 1

The functional effect of Ala^{348} to Thr in transfected HEK-293 T cells. (A) P2X7-dependent ethidium^{+} uptake induced by 1 mM ATP (applied at arrow) in Ala^{348} to Thr, compared with wild type (WT). (B) Mean ATP-induced ethidium^{+} bromide uptake quantified by calculating area under dye uptake curve for cells expressing in Ala^{348} to Thr. ***p<0.001

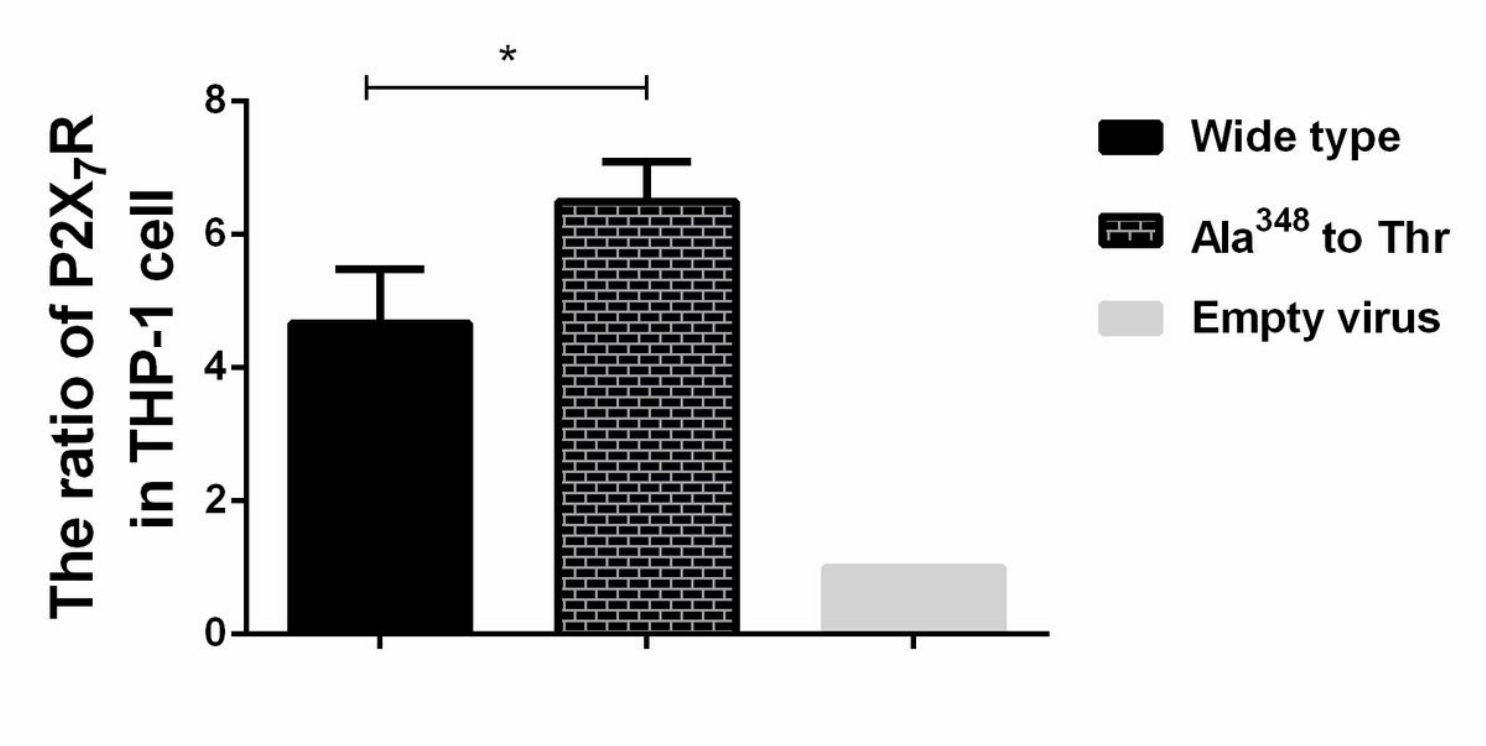

Figure 2

mRNA expression levels of P2X7R in THP-1 cells carrying $Ala^{348}$to Thr□wild type and empty virus *p < 0.05

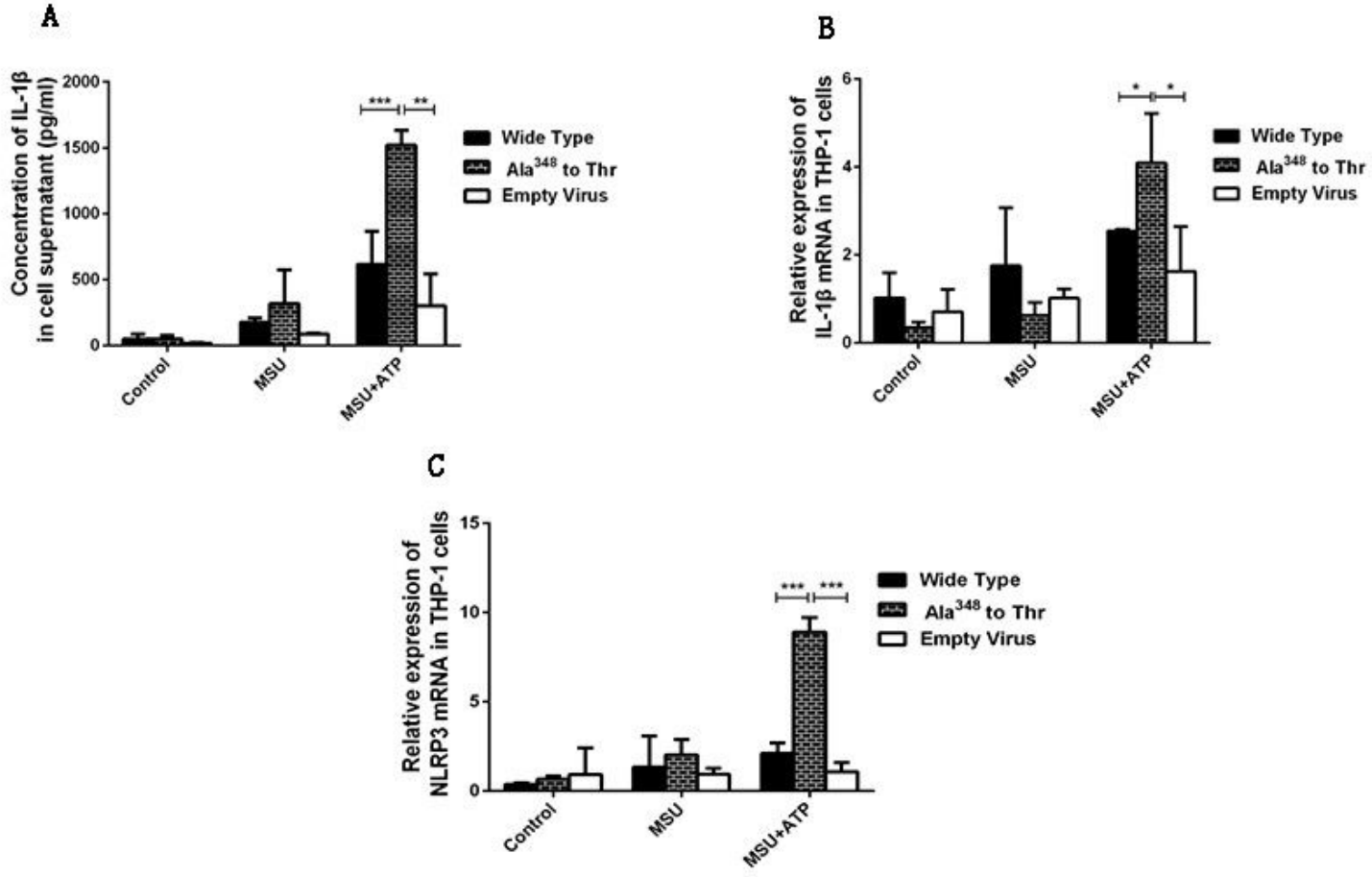

Figure 3

The IL-1β and NLRP3 levels in Ala^{348}to Thr mutation. (A) IL-1β levels in Ala^{348}to Thr□wild type and empty virus in serum. (B) IL-1β mRNA expressions in Ala^{348}to Thr□wild type and empty virus. (C) NLRP3 mRNA expressions in Ala^{348}to Thr□wild type and empty virus

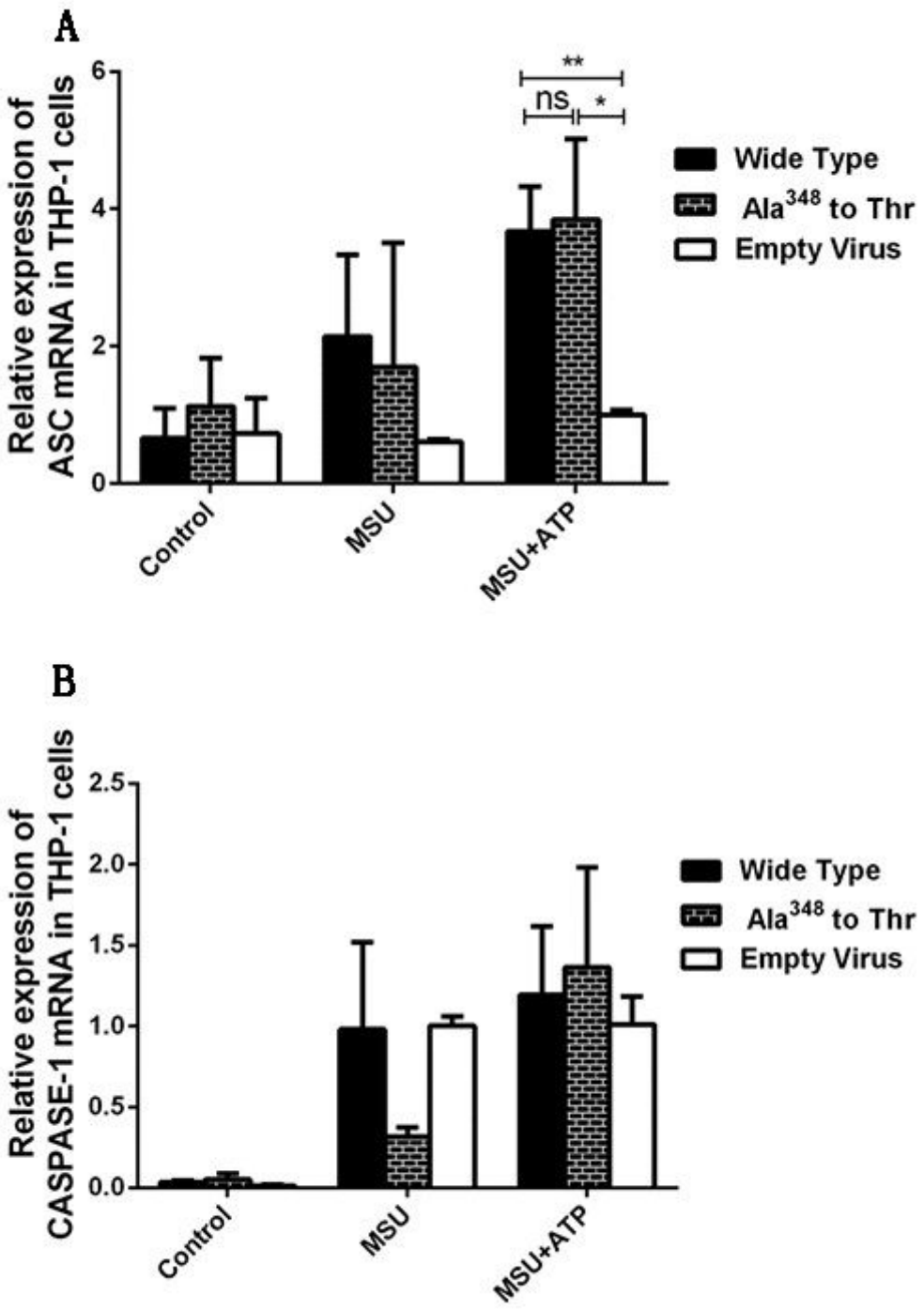

Figure 4

The ASC and Caspase-1 levels in Ala^{348}to Thr mutation. (A) ASC mRNA expressions in Ala^{348}to Thr□wild type and empty virus. (B) Caspase-1 mRNA expressions in Ala^{348}to Thr□wild-type and empty virus