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Research Article
NLRP3 Inflammasome Gene Polymorphisms Variably Associated with its Serum Levels in Acute Myocardial Infarction

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Abstract

Background and Objective: Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) plays a pivotal role in initiation of inflammation. genetic variation in NLRP3 gene have been proposed to predispose several inflammatory diseases. This study aimed to evaluate the risk of NLRP3 (exon 3) gene polymorphisms and its relation with serum NLRP3 among myocardial infarction. **Materials and Methods:** Case-control study involved 69 patients with Myocardial Infarction and 53 controls, from each subject 3 mL were collected and used for DNA extraction then the amplified exon 3 genes were sequenced by Sanger method. Serum NLRP3 was quantified using sandwich ELISA. **Results:** According to the results Q705K found to possess a 16.21 times risk for MI incidence compared with controls. In addition, 44 novel single nucleotide polymorphisms have been identified at the position 14347, 14261, 14240 and 14229 and their allelic variants as a risk factor for MI incidence as 3.92, 8.6, 2.04 and 4.57 when compared with their relevant allele in controls respectively. Statistically high level of serum NLRP3 (1.7 ng mL^{-1}) among MI patients compared to controls (0.71 ng mL^{-1}). The 0.75 ng mL^{-1} considered as a good predictor for MI with ECG findings. Only Q705K and 14229 genetic variant alleles were significantly associated with high NLRP3 protein serum level among MI patients. **Conclusion:** Four novel SNPs in exon 3 of NLRP3 gene in addition to previously reported Q705K conferring risk for development of MI among Iraqis. Only variants allele of Q705K and gene position 14229 was associated with elevated serum NLRP3 protein among MI patients.

Key words: Inflammasome, polymorphism, myocardial infarction, nucleotide, leucine, pyrin, genetic variation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inflammasome considered as basic structural protein complex in the innate immune responses to infection and cell stress. Its activation resulted in maturation of pro inflammatory cytokines pro-interleukin-1β (pro-IL-1β) and (pro-IL-18)[1]. NLRP3 gene polymorphisms have been recognize as possible predisposing factors for development of a variety of autoimmune and/or inflammatory diseases, such as RA, Crohn's disease, myocardial infarction[2]. The rs35829419 (Q705K) polymorphism in NLRP3 refer to gain-of-function mutation leading to an overactive NLRP3 inflammasome (hyper activation of pro-inflammatory cytokine: IL-1β and IL-18)[3]. Single nucleotide polymorphism of NLRP3 in atherosclerosis or MI patients was evaluated by assess several SNPs such as: rs4353135, rs4266924, rs6672995 and rs10733113, located within the downstream regulatory region of the NLRP3 gene, also NLRP3 gene rs10754558 locus polymorphism is considered as an important factor causing the increased formation of IL-1β and IL- 18 as well as the abnormal activation of inflammatory reaction in ACS patients[4-6]. There is one study from China submit that genetic polymorphisms in NLRP3 may impact the risk of atherosclerosis (MI) in the Chinese population[5].

Many of evidence suggest that the myocardial response to tissue injury is regulated by the innate immune system, including several families of pattern recognition receptors (PRRs), the most important PRR family is the toll-like receptors (TLRs) and nod-like receptors[7]. During acute MI, DAMP-induced activation of TLRs may initiate a signaling cascade leading to enhanced synthesis of inflammatory cytokines[8,9] IL-1β is The first inflammatory cytokines of IL-1 family produced which is represented exclusively the most important signal amplifier due to its perfect ability to induce secretion of other cytokines. The current study objective to study the single nucleotide polymorphisms in the NLRP3 exon 3 gene in Iraqi Myocardial infraction patients in comparison with controls and their association with serum level of NLRP3 protein.

MATERIALS AND METHODS

Study design: Case-controlled study design was directed from October, 2018 to March, 2019. Informed agreement was written achieved from all participants and the study was permitted by the ethical committees of the ministry of health and institute of Review Board of collage of medicine, AL-Nahrain University (No: 5/1/52/2355 in 22/11/2018).

This study was accompanied on (69) Myocardial infarction patients they were diagnosed by cardiologists in the center for cardiac surgery depending to ECG and cardiac biomarker (inclusion criteria)[10]. While exclusion criteria include (active chronic inflammatory diseases infections) the patient including 23 female and 46 male who visited the Ibn-Al Bitar Specialist Center for cardiac surgery.

The patient's age range between (30-70) years old, this study also includes a group of 55 healthy controls, seemly age-matched for comparison scientific benchmarks.

Sample processing: Five milliliter of blood sample were occupied from all participants, 2 mL used in EDTA tube for DNA extraction. Three milliliter of remaining blood was allowed to clotted, serum was separated and used for cardiac biomarker measurements (Troponin and hsCRP) and Human NACHT, LRR and PYD domains-containing protein 3 (NLRP3 quantification by sandwich enzyme linked immunosorbent assay CSB-E16885h, Cusabio®, China.

NLRP3 gene sequencing: Genomic DNA was extracted blood sample using gSYNC™ DNA Extraction Kit, Geneaid®, Taiwan. The PCR reaction amplified in thermal cycler (PCR) for target region of NLRP3 gene with specific forward and revers NLRP3 primers NLRP3 foreword primer 5-CAGGAGGAGGACTTCGTGCAAA-3 and NLRP3 Reverse primer 5- ATACCTTGTGCTAAACTGGCAT-3 and Accupower premix, Bioneer® Korea. A Pre-Denaturation step at 95°C for 5 min, followed by 35 cycles of 20 sec of denaturation at 95°C, Annealing step at 60°C for 20 sec and extension step at 72°C for 45 sec, the final step of extension at 72°C for 7 min. The product samples send to Korea for income typical raw sequence data produced from a Sanger sequencing run. The result of sequencing data analyzed by specialized software Geneious Prime 2019.

Statistical analysis: The statistical issue of analysis results is expressed as median and 25-75 confidence intervals. For comparison between groups for abnormally distributed specific Mann Whitney test are used. Pearson correlation

made to estimate the association between each pair of variables. The p-value less than 0.05 was reflected statistically significant. All analysis were approved by SPSS 20.0 software (SPSS Inc, Chicago, Illinois, USA). Receiver operating curve (ROC) was used to estimate the cutoff for NLRP3 serum value. Diagnosis and performance of analyzes according to their sensitivity, specificity, positive predictive value and negative predictive value.

RESULTS

NLRP3 protein as serum biomarker for MI: The results in Table 1 clearly shown that there is no statistical significance in the mean age and sex of MI patients in comparison with control group (p = 0.065 and 0.196) respectively. A statistically significant high systolic and diastolic blood pressure among MI patients compared with controls (p = 0.016 and 0.015) respectively. The existing results highlighted that a higher statistically significant higher CRP, troponin and serum NLRP3 levels among MI patients when compared with controls.

Genotyping of NLRP3 gene and risk assessment for MI predisposition: In this study 43 MI patients and 46 controls were successfully genotyped for NLRP3 Gene exon 3 polymorphism by Sanger DNA sequencing. After gene alignments with the reference gene, the result showed that there were 4 new SNPs in addition to previously registered rs35829419 conferring risk to MI development among Iraqis rather controls.

At the position 14347, the homozygous wild genotype (GG) were 31 (72.09%), heterozygous genotype (GA and/or AG) were 11 (25.58%) and homozygous mutant genotype (AA) were present in one patient (2.33%). Compared to controls the variant allele were 13 (15.12%) among MI and 4 (4.35%) among controls with statistically significant association with MI patients (p = 0.020), the variant allele A possess 3.92 times to predispose MI than those in Iraqi healthy people.

The second SNP at 14261 loci, the heterozygous AC genotype in patients found 7 (16.28%) compare to controls were found only 1 (2.17%), the result shows the genotype ratio of controls group were statistically not significant than those of patients group in which the p-value (0.068) at the allelic level, the results reveal that the allele (C) constitutively higher in MI patients 7 (8.14) rather than controls 1 (1.09%) indicate as statically significant p-value (0.030), Odd ratio 8.06 and 95% CI as a wild allele in genotyped.

Table 1: Descriptive statistics of demographic and laboratory data in study groups

	Study groups		
Parameters	Patients	Controls	p-value
Age (years)	53.65±8.06	50.47±10.73	0.065^{NS}
Male sex	46 (66.7)	40 (75.5)	0.196^{NS}
Systolic blood pressure	126.77±11.03	122.83±4.52	0.016*
Diastolic blood pressure	88.26±8.49	84.91±5.73	0.015*
CRP (mg dL^{-1})	31.00 (3-150)	2.00 (2-5)	<0.001**
Troponin (%)	51 (73.91%)	0 (0%)	<0.001**
Serum NLRP3 (ng mL^{-1})	1.77 (0.7-5.79)	0.71 (0.44-0.9)	<0.001**

*Significant difference ($p \leq 0.05$), **Highly significant difference ($p < 0.001$), NS: Non-significant

At the 14240, the allelic variant homozygous mutant TT genotype in MI patients was 22 (51.16%) compare to controls were found in 2 (4.35%), the result shows the genotype ratio of MI patients group were statistically significant higher than those of control group in which the p-value (0.001). The variant allele (T) were highly frequent in MI patients 55 (63.95%) rather than controls 35 (38.04%) indicate as statistically significant the p-value (0.020), conferring risk (Odd ratio) 2.04 for MI among Iraqis.

At the 14229 loci, the AA homozygous variant genotype in patients was 4 (9.3%) compared to controls were found 1 (2.17), the result show the genotype distribution in both MI patients and controls groups were statistically non-significant (p-value = 0.342). The allelic variant (A) were present in 11.63% among MI patients than controls 5.43% indicating this association was statistically significant the p-value (0.049) and have a risk for developing MI as 4.571 times than those controls.

In Table 2, the result of nlrp3 gene sequencing documented that at the position 14384 loci (Q705K or rs35829419) was present as (AA) homozygous genotype in patients 2 (4.65%), while its not present among controls with statistically significant according to p-value (0.005). The allelic variant in this SNP (Allele A) higher in patient 13 (15.12%) rather than controls 1 (2.17%) this was statistically significant association (p-value<0.001). The Q705K variation conferring risk as 16.21 times for MI incidence than controls.

Association of NLRP3 gene polymorphisms and serum NLRP3 level among MI: The existing results described in Fig. 1 showing the median levels of NLRP3 serum protein in MI patients and control group, part a showing highly statistically significant elevated serum NLRP3 protein in patient group (1.76) compare to controls group (0.77). Further comparisons were made between MI patients according to the variation in

Table 2: Association of NLRP3 genotype polymorphisms with MI patients and controls

14347 position	Patients group (n = 43)	Control group (n = 46)	p-value	Odd ratio (CI 95%)
Genotype				
AA	1 (2.33)	1 (2.17)	0.018*	-
GA	11 (25.58)	2 (4.35)		
GG	31 (72.09)	43 (93.48)		
Allele				
Allele A	13 (15.12)	4 (4.35)	0.020*	3.92 (1.23-11.32)
Allele G	73 (84.88)	88 (95.65)		
14261 position				
Genotype				
AA	36 (83.72)	45 (97.83)	0.065^{NS}	-
AC	7 (16.28)	1 (2.17)		
Allele				
Allele C	7 (8.14)	1 (1.09)	0.030*	8.06 (1.35-91.72)
Allele A	79 (91.86)	91 (98.91)		
14240 position				
Genotype				
CC	8 (18.6)	13 (28.26)	<0.001**	-
CT	11 (25.58)	31 (67.39)		
TT	22 (51.16)	2 (4.35)		
Allele				
Allele T	55 (63.95)	35 (38.04)	0.020*	2.04 (1.15-3.59)
Allele C	27 (31.4)	57 (61.96)		
14229 position				
Genotype				
AA	4 (9.3)	1 (2.17)	0.342^{NS}	-
AT	5 (11.63)	3 (6.52)		
TT	34 (79.07)	42 (91.3)		
Allele				
Allele A	13 (15.12)	5 (5.43)	0.049*	4.57 (1.05-21.8)
Allele T	73 (84.88)	87 (94.57)		
rs35829419				
Genotype				
AA	2 (4.65)	0 (0)	0.005*	
AC	9 (20.93)	1 (2.17)		
CC	32 (74.42)	45 (97.83)		
Allele				
Allele A	13 (15.12)	1 (1.09)	<0.001**	16.21 (2.46-174.6)
Allele C	73 (84.88)	91 (98.91)		

*Significant difference ($p \leq 0.05$), **Highly significant difference ($p<0.001$), NS: Non-significant

Table 3: Cut off point, sensitivity and specificity for NLRP3 plasma protein

Serum NLRP3 protein	Study groups				Positive predictive value (CI)	Negative predictive value (CI)
	Patients (%)	Controls (%)	Sensitivity (CI)	Specificity (CI)		
Positive	59** (85.51)	21 (39.62)	85.51	60.38	73.75	76.19
Negative	10 (14.49)	32 (60.38)	75.34-91.93	46.94-72.42	63.18-82.14	61.47-86.52
Total	69	53				

Cutoff value: 0.75 ng mL^{-1}, **Highly significant difference ($p<0.001$)

NLRP3 gene, MI patients whom carry variant allele at the position 14229 and 14384 (Q705K) were demonstrated a statistically significant elevation in NLRP3 serum level than those with dominant allele carriers (Fig. 1e-f) while genetic variants at other position were not statically different (Fig. 1b-d).

In Table 3, the validity of NLRP3 protein as a determinant marker for MI rather than control group was measured by determination of area under the curve (AUC) by receiver operating curve. NLRP3 plasma protein was shown to be as a potential biomarker for MI with AUC = 0.872 and CI between 0.811-0.933. Furthermore, the

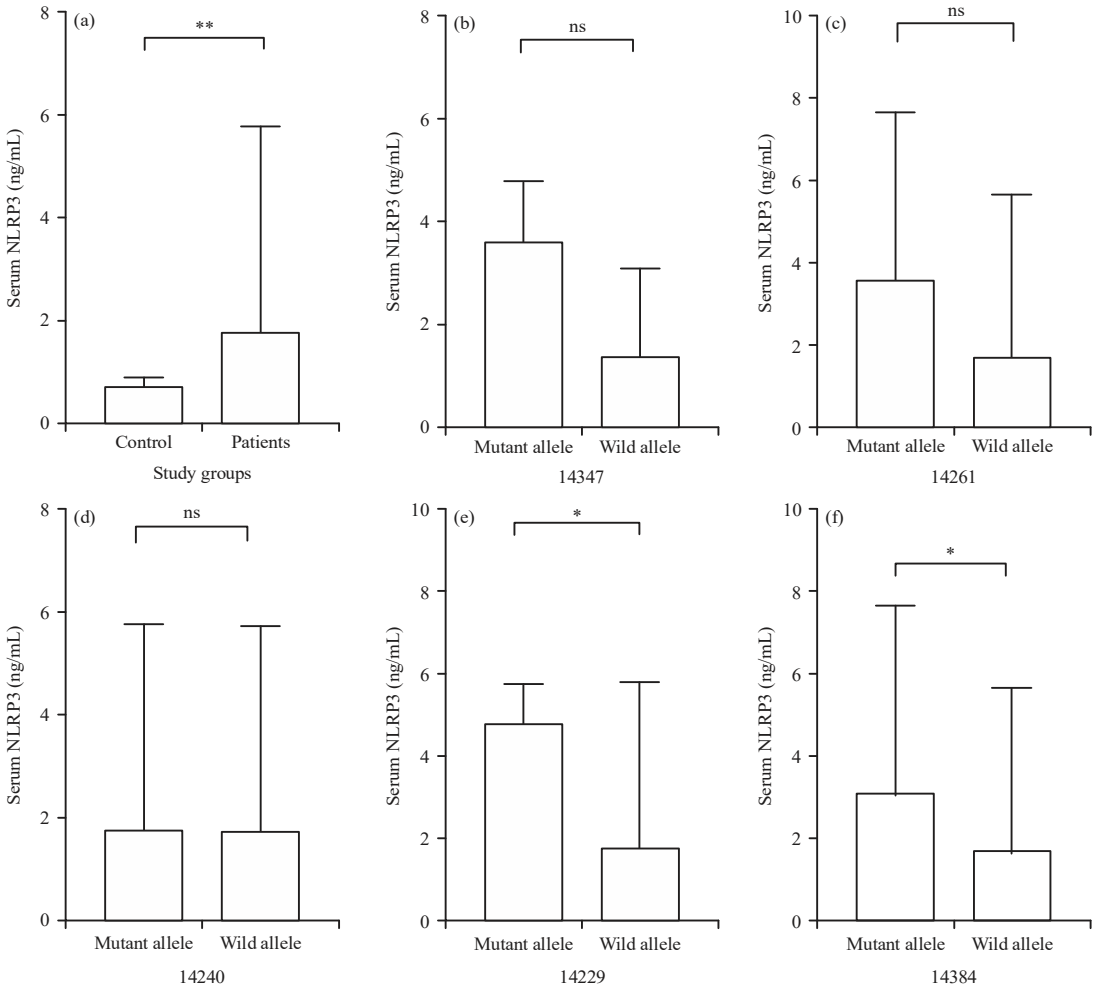

Fig. 1(a-f): Bar-chart representation of serum NLRP3 protein level according to allelic variation of recorded SNPs, (a) Comparison between patients and control groups and (b-f) Comparisons according to wild type and mutant alleles of recorded SNPs in MI patients

*Significant difference ($p \leq 0.05$), **Highly significant difference ($p < 0.001$), ns: Non-significant

cut-off point for plasma NLRP3 protein = 0.75 ng mL^{-1}. The sensitivity was 85.51%, specificity was 60.38% with positive predictive value 73.75% and negative predictive value as 76.19%.

DISCUSSION

Atherosclerosis is the hallmark pathology in coronary artery disease. There is an agreement of recent study highlighted the involvement of NLRP3 activation in the initiation of cardiomyopathy via IL-1β and genetic deletion of NLRP3 will abrogate tissue pathology[11]. Furthermore, activation of NLRP3 inflammasome contribute in the progression of atherosclerosis-related inflammation via Cathepsin B and potassium efflux as a response of cardiac fibroblast and cardiomyocyte for cholesterol crystals[12-14]. Another study highlighted the essential role of inflammasome activation in ischemia/reperfusion injury. It have been demonstrated that both ASC and Caspase-1 were expressed in the cardiomyocyte cellular injury and their inhibition will reduce inflammatory cell infiltration, cytokine expression and tissue injury[9,15].

Here in this study, serum NLRP3 level was measured using sandwich ELISA kit. The median level was significantly higher among MI patients in comparison with control group. This finding was supported by Afrasyab *et al.*[16] study whom measured NLRP3 protein in cellular components of peripheral circulation with higher level among CAD patients in comparison with controls. The median cutoff value was 0.75 ng mL^{-1} was selected as predictor as a diagnostic test for MI, 85.51 sensitivity and lower 60.38% specificity were recorded. The low specificity was clearly argued to the several

stimulation pathways that cause activation and expression of NLRP3 inflammasome such as: infection, stress etc. Several clinical studies have shown that the mRNA level of NLRP3 was elevated peripheral blood of CAD patients[17] and its correlated with the severity and progression of coronary artery syndrome[16]. It was positively correlated with the peripheral mRNA and protein levels of IL-1β, IL-18 and TNF-α[16,18].

The present study first ever report of multiple single nucleotide polymorphisms in exon 3 of NLRP3 gene that confer risk for developing MI among Iraqis. In addition to that higher serum NLRP3 among MI patients suggest its usefulness as an inflammatory mediator in the pathogenesis of MI. The reported polymorphisms including rs35829419 (Q705K) polymorphism in the NLRP3 gene conferring risk for MI. The potential risk allele A is 16.21 for MI rather than control. Followed by allele C at the position 14261 in exon 3 (OR = 8.06), allele A at the position 14229 (OR = 4.57), allele A at the position 14347 (OR = 3.92), then allele C at the position 14240 (OR = 2.04). In contrary, Varghese *et al.*[19] reported no significant association between variant rs35829419 and MI in Northern Sweden.

The current study analyzed the possible genetic association with serum NLRP3 level among MI patients. The results showed variable association by detected risk factor SNPs with elevated level of NLRP3. The recessive allele (A) gain of function mutation (Q705K) and SNP at the 14229 position were associated with higher concentration of NLRP3 suggesting a gain of function mutations. The minor alleles of other SNPs at the positions (14347 and 14261) were recorded higher levels of NLRP3 without statistical significance. This might be related to small sample size.

CONCLUSION

This study recorded 4 novel SNPs at the exon 3 (an active region in NLRP3 gene) are suggested to be gain of function mutation leading to excessive NLRP3 gene activation and expression. This activity was clearly manifested by inflammatory bases of MI lesion among Iraqi patients. Further analysis and validation of novels SNPs are required to explore the exact their functional profile.

SIGNIFICANCE STATEMENT

The study reported for the first time ever in Iraqi MI four novel single nucleotide polymorphisms in exon 3 of NLRP3 gene that variably associated with serum NLRP3 level as a predisposing risk factor for development MI. Thus, these SNPs might recognize people whom at risk for developing MI. attenuation of NLRP3 activity might be a good pharmacological target in development of atherosclerotic lesions.

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