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Interleukin-1 Receptor Antagonist (*IL1-Ra*) Gene Polymorphism as a risk factor for coronary artery disease

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Cytokine gene polymorphism contribute in inflammatory pathology. We aimed to investigate interleukin-1 receptor antagonist (IL-1Ra) gene polymorphism in coronary artery disease (CAD) and to analyze any possible relationship to biomarkers of oxidative stress and/or cardiac injury.Two groups of CAD patients were enrolled comprising 75 patients (35 angina and 40 myocardial infarction MI). A control group of 124 healthy individuals was included. Two blood samples were withdrawn from each individual; one for serologic assay of biomarkers of oxidative stress (total antioxidant capacity; TAC, malonyldialdehyde; MDA, and nitric oxide; NO) and cardiac injury (LDH and CK). Another 2 ml blood on EDTA for DNA extraction and amplification of IL-1Ra intron 2 and analysis of its polymorphism.Genotype A1/A1 was a risk factor for angina and MI (p= 0.0001, p=0.012; respectively). However, A1/A2 genotype showed significant protection from CAD (70.97% in control vs. 34.29% in angina, and 30.0% in MI; p= 0.0001). Genotypes A1/A3 and A2/A3 were found only in MI patients. A1 allele is a risk factor for CAD. On the other hand, allele A2 seemed to be more protective. The exclusive presence of genotypes A1/A3 and A2/A3 among MI patients and their absence in the control needs further studies.

Keywords: Angina; Myocardial infarction; *IL-1Ra*; Oxidative stress

INTRODUCTION

In view of lack sensitivity and specificity of chest pain, history, and ECG, diagnosis of myocardial infarction (MI) is confirmed by elevation of serum level of cardiac-specific enzymes creatine phosphokinase (CK) and lactate dehydrogenase (LDH) (Valentin et al. 1998)

Genetics of coronary artery disease (CAD) may arise from a gene having a direct effect on the inflammatory process or modifying this process after its start (Francis et al. 1990) Interleukin-1 receptor antagonist (IL-1Ra) is a counter inflammatory cytokine produced locally in various tissues in response to infection or inflammation and presented in high levels in the circulation secondary to hepatic production as an acute-phase protein (Gabay et al. 1997) Cytokine IL-1Ra is encoded by a polymorphic gene *IL-1Ra* in the IL-1 gene family; it is located on the long arm of chromosome 2 in human (2q14-21) (Steinkasserer et al. 1993). The second intron of this gene is 86 bp of tandem repeat polymorphism that forms five alleles; a number of tandem repeats vary from two to six in different persons, and the frequency of individual alleles also varies among different ethnics or geographic populations (Bid et al. 2004). Because of the biological

plausibility of *IL-1Ra* gene for involvement in atherosclerosis; its association with inflammatory disease through cytokine production namely IL-1Ra remains an interesting research point (Hurme and Santtila, 1998).

Also, oxidative stress and free radicals are known to have an essential role in the development of atherosclerosis which leads to CAD (Holvoet, 1998). Oxidative modification of LDL by free radicals results in adverse effects on vascular function such as endothelial apoptosis, decrease in nitric oxide (NO) levels, increase in smooth muscle cell proliferation, and synthesis of proinflammatory molecules (Steinberg, 1997). Malondialdehyde (MDA), a carbonyl compound produced during lipid peroxidation, is used widely to determine the oxidative stress (Serdar et al. 2007). Nitric oxide (NO), a vasodilator agent derived from the endothelium, is synthesized by NO synthase and converted to more stable forms such as nitrite (NO_2^-) and nitrate (NO_3^-) on exposure to oxidative stresses. The decreased synthesis and bioavailability is an important step in the development of atherosclerosis (Furchgott and Zawadzki, 1980).

So, the present study aimed to investigate, for the first time among Egyptians, the *IL-1Ra* gene polymorphism in CAD and the possible relationship between this polymorphism and the biomarkers of oxidative stress and/or cardiac injury (CK, LDH)..

MATERIALS AND METHODS

The utilized methodology was conducted on 75 patients with angiographically-documented CAD admitted to the Cardiology department in Mansoura University Hospital. The sample was collected under the supervision of cardiology physician. A written informed consent was obtained from all individuals who participate in the study after knowing the nature of the disease and the diagnostic procedures involved. These patients were classified into two groups: the first group included 35 patients suffering from angina (20 males, 15 females, with a mean age of 55.5±10.1 years) and the second group included 40 patients suffering from myocardial infarction (MI) (28 males, 12 females, with a mean age of 59.7±8.9 years). All patients were examined clinically and information related to age, gender, habits, and health status was followed by a series of laboratory investigations. A group of 124 healthy adults with no known history of any disease like diabetes, hypertension or dyslipidemia (110 males and 14 females, with a mean age of 25.98±5.7 years) was enrolled as a negative control (Table 1)

Biomarkers of oxidative stress and cardiac injury:

Five mL blood samples were used for assay of biomarkers of oxidative stress and cardiac injury; total antioxidant capacity (TAC) content was determined by a commercially available kit (Biodiagnostic, Cairo, Egypt) (Koracevic et al. 2001). The Draper and Hadley method was used to measure the Malonyldialdehyde (MDA) content (Draper and Hadley, 1990). Colorimetric determination of serum nitric oxide (NO) was done using the method described by Montgomery and Dymock (Montgomery and Dymock, 1961). a commercially available kit (Biodiagnostic, Cairo, Egypt). Finally, creatine phosphokinase (CK) was assessed by a commercially available kit (POINTE SCIENTIFIC, INC., USA) (Oliver, 1955). However lactate dehydrogenase (LDH) was done by a commercial kit (POINTE SCIENTIFIC, INC., USA) (Wroblewski and Ladue, 1955).

IL-1Ra gene polymorphism:

Another 2 mL blood samples were drawn by venipuncture into EDTA-containing (ethylenediaminetetraacetic acid) tubes. DNA was extracted from the blood samples of both patients and control groups, then DNA was purified using DNA purification capture column kits (Gentra system, USA). Amplification via PCR technique was carried out for *IL-1Ra* intron 2 contained the 86 bp VNTR. Each PCR was carried out in 25 µL reaction mixture containing 10 µl Master Mix (Fermentas, Germany), 8 µl PCR grade water, 2 µl *IL-1Ra* forward (F) primer, 2 µl *IL-1Ra* reverse (R) primer (Bio Basic Inc., Canada), and 3 µl extracted DNA according to the method described in literature (Wilkinson, 1999).

Primer sequence

(Forward): 5'-TCCTGGTCTGCAGGTAA-3'
(Reverse): 5'CTCAGCAACACTCCTAT-3'

PCR conditions:

Initial denaturation cycle of 95°C for 5 minutes followed by 35 cycles in the form of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing) and 72°C for 1 minute (extension) with a final extension cycle of 5 minutes at 72°C. The amplified segment was run on agarose gel (3%) electrophoresis and photographed on ultraviolet transilluminator (Zhang et al. 2004).

Table 1: Descriptive data of control and patient groups

Characteristics		Control (N=124)	Patient groups	
			Angina (N=35)	MI (N=40)
sex	Male	110 (88.71%)	20 (57.14%)	28 (70%)
	Female	14 (11.29%)	15 (42.85%)	12 (30%)
Age (mean±SD)		25.98±5.7	55.5±10	59.7±8.9
Diabetes N(%)		--------	26 (74.28%)	28 (70%)
Hypertension N (%)		--------	28 (80%)	27 (67.5%)
Dyslipidemia N (%)		--------	21 (60%)	22 (55%)
Smoker N(%)		--------	15 (42.85%)	25 (62.5%)
Non- smoker N (%)		124 (100%)	20 (57.14%)	15 (37.5%)

Statistical analysis:

Statistical analysis was carried out using statistical package of social sciences (SPSS) for windows (USA, Chicago, IL: SPSS Inc. 1999). The association between categorical variables was done by applying Chi-square test and Fisher Exact for continuity correction in critical sample size. Mean and standard deviations were used to express the central tendency and dispersion given that the data was normally distributed. The difference in frequencies of different genotypes among studied groups was determined using the odds ratio (OR) and 95% confidence interval.

RESULTS

The present study revealed three alleles including allele A1 (four repeats, 410 bp), allele A2 (two repeats, 240 bp), and allele A3 (five repeats, 500 bp). In addition, five genotypes of *IL-1Ra* including A1/A1, A1/A2, A2/A2, A1/A3, and A2/A3 could be recognized among CAD patients (Figure: 1a and 1b). Patients with CAD were showing clearly a significant lower frequency of A1/A2 genotype compared with control (32.0% vs. 70.97%, p= 0.0001, OR= 0.21). This means that A1/A2 genotype might be considered a protective factor against CAD, whereas allele A2 frequency was responsible for this protective effect (44.35% in control vs. 29.3% in CAD patients, p= 0.005, OR= 0.524). On the other hand, genotypes A1/A1 (homozygote) and A2/A3 (heterozygote) were demonstrating significant increases in patients compared with control (46.67% vs. 20.16%, p= 0.0003 and 5.33% vs. 0.00%, p= 0.052 respectively, Table: 2 and Figure: 2).Both angina and MI groups showed a significant lower frequency of A1/A2 genotype when compared with control (34.29%, p=0.0001, OR=0.18 and 30%, p=0.0001, OR=0.19, respectively), whereas a significant higher frequency of A1/A1 genotype, when compared with control (48.57%, p=0.0001, OR=5.72 and 45.0%, p=0.012, OR=2.86, respectively). However homozygote A2/A2 was not consistently different in angina and MI patients compared to control (17.14%, p=0.97, OR=0.98 and 5.0%, p=0.87, OR=0.88, respectively, Table: 2 and Figure: 2).A significant decrease in serum activities of TAC had been observed in all patients (1.14±0.09 mM/L), when compared with control (1.35±0.46 mM/L; p=0.002), while significant higher values for MDA (13.1±3.46 nmol/ml) when compared with control (4.1±2.58 nmol/ml; p=0.0001). Similar increments were observed in NO and LDH in patients compared with control (for NO 35.9±5.44 vs. 18.75±3.62µmol/L; p=0.0001, for LDH 583.36±148.2 vs. 379±115.6 U/L; p= 0.0003 respectively).

Table 2: Comparison between allele frequencies of *IL-1Ra* polymorphism in different genotypes in studied cases

IL-1Ra Genotype	Control (N= 124)	All Patient (N= 75)	Angina (N= 35)	MI (N= 40)
A1/A1 Genotype				
N (%)	25 (20.16%)	35 (46.67%) **	17 (48.57%) **	18 (45.0%) *
P		0.0003	0.0001	0.012
OR (95% CI)		3.3 (1.72-6.34)	5.72 (2.2-14.89)	2.86 (1.24-6.61)
A1/A2 Genotype				
N (%)	88 (70.97%)	24 (32.0%) **	12 (34.29%) **	12 (30.0%) **
P		0.0001	0.0001	0.0001
OR (95% CI)		0.21 (0.11-0.39)	0.18 (0.08-0.37)	0.19 (0.08-0.47)
A2/A2 Genotype				
N (%)	11 (8.87%)	8 (10.67%)	6 (17.14%)	2 (5.0%)
P		0.81	0.97	0.87
OR (95% CI)		1.14 (0.39-3.29)	0.98 (0.25-3.75)	0.88 (0.18-4.24)
A1/A3 Genotype				
N (%)	0	4 (5.33%)	—	4 (10.0%) *
P		0.052	—	0.0024
OR 95% CI)		7.94 (0.87-72.52)	—	14.47 (1.57-133.77)
A2/A3 Genotype				
N (%)	0	4 (5.33%)	—	4 (10.0%) *
P		0.052	—	0.0024
OR (95% CI)		7.94 (0.87-72.52)	—	14.47 (1.57-133.77)

MI= myocardial infarction, N= number of cases, (%)= percentage, OR= odds ratio, CI= confidence interval, *p*= probability and it is obtained from chi-square test for cells which contain expected values >5 and Fisher's exact test for cells contain expected value <5. Genotypes are expressed as a number of cases (proportion in % within brackets).

* significant difference from control *P* less than 0.01, ** highly significant difference from control *P* ≤0.001 OR significant if more than one.

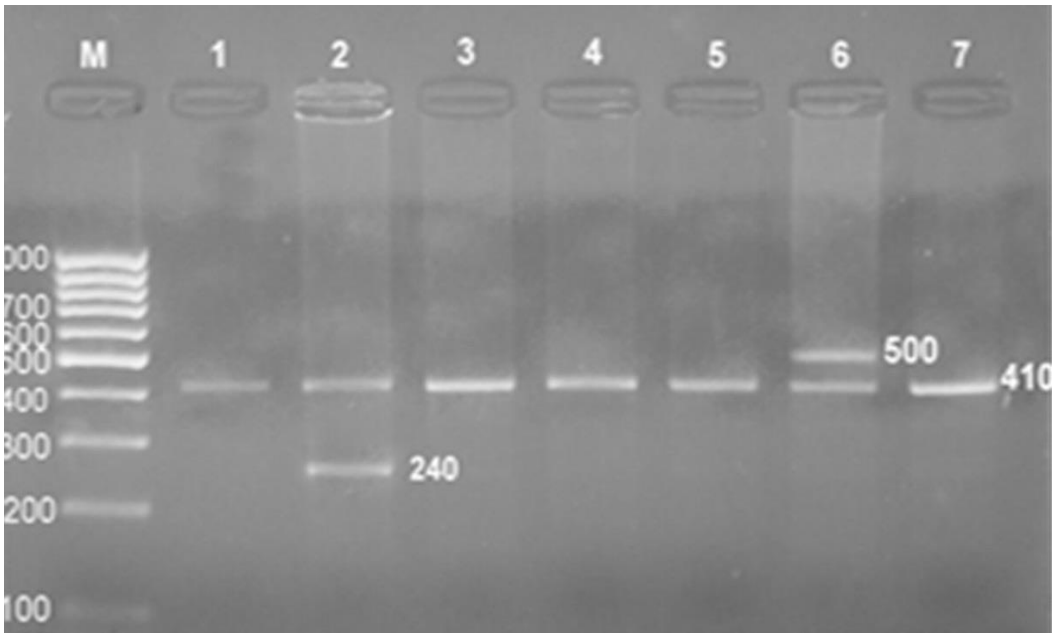


Figure 1a

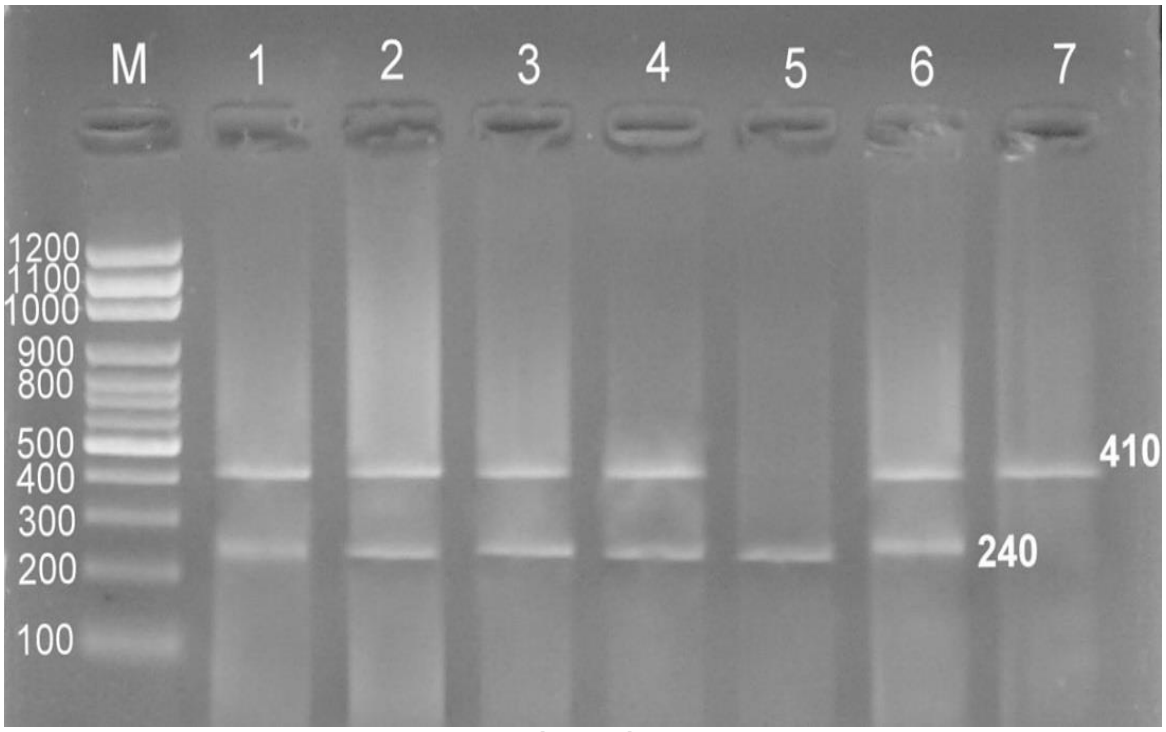
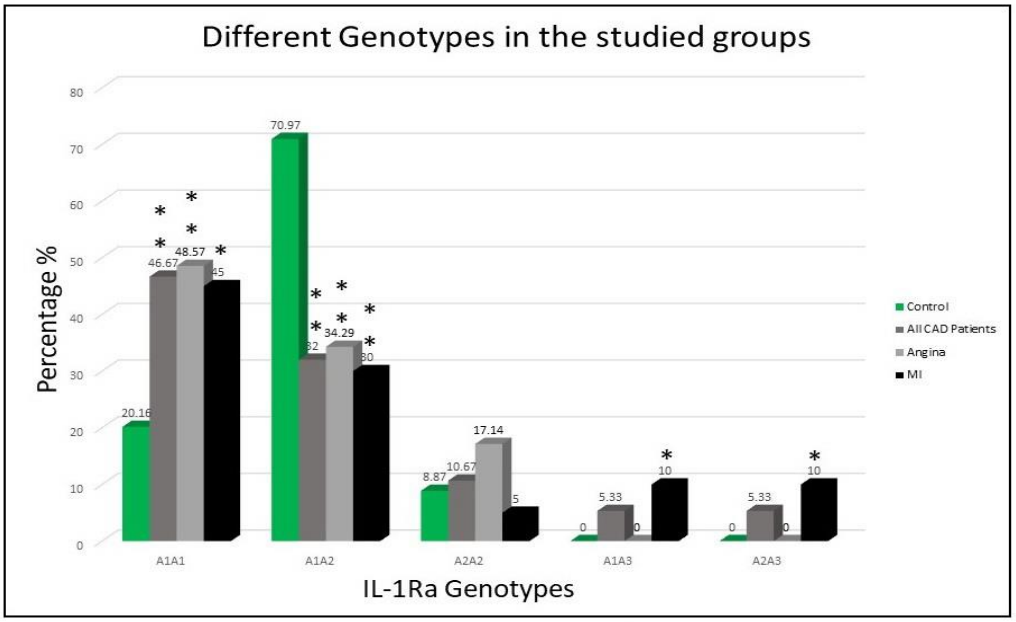


Figure 1b

Figure 1: Agarose gel electrophoresis showing PCR products for *IL-1Ra* gene intron 2 polymorphism.

<u>Figure: 1a → for CAD patients;</u> Lane (M): DNA marker, Lanes 1, 3, 4, 5, 7: homozygous A1/A1 at 410 bp, Lane 2: heterozygous A1/A2 (A2: 240 bp), Lane (6): heterozygous A1/A3 (A3: 500 bp).

<u>Figure: 1b → for control;</u> Lane (M): DNA marker, Lane (1, 2, 3, 4, and 6): heterozygous A1/A2; Lane (5): homozygous A2/A2, Lane (7): homozygous A1/A1.



* Significant if P ≤ 0.01 ** Highly significant if P < 0.001

Figure 2: Distribution of *IL-1Ra* genotypes among the different studied groups

Table 3: Serum contents of total antioxidant capacity (TAC), malondialdehyde (MDA), nitric oxide (NO) and activities of lactate dehydrogenase (LDH) and creatine phosphokinase (CK) in patient groups compared to control (T test).

	All Genotypes of *IL–1Ra*			
Parameters	**Control (N= 100)**	**Total Patient (N= 75)**	**Angina (N= 35)**	**MI (N= 40)**
Serum TAC (mM/L)				
mean±SD	1.35±0.46	1.14±0.09 **	1.18±0.07 *	1.12±0.09 **
P	—	0.002	0.015	0.001
Serum MDA (nmol/ml)				
mean±SD	4.1±2.58	13.1±3.46 ***	11.8±3.03 ***	13.7±3.65 ***
P	—	0.0001	0.0001	0.00***01***
Serum NO (µmol/L)				
mean±SD	18.75±3.62	35.9±5.44 ***	35. 6±3.7 ***	34.09±6.37 ***
P	—	0.00***01***	0.0001	0.00***01***
Serum LDH (U/L)				
mean±SD	379±115.6	583.36±148.2 ***	584.3±141.39 ***	583.4±148.18 ***
P	—	0.0003	0.0001	0.0002
Serum CK (U/L)				
mean±SD	165.9±19.8	194.6±161.12	164.3±160.2	250.9±162.9 *
P	—	0.053	0.967	0.04
	For Genotype A1/A1 of *IL–1Ra*			
	Control (N= 25)		**Angina** (N= 17)	**MI** (N= 18)
TAC mean±SD	1.45±0.73		1.19±0.07 *	1.12±0.09 **
P	—		0.015	0.003
MDA mean±SD	4.15±2.87		12.5±3.0 ***	12.4±4.1 ***
P	—		0.0001	0.0001
NO mean±SD	18.98±2.49		36.14±3.34 ***	31.43±6.16 **
P	—		0.0001	0.0016
LDH mean±SD	365±121.43		628.8±158.6 ***	532.8±131.74 **
P	—		0.0001	0.006
CK mean±SD	163.4±19.2		230.1±191.85	358.57±303.52
P	—		0.393	0.14
	For Genotype A1/A2 of *IL–1Ra*			
	Control (N= 88)		**Angina** (N= 12)	**MI** (N= 12)
TAC mean±SD	1.25±0.41		1.18±0.11	1.086±0.11*
P	—		0.79	0.32
MDA mean±SD	3.81±2.52		13.28±2.35 ***	13.67±3.7 ***
P	—		< 0.0001	0.0001
NO mean±SD	18.29±4.07		33.61±5.9 ***	35.43±6.64 ***
P	—		0.0004	0.0001
LDH mean±SD	397.14±112.3		567.67±85.71*	626.17±140.9 ***
P	—		0.02	0.0001
CK mean±SD	169.71±20.44		158.67±9.02	224.29±28.6 **
P	—		0.306	0.004

MI = myocardial infarction, N= number of cases, TAC= total antioxidant capacity, MDA= malondialdehyde, NO= nitric oxide, LDH= lactate dehydrogenase, CK= creatine phosphokinase. SD= standard deviation, *P* = probability obtained from t-test. *P* is not significant >0.05, * *P* is significant difference from control ≤0.05,

** *P* is very significant difference from control ≤0.01 and *** *P* is highly significant difference from control ≤0.001.

On the other hand, CK did not show any significant change in patients compared to control (194.6±161.12 vs. 165.9±19.8 U/L, p= 0.053). The results obtained from applying t-test on the two groups of patients (MI and angina) were the same except for CK in MI patients, that showed some significance (P=0.04) (Table: 3).The relationships between biomarkers of oxidative stress and cardiac injury on one side and *IL-1Ra* genotyped in the current work, showed that: for A1/A1

genotype, TAC was decreased in CAD; 1.19±0.07 (p=0.015) for angina, 1.12±0.09 (p=0.003) for MI. However, MDA values were increased in the A1/A1 genotype of CAD; 12.5±3.0 (p=0.0001) for angina and 12.4±4.1 (p=0.0001) for MI. Similar effects appeared with NO and LDH (Table: 3). However, no significant change was observed in CK with A1/A1 genotype.For A1/A2 genotype, TAC did not change significantly in any patient group compared to control; 1.18±0.11 (p=0.79) for angina and 1.09±0.11 (p=0.32) for MI. MDA showed a significant increase in both patients' groups; 13.28±2.35 (p=0.0001) for angina and 13.67±3.7 (p=0.0001) for MI. NO and LDH showed the same significant effects (for NO 33.61±5.9 and 35.43±6.64, for LDH 567.67±85.71 and 626.17±140.9). In genotype A1/A2, CK was only affected in cases with MI compared with control 224.29±28.6 vs. 169.71±20.44 (p= 0.004). (Table: 3).

DISCUSSION

This study examined closely patients from two different groups; angina and myocardial infarction (MI). Genotype A1/A1 was significantly increased (48.57%; p= 0.0001 for angina and 45.0%; p<0.012 for MI) when compared with control (20.16%). This signifies that allele A1 could be a risk for CAD in homozygotes; a finding which agreed with other researchers whose published frequency of A1/A1 genotype is much higher among stroke survivors when compared with control (77.2% and 45.5%, respectively; p< 0.001) (Seripa et al. 2003). Also, in the tested samples, heterozygotes A1/A3 and A2/A3 were found only in MI patients; thus these heterozygotes could behave as risk genotypes (p= 0.0024). These observations had not been confirmed yet by any publications.On the other hand, genotype A1/A2 was acting as a resistant factor for CAD as shown in Table (1) in agreement with the results from Arman et al. 2008 Genotype A1/A2 showed another difference between risk and protective haplotypes due to the presence of A1 in the risk haplotype and A2 in the protective haplotype. The role of this polymorphism as a susceptibility marker for CAD is controversial, with positive and negative results reported in the literature. For instance, Francis et al. reported the association of allele A2 in single vessel coronary disease (SVD), but not in multi-vessel coronary disease (Francis, 1999). Furthermore, it has been reported that allele A2 is significantly associated with CAD in type 2 diabetes (Marculescu et al. 2002, Arman et al. 2008). Regarding plasma levels of IL-1Ra, Tolusso et al. reported that healthy blood donors homozygous for A2 had decreased plasma levels of IL-1Ra as compared with non-allele A2 carriers (Tolusso et al. 2006). In contrast, Hurme and Santtila found that individuals with A2 have 1.2-fold increased IL-1Ra plasma levels (Hurme and Santtila, 1998). Also, subjects with A2 allele or A2/A2 genotype produced statistically higher serum levels of IL1Ra when compared with the A1 allele or A1/A1 genotype (Awasthi et al. 2018). Besides, in vitro studies suggested a high production of IL-1Ra in individuals with A2 allele (Danis et al. 1995, Vamvakopoulos et al. 2002). These findings confirms the previous shown results, since the individuals with these haplotypes could produce more IL-1Ra, thus diminishing the pro-inflammatory effects of IL-1.As mentioned earlier, the present study did not show any difference between CAD patients and control regarding A2/A2 genotype, and this is because in every population studied to date, most persons are either A1/A1 homozygous or A1/A2 heterozygote. However, some studies found A2/A2 genotype more significantly associated with SVD among British (Sheffield) and Turkish populations (Mendis et al. 1995, Awasthi et al. 2018). In one study, no significant association had been proved between *IL-1Ra* polymorphism and the risk of CAD (Vohnout et al. 2003), moreover this association is further denied in a recent meta-analysis (Zhou et al. 2012). In the current study, serum TAC was statistically lower while MDA, NO, LDH, and CK were significantly higher in CAD patients as a whole when compared with control (Table: 2). Trying to correlate these biomarkers to *IL1Ra* polymorphism; these biomarkers in A1/A1 genotype showed the same difference between CAD patients and control (Table: 2). On the other hand, no statistically significant difference was noted in TAC between A1/A2 genotype of CAD patients and control, but still the other serological parameters were significantly different. The decreased TAC in our CAD patients agreed with many studies (Nojiri et al. 2001, Bastani et al. 2018) Moreover, Demirbag et al. observed also the increased DNA damage in the nucleus of coronary cells (Demirbag et al. 2005). Also the increased MDA in CAD patients agreed with many others (Mendis et al. 1995, Surekha et al. 2007, Bastani et al. 2018). The significant elevation of NO in CAD patients is probably caused by the oxidative stress and increased superoxide production (ROS) to reduce NO bioactivity; otherwise oxidative stress will lead to vasoconstriction and endothelial damage which

may play a role in the pathogenesis of cardiovascular events (Shaikh and Suryakar, 2008). The disease pathology could explain the significant elevation of LDH activity in CAD patients as a whole compared to control, however CK is elevated only in MI group. In order for these biomarkers to become positive for identification, its proteins have to leak out of monocytes in considerable amounts after a large number of cell death (Fagan et al. 2002). The fluctuation of CK and LDH in CAD patients was observed in many studies (Jurlander et al. 2000, Dhruva et al. 2012). Some limitations for our study have to be addressed; firstly LDH, MDA and CK as biomarkers for myocardial injury and the standardized timing for its analysis was not fixed. Secondly, subgroup analysis weakened the statistical inferences in some analyses.

To sum up, CAD with genotype A1/A1 showed significant changes in the mean level of TAC, MDA, NO, and LDH, but in genotype A1/A2, TAC was not affected compared to control. ROS production modulates the release of other inflammatory mediators that are related mainly to the constitutive expression of NAD(P)H oxidases in various tissues (Griendling et al. 2000). ROS also increases chemokine and cytokine expression, intracellular ROS also act as second messengers in inflammatory signal transduction, leading to further enhancement of the inflammatory response. At least part of these effects results from the ability of ROS, in particular H_2O_2, to stimulate MAP-kinases activity which leads to activation of several transcription factors (Blanc et al. 2004).

CONCLUSION

In conclusion, we speculated that subjects having the A1 allele of *IL-1Ra* was a risk factor for CAD. On the other hand, A2 allele seemed to be more protective against such affection. The exclusive presence of genotypes A1/A3 and A2/A3 among MI patients in our study and their absence in the control needs further studies on larger populations, especially from different ethnics. MDA values were also increased in the A1/A1 genotype subjects. Similar effects appeared with NO and LDH

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Mohammad Mahmoud Tarabay → Share in the study design, share in molecular testing and writing the manuscript
Mohammad Saleh Al-Haggar → study design, statistical analysis and write manuscript
Abdel-Aziz Fotouh Abdel-Aziz → Share in the study design, write and review manuscript
Marwa Hassan → collection of samples, share in molecular testing and writing the manuscript
Afaf Mohamed AlSaeid → collection of samples, molecular tests and share in writing the manuscript

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