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- 251 A/T Single Nucleotide Polymorphism of Interleukin 8 and Susceptibility to Acute Myocardial Infarction

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Authors' contributions

This work was carried out in collaboration between all authors. Author HAY participated in the planning of the study, performed the statistical analysis, wrote the protocol, recruited the cases and critical review of the manuscript. Author SAMA participated in the planning of the study, performed the laboratory work, managed the literature searches, managed the analyses of the study and wrote the first draft of the manuscript. Author MKA recruited the cases, shared in management of the literature searches and critical review of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Acute myocardial infarction (AMI) remains a leading cause of morbidity and mortality worldwide A well-characterized IL8 gene single-nucleotide polymorphism (SNP) at position −251 A/T is the only one known to influence its expression and was associated with several diseases. **Aim:** To investigate the possible correlation of the −251 A/T polymorphism of *IL-8* gene with susceptibility to Acute Myocardial infarction and its correlation with Plasma *IL-8* levels in two case-control populations, the Saudi and Egyptian populations.
Methods: 20 AMI patients and 20 asymptomatic controls from Saudi population in addition to 20 AMI patients and 20 asymptomatic controls from Egyptian population were included in the current study. The *IL-8* plasma level was assayed using the human *IL-8* Quantikine ELISA Kit. The *IL-8*

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−251 A/T genotypes were determined by using a polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP).
Results: Patients with AMI were in old age and had a significantly higher number of cardiovascular risk factors (i.e. male gender, diabetes, hypertension, hyperlipedemia and smoking) with higher level of CKMB, troponin and plasma level of *IL-8* with definite ECG diagnosis of AMI as compared to asymptomatic controls. The *IL-8* -251 A/T polymorphism and Plasma *IL-8* level were significantly higher in the AMI patients than asymptomatic controls ($P < 0.001$) in both Saudi and Egyptian populations with AA genotype carriers having significantly higher levels than AT or TT genotypes.
Conclusion: Our findings suggested that the *IL-8* gene polymorphisms may play an important role in susceptibility of AMI, and patients with AA genotype have higher levels of *IL-8* compared to individuals with AT or TT genotypes in that sample of both Saudi and Egyptian populations.

Keywords: Interleukin-8; polymorphism; coronary artery disease; acute myocardial infarction and PCR-RFLP.

1. INTRODUCTION

Interleukin-8 (*IL-8*) is a pro-inflammatory cytokine, related with the initiation and intensification of acute and chronic inflammatory processes. It is typically released by numerous cells such as lymphocytes, monocytes, macrophages, fibroblasts and epithelial cells [1]. It also has a substantial effect on regulating the function of neutrophils. *IL-8* not only induces neutrophil adhesion to endothelial cells, but it also stimulates chemotaxis and neutrophil granule exocytosis, contributed by releasing lysosomal enzymes. Hence, it is an important factor in inflammation induction [2].

Single-nucleotide polymorphisms (SNPs) are DNA sequence variations that result from the alteration of a single nucleotide, which do not result in amino-acid substitution, but they can alter gene function and/or affect gene expression. More than 10 million SNPs have been identified in the human genome [3].

A well-characterized IL8 gene SNP at position −251A/T is the one of the known to influence its expression, and it was associated with several diseases as gastric cancer, bronchiolitis, breast cancer, macular degeneration, oral squamous cell carcinoma , prostate cancer and generalized chronic periodontitis [4–11]. Analyses have shown that the A allele increases the *IL-8* levels after stimulation with lipopolysaccharide (LPS) [12].

Coronary artery disease (CAD) is the most common form of heart disease and is the reason for mortality worldwide [13]. Patients with CAD can be asymptomatic or present as acute coronary syndrome (ACS), which could be due to unstable angina (UA) or acute myocardial infarction (AMI). Acute Myocardial infarction is the most serious event and always results in sudden death or permanent disability in a substantial proportion of patients [14]. Acute Myocardial infarction occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis [15]. Most myocardial infarctions are caused by a disruption in the vascular endothelium associated with an unstable atherosclerotic plaque that stimulates the formation of an intra-coronary thrombus, which results in coronary artery blood flow occlusion [16].

In light of the all above information, the main goal of the current study was to look for a possible correlation of the (-251A/T) polymorphism of *IL-8* with Acute Myocardial infarction and also with plasma *IL-8* levels, in two case-control populations: Saudi and Egyptian.

2. PATIENTS AND METHODS

2.1 Patients and Controls

The current investigation was carried out between January and August 2014. A total of 40 patients and 40 age-matched asymptomatic controls participated in the study from Al ansar hospital-Madina, KSA and Coronary Care Unit Menoufia university hospital, Egypt. Twenty AMI patients (17 male and 3 female) with mean age 73.20±12.42 years and 20 age-matched asymptomatic controls (10 male and 10 female) from Saudi populations in addition to 20 AMI patients (18 male and 2 female) and 20 age-matched asymptomatic controls (11 male and 9 female) from Egyptian population were included

in this study. 12 lead ECGs were done to all patients on arrival to ER (Table 1 and Fig.1) and also ECGs were done to all control individuals. Cardiac enzyme was done using AQT90 FLEX immunoassay analyser (Radiometer) with the following normal ranges TroponinT Less than 0.01 ng/mL and CKMB 55–170 U/L also kidney function tests and lipid profile were done using the Hitachi 7600 DDP modular chemistry analyzer (Hitachi High- Technologies, Tokyo, Japan). With the following normal reference range urea 20-40 mg/dL, creatinine (0.6 to 1.2 mg/dL) total cholesterol (2.7-5.7 mmol/L), triglyceride (0-2.3 mmol/L), high density lipoprotein cholesterol (0.9-1.5 mmol/L), low density lipoprotein (1.7-4.2 mmol/L). The demographic characteristics of studied AMI patients and asymptomatic controls are shown in Table 2.

2.2 Definitions

Acute myocardial infarction is defined as the rise and/or fall of cardiac biomarker values (preferably troponin) with at least one value above the 99th percentile of the upper reference limit and with at least one of the following: Symptoms of ischaemia; New or presumably new significant ST-T changes or new LBBB; Development of pathological Q- waves in the ECG; Imaging evidence of new loss of viable myocardium, or new regional wall motion abnormality; Identification of an intracoronary thrombus by angiography or autopsy .

Classification scheme of AMI was based on electrocardiographic findings as a means of distinguishing between two types of MI, one that is marked by ST elevation (STEMI) and one that is not (NSTEMI). The distinction between STEMI and NSTEMI does not distinguish a transmural from a nontransmural MI. The presence of Q-waves or ST-segment elevation is associated with higher early mortality and morbidity; however, the absence of these two findings does not confer better long-term mortality and morbidity [17].

2.3 Diagnosis of Myocardial Infarction

A diagnosis of myocardial infarction was made by integrating the history of the presenting illness and physical examination with electrocardiogram (ECG) findings and cardiac markers (blood test for troponin and CK-MB) [18]. The cases were defined as having AMI according to standard clinical criteria [19]. All the patients included in this study whether Saudi or Egyptian were diagnosed as acute STEMI. The type of infarction as diagnosed by ECG were summarized in Table 1 and Fig. 1 the most common type of lesion were anterio infarction in both Saudi (45%) and Egyption (35%) populations. The asymptomatic control individuals underwent in the same recruitment period the same clinical and investigational assessment as the patients. Subjects with severe liver or kidney disease were excluded.

2.4 Determination of Plasma *IL-8* levels

Blood samples from patients and control individuals were extracted for the determination of *IL-8*. EDTA-anti-coagulated peripheral blood leukocytes were collected, after that they were centrifuged at 3000g for 15 min at 4ºC to isolate the plasma. Plasma samples were then frozen at -70ºC for storage. The *IL-8* plasma level was assayed using the human *IL-8* Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions with assay range:31.2 - 2,000 ng/L and sensitivity 7.5 ng/L. All samples were analyzed twice, and mean values were used for further statistical analysis.

2.5 DNA Extraction

Genomic DNA of patients and control individuals was extracted from EDTA-anticoagulated peripheral blood leukocytes using a commercially available Qiagen Genomic DNA Purification kit (QIAamp DNA mini kit; Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

2.6 Determination of *IL-8* Genotype

The *IL-8* −251 A/T promotor rs4073 polymorphism was determined by using a polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP), and the PCR primers were designed as described previously [20] using the primer pairs: (forward) 5′-CCATCATGATAGCATCTGTA 3′ and (reverse) 5′-CACAATTTGGTGAATTATTAA -3′. PCR was carried out in a total volume of 10 µl, containing 1 µl genomic DNA, 40 mM of dNTP and 0.2 U of Taq polymerase,1.5 mM MgCl2 and 1.5 µl of each primer. Amplifications were carried out in a thermal cycler (Swift™ MiniPro Thermal Cycler, Esco Micro Pte. Ltd). The program consisted of an initial heating step at 94ºC for 5 minutes; followed by 35 cycles of 30 seconds

denaturation step at 94ºC, 55 seconds annealing step at 57ºC and 1 minute extension at 72ºC; and a final 8-minutes extension step at 72ºC. After PCR, the product was digested with 1 U of *Ase*I (New England Biolabs, Beverly, Mass)for 4 hours at 37ºC, producing fragments of 152 and 21 bp for allele A, or 173 bp for allele T; the fragments were visualized by electrophoresis on a 3% agarose gel stained with 0.1% ethidium bromide (Fig. 2).

2.7 Statistical Data Analysis

The statistical analyses were performed using SPSS version 17 computer statistical software package (SPSS Inc., Chicago, IL, USA). The results were expressed as mean±SD. The frequencies of alleles and genotypes of the whole group of patients were compared to the respective frequencies of the control group using the Fisher's exact test and odds ratios. The paired t-test was used to determine significant difference between test and control subjects. The Maentel - Haenzel method was used for the calculation of all odds ratios with a 95% confidence interval (CI). Generally a two-tailed P value <0.05 was considered significant. The Hardy-Weinberg equilibrium (HWE) was calculated using the Online Encyclopedia for Genetic Epidemiology (OEGE) software (http://www.oege.org/software/hwe-mrcalc.shtml) and tested by χ 2 test to compare the expected genotype frequencies among patient and control groups.

3. RESULTS

3.1 Baseline Characteristics of the Study Participants

Demographic and Laboratory characteristics in patients with AMI and asymptomatic controls are shown in Table 2 The data for the two tested populations (Saudi and Egyptian) were analyzed together, since there were no significant differences of allele frequencies of the -251A/T polymorphism among the two populations. Patients with AMI were significantly older and suffered from a significant higher number of cardiovascular risk factors (i.e. Male gender, diabetes, hypertension, hyperlipidaemia and smoking) as compared to asymptomatic controls. A statistical significant difference was found also between patient and asymptomatic controls regarding blood level of CKMB, troponin and plasma level of *IL-8* (Table 2).

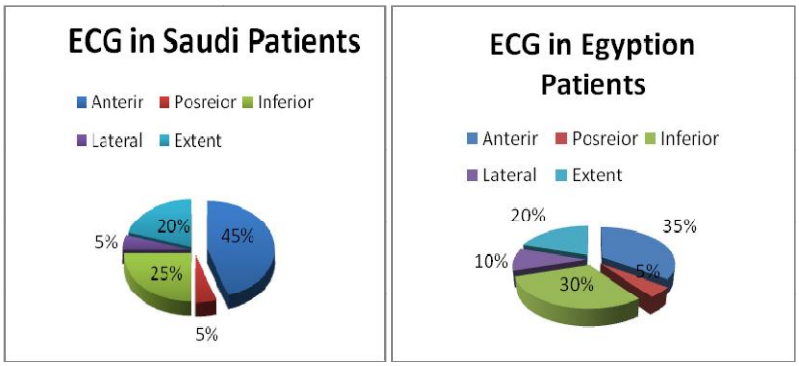

Fig. 1. Types of AMI lesions in Saudi and Egyption patients according to the ECG findings. The most common type was anterior infarction in both Saudi (45%) and Egyption (35%) populations

Table 1. Comparison of types of AMI lesions in Saudi and Egyption patients according to the ECG findings

Patients	ANT-MI	Extent-MI	INF-MI	Post-MI	Later-MI
Saudi male(17)	8	4	3	1	1
Egyptian male(18)	7	4	4	1	2
Saudi female(3)	1	0	2	0	0
Egyptian emale(2)	0	0	2	0	0

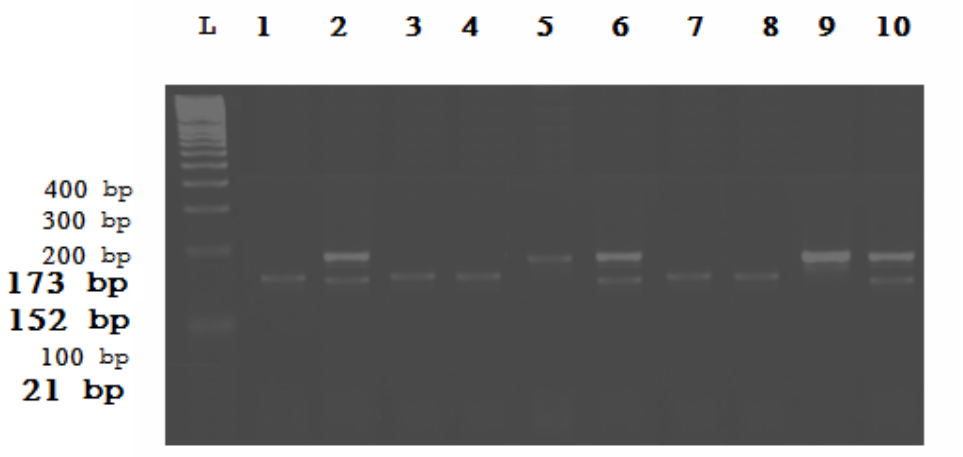

Fig. 2. PCR-RFLP assay for analyzing the −251 A/T polymorphism of *IL-8*. Lanes 2,6 and 10 of heterozygous AT genotype showed three fragments of 173, 152, and 21 (not shown) bp; lane 5 and 9 homozygous of TT genotype showed only one fragment of 173 bp; lanes 1, 3 , 4 , 7 and 8 of homozygous AA genotype showed two fragments of 152 and 21 (not shown) bp. Lane L was loaded with 100–600 bp DNA markers

Table 2. Comparison of demographic data and laboratory findings between cases of AMI and controls in Saudi and Egyptian population

Variable	Saudi population			Egyptian population		
	Cases(20)	Control(20)	p	Cases(20)	Control(20)	p
Age (years)	73.20±12.42	68.25±8.16	0.1379	65.05±10.89	67.35±10.26	0.4960
Male gender (%)	17 (85%)	10 (50%)	0.042*	18 (90%)	11 (55%)	0.033*
Hypertension (%)	15 (75%)	5 (25%)	0.004*	14 (70%)	6 (30%)	0.026*
DM (%)	12(60%)	4(20%)	0.010*	7 (35%)	1 (5%)	0.018*
Smoking (%)	15 (75%)	5 (25%)	0.004*	13 (65%)	5 (25%)	0.026*
urea (mg/dL)	38.25±14.65	32.45±7.01	0.117	40.10±22.20	30.90±7.59	0.093
Creatinine (mg/dL)	1.05±0.31	0.89±0.18	0.053	1.09±0.72	0.85±0.18	0.158
TC (mmol/L)	6.12±0.4	3.44±0.2	<0.001*	5.9±0.3	3.11±0.2	<0.001*
TGL(mmol/L)	4.2±0.3	1.4±0.1	<0.001*	4.4±0.5	2.1±0.1	<0.001*
HDL-C (mmol/L)	1.2±0.23	1.6±0.20	<0.001*	1.1±0.2	1.8±0.3	<0.001*
LDL-C (mmol/L)	4.36±1.16	3.54+0.97	0.020*	4.77±0.82	3.98±0.21	0.0002*
CKMB (U/L)	110.45±63.06	11.45±1.5	<0.001*	114.5±67.09	10.65±1.66	<0.001*
Treponin (ng/mL)	2.67±1.97	0.004±0.003	<0.001*	2.54±2.39	0.004±0.0003	<0.001*
IL-8 (ng/L)	413.70±63.18	318.00±19.13	<0.001*	416.1±55.77	328.85±25.46	<0.001*

DM, diabetes mellitus; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein chlolsterol and CKMB: Creatine kinase myocardial band
*P values were for the comparison between Cases and Control Subjects. *= statistically significant*

3.2 The Genotype Distribution of *IL-8* Polymorphisms

The genotype distribution of *IL-8* polymorphisms in both Saudi and Egyptian populations are shown in Table 3. The genotype distribution in cases was in Hardy–Weinberg equilibrium while the controls showed deviation from Hardy–Weinberg equilibrium in both Saudi and Egyptian population. The distribution of the *IL-8* -251 A/T genotype was significantly different between the AMI patients and asymptomatic controls (P < 0.001) and (P = 0.007) for Saudi and Egyptian population, respectively. The detected genotypes in Saudi population were: AA=8 (40%), AT=12 (60%), TT=0 (0 %) for the patients' group. In the control group, the following genotype frequencies were observed AA=10 (50%), AT=2 (10%), TT=8 (40%). while the in Egyptian population, The detected genotypes in the patients' group were AA=8 (40%), AT=10 (50%), TT=2 (10%) while in the control group were AA=9 (45%), AT=2 (10%),

TT=9 (45%):, resulting in significantly increased mutant allele A compared to the equivalent ones in the control group for Saudi and Egyptian population.

In both Saudi and Egyptian populations the A allele frequency was higher in AMI patients than in asymptomatic control subjects but without significant statistical difference (P = 0.166; OR = 1.91; CI: (0.76 – 4.79) for Saudi populations and (P= 0.175; OR = 1.86; CI: (0.76 – 4.56) for Egyptian population.

The origin was determined in Egyptian and Saudi groups; by survey: individuals with three generations included their own (Table 3).

3.3 Plasma *IL-8* level in Patients and Controls

Plasma *IL-8* levels were measured in AMI patients and asymptomatic controls and results were shown in Table 4 and Fig. 3, plasma *IL-8* levels were significantly higher in AMI patients than asymptomatic control subjects (P < 0.001). Plasma *IL-8* levels were correlated with *IL-8* -251 A/T polymorphism; the level was higher among subjects carrying the *IL-8* -251 AA genotype, and lower among subjects carrying the -251 AT and TT genotype (P < 0.001) in both Saudi and Egyptian populations (Table 4 and Fig. 3).

4. DISCUSSION

Acute myocardial infarction is the leading cause of mortality worldwide [13]. L-8 -251 (A/T) single nucleotide polymorphism affects the function of promoter gene. Hence, this type of polymorphism is considered as a rational source of investigation to study the role of genetic factors in the incidence and severity of many diseases [21].

We investigate whether the (-251A/T) polymorphism which affects *IL-8* gene expression is associated with susceptibility to AMI. The subjects under investigation were Saudi and Egyptian individuals with AMI (STEMI) whose genotypes were compared to those from healthy subjects. The overall obtained data revealed that the (-251A/T) polymorphism which affects *IL-8* gene is significantly higher in AMI (STEMI) than asymptomatic subjects in both studied populations.

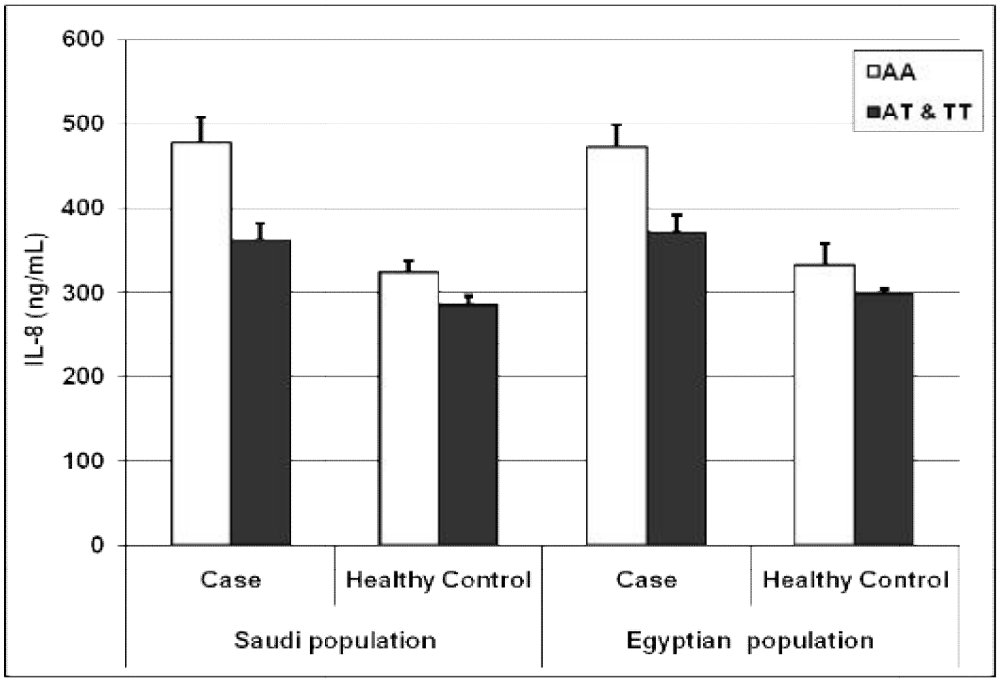

Fig. 3. Correlation between plasma *IL-8* levels (ng/L) with *IL-8* - 251 A/T polymorphism; plasma *IL-8* levels were significantly higher in AMI patients than asymptomatic control subjects and subjects carrying the *IL-8* -251 AA genotype have higher level than subjects carrying the -251 AT and TT genotypes

Table 3. The association between the *IL-8* -251A/T genotype and AMI cases and controls

IL-8 genoype	Saudi population				Egyptian population			
	Cases (n=20)	Control (n=20)	p	OR	Cases (n=20)	Control (n=20)	p	OR
A/A	8 (40%)	10 (50%)	<0.001		8 (40%)	9(45%)	0.007	
A/T	12 (60%)	2(10%)			10(50%)	2(10%)		
T/T	0 (0%)	8 (40%)			2 (10%)	9 (45%)		
A allele	28(70%)	22 (55%)	0.166	1.91 (0.76 – 4.79)	26(65%)	20(50%)	0.175	1.86 (0.76 – 4.56)
T allele	12 (30%)	18(45%)			14(35%)	20(50%)		

P values were for the comparison between Cases and Control Subjects.
P values were given by univariate logistic analysis for the IL-8 -251A/T polymorphisms

Table 4. Plasma *IL-8* level according to *IL-8* -251A/T genotype

Group	Plasma *IL-8* level (ng/L) in saudi			Plasma *IL-8* level (ng/L) in Egyptian		
	AA	AT &TT	p	AA	AT &TT	p
Cases of AMI	476.67±30.21	362.18±19.15	<0.001*	470.78±27.24	371.36±20.57	<0.001*
Control	323.65±14.0	286.0±10.15	<0.001*	332.17±24.62	299.0±5.66	0.080
p	<0.001*	<0.001*		<0.001*	0.001*	

In our study the frequency of genotype distribution in cases was in Hardy–Weinberg equilibrium while the controls showed deviation from Hardy–Weinberg equilibrium in both Saudi and Egyption population. Schaid et al. [22] reported that deviations from HWE can be caused by multiple reasons, such as small population variation (random genetic drift), and the structure of the population. Although, our results demonstrated that the (-251A/T) polymorphism of *IL-8* gene is significantly higher in AMI (STEMI) than asymptomatic subjects, we could only suggest that the *IL-8* gene polymorphisms may play an important role in susceptibility of AMI. According to Xu et al. [23], It is possible that a departure from HWE for some SNPs in control subjects is due to some unknown factors other than genotyping errors and deviation from HWE has been shown to inflate the chance of a false-positive association.

In line with our results, the data, obtained by Zhang et al. and Kucherenk et al. concluded that *IL-8* -251 A/T polymorphism is associated with ACS risk in the Chinese Han and Ukraine populations, respectively [24,25]. In contrast to the findings of the present study, an investigation with the same SNP by Konstantina et al. [26] in Chinese population failed to demonstrate a positive association of that polymorphism with ACS. The discrepancy in the results may be related to genetic differences in populations and sample size. An important point is the difference in the distribution of ethnics between the separate studies.

The literature demonstrates that genetic associations may not be uniformly consistent across populations and that the frequency of occurrence of polymorphisms appears to vary extensively between the ethnic groups. Moreover, the literature shows that the subject samples involved in studies are generally of small size [10].

Acute myocardial infarction is the result of erosion or rupture of vulnerable plaques. The plaques which are susceptible to erosion or rupture have a large lipid core enriched with inflammatory cells and a thin fibrous cap with smooth muscle cell content. Vulnerable plaques show marked inflammation, and inflammatory mediators play an important role in the procession of plaque disruption [27]. It is conceivable that genetic variations that influence the expression of inflammatory mediators could influence susceptibility to AMI. *IL-8* is a strong chemo-attractant for neutrophils, macrophages and T-lymphocytes in response to various inflammatory stimuli [28-30].

The fact that some but not all the patients in the current study had a mutant *IL-8* allele may be explained with the possible contribution of similar polymorphisms affecting gene expression of other interleukins [31]. As a consequence, it is of great importance to perform further genetic studies regarding the contribution of factors related to inflammation and thrombosis in predisposition to AMI .

In the present investigation, the *IL-8* -251 A allele is higher in AMI than in asymptomatic subjects. Nearly same result has been obtained by Zhang et al 2011 who have concluded that the A allele of *IL-8* - 251 A/T may be an independent predictive factor for ACS [24].

Our results demonstrated that the plasma *IL-8* levels were significantly higher in AMI patients than in healthy controls ($P < 0.001$); the level was higher among carriers of the *IL-8* -251 AA genotype, and lower among subjects carrying the - 251 AT and TT genotype ($P < 0.001$) in both Saudi and Egyptian populations. Nearly similar results were obtained by other researchers, Rus et al. [32] who have reported for the first time high levels of *IL-8* in the human arterial atherosclerotic wall, as well as intra- and extra-cellular deposits in the connective tissue matrix. Similarly, Qi et al. found that the *IL-8* level was markedly increased in an UA group pre- and post-(percutenous coronary intervention) PCI, and that elevated pre-procedural *IL-8* levels were associated with a high incidence of cardiac events and restenosis [33,34]. These studies linked plasma *IL-8* levels with atherosclerotic plaques which may help us explain the possible effect of elevated plasma *IL-8* in patients with AMI [35,36]. Several reports have demonstrated significantly higher values in patients with UA or AMI, suggesting that *IL-8* is involved in the process of atherosclerosis by strongly expression of CXCR2 receptor on macrophage that infiltrate the atherosclerotic lesion which is a receptor for *IL-8* [37,38], and it may be a useful clinical predictor of unstable CAD [39]. *IL-8* -251 (A/T) single nucleotide polymorphism affects the function of promoter gene and cases with AA genotype produce and release higher levels of *IL-8* compared to individuals with AT or TT genotype [21].

5. CONCLUSION

Our findings suggested that the *IL-8* gene polymorphisms may play an important role in susceptibility of AMI, and patients with AA genotype have higher levels of *IL-8* compared to individuals with AT or TT genotypes in that sample of both Saudi and Egyptian populations.

6. RECOMMENDATIONS

1. As this polymorphism is biologically important in the pathogenesis of many diseases and as our research was the first to be applied on Saudi and Egyptian populations, An extended researches on a large number of the same studied populations and in different populations will be required to detect presence of that association and to better define the functional significance of -251 A/T polymorphism.
2. Attention should be paid to perform an Hardy–Weinberg equilibrium test for each SNP before testing any hypothesis to increases the ability to identify small but real associations between SNPs and complex diseases.

ETHICAL APPROVAL

Adherence to Saudi Arabia and Egypt regulations concerning the welfare of human subjects was maintained throughout the study performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The participant's rights, privacy, health, and well-being were safeguarded through informed consent forms that they asked to read and sign if they agreed to participate in the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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