Evaluation of Some Biomarker and Genetic Marker in Myocardial Infraction Patients

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Abstract

Acute coronary syndrome (ACS) consists of unstable angina pectoris, non-ST segment elevation myocardial infarct ion and ST segment elevate ion myocardial infarction. The aim of the study was to compare the vibration some hormones levels in the Acute coronary syndrome patients and control groups and This study aimed to investigate the association between two single nucleotide polymorphisms (−1562C>T, of the MMP-9 gene in patients with ACS in the Iraq population.

Subjects and methods: This study was conducted between / mrjan teaching hospital in Babylon Province between November 2012- Aprial 2013and it was carried out at the coronary care unit / in Babylon province/Iraq . This patient-control study was composed of 60 ACS patients with age 53.38 ± 9.51 and 30control withe age 51.43 ± 7.81subjects, The present study is divided into two main parts: physio-biochemical and molecular parts. The physiological part involved hormonal assay(troponin and endothelin-1) while molecular part included The genotypes of the selected SNPs were determined by the method of polymerase chain reaction and restriction fragment length polymorphism (RFLP-PCR). The relationship between the polymorphism of the MMP-9 gene and the severity of Acute coronary syndrome with negative medical history.

The result of this study showed that patients with Acute coronary syndrome both males and females had significantly troponin and elevated Endothelin -1 in patient than control**<0.001**(**p value $\leq$ 0.01 was significant).Analysis of the SNPs showed that the frequency of CT and TT genotypes in patients with ACS was significantly higher than that in the control group (ACS vs. controls; CT+TT: (85%Vs 36%).

Keywords:single nucleotide polymorphisms, troponin, endotheline, MMP-9

Introduction

ACS is mainly caused by coronaryatherosclerotic plaque rupture or erosion and subsequentintracoronary thrombus formation (Davies MJ,1996). Age, gender,smoking, hypertension, hypercholesterolemia, diabetesmellitus, obesity and sedentary lifestyle are reported tobe associated with ACS but the exact mechanismof ACS is still not clear (Anderson *et al*.,1991). Acute coronary syndrome (ACS) consists of unstableangina pectoris, non-ST segment elevation myocardialinfarct ion and ST segment elevat ion myocardialinfarction. The pathogenic mechanism of ACS ismost often based on thrombosis secondary to plaquerupture in atherosclerosis (ASC) (Liu *et al*.,2006).Vascular diseases, including coronary artery disease, are the leading cause of death in developed countries. The data indicate that persons under 45 years of age constitute approximately 10% of all patients with myocardial infarction (Doughty *et al*., 2002). Among young people, the leading factor for myocardial infarction (besides the classic risk factors of hypercholesterolemia, arterial hypertension, smoking, and diabetes mellitus) is a positive family history among first-degree relatives (Garoufalis*et al*., 1998) (Sakowicz*et al*., 2010b), perhaps because environmental factors have had less time to exert a dominating influence on the disease process in young people (Chaer*et al*., 2004).

Approximately 90% of all cases of myocardial infarction are the result of acute thrombus, causing obstruction of an artery vessel in places of atherosclerosis plaque rupture (Sakowicz*et al*. 2010b). The etiopathogenesis of atherosclerosis plaque is still not clear. Several theories or hypotheses about atherogenesis have been proposed. The most probable is the unified theory in which atherosclerosis is caused by complicated interactions among cells of the endothelium, blood cells, and lipoprotein. Damage to the endothelium leads to its dysfunction and activation (Libby 2002). This activation is related to the increased expression of chemoattractant cytokines, lecithin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and adhesion molecules such as selectin E, intracellular adhesion molecule type 1 (ICAM1), vascular adhesion molecule (VCAM), and platelet endothelial cell adhesion molecule type 1 (PECAM1) (Libby 2002). There is a genetic associationbetween polymorphic variants in candidate genes andatherosclerosis.

The matrix metalloproteinase (MMP)family is one of the potential candidate gene systems.matrixmettalopritenase(MMP) family are involved in the breakdown of the extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis (Visse and Nagase ,2003). mmp_9 play significant role in the progression of atherisclerrosis ,plaque rupture and ischemic heart disease (Visse and Nagase ,2003). Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases.(Henney*et al* 200) reported that genetic change which affects the expression of MMPs may

contribute to the occurrence of cardiovascular disease. Matrix metallopeptidase 9 (MMP-9), also known as 92 kDa type IV collagenase, 92 kDagelatinase or gelatinase B (GELB), is an enzyme that in humans is encoded by the MMP9 gene. MMP-9 is highly expressed in the vulnerable regions of atherosclerotic plaques. For this reason, MMP-9 plays a key role in vascular remodeling and development of atherosclerotic lesion, and plays a potential role in arterial plaque rupture (Galis and Khatri ,2002). The rol of MMP_9 gene are relatedto presence and severity of CAD (Morgan *el at* 3003). (Zhang*et al.,*1999) have identified several single nucleotide polymorphisims in the mmp_9 gene including the C . the 1562T allele has a higher promoter activity than the C allal and may influence the severity and extent of coronary artery stenosis (Morgan *et al.,* 2003).

 ET-1 is a 21-amino acid peptide and represents the major isoform of the endothelin peptide family (Khan IA.,2005) which also includes ET-2, ET-3 and ET-4. The endothelin peptides are produced by endothelial and smooth muscle cells, neural, renal, pulmonary and inflammatory cells. ET-1 is the most potent vasoconstrictor known. The inactive precursor is converted into its active form by ET converting enzyme. ET-1 acts through two receptors, ET-A and ET-B. ET-A receptors lead to increased intracellular calcium concentrations and induce vasoconstriction and cell proliferation. ETB receptors mediate the release of nitric oxide and prostacyclin and therefore cause vasodilatation. They are also involved in the clearance of ET-1 and inhibit the action of ET converting enzyme (Spieker*et al*.,2001) ET-1 has also been found to play an important role in the pathogenesis of hypertension, congestive heart failure and regulation of renal function(Spieker*et al*.,2001).

ET-1 levels are positively correlated with the extent of atherosclerosis. Other studies have shown that in patients with coronary artery disease tissue ET-1 levels are related to the extent of angina(Berger and Pacher .,2003) . ET-1 promotes direct vasoconstriction, induces smooth muscle cell proliferation, stimulates adhesion of neutrophils to the endothelium and platelet aggregation. (Knofler*et al.,*1995) It also antagonises the action of nitric oxide.

Cardiac troponin is composed of three subunits T, I, and C, which are the products of different genes. The total mass of the troponin complex is minuscule when compared with the protein mass of other myofibrillar proteins like actin and myosin. However, both pattern of cardiac troponin (cTn) T when compared with the monophasic pattern seen with cTnI (Thygesen*et al* .,2010). The release pattern of cTnT is different in non-reperfused MI and may vary with small MIs. Although the exact reason for this different release kinetics is still illusive, cTnT differs from cTnI with respect to higher molecular weight, higher fraction of unbound cTnT, less degradation, whereas cTnI is more frequently found as binary or tertiary complex in blood. The diagnosis of MI has thus evolved following the introduction of routine troponin testing, resulting in the redefinition of MI by the joint European Society of Cardiology (ESC)/American College of Cardiology (ACC) in 2000 (Alpert *et al*.,2000) . Based on this consensus document, any amount of myocardial damage, as detected by serum troponin and associated with evidence of ischemia, should be considered an MI. It is increasingly recognized that elevated troponin levels occur in many patients who do not have evidence of flow-limiting coronary artery disease (Ammann*et al*., 2009). In addition to nonthrombotic cardiac conditions (myocardial contusion, infiltrative myocardial diseases), nonthromboticnoncardiac diagnoses (sepsis, pulmonary embolism, stroke, renal failure) are also associated with elevated levels of troponin. Given this observation, it is generally considered inappropriate to use elevated troponin levels as the only diagnostic criterion for MI (Luepker*et al* .,2003).

Subjects and Methods

Study Design, Setting and Data Collection Time

 Case-control study was conducted between November 2012- Aprial 2013and it was carried out at the coronary care unit / in Babylon province/Iraq .

Study Population

 The study subjects comprised from 60 patients suffer frome ACS randomly selected fromemrjan teaching hospital (50 male and 10 female) as ACS patients group with age average (3o-65 year), the control group study included 30 people apparently healthy that included 20 male and 10 female with age average (33-65 year) ,this control group matched with patient group. All subjects in this study were taken written consent beforparticipatian in this study.

Exclusion Criteria

The excluded include patients DM, hypertension ,hepatitis , heart failure, renal failure, liver disease, malignant disease ,patients on chemotherapy , etc. and excluded patients who suffer from complication MI. Questionnaire taken from the patient included : age, sex ,occupation ,types of MI , smoking habit, alcohol intake, pervious attack, blood pressure, , and family history of MI,past medical history

DNA extraction

DNA was extracted from freezing blood according to kit leaflet (genaid, genomic DNA extraction kit).

PCR of *MMP-9* **

 Amplification of gene by PCR performed according to table A using sequence of primers in table B

Table (A) program was used for amplification the target DNA fragments(for MMP-9 gen)

Stage	Temperature(^{O}C)	Time(min)	Function	Cycles
1	94	0.30	Initial denaturation	
2	94	0.30	DNA denaturation	
	67	1	Primer annealing	30
	72	1	Template elongation	
3	72	5	Final elongation	
4	4	-	Incubation	Hold

Table (B) sequence of *MMP-9* ** primers

Primer gen name	Sequences
MMP-9 **	**F:5'-GCC TGG CAC ATA GTA GGC CC-3'**
	R:5'-CTT CCT AGC CAG CCG GCA TC-3'

RFLP-PCR

1- Prepare the Sph I enzyme and dilute the buffer enzyme 10X to 1X.

2- taked 100 µl from buffer enzyme (10X) and add 900 µl d.d.w in Eppindorf tube to prodused buffer 1X .

3- 8 µl of amplified product (422 pb fragment) with 2.5 µl from enzyme and add 39.5 µl with buffer enzyme (1x).

4- put the mixture Eppindorf tubes and incubated at 37 C^o for 1-2 hour by using thermocycler (Cleaver Scientific).

5- Digested amplified DNA fragments were electrophoresed on 1% agaros, ,(1X) TBE buffer (40 min at 75 V) and the bands visualized after staining with ethidium bromide under UV light. A 100 base-pair ladder were used as a size marker for estimation of fragment sizes.

Blood collection

About five milliliters of venous blood were collected from each subject in the study. blood were separated by centrifugation at 3000 rpm for 15 min for separated serawhich remaining stored frozen at -20 ºC until hormonal assayed. Troponin Kit Biomerieux (France) msurmeant by Vides and endothelin Kit enzolifesincese(USA) msurmeant by Eliza reader & washerBiotek – USA.

Results

Table1 shows the association of study groups (Cases Vs controls) by sex , residence, occupation, family history , smoking habit, alcohol intake , history of previous attacks and blood pressure level . There was significant association between development of myocardial infarction and occupation, presence of smoking habit , presence of history of previous attacks and blood pressure level , meanwhile there was no significant association between development of myocardial infarction and sex , residence , family history and alcohol intake. Majority of patients with MI were male (83.3%) , (70%) were not employed and (78.3%) came from urban area .

Table (1) The association of study groups by study variables

Variable	Case	Control	χ^2	df	P-value	Odds ratio	95% CI
Sex							
Male	50(83.3%)	23(77%)					
Female	10 (16.7%)	7(23%)	0.58	1	0.446	1.52	0.514-4.503
residence							
Rural	13 (21.7%)	3 (10%)					
Urban	47 (78.3%)	27(90%)	1.86	1	0.172	2.48	0.651-9.523
Occupation							
Not employed	42 (70%)	5(17%)					
Empolyee	18 (30%)	25(83%)	22.8	1	**<0.001****	**11.66**	**3.85-35.317**
family history							
Present	22 (37%)	13(43%)					
Absent	38 (63%)	17(57%)	0.37	1	0.541	0.75	0.31-1.849
Smoking habit							
Present	33 (55%)	6(20%)					
Absent	27 (45%)	24(80%)	9.97	1	**0.002****	**4.88**	**1.747-13.651**
History of previous attacks							
Present	14 (23%)	1(3%)					
Absent	46 (77%)	29(97%)	5.76	1	**0.016***	**8.82**	**1.1-70.7**
Alcohol intake							
Present	2 (3%)	0 (0%)					
Absent	58 (97%)	30(100%)			0.551[a]		
Blood pressure							
Normal	23 (38%)	2 (7%)					
Pre-hypertensive	21 (35%)	25 (83%)	18.99	2	**<0.001****		
Hypertensive	16 (27%)	3 (10%)					

* Normal blood pressure (<120 and <80) , pre-hypertension (120-139 or 80-89) and hypertensive (≥140 or ≥90).

Table 2 show the mean differences of age, heart rate and BMI for both gender of myocardial infarction and control subjects . There were significant mean differences of heart rate and body mass index between myocardial patients and control subjects ,meanwhile there were no significant mean differences of age between case and control groups and no significant mean differences of age ,heart rate and BMI for both male and female gender .

Table (2) Mean differences of age, heart rate and BMI for both gender of myocardial infarction and control subjects

Group / Indices	Case (Mean ± SD)		Control (Mean ± SD)		P value of gender	P value of group
	Male	**Female**	**Male**	**Female**		
Age (years)	51.86 ± 9.33	61.00 ± 6.42	52.21 ± 8.15	48.85 ± 6.46	0.09	0.335
Heart rate (beat/minute)	79.46 ± 13.67	84.30 ± 16.58	75.65 ± 1.64	74.14 ± 1.21	0.562	**0.009****
BMI (Kg/M^2)	25.97 ± 3.32	25.98 ± 3.40	29.84 ± 4.15	29.28 ± 4.76	0.891	**<0.001****

Table 3 shows the association of study groups (Cases Vs controls) by physiological markers of heart disease (endothelin , troponin) . There was significant association between development of myocardial infarction and increment of these markers .

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Table (3) The association of study groups (Cases Vs controls) by physiological markers

Variable	Case	Control	χ^2	df	P-value
Endothelin					
Normal value (1.2-2.5 pg/ml)	3 (5%)	21 (70%)	52.18	2	**<0.001****
High (>2.5 pg/ml)	45 (75%)	1 (3%)			
Low (<1.2 pg/ml)	12 (20%)	8 (27%)			
Troponin					
High (0.01) and above	50(83%)	0 (0%)	56.25	1	**<0.001****
Normal value (< 0.01)	10(17%)	30(100%)			

Figure 1 shows the distribution of the cases by types of myocardial infarction .Majority (42%).
of the cases presented with inferior MI
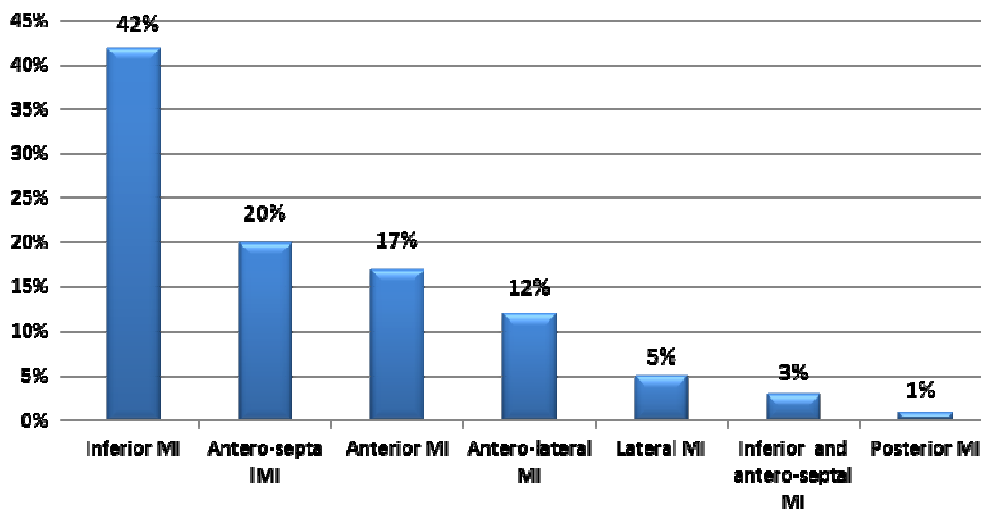
Figure(1) Distribution of cases by types of MI

Figure 2 shows the distribution of the study population by Body mass index . Majority (48%) of the cases were
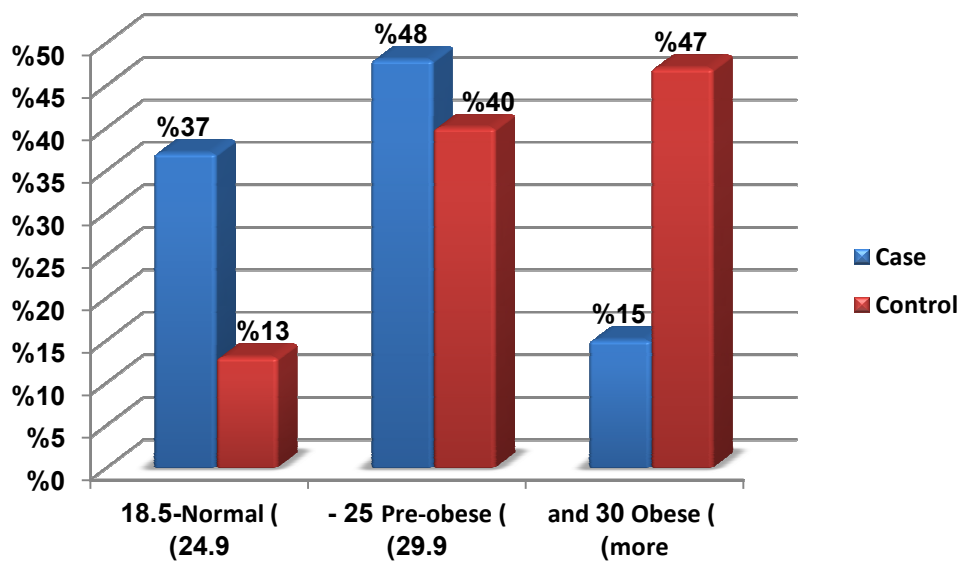pre-obese while majority (47%) of control were obese .

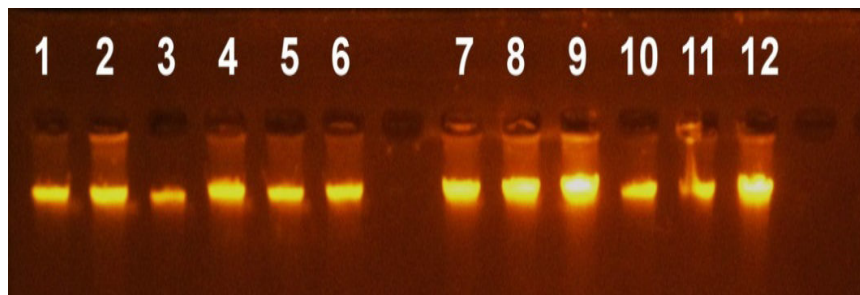**Figure (2) Distribution of study population by BMI**

figure (3) Electrophorese patteren of DNA extraction from blood for patient ACS and control % agarose 75V, 20 AM, for 1h (10 µl in each well).
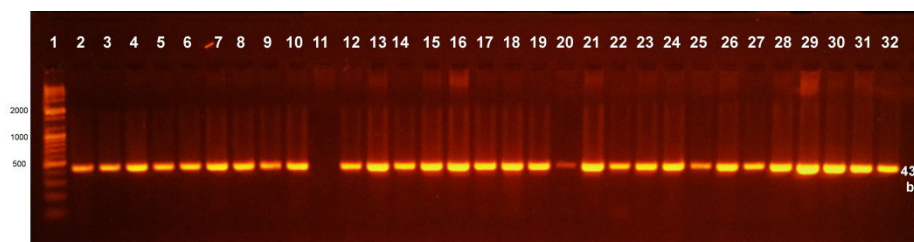

figure (4) Electrophorese patteren of PCR product for MMP-9 gene , this amplification prduse one band 435 bp both patient and and control 1% agarose 75V, 20 AM, for 1h (10 µl in each well).
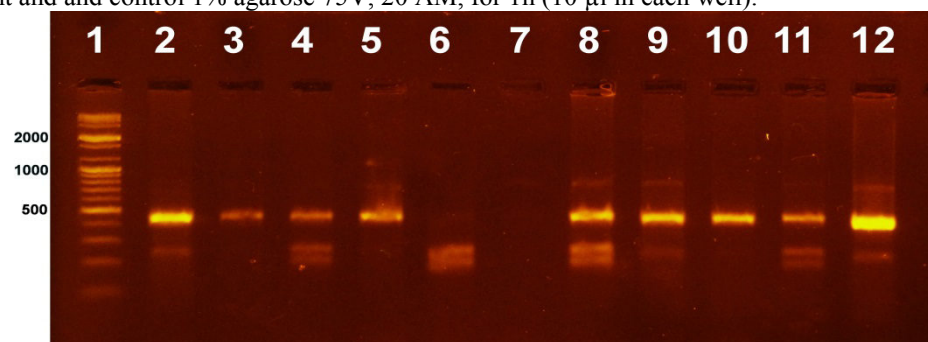

figure (5) Electrophorese patteren of RFLP- PCR product for 435 bp both with restriction enzyme Spha I, 1% agarose 75V, 20 AM, for 120 m (10 µl in each well).
Lane 1:- DNA lader 100 bp
Lane,3,5,10,12:-showing wild type (CC) genotype.
Lane 6:- showing mutant homozygote (TT) genotype.
Lane 2,4,8,9,11,12:- showing mutant homozygote(CT) genotype.

Discussion

The diagnosis of acute myocardial infarction (AMI) has traditionally been based on the characteristic clinical history, electrocardiographic abnormalities and increased serum concentrations of cardiac marker enzymes. As the differential diagnostic value of chest pain is limited and the electrocardiographic changes have various degrees of sensitivity and specificity (Rude *et al*., 1983),

The results of the present study show that the patients with ACS of ECG is higher type MI inferior 42% other than type MI as show in the(figure 1),ECG diagnosis of myocardial infarction may not be evident during initials hours at all the times and it is always essential to diagnose myocardial infarction earlier for timely therapeutic intervention. Because cardiac troponin is a sensitive and specific measure of myocardial necrosis, it is the preferred biomarker for use in the diagnosis of acute MI. Although an elevated troponin indicates myocardial necrosis so in the present study show that the patients with ACS whether male or female, had significantly higher troponin concentration than control group as show (table 3).

According to Katus*et al* (1993) Troponin T is elevated more than twice the analytical sensitivity of the assay (0.5 ng/ml) in all patients with infarction Troponin T appeared in the serum as early as 3 hours after the onset of chest pain in 50% of patients and remained elevated JIn all patients for more than 130 hours. Thus cardiac Troponin T measurement is particularly useful in clinical ircumstances in which traditional enzyme determiations fail to diagnose myocardial cell damage efficiently(Gerhardt *et al*.,1992).

The incidence and prevalence rates of elevated levels of troponincited in the literature vary widely, ranging from

15% to 70% of patients (Noble *et al*.,1999) Recent studies have examined thefrequency of elevated troponin levels excluding those patients with underlying coronary heart disease (Ammann*et al*.,2003).

The results of the present study show that the patients ACS ,whether male or female, had elevated endothlin-1 concentration than control group and higher significantly between them as show(table 3).

Experimental studies have shown that endothelin may be produced by activated human macrophages (Ehrenreich*et al* .,1990) and has been also implicated in cytokine(Ruetten*et al*.,1997) and vascular cell adhesion molecule expression(Ishizuka et al .,1999).

studies have suggested that plasma endothelin is increased in patients with acute coronary syndromes(Wieczorek*et al* .,1994). ET antagonists have an adverse effect on myocardial contractility in healthy individuals with normal ET-1 plasma levels but improve contractility in patients with advanced ventricular dysfunction(Khan ,2005).

ET-1 levels are positively correlated with the extent of atherosclerosis. Other studies have shown that in patients with coronary artery disease tissue ET-1 levels are related to the extent of angina(Berger and Pacher .,2003) . The effects of ET-1 on the heart are multiple(Khan IA.,2005) Normal ET-1 plasma levels produce a positive inotropic effect through an increase in intracellular calcium, whereas elevated plasma levels of ET-1 result in a decline in cardiac output. This is because ET- 1 has a predominantly vasoconstrictive effect, at both peripheral and coronary level, thus resulting in increased afterload and reduced myocardial perfusion. ET antagonists have an adverse effect on myocardial contractility in healthy individuals with normal ET-1 plasma levels but improve contractility in patients with advanced ventricular dysfunction(Khan ,2005).

This is the first study on the two potentially functional polymorphisms of the MMP-9 gene in relation to ACS(MI) susceptibility in the Iraq population. The data of this study demonstrated that the −1562 T allele of the MMP-9 gene is significantly associated with an increased risk of ACS. The frequencies of C/C,C/T and T/T ofMMP-9 (−1562C>T) polymorphism were 27(45%),24(40%),9(15%) in the ACS group, and 19(63.3%),8(26.7%),3(10%) in the control group The homozygote TT and the heterozygote CT were more frequent in the ACS than in the control group(85%) Vs(36%).

MMP-9 possesses proteolytic activity on type IV collagen, a major constitute of the basement membrane that surrounds every vascular smooth muscle cell and underlies the endothelium in the blood vessel wall(Newby AC 2005).

The human MMP-9 gene is located on chromosome b20q12.2-13.1, (Zhang *et al*.,1999)found a number of single nucleotide polymorphisms (SNPs) in the promoter, coding and untranslated regions. Of these, two polymorphisms, namely promoter −1562C>T polymorphism and codon 279 polymorphism (R279Q), are of special significance. Functional studies indicate that the −1562C>T polymorphism has an al lelespecific effect on MMP-9 transcription. DNA-protein interaction assays have revealed that the sequence between nucleotide position −1567 and −1559 relative to the transcription start site of the MMP9 gene, which encompasses the −1562 polymorphic site, can interact with a nuclear protein, but its mechanism is still unknown(Zhang *et al*.,1999).

Functional studies indicate that the −1562C>T polymorphism has an allele-specific effect on MMP- 9 transcription. In this study we found that −1562CT/ TT genotypes were associated with a significantly increased risk of ACS. A genetic epidemiological study(Morgan *et al*.,2003) indicated that T-1562 allele carriers are predisposed to the development of coronary atherosclerosis, which causes coronary stenosis. (Zhang *et al*.,1999). reported a functional 1562C>T polymorphism in the promoter region of MMP- 9. Transfection experiments and DNA-protein interaction assays indicated that the T allele had a higher activity. In addition,(Blankenberg*et al*.,2003) reported that plasma MMP- 9 levels were also higher in −1562T allele carriers than in non-carriers. A study(Medley *et al*., 2004) on aortic tissues showed that MMP-9 mRNA levels, MMP-9 protein levels and MMP- 9 activity were higher in −1562 T allele carriers than in non-carriers. MMP-9 knockout studies (Galis*et al*., 2002; Johnson and Galis ,2004)in mice also demonstrated a role of MMP-9 in the development of atherosclerosis. Compared with MMP-9 wild-type mice, MMP-9 deficient mice had fewer and smaller atherosclerotic lesions (Johnson and Galis ,2004) .

Thus, increased vascular smooth muscle migration and macrophage infiltration are likely to explain increased coronary atherosclerosis in carriers of the MMP-9 high expression −1562T allele in humans. These findings suggest that the −1562 C>T polymorphism not only affect the MMP-9 promoter activity *in vitro* experiments, but also influence the MMP-9 transcription *in vivo*, and this effect is translated into differences in MMP-9 protein level and activity between individuals with different MMP-9 genotypes.

Our study result showed that serum MMP-9 level was significantly increased in STEMI, indicated greater damage or degradation of extracellular matrix from plaque rupture. MMP-9 contributes to the vulnerability of coronary atherosclerotic plaques which tends to rupture or erode and precipitates acute coronary events(Galis and Khatri ,2002) . (Fiotti et al.,2006) reported that the MMP-9 promoter microsatellite polymorphism associated with thin fibrous caps and large lipid core of coronary atherosclerosis.

Study by(Koh*et al*.,2008) found that MMP-9 (-1562C>T) polymorphism was significantly and independently

associated with acute myocardial infarction. Our study indicated heterozygote C>T was higher in STEMI subjects as compared to NSTEACS.(Morgan *et al*.,2003) showed in -1562C>T polymorphism, -1562T allele had a higher transcriptional activity as compared to that of -1562C allele. Similarly,(Kim *et al*., 2002) reported that the substitution of C>T at -1562 promoter will provide a higher promoter activity of the T-allelic promoter. This fact contributes to elevated MMP-9 in the plaque which is detected in serum during plaque rupture in subjects with MMP-9 (-1562C>T) polymorphism. The mechanism whereby the MMP-9 allele contributes to CAD and atherosclerosis is unknown. Atherosclerosis is apparently initiated in response to arterial endothelial injury, which allows increased permeability to lipid, monocytes and lipid-laden macrophages. The MMP-9secreted from macrophages apparently helps medial smooth muscle to migrate into the intima by degrading extracellular matrix(Robertson et al .,2007).

(Zhi*et al* .,2010)reported that −1562 CT/TT genotypes may contribute to CAD in diabetics and MI in CAD patients in a Chinese population. (Fallah*etal*.,2010)found that the −1562 C>T polymorphism in the MMP- 9 gene potentially plays a role in the manifestation of coronary atherosclerosis but does not affect the number of diseased vessels. The results of their study were not consistent. Three published studies on 788 Caucasians, 248 Koreans and 2731 German men with angiographically documented CAD failed to confirm an association with the T allele(Kim et al.,2002; Haberbosch and Gardemann,2005).

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