The Role of Oxidative Stress and NADPH Oxidase P22phox Polymorphism in Acute Kidney Injury Patients

Ashraf Dawood[*1], Rania Azmy[1] and Mahmoud Emara[2]

[1]Medical Biochemistry, and [2] Internal Medicine Departments, Menofia University, Egypt
[*]kingashrafdawood@yahoo.com

Abstract: Acute kidney injury (AKI) is a common condition with significant associated morbidity and mortality. Although several studies have been done to understand the molecular and biochemical mechanisms of kidney injury. Results were static over the last 30 years. Increased production of reactive oxygen species (ROS) is thought to play a major role in the pathogenesis of AKI and its complications. The NADPH oxidase complex is an important source of ROS in AKI. Its p22 subunit is polymorph with a C242T variant that changes histidine-72 for a tyrosine in the potential heme binding site. The aim of this study was to investigate the occurrence of this polymorphism in 75 patients with AKI and correlate the genotype to the extent of the load of the circulating ROS and its association with the unfavorable course of the disease. This study included 75 hospitalized patients in the Internal Medicine Department Menofiya University Hospital with established AKI. We had done genotyping for the C242T polymorphism of the p22 subunit of the NADPH oxidase gene using restriction fragment length polymorphism (RFLP) technique. The extent of the ROS load was estimated by measuring the level of plasma nitrotyrosine level and then correlation to the severity of the course of the disease and its outcome was made. The results revealed that, compared to CC group, the T- allele carrier was associated with higher levels of serum urea, creatinine and plasma nitrotyrosine ($p<0.01$) and the levels of plasma nitrotyrosine are correlated with age of the patient and length of hospital stay. Also the T- allele carries (CT and TT groups) showed higher cumulative probability of remaining hospitalized, while there was no significant difference concerning requirement for dialysis, for ICU admission and dialysis dependency. It is concluded that the polymorphism C242T in the gene encoding p22phox of NADPH oxidase is associated with the severity of the course of the disease and the adverse outcome in cases with AKI.
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1. Introduction

Oxidative stress (OS) means damage to cells, tissues and organs caused by reactive oxygen species (ROS). ROS were generated exogenously and intracellularly and include superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) hydroxy radicals ($OH^{\cdot}$) and peroxynitrite ($ONOO^{-\cdot}$) ***(Djamali, 2007)***. The principal intracellular sources of ROS include the mitochondrial electron transport system, peroxisomes, cytochrome P450 and NADPH oxidase enzymes ***(kim et al., 2009).***

Whereas ROS plays an import role as signaling and regulatory molecules in cell proliferation, differentiation and apoptosis, a prooxidant milieu can alter and denature nucleic acids, carbohydrates, lipids and proteins resulting in cell damage ***(Gutierrez et al.,2006).***

The balance between ROS production and antioxidant defenses defines the degree of OS in a given tissue. The imbalance between them has been considered as an interesting candidate at the intersections between injury and histopathology in kidney injury ***(Djamali, 2007).***

NADPH oxidase systems, which constitute the most important source of superoxide anion in the vessel wall, are present in endothelial cells, and fibroblasts. Phagocytic NADPH oxidase is a membrane – bound enzyme catalyzes the single election reduction of molecular oxygen to form $O_2^{\cdot -}$ ***(Ivanov et al., 2008)***.

The gene coding for the p22 subunit of NADPH oxidase is polymorphic, including a C242T transition that results in replacement of histidine by tyrosine at amino acid 72 of the putative heme binding site ***(Hodgkinson et al., 2003)***. The polymorphism appears to have effect on the activity of NADPH oxidase to produce superoxide anion. It is possible that functional polymorphism in this P22 phox (phox is the short term for phagocyte- oxidase) subunit may contribute to the imbalance that leads to OS in patients with AKI ***(Doi et al., 2005).***

In this study we have investigated the frequency of C242T variants in a population of patients with AKI , the extent of ROS load in those patients, the effect of the genetic variant on the severity of disease and the outcome of its course.

2. Subject and Methods

A population sample of Egyptian individual living in Shebin El Kom on the Nile Delta was studied. A total numbers of 75 patients with established AKI who

were hospitalized in Internal Medicine Department of Menoufiya University hospital. All the patients participating in the study had given informed written consent before blood sampling. Approval was obtained from the Research Ethics Committee of Menoufiya Faculty of Medicine.

AKI was defined as an abrupt (within 48hrs) reduction in kidney function defined by absolute increase in serum creatinine level ≥26.4 µmol/L (0.3 mg /dL) or a percentage increase in serum creatinine level ≥ 50% (1.5 fold from baseline) or a reduction in urine output (documented oliguria of <0.5ml /Kg/h for > 6hrs) *(Mehta et al., 2007).*

All the patients included in the study under went full history taking, general examination, MODS scoring, blood sample was taken for measuring serum urea, creatinine, plasma nitrotyrosine and for genotyping.

Follow up of the patients were done by monitoring the development of sepsis, the need for ICU admission, the requirement for dialysis, the length of hospital stay, the dialysis dependency after discharge and hospital death.

Blood sampling:

10 ml of venous blood were withdrawn from the cubital vein of every subject.5 ml was transferred slowly into vacunated EDTA tube for isolation of White Blood Cells for genotyping. 2 ml was transferred slowly into vacunated EDTA tube and centrifuged for 10 min at 4000 r.p.m. The plasma obtained for determination of plasma nitrotyrosine frozen at - 20 ºC till analysis.

3 ml was transferred slowly into a plain tube for determination of serum urea and creatinine left for 30 min for clotting and centrifuged for 10 min at 4000 r.p.m. The serum obtained for determination of serum urea and creatinine frozen at - 20 ºC till analysis.

Plasma nitrotyrosine was estimated by OxisResearch™ Enzyme Immunoassay *(Monezen and Augustus, 2001).*Serum urea is estimated by enzymatic end point method *(Taylor and Vadgama, 1992)*. Serum creatinine is estimated by enzymatic fixed rate colorimetric test *(Perrone et al., 1992)*.

Peripheral Blood Mononuclear Cells Isolation (PBMCs): For separation of PBMCs, Lymphoflot solution was used *(Bio test AG, Dreieich, Germany) (Sirchia et al., 1972)*.

DNA Extraction:

By Using QIAamp® DNA Blood Mini Kits (QIAGEN HILDEN, Germany).

NADPH Oxidase p22phox +242(C to T) Genotyping (Hodgkinson et al., 2006):

The amplification reaction for C242T was performed in 25 µl volumes (10 µl DNA template + 15 µl Master Mix {containing 2.5 µl of 10x PCR buffer, 0.25 µl MgCl 25mM, 1.0 µl dNTPs mM, 1.0 µl Forward primer (F5'-TGCTTGTGGGTAAACCAA G GCCGGTG-3'),1.0 µl reverse primer (R5'-AACA CTGAGGTAAGTGGGGGTGGCTCCTGT-3'), 0.30 µl Taq polymerase 5µ/ µl, 8.95 µl distilled water}. using an initial denaturation (5min at 95°C), denaturation (1min at 95°C), annealing (1min at 60°C), extension (1min at 72°C), number of cycles: 35 cycles and final extension (7min at 72°C). using Perkin Elmer thermal cycler 2400 (USA).

Identification of the C242T polymorphism:

The C242T polymorphism results in the creation of the *Rsa*I recognition site through a cystenine-to-thymine transition.

Recognition Site:

5'... GT▼AC... 3'
3'... CA▲TG ... 5'

The PCR product then was digested with RsaI at 37°C for 3 hrs (2µl 10x buffer, 1µl RsaI, 7µl distilled water and 10 µl PCR product). The RsaI digestive products were run by 2% agarose gel electrophoresis for 30 minutes and stained with ethidium bromide, and the bands were visualized under ultraviolet light. Digested PCR products yielded 348-bp bands in CC homozygotes, 188- and 160-bp bands in TT homozygotes, and all three bands in CT heterozygotes.

Statistical analysis:

Statistical analysis was made by IBM computer with the aid of Microsoft Excel and some statistical software programs like statistical program of social sciences version 11.5 (SPSS V. 11.5), Epicalc 2000, Epiinfo 2000.

Qualitative data were expressed in the form of number and percentage. Quantitative data were expressed as mean and standard deviation. The following statistical tests were used: Anova (F) test, Chi-square test, Pearson's correlation, Kaplan-Meier survival analysis and the log-rank test was used to test differences between groups. General linear model univariate procedure provides regression analysis and analysis of variance for one dependent variable by one or more factors and/or variables. Binary logistic regression analysis and Logistic regression coefficients.

3. Results

In this study, concerning the base line characteristics of the patients with acute AKI, the patients were classified according to their genetic variants into 3 groups: CC, CT and TT groups. From the 75 patients included in this study, 25 patients (33.3%) were of CC variant, 32 patients (42.7%) were CT and 18 patients (24%) were of TT variant. The mean age of the CC group was 56 3± 4.8, of the CT

group was 63± 4.1 while that of the TT group was 70.7± 5.7. The mean age of the CT group was significantly higher than that of CC group and the mean age of the TT group was significantly higher than those of the CC and CT groups.

Of the CC group, there were 19 males (76%) and 6 females (24%). Of the CT group, these were 19 males (59.4%) and 13 females (40.6%) while the TT group included 6 males (33.3%) and 12 females (66.7%) the number of females were significantly higher in the TT group more than the other two groups. The causes of the AKI in this study varied, of the CC group 5 patients (20%) were ischemic, 8 patients (32%) were nephrotoxic, 4 patients were obstructive and 8 patients (32%) were suffering from glomerulonephritis. Whereas of CT group, 10 patients were ischemic (31.3%), 4 patients (12.5%) were nephrotoxic, 13 patients were obstructive (40.6%) and 5 patients (15.6%) were suffering from glomerulonephritis. Of the TT group, 6 patients (33.3%) were ischemic, 4 patients (22.2%) were nephrotoxic, 7 patients were obstructive (38.9%) and 1 patient (5.6%) was suffering from glomerulonephritis. There was no significant statistical deference concerning the distribution of the etiology of the AKI between the three groups.

Regarding the biochemical markers, the serum urea and creatinine levels were higher in CT and TT groups (T allele carriers) compared to CC group.

Plasma nitrotyrosine levels is showed its highest level in the TT group compared to the other two groups ($p<0.001$) concerning the course of the disease, sepsis developed in 3 patients of the CC groups (12%), 8 patients of the CT group (25%) and 5 patients of the TT group (27.8%) with no statistical difference between the 3 groups concerning the development of sepsis.

According to MODS (multiple organ Dysfunction score), 19 patients of the CC group were <2 (76%) while 6 patients were ≥2(24%). Of the CT group, 16 patients were <2 (50%) and 16 patient were ≥2(50%). While the TT group included 5 patients <2 (27.8%) and 13 patients (72.2%) ≥ 2. The TT group was significant higher than the other two groups concerning the numbers of patients who had MODS above 2.

The TT group had the longest hospital stay ($P<0.0001$) while there was no significant statistical difference between the 3 groups regarding the requirement for dialysis and the dialysis dependency after discharge from the hospital.

The 3 groups didn't show significant statistical difference regarding the ICU admission ($p>0.05$) while the number of deaths was significantly higher in TT group compared to the other two groups ($p<0.005$).

There was positive correlation between the plasma nitrotyrosine level and the age of the patient, the length of hospital stay and serum level of urea and creatinine while there was negative correlation between the plasma nitrotyrosine level and the glomerular filtration rate (GER).

Kalpan – Meier analysis demonstrated that compared to CC genotype the – T allele group had a higher cumulative probability of remaining hospitalized.

By the general linear model for the association between the length of hospital stay and plasma nitrotyrosine level after adjustment for age, sex, MODS and sepsis, results showed that age ($P=0.004$) and length of hospital stay ($P=0.0001$) remained independent variables that are associated with the variation of plasma nitrotyrosine level. The binary logistic regression analysis showed that MODS was the only independent factor which affects the relationship between plasma nitrotyrosine level and dialysis dependency.

4. Discussion

Acute kidney (AKI) is a complex disorder comprising several etiological factors and occurring in multiple settings. The disorder has a variety of manifestations that range from minimal elevation in serum creatinine to anuric renal failure *(Molitoris et al., 2007).*

The most recent proposal for a uniform definition of AKI includes criteria for three categories of injury. Risk of acute renal failure (R), Injury to kidney (I) and failure of kidney function(F) with increasing severity and two classes of kidney outcomes [loss of kidney function (L) and end-stage Kidney disease (E)] *(Li et al.,2009).*

Patients who develop acute kidney injury experience a high mortality rate that is not entirely explained by sepsis, advanced age, or underlying combined conditions *(Himmelfarb et al., 2004),* Metabolic derangements related to hypercatabolism, dysregulated inflammation and multiple organ system failure are highly prevalent with consequence of an increase oxidative stress *(Abdel- Raheem et al.,2010).*

Evidences over the last decades showed that reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide and their reaction products play crucial roles in the pathogenesis of AKI.

Oxidative stress (OS) may be measured by using one of 3 primary methods, measuring the reactive oxygen species (ROS) directly such as it H_20_2 or measuring the presence of antioxidants such superoxide dismutases, catalase and total antioxidant capacity or measuring the degree of the resulting damage to lipids (Lipid peroxidation as malondialdehyde) proteins (3-nitrotyrosine advanced glycation products) and DNA/RNA damage (double- strand DNA breaks) *(Paromov et al., 2008).*

The modification of tyrosine residues in protein to 3-nitrotyrosine by peroxynitrite has been detected in

biological systems that have been subjected to OS *(Bian et al., 2006).*

In AKI, there are several potential sources of ROS, including the mitochondrial respiratory chain, xanthine oxidase activation as a result of ischemia/perfusion and endothelial and neutrophil – associated respiratory burst through activation of NADPH oxidase *(Perianayagam et al., 2007).*

The NADPH oxidase is a multicomponent complex that catalyze the production of superoxide anion ($O_2^{\cdot-}$). It has a heterodimer membrane component that consists of a large glycoprotein gp 91phox, a small protein p22 phox and cytosolic components p47 phox, p67 phox and p40 phox and Rac *(Doi et al., 2005)*

P22phox (phox is the short term for phagocyte-oxidase) also called α light chain has an essential role in $O_2^{\cdot-}$ production. The gene encoding the p22phox is polymorphic and shows several polymorphisms among them is C242T polymorphism, located in exon 4, which is considered the most interesting because the structural change it causes in enzyme structure, amino acid substation (histidine to tyrosine) at position 72 of the protein is believed to affect heme binding affinity *(Meng et al.,2005).* Mutation affecting this site not only disturbs the heme binding but also affect the stability of the entire subunit *(Biberstine- Kinkade et al., 2002).*

In the current study , 75 patients with established AKI of different cause and varying severity were classified according to the C242 T polymorphism of the p22phox subunit of NADPH oxidase into3 groups CC, CT and TT groups concerning the baseline characteristics of the patient, there was significant difference between the 3 groups concerning age and gender distribution while there was no statistical difference concerning the etiology of AKI, the presence of oliguria and the occurrence of sepsis. *Perianayagam et al.(2007)* found that the baseline characteristics did not differ significantly among the genotype groups.

The assessment of morbidity during hospital stay was done by multiple organ dysfunction scoring, which is defined as the presence of altered organ in acutely sick patients MODS was based on a literature review by *Marshall et al., (1995)* with a score system from zero to 4 based on six organs failure (cardiovascular, respiratory, hematologic, liver, renal and neurological system *(Khwannimit, 2007)*

In this study, there was a significant statistical difference between the 3 groups concerning MODS. The T- allele carriers (CT and TT groups) were significantly higher than the CC group while *Perianayagm et al. (2007)* did not find significant difference between the genotype groups.

The study of plasma nitrotyrosine level, showed significant statistical increase between the T- allele carries and CC group with its highest levels in TT group. *San Jose et al. (2008)* stated that the C242T polymorphism has functional effect on enzyme activity with evidences supporting that T- allele was associated with reduced basal and NADH stimulated superoxide production. *Wyche et al. (2004)* showed that adults with TT genotype had a significant reduction in the NADPH oxidase activity due to reduced expression of the p22phox protein, the level of p22phox were also assessed by Western blot analysis . Results showed that by the same protein levels of all genotypes, TT carriers had only 30% $O_2^{\cdot-}$ production compared to CC carriers. In contrast *Schirmer et al. (2008)* stated that the effects of C242 T polymorphism were opposite with increasing activity of NADPH oxidase. *Perianayagam et al. (2007)* found that the T- allele carriers were associated with higher plasma nitrotyrosine levels and as attempt to explain this contradictory finding, they assumed that nitrotyrosine is a circulating marker of both superoxide anion and nirtric oxide generation so there might be a linkage disequilibrium between this NADPH oxidase p22phox polymorphism and the gene that encodes for one of the three nitric oxide synthase isoforms particular inducible nitric oxide synthase, an enzyme that is typically induced by proinflammatory stimuli that occurs in the context of AKI.

Concerning the course of the disease, the T- allele carriers were significantly higher than CC group regarding the length of hospital stay with the longest period for TT group and also regarding the hospital death. While there was no significantly statistical difference regarding the requirement for dialysis, dialysis dependency after discharge from the hospital and the need for ICU admission ($p>0.05$) *Perianayagam et al. (2007)* stated the T- allele carriers had a significant trend towards prolonged hospital stay, higher dialysis requirement and hospital death compared with those with the CC genotype.

Hodgkinson et al. (2003) tend to explain the unfavorable cause associated with the T242 allele by the fact that nature of the balance between pro-and antioxidant defense has not been fully elucidated and that there is a growing list of genes that are regulated through redox-sensitive pathways that are not always due to an increase in ROS or there is a possibility that the association is not related to T242 allele and that the entire NADPH oxidase haplotype is important and this raises the possibility that additional polymorphism may exist within the gene that contribute to the association. However, *Doi et al. (2005)* had shown that the T-allele carriers are protected against kidney failure in non- diabetic Japanese people.

Table (1): Baseline Characteristics of patients with acute renal failure (ARF) by NAPDH genotypes.

Parameters	Genotype distribution						Total		Test of significance	p-value
	CC		TT		CT					
	No.	%	No.	%	No.	%	No.	%		
Age in years ($\bar{X}\pm$ SD)	56.3± 4.8		70.7± 5.7		63.4± 4.1		----		** Anova (F) test = 48.2	< 0.001
Gender:										
Male	19	76.0	6	33.3	19	59.4	44	58.7	χ^2= 7.9	< 0.05
Female	6	24.0	12	66.7	13	40.6	13	41.3		
Cause of ARF:										
Ischemic	5	20.0	6	33.3	10	31.3	21	28.0		
Nephrotoxic	8	32.0	4	22.2	4	12.5	16	21.3	χ^2= 10.6	> 0.05
Obstructive	4	16.0	7	38.9	13	40.6	24	32.0		
Glomerulonephritis	8	32.0	1	5.6	5	15.6	14	18.7		
Oliguria:										
Present	5	20.0	8	44.4	13	40.6	26	34.7	χ^2= 3.6	> 0.05
Absent	20	80.0	10	55.6	19	59.4	49	65.3		
Sepsis:										
Present	3	12.0	5	27.8	8	25.0	16	21.3	χ^2= 2.0	> 0.05
Absent	22	88.0	13	72.2	24	75.0	59	78.7		
MOF score:										
< 2	19	76.0	5	27.8	16	50.0	40	53.3	χ^2= 10.0	< 0.01
≥ 2	6	24.0	13	72.2	16	50.0	35	46.7		
Total	25	100.0	18	100.0	32	100.0	75	100.0	----	----

** LSD post-hoc test was applied. All groups were significantly different alternatively.

Table (2): Outcome of patients with ARF by NAPHH genotypes.

Parameters	Genotype distribution						Total		Test of significance	p-value
	CC		TT		CT					
	No.	%	No.	%	No.	%	No.	%		
Length of hospital stay ($\bar{X}\pm$ SD)	11.8± 3.7		20.9± 6.0		15.8± 3.6		----		** Anova (F) test= 23.5	< 0.0001
Requirement for dialysis:										
Present	3	12.0	5	27.8	10	31.3	18	24.0	χ^2= 3.0	> 0.05
Absent	22	88.0	13	72.2	22	68.8	57	76.0		
Dialysis dependency:										
Present	2	8.0	5	27.8	8	25.0	15	20.0	χ^2= 3.4	> 0.05
Absent	23	92.0	13	72.2	24	75.0	60	80.0		
ICU admission:										
Present	7	28.0	11	61.1	14	43.7	32	42.7	χ^2= 4.7	> 0.05
Absent	18	72.0	7	38.9	18	56.3	43	57.3		
Death:										
Present	0	0.0	7	38.9	5	15.6	12	16.0	χ^2= 11.8	< 0.005
Absent	25	100.0	11	61.1	27	84.4	63	84.4		
Total	25	100.0	18	100.0	32	100.0	75	100.0	----	----

** LSD post-hoc test was applied. All groups were significantly different alternatively.

Table (3): Differences in the laboratory investigations among the different NAPDH genotypes.

Parameters	Genotype distribution			Anova (F) test	p-value	LSD*
	CC	TT	CT			
GFR ($\bar{X}\pm$ SD)	18.0± 6.5	8.6± 3.8	10.9± 5.9	16.9	< 0.0001	CC VS. TT CC VS. CT
Blood urea in mg/dl ($\bar{X}\pm$ SD)	94.6±29.1	132.2±70.1	139.9±62.5	4.9	< 0.01	CC VS. TT CC VS. CT
S. creatinine in mg/dl ($\bar{X}\pm$ SD)	4.1± 2.3	6.6± 2.9	6.1± 2.8	5.6	< 0.01	CC VS. TT CC VS. CT
Nitro tyrosine in nmol ($\bar{X}\pm$ SD)	21.7± 13.0	69.6±18.7	41.2±11.6	61.3	< 0.0001	All groups

* LSD= Least significant difference.

Table (4): Pearson's correlation between plasma nitrotyrosine level and each of age, length of hospital stay, and laboratory investigations.

Correlations	Plasma nitrotyrosine level in (nM)	
	Correlation coefficient (r)	p-value
Age in years	0.59	< 0.0001
Length of hospital stay in days	0.79	< 0.0001
Glomerular filtration rate	- 0.64	< 0.0001
Blood urea in mg/dl	0.50	< 0.0001
Serum creatinine in mg/dl	0.57	< 0.0001

Table (5): General linear model for the association of the length of hospital stay with plasma nitrotyrosine level (nmol).

Parameters	B	S.E***	t	p-value	95 % confidence interval	
					Lower bound	Upper bound
Age	0.74	0.25	3.02	0.004	0.25	1.23
Sex	5.30	3.14	1.69	0.10	-0.96	11.55
MOF score	4.86	3.71	1.31	0.20	-2.55	12.27
Sepsis	6.77	3.89	1.74	0.09	-0.99	14.53
Length of hospital stay	2.57	0.39	6.65	0.0001	1.80	3.34

* Dependent variable: plasma nitrotyrosine level (nmol).
** The model was fit after adjustment for age, sex, MOF score, and sepsis. *** S.E= standard error.

Table (6): Binary logistic regression testing the association of the plasma nitrotyrosine level with the dialysis dependency.

Parameters	B	S.E	Odds ratio (OR)	p-value
Age	0.045	0.059	1.05	0.44
Sex	-0.120	0.746	0.89	0.87
MOF score	-2.717	1.129	0.07	**0.02**
Sepsis	0.389	0.791	1.48	0.62
Length of hospital stay	0.017	0.096	1.02	0.86
Nitrotyrosine level	-0.038	0.027	0.96	0.16

* The model was adjusted for age, sex, MOF score, sepsis, and length of hospital stay.

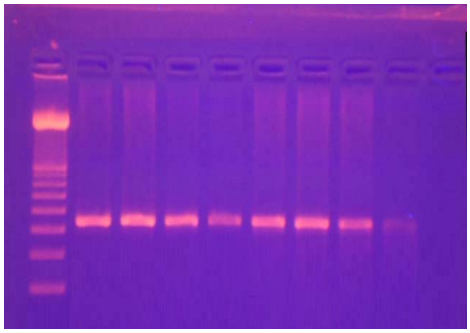

Figure (1): PCR products yielded 348-bp bands

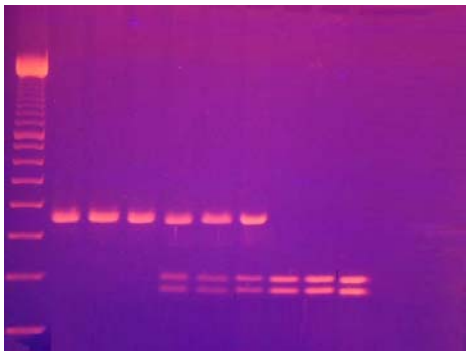

Figure (2):
Lane (1) DNA Ladder 100 bp.
Lane (2,3 and 4) CC homozygotes, 348 bp bands.
Lane (5,6 and 7) all three bands in CT heterozygotes
Lane (8,9 and 10) TT homozygotes 160 and 188-bp bands.

Corresponding author
Ashraf Dawood
Medical Biochemistry Departments, Menofia University
kingashrafdawood@yahoo.com

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