Total Antioxidant Status and Genetic Polymorphism of SOD2 and SOD3 genes Associated to Diabetic patients in Major β-Thalassemia

Moaed E. Al-Gazal ly
Department of Clinical biochemistry. College of Medicine. University of Babylon,.
E- mail : moaedalgazally @yahoo.com
P.O.Box. 473, IRAQ

Abstract

Oxidative stress can be induced in different ways, some of which are connected with known pathologies. Deficiencies in antioxidant or related enzymes are the factors which can result in oxidative stress. Genetic polymorphism is the most common cause of these deficiencies. There were statistically a highly significant elevated ($p<0.001$) in total antioxidant status(TAS) , Bilirubin(Bil.) and uric acid(UA) in β- thalassemia major with diabetic patients(No.51), when compared with β- thalassemia major(No.55) and control groups (No.50). The results showed a highly significant increase ($p<0.001$) of Mn-SOD (SOD2) and EC-SOD(SOD3) activities in β-thalassemia major with diabetic patients when compared with β-thalassemia major patients and control groups. The present study was performed assess whether there is a genetic association between a functional polymorphism (Ala-9Val) in the human Mn-SOD (SOD2) and EC-SOD(SOD3) genes in major β- thalassemia, major β-thalassemia with diabetic and control using PCR/RFLP method.

The results shows frequencies of the Ala allele (64.1% vs. 51.1%, $p < 0.001$) and the Ala/Ala genotype (29.39% vs. 23.51%, $p < 0.001$) of the SOD2 gene were significantly lower in major β-thalassemia with diabetic patients than in major β- thalassemia. In contrast, the Val allele (35.9% vs. 48.9%, $p < 0.001$) of the SOD2 gene were significantly increased in major β- thalassemia with diabetic patients than in major β- thalassemia and the Val/Val genotype (13.76% vs. 13.76%, $p < 0.01$) were no significantly between groups. Significance difference in the frequencies appears in Arg213GlySOD3 ,Arg/Arg, Gly/Gly genotype between major β- thalassemia with diabetic and major β- thalassemia. The present study provides some evidence that genetic polymorphism in the SOD2 and SOD3 genes associated with increased risk of major β- thalassemia with diabetic among major β- thalassemia with high levels of oxidative stress .This suggests that the Ala(-9)Val dimorphism in the SOD2 gene is associated with major β- thalassemia with diabetic.

Keywords: Total antioxidant,Major β-thalassemia,diabetic,genetic polymorphismSOD2and SOD3 genes

الخلاصة :

جهد الاكسدة من الممكن أن يتسبب بطرق مختلفة، ويرتبط مع بعض الأمراض المعروفة حيث ان النقصان في المواد المضادة للأكسدة أو الأنزيمات المضادة للأكسدة هي التي تشير إلى الإجهاد التأكسدي. وتعدد الأشكال الجينية هو السبب الأكثر شيوعا لهذا النقصان.إحصائية النتائج اضطرت ارتفاع عالي المعنوية (P <0.001) عند قياس كل من مضادات الاكسدة الكلية (TAS)، البيليروبين (.Bil) وحامض اليوريا (UA) في مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري (العدد52) عند مقارنتهم مع مرضى فقر الدم البحري الكبير نوع بيتا (العدد55) ومجموعة السيطرة (العدد50).أظهرت النتائج حدوث زيادة معنوية (P<0.001)لفعالية كل من انزيم SOD2 وانزيم SOD3 في مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري،عند مقارنتهم مع مرضى فقر الدم البحري الكبير نوع بيتا ومجموعة السيطرة .

وقد أجرت الدراسة تقييم في ما إذا كان هناك ارتباط جيني بين تعدد الأشكال ل (Ala−9Val) الموجودة في الإنسان لجينات كل من انزيم (SOD2) Mn−SOD وانزيم (SOD3)EC−SOD في مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري ومجموعة السيطرة باستخدام طريقة PCR / RFLP .النتائج تظهر ترددات الأليل للحامض الاميني الانين(64.1٪ مقابل 51.1٪،P<0.001) واظهر الكشف الجيني الى الانين / الانين (29.39٪ مقابل 23.51٪ ، P <0.001) للجين SOD2 , حيث كانت منخفضة بشكل ملحوظ في مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري عند مقارنتهم مع مرضى فقر الدم البحري الكبير نوع بيتا ومجموعة السيطرة ،في المقابل، ترددات الأليل للحامض الاميني الفالين (35.9٪ مقابل 48.9٪،P <0.001) للجين SOD2 ازدادت بشكل كبير في مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري، اما الكشف الجيني الى فالين فالين(13.76 ٪ مقابل 13.76٪،P<0.01) لم يظهر اي تغير معنوي بين المجموعات .اختلافات معنوية ظهرت في الترددات

Arg213GlySOD3، الارجنتين /الأرجنتين، كلايسين /كلايسين بين مرضى فقر الدم البحري الكبير نوع بيتا ولديهم مرض السكري و مرضى فقر الدم البحري الكبير نوع بيتا .

هذه الدراسة اثبتت بعض الأدلة على أن تعدد الأشكال الجينية في جينات انزيم كل منSOD2 وانزيم SOD3 تكون مرتبطة مع زيادة خطر الاصابة في مرض السكري لمرضى فقر الدم البحري الكبير نوع بيتا مع مستويات عالية من الإجهاد التأكسدي لهذا النوع من المرضى .هذا يشير إلى أن الأليل للحامض الاميني الانين في جين SOD2 يرتبط بالسكري لمرضى فقر الدم البحري الكبير نوع بيتا.

الكلمات المفتاحية: إجمالي المضادة للأكسدة، والرائد β–الثلاسيميا، السكري، الجينات الوراثية polymorphismSOD2and SOD3

Introduction

Thalassemia will remain to be one of the major health problem for at least the next few decades, particularly in the developing countries (Kuldeep *et al.*,2011). β - Thalassemia major is an autosomal disorder caused by mutations in the HBB gene in chromosome 11 (β) which leads to underlie deficiency in β- globin subunits of hemoglobin (Eliezer and Patricia,2011). The reactive oxygen species (ROS), generated in normal cellular respiration and during metabolism of xenobiotics, cause damage to membranes, mitochondria, and macromolecules including DNA, and thus are potential causes for carcinogenesis(Valko *et al*,2004;Shi *et al*,2004;De Bont,2004 and Klaunig 2004).A large number of antioxidant systems are involved in the scavenging of ROS, including the superoxide dismutase (SOD) family of proteins(Beatti ,2002). These enzymes catalyze the dismutation of O_2-, the superoxide anion, to form hydrogen peroxide (H_2O_2), which is further converted to water by the action of two other antioxidant enzymes,catalase and glutathione peroxidase(BeattI,2002).

All mammalian tissues contain three forms of superoxide dismutase (Beyer *et al*,1991): Cu/ZnSOD (SOD1), Mn-SOD (SOD2) and extracellular superoxide dismutase (ECSOD or SOD3). Cu/ZnSOD is localized in cytosol, Mn-SOD in mitochondria and ecSOD in extracellular space. Importantly, ECSOD activity is10-fold higher in the vessel wall than in other tissues, where Cu/ZnSOD and Mn-SOD constitute the majority of SOD activity (Beyer *et al*,1991 and Stra *et al*,1995). The predominant site of production of EC-SOD is the smooth muscle cell in healthy vessels (Stra *et al*,1995). Studies from other laboratories, and our own, indicate that endothelial cells do not produce EC-SOD (Stra *et al*,1995). Despite the fact that the EC-SOD is predominantly made by vascular smooth muscle rather than endothelial cells, it binds to the heparin sulfates on the endothelial cell surface and can be internalized by endothelial cells (Oth *et al*,1994)

Genetic polymorphism is frequently related to large number of pathologies. Enzymes involved in defense against ROS are not an exception. There has been much interest and research on single nucleotide substitutions (SNPs) in order to understand the maintenance of such polymorphisms in human populations(Cassia *et al*,2011). Several SNPs have been reported to result in changes in the levels or the activities of antioxidant enzymes, which can lead to reduction in protection against oxidative stress(Forsberg *et al* ,2001)less mutations were found in the gene coding human manganese containing superoxide dismutase (SOD2). Substitution of alanine-16 to valine (so called "Ala variant") is the most known mutation (Lightfoot *et al*,2006). This mutation associated with cancers of breast, prostate, ovaries and bladder, as well as non-Hodgkin lymphoma, mesothelioma and hepatic carcinoma (Lightfoot *et al*,2006). The Mn-SOD gene, which is composed of five exons and four introns, is localized to chromosome 6q25 (Church,1992 *et al* and Zelko *et al* ,2002). A T → C substitution, resulting in a Val → Ala change at the -9 position (Val-9Ala), which alters the secondary structure of the protein (Shimoda *et al*,1996), has been noted to

affect transport of Mn-SOD into the mitochondria(Rosenblum *et al*,1996 and Sutton *et al* ,2003).

Genetic studies have described that a single AA polymorphism exists in manganese- dependent superoxide dismutase (Mn-SOD). A change of alanine (Ala) to valine (Val) at the 16th amino acid (Ala16Val) of the signal sequence of the Mn-SOD (ninth amino acid from the first amino acid of the mature protein) has been suggested to change the secondary structure of the protein and therefore the mitochondrial targeting of the enzyme (Shimoda *et al* ,1996).The genes encoding these enzymes are located in different chromosomes and in all of them polymorphisms have been described. Copper-zinc SOD (Cu ZnSOD, SOD1) is encoded on 21q22.1 manganese SOD (Mn-SOD, SOD2) on 6q25.3 and extracellular SOD (ECSOD, SOD3) on 4p16.3–q21(McKusik ,1998) All these enzymes are expressed in human bronchial epithelium and alveolar macrophages(Lakari *et al*,1998).Another polymorphism (Ile58Thr) in exon 3 affects the stability of Mn-SOD and reduces protein amount and enzyme activity (Ho Y-S,1988 and Borgstahl *et al*,1996). Cells that over-expressed the Ile58 allele had higher Mn-SOD activity than did cells that overexpressed the Thr58 allele (Zhang *et al*.,1999).It is biologically plausible that the Val-9Ala and Ile58Thr polymorphisms play an important role in ROS detoxification, thus affecting the risk for developing cancer, particularly among individuals with a higher level of oxidative stress or who are deprived of other antioxidative protection, such as through a low level of antioxidant intake.

The aim of study focus on the study the oxidative stress in patients with major β- Thalassemia, and major β- Thalassemia with diabetic and study the genetic polymorphism in Ala(-9)Val and Ile58Thr polymorphisms of the SOD2 gene and Arg 213Gly dimorphism of the SOD3

Materials and methods

Samples

This study included 106 patients suffering from β- thalassemia major (58 male, and 48 female) aged between 3-18 years with mean± SD were 11.36 ± 1.53 years included in this study, who were consecutively referred to the center of inherited blood diseases in General Al-Habuby Hospital in Nasseryia city in Thiqar province at period between 1st of December 2011 up to 30th of June 2012,were investigated. The diagnosis of β- thalassemia major based on peripheral blood film and hemoglobin electrophoresis ,and transfusion dependent ,all patients were checked for none suffered from other disease. 51 patients suffering from β-thalassemia major with diabetic patients (28 male, and 23 female) were diagnosed on the basis of WHO criteria and not given any medication only treatment with insulin, any subject that have not these criteria are excluded from this study. Eight milliliters blood (divided two parts) was obtained after 8-12 hours, fasting from each subject in thalassemia patients sample was done just before the transfusion. Five milliliters used in the analysis of total antioxidant, albumin, bilirubin ,uric acid and superoxide dismutase, the blood samples were centrifuged at 3000×g for minutes and sera were kept in -50 ^{0}C until analysis. Three milliliters from blood used in the isolation of genomic DNA.

Estimation of total antioxidant status:

The total antioxidant status ,was determined according to the following formula (Bonnefont *et al.,*2000): (0.63×albumin mmol/l) + (1.02×UA mmol/l) +(1.5×bilirubin mmol/l).

Estimation of Albumin(Alb.): Albumin was measured spectrophotometrically according to Cooper and Morgan (Cooper and Morgan,1984).

Estimation of bilirubin(Bil.): Bilirubin was measured by sulfanilic acid method by BIOLABO kit(France) (Henry ,1965).

Estimation of uric acid (UA): Uric acid was measured by urease method with BIOLABO kit(France)(Fossait *et al*.,1980)

Estimation of plasma Mn-SOD(SOD2) activity: The Mn-SOD plasma level determination in the case and control samples were realized using the sandwich enzyme linked immunosorbent assay (ELISA) methodology as described by Taniguchi *et al*(Taniguchi *et al.*,1992)

Estimation of blood EC-SOD(SOD3) activity: Serum EC-SOD enzyme activity was assayed using a two steps enzyme –linked immunosorbent assay with a monoclonal antibody as described by Adachi *et al* (Adachi *et al.,*2012)

Isolation of genomic DNA from whole blood: Genomic DNA was isolated from whole human blood samples Genomic DNA Mini kit(Vogelstein and Gillespie,1979)**.**

Step 1 : RBC Lysis: Three hundred microliter of whole blood(Collect fresh blood in EDTA) was taken in 1.5 ml micro centrifuge tube. Add 900 µl of cell lysis buffer and mix with inversion, and incubate the tube for 10 minutes at room temperature(ice).Centrifuge for 5 minutes at 3000×g and remove the supernatant completely. Add 100 µl of RBC lysis buffer to re-suspend the cell pellet .

Step 2: Cell Lysis: A volume of 200 µl from GB buffer add to the 1.5 ml microcentrifuge tube and mix by shaking vigorously. Incubate at 60ºC for at least 10 minutes to ensure the sample lysate is clear. During incubation, invert the tube every 3 minutes. At this time, pre-heat the required elution buffer (200 µl per sample) to 60ºC (for Step 5 DNA Elution).

Step 3:DNA Binding: A volume of 200 µl of absolute ethanol added to the clear lysate and immediately mixed by shaking vigorously for 10 seconds. Place a GD column in a 2 ml collection tube. Transfer all of the mixture (including any precipitate) to the GD column centrifuge at 15000 ×g for 5 minutes. Discard the 2 ml collection Tube containing the flow-through and place the GD. Column in a new 2 ml collection tube.

Step 4:Washing: A volume of 400 µl from W1 buffer to the GD column. Centrifuge at 15,000 x g for 30 seconds. Discard the flow-through and place the GD column back in the 2 ml collection tube. Add 600 µl of wash buffer (ethanol added) to the GD Column. Centrifuge at 15000 ×g for 30 seconds. Discard the flow-through and place the GD column back in the 2 ml collection tube. Centrifuge again for 3 minutes at 15,000 x g to dry the column matrix.

Step 5 :DNA Elution: Transfer the dried GD column to a clean 1.5 ml microcentrifuge tube, Add 100 µl of pre-heated elution buffer or TE to the center of the column matrix. Let stand for at least 3 minutes to ensure the elution buffer or TE is absorbed by the matrix. Centrifuge at 15000 ×g for 30 seconds to elute the purified DNA.

Genotyping of Mn-SOD: After DNA extraction from blood samples. The Mn-SOD genotyping were determined with polymerase chain reaction (PCR) (Bonnefont *et al.,*2000).

1-To amplify the polymorphic Ala(-9)Val region ,the primer sequences were SOD2-16 were F 5-CCAGCAGGCAGCTGGCACCG-3 and SOD2-16 5- TCCAGGGCGC CGTAGTCGTAGG-3.

2-To amplify the polymorphic Ile 58 Thr region of Mn-SOD gene, the primer sequences were SOD2-58 F 5-AAGCTCCTCCCATTATCTAATAGC-3 and SOD2-58R 5-TCAGTGCAGGCTGAAGAGAT-3.

3-Master mix consisting of 67mM Tris-HCL(pH 8.8),16.7 Mm ammonium chloride,1 mM magnesium chloride,0.1 % Tween-20,10% dimethyl sulfoxide,0.2 mM of each dNTP ,5pmol of each primer ,100ng of genomic DNA ,and 2.5 units of Taq polymerase.

Detection of Ala(-9)Val and Ile 58 Thr dimorphism(Bonnefont *et al.*,2000)

1-The PCR product was digested with *Bsh* TI for the Ala(-9)Val.

2-The PCR product was digested with *Eco*32I for the Ile 58 Thr.

17 µL of PCR product,2 µL of buffer O^{+}/Tango ^{TM} (*Bsh*TI) and 1 µL restriction endonuclease (1 unit/ µL) were mixed and incubated for 12 h at 37 ^{0}C .

Analysis of amplified product: Successful PCR amplification (digested DNA product) was performed and confirmed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining (Budowle *et al.,*1991)

Genotyping of EC-SOD(Bonnefont *et al.*,2000)

1-To amplify the polymorphic Arg 213 Gly EC-SOD region ,the primer sequences of SOD3-213 were F 5-GGCTGGCCTGCTGCGTGGTGG-3 and SOD3-213R 5-CCTTGCACTCGCTCTCGCGCG-3.

2-Master mix consisting of 10mM Tris-HCL(pH 8.8),50 mM KCL,1.5 mM magnesium chloride,0.1 % Tween-20,10% dimethyl sulfoxide,0.2 mM of each dNTP,5pmol of each primer ,100ng of genomic DNA ,and 2.5 units of Taq polymerase.

Statistical analysis: The data of the study was subjected to statistical analysis is expressed as mean ± SD. Statistical analyses were done on a computer by using the Student 't' test. Conditional logistic regression models applied to estimate odds ratios and 95% confidence interval (95% CI) were measure the strength of the relationship between the Mn-SOD or EC-SOD polymorphisms and β- thalassemia major and β-thalassemia major with diabetic patients.

Results

Part one : Table 1 show the total antioxidant status(TAS), Albumin(Alb.) bilirubin(Bil.) and uric acid (UA) were determined in control(C), β- thalassemia major(BTM) and β- thalassemia major with diabetic patients(BTM+DM).

The results showed a highly significant increase ($p<0.001$) in TAS , Bil. and UA in β- thalassemia major and β- thalassemia major with diabetic patients, when compared with control group .Also a highly significant increase ($p<0.001$) in TAS , Bilirubin. and UA in - thalassemia major with diabetic patients, when compared with β- thalassemia major. The result show a highly significant increase ($p<0.001$) of SOD2 and SOD3 activities in β- thalassemia major with diabetic patients when compared with β- thalassemia major patients and control groups. Also cTAS show a highly significant increase when compared with the other groups in patients. No significant difference is observed in Alb. concentration in patients groups when compared with control group. Also there is a highly significant increase ($p<0.001$) in TAS and Bli. levels in male patients when compared with females.

Table (1): Biochemical parameters of control(C), β- thalassemia major(BTM) and β- thalassemia major with diabetic (BTM+DM) patients in male(M) and female (F).

TAS (mmol/l)				
p<0.001	**BTM+DM=** 2.07±0.36	p<0.05	**BTM=** 1.60±0.43	C= 1.47±0.34
p<0.001	**M**=2.37±0.24	p<0.05	**M**=1.76±0.32	M=1.58±0.27
p<0.001	**F**=1.78±0.25	p<0.05	**F**=1.44±0.21	F=1.36±0.32
	Alb. (g /dl)			
-	**BTM+DM=** 4.68±0.32	-	**BTM=** 4.89±0.45	C= 5.07±0.53
-	**M**=4.73±0.41	-	**M**=4.80±0.51	M=5.16±0.34
-	**F**=4.63±0.37	-	**F**=4.79±0.34	F=4.99±0.42
Bli. (mg/dl)				
p<0.001	**BTM+DM=** 1.58±0.25	p<0.001	**BTM=** 1.23±0.11	C= 0.57±0.12
p<0.001	M=1.62±0.24	p<0.001	M=1.30±0.21	M=0.62±0.27
p<0.001	F=1.55±0.32	p<0.001	F=1.26±0.26	F=0.53±0.36
UA (mg/dl)				
p<0.001	**BTM+DM=** 5.89±0.34	p<0.001	**BTM=** 5.02±0.36	C= 4.04±0.54
p<0.001	**M**=6.13±0.26	p<0.001	**M**=5.47±0.41	M=4.26±0.43
p<0.001	**F**=5.65±0.29	p<0.001	**F**=4.58±0.24	F=3.82±0.39
SOD2 (ng/ml)				
p<0.001	**BTM+DM=** 109.24±0.41	p<0.001	**BTM=** 96.34±0.34	C=63.65±0.37
p<0.001	**M**=108.43±0.47	p<0.001	**M**=97.42±0.32	M=62.32±0.26
p<0.001	**F**=107.64±0.52	p<0.001	**F**=96.35±0.52	F=62.15±0.42
SOD3 (ng/ml)				
p<0.001	**BTM+DM=** 102.78±13.1	p<0.001	**BTM=** 97.94±12.21	C= 67.08±11.13
p<0.001	**M**=102.34±12.2	p<0.001	**M**=98.45±10.99	M=67.34±10.21
p<0.001	**F**=103.23±11.4	p<0.001	**F**=97.44±9.27	F=66.83±8.48

Part Two : Ala(-9)Val SOD2(MnSOD) allele and genotypes in patients with control and β- thalassemia major. Genotype data for Ala(-9)Val SOD2 were available from 55 patients with β- thalassemia major and 50 as a control. In the control and β-thalassemia major Ala allele and Ala/Val genotype were the most common in control and patients groups. The Ala allele (odd ratio 0.8913 ;95%CI) and Ala/ Ala genotype (odd ratio1.587 ;95%CI) as shown in table 2.

Table(2) : Ala(-9)Val SOD2 allele and genotype frequencies in control, and beta-thalassemia major .

Ala(-9)Val SOD2 Allele distribution			
OR (95%CI)	**Beta- thalassemia major(55)**	**Control(50)**	
0.8913	64.1 %(64)	65.3%(73)	Ala
0.724	35.9 % (39)	34.7%(34)	Val
Ala(-9)Val SOD2 genotype distribution			
OR (95%CI)	**Beta- thalassemia major(55)**	**Control(50)**	
1.587	29.39 %(15)	33.42 %(17)	Ala/ Ala
1.013	56.85 (31)	52.93 %(27)	Ala/ Val
0.918	13.76%(8)	13.65 %(6)	Val/ Val

The results of restriction analysis of Ala(-9)Val polymorphism in the SOD2 gene after digestion with *Bsh*Tl as shown in figure 1.

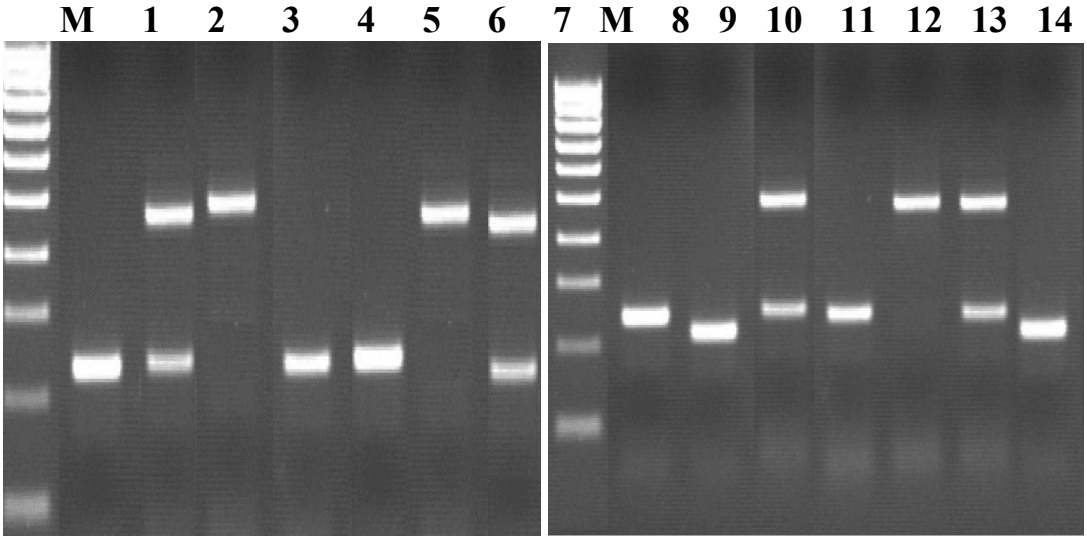

Figure 1:Genotyping of SOD2 gene,for analysis of Ala(-9)Val polymorphism, ; lanes 1,4,5,8,9,11,and 14, Ala/Ala genotype; lanes 2,7,10 and 13, Ala/Val genotype; lanes 3,6, and 12, Val/Val genotype. Lanes M: standard size reference marker(50 bp). (sample after digestion with *Bsh*Tl) electrophoresis in a 2% agarose gel.

Table 3 shows the frequencies of the Arg / Arg and Gly / Gly genotype were significantly lower in patients with beta- thalassemia major when compared with control groups($p<0.05$) ,while the frequencies of the Gly allele and heterozygous Arg/Gly genotype was highly significant higher in patients with beta- thalassemia major patients ($p<0.001$) when compared with control.

Table(3) : Arg 213 Gly SOD3 allele and genotype frequencies in control, and beta- thalassemia major .

Arg 213 Gly –SOD3 Allele distribution			
OR (95%CI)	**Beta- thalassemia major(55)**	**Control(50)**	
0.765	39.85 %(41)	63.7% (71)	Arg
0.624	60.15 % (58)	36.3% (35)	Gly
Arg 213 Gly –SOD3 genotype distribution			
OR (95%CI)	**Beta- thalassemia major(55)**	**Control(50)**	
1.587	23.21 %(15)	31.5 %(19)	Arg / Arg
1.013	65.89 (32)	55.6 %(27)	Arg / Gly
0.918	10.9%(7)	12.9 %(4)	Gly / Gly

Table 4 show the results of Ala(-9)Val SOD2 allele and genotypes in patients with control and beta- thalassemia major +diabetic. The result show a highly significantly difference in Val allele(48.9%) and Ala/Val genotype (62.73%) in patients with β- thalassemia major+diabetic when compared with β- thalassemia major(35.9% ,56.85% respectively) and control groups (34.7%,52.93% respectively).While a lower significant difference in Ala allele and Ala/ Ala genotype frequencies in patients with β- thalassemia major +diabetic(51.1%,23.51% respectively) when compared with β- thalassemia major (64.1% ,29.39% respectively) and control groups (65.3%,33.42% respectively).No significance difference is observed in Val/ Val genotypes between different groups.

Table(4) : Ala(-9)Val SOD2 allele and genotype frequencies in control, and beta-thalassemia major +diabetic

Ala(-9)Val –SOD2 Allele distribution			
OR (95%CI)	**Beta thalassemia major with diabetic (51)**	**Control(50)**	
0.765	51.1 %(52)	65.3%(73)	Ala
0.624	48.9 % (50)	34.7%(34)	Val
Ala(-9)Val –SOD2 genotype distribution			
OR (95%CI)	**Beta- thalassemia major with diabetic (51)**	**Control(50)**	
1.587	23.51 %(11)	33.42 %(17)	Ala/ Ala
1.013	62.73 %(32)	52.93 %(27)	Ala/ Val
0.918	13.76 %(8)	13.65 %(6)	Val/ Val

Table 5 showed the results of Arg 213 Gly-SOD3 allele and genotypes in patients with control and beta- thalassemia major with diabetic. The results showed a highly significantly difference in Gly allele(50.0%) and Arg /Gly genotype (58.8%) in patients with β- thalassemia major with diabetic and in β- thalassemia major(60.15% ,65.89% respectively) when compared with control group (36.3%,55.6% respectively).Also the result show a lower significant difference in Arg allele and Arg/Arg genotype frequencies in patients with β- thalassemia major with diabetic (50.0%,19.84% respectively) and in β- thalassemia major(39.85% ,23.21% respectively) when compared with control groups (63.7%,31.5% respectively).

Table(5) : Arg 213 Gly-SOD3 allele and genotype frequencies in control, and beta- thalassemia major+diabetic

Arg 213 Gly-SOD3 Allele distribution			
OR (95%CI)	**Beta- thalassemia major with diabetic (51)**	**Control(50)**	
0.765	50.00 %(51)	63.7% (71)	Arg
0.624	50.00 % (51)	36.3% (35)	Gly
Arg 213 Gly-SOD3 genotype distribution			
OR (95%CI)	**Beta- thalassemia major with diabetic (51)**	**Control(50)**	
1.587	19.84 %(10)	31.5 %(19)	Arg/Arg
1.013	58.8 (30)	55.6 %(27)	Arg /Gly
0.918	21.36%(11)	12.9 %(4)	Gly/Gly

The results of restriction analysis of Arg 213Gly polymorphism in the SOD3 gene after digestion with Eco521 as shown in figure 2.

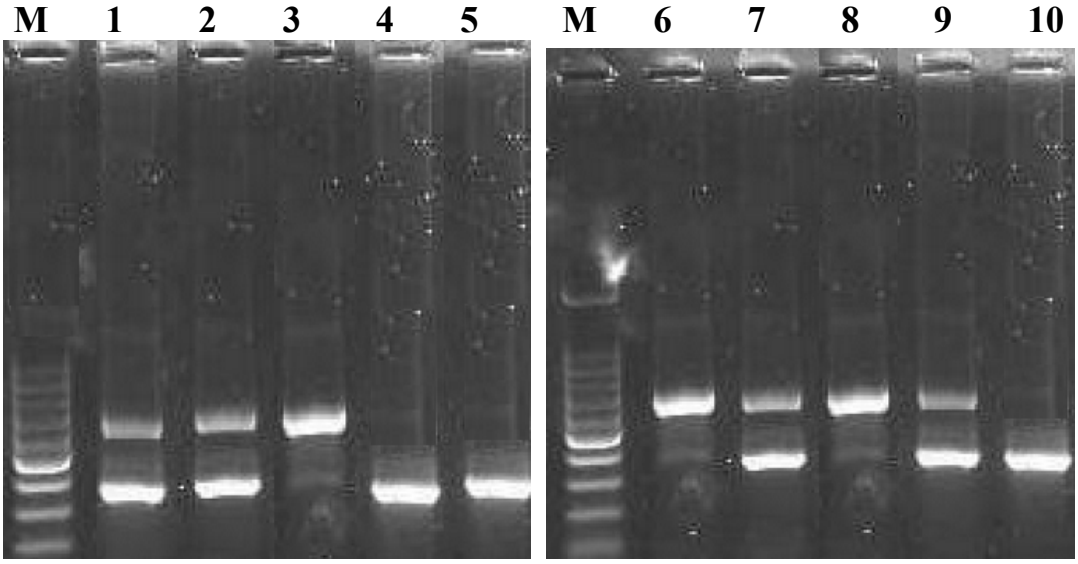

Figure 2:Genotyping of SOD3 gene, for analysis of Arg213Gly polymorphism, ; lanes 4,5,and 10, Arg/Arg genotype; lanes 1,2,7 and 9, Arg/Gly genotype; lanes 3,6, and 8, Gly / Gly genotype. Lanes M : standard size reference marker(50 bp) .(sample after digestion with *Eco*521) electrophoresis in a 2% agarose gel

Discussion

The antioxidant enzyme play an important role in scavenging ROS produced under oxidative stress(Ramchandra *et al*.,2012).Oxidative stress is defined as the interruption of balance between oxidants and reluctant within the body due to the excess production of peroxides and free radicals. This imbalance will cause damage to cellular components and tissues in the body leading to oxidative stress(Q.Shazia *et al.*,2012).In patients with β- thalassemia major where frequent blood transfusion are required due to severe anemia.

In the present study total antioxidant, superoxide dismutase in patients with β-thalassemia major and showed elevated when compared to control group. This increased may be a compensatory mechanism in response to increase oxidative stress in these patients. Also the results showed a highly significance increased in the total antioxidant and superoxide dismutase in patients with β- thalassemia major +diabatic,this indicated that oxidative stress induced by high level of glucose may

increase superoxide radical production with diabetic this findings was agreement with the other studies.(Ramchandra *et al*.,2012;Seghrouchni *et al*.,2002;Domingues et al.,1998). Some investigators suggested that assessment of total antioxidant capacity of plasma may be more useful than measuring antioxidants individually since their synergistic interaction could be determined [Kampa *et al*.,2002;Erel 2004;Fatemeh *et al*.,2011).Cakmak *et al* (Cakmak *et al*.,2010) reported that no significant differences in TAC between thalassemia and control groups, in spite of increase level of Oxidant Status (OS) and Oxidative Stress Index(OSI).

This study showed an increased concentration in bilirubin and uric acid in thalassemia patients, increased due to the hemolysis that occurs in patients ,also may be due to the decrease in the activity of cytochrome C oxidase. Also the study showed markedly greater bilirubin concentration was found in males when compared with females ,may be due to hormonal changes due to metabolism of bilirubin(Fatemeh *et al*.,2011).Previous studies have shown the effect of uric acid on total antioxidant capacity in patients with renal dysfunction .Therefore, in order to attenuate this effect, in this study used cTAS(Bonnefont *et al*.,2000)**,**from result suggest that elevation of TAS cannot be solely explained as a result of increased uric acid and bilirubin. Compensatory excess of TAS to oxidative stress could also be the reason for difference between our findings and previous studies.

In the genetic study the results showed the Ala allele of the SOD2 gene was more widespread than the Gly allele in control . This feature is common to all other Asian populations (Chinese and Japanese) than in most European populations (Cakmak *et al*.,2010;Hori *et al*.,2000;Hiroi *et al*.,1999). In contrast, the frequency of the Ala variant is significantly higher in Caucasian populations (Germans, Swedes, Lithuanians, Finns,Russian and Saamis) (Van ,1999;Grasbon *et al*.,1999;Dimitry *et al*.,2001).Populations of the world vary considerably in their predisposition to diseases and in allele frequencies at pharmacogenetically important loci, probably as a result of genetic drift, and also because of adaptation to local selective factors such as climate and available nutrients(Suarez-Kurtz ,2004).The Ala allele varies among ethnic groups (Zhao *et al*.,2005) and has been associated with increased risk of different diseases related to oxidative stress and abnormal free radical defense mechanisms (Choi et al.,2008).The Val form (change from alpha-helix to beta-sheet) is less efficiently transported into mitochondria than the Ala form (alpha-helical structure)of the enzyme (Shimoda ,1996).The Val variant of the Mn-SOD may be present in a lower concentration in mitochondria. If this is the case, then homozygous Val/Val should have lower resistance to oxidative stress than patients with other Mn-SOD variants(Suarez-Kurtz ,2004).

The Ala/Val variation in the Mn-SOD leader signal affects the processing efficiency of the enzyme. Studies of processing have suggested basal level of the Mn-SOD activity may be highest for Ala/Ala, followed by Ala/Val, and then Val/Val (Hiroi *et al*.,1999).This study showed in the β- thalassemia major patients, the Val Mn-SOD allele predisposed patients to the development to diabetes mellitus. MnSOD is the major antioxidant in the mitochondria. Because the mitochondria carry out oxidative phosphorylation, which is essential to the vitality of the cell, they are a major source of endogenous ROS generation. (Wang *et al*.,2001). MnSOD is transcribed in the nuclear matrix and transported into the mitochondria via a mitochondrial signal sequence (MSS), where it exerts its antioxidant function (McKusik ,1998).Inefficient targeting of Mn-SOD may leave mitochondria inadequately defended against superoxide radicals. This may lead to protein oxidation, mitochondrial DNA mutations and damage, common in the pathogenesis of

diabetic neuropathy [Q.Shazia *et al.*,2012; Seghrouchni *et al.*,2002; Dimitry *et al.*,2001)].

Conclusion

1- Increased endogenous antioxidants such as uric acid , bilirubin ,SOD2,SOD3 in the patients can also lead to increase level of TAS. Regular monitoring of antioxidant defense and administration of proper antioxidant may protect thalassemia patients from consequences of oxidative damages. These finding suggest that serum SOD2,SOD3 activities may be as marker of severity of β- thalassemia major to develop the β- thalassemia major with diabetic.

2- The present study provides some evidence that genetic polymorphism in SOD2 and SOD3 genes and associated with increased risk of diabetic among β- thalassemia major patients with high level of oxidative stress.

3- Ala(-9)Val substitution in the SOD2 gene was associated with β- thalassemia major with diabetic millets.

Acknowledgements

Thank to Dr.Naeem Salih Al-Zaidi (Assistant lecture in clinical biochemistry) for collection blood samples.

References

Adachi T, Ohta H, Yamada H, Futenma A,Kato K, and Hirano K .1992 : Quantitative analysis of extracellular-superoxide dismutase in serum and urine by ELISA with monoclonal antibody. Clin Chim Acta 2012:89–102.

Budowle B, Cakraborty T, RiuGsti M, Eenberg J, and AllAen C. 1991: Analysis of the VNTR locus D1S80 by PCR followed by high-resolution PAGE. Am J Hum Genet, 48:137-144.

Beattie D. 2002: Bioenergetics and oxidative metabolism. In: Devlin TM (ed.). Textbook of Biochemistry: With Clinical Correlations. New York: Wiley-Liss; 590–592.

Beyer W, Imlay J, and Fridovich I. 1991 :Superoxide dismutases. Prog Nucl Acid Res Mol Biol;40:221–253.

Bonnefont D, Lehmann E, and Jaudon MC,*et al*. 2000. :Blood oxidative stress and lipoprotein oxidizability in haemodialysis patients: effect of the use of a vitamin E-coated dialysis membrane. Nephrol Dial Transplant, 15:2020-8.

Borgstahl G, Parge H, Hickey M, Johnson M, Boissinot M, Hallewell R, Lepock J, Cabelli D, and Tainer J. 1996: Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. Biochemistry, 35:4287-4297.

Cakmak A, Soker M, Koc A, and Aksoy N. 2010:Prolidase activity and oxidative status in patients with thalassemia major. J Clin Lab Analysis; 24:6–11.

Cássiade O., Ana L., Dulce M., Silviene F., Ana H. and Mariade N . 2011: Superoxide dismutase, catalase, glutathione peroxidase and gluthatione S-transferases M1 and T1 gene polymorphisms in three Brazilian population groups.Genetics and Molecular Biology; 34: 1, 11-18 .

Choi J, Neuhouser M, Barnett M, Hong C, Kristal A, Thornquist M, King I, Goodman G and Ambrosone C ,2008. :Iron intake, oxidative stress-related genes (MnSOD and MPO) and prostate cancer risk in CARET cohort. Carcinogenesis 29:964-970.

Church S, Grant J, Meese E, and Trent J,1992: Sublocalization of the gene encoding manganese superoxide dismutase (MnSOD/SOD2) to 6q25 by fluorescence in situ hybridization and somatic cell hybrid mapping. Genomics, 14:823-825.

Cooper E. ,and Morgan D1.984: Specific protein laboratory improvement program .Am Assco Clin Chem.;1:1-11.

De Bont R, and van Larebeke N. 2004:Endogenous DNA damage in humans: A review of quantitative data. Mutagenesis; 19: 169–185.

Dimitry A., Kirill V., Elena V. and Valery V. 2001: Polymorphisms in the Mn-SOD and EC-SOD genes and their relationship to diabetic neuropathy in type 1 diabetes mellitus.BMC Medical Genetics , 2:4.

Dominguez C, Rniz E, Gossinye M, and Carrascosa A. 1998: Oxidative stress at onset and in early stage of type 1 diabetes in children and adolescents. Diabetes Care; 21:1736-1742.

Eliezer A, and Patricia G. How I treat thalassemia. 2011: Blood;118:3479-3488.

Erel O. 2004: A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem; 37; 277–85.

Fatemeh B. Sedigheh S. Mahtab B. ,Lili K., Maryam M. , Mohammad - Taghi H., and Nima R. 2011: Total Antioxidant Status in Patients with Major β-Thalassemia,Iranian Journal of Pediatrics,, Volume 21: 2, 159-165.

Forsberg L, Faire U and Morgenstern R .2001: Oxidative stress, human genetic variation and disease. Arch Biochem Biophys 389:84-93.

Fossait P., Prencipe L.,and Berti G. 1980:Use of 3,5-dichloro-2-hydroxybenzene sulfonic acid /4 Amino phenazone chromogenic system in direct enzymatic assays of uric acid in serum and urine.Clin.Chem;26,227-231.

Grasbon-Frodl E, Kosel S, Riess O , Muller U, Mehraein P, and Graeber MB. 1999: Analysis of mitochondrial targeting sequence and coding region polymorphisms of the manganese superoxide dismutase gene in German Parkinson disease patients. Biochem Biophys Res Commun, 255:749-752.

Hori H, Ohmori ,O Snhkai , T oKjima H, Oanko , CSuzuki , and TNakamura J. 2000: Manganese superoxide dismutase gene polymorphism and schizophrenia. Relation to tardive dyskinesia. Neuropsychopharmacol, 23:170-177.

Henry R. 1965: Principles and technics. Harper and Row ,592.

Hiroi S., aHrada H, isNhi , HSatoh M, aNgai , and RKimura AP. 1999 : Polymorphisms in the SOD2 and HLA-DRB1 genes are associated with nonfamilial idiopathic dilated cardiomyopathy in Japanese. Biochem Biophys Res Commun, 261:332-339.

Ho Y-S, and Crapo JD.,988: Isolation and characterization of complementary DNAs encoding human manganese-containing superoxide dismutase. FEBS Lett, 229:256-260.

Kampa M, Nistikaki A, and Tsaousis V, *et al.* 2002: A new automated method for the determination of the Total Antioxidant Capacity (TAC) of human plasma, based on the crocin bleaching assay. BMC Clin Pathol; 2(1):3.6.

Klaunig J., and Kamendulis L. 2004: The role of oxidative stress in carcinogenesis . Annu Rev Pharmacol Toxicol; 44: 239–267.

Kuldeep K., Amit M., and Archana T. 2011: Production of reactive oxygen species, its effects, drugs and plant extract used as an antioxidant, chelator on thalassemic patients: A Review. International J of Pharma Sciences and Research; 2(9):2278-2285.

Lakari E., Paakko P, and Kinnula VL. 1998: Manganese superoxide dismutase, but not CuZn superoxide dismutase, is highly expressed in the granulomas of

pulmonary sarcoidosis and extrinsic allergic alveolitis. Am J Respir Crit Care Med;158:589–96.

Lightfoot, T., Skibola, C., Smith, A., Forrest, M., Adamson, P., Morgan, G., Bracci, P., Roman, E., Smith, M. and Holly, E. 2006: Polymorphisms in the oxidative stress genes superoxide dismutase, glutathione peroxidase and catalase and risk of non-Hodgkin's lymphoma, Haematologica, Vol. 91, No. 9, pp. 1222-1227.

McKusik V. 1998.: Mendelian inheritance in man. 12th ed. Baltimore, MD: John Hopkins University Press,

Ohta H., Adachi T., and Hirano K. 1994: Internalization of human extracellular-superoxide dismutase by bovine aortic endothelial cells. Free RadBiol Med;16:501–507.

Q. Shazia, Z. H.Mohammad, Taibur R., and Hossain U., 2012:Correlation of Oxidative Stress with Serum Trace Element Levels and Antioxidant Enzyme Status in Beta Thalassemia Major Patients: A Review of the Literature;Anemia Volume, Article ID 270923, 1-7 .

Ramchandra K ., Ashok V .,and Sangita M . 2012:Lipid profile, serum malondialdehyde, superoxide dismutase in chronic kidney diseases and Type 2 diabetes mellitus; Biomedical Research;23:207-210.

Rosenblum J., Gilula N., and Lerner R.1996: On signal sequence polymorphisms and diseases of distribution. Proc Natl Acad Sci USA, 93:4471-4473.

Seghrouchni J., Drani J., Bannier F., Riviere J., and Calmard P. *et al*. 2002:Oxidative stress parameters in type 1, type 2 an insulin treated diabetes mellitus, insulin treatment efficiency. Clin Chim Acta; 321: 89-96.

Shi H., Hudson L., and Liu K. 2004:Oxidative stress and apoptosis in metal ion-induced carcinogenesis. Free Radic Biol Med; 37: 582–593.

Shimoda S., Matsumine H., Kobayashi T., Nakagawa Y., Shimizu Y., and Mizuno Y. 1996: Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. A predictive evidence for conformational change to influence mitochondrial transport and a study of allelic association in Parkinson's disease. Biochem Biophys Res Commun, 226:561-565.

Shimoda-Matsubayashi S, Matsumine H., Kobayashi T, and Nakagawa-Hattori Y. 1996: Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. Biochem Biophys Res Commun 226:561–565. doi:10.1006/ bbrc..1394.

Shimoda-Matsubayashi Sa,tsumMine H, obKayashi Ta,k agawNa-Hattori Y, Shimizu Y, Mizino Y. 1996: Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. Biochem Biophys Res Commun, 226:561-565.

Stralin P., Karlsson K., Johansson B., and Markland SL. 1995: The interstitiumof the human arterial wall contains very large amounts of extracellu-lar superoxide dismutase. Arterioscler Thromb Vasc Biol;15:2032–2036.

Suarez-Kurtz G .2004: Pharmacogenomics in admixed populations:The Brazilian pharmacogenetics/pharmacogenomics network-REFARGEN. Pharmacogenomics J 4:347-348.

Sutton A., Khoury H., Prip-Buus C., Cepanec C., Pessayre D., and Degoul F.: The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. Pharmacogenetics, 13:145-157.

Taniguchi N., Ishikawa M., Kawaguchi T., Fujii J., Suzuki K., and Nakata T. 2003:Expression of Mn-superoxide dismutase in carcinogenesis. Tohoku J Exp Med 1992; 168: 105–111.

Valko M., Izakovic M., Mazur M., Rhodes C., and Telser J. 2004: Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem; 266: 37–56.

Van Landeghem G., Tabatabaie P., Kucinskas V., Saha N., and Beckman G. 1999: Ethnic variation in the mitochondrial targeting sequence polymorphism of MnSOD. Hum Hered, 49:190-193.

Vogelstein B., and Gillespie D. 1979, Proc. Natl. Acad. Sci. USA 76.

Wang L., Miller D., and Sai Y *et al*. Manganese superoxide dismutase alanine-to-valine polymorphism at codon 16 and lung cancer risk. J Natl Cancer Inst 2001; 93: 1818–1821.

Zelko I., Mariani T., and Folz R. 2002: Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med, 33:337-349.

Zhang H., Yan T., Oberley T., and Oberley L . 1999: Comparison of effects of two polymorphic variants of manganese superoxide dismutase on human breast MCF-7 cancer cell phenotype. Cancer Res., 59:6276-6283.

Zhao H., Liang D., Grossman H., and Wu X .2005: Glutathione peroxidase 1 gene polymorphism and risk of recurrence in patients with superficial bladder cancer. Urology 66:769-774.