Renal Disease in β-Thalassemia. Is there a Relation to ApoE Gene Polymorphism? A Study in β-Thalassemia Egyptian Patients

MAGGIE S. EL NAHID, M.D.*; YASMINE M. AMROUSY, M.D.**; MOHAMMED I. MOSTAFA, M.D.*** and MAHA F. YACOUB, M.D.*

The Department of Internal Medicine*, Faculty of Medicine, Cairo University, Department of Clinical & Chemical Pathology**, Faculty of Medicine, Helwan University and Department of Clinical Pathology***, National Research Centre

Abstract

Background: β-thalassemia is acommon haemolytic anaemia in Egypt. Renal complicationsare an underestimated problem of β-thalassemia. Renal injury has been attributed to the anaemia, the haemolysis, iron overload, or iron chelators. ApoE gene polymorphism has been studied in many settings in β-thalassemia.

Aim of Study: In our study, we aimed to examine the possible relation of ApoE gene polymorphism to renal disease in β-thalassemia patientsand whether the APO E4 allele can be a potential genetic risk factor for the development of proteinuria in that population.

Patients and Methods: Forty patients with β-thalassemia were recruited from the Internal Medicine outpatient clinic at the Kasr Al-Ainy Hospital, Cairo University and compared to 45 healthy control subjects, age and sex-matched. β-thalassemia patients were further subdivided in two group. Group I with ACR less than 30 μg/mg (20 patients) and group II with ACR more than or equal 30 μg/mg (20 patients). ApoE Polymorphisms genotyping was performed by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP).

Results: Our results showed that there was a statistically significant difference between cases and control regarding serum creatinine, eGFR and ACR. The distribution of ApoEin β-thalassemia cases is E2/E3 10%, E3/E3 87.5% and E3/E4 2.5% and in control is E2/E3 4.4%, E3/E3 88.9% and E3/E4 6.7% with a statistically significant difference (*p*-value <0.001).

Conclusion: Our study demonstrated a significant difference in eGFR, Albumin Creatinine Ratio and ApoE genotyping between β-thalassemia cases and control. Although the distribution of ApoEin β-thalassemia cases is statistically different from control, it was not correlated to eGFR or proteinuria.

Key Words: β*-thalassemia – ApoEgene polymorphism – Iron overload – Iron chelators – Renal disease in* β*-thalassemia.*

Correspondence to: Dr. Maggie S. El Nahid, The Department of Internal Medicine*, Faculty of Medicine, Cairo University

Introduction

β-**THALASSEMIA** disease is considered one of the most common inherited monogenic disorders worldwide. About 1,000/1.5 million births are diagnosed with thalassemia, yearly, in Egypt [1]. Haemolytic anaemia patients survive on frequent blood transfusions and consequently face the problem of iron overload, requiring iron chelation therapy early in the disease [2].

Renal complications are considered an underestimated problem of β-thalassemia. Renal tubular dysfunction, glomerular hyperfiltration and renal stones are all encounteredin β-thalassemia [3,4,5]. Other studies, however, show that the use of iron chelators is also a cause of the rise in serum creatinine [6].

Several genetic factors lie behind the propensity of renal involvement in β-thalassemia and can determine disease severity [7].

In our study, we aimed to examine the possible renal complications in a group of Egyptian β-thalassemia patients. We also aimed to explore the possible involvement of ApoE gene polymorphismin renal disease in β-thalassemia.

Patients and Methods

Forty patients with β-thalassemia were recruited from the Internal Medicine outpatient clinic at the Kasr Al-Ainy Hospital, Cairo University from May to October 2018, and compared to 45, age and sex-matched, healthy control subjects. Inclusion criteria were any patient with a confirmed diagnosis of β-thalassemia above the age of 18. Exclusion criteria included an age less than 18 years old, the presence

of diabetes, hypertension or any evidence of previous renal diseases (By history, clinical examination or lab investigation of urine and renal function tests). Participants were selected after the study protocol was approved by the Medical Research Committee in the Internal Medicine Department, Faculty of Medicine, Cairo University. Consent forms were obtained from the patients and participants, in accordance with Helsinki Declaration II [8].

Diagnosis of β-thalassemia was based on full medical history, laboratory investigations and haemoglobin electrophoresis studies. Assessment of renal affection in β-thalassemia patients was done through the combined assessment of GFR and albuminuria status [9]. The patient group was further sub-grouped according to the result of albumin creatinine ratio (ACR), with cut-off 30 μg/mg into 2 groups; the group I with ACR less than 30 μg/mg (20 patients) and group II with ACR more than or equal 30 μg/mg (20 patients) [9].

Biochemical analysis:

CBC was done using the automated cell counter (Cell Dyn-1700). Serum creatinine was analysed on Dimension EXL® b (Siemens Healthcare, Germany). Urinary Albumin creatinine Ratio (ACR) was determined by immunoturbidimetry method on Mindray BS-200 chemistry analyser (Mindray, China). Estimated glomerular filtration rate (eGFR) was calculated according to automatic eGFR calculation [10]. Serum ferritin by electrochemiluminescence immunoassay on Cobas e411 (Roche Diagnostics, North America).

Molecular analysis:

DNA extraction: 5ml of ethylene diamine tetra acetic acid EDTA-anticoagulated venous blood samples were collected from all participants, either used directly or stored at –20°C. Qiagen extraction kitwas used for DNA extraction according to their instructions. The qualityand quantity of DNA were determined using Nanodrop spectrophotometer (2000c) (Thermofisher ScientificTM, USA). APOE Polymorphisms genotyping was performed by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP). Primers F (5'-GCGGGCCCCGGCCTGGTACAC-3') and R (5'-GACGC GGGCACGGCTGTCCAAGGA-3') (Applied BiosystemsTM).The total volume of the amplified DNA was 25 μl, 5μl (50ng) of the isolated DNA, 1 μl (20 pmole) of both primer, and 12.5 μl Taq DNA Polymerase (Thermofisher ScientificTM, USA) and 5.5 μl of Nuclease-free water. PCR conditions: 5min at 94°C for Initial denaturation, then denaturation at 94°C for 1min for 35 cycles of, 1min at 60°C for annealing, and 2min at 72°C for elongation then 5min at 72°C. The amplified PCR product was of 238 bp, PCR products were visualized by electrophoresis on 2% agarose gel containing 5 μe ethidium bromide to confirm successful amplification. The amplified PCR product was digested with 10U of the restriction enzyme Hha1 (20) for 15min at 37°C. The digested PCR products were separated on 4% agarose gel at 120 V for 1 hr. and were analyzed using a gel documentation system (Bio-Rad). Ethidium bromide was used for visualization of DNA fragments. APO E2 is found as 91 and 81bp fragments. APO E3 allele as 91 and 48bp fragments, while 72 and 48 bp fragments were indicative of APO E4 allele (2 1) (Fig. 1).
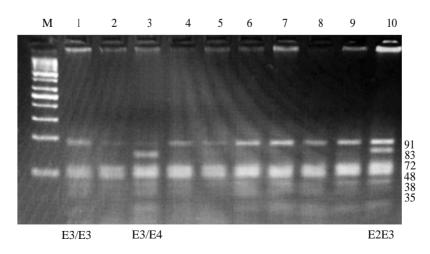

Fig. (1): PCR-RFLP gel electrophoresis of APO E genotyping. M; DNA ladder. Lane 1, 2 & 4-9: E3/E3 homozygous allele. Lane 3: The heterozygous allele E3/E4. Lane10: The heterozygous allele E2/E3.2.

Statistical analysis:

The collected data was revised, coded, and tabulated using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Kolmogorov-Smirnov test was done to test the normality of data distribution. Student *t*-Test was used to assess the statistical significance of the difference between two study group means. For the comparison of more than two groups' means, one-way analysis of variance (ANOVA) was used. Mann Whitney Test was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. The Kruskal-Wallis test is was used to assess the statistical significance of the difference between more than two study group nonparametric variables, For comparing categorical data, Chi-square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlation between various variables was done using Pearson moment correlation equation for linear relation of normally distributed variables and Spearman rank correlation equation for non-normal variables/non-linear monotonic relation. *p*-values less than 0.05 were considered statistically significant. Odds ratio and 95% confidence interval were calculated.

Results

This study was conducted on 40 β-thalassemia patients and 45 healthy control subjects matched in age and sex. The β-thalassemia patients included Thalassemia major 12 (30%), Thalassemia intermediate 21 (52.5%) and Thalassemia trait 7 (17.5%). Fourteen (35%) of the β-thalassemia patientsreceived iron chelation therapy (78.6% were on deferasirox (DFX) and 21.4% on deferipone (DFP).

Regarding lab parameters:

The mean ± SD value for haemoglobin, creatinine, eGFR and ferritin in β-thalassemia cases and control subjects are shown in Table (1).

- There was a statistically significant difference between β-thalassemia cases and control subjects, regarding Hb level, serum ferritin, serum creatinine, eGFR and Urinary Albumin creatinine Ratio (ACR), (*p*-value <0.001), Table (1).

- There was no significant correlation between eGFR or ACR to Hb, serum ferritin or dose and duration of iron chelation.

Table (1): Lab parameters of β-thalassemia cases and control subjects.

	β-thalassemia Cases n=40	Control n=45	*p*-value
Age (years)*	29 (±9)	28 (±5)	0.65
Sex n (%):			
Male	13 (32.5%)	23 (51.1%)	0.083
Female	27 (67.5%)	22 (48.9%)	
Hb (g/dl)*	8.4±1.3	13.8±1.1	<0.001
Ferritin (ng/ml)*	859.0 (55-12503)	58.5 (15.4-265.9)	<0.001
Creatinine (mg/dL)*	0.6±0.2	0.8±0.2	<0.001
ACR (μg/mg)* [a]	29.4 (6.8-850)	10.0 (6-27.9)	<0.001
eGFR (mL/min/1.73m^2)* [b]	169.8 (84.5-727.9)	119.6 (84.8-203.4)	<0.001

* Mean ± SD median (range).
[a] ACR : Albumincreatinine ratio in urine
[b] eGFR: Estimated glomerular filtration rate.

Regarding APO E:

The distribution of APO E in β-thalassemia cases and the control subjects are shown in Table (2).

I- Regarding the ApoE gene polymorphism studies, results showed that the distribution of ApoE-in β-thalassemia cases is E2/E3 10%, E3/E3 87.5% and E3/E4 2.5% and in control is E2/E3 4.4%, E3/E3 88.9% and E3/E4 6.7% with a statistically significant difference (*p*-value <0.001), Table (2).

Table (2): Distribution of APOE genotyping among β-thalassemia cases and control subjects.

APOE	β-thalassemia Cases n=40		Control n=45		*p*-value
	N	%	N	%	
E2/E3	4	10	2	4.4	<0.001
E3/E3	35	87.5	40	88.9	
E3/E4	1	2.5	3	6.7	

II- No statistically significant difference could be detected on comparing E2, E3, E4 allele frequency between group I, group II and control with a p-value >0.5. E4 allele was 2.5% in group II and 0% in group I, 3.3% in control subjects, Table (3).

Table (3): Distribution of APOE genotyping and Allele frequency in all studied groups with Odds ratio 95%CI.

APOE	Group I (N=20)		Group II (N=20)		Group III (N=45)		*p*-value	Odds ratio 95% CI		
	N	%	N	%	N	%		Group I VS Control	Group II VS Control	Group I VS Group II
E2	1	2.5	3	7.5	2	2.2		1.09 (0.096-12.3)	3.7 (0.57-24.5)	3.2 (0.3-32.6)
E3	39	97.5	36	90	85	94.4	0.437			
E4	0	0	1	2.5	3	3.3		1.05 (0.090-12.3)	0.78 (0.079-7.8)	
E2/E3	1	15	3	15	2	4.4	0.418		3.75 (0.57-24.5)	3.5 (0.33-37.6)
E3/E3	19	95	16	80	40	88.9				
E3/E4	0	0	1	5	3	6.7			0.83 (0.081-8.6)	

Discussion

Renal complications in β-thalassemia is an area of great interest. Studies identified renal disease in 2% of patients. Cunningham et al., demonstrated decreased creatinine clearance in almost 8%, and albuminuria in almost 60% of thalassemia patients [11,12].

Interestingly, β-thalassemia patients who received hematopoietic stem-cell transplantation, demonstrated better renal function, as tested by proteinuria, urine osmolality and urinary markers as NAG and β2M [13]. However, there are no large studiesto clarify themagnitude of kidney disease in β-thalassemia and the effect of the blood disease on the kidney is not fully elucidated [14].

In our study, we testedrenal involvement (eGFR and the presence of proteinuria as ACR) in 40 thalassemia β-thalassemia patients and compared them to age and sex matched control subjects. We also aimed to evaluate the relation of ApoE gene polymorphismto renal involvement in the same group of patients.

Results showed that the median and range for eGFR is 169.8 (84.5-727.9) in the β-thalassaemia patients and 119.6 (84.8-203.4) in the control group with a statistically significant difference (p<0.001). The median and range for ACR (μg/mg) in the β-thalassaemia patients is 29.4 (6.8-850) μg/mg and in the control, group is 10.0 (6-27.9) μg/mg, with a statistically significant difference (p<0.001). Regarding the ApoE gene polymorphism studies, results showed that the distribution of ApoEin β-thalassemia cases is E2/E3 10%, E3/E3 87.5% and E3/E4 2.5% and in control is E2/E3 4.4%, E3/E3 88.9% and E3/E4 6.7%with a statistically significant difference (p-value <0.001).

Regarding eGFR and Albumin/Creatine ratio:

Previous studies employed different techniques for measuring or estimating GFR in thalassemia patients. Some studies, measuring serum creatinine or creatinine clearance revealed glomerular hyperfiltration [15], while others, using inulin clearance-and cystatin C, described a decrease in GFR [16], [17]. Reduced estimated GFR, using MDRD, has also been demonstrated in patients treated with iron chelation [3].

Collectively, measurement of GFR can differ from one study to another, in view of the different parameters used in each study [18].

Regarding proteinuria, tubular or glomerular proteinuria can be a complicationof β-thalassaemia. Anaemia and hypoxia are accompanied by oxidative stress, which can lead to tubular dysfunction [19].

Hypoxia, alone, changes the metabolism of renal cells, affects tubular function and can alter peritubular capillaries [20]. Previous studies demonstratedhigher proteinuria and lower urine osmolarity in patients with severe versus moderate anaemia [21,22]. Hyperfiltration, caused by anaemia can lead to progressive renal dysfunction [23].

Renal abnormalities have also been related to the coexisting iron overload in β thalassaemia patients [14]. Clinical studies demonstrated relationships between serum ferritin and markers of tubular injury [23]. Surprisingly, many studies attributed renal dysfunction in β thalassemia to the use of iron chelation therapy, demonstrating their deleterious effect on tubular functionand increases in serum creatinine [24]. In most cases, however, the increase is transient [25,26]. In our study, results showed that there was no significant

correlation between serum creatinine, eGFR or ACR and serum ferritin, dose or duration of iron chelation therapy. This is in disagreement with [25-28].

Regarding ApoE:

Attempts should be made to counteract the unfavourable effects of haemolysis on the kidney [29]. Many genetic factors are considered modifiers of β-thalassemia. Examples include; genetic variants ofmetabolic processes involving iron and bilirubin, as well as cardiac and bone disease in β-thalassemia [30]. In a study by Salah et al., patients with TT genotype of the BCL1 1A gene polymorphism had significantly more severe β-thalassemia disease scores than other genotypes [31].

Apolipoprotein E (ApoE), a major constituent of plasma lipoproteins, is not only related to the atherosclerosis associated with renal diseases, but also to the development and progress of many renal diseases [32]. Apo E4 allele was associated with lower chances of Chronic Kidney Disease (CKD) [33]. Apo E2 allele was linked to the risk of End Stage Renal Disease [34-36]. Many studies relate APO E2 to renal disease [37-39], while others report Apo E4 to be preventive [40].

Our results showed that the distribution of ApoE in β-thalassemia cases is E2/E3 10%, E3/E3 87.5% and E3/E4 2.5% and in control is E2/E3 4.4%, E3/E3 88.9% and E3/E4 6.7% with a statistically significant difference (*p*-value <0.001). However, no statistically significant difference could be detected on comparing E2, E3, E4 allele frequency between group I, group II and control with a *p*-value >0.5. E4 allele was 2.5% in group II and 0% in group I, 3.3% in control subjects.

In β-thalassemia patients, iron overload is associated with the formation of oxygen free radicals. Apo Eacts as the scavenger of free radicals. Many studies showed that Apo E4 allele was significantly higher among β-thalassemia patients with left ventricular abnormalities than in controls [41-44]. Apo E2 allele is a risk factor to oxidative stress-induced cardiac injury. In oxidative stress, ApoE4 is the least efficient [45]. Constadina et al showed that the lower level of ApoE in β-thalassemia major patients, is associated with endothelial dysfunction [46].

Different genotype and allele frequency can be attributed togeographical, behavioural or environmental differences [47].

Conclusion:

Our study shows that renal involvement in β-thalassemia is evident, in the form of a significant difference in eGFR and Albumin Creatinine Ratio between β-thalassemia cases and control.

These results were not correlated to iron overload or iron chelation therapy. The distribution of ApoE in β-thalassemia cases is statistically different from control. However, not correlated to renal disease. To our knowledge there had been different genetic studies on β-thalassemia patients, but this is the first study to address the ApoE gene polymorphism.

Limitations:

The sample size was small to have a wider conclusion.

Declarations:

- No grants or funds.

- Consent was obtained from participants.

- Ethical approval was obtained from the Internal Medicine Ethical Committee, Kasr Al-Ainy, Cairo University.

- No conflict of interest.

- Consent for publication was obtained from authors.

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المرض الكلوى فى β-ثلاسيميا
هل هناك علاقة بتعدد الأشكال لجيـن ApoE؟
دراسة فى مرضى β-ثلاسيميـا المصريين

β-ثلاسيميا هو فقر دم انحلالى شائع فى مصر. المضاعفات الكلوية هى مشكلة لا يستهان بها من β-ثلاسيميا. تعزى الإصابة الكلوية إلى فقر الدم، وانحلال الدم، والحمل الزائد للحديد، أو خالب الحديد. وقد تمت دراسة تعدد الأشكال الجينى لـ ApoE فى العديد من الأماكن فى β-ثلاسيميا.

فى دراستنا، هدفنا فحص العلاقة المحتملة لتعدد الأشكال الجينى لـ ApoE مع أمراض الكلى فى مرضى β-ثلاسيميا وما إذا كا الـ E4 APO يمكن أن يكون عامل خطر وراثياً محتملاً لتطوير التسريبات البروتينية فى تلك المجموعة.

تم اختبار أربعين مريضاً مصاباً بـ β-ثلاسيميا من العيادة الخارجية للطب الباطنى بمستشفى القصر العينى بجامعة القاهرة ومقارنة بـ ٤٥ شخصاً من الأصحاء.

تم تقسيم مرضى β-ثلاسيميا إلى مجموعتين المجموعة الأولى مع ACR أقل من ٣٠ ميكروغرام / مجم (٢٠ مريضاً) والمجموعة الثانية مع ACR أكثر من أو يساوى ٣٠ ميكروغرام / مجم (٢٠ مريضا). تم إجراء التنميط الجينى لتعدد الأشكال لـ ApoE عن طريق تعدد الأشكال فى طول جزء تقييد تفاعل لبو ليميراز المتسلسل (PCR-RFLP).

أظهرت نتائجنا وجود فروق ذات دلالة إحصائية بين الحالات والأصحاء فيما يتعلق الكرياتينين و eGFR و ACR. توزيع ApoE فى حالات β-ثلاسيميا هو E2/E3 10%, E3/E3 87.5% و E3/E4 2.5% وفى الأصحاء هو E2/E4 4.4%, E3 88.9%, E3/E4 6.7% مع فرق ذو دلالة إحصائية (قيمة p<0.001).

أظهرت دراستنا اختلافاً كبيراً فى معدل الترشيح البروتينى، ونسبة الكرياتينين الزلالى، والتنميط الجينى لـ ApoE بين حالات β-ثلاسيميا الأصحاء.