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Research Article

Comparative evaluation of cystatin C and neutrophil gelatinase-associated lipocalin in patients with thalassemia major versus thalassemia intermedia

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Abstract

Kidney disorders are long-term complications in thalassemia patients, especially with the high life expectancy of these patients. Proper evaluation of kidney impairment in β-thalassemia patients can be difficult due to higher intake of iron chelators, resulting in renal impairment. Early biomarkers of renal disease are used for the diagnosis of tubular and glomerular abnormalities. The current study was conducted on 88 individuals, 25 healthy people and 63 β-thalassemia patients. Circulating levels of urea, creatinine, cystatin C and neutrophil gelatinase-associated lipocalin were measured in all groups. Compared to healthy control, patients with thalassemia major and intermedia showed a significant increase in both cystatin C and NGAL levels, with no effects on creatinine levels. Furthermore, urea levels were markedly higher in patients with thalassemia major compared to control. As early renal dysfunction markers, cystatin C and NGAL should be routinely evaluated in thalassemia patients major and intermedia.

Keywords

β-thalassemia, Cystatin C, Neutrophil gelatinase-associated lipocalin, Urea

Introduction

Thalassemia is a genetic autosomal recessive disease widespread in the world, caused by a defect in globin synthesis that results in a reduced (β^+) or absence (β^0) production of β globin chain in haemoglobin (Hb) synthesis. Depending on the globin chains of Hb, thalassemia is categorized into α and β thalassemia (Salomon-Andonie et al. 2013). β-thalassemia major is a severe autosomal recessive disease caused by the absence of β^0 due to homozygous or double heterozygous mutations of the β globin chain, accumulation of α globin chain in red blood cells, and chain ratio imbalance (Saxon 2004). In vital organs like the liver, endocrine glands, heart and kidney, progressive iron

accumulation is noticed due to continuous blood transfusions in thalassemia patients. β-thalassemia intermedia is an intermediate recessive disease characterized by inefficient erythropoiesis, iron overload and severe anaemia (Asadov et al. 2018).

Kidney disorders are long-term complications in thalassemia patients, especially with the high life expectancy of these patients. Proper evaluation of kidney impairment in thalassemia patients can be difficult due to higher intake of iron chelators, resulting in renal impairment. Early biomarkers of renal disease are used for the diagnosis of tubular and glomerular abnormalities. Nevertheless, conflicting results render it difficult to obtain definitive correlations and there are also no strong statistical diagnostic parameters for such biomarkers. For example, the ratio of albumin to creatinine may identify the initial signs of glomerulular disorder.

Cystatin C is a low-molecular-weight, non-glycosylated protein that is synthesized by all human cells and has the potential to inhibit lysosomal cysteine proteinases and papain (Bobek and Levine 1992). Because of being a potential biomarker for glomerular filtration rate (GFR), cystatin C is not reabsorbed into the serum or secreted by the renal tubules. Moreover, it is superior to serum creatinine as an early diagnostic marker for renal dysfunction because it is unaffected by gender, height, muscle mass and food (Mohammed Abdulrazzaq 2020).

Neutrophil gelatinase associated lipocalin is a 25 kDa protein which has been released by damaged nephron epithelia. It is considered a potential early renal biomarker for epithelial injury (Singer et al. 2013). Compared to creatinine levels and urine output, NGAL is specifically produced by proximal tubular injury which interferes with NGAL reabsorption and increases its synthesis. This injury leads to release of NGAL in the blood and urine and is easily evaluated. Recently, many studies assessed NGAL's role in acute renal damage, where NGAL is increased in 2–6 hrs following acute kidney damage (Devarajan 2010; Haase et al. 2011; Soliman et al. 1999). After kidney injury, the elevation in NGAL production precedes the increase in conventional biomarkers such as serum levels of creatinine and urinary N-acetyl glucosaminidase levels (Cappellini et al. 2017). Therefore, serum cystatin C has been given more attention than serum creatinine and estimated glomerular filtration rate (eGFR) as improved instruments for evaluation of GFR and creatinine clearance in β-thalassemia patients. Excretion of neutrophil gelatinase-associated lipocalin in urine results from proximal tubular damage, producing increased NGAL synthesis or disruption in NGAL reabsorption.

The aim of the current study is to compare the serum levels of urea, creatinine, cystatin C and NGAL in patients with β-thalassemia major versus intermedia, in addition to finding a significant correlation between these parameters. However, these early renal injury biomarkers have not yet been compared in these patients.

Material and methods

Patients

The current cross-sectional study was conducted on forty patients with thalassemia major, twenty-three patients with thalassemia intermedia and twenty-four healthy subjects as a control. The ages of the study groups ranged from 10 to 28 years, referred from Ibn Al-Atheer Centre in Mosul, between September 2019 and March 2020.

Patients with thalassemia major or intermedia who were over the age of 29 and receiving iron chelation therapy, such as deferoxamine and deferasirox, were excluded from the current study. Furthermore, thalassemia major patients with organ damage caused by drugs such as trimethoprim, steroids, and cephalosporins, or by other diseases such as diabetes mellitus, as well as patients with kidney or urinary tract disorders, were excluded. The healthy subjects did not have any diseases, such as autoimmune, infectious, neurological and pulmonary.

Biochemical analysis

To measure the serum levels of urea and creatinine, blood samples were collected from the control and patient groups and immediately analysed. Whereas, to measure the serum levels of cystatin C and NGAL, blood samples were collected from the control and patient groups and serum was separated by centrifugation at around 3000 rpms for 10 mins at 4 °C. The obtained serum was stored at -80 °C for later use. The concentration of cystatin C and NGAL were estimated by enzyme linked immunosorbent assay (ELISA) using human Cys-C (cystatin C) and human NGAL (neutrophil gelatinase associated lipocalin) ELISA Kit (Elabscience, USA)

Statistical analysis

All values were set as mean ± SD. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test were used for data analysis of multiple comparisons, using GraphPad Prism version 8.0.2 (San Diego, USA). $P < 0.05$ were set statistically significant.

Results

Demographic characteristics of control, β-thalassemia major and intermedia

Forty patients (17 male and 23 female) with β-thalassemia major, twenty-three patients with (10 male and 13 female) with β-thalassemia intermedia and 25 subjects (12 male and 13 female) healthy control subjects were conducted in the present study, from September 2019 to March 2020. The mean age of the patients with β-thalassemia major was 17.28 ± 4.798 years, β-thalassemia intermedia was 16.22 ± 5.351 years and the control group was 18.80 ± 5.431 years.

Table 1. Demographic characteristics of study groups.

Parameters	Control (n = 25)	Thalassemia major (n = 40)	Thalassemia intermedia (n = 23)
Age (years)	18.80 ± 5.431	17.28 ± 4.798	16.22 ± 5.351
Sex			
(male)	12	17	10
(female)	13	23	13

No significant variations (P > 0.05) were found between study groups with regard to age and sex.

Validation of serum levels of urea, creatinine, cystatin C and NGAL

Compared to control, serum levels of cystatin C and NGAL were markedly increased in both β-thalassemia major and intermedia. Moreover, serum levels of urea were significantly increased in thalassemia major compared to control, with no significant difference in β-thalassemia intermedia. However, no significant variation was noticed between thalassemia patients and control group in terms of serum creatinine levels. Compared to thalassemia intermedia, thalassemia major showed a significant increase in serum levels of NGAL and creatinine (Table 2). Interestingly, no significant correlations have been found between cystatin C and either the parameters in the study groups.

Table 2. Renal function parameters in β-thalassemia patients and control group.

Parameters	Control	β-thalassemia major	β-thalassemia intermedia
Urea (mmol/l)	4.016 ± 0.8184	4.588 ± 0.9143 ^{a*}	4.191 ± 1.071
Creatinine (mmol/l)	52.84 ± 11.62	55.25 ± 10.26 ^{b**}	47.35 ± 6.307
Cystatin C (ng/ml)	306.7 ± 69.03	385.4 ± 78.93 ^{a**}	371.3 ± 101.8 ^{a*}
NGAL (ng/ml)	10.23 ± 1.728	18.40 ± 2.459 $^{a**** b****}$	15.49 ± 1.219 ^{a****}

Data set as mean value ± SD. [a] show variations between β-thalassemia major and β-thalassemia intermedia patients versus healthy control group; [b] show variations between β-thalassemia major and β-thalassemia intermedia patients. *p < 0.1; **p < 0.01; ****p < 0.0001 represents statistically significant variations, as set by one-way ANOVA followed by a Tukey's multiple comparisons test.

Discussion

The life expectancy of thalassemia patients, in particular β-thalassemia major, has been recently improved with the appropriate therapeutic options, but new complications have been developed. The kidney is one of the vital organs affected by the regular blood transfusions in these patients, causing progressive iron accumulation and renal dysfunction. Moreover, subclinical renal impairment is known to develop early in thalassemia patients (Haitham et al. 2013). Thus, in order to reduce mortality and morbidity in these patients, early diagnosis of renal dysfunction may have a crucial role in determining the optimal therapy. Consequently, identifying reliable and accurate biomarkers is extremely important for diagnosing asymptomatic renal dysfunction in β-thalassemia patients. Recently, several findings have been established the glomerular and tubular injury in β-thalassemia patients (Musallam and Taher 2012; Bekhit et al. 2017; Mahmoud et al. 2021). Accordingly, Şen et al. (2015) revealed that the urinary levels of NGAL may be used as a reliable biomarker to monitor renal impairment in β-thalassemia major patients, and thus predicting patients with a possible risk for future renal function. Moreover, Patsaoura et al. (2014) reported that serum levels of NGAL were markedly increased in thalassemia intermedia patients compared to control. Furthermore, this elevation is consistent with increased expression of NGAL in mice with β-thalassemia intermedia. On the other hand, two studies by Behairy et al. (2017a) and Mohammed Abdulrazzaq (2020) revealed that patients with β-thalassemia major had significantly higher levels of serum cystatin C compared with control, suggesting that cystatin C is specifically a sensitive marker to monitor tubular and glomerular function in these patients. Therefore, our current study was conducted to identify the possibility of use cystatin C and NGAL compared with conventional biomarkers (urea and creatinine) in the diagnosis of early renal dysfunction in patients with β-thalassemia major versus β-thalassemia intermedia.

The accumulation of degradation products of Hb in the renal tubules in β-thalassemia patients results in renal tubular dilation, with subsequent mild to moderate proteinuria, haematuria and interstitial nephritis (Musallam et al. 2014). Aldudak et al. (2000) revealed that patients with β-thalassemia major had normal serum levels of creatinine, with a simultaneous increase in serum levels of urea, uric acid, potassium and phosphorous resulting from the rapid turnover of erythrocytes. Although there was no significant variation in serum levels of creatinine between the study groups and control, urea levels in β-thalassemia major patients were significantly higher compared to control. In β-thalassemia major patients, the non-significant increase in serum creatinine was attributed to the anaemia-induced renal dysfunction and subsequent chronic hypoxia which is compensated by low creatinine production induced by the low muscle mass due to growth retardation in these patients (Behairy et al. 2017a; Karaman et al. 2019).

Lipocalin-2 (24p3r) and megalin are two receptors that allow NGAL to bind and produce its action on target cells. The 24p3r receptor is expressed in distal nephron and is responsible for protein endocytosis by NGAL uptake into the cell (Langelueddecke et al. 2012). In renal tubular epithelial cells, megalin receptors are expressed and bind with a high affinity for NGAL (Hvidberg et al. 2005). Accordingly, NGAL is directly excreted from the renal tubular epithelial cells and thus nephron damage results in rapid excretion of this biomarker which can help to diagnose renal injury at many clinical levels (Pickering and Endre 2013; Singer et al. 2016). Several findings have established that increased levels of NGAL occurred prior to diagnosis, and hence determines higher levels of creatinine or lower eGFR. Interestingly, Buelow et al. (2012) reported that the increase of urinary levels of NGAL and

interleukin-18 reached its peak 6 hrs after cardiopulmonary bypass, while changes in creatinine or a decrease in eGFR were observed at least after 24 hrs. Moreover, Nishida et al. (2010) revealed that levels of urinary NGAL significantly increased in children with various chronic disorders of the kidney, including nephrotic syndrome, proliferative glomerulonephritis and tubular dysfunction. Similarly, several studies reported that β-thalassemia major patients had higher levels of urinary NGAL compared to control (Roudkenar et al. 2008; Ozek et al. 2014; Şen et al. 2015). The principal finding of the current study is that patients with β-thalassemia major and intermedia had considerably elevated levels of serum NGAL compared to control independent of age and sex. In addition, these levels are markedly higher in β-thalassemia major compared to those with β-thalassemia intermedia.

Several possible explanations can be contemplated for increased levels of NGAL in β-thalassemia patients, such as anaemia/chronic hypoxia, renal injury, and homeostatic iron disorders. Increased systemic levels of NGAL can be attributed to anaemia by causing direct inhibition of erythrocyte maturation. Hence, the autocrine regulatory pathway generated by local synthesis of NGAL by progenitors of immature medullary erythroid stem cells, and thus promoted by interleukin-1 that causes erythropoiesis inhibition by arresting of differentiation activation of apoptosis (Miharada et al. 2005). Elevation of systemic levels of NGAL would negatively influence erythrocyte homeostasis in the bone marrow, hence by decreasing NGAL production by progenitors of erythroid stem cells and by increasing the survival mechanism of the same cells, the bone marrow is counteracting this potential negative effect (Spiropoulos et al. 2010). Interestingly, there were no correlations between NGAL and other indices of renal injury, which may be due to the small number of β-thalassemia patients, in addition to their heterogeneity.

In the present study, levels of serum cystatin C were significantly increased in patients with β-thalassemia major and intermedia compared to control. We also found that cystatin C levels did not correlate with other biomarkers of renal dysfunction, suggesting a lack of activation of tubuloglomerular feedback (Kuwabara et al. 2009). Several findings are in line with our study regarding β-thalassemia major (Behairy et al. 2017b; Mohammed Abdulrazzaq 2020), with no studies revealing serum levels of cystatin C in β-thalassemia intermedia. Compared to serum creatinine, cystatin C is expected to be a more potent and reliable endogenous biomarker for GFR, since it is thought to be synthesized at a constant amount in all nuclear cells, slightly bound to protein, largely filtered by glomerulus and completely metabolized and reabsorbed in the proximal tubule (Elbedewy et al. 2015). However, Papassotiriou et al. (2010) revealed that little variations in cystatin C levels after deferasirox therapy did not reflect kidney damage. Interestingly, a study by Badeli et al. (2019) suggested that after deferoxamine therapy, there was no significant correlation between renal injury and NGAL levels in thalassemia patients.

Conclusion

In the current study, we concluded that cystatin C and NGAL could be used as specific, reliable and sensitive early predictors for renal dysfunction in patients with β-thalassemia major and intermedia, compared to conventional biomarkers (urea and creatinine).

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