Pediatr Nephrol
DOI 10.1007/s00467-015-3172-9

ORIGINAL ARTICLE

Novel marker for the detection of sickle cell nephropathy: soluble FMS-like tyrosine kinase-1 (sFLT-1)

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Received: 21 April 2015 / Revised: 5 July 2015 / Accepted: 9 July 2015

Abstract
Background Given the burden and poor outcome of end-stage renal disease in sickle cell disease (SCD), early markers of sickle cell nephropathy (SN) are desirable. Disordered angiogenesis underlies many complications of SCD. We aimed to determine the relationship between serum FMS-like tyrosine kinase-1 (sFLT-1) and other biomarkers of renal damage for the early diagnosis of SN.
Methods Forty-seven SCD patients and 49 healthy controls were enrolled. Microalbuminuria was determined in patient urine samples. Blood samples were tested for sFLT-1, serum creatinine, and various hemolysis and inflammation markers. Peripheral blood monocyte expression of sFLT-1 was measured using real-time polymerase chain reaction (PCR).
Results The serum level of sFLT-1 (pg/ml) in SCD patients was higher than controls (median/range/IQR=142/ 60–1300/ 61 pg/ml vs. 125/ 110–187/52 pg/ml, respectively) (p=0.006). Median (range) of sFLT-1 level was higher in SCD patients with microalbuminuria compared to SCD patients with normoalbuminuria, 185 (140–1300) vs. 125 (60–189) mg/g, respectively) (p=0.004). There was a significant positive correlation between serum sFLT-1 and microalbuminuria, lactate dehydrogenase (LDH), and indirect bilirubin (r=0.59, 0.39, 0.30, and p=<0.001, 0.007, 0.041, respectively). sFLT-1 sensitivity in early detection of renal affection in SCD was 93.6 %, while specificity was 68.6 %. Finally, peripheral blood monocytes (PBM) sFLT-1 expression was significantly higher in SCD patients compared to controls (p=0.05).
Conclusions sFLT-1 may contribute to pathogenesis of albuminuria in SCD patients and constitute a novel renal biomarker of SN.

Keywords Sickle cell disease · Microalbuminuria · Soluble FMS-like tyrosine kinase · Sickle cell nephropathy

Introduction

Sickle cell disease (SCD) is an inherited monogenic disease that leads to a vasculopathy which may manifest as one or more of several complications; sickle cell nephropathy (SN), pulmonary hypertension (PHT), leg ulcers, and stroke [1]. Such pathologic processes are driven to a certain extent by hypoxia and inflammation-induced angiogenesis [2].

SN is one of the serious complications of SCD that is frequently reported. As the disease progresses, nephrotic syndrome and end-stage kidney disease can develop [3]. SN that is induced by insufficient angiogenesis is due to an imbalance between pro-angiogenic and anti-angiogenic processes and may lead to endothelial dysfunction. Soluble FMS-like tyrosine kinase-1 (sFLT-1) is a member of the vascular endothelial growth factor receptor family (VEGFR) and has an anti-angiogenic effect. Soluble FLT-1 is increased in SCD due to its over-expression by vascular endothelial cells, vascular smooth muscles, activated blood monocytes, and proximal tubular cells of the renal epithelia [4].

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Published online: 04 August 2015

In SN, the classic biomarkers of renal damage (plasma creatinine and estimated glomerular filtration rate (eGFR)) are not informative in the early stages of the disease [5]. Thus, novel biomarkers are needed for early diagnosis of SN before damage to the kidneys becomes irreversible [4]. The purpose of this study is to determine the relationship between serum levels of sFLT-1 and other conventional biomarkers of renal damage (microalbuminuria, serum creatinine, and eGFR), as well as with measurements of hemolysis and inflammation, to identify novel renal biomarkers for early diagnosis of SN. We also tested the expression of sFLT-1 in blood monocytes.

Patients and methods

Forty-seven patients with SCD were included in this cross-sectional study during their regular follow-up visits to the hematology clinic, New Cairo University Children Hospital (NCUCH) and after giving their consent. Their age ranged from 5–19 years. They were diagnosed with SCD at a mean age of 1.95±1.24 years. Thirty-nine patients (83.0 %) had SS genotype and eight patients (17.0 %) had Sβ genotype (five patients with $S\beta^0$ and three patients with $S\beta^+$). Most of the enrolled patients 29 patients (61.7 %) were on deferiprone (iron chelation therapy), while 18 patients (38.3 %) were not on chelation therapy. Hydroxyurea was taken by 41 patients (87.2 %).

The following patients were excluded from the study: patients who had vaso-occlusive crisis or were in pain at the time of their visit to the clinic, those with a family history of inherited renal disease or severe renal symptoms, patients who were on any medications that may affect renal function, patients who had other underlying renal disease not secondary to SCD, and patients with a history of blood transfusion within the last 90 days prior to enrollment.

Moreover, 49 age- and sex-matched healthy cases were recruited from the outpatient clinic during check-up as a control for the level of serum sFLT-1 and expression of sFLT-1 in blood monocytes.

Patients were tested for the presence of microalbuminuria in morning urine samples by the urinary albumin:creatinine ratio. Microalbuminuria was defined as albumin/creatinine >30 mg/g. Patients were classified into two groups according to the presence or absence of microalbuminuria: Group A with microalbuminuria and Group B with normoalbuminuria. The determination of microalbuminuria was accomplished using a commercially available kit (BioSystems S.A. Costa Brava 30, Barcelona, Spain).

Blood samples were collected from patients in the same setting for determination of complete blood count (CBC), erythrocyte sedimentation rate (ESR), serum creatinine, serum ferritin, total serum bilirubin (TSB), indirect serum bilirubin (ISB), and lactate dehydrogenase (LDH). Estimated glomerular filtration rate (eGFR) was estimated by the Schwartz formula [6].

The serum quantitation of sFLT-1 was done for patients and controls using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA).

sFLT-1 expression in PBMs was tested in only 30 cases of the enrolled SCD patients and controls. Monocytes were obtained by Ficoll-Hypaque density gradient centrifugation and isolated by exploiting the ability of monocytes to adhere to glass or plastic. *sFLT-1* expression was relatively quantitated by real-time polymerase chain reaction (PCR) using SYBR Green PCR Master Mix (Applied Biosystems, Germany) with the ABI PRISM 77500 Sequence Detection System. Relative gene expression values were evaluated with the $2^{-\underline{AA}}$CT method using *GAPDH* as a housekeeping gene. Human *sFLT-1* was amplified using the following primer sequences: forward, 5′-GGC TGT TTT CTC TCG GAT CTC-3′; reverse, 5′-CAT CTC CTC CGA GCC TGA AAG-3′ [7], under the following conditions: stage 1: 50 °C for 2 min; stage 2: 95 °C for 10 min; stage 3: 40 repeats of 95 °C for 15 min and 60 °C for 1 min; stage 4: 95 °C for 15 min, 60 °C for 15 min and 95 °C for 15 min.

Statistical analysis

Categorical data are presented as frequency (and percentage). Continuous variables of skewed distribution are presented as median and interquartile range (IQR), while normally distributed continuous variables are presented as mean±standard deviation (SD). Comparison between groups was done using Student's *t* test for parametric data and the Mann–Whitney test for non-parametric data. Correlation between variables was done using Pearson's coefficient (*r*) for parametric data and Spearman's correlation for non-parametric data. Multivariate linear regression analysis models were used to test for the preferential effect of the independent variable(s) on sFLT-1 level. The significance level was set at $p<0.05$. Statistical analysis was performed with SPSS 16.0 (Statistical Package for Scientific Studies) for Windows.

Results

We studied 47 patients with SCD and 49 controls. Demographic and laboratory data of studied patients and the control group are shown in Table 1.

The serum level of sFLT-1 in the studied SCD patients was significantly higher than controls ($p=0.006$). The median level of serum sFLT-1 showed no significant difference when comparing patients with SS and Sβ genotypes, patients given and not given hydroxyurea therapy, chelated and not chelated patients (195 and 191 pg/ml, 192 and 210 pg/ml, 192 and 194 pg/ml, respectively); $p>0.05$ for all. In the studied SCD

Table 1 Demographic data of studied sickle cell disease (SCD) patients and control group

	Patients (n=47)	Control group (n=49)	p value
Age (years)	13 (5–19)	12 (6–19)	0.21
Gender			
Male	26 (55.3 %)	27 (55.1 %)	0.82
Female	21 (44.7 %)	22 (44.9 %)	
Hemoglobin (gm/dl)	7.5 (5.4–11.1)	12.1 (10.3–14.7)	<0.001
BUN (mg/dl)	11 (7–13)	9.5 (5–15)	0.9
Serum creatinine (mg/dl)	0.58 (0.2–0.9)	0.74 (0.4–1.1)	0.031
eGFR (ml/min/1.73 m^2)	118.33 (83.3–262.5)	112 (94–130)	0.004
ESR (mm/h)	20 (5–65)	8 (5–20)	0.002
Indirect bilirubin (mg/dl)	1.4 (0.3–8.5)	0.7 (0.5–1)	<0.001
LDH (IU/l)[a]	495 (156–1411)	184 (125–344)	0.005
Serum ferritin (ng/dl)[b]	910 (127–5857)	64 (32–125)	<0.001
Microalbuminuria (mg/g)	25.2 (3.1–75)	14.4 (6.1–29)	0.01
sFLT-1 (pg/ml)	142 (60–1300)	125 (110–187)	0.006
sFLT-1 expression (fold increase in comparison to control group)	1.4±0.36	1±0.3	0.05

BUN blood urea nitrogen, *eGFR* estimated glomerular filtration rate, *ESR* erythrocyte sedimentation rate, *LDH* lactate dehydrogenase, *sFLT-1* soluble FMS-like tyrosine kinase-1

[a] Normal range of LDH=125–350 IU/l

[b] Normal range of ferritin=20–140 (ng/dl)

patients, there were significant positive correlations between serum levels of sFLT-1 and microalbuminuria (r=0.59, p<0.001) (Fig. 1), LDH (r=0.39, p=0.007) and indirect bilirubin (r=0.30, p= 0.041). Meanwhile, there were no significant correlations between serum levels of sFLT-1 and creatinine (r=0.017, p=0.91), eGFR (r=−0.005, p= *0.97*), serum ferritin (r=−0.082, p=0.58) or ESR (r=0.017, p=0.91).

Microalbuminuria (group A) was detected in 21 patients (44.7 %) with median/range/IQR=42.4/39.9–75/8.15 mg/g, while normoalbuminuria (group B) was found in 26 patients (55.3 %) with median/range/IQR 16.05/3.1–29/13.93 mg/g. Comparison between the two groups is shown in Table 2.

As analyzed by real-time PCR, PBM sFLT-1 expression was significantly higher in SCD patients when compared to controls (p=0.05), while there was no statistically significant difference in SCD patients with microalbuminuria and normoalbuminuria in comparison to the control group (p=0.212).

ROC curve (receiver operating characteristic curve analysis) for sFLT-1 revealed that the sensitivity of sFLT-1 in early detection of SN was 93.6 %, while the specificity was 68.6 % (Fig. 2). The diagnostic cutoff value of sFLT-1 was 137 pg/ml with positive predicted value (PPV)=80.0, negative predicted value (NPV)=88.9 and accuracy (area under curve)=0.714.

By using multivariate regression analysis, we found that both indirect bilirubin and microalbuminuria were independent predictors of sFLT-1 (Table 3).

Discussion

Increased creatinine clearance and eGFR have been well described in patients with SCD. These have been attributed to compensatory hypersecretion of vasodilator prostaglandins [8] and/or increased nitric oxide synthesis in response to hypoxia induced by sickling [9]. In this study, we confirm that SCD patients have increased eGFR. Consequently, creatinine clearance and serum creatinine could be seen as unreliable markers of early kidney effects in SCD.

Given the insight into angiogenesis as an important factor in the pathophysiology of SN, we set out to characterize sFLT-1 in relation to other classic markers of renal damage, such as microalbuminuria, serum creatinine, and eGFR.

In the current study, we found that sFLT-1 levels were higher in SCD patients than healthy controls. Similarly, a previous study reported that the level of sFLT-1 was higher in patients with SCD compared to healthy subjects [10]. Moreover, they reported correlations between sFLT-1 and various measures of hemolysis [10]. Also, in another study, it was shown that sFLT-1 increased significantly in clinically asymptomatic SCD patients [2].

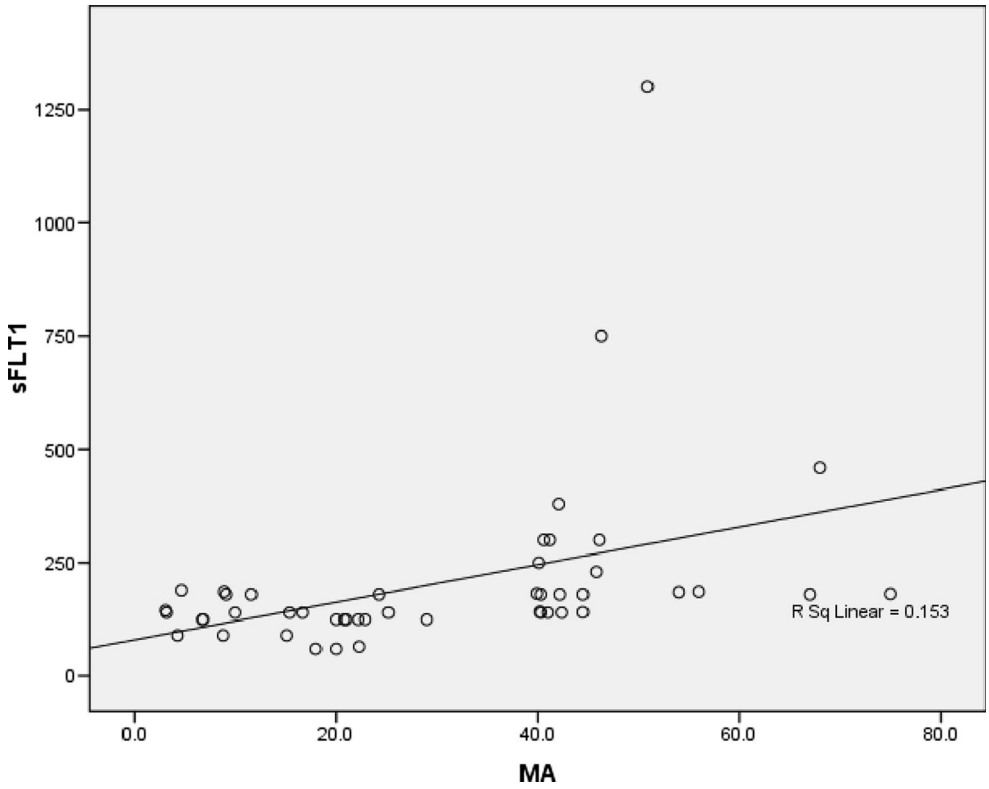

Fig. 1 Correlation between serum levels of soluble FMS-like tyrosine kinase-1 (sFLT-1) and microalbuminuria (MA)

Soluble FLT-1 production is increased in SCD patients due to misregulation of FMS-like tyrosine kinase in endothelial cells and PBMs resulting in over-expression of the soluble splice variant of FLT-1 in the plasma. Insufficient angiogenesis in SN results from sequestration and inhibition of VEGF by sFLT-1, which is a potent antagonist [11].

Our data reveal that sFLT-1 levels were significantly higher in SCD patients with microalbuminuria compared to SCD patients with normoalbuminuria. This association, combined with the association of sFLT-1 with soluble vascular cell adhesion molecule (VCAM) in prior studies [10], suggests that sFLT-1 may contribute to the pathogenesis of albuminuria in SCD by promoting endothelial dysfunction. This is in agreement with several studies that have reported the role of sFLT-1 in the development of albuminuria in SCD [10, 12], and in other disease states such as preeclampsia [13], diabetes, and essential hypertension [14, 15].

Table 2 Comparison of different variables in relation to presence or absence of microalbuminuria

	Microalbuminuria (n=21)	Normoalbuminuria (n=26)	p value
eGFR (ml/min/1.73 m^2)	124 (84.4–260)	113 (83.3–262)	0.467
ESR (mm/h)	20 (5–65)	20 (5–59)	0.637
Indirect bilirubin (mg/dl)	2.6 (0.7–8.5)	1 (0.3–5.5)	0.003
LDH (IU/l)[a]	596 (335–985)	408 (156–1411)	0.001
Serum ferritin (ng/dl)[b]	935 (140–3447)	814 (127–5857)	0.732
Microalbuminuria (mg/g)	42.4 (39.9–75)	16.05 (3.1–29)	<0.001
sFLT-1 (pg/ml)	185 (140–1300)	125 (60–189)	<0.001
sFLT-1 expression (fold increase in comparison to control group)	1.27±0.28	1.5±0.4	0.212

Data presented in median (range)

eGFR estimated glomerular filtration rate, *ESR* erythrocyte sedimentation rate, *LDH* lactate dehydrogenase, *sFLT-1* soluble FMS-like tyrosine kinase-1

[a] Normal range of LDH=125–350 IU/l

[b] Normal range of ferritin=20–140 (ng/dl)

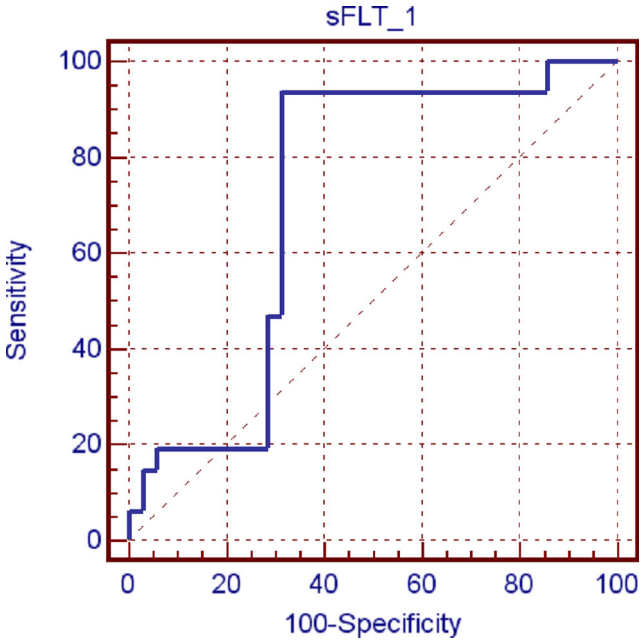

Fig. 2 Receiver operating characteristic (ROC) curve between the studied groups (sickle cell disease (SCD) patients and controls) with regard to soluble FMS-like tyrosine kinase-1(sFLT-1)

Early glomerular selectivity damage has been found in SCD children, which is secondary to both charge selectivity and size selectivity impairment [16]. Becton et al. found that microalbuminuria is a simple screening biomarker of early kidney injury in children with SCD [17]. In view of these data together with our results, we may suggest that sFLT-1 is a potential novel marker of glomerular damage in children with SCD.

In this study, the prevalence of microalbuminuria was found to be 44.7 %, which is higher than the rates of 27 % and 26 % reported from the United States and Jamaica, respectively [18, 19]. Different genotypes in different races and age variation among different studies may be partly responsible [18–22]. In this present study, the serum level of sFLT-1 in SCD patients showed a significant positive correlation with microalbuminuria and markers of hemolysis (LDH and indirect bilirubin). This is in agreement with other researchers who reported a significant association of hemolytic markers (LDH and indirect bilirubin) with sFLT-1 [10].

We show that PBMs expressed higher levels of sFLT-1 than cells isolated from the control group. Moreover, its expression trended higher in SCD patients with microalbuminuria than in those with normoalbuminuria; yet this difference could not be proven to be statistically significant. Previous studies have also described that PBMs are an important alternative source of sFLT-1 in preeclampsia [23] and in early renal injury [24]. We suggest that the possible source of increased sFLT-1 in SCD patients is PBMs. However further studies on a wide range of patients are needed to confirm this.

Although this current study provides new information regarding a novel potential biomarker of SCN, our results should be interpreted in the context of some limitations. First, the size of the study population was small, meaning that multivariate regression analysis was not be the most appropriate procedure. Second, we failed to prove definitively that PBMs are the source of sFLT-1 in SCD patients.

We conclude that sFLT-1 may contribute to the pathogenesis of albuminuria in SCD patients and constitute a novel renal biomarker of SN. Moreover, PBMs are overexpressed in SCD patients compared to controls, which, in the context of previous studies, suggests that PBMs could be the possible source of sFLT-1.

Table 3 Multivariate regression analysis to detect predictors of soluble FMS-like tyrosine kinase-1 (sFLT-1)

	ß	*p*
Hemoglobin	0.153	0.346
BUN	−0.116	0.417
Creatinine	−0.293	0.276
eGFR	−0.314	0.234
Indirect bilirubin	0.419	0.008
ESR	0.119	0.442
LDH	−0.008	0.956
Ferritin	−0.096	0.498
Microalbuminuria	0.289	0.047

BUN blood urea nitrogen, *eGFR* estimated glomerular filtration rate, *ESR* erythrocyte sedimentation rate, *LDH* lactate dehydrogenase

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The study protocol was approved by the Institutional Review Board.

Informed consent Informed consent was obtained from the patients or the patients' guardians.

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