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Identification of Biomarker Profile for Chronic Kidney Disease of uncertain aetiology in Sri Lanka

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Abstract

Background: Chronic Kidney Disease of uncertain aetiology (CKDu) is a major health problem in Sri Lanka. Current laboratory markers are not sensitive enough for early detection of CKDu. It is evident that a more efficient, sensitive and specific diagnostic procedure is needed for early detection and to confirm the diagnosis of CKDu.

Objectives: To identify a representative biomarker profile for CKDu, Sri Lanka and to study the applicability of these biomarkers in identifying at risk population for screening and diagnosis of CKDu, Sri Lanka

Methods: Girandurukotte and Wilgamuwa which are considered as CKDu endemic areas were selected for the study to recruit definite non-dialysis CKDu cases (n = 119), endemic CKD (n = 82) and endemic healthy controls (n = 79). Non-endemic CKD group (n = 85) and healthy controls (n = 85) were recruited from Kandy. Routine markers and novel biomarkers for CKDu were measured using serum and random urine of CKDu patients. The eight selected renal biomarkers were measured using multiplex biomarker assay, and the data were analyzed using logistic regression algorithm aiming to extract the best marker combination that could distinctly identify the disease groups noninvasively from the healthy controls. Data were analyzed using SPSS and R software.

Results: Among the selected patients, 97 (81.5%) were males while 22 (18.5%) were females. Under routine markers, hyperuricemia, acidosis, hypomagnesemia, vitamin D deficiency, anemia, increased level of serum osmolality, amylase, Lactate Dehydrogenase and Alkaline Phosphatase were identified. Alpha1 microglobulin (A1M) stood out as the single strong candidate marker that was highly specific (84.7%) in identifying CKDu from healthy controls. Combination of A1M+ Kidney Injury Molecule 1 (KIM1) + Retinol Binding Protein 4 (RBP4) was able to accurately differentiate the disease groups (CKDu/CKD), from healthy controls. Combination of Osteopontin + KIM1+ RBP4 accurately predicted CKDu with high performance from a CKD background. Higher mean (±SD) value (69587 ng/mL) of Transforming Growth factor beta 1was obtained from the CKDu group compared to the other controls with a significant negative correlation ($r = -0.293$, $p < 0.01$) with the serum creatinine.

Conclusions: A representative biomarker profile has been identified for identification of risk population for screening and diagnosis of CKDu. Biomarker combinations are helpful to diagnose CKDu effectively and non-invasively.

Keywords: *Chronic kidney disease of uncertain aetiology, Chronic kidney disease, Biomarkers, Alpha1microglobulin*

Background

From 1990s, an increase in Chronic Kidney Disease (CKD) prevalence and emergence of a Chronic Kidney Disease of uncertain aetiology (CKDu) was observed in several tropical and subtropical countries including El Salvador, Egypt, India, Central America, Nicaragua and Sri Lanka (Weaver *et al*., 2015). Among them, Sri Lanka reports the highest occurrence of CKDu, in rural dry zone where extensive farming is carried out. CKDu was first reported in Sri Lanka in early 1990s, and over the last 10 years its prevalence has progressively increased to epidemic levels (Jayatilake *et al*., 2013). A common observation has been that CKDu is caused by multiple factors, likely linked to a combination of environmental factors, diet and nutritional practices, and genetics (Elledge *et al*., 2014).

The CKDu is an environmental nephropathy reported from Central America, India, Taiwan and Sri Lanka causing significant morbidity and mortality. This is the first study which describes the biomarker profile of non-dialysis patients with the diagnosis of definite CKDu, in Sri Lanka. Many Risk factors have been postulated for CKDu by different studies, which suggest the possibility of a multifactorial causation of the disease (Bandara *et al*., 2008). Family history of CKD is especially important in diagnosis of CKDu because positive family history of CKD indicates a genetic tendency of CKDu and a higher probability to have CKDu. Hence, this needs further exploration with proper medical data. Previous studies have reported a family history of chronic kidney disease as significant predictors for CKDu (Wanigasuriya *et al*., 2008; Wanigasuriya *et al*., 2011; Athuraliya *et al*., 2011).

Studies on complications of CKDu are rare up to date. According to Fernando *et al* (2019a), a significant association of the prevalence of anemia with the CKDu severity has been observed (Fernando *et al*., 2019a). Earlier, CKDu case definition in Sri Lanka was based on persistent albuminuria defined as an albumin to creatinine ratio (ACR) of > 30 mg/g in an initial urine sample and at a repeated visit (Jayatilake *et al.*, 2013). Noble *et al* (2014) described a criterion including exclusion of all known aetiology of CKD, with proteinuria < 300 mg/L for 24-hour urine in three consecutive months, no hematuria, increased echogenicity in ultra sound scan, biopsy with tubular interstitial pathology and small size of kidney, living in a CKDu endemic area more than for five years for classification of CKDu patients (Noble *et al*., 2014). Some factors in above methods are not sensitive enough to detect CKDu patients in early stages at the moment. Of note, a link between serum creatinine level and eGFR has been established wherein serum creatinine concentrations increase only when approximately 40-50% of renal parenchyma is damaged (Steubl *et al*., 2016).

Evolving criteria have been applied in the diagnosis of CKDu but still it is a diagnosis of exclusion of known causes like Diabetes Mellitus (DM), chronic or severe hypertension, snake bite with systemic envenomation, glomerular nephritis obstructive nephropathies and other renal diseases (Fernando *et al*., 2019). Recently Wijewickrama *et al* (2019) have described a new epidemiological case definition to identify CKDu in Sri Lanka (Wijewickrama *et al*., 2019). This study focused to identify representative biomarker profile (routine and novel biomarkers) for CKDu in Sri Lanka and study the applicability of these biomarkers in identifying at risk population for screening and diagnosis of CKDu in Sri Lanka. It will help to detect CKDu in early stages in patients in Girandurukotte and Wilgamuwa and other affected areas. Identifying the unique features of CKDu compared to the other forms of CKD will give a chance to screen and monitor the patients.

Materials and Methods

Girandurukotte and Wilgamuwa which considered as endemic areas were selected for the study to recruit non-dialysis, definite CKDu cases (n = 132), endemic CKD (ECKD: n = 82) and endemic healthy controls (EC: n = 79). Non-endemic CKD group (NECKD: n = 85) and Non-endemic controls (NEC: n = 85) were recruited from, Kandy. Healthy controls were apparently healthy people without any diagnosed medical conditions by clinical examination and past medical history along with the normal serum creatinine and no proteinuria.

The ethical clearance for the study was obtained from ethical review committee of the Faculty of Medicine, University of Peradeniya (2016/EC/28). Written informed consent was obtained from the participants of the study and clinical details were recorded. The height and weight were measured for the Body Mass Index (BMI, kg/m^2) calculation according to the standard protocols. Blood and random urine samples were taken for the analysis. In the pilot study done on serum and urine of definite CKDu cases, 27 novel biomarkers were analyzed using Luminex Magpix machine. According to the results of the pilot study, one serum marker Transforming Growth Factor (TGF beta 1) and eight urinary markers [Kidney injury Molecule 1 (KIM 1), Neutrophil Gelatinase-associated lipocalin (NGAL), Tissue Inhibitor Matrix metalloproteinase 1 (TIMP 1), Alpha 1 microglobulin (A1M), Beta 2 microglobulin (B2M), Osteopontin (OPN), Cystatin C (Cys C) and Retinol Binding Protein 4 (RBP4)] were shortlisted using their level of expression and clinical significance. Selected markers from the pilot study were applied for the case-control study. The urinary levels of the eight selected renal biomarkers were quantified using multiplex biomarker assay using xMAP technology, and the data were subjected to systematic analysis using logistic regression algorithm aiming to extract the best marker combination that could distinctly identify the disease groups non-invasively from the healthy controls (Fernando *et al*., 2019b). Some samples were rejected due to inadequate sample volume, unclear labeling and hemolysis of the sample. Hence 75 CKDu samples were selected for the biomarker analysis. To increase the power of the results, at least 50% or more than 50% of the number of cases were selected as controls. The urinary levels of eight selected renal biomarkers, KIM1, NGAL, B2M, A1M, CST3, OPN, TIMP1 and RBP4, were quantified using Milliplex multiplexing protein assay. In addition, urine and serum creatinine levels were measured in every sample and protein measurements were adjusted to urine creatinine level. For differentiating the CKDu/ CKD from healthy controls, distribution of protein markers across different categories was assessed using Receiver Operating Characteristic Curve (ROC) analysis. Routine markers were measured using serum and random urine. For the case group, initially 132 cases were recruited according the inclusion and exclusion criteria, only 119 CKDu cases participated for the assessment of full biochemical profile at the Teaching Hospital Kandy. Biochemical parameters were analyzed using Indiko plus Analyzer, osmometer. Hemoglobin was measured using a hematology analyzer.

Non-dialysis, definite CKDu patients were recruited according to the criteria developed by Sri Lankan Society of Nephrology (SLSON) (Wijewickrama *et al*., 2019). According to eGFR ($mL/min/1.73\ m^2$), CKDu was graded as stage 1 (eGFR $\geq$ 90), 2 (eGFR 60 – 89), 3a (eGFR 45-59), 3b (eGFR 30-44), 4 (eGFR 15-29) and 5 (eGFR < 15) (KDIGO, 2013). The Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation was used for the estimated eGFR (Levey *et al*., 2009). Stage 1, 2 and 3a were taken as early stage and stage 3b, 4 and 5 were taken as late stage of the CKDu. Data were analyzed using SPSS (IBM statistics version 23.0) and R software.

Results

Out of 132 recruited patients, only 119 patients participated for all laboratory investigations. Among the selected patients, 97 (81.5%) were males while 22 (18.5%) were females. Demographic data, lifestyle and health characteristics of the non-dialysis patients with definite CKDu are shown in Table 1.

Table 1: Demographic and clinical characteristics of the study subjects (n = 119)

Variable	Mean ±SD (Range) or Number (Percentage %)
Age (years)	51 ± 9 (19 -76)
Sex distribution: Male	97 (81.5%)
Female	22 (18.5%)
Weight (kg)	58.6 ±12
Height (m)	1.62 ±0.22
BMI (kg/m^2)	22.1 ±3.6 (15 – 33.3)
Systolic BP (mmHg)	124 ±13 (80 – 160)[a]
Diastolic BP (mmHg)	78 ±7 (60 – 100)
Occupation as a farmer	88 (74%)
Family history of CKD	61 (51%)
Smoking (males only)	48 (40%)
Chewing betel	93 (78%)
Alcohol (males only)	50 (42%)

Note: Values are expressed as numbers with proportion of populations for each characteristic (%), or as mean ±SD as appropriate.

[a] Four-patients had increased systolic blood pressure; SD, standard deviation; BMI, body mass index; BP, blood pressure; CKD, chronic kidney disease

The 74% of the patients' age were 40 to 60 years. Out of the total, 74% were farmers. Average BMI in all CKDu patients was within the normal range. Higher BMI (> 25 kg/m^2) was observed in 20.2% of CKDu patients while it was 14% for lower BMI (< 18 kg/m^2). According to the urine analysis, urine Sulfosalicylic acid test showed abnormal urine protein (+, ++, +++ or > +++) in 31 patients (26%). The CKDu patients had the highest percentage of family history of CKD (51%) and chewing betel (78%).

The prevalence of anaemia was 72.3% and it was significantly higher in females (95.4%) than males (67.01%) ($p < 0.001$). Among CKDu cases, 76.7% had mild anemia while 23.3% had moderate anemia. Severe anemia (Hb <8.0 g/dL) was not observed in any patient. The prevalence of anemia increased with worsening renal function; 66.7% in stage 1, 60% in stage 2, 50% in stage 3a, 95% in stage 3b, 79.2% in stage 4, 100% in stage 5.

Average levels and clinical significance of biochemical laboratory findings in serum of CKDu patients are presented in Table 2 (Fernando *et al*., 2020). The mean for all the blood investigations are within the reference interval except for the raised serum creatinine, uric acid, amylase, alkaline phosphatase, and decreased eGFR (Table 2). Only some CKDu patients had hyperuricemia (34.5%), hypophosphatemia (5%), hypocalcemia (13.4%) and acidosis (16.8%). Alkaline phosphatase (62.2%) and amylase (79.8%) were increased in a majority. Under these investigations, hyperuricemia, acidosis, hypomagnesemia, vitamin D deficiency, anemia, increased level of serum osmolality, amylase, Lactate dehydrogenase (LDH) and Alkaline Phosphatase (ALP) were identified.

Table 2: Biochemical laboratory findings in serum of CKDu patients in Girandurukotte and Wilgamuwa (n = 119)

Parameter	Mean ± SD	Normal N (%)	Low N (%)	High N (%)	Reference Range
Sodium (mmol/L)	140.4 ±6	64 (53.8)	28 (23.5)	27 (22.7)	136 - 145
Potassium (mmol/L)	4.5 ±0.6	98 (82.4)	5 (4.2)	16 (13.4)	3.5 – 5.1
Calcium (mg/dL)	9.1 ±0.5	98 (82.4)	19 (16)	2 (1.6)	8.6 – 10.3
Phosphorous (mmol/L)	1.06 ±0.24	96 (80.7)	17 (14.3)	06 (5.0)	0.87 – 1.45
Creatinine (µmol/L)	178.1 ±122	40 (33.6)	NA	79 (66.4)	M < 113 F < 96
Urea (mg/dL)	31 ±14	95 (79.8)	4 (3.4)	20 (16.8)	13 – 43
Uric Acid (mg/dL)	6.6 ±1.7	78 (65.5)	NA	41 (34.5)	M - 3.5 – 7.2 F - 2.6 – 6.0
Total Protein (g/L)	72 ±4	116 (97.5)	2 (1.7)	1 (0.8)	64 – 83
Albumin (g/L)	44 ±2	118 (99)	NA	1 (0.8)	35 – 52
Amylase (U/L)	153 ±64	24 (20.2)	NA	95 (79.8)	< 100
LDH (U/L)	220 ±38	70 (58.8)	NA	49 (41.2)	135 – 225
Bicarbonate (mmol/L)	25.4 ±3.3	83 (69.7)	20 (16.8)	16 13.5)	22 – 29
GGT (U/L)	35 ±29	102 (85.7)	NA	17 (14.3)	M < 55 F < 38
SGPT (U/L)	29 ±21	97 (81.5)	NA	22 (18.5)	M < 45 F < 34
SGOT (U/L)	28 ±14	98 (82.4)	NA	21 (17.6)	M < 35 F < 31
ALP (U/L)	162 ±204	44 (37)	1 (0.8)	74 (62.2)	35 – 105
Vitamin D (ng/mL)	31.9 ±15.3	44 (37)	74 (62.2)	1 (0.8)	30 – 100
T.Cholesterol (mmol/L)	5 ±1	69(58.0)	36 (30.2)	14 (11.8%)	<5.2 Desirable 5.2-6.2 Borderline high >6.2 High
Magnesium (mmol/L)	0.93 ±1.25	79 (66.4)	35 (29.4)	5 (4.2)	0.66 – 1.07
Hemoglobin (g/dL)	12.1 ±1.5	33 (27.7)	86 (72.3)	NA	M < 13 F < 12
Serum Osmolality, (mOsm/kg) (n=118)	300 ±23	56 (47.4)	4 (3.4)	58 (49.2)	275 – 295
Urine Osmolality (mOsm/kg) (n=118)	385 ±160	74 (62.7)	42 (35.6)	02 (01.7)	300 – 900

SD, standard deviation; LDH, lactate dehydrogenase; GGT, gamma glutamil transferase; SGPT, serum glutamate-pyruvate transaminase; SGOT, serum glutamate-oxaloacetate transaminase; ALP, alkaline phosphatase; M, male; F, female; NA, not available

From the evaluation of the markers across all comparisons, A1M was identified as the single best candidate marker with the highest performance (CKDu vs NEC; Sensitivity = 92%, specificity = 847%). The Area Under Curves (AUC) were comparably high with the highest sensitivity (92%) achieved for CKDu. The AUC for the A1M in CKDu, ECKD and NECKD groups against NEC were 0.914, 0.913 and 0.891 (Fernando *et al*., 2019b).

According to the findings of this study, a biomarker signature panel, representing diverse CKD scenarios is ideal instead of a single marker to improve the efficiency of CKD diagnosis in community screening. Towards this goal, logistic regression models were trained using the most discriminated proteins that can stratify the disease from the control group for all three comparisons as described in methods. Two different marker combinations were identified that can discriminate the disease group from the healthy controls. Among them, A1M+KIM1+RBP4 combination showed higher sensitivity than serum creatinine for all disease types (CKDu, NECKD and ECKD) against the healthy control group (NEC) and displayed a high AUC of 0.903 (Figure 1). The second marker combination OPN+KIM1+RBP4 showed a good AUC in discriminating the CKDu from other CKD diseases (Figure 2). This accentuates the need for developing high-performing new marker signature that can accurately identify CKDu subjects for disease stratification and proper patient management, thus emphasizing on the usefulness of our proposed 3-marker signature (Fernando *et al*., 2019).

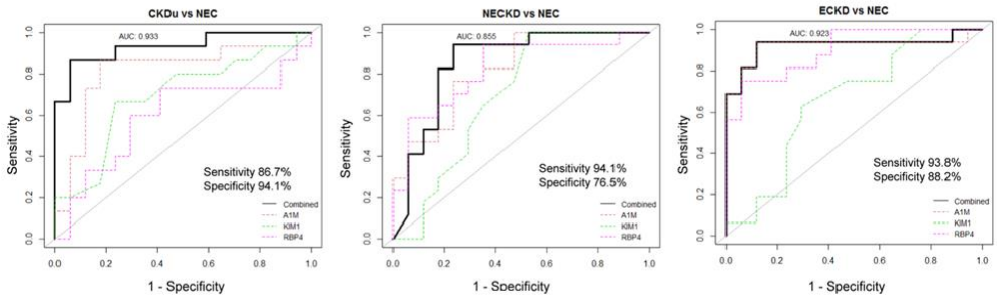

Figure 1: Best marker combination A1M+KIM1+RBP4 in distinguishing CKD/CKDu from NEC

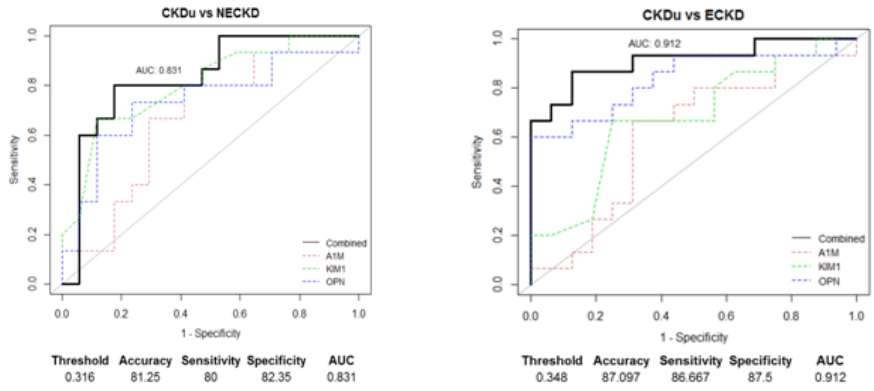

Figure 2: Best marker combination OPN+KIM1+RBP4 in distinguishing CKDu from other CKD disease groups

According to the results of the pilot study of novel biomarkers, the only serum biomarker selected was TGF beta 1. There was a significant difference (p <0.001) in mean TGF beta1 (69587 ng/mL) of CKDu with other four control groups. But there was no significant difference between the healthy control groups (EC & NEC) and CKD groups (ECKD &

NECKD). In CKDu group, there was a significant negative correlation of TGF beta 1 with the serum creatinine ($r = -0.293$, $p < 0.01$) and significant positive correlation with the eGFR ($r = 0.332$, $p < 0.01$).

Discussion

Results of the current study were conducted in CKDu definite cases, compatible with most of the previously described manifestations of CKDu. Moreover, some specific patterns including hyperuricemia, acidosis, hypomagnesemia, anemia, increased level of serum osmolality, amylase, LDH and ALP have been identified which need to be validated in a larger group. It was observed that chewing betel was significantly high among CKDu patients.

In this study, we took advantage of the renal biomarkers previously reported for CKD and assessed their predictive performance in the context of CKDu. Potential marker combinations that can be used to distinguish CKD population from healthy controls and to stratify CKDu patients from all subjects diagnosed with CKD were identified. Since the diagnostic signatures we propose in this study are based on urine biomarkers, the screening process is non-invasive to be implemented in at-risk populations. Currently, the identification of CKDu relies on biomarkers used to diagnose CKD in general, which includes serum creatinine and urinary protein. Recently, several surrogate markers have been reported to perform better in diagnosing CKD and CKDu. For example, CST3 is reported to better predict the clinical outcomes of CKD than creatinine (Qiu *et al*., 2017). CKDu characteristically affects tubulointerstitium and notably, A1M is an indicator of renal tubular function.

In comparison to using single marker, our analysis suggested higher predictive performance of combination biomarker signature consisting of A1M+KIM1+RBP4 in accurately identifying the disease groups, particularly CKDu and NECKD. Of note, this marker panel was found to be uniquely predictive for CKDu and not for ECKD. CKDu and ECKD both occur in the endemic region and because of the unidentified etiology, CKDu patients are often misdiagnosed as diabetic or hypertensive kidney disease if they have concomitant diseases. The novel biomarker signature thus holds a greater potential in improving clinical decisions, leading to better patient management and clinical care. There may be some unique feature to increase TGF beta 1 in CKDu than the control groups. The negative correlation of TGF beta 1 with the serum creatinine led to the increasing of TGF beta 1 in the early stages provides a good clue for early diagnosis of CKDu. There are multiple challenges for the development of clinically useful biomarkers with their discovery, confirmation, validation and clinical assessment.

Conclusion

In the present study, a representative biomarker (routine and novel) profile has been identified to screen the population at risk and for diagnosis (with unique patterns) of CKDu. A1M is the single strong candidate marker that was highly specific in identifying CKDu/CKD from healthy controls. The study reveals that combination of biomarker panel is more useful in diagnosis of CKDu. The serum biomarker, TGF beta 1 can be used for early detection of CKDu. Further studies should be conducted in larger validation group for better identification of clinically useful novel biomarkers for CKDu.

Conflict of interests

The authors declare that there is no conflict of interest.

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