How to Cite:
Rajyalakshmi, N., Vijayaraghavan, R., Senthilkumar, S., & Sekar, D. (2022). Evaluation of circulatory microRNA-196 levels during the progression of chronic renal (kidney) disease of unknown etiology. *International Journal of Health Sciences*, *6*(S2), 5624–5630. https://doi.org/10.53730/ijhs.v6nS2.6406

Evaluation of circulatory microRNA-196 levels during the progression of chronic renal (kidney) disease of unknown etiology

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***Abstract*---**Chronic renal disease of unknown etiology is prevalent & clinically silent until its late stages at which point patients may suffer significant irreversible damage or mortality in the lack of early screening & intervention. If diagnosed early stage 1-3 the progression of chronic renal disease can be altered and complications reduced. Current diagnosing markers of renal function, serum creatinine, blood urea and uric acid have been shown to be lacking high predictive value. So, there is a need for next generation biomarkers suitable for Chronic renal disease of unknown etiology and should not be influenced by age, sex, nutritional status, current health concern. microRNAs appears to be very stable in tissues & biological fluids even under adverse conditions like extreme p^H, long term room temperature storage, multiple freeze thaw cycles & RNAase activity. microRNAs elaborate in renal development, maintenance of renal function & progression of renal disease. miRNA-196 is a renal derived biomarker, extensively distributed in renal, shows an important role in renal diseases & superior to other renal biomarkers. The aim of present study is to evaluate circulatory miRNA-196 during progression of

chronic renal disease of unknown etiology. The results showed there was a significant higher expression of miR-196 in CRD_U patients (mean±SD, 2.942±0.05709) compared to control (mean±SD, 1.585±0.08805) and also there was significant higher expression in all the stages of Chronic renal disease of unknown etiology compared to controls (p=0.001).

***Keywords*---**chronic renal disease (CRD), chronic renal disease of unknown etiology (CRD_U), microRNA-196, RT-qPCR.

Introduction

Chronic renal disease of unknown etiology (CRD_U) a new form of severe CRD which is not due to traditional risk factors such as diabetes & hypertension has been reported from Central America, Srilanka, Egypt & India during the past 2 decades[1]. These have been named as Balkan endemic nephropathy(BEN), Itai-Itai disease in Japan, Mesoamerican nephropathy(MeN), Srilankan nephropathy, Indian CRD_U named as Uddanam endemic nephropathy[2]. They have in common chronic tubulointerstitial disease pattern[2,3], disease is among poor agricultural working communities[4] seen in young and middle aged adults in the age group of 25-42 years[2] late presentation, a long asymptomatic phase[5], with serum creatinine value ranging from 1.4 to 2.6mg/dL and minimal or no proteinuria. CRD_U patients typically are non diabetic & have either normal blood pressure[4,5] or absence of hypertension in early phases disease prior to reduction in glomerular filtration rate[6]. In 2017 the global prevalence of CRD was 9%, 12th leading cause of deaths. In India it was 11-18% and 8th leading cause of deaths[7]. In Uddanam region, Southern Indian state of AndhraPradesh prevalence of CRD was 18.23%. 73% of patients with CRD in Uddanam were identified as CRD_U[1]. The cause of these regional nephropathies remain elusive to date. Etiology has been established for Itai-Itai disease was due to Cadmium and BEN due to Aristolochic acid[5]. Etiological hypothesis for remaining nephropathies are heat stress, agrochemicals, heavy metals, genetic predisposition[5]. The traditional laboratory indicators for renal disease like serum creatinine, blood urea, uric acid, urinary electrolytes, glomerular filtration rate are insensitive, nonspecific and tedious[8,9]. So there is a need for next generation novel biomarker for CRD_U. microRNAs(miRNAs) are small non coding RNAs with 22 nucleotides in length[10] involved in various biological processes including cell proliferation, cell death, stress resistance & metabolism[11]. The first miRNAs were discovered by Lee and colleagues in nematodes Caenorhabditis elegans in 1993[12,13]. There are over 21,000 miRNAs informed in 168 species to date from plants to animals[14] and over 2000 of miRNAs have been identified in human[15]. miRNAs form a vital part of the regulatory cascade in renal development, maintenance of renal function & progression of renal disease. miRNAs like miR-10a/b, miR-21, miR-30, miR-130, miR-143, miR-192, miR-194, miR-196a/b, miR-200a, miR-204, miR-215, miR-216, miR-872 are expressed in the renals[16]. miR-196 was identified by Mariana langos Quintana et al in 2003 in mice from osteoblast sarcoma cell line SaOS-2 by using Lagos Quintana et al at 2001, 2002 Method[17]. Urinary miR-196a is a renal derived biomarkers for renal damage also demonstrated that urinary miR-196a may function as a biomarkers for pretending the disease progression in

patients with FSGS also known as that expression of miR-196a in inflammatory cells is much lesser than that in renal tubular cells[18]. Time dependent ROC analysis also displayed that addition of urinary miR-196a was superior to proteinuria and eGFR. miR-196a has also been presented to play a role in other renal diseases[11]. To the best of our knowledge, this is the first study that evaluated serum levels of miRNA-196 in CRD_U patients in all stages to explore whether changes in miR-196 can predict the progression of CRD_U.

Material and Methods

Study design & participants

The present study was considered as cross-sectional study involving a total of 42 CRD_U patients and 30 healthy volunteers attending the outpatient ward of General Medicine, Government General Hospital and Medical College, Srikakulam, Andhrapradesh, India from 27th July to 19th September 2019 after approval by Institutional Ethical Committiee. Inclusion criteria were as follows: 1.Patients above 18 years and below 65 years with serum creatinine>1.2mg/dL including male and female 2.Blood pressure SBP/DBP<140/90 mmHg 3.Random blood sugar <200mg/dL. Exclusion criteria were 1. Pregnant & lactating women 2.Chronic systemic illness 3.Family H/o renal stones.

Data collection

Complete data was collected with an open ended structured questionnaire from all the participants after taking consent which included demographic, clinical and laboratory outcome information prior to the analysis.

Laboratory examination

5mL of venous blood was collected from all the CRD_U patients and controls and was centrifuged at 3000rpm for 10 minutes and serum was separated and stored at -4^{0}C. Real time RT-qPCR was implemented. CFX96 real time system C1000 thermal cycler (BIO-RAD, USA) was used for estimating serum miRNA-196. All laboratory tests were conducted in accordance with the product manual. The extraction of miRNAs from each serum sample was performed using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). The quantitative expression analysis of miR-196 was performed using SYBR Green-based RT-qPCR in all the patient samples compared to controls relative to endogenous control U6. Primer sequences specific to human miR-196a (Forward: 5′-ACCTGCGTAGGTAGTTTCATGT-3′, Reverse:5′-CGTCAGAAGGAATG ATGCACAG-3′) and U6 (Forward: 5’-CGCTTCGGCAGCACATA TACTA-3’, Reverse: 5’-CGCTTCACGAATTTGCGTGTCA-3’) were used to perform RT-qPCR using Real-time PCR system (S1000 BioRad, USA). The mean Cq value of each sample for both the targets was exported and calculated for expression of miR-196 in relation to U6 separately in patients and control samples using Livak method[19].

Analysis of Statistics

The data was analysed by SPSS (version.19, IBM Corp.2010) and represented as mean±SD. Student t-test was used to compare values and define statistical significance between controls and patients. One-way analysis of variance was used to compare and define statistical significance among difference stages of CRD_U and controls using Bonferroni's multiple comparison test.

Results

The expression of circulatory miR-196 in overall CRD_U patients was higher (mean±SD, 2.942±0.05709) compared to control individuals (mean±SD, 1.585±0.08805). Statistical analysis of relative expression of miR-196 showed significantly increased levels in CRD_U patients compared to control individuals with mean difference -1.357±0.1004; 95% CI, -1.557-1.156; p=0.0001 (Figure 1).

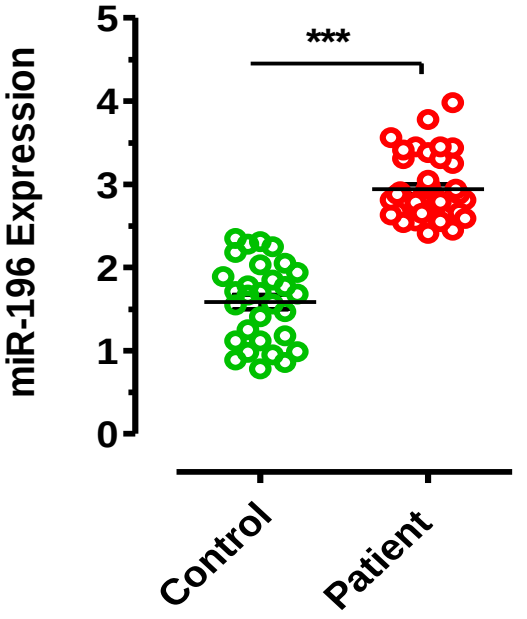

Figure 1: Expression analysis of miR-196 relative to U6 in total CRD_U patients and control individuals (***p=0.0001)

Stage-wise analysis of circulatory miR-196 expression showed significantly higher expression in all the stages compared to controls (stage 1, p=0.001; all other stages, p=0.0001).Intergroup analysis showed significantly increased miR-196 levels in stage IV (95% CI, -1.470 to -0.06611; p=0.01) and stage V (95% CI, -2.066 to -0.4192; p=0.0001) compared to stage I, in stage V (95% CI, -1.805 to -0.2803; p=0.001) compared to stage II, in stage V (95% CI, -1.628 to -0.2769; p=0.0001) compared to stage IIIa, and in stage IV (95% CI, -0.9027 to -0.04697; p=0.01) and stage V (95% CI, -1.556 to -0.3426; p=0.0001) compared to stage IIIb (Figure 2).

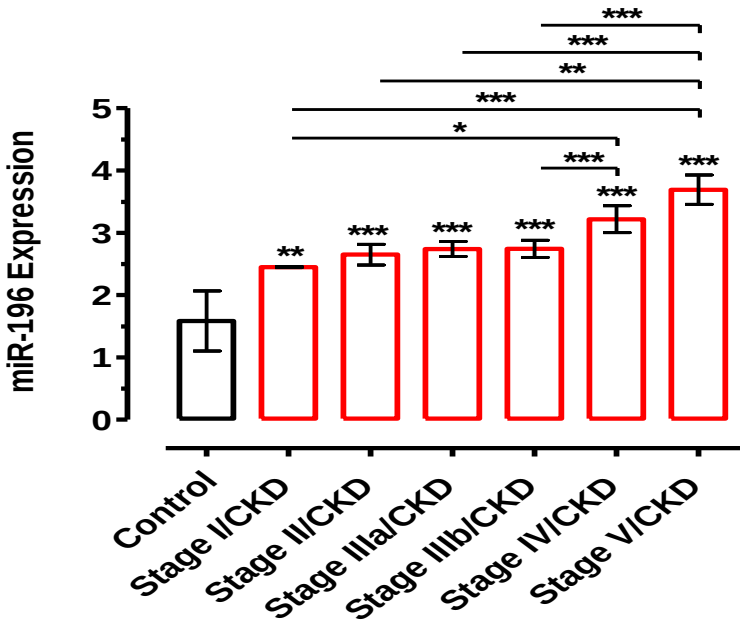

Figure 2: Expression analysis of miR-196 relative to U6 in different stages of CRD_U patients distributed according to eGFR and control individuals (*p=0.01, **p=0.001, ***p=0.0001)

Discussion

In the present-day work, we studied serum miR-196 levels in CRD_U patients (stage I, II, IIIA, IIIB, IV and V) and healthy controls to explore whether changes in miR-196 can predict the progression of CRD_U. The results indicate that circulatory miR-196 can serve as a renal-derived biomarker for renal damage. The RT-qPCR results showed significant increase in the expression of miR-196 in CRD_U compared to healthy controls and moreover the fold change expression of miR-196 increased proportionately with the severity of CRD_U (from stage I to V). There was a positive correlation between urinary miR-196 level & disease activity of focal segmental glomerulo sclerosis a common cause of CRD & ESRD and recent study presented that miR-196a is primarily communicated in renal with 74.3% of miR-196a distributed in renal & plays an important role in renal fibrosis[11]. The present findings are interesting and implicate the importance of studying miR-196 for assessing the progression of CRD_U. We conclude that miR-196 can serve as a renal-derived biomarker for CRD_U.

Acknowledgements

We are thankful to DR. Dodda Vishnumurthy Dean, Aditya Institute of Technology and Management, Srikakulam, Andhrapradesh, India for his support in data and sample collection.

Declarations

Funding: No source of funding.
Conflict of interest: No conflict of interest.
Ethical approval: The protocol of the study was approved by the Institutional Ethics Committee of Govt. Medical College, Srikakulam with reference no:ECR/492/inst/AP/2013/RR-16 & written informed consent was taken from all the participants.

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Figure 1: Expression analysis of miR-196 relative to U6 in total CKD_U patients and control individuals (***p=0.0001).
Figure 2: Expression analysis of miR-196 relative to U6 in different stages of CKD_U patients distributed according to eGFR and control individuals (*p=0.01, **p=0.001, ***p=0.0001).