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A Comparison of Mirna-196 in Chronic Kidney Disease and Chronic Kidney Disease with Unknown Cause and its Relationship to Neutrophil Gelatinase Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (Kim-1)

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Abstract:
MicroRNAs, also known as miRNAs, are tiny, non-coding RNAs that regulate gene expression by base pairing with target miRNAs in the 3' untranslated region. MiRNAs are degraded or translation is suppressed as a result. MiRNAs have been linked to a wide range of biological functions, including cell proliferation, cell death, stress tolerance, and metabolic regulation. Extreme pH, long-term room temperature storage, numerous freeze thaw cycles, and RNAase activity do not seem to affect the stability of miRNA in tissues and biological fluids. Relationships between neutrophil gelatinase associated lipocalin (ngal) or kidney injury molecule-1 (kim-1) are examined, as well as the role that mirna-196 plays in CKD and CKD of unclear aetiology.

1. Introduction

The global burden of illness is shifting, with chronic kidney disease becoming second only to infectious virus diseases as the leading cause of death worldwide. The majority of deaths and disabilities in the twentieth century were brought on by infectious illnesses. With the exception of the CovD-19 pandemic, noncommunicable and noninfectious illnesses have emerged as the leading causes of death and disability in the modern world. NKFdefines CKD as either a decline in GFR to 60mL per 1.73m2 for at least 3 months or the presence of kidney damage. CKD is a complex and progressive condition that can be caused by both communicable and infectious diseases. Primary causes of CKD include diabetes mellitus and high blood pressure. Metabolic syndrome, cardiovascular disease, and obesity are other prevalent risk factors. Infectious disorders including malaria, schistosomiasis, HIV, and hepatitis-B are other risk factors, as are advanced age (older than 60 years), urinary tract infections, autoimmune diseases, and family histories of kidney disease. [1]

Gross hematuria, frothy urine, nighttime urination, flank discomfort, and/or a decrease in pee production are the symptoms. In advanced stages, patients may have symptoms including tiredness, low appetite, nausea, vomiting, metallic taste, unintended weight loss, pruritus, mental status changes, dyspnea, or peripheral edoema.[2]

CKDU, which is prevalent in nations of Asia and Sub-Saharan Africa, is not caused by conventional risk factors such as diabetes and hypertension, but rather by glomerulonephritis and tubulointerstitial damage.

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Proteinuria is a common sign of kidney injury, although individuals whose damage is limited to the renal tubules rather than the glomeruli may not show any signs of proteinuria at all.[3]

The incidence of CKD is increasing worldwide. These nations do not provide clear estimations. The prevalence and distribution of CKD in India remain unknown. Rare research has been done on chronic kidney disease. Across south Asian countries, the rate of CKD prevalence was 5.01-13.24%. The rates of CKD-related mortality and disability-adjusted life years were greatest in India and Pakistan. Noncommunicable illnesses have surpassed infectious diseases and reproductive problems as the leading causes of mortality and disability in the South Asian area in recent years. In 2017, the number of fatalities caused by CKD rose by 42% globally, as reported by the Global Burden of Disease Study. [4-5]

In 2017, CKD was responsible for 9,459,473 DALYs in South Asia, which is equivalent to 26% of all DALYs globally. The incidence of CKD across all age groups worldwide rose by 29.3 percent and the death rate by 41.5 percent between 1990 and 2017, according to the GBD partnership. Between 2001-2003 and 2010-2013, renal failure was responsible for 38% more fatalities in India than any other cause of mortality. The prevalence of CKD in India was 17.2%, according to SEEK-India research. Chennai had an 8.7% prevalence of CKD, Delhi had an 8% prevalence, the coastal part of Saurastra, Gujarat had a 15.7% prevalence, the non-coastal region of north Maharastra had a 26% prevalence, and Andhrapradesh had a 46.8% prevalence. In addition to being a leading cause of death worldwide, chronic kidney disease is also a key contributor to the development of cardiovascular disease. [6]

There seems to be a combination of factors that leads to CKD. Comorbidities such as diabetes, hypertension, obesity, and cardiovascular illnesses have increased with the CKD burden as a direct result of the changing lifestyles brought on by socioeconomic transition and the ageing trend of the population. Many nations have seen a rise in the frequency of CKD due to factors such inadequate sanitation, overcrowding, illiteracy, infections, low socioeconomic level, and a lack of access to excellent medical treatments in outlying regions. Traditional risk factors for chronic kidney disease include diabetes and high blood pressure in both industrialised and many developing nations. Risk factors include impaired fasting plasma glucose, high blood pressure, a high body mass index, and a diet rich in salt and lead). These GBD-measured parameters accounted for 57.6%, 43.2%, 26.6%, 9.5%, and 3.6% of the risk for CKD, respectively.[7]

Whereas high fasting plasma glucose was the primary risk factor for CKD in East Asia, Eastern Europe, Tropical Latin America, and Western Sub-Saharan Africa, high blood pressure accounted for the biggest percentage of the CKD burden in these regions (Bikbov et al, 2020). Remarkably, 36% of Indian young people were found to be obese, while the frequency of proteinuria was found to be 6% (Rao et al, 2017). Unknown aetiology for CKD was the second most common cause of CKD among the 52,273 people with CKD included in the Pan-India CKD registry study. Researchers in India found that diabetes was the leading cause of CKD, accounting for 24.9% of cases. Chronic interstitial nephritis and an unknown cause each accounted for 23.2% and 19.5% of cases, respectively.

The most common causes of CKD were hypertension (87%), diabetes (37.5%), alternative medication use (22.9%), nephrolithiasis (11.8%), and recurrent urinary tract infections (11.1%). Acute kidney injury (AKI) was reported by 6.7% of the study group, and a family history of renal illness was present in around 9%. People in rural regions were more likely to be exposed to toxic chemicals at work, smoke more cigarettes, and had lower levels of education. Diabetic kidney disease and chronic glomerulonephritis were more common in the urban group, whereas contrast-induced nephropathy and unknown aetiologies were more common in the rural 4 group.[9]

In 1993, miRNA was found for the first time in the worm Caenorhabditiselegans. The transition from L1 to L2 of the larval stage in these species required transcription of the second gene, Lin4, and was accompanied by a downregulation of the Lin-14 protein. Let- 7 was first identified as a crucial factor in C. elegans growth from the larval to adult stages in the year 2000. The subsequent discovery of homologues of this gene in many other species, including humans, was a major breakthrough in the field. Over 21,000 microRNAs have been identified in 168 plant and animal species. There are around 2,000 miRNAs

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CODEN: JCLMC4
Journal of Coastal Life Medicine

known to exist in humans. The regulation of renal development, function, and disease progression is dependent on microRNAs. Several microRNAs (miRNAs) are expressed in kidneys. These include miRNA-10a/b, miRNA-21, miRNA-30, miRNA-130, miRNA-143, miRNA-192, miRNA-194, miRNA-196a/b, miRNA-204, miRNA-215, miRNA-216, miRNA-200a, and miRNA-872. The osteoblast sarcoma cell line SaOS-2 was used to discover miRNA-196 in mice.[10]

HOX clusters in the vertebrate genome are where miRNA-196 may be found. Developmental processes rely heavily on the homeodomain containing transcricption factors encoded by HOX genes. There are three known genes for microRNA-196. There are three of them, and they go by the names miRNA-196a-1, miRNA-196a-2, and miRNA-196b. The miRNA-196a-1 gene is situated between the homeobox genes B9 and B10 on chromosome 17. Located on chromosome 12 between HOXC10 and HOXC9 is the miRNA-196a-2 gene. miRNA-196 b is found on human chromosome 7 between the HOXA9 and HOXA10 genes, and on mouse chromosome 6 between the HOXA9 and HOXA10 genes. HMGA proteins may directly attach to the upstream region of miRNA-196a-2, hence controlling miRNA-196 production. Recent miRNA profiling studies have shown that miRNA-196 plays a crucial role in development and immunity, and is highly expressed in a variety of cancer tissue types. MiRNA-196 has just been found, and there is reason to believe that it may play a significant role in the treatment of a number of human disorders. Mice with DN have significantly lower levels of miRNA-196a because it targets p27.[11]

In various cell types and environmental settings, miRNA-196 may control a wide variety of genes. miR-196a suppresses HOX B8 expression during HL60 myeloid development. Since HMGA1 may upregulate miRNA-196a-2 expression, this suggests that miRNA-196a-2 may function as a negative regulator of HMGA family members' expression. Ren et al. (2019) found that miRNA-196a might suppress protein production of HMGA2. Targetgene of microRNA-196a is annexin A1, also known as lipocortin, p35, or calcium and phospholipid binding protein 35.miR-196 regulates the expression of genes for calcuim binding protein A9, small proline rich protein 2C, kertain 5, CLCA family members 2 (a chloride channel regulator), cytochrome P450, keratin 4, LDOC1, leukotriene A4 hydrolase, and pleotrophin. Oesophageal adenocarcinoma, pancreatic cancer tissue, and breast cancer all have increased levels of miRNA-196a. Myeloid lineage leukaemia, colon cancer, and Hepatocellular carcinoma (HCC) all have high expression levels of miR196b. Melanoma cells express less miRNA-196a than normal cells.[12]

Our goal here is to examine the relationship between miRNA-196, KIM-1, and NGAL in patients with CKD and CKD of unclear aetiology, and to compare these values to those in healthy controls.

2. Material and Methods

Study population: From April through September of 2021, research participants visited the outpatient clinic of the Department of General Medicine at S.N Medical College (Agra). There were 35 healthy controls, 46 patients with CKD, and 79 CKDU in this prospective cross-sectional research. Sample sizes (control = 30, CKD = 23, and CKDU = 42) were used to calculate miRNA-196 levels. Specifics on how to determine whether you have chronic kidney disease or chronic kidney disease of unclear cause. S.N. Medical College Lucknow's Institutional Ethics Committee gave its permission to this work.

Blood sample collection and preparation: Centrifugation at 3000 rpm for 10 minutes of 5 mL of venous blood from healthy controls, CKD patients, and CKDU patients yielded similar results. Separated serum was frozen in the -40 degree Celsius freezer. The RT-qPCR was done in real time. Estimation of serum miRNA-196 was performed using a CFX96 real time system C1000 thermal cycler (BIO-RAD, USA). The lab tests were carried out as specified in the user guide.

ESTIMATIONOFMIRNA-196

MicroRNA (miRNA) Isolation from Serum:

Each serum sample had its miRNAs isolated with the use of the miRNeasy Serum/Polyplasmic Extraction Kit (Qiagen, Hilden, Germany). Before miRNA extraction, we allowed each frozen serum sample to thaw at room temperature. After adding 1mL of QIAzol Lysis reagent to 200L of serum sample and briefly vortexed, the mixture was left to incubate at room temperature (15-25oC) for 5 minutes. After adding 200 L of chloroform to the sample mixture, the

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CODEN: JCLMC4
Journal of Coastal Life Medicine

tube was securely sealed and vortexed for 15 seconds. Each sample combination was incubated for 2-3 minutes at room temperature before being centrifuged at 12,000 x g for 15 minutes at a cold temperature of 4 degrees Celsius.

After carefully removing the interphase, the upper aqueous phase of each sample was transferred to fresh collection tubes. Each aqueous phase had 1.5 litres of 100% ethanol added to it, and the mixture was pipetted until it was completely homogenous. Any precipitate was pippetted along with 700 L of sample into a 2 mL collection tube containing an RNeasy elute spin column. After sealing the container, centrifugation was performed at room temperature at 8000 x g for 15 seconds. The flow-through was thrown away, and the procedure was carried out again with the remaining material.

After adding 500 L of buffer RPE to the RNeasy Elute spin column, the lid was sealed and the centrifuge was set to 8000 x g for 15 seconds. Flow-through was discarded, and 500 L of 80% ethanol was poured to the RNeasy Elute spin column before being centrifuged at 8000 x g for 2 minutes. Thrown away the flow-through and the collecting tube together. After transferring the RNA from the RNeasy Elute spin column into a fresh 2 mL collection tube, we centrifuged it at 12000 rpm for 5 minutes to dry the membrane.

We threw away the collecting tube together with the flow-through. To elute the miRNA, we put the RNeasy Elute spin column in a fresh 1.5 mL collection tube, poured 14-20 L RNase-free water directly to the centre of the spin column membrane, carefully closed the lid, and centrifuged at 12000 rpm for 1min. In either case, the miRNA from each sample was transformed into cDNA right away or frozen at -20 degrees Celsius until cDNA preparation was ready.

PreparationofcomplementaryDNA:

Each extracted miRNA sample was used to generate complementary DNA (cDNA) using universal stem loop micro RNA primer (5'GAAAGAAG

In order to execute cDNA synthesis, 10 L of the extracted miRNA sample was employed as a template in a thermal cycler. First, we denaturated 10 L of miRNA at 65 degrees Celsius for 10 minutes with 2 L of the aforementioned stem loop miRNA primer, and then we sped the reaction up by cooling it for 2 minutes in ice. This was followed by starting up each cDNA reaction using 4 L of 5X RT buffer, 1 L of a 10 mM dNTPs combination, 0.5 L of reverse transcriptase enzyme, and 2.5 L of molecular biology grade milliq water. The whole reaction mixture was incubated at 42 degrees Celsius for 45 minutes, followed by a 10-minute incubation cycle at 72 degrees Celsius. After being used, the reaction was kept at -20 degrees Celsius until it was cooled to 4 degrees Celsius in a thermal cycler.

Real-time PCR (RT-qPCR) quantification of microRNA-196: All patient samples and control samples were analysed for miRNA-196 expression levels relative to endogenous control U6 using SYBR Green-based RT-qPCR. Using a Real-time PCR equipment, we conducted RT-qPCR using primers designed to amplify expression of human miRNA-196a and U6. Table 6.3 shows the sequences of primers designed to target human miRNA-196a and U6. In 96 well microplates, 2 L of cDNA from each sample was used to make two reaction mixes, one for miRNA- 196 and one for U6. Each reaction mixture included 0.5 L of each primer, 10 L of SYBR Green mix, and 7 L of dH2O. The RT-PCR system was used to incubate the reaction plates at 95 degrees Celsius for 5 minutes to initiate denaturation, followed by 40 cycles of 60 degrees Celsius, 95 degrees Celsius, and 72 degrees Celsius. Melt curve analysis was used to differentiate primer-dimers from genuine miRNA-196 and U6 amplicons at the conclusion of each cycle. For miRNA-196 expression in respect to U6 in cases and controls independently, the mean Cq value of each sample for both targets was exported and computed.

Table1: Human miRNA-196a and U6-specific primers

miRNA-196	**Forward**	5'-ACCTGCGTAGGTAGTTTCATGT-3'
	Reverse	5'-CGTCAGAAGGAATGATGCACAG-3'

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Journal of Coastal Life Medicine

U6	**Forward**	5'-CGCTTCGGCAGCAGCACATATACTA-3',
	Reverse	5'-CGCTTCACGAATTTGCGTGTCA-3'

Statistical analysis

The information is shown as a meanSEM. Multiple comparisons across groups were performed using one-way ANOVA followed by the Bonferroni 't' test. The effect of age and gender on miRNA-196 concentration was examined using a two-way analysis of variance. It was considered statistically significant if the probability was less than 0.05. For statistical processing and graphing of correlations, SigmaPlot 14.5 was utilised.

3. Results

Expression of miRNA-196 ranged from 1.539+0.088 (control) to 2.290+0.127 (CKD) to 2.919+0.064 (CKDU). There was a statistically significant difference (P 0.001). There was a statistically significant (P 0.001) difference between those with CKD and CKDU and the control group. The expression levels of miRNA-196 were significantly greater in CKDU than in CKD (P 0.001).

Table 2: Micro RNA-196 (miRNA-196) mean values compared between controls, CKD, and CKDU

Parameter	**Group**	**Mean**	**SEM**
miRNA-196 (Foldchange)	C	1.539	0.088
	CKD	2.290^{a}	0.127
	CKDU	2.919^{ab}	0.064

In CKD, but not in controls or CKDU, a gender gap was found using a two-way analysis of variance. The miRNA-196 level was considerably higher in CKD females compared to controls. The results showed no statistically significant differences across the ages.

Using KIM-1 and NGAL, researchers found that miRNA-196 is connected with KIM-1 in CKD. The levels of NGAL and miRNA-196 are linked in CKDU but not in controls. .

Table3: Two-way ANOVA reveals the impact of age and gender on miRNA-196 expression. ANOVA

S.No.	**Groups**	**Gender**		**Age(years)**	
		Male	**Female**	**< 40**	**> 40**
1	Control	1.556 ±0.108	1.513 ± 0.133	1.558 ±0.092	1.265 ±0.344
	CKD	2.214 ±0.098	3.980 ±0.460	2.072 ±0.198	2.368 ±0.118
	CKDU	2.862 ±0.105	2.967 ±0.096	2.716 ±0.184	2.960 ±0.082

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
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Journal of Coastal Life Medicine

2	Significancewithin Group	F=81.495 P <0.001	F=26.132 P <0.001
	Significancewithin genderorage	F=12.374 P <0.001	F=0.277 P= 0.600
	Significanceofinteraction Groupwithgenderorage	F=6.582 P= 0.002	F=1.062 P= 0.350
3	Significanceamonggroups ControlandCKD	t= 6.247 P<0.001	t= 3.812 P<0.001
	Significanceamonggroups ControlandCKDU	t= 12.386 P <0.001	t=6.980 P <0.001
	Significanceamonggroups CKDandCKDU	t=0.743 P= 1.000	t=4.038 P <0.001
	Significanceamonggenderorage– maleandfemaleor<40and >40	t=3.518 P <0.001	t=0.526 P= 0.600
4	Significancewithincontrol maleandfemaleor<40and >40	t=0.246 P= 0.806	t=0.824 P= 0.412
	SignificancewithinCKD maleandfemaleor<40and >40	t=3.759 P <0.001	t=1.283 P= 0.203
	SignificancewithinCKDU maleandfemaleor<40and >40	t=0.740 P= 0.462	t=1.214 P= 0.228
5	Significancewithinmaleor<40 ControlandCKD	t=4.505 P< 0.001	t=2.349 P= 0.063
	Significancewithinmaleor<40 ControlandCKDU	t=8.639 P< 0.001	t=5.637 P <0.001
	Significancewithinmaleor<40 CKDandCKDU	t=4.501 P< 0.001	t =2.382 P= 0.058
6	Significancewithinfemaleor>40 ControlandCKD	t=5.156 P <0.001	t=3.035 P= 0.009

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ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

	Significancewithinfemaleor>40 ControlandCKDU	t=8.881 P <0.001	t=4.798 P <0.001
	Significancewithinfemaleor>40 CKDandCKDU	t =2.158 P= 0.101	t=4.123 P <0.001

4. Discussion

To investigate whether changes in miRNA-196 may predict the course of CKDU, serum miRNA-196 levels in CKD, CKDU patients, and healthy controls were analysed here. The findings suggest that miRNA-196 in blood circulation may be used as a biomarker for renal damage that originates in the kidneys. When comparing CKD and CKDU to healthy controls, RT-qPCR findings indicated that the expression of miRNA-196 was significantly higher in both conditions. Furthermore, the fold change expression of miR-196 increased proportionally with the severity of CKDU.

MiRNA-196 was shown to have strong correlations with both KIM-1 and NGAL in the current investigation, with KIM-1 and miRNA-196 being more strongly linked in CKD and NGAL and miRNA-196 being more strongly linked in CKDU. There is no link when compared to the norm. All three of the miRNAs studied had strong correlations with one another but only a moderate relationship with KIM-1 and serum creatinine in a longitudinal cohort research. In a study comparing serum NGAL and three miRNAs for the diagnosis of acute kidney injury after acute myocardial infarction, the miRNAs were more discriminating when used together. Using RT-PCR, Zhang et al. analysed urine miRNA196a levels to predict CKD development and found that it may serve as a noninvasive biomarker for diverse kidney illnesses to evaluate disease severity and track the pathogenesis of kidney disorders. Only FSGS patients were included in this investigation, and urine samples were taken at the time of renal biopsy.It has been shown that miRNA-196a contributes to tissue fibrosis. Levels of miRNA-196a expression are directly correlated with NF-B inflammatory signalling pathway activation. This demonstrates that miR- 196a in fibroblasts directly interacts with its target genes, IKK and IKK.[13]

In a mouse model of FSGS, researchers found conflicting findings . MiRNA-196a inhibits p27 expression and diabetic nephropathy in mice. There was a report of miRNA-196a in the kidneys being downregulated. Renal fibrosis severity is inversely correlated with miRNA-196a expression inside the kidney.Both breast cancer and hepatocellular carcinoma have shown miRNA-196a as a promising biomarker.MiRNA-196's methods of action in various cancers have yet to be fully investigated. However, miRNA-196 seems to have a dominating impact on the suppression of oncogenic molecules, demonstrating its role as a tumour suppressor.In this investigation, there was no statistically significant variation in age brackets between the groups. However, there was a statistically significant difference between male and female CKD values, albeit not CKDU. MiRNA-196 expression was significantly higher in CKD compared to CKDU in females. Women were shown to be more vulnerable to cardiovascular risk factors because their miRNA levels were expressed at higher levels than men's. [14]

The enhanced expression of miRNA was shown to have a favourable correlation with high blood pressure and poor HDL levels (Sharma et al, 2014). In a recent research conducted in South Africa, miRNA levels were shown to be significantly dysregulated in hypertensives. MiRNA expression levels below the median were not related to systolic, diastolic, or mean arterial pressure. However, increased expression of miRNA is linked to those previously mentioned characteristics. This occurs because endothelial cells work to keep blood vessels healthy. It has been suggested that miRNA sexual dimorphism may impact a wide range of physiological and pathological processes. However, in a cross-sectional investigation , miRNA was not associated with either sex.[15]

5. Conclusion

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

These results provide credence to the idea that miR-196 is a significant measure of exploration in CKDU patients and highlight the need for more research into its function in determining the course of the disease. The reduced sample size for miRNA prevented ROC curves from being drawn with creatinine, KIM-1, and NGAL.

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