Original article

Exploring the Interplay of HIF-1α Expression and its Correlation with Selected Biomarkers in Acute Kidney Injury Patients

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ABSTRACT

Background: Acute Kidney Injury (AKI) is a complex and heterogeneous clinical syndrome, characterized by the sudden decline in renal function, and has greatly contributed to worldwide mortality and morbidity. Its incidence is 68.55% in Pakistan and is still on the rise. The study intended to the analysis of gene expression of HIF-1α and correlate it with the expression of a chosen kidney injury biomarker panel comprising of NGAL, KIM-1 and L-FABP in AKI patients.

Materials and methods: Out of blood samples of 50 patients enrolled with AKI in our study, 14 patients with superimposition of acute-on-chronic kidney injury (males n=5; females n=9) were selected along with blood samples of 4 healthy controls (males n=2; females n=2). Their RNA was isolated and mRNA gene expression was quantified by RT-PCR.

Results: Analysis showed that HIF-1α expression significantly correlated positively with NGAL (r=0.8829, p=<0.0001) and KIM-1 (r=0.9199, p=<0.0001) mRNA gene fold expression in all male and female AKI on CKD patients, whereas, only the expression of L-FABP in males (r=0.9535, p=0.012) positively correlated with HIF-1α and there was no correlation of it in females (r=0.4067, p=0.2774).

Conclusion: We concluded that a positive correlation of all these kidney injury biomarkers in all patients and L-FABP in males suggested that comorbidities such as heart disease, hypertension, diabetes mellitus, sepsis and liver disease produce hypoxic cellular conditions in the sensitive medulla of the kidney, damaging it easily, and can worsen renal function unless treated properly.

Keywords:

HIF-1α, acute kidney Injury, Renal Pathology, Injury Biomarkers, NGAL, KIM-1, L-FABP, Expression Analysis

INTRODUCTION

Acute Kidney Injury (AKI) is a rapid decline in kidney function in seven days or less than three months. This sudden deterioration includes, both, the structural damage because of injury, and loss of function due to impairment of the kidney leading to the accumulation of nitrogenous waste products in the body, such as urea, creatinine, and other products.[1] Comorbidities such as heart failure, liver failure, proteinuria, hypoalbuminaemia, rhabdhomyolysis, pre-eclampsia (PE), post-partum haemorrhage (PPH), hypotension, hypovolemia, anaemia, dehydration, renal ischemia/reperfusion (I/R), hypoxia and exposure to any kind of contrast media, such as iodine, are strong risk factors for AKI. The current way of diagnosis is based on the KDIGO (Kidney Disease: Improving Global Outcomes) criteria, proposed by the Acute Kidney Injury Working Groups based on serum creatinine (SCr) and urinary output (UO).[2] The aim of treating AKI is to prevent the further reduction of glomerular filtration rate and serum creatinine. It has been hypothesized that various mechanisms such as maladaptive repair of kidney and nephrons, hyperfiltration by the kidney due to intrarenal hypertension, glomerulosclerosis, tubular interstitial hypertrophy, fibrosis of the tubules, and arteriosclersosis due to hypertension might also contribute to the development of CKD after an episode of AKI.[3]

Hypoxia Inducible Factor-1α (HIF- 1α) protein is elevated during renal fibrosis or tubulointerstitial disease. Since any risk factor like endothelial injury or vascular rarefaction can cause ischemia.[4] Its role has also been found during cell death, inflammation and regulation of fibrosis. Previous studies emphasizing the role of HIF-1 proteins mainly working to promote those cellular processes and adaptations during hypoxia which can increase oxygen concentrations in the cell, such as angiogenesis, erythropoiesis, iron metabolism, and anaerobic glucose metabolism.[5-7] HIF also executes fibrosis by regulation of fibrotic genes by transcription,

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having a chief role in the epithelial-to-mesenchymal-transition (EMT), interacting with other genes that participate in fibrosis, and epigenetic regulation.[8,9]

Neutrophil gelatinase-associated lipocalin (NGAL) is a gelatinase protein linked to the neutrophils and its secretion is induced in the kidney tubules (the thick ascending limb, loop of Henle, collecting duct) following soon after an ischemic reperfusion or injury, or due to nephrotoxins. During renal tubular damage and stress, NGAL is activated by the TLR-4 and NF-kB pathway.[10] Liver Fatty Acid Binding Protein (L-FABP) is a 14-kDa protein expressed predominantly in the renal cortex and proximal tubules. Its primary function is to bind, transport and metabolize long chain and very long chain fatty acid molecules. In addition, L-FABP can also act as a renoprotective molecule to reduce oxidative stress and human L-FABP proteins also respond to hypoxia by inducing the production of HIF-1α. Since they are efficiently absorbed by the proximal tubules by endocytosis, they are undetectable in the urine and plasma in normal circumstances.[11] However, it is reported that their expression peaks 6 hours after development of AKI and correlates with its severity. Moreover, it also correlates with the severity and duration of ischemic renal injury, especially during AKI and CKD. This can be because it binds to the lipid peroxides and transports them to the lumen of the tubules from the cytosol to protect epithelial cells from these reactive oxidative species (ROS).[12,13]

Kidney Injury Molecule-1 (KIM-1) is a transmembrane glycoprotein of molecular weight 38kDa, and is primarily expressed in the renal proximal tubules. It has a low expression in normal circumstances. However, after an ischemic/reperfusion (I/R) injury, or tubular damage, especially in AKI, its levels have reported to be elevated. Moreover, KIM-1 proteins also play a role in tubular regeneration and renal recovery. They promote the phagocytosis of debris left after apoptosis and necrosis. Due to this role, their elevated levels approximately 2-3 days after (AKI) injury can be appreciated.[14, 15]

MATERIALS AND METHODS

Samples Collection and Transportation: After an informed consent, 50 EDTA blood samples of patients diagnosed with AKI were collected from the Dialysis Ward of Nephrology Department, the Medical Wards, Surgical Wards and Gynaecology Wards from Jinnah Hospital, Lahore. After collection, the samples were placed in an ice box and transported to Forman Christian College, Lahore. For the purpose of control group, 24 blood samples of healthy individuals, comprising of 12 males and 12 females, were taken from Forman Christian College, Lahore. The sampling was done using randomized probability sampling.

Patients of both genders, age 18-80 years, patients suffering from AKI, AKI on CKD patients during dialysis were included in the study. While, patients suffering from ESRD, with renal cancer, who are dialysis-dependent, and patients who have previously undergone kidney transplantation were excluded from the study.

RNA Isolation, Quality Check and cDNA Synthesis: RNA was extracted using (Invitrogen TRIzol reagent: Catalog #15596026, USA) as per manufacturer's instructions. Quality and quantity was checked by Nanodrop 2000/2000c spectrophotometer (Thermo Scientific). Only RNAs giving 260/280 and 260/230 ratio more than 1.5 along with depiction of clear bands on agarose gel electrophoresis were processed for cDNA synthesis. Otherwise re-precipitated or extracted again. cDNA was synthesized by using a Thermo Scientific kit (RevertAid First Strand cDNA Synthesis Kit: Catalog #K1622, USA) as per the manufacturer's protocol.

Primer Designing: Primers sequences of genes for all the biomarkers were designed using NCBI, NCBI BLAST, InSilico PCR of UCSC Browser and Serial Cloner. The primers sequence are given in **Table 1.** Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR): RT-qPCR was carried out using Bio-Rad's CFX 96 qPCR system to analyze the expression of mRNA HIF-1α, VEGF-A, ANG-2, FGF-2 and uPA. It was done by using (ThermoFisher Scientific SYBR Green qPCR Master Mix: Catalog# 4309155, USA) as instructed by the manufacturer.

Estimated Glomerular Filtration Rate (eGFR) Calculation: The estimated glomerular filtration rate (eGFR) was calculated by the MDRD equation, requiring the respective age, gender, race and creatinine value of the patients: GFR (mL/mim/1.73m^2) = 175 x $(SCr)^{-1.154}$ x $(Age)^{-0.203}$ x (0.742 if female) x (1.212 if African American)

The data was analysed by Graphpad Prism 6.0. Demographic data was plotted through bar charts and pie graphs. Frequencies of relative morbid conditions were plotted as bar charts. Pearson correlation coefficient was used to document correlation in between samples. Student t-test was used to find out

Table 1. **Primers sequence**

Primer		Primer Sequence
NGAL (NM_005564.5)	Forward	5' F-CTAGGTCTCCTGTGGCT '3
	Reverse	5' R-CACATACCACTTCCCCTG '3
L-FABP (NM_001443.2)	Forward	5' F-CCGGAAGAGCTCATCCAGAA '3
	Reverse	5' R-CCACTGTCTTGACTTTCTCCCC '3
KIM-1 (NM_001173393.2)	Forward	5' F-CCTCCAATGCCTTTGCCC '3
	Reverse	5' R-CTCTGTCACGGTGTCATTCCC '3
HIF-1α (NM_001243084.1)	Forward	5' F-GTATTGCACTGCACAGGCC '3
	Reverse	5' R-TCCAGGCTGTGTCGACTGAG '3

significance between groups. A p-value ≤0.05 was taken as significant.

RESULTS

Ethical Statement: The study was approved by the Institutional Review Board (IRB-163/06-2019) of FCCU was taken initially before the commencement of the study.

Demographic Profiles, Clinical History and Routine Testing Profiles of AKI Patients: 50 patients were enrolled in this study. Blood samples were taken from all the patients to study gene expression of the selected biomarker panel. To glean a better comprehension of the impact various comorbid conditions, as well as other diseases or disorders can have on AKI patients, knowing their past and current medical history is essential as shown in **Table 2.**

RIFLE Classification: According to the RIFLE Classification, out of the enrolled AKI subjects, 8% were in the category of Risk, 10 % were in the category of Injury, Failure comprised of 50% of the patients, 20% had experienced Loss of function and 2% were in the category of ESRD as summarised in **Table 3**.

Estimated Glomerular Filtration Rate (eGFR) and classification of AKI patients: The values of serum creatinine and urea alone are not adequate enough to reflect the functional condition of the kidney. Therefore, the estimated glomerular filtration rate (eGFR) is calculated from the MDRD equation as shown in **Table 4**, based on the value of serum creatinine, age, gender and race of the patient. According to the National Kidney Foundation, the normal eGFR in adults is above 90 mL/min/1.73m^2. According to the CKD Classification as proposed by KDIGO, out of the enrolled subjects, there was no patient in the G1 category, 2% were present in the G2 category, there was no patient in the G3a category, 2% were present in the G3b category, G4 comprised of 18% and the last category of G5 included 76% patients.

Expression of HIF-1α and NGAL, in AKI on CKD male and female patients: No significant fold gene expression of HIF-1α in male and female AKI on CKD

Table 2: Demographic features, clinical history and routine testing profile of patients (n=50)

Patients	**Age (years)**	**Mean Age**	
Total (n=50)	20-76	45.44 ±17.61	
Males (n=24)	20-75	49.67 ±17.49	
Females (n=26)	20-76	41.53 ±17.13	
Previous Clinical History			
Condition	**Males (n=24)**	**Females (n=26)**	
Diabetes Mellitus	54.16%	9 (34.6%)	
Hypertension	54.16%	14 (53.8%)	
Complete Blood Count (CBC)	**Average in Males**	**Average in Females**	**Normal Value**
WBC (cells/mm^6)	8.29 ± 3.45	6.58 ± 4.07	4x10^6
Total RBC (cell/mm^3)	4.01 ±1.66	3.15 ± 0.71	4.5-6x10^6
Haemoglobin (Hb) (g/dL)	10.91 ± 2.43	8.73 ± 2.47	13-18
Platelets (mm^3)	308.29 ± 241.45	223.7 ± 180.98	150-450x10^3
Haematocrit (%)	46.0 ± 50.7	26.82 ± 6.71	
Mean Corpuscular Haemoglobin (MCH) (%)	27.0 ± 3.84	38.98 ± 47.62	27-32
Mean Corpuscular Volume (MCV) (%)	83.34 ± 9.53	79.51 ± 15.12	77-93
Neutrophils (%)	76.38 ± 7.61	70.02 ± 22.48	40-60
Lymphocytes (%)	21.59 ± 17.36	16.39 ± 12.8	20-40
Liver Function Tests (LFTs)			
Bilirubin (U/L)	0.73 ± 0.60	1.82 ± 4.42	0.3-1.2
Serum Electrolytes (SE)			
Serum Sodium Level (mg/dL)	140.1 ± 7.95	132.35 ± 27.0	135-145
Serum Potassium Level (mg/dL))	3.99 ± 0.80	4.51± 0.95	3.5-5.0

Table 3: Classification of AKI Patients according to the RIFLE Criteria by staging and severity

Stage	GFR Criteria	Urinary Output Criteria	Study Population
Risk	Serum Creatinine (SCr) increased 1.5-2 times baseline 'or' GFR decreased >25%.	UO<0.5ml/kg/h <6h	8%
Injury	SCr increased 2-3 times baseline 'or' GFR decreased >50%.	UO<0.5ml/kg/h >12h	10%
Failure	SCr increased >3 times baseline 'or' GFR decreased 75% 'or' SCr ≥4mg/dL; acute rise ≥0.5mg/dL	UO<0.3mL/kg/h 24h (oliguria) 'or' anuria 12h.	50%
Loss of function	Persistent acute renal failure: complete loss of kidney function >4week (requiring dialysis)		28%
ESRD	Complete loss of kidney function> 3 mo (requiring dialysis)		2%

Table 4: Gender-wise eGFR values (range and mean) for classification of AKI patients.

Characteristics	eGFR (mL/min/1.73m²)	
	Range	Average
Male Controls (n=12)	93-105	99 ±6
Male AKI Patients (n=24)	3-36	14.57 ±12.50
Female Controls (n=12)	87-124	105.5 ±18.5
Female AK patients (n=26)	4-62	9.57 ±7.53
Description and Range	**Categories**	**Study Population**
≥90; Normal and high	G1	0%
60-89; Mild reduction related to normal range for a young adult	G2	2%
45-59; Mild-moderate reduction	G3a	0%
30-44; Moderate-severe reduction	G3b	2%
15-29; Severe reduction	G4	18%
<15; Kidney failure	G5	76%

patients as compared to controls, was observed. Thefold expression of NGAL in male AKI on CKD patients showed a slight increase as compared to male controls, whereas in females, difference in controls and patients was little significant as shown in **Figure 1**.

Expression of KIM-1 and L-FABP in AKI on CKD male and female patients: The fold expression of KIM-1 in male patients again shows a mild increase, as compared to the controls; whereas, in females, there is no significant difference between controls and patients. As can be seen, there was a slight increase in the fold expression of L-FABP in male AKI on CKD patients as compared to their controls; however, surprisingly, there was a moderate decrease of expression in female patients as compared to their controls as shown in **Figure 2**.

Correlation analysis of HIF-1a with NGAL, KIM-1 and L-FABP in AKI on CKD patients: According to the Pearson correlation, with a significant p-value being 0.05, overall there was a significant positive correlation between the gene expressions of HIF-1α with NGAL (r value= 0.8829) and HIF1a with KIM-1 (r value=0.9199) in the enrolled patients with the same trend in both male and female subjects. Whereas with L-FABP no correlation was observed overall (r=0.4379); however, positive correlation was observed only with HIF1a in enrolled male patients (r value=0.9535) as compared to females (r=0.4067) as shown in **Figure 3**.

DISCUSSION

The superimposition of Acute-on-Chronic kidney injury has also become a serious cause of concern in patients with long-term renal function decline. Our results showed slight elevated expression in male population as compared to females. During a pathogenic infection or sepsis in extreme cases, HIF-1α is up-regulated in response to hypoxic conditions produced in the cell due to more oxygen consumption by pathogens, and to exhibit a protective effect like phagocytosis and antimicrobial activities in the cell by reprogramming of monocytes.[16] Furthermore, according to Huang and group, HIF-1α is activated in oxygen homeostasis enhancing mechanisms such as angiogenesis, to promote activity of genes for glycolysis' enzymes to increase metabolism in anaerobic conditions, and inflammatory and tumour proliferation sites, while its overproduction can also lead to apoptosis.[17] The expression of HIF-1α is down-regulated in normoxic conditions by Factor Inhibiting HIF-1α (FIH), degraded by the proteasome, by p53, activation of hydroxylases, and small molecule inhibitors such as topotecan. Most probably women have better control of the dialysis conditions as compared to stress induced in men.[18]

NGAL is up-regulated in conditions of sepsis, due to its siderophore-chelating activity, for dedifferentitation of epithelial to mesenchymal structures such as the glomeruli, proximal tubules and distal tubules, injury of epithelial cells and prognosis of cancer. Our results for NGAL are in consensus with the aforementioned study by Devarajan. Since 80% of the males suffered from sepsis and only 44% of females in our research.[14] NGAL is not expressed in normal kidneys. NGAL is up-regulated in patients with only AKI, rather than those with underlying previously present renal disease or in patients with GFR <60 ml/min/1.73m².[19] Furthermore, in our study, low fold

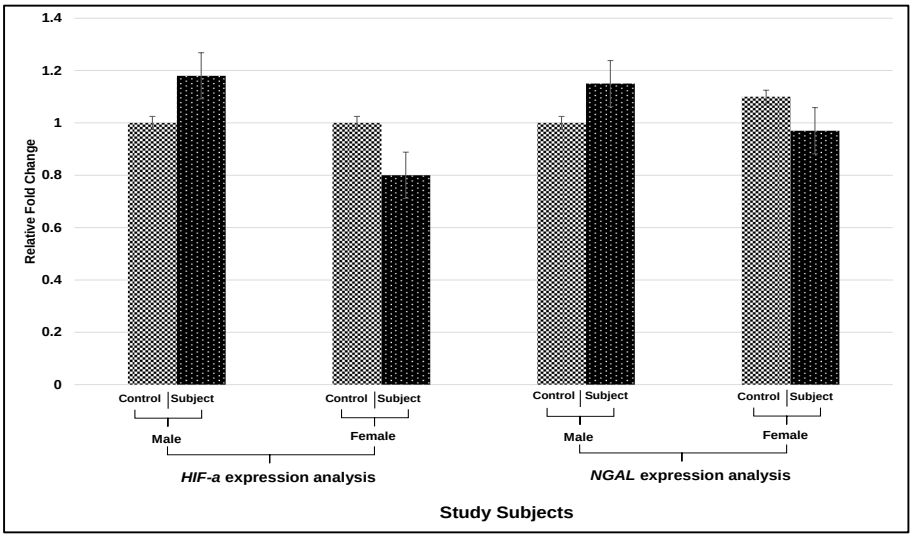

Figure 1: Mean HIF-1α and NGAL Fold Expression in AKI on CKD in male and female patients with their respective controls. p>0.05 was taken as significant.

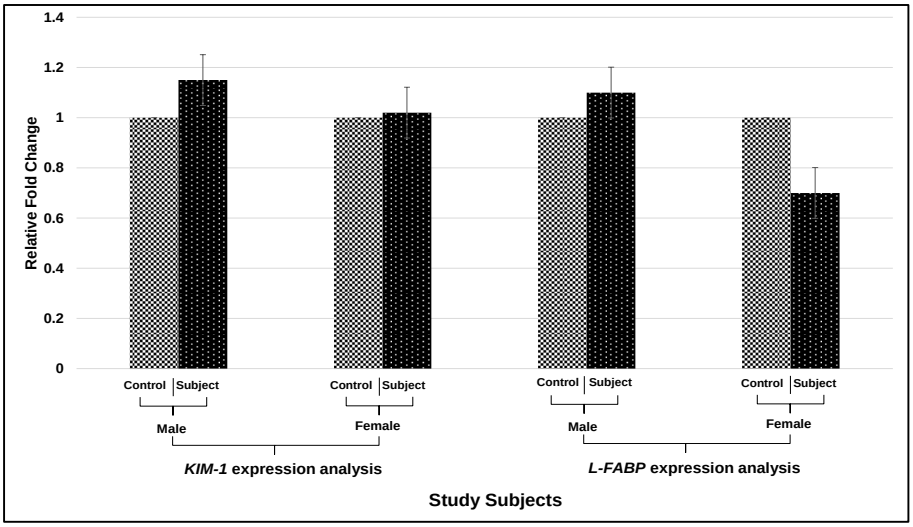

Figure 2: Mean KIM-1 and L-FABP Fold Expression in AKI on CKD male and female patients with their respective controls. P>0.05 was taken as significant.

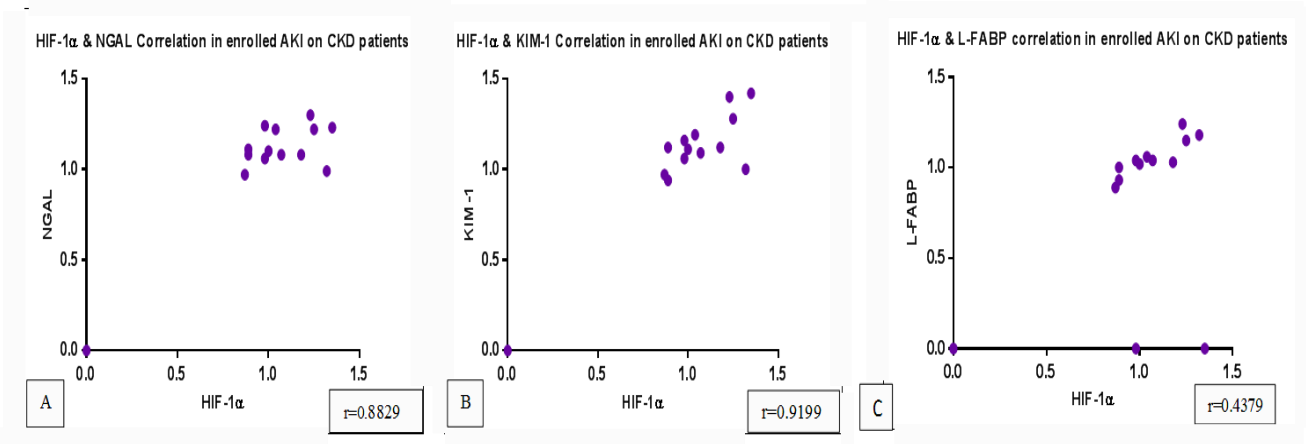

Figure 3: **A)** HIF-1α and NGAL displaying positive correlation in AKI on CKD enrolled subjects. **B)** HIF-1α and KIM-1 showing positive correlation in AKI on CKD enrolled subjects. **C)** HIF-1α and L-FABP showing no correlation in AKI on CKD enrolled subjects.

expression of NGAL in blood as compared to urine in females can also be attributed to studies which suggest that plasma NGAL, respectively, is secreted more pronouncedly into the ureters, than being introduced into blood circulation; it can also be said that most of urinary NGAL comes from loop of Henle and the collected ducts, while most of plasma NGAL comes from other sources, such as neutrophils.[20] These reasons can contribute to our overall results of not a very significant fold-expression of NGAL in patients.

KIM-1 is up-regulated in a variety of conditions in the human body, such as during autoimmune diseases, tolerance of the immune system and immune dysfunction diseases, viral infections and atopic diseases such as eczema. KIM-1 gene expression in normal kidneys is rare, but is elevated in the proximal tubules and outer medulla during acute renal injury due to toxicity, ischemia, hypoxia, polycystic kidney disease or any disease relating to the tubulointerstitium, by the ERK ½ and STAT 3 pathways known to up-regulate transcription of its genes. When cellular conditions are exposed to toxicity, ischemia or hypoxia, its gene expression is elevated and is transformed into 'semi-professional' phagocytes to promote phagocytosis and ultimately repair of the renal cells.[21,22] These studies go in line with our results of slightly increased fold expression of KIM-1 in males, because 80% of them had sepsis, whereas, only 44% females, so there was no significant increase in its fold expression. While it is only negligibly expressed in normal healthy kidneys, its expression is also down-regulated by decreased proteinuria.[23] Similar to NGAL, research shows a strong positive correlation between the gene expression of HIF-1 and KIM-1 or the latter's elevated levels in hypoxic or ischemic conditions and supports our results. One study reported increased KIM-1 levels in I/R and Lipopolysaccharide-induced sepsis renal injury; they found gene expression of KIM-1 to be up-regulated by the ERK/MARK signalling pathway to promote proliferation of the proximal tubule cells and facilitate their repair.[24]

L-FABP is primarily produced by the liver but is also expressed in kidneys, lungs, pancreas and intestines. It is produced in the tubulointerstitium to reflect damage. Furthermore, Suzuki, it has been reported that they found a significant association between L-FABP expression and diabetic nephropathy patients with Type 2 DM.[25] Similar results probably indicate that presence of diabetes is a huge contributing factor to expression of L-FABP in renal disease, which supports our results of significant correlation observed in males, but not in females, because all the five male patients suffered from DM, whereas only 55% females had this disease. Another reason for no correlation in of L-FABP and HIF-1α in females in our study can be the fact that there were no values of L-FABP for 3 out of 9 females during the experiment. Some other studies also indicate the down-regulation of L-FABP in renal disease. Jin, *et al.,* reported that they found low expression of Peroxisome proliferator-activated receptor alpha (PPRAα), involved in the signalling of L-FABP, due to which no significant gene expression of L-FABP was seen in patients with CKD.[26]

The gene expression of L-FABP correlated with elevated expression of HIF-1α in males, but not in females. Research also supports a positive correlation of both the genes. According to many studies the promoter region of L-FABP contains a HIF-1 binding site, is associated with secretion in hypoxic and septic cellular conditions, in patients after cardiac surgery, and in response to nephrotoxic agents for the kidney (such as ACEs, which might promote renal damage).[27,28] A panel of injury biomarkers can also facilitate detection of hypoxic AKI. KIM-1, and NGAL were up-regulated in both the distal and proximal tubules in mouse models with induced hypoxic AKI, which strongly suggests that their gene expression would positively correlate with the up-regulation of HIF-1α.

CONCLUSION

Our data suggests that 50% of AKI patients enrolled in our study were in the category of Failure according to RIFLE Criteria. Moreover, estimation of their GFR showed that 76% of the patients had renal failure. Although these criterion determine kidney failure in more than 50% of the patients, their serum creatinine values are not increased very significantly, indicating that serum creatinine should not be used as the 'gold standard' for diagnosis and classification of AKI. Moreover, our data also suggests that proximal injury markers like NGAL, KIM-1 and L-FABP are elevated in AKI on CKD patients, most probably because various comorbid conditions produce hypoxic conditions in the cells. Furthermore, gene fold expression of NGAL and KIM-1 of all patients with AKI on CKD showed a positive correlation with the elevation of HIF-1α, a hypoxic marker of the cell, whereas, L-FABP gene expression only in males showed a positive correlation with it, and not females. Therefore, the presence of various comorbid conditions

promote hypoxia in the renal cells and worsen renal failure, unless treated properly. Firstly, due to shortage of time, our sample size was small. Secondly, due to shortage of resources, we further reduced the number of samples and brought down our final study criteria to only Acute-on-Chronic kidney injury patients. Thirdly, most of the procedures were performed manually, which could produce some alterations in results. In addition, there was no follow-up on the condition of patients, which could have indicated recovering or worsening of their state, hence, suggesting a change in the expression of these genes.

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