ARTICLE

Hypoxia Inducible Factor 1: A Urinary Biomarker of Kidney Disease

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Identifying noninvasive biomarkers of kidney disease is valuable for diagnostic and therapeutic purposes. Hypoxia inducible factor 1 (HIF-1) expression is known to be elevated in the kidneys in several renal disease pathologies. We hypothesized that the urinary HIF-1α mRNA level may be a suitable biomarker for expression of this protein in chronic kidney disease (CKD). We compared HIF-1α mRNA levels from urine pellets of CKD and healthy subjects. To ensure that urinary HIF-1α mRNA is of kidney origin, we examined colocalization of HIF-1α mRNA with two kidney specific markers in urine cells. We found that HIF-1α mRNA is readily quantifiable in urine pellets and its expression was significantly higher in CKD patients compared with healthy adults. We also showed that the urinary HIF-1α mRNA comes primarily from cells of renal origin. Our data suggest that urinary HIF-1α mRNA is a potential biomarker in CKD and can be noninvasively assessed in patients.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Currently, evidence suggests a close relationship between hypoxic signaling via HIF-1 and pathogenesis of CKD. However, no biomarkers have yet been identified to noninvasively assess HIF-1α expression levels in patient kidneys.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ This study addresses the feasibility and validity of using urinary HIF-1α mRNA levels as a noninvasive biomarker of kidney HIF-1α expression levels.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
✔ The study shows that urinary HIF-1α mRNA is readily measurable in urine and is significantly elevated by 3.91-fold in patients with stage 3–5 CKD compared with healthy adults. Together, these findings suggest that urinary HIF-1α mRNA index could be utilized as a biomarker for HIF-1α levels in the kidneys in CKD.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
✔ Using HIF-1α mRNA levels in urine can guide and assess the effectiveness of HIF-1-modulating therapies currently in development for treatment of CKD and other renal pathologies.

Among many factors that precipitate kidney failure, hypoxia appears to be a critical underlying feature. Histological samples of diseased kidneys show extensive tubulointerstitial injury, damaged arterioles, and loss of blood flow to the kidney and hypoxia is a key feature of tubulointerstitial disease.1,2 Many studies suggest that hypoxic signaling plays a critical role in tissue response to injury at the early and late stages of kidney disease.

A key mediator of hypoxic signaling is hypoxia inducible factor 1 (HIF-1). HIF-1 is a heterodimer protein complex composed of an alpha and a beta subunit. Under normoxia, multiple oxygen-dependent catalytic steps degrade HIF-1α subunits making the protein inactive. During hypoxia, however, degradation of HIF-1α is impaired, leading to accumulation and coupling of the HIF-1α-β subunits, which allows for translocation of the protein to the nucleus and binding to hypoxia response element of DNA. HIF-1 signaling then facilitates transcription of proteins and hormones responsible for proliferation, angiogenesis, erythropoiesis, and glycolysis.3,4

HIF-1 has been extensively studied in both acute and chronic kidney diseases.5,6 At early stages of kidney disease HIF-1 activation has been shown to be protective against injury, while in chronic states the benefit of HIF-1 elevation is at best controversial, with some evidence of increased fibrosis and tissue remodeling.7 Nevertheless, it has been suggested that HIF-1 induction therapies may protect against anemia of chronic kidney disease (CKD), AKI, diabetic nephropathy, and possibly specific chronic
conditions where HIF-1 levels are altered via transcriptional and translational pathways.\textsuperscript{6,9} HIF-1 induction can be either through stabilization of the protein or gene therapy and increased expression of the protein.\textsuperscript{10,11} The effectiveness of HIF-1 therapy is weighed down by potential risks of over-activation of HIF-1 systemically. Determining the HIF-1 level and activity would best assess the effectiveness of HIF-1-modulating agents.

Quantification of HIF-1 activity is challenging in clinical studies. Undegraded HIF-1 protein is very unstable and is primarily found via immunoassays or western blot analysis of kidney tissue.\textsuperscript{12} In human studies kidney tissue is only obtained through kidney biopsies, which could be an invasive approach. In this study we propose an alternative noninvasive approach to quantify HIF-1a expression levels from kidney cells collected from urine. Use of urine cells has previously been suggested as a diagnostic tool for detection of kidney injury and allograft rejection biomarkers and methods for mRNA isolation in urine have been previously developed.\textsuperscript{13} Using a similar approach, we examined whether urinary HIF-1a expression can be readily quantified in urine. We showed a significant difference in urinary HIF-1a mRNA levels between healthy subjects and CKD patients. Our findings validate that urinary HIF-1a can be used as a marker of kidney disease for diagnostic and therapeutic purposes.

METHODS
Subject recruitment
Upon approval from the Institutional Review Board of the Bernard J. Dunn School of Pharmacy, healthy volunteers as well as CKD patients (stage III to V) were recruited into the study. Healthy subjects were recruited at the Bernard J. Dunn School of Pharmacy in Ashburn, VA, through in-person and email outreach. Patient recruitment took place at Kidney and Hypertension Specialists clinics in Manassas, VA. Patients were initially screened by healthcare providers for eligibility and a written consent was obtained from patients at their routine office visits for enrollment into the study. The study adhered to the Declaration of Helsinki. The study inclusion criteria consisted of age range of 18–95 years old and CKD stage 3–5 (estimated glomerular filtration rate (eGFR) <60 ml/min/1.73m\textsuperscript{2}) for patients and no active CKD level and activity would best assess the effectiveness of HIF-1-modulating agents.

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RNA isolation
RNA isolation was performed in a QIAcube system using the Qiagen RNeasy Plus Mini Kit. RNA quality was determined through purity and concentration measurements using a Pico100 Picodrop Spectrophotometer.

cDNA synthesis
Total RNA of each sample was converted to cDNA using the high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA). A total of 10 \(\mu\)l of RNA and 10 \(\mu\)l of reaction master mix were used in the reverse transcription experiments.

Real-time reverse-transcription polymerase chain reaction (RT-PCR)
cDNA samples were amplified using Applied Biosystems 7300 PCR (Applied Biosystems, Calsbad, CA) and the TaqMan gene expression assays protocol by Applied Biosystems. Samples were loaded onto 96-well plates in triplicate. For housekeeping gene screening a predesigned panel of 32 human endogenous control assays (TaqMan Array 96 – Well Plate Endogenous Control, Applied Biosystems) was utilized. In order to determine gene stability, pairwise comparisons were made between genes using methods described by Vandesompele et al.\textsuperscript{17} in conjunction with qbase+ (geNorm) software produced by Biogazelle (Belgium).\textsuperscript{17} To take into account the effect of varying RNA quality and amount between samples, the previously described 2\textsuperscript{−\Delta\text{Ct}} method was utilized whereby each HIF-1a sample is normalized to its corresponding ACTB values and average of individual delta Ct values were used for comparison between groups.\textsuperscript{18}
**Simultaneous fluorescence in situ hybridization (FISH) and immunofluorescence**

Simultaneous FISH and immunofluorescence was performed following the protocol provided by (Biosearch Technologies, CA). Urine pellets were washed in 1 ml 1X phosphate-buffered saline (PBS) two times and fixed with formaldehyde (3.7% in 1X RNase free PBS) for 10 min. After the fixation, buffer was removed and washed with 1X PBS the cells were then permeabilized with 70% ethanol at 4°C overnight. The following day the ethanol was removed and the cells were suspended in wash buffer (10% formamide in 2X saline-sodium citrate (SSC)) and incubated for 2–5 min. After removal of the wash buffer 100 μl of HIF-1a or Kidney Injury Protein 1 (KIM-1) Stellaris FISH probe (1 μl from 25 μM stock solution) along with Kidney Specific Protein; Cadherin-16 (KSP) primary antibody (0.5 μg/ml as final concentration) was diluted in hybridization buffer (10% formamide in 2X SSC plus 100 μg/ml of dextran sulfate) and added to the samples. The samples were then incubated at 37°C overnight. After the aspiration of hybridization buffer, the samples were suspended in wash buffer containing 10 μg/ml fluorescently labeled secondary antibody and incubated for 30 min at 37°C in the dark. One drop of mounting medium containing 1.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) was used as a DNA counterstain and mounting media. The panel of fluorescently labeled FISH probes for HIF-1a and KIM-1 were custom-designed, each composed of 30–48 oligos (20-bp) complementary to the coding sequences of these genes. The cells were imaged using an EVOS inverted fluorescent microscope and FL auto software at 10–20X magnification (Life Technologies, Bethesda, MD). Additional imaging studies were performed using wide-field confocal microscopy. Images were taken with a Zeiss 510 confocal microscope using an oil-immersed 40X objective and captured using Zen 9 software.

**Data and statistical analyses**

Relative quantification of HIF-1a was determined using the 2−ΔΔCt method. Differences between groups were determined using Student’s t-test on the delta Ct. mean values. Correlation analysis of KSP and HIF-1a expression values was performed using Pearson correlation. All P-values were two-sided, and P < 0.05 was considered statistically significant. Calculations were carried out using the SPSS 22.0 statistical software (IBM, Armonk, NY).

**RESULTS**

Characteristics of study population

(Table 1) describes the baseline characteristics of the healthy (N = 27) and CKD patient (N = 50) populations whose samples were included in all data analyses. All patients and healthy subjects met the inclusion criteria and were enrolled over a period of 2 years. Numbers are broken down based on age, race, eGFR, and comorbid conditions. Patients were of CKD stages III–V with mean eGFR of 37 ± 16 in comparison with healthy subjects with mean eGFR of 97 ± 20. Due to weighing of age in the eGFR calculation, inclusion of healthy subjects resulted in a significantly younger age than the CKD population (32 ± 8 vs. 70 ± 10).

### Table 1 Patient Demographics

| Feature            | Healthy (n = 27) | CKD (n = 50) |
|--------------------|-----------------|--------------|
| Gender, n (%)      |                 |              |
| Male               | 13 (48)         | 29 (58)      |
| Female             | 14 (52)         | 21 (42)      |
| Age (yr)           | 32 ± 8          | 70 ± 10      |
| Race (%)           |                 |              |
| Caucasian          | 15 (55)         | 29 (58)      |
| African American   | 1 (4)           | 13 (26)      |
| Asian              | 8 (30)          | 2 (4)        |
| Hispanic           | —               | 2 (4)        |
| Other              | 3 (11)          | 4 (8)        |
| GFR (ml/min/1.73m²)| 97 ± 20         | 37 ± 16      |
| Comorbidities n (%)|                 |              |
| Diabetes           | —               | 28 (56)      |
| Hypertension       | —               | 45 (90)      |
| Anemia             | —               | 19 (38)      |
| Cardiovascular     | 1 (6)           | 16 (32)      |
| Respiratory        | —               | 5 (10)       |

CKD, chronic kidney disease; GFR, glomerular filtration rate.

**Detection of HIF-1A mRNA in urine**

Urine mRNA levels of HIF-1A were determined by total RNA isolation from urine pellets and real-time RT-PCR experiments. We examined HIF-1A mRNA isolated from urine samples obtained from healthy individuals as well as CKD patients. HIF-1A mRNA levels were consistently detectable with average cycle threshold (Ct) values for HIF-1A ranging from 31.47 ± 1.82 and 31.34 ± 1.57 in healthy and CKD urine, respectively. On average, the concentration of urinary RNA was 8.73 ± 3.52 ng/μl, for healthy urine and 10.7 ± 5.36 ng/μl for CKD urine (N = 45 and 79, respectively).

**Identification of the most stable urinary reference genes**

A number of reference genes have been reported in published studies evaluating gene expression by RT-PCR analysis utilizing urine samples. However, we encountered variable results using these previously reported reference genes, particularly in frozen samples. In order to determine which reference genes would be the most stable under our experimental conditions we utilized a predesigned panel of 32 human endogenous control assays (TaqMan Array 96 – Well Plate Endogenous Control, Applied Biosystems). Supplemental Table 1 lists the 32 genes included in triplicate in this panel. The potential reference genes were first evaluated in both fresh samples from three healthy individuals. Among the 32 genes evaluated, only 6 genes (ACTB, B2M, GAPDH, POLR2A, PES1, and UBC) had consistently detectable Ct values in fresh urine samples, with a standard deviation of less than 1.5 Ct units. We then evaluated these six genes in frozen urine samples from 10 healthy individuals. The samples were frozen at −80°C for 2–8 days and then processed. The six genes were also evaluated in a similar manner in frozen samples from 10 patients with CKD. The average raw Ct values for the six potential reference genes ranged from 29.13 ± 2.69 to 33.58 ± 4.64 for frozen urine from 10 healthy subjects, and 30.08 ± 3.02 to 30.79 ± 3.87 cycles for the 10 CKD patients (Figure 1a,b).
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Figure 1 Average raw Ct values from six candidate housekeeping genes and HIF-1α obtained in urine samples. (a) Ct values for freeze/thaw processed urine from healthy subjects. (b) Ct values for freeze/thaw processed urine from CKD patients. N = 10 in each group. Boxes represent lower and upper quartiles of cycle threshold. Whiskers indicate 10th and 90th percentiles. (c) Reference gene stability in healthy and CKD frozen urine. Stability M values were obtained using pairwise comparisons of endogenous control genes for six genes (UBC, PES1, POLR2A, B2M, GAPDH, ACTB).

To further establish the stability of the six potential reference genes we used the methods described by Vandesomple et al. and used in qbase+: (geNorm) software produced by Biogazelle, with threshold M value of 1.5 as the upper limit of gene stability. Evaluation of the frozen samples based on pairwise comparison resulted in the exclusion of PES1, POLR2A, and B2M, leaving only ACTB and GAPDH with an M value of less than 1.5 in healthy subjects. However, in the CKD patients the M values of ACTB and B2M were lower than GAPDH (M values 1.1 vs. 1.7 for GAPDH) (Figure 1c). As a result, we concluded that among the three remaining genes, ACTB showed the most consistent expression stability in frozen samples of both patients and healthy subjects and would be suitable as the reference gene for relative quantification of HIF-1α in urine.

Comparison of HIF-1α mRNA in healthy and CKD subjects
Among the enrolled subjects we were able to successfully quantify mRNA for 27 healthy adults and 50 patients. To compare HIF-1α expression levels in healthy and CKD patient populations, we performed real-time RT-PCR quantification of urine HIF-1α mRNA. Figure 2a,b depicts the difference in urinary HIF-1α expression between groups. CKD patients had a 3.91-fold higher expression of HIF-1α mRNA in their urine with mean RQ value of 1.33 ± 0.29 compared with 0.34 ± 0.07 in healthy subjects. Statistical analysis was performed on the mean delta Ct. Mean values between the CKD and the control groups and P value was shown to be 0.02.
immunofluorescent staining produced the anticipated pattern of mRNA and protein localization, including the intense nuclear and cytoplasmic spots indicative of active transcription from mRNA; and protein immunostaining indicative of kidney-specific tubular cells shedded in urine (Figure 3a–d).

The spots corresponding to mRNAs and protein overlapped only in cells with morphology similar to those identified by KIM-1 FISH probes (Figure 4e,f). Cells that did not have strong fluorescent signals were primarily of transitional epithelial morphology (Figure 4a–d). Taken together, our data suggest that the expression of KIM-1 and HIF-1a strongly correlates with tubular cells as compared with transitional epithelial cells of bladder and urogenital origins.

**Correlation of KSP and HIF-1a urine mRNA**

To further establish the degree of correlation of HIF-1a urinary expression with the kidney marker KSP, in urine shed cells we performed real-time RT-PCR analysis of patient urine samples using KSP and HIF-1a primer-probes. Relative quantification was performed using ACTB as endogenous control using the previously defined $2^{-\Delta\Delta Ct}$ method. HIF-1a and KSP were closely correlated with a correlation coefficient $r = 0.58$, $N = 14$ (Figure 3e).

**Correlation of HIF-1a mRNA with serum creatinine**

We additionally performed a comparison between serum creatinine levels of CKD patients and their corresponding urinary HIF-1a mRNA values. Figure 5 depicts these results, showing a weak correlation between the two markers ($r = 0.26$).

**DISCUSSION**

In this study we demonstrated that HIF-1a mRNA is differentially expressed in healthy vs. CKD patient urine and can be utilized to extrapolate HIF-1a expression levels in the kidneys for diagnostic and therapeutic purposes.
HIF-1α mRNA in human urine

An important observation we made was that HIF-1α mRNA was consistently quantifiable in urine. One limiting factor in using urine as a source of mRNA is viability of the samples for pellet and RNA isolation. Factors such as urine clarity, concentration of shed cells, as well as freeze-thaw processing of urine affect sample viability. The other limiting factor is stability of housekeeping genes in frozen urine for normalization. We found that several commonly used housekeeping genes had variable expression levels in cells shed in urine in CKD and healthy urine samples. Immediate processing of the urine samples may in part help both HIF-1α and housekeeping gene stability. Recently, other urine mRNA isolation kits have become commercially available that allow for more rapid mRNA isolation from urine in clinical settings. These new methodologies will increase yield of urine mRNA analysis for diagnostic purposes. In our study we conducted a thorough analysis of a number of housekeeping genes found in urine pellets of both healthy and CKD patients and found ACTB to be the most stably expressed housekeeping gene under frozen conditions for normalization (Figure 2).

Quantification of HIF-1α mRNA in urine accords with high expression levels of HIF-1α in the kidneys and confirms that urine is a valuable source for quantifying renal HIF-1α expression in both healthy and kidney disease conditions. Our imaging studies confirmed that HIF-1α mRNA is colocalized with kidney markers KSP (Figure 3) and KIM-1 (Figure 4) in urine shed cells. Although urinary cells are composed of various cell types, including epithelial cells of the bladder and urethra as well as hematopoietic cells, studies suggest that HIF-1α is overexpressed in hematopoietic cells during active cancer or infections. Similar observations have been made in bladder cancer or bladder outlet obstruction. In our study population, active cancer, conditions of bladder, or active infection were excluded. We specifically showed in our imaging analysis that transitional epithelial cells of most likely of bladder or urethral origin do not express HIF-1α as readily. In Figure 4a-f we show that the FISH signals for both KIM-1 and HIF-1α are primarily observed in kidney tubular morphology compared with transitional epithelial morphology. To further support our hypothesis that the quantified HIF-1α is primarily from the kidney, in a subgroup of CKD patients we correlated HIF-1α mRNA relative expression with that of KSP and found a strong correlation between HIF-1α and KSP mRNA levels (Figure 3e). These data indicate that the quantified HIF-1α mRNA levels in urine are most likely of kidney origin.

Increased expression of HIF-1α mRNA in CKD patient urine

In this study, we showed that HIF-1α mRNA expression is significantly higher in CKD patients in comparison with healthy individuals. CKD in our patient population was primarily diagnosed secondary to hypertension and diabetes, with the most common other comorbidities being cardiovascular and respiratory diseases. It is noteworthy that we enrolled control individuals with average age of 32 ± 20 years who were significantly younger than our patient group age (70 ± 16 years). Although this may introduce an unmatched age limitation to our study, in order to establish a true baseline for urinary HIF-1α mRNA and to adhere to the definition of eGFR of ≥90 ml/min/1.73m², we limited our control group to healthy kidneys with no comorbidities or age-related eGFR decline.

Previous studies have shown induction of HIF-1 in models of acute and chronic kidney injury. It has been shown that HIF-1 induction produces variable effects at different stages of kidney injury. Acute kidney injury studies show induction of HIF-1 may promote adaption to acute hypoxia and protection of renal tubular tissue. On the contrary, in models of chronic kidney injury, prolonged HIF-1 induction has been shown to induce progression of tubulointerstitial damage and fibrosis. The progressive physiological effects of HIF-1 in kidney injury have important diagnostic and therapeutic implications. In this study for the first time we showed that urinary mRNA expression HIF-1α subunit of HIF-1 complex is increased by 3.91-fold in CKD patients of stage III–V compared with controls. This finding is consistent with previous animal models of chronic kidney injury and suggests the potential involvement of HIF-1 in progression of chronic kidney disease. Additionally, transcriptional regulation of HIF-1α mRNA via molecules such as angiotensin II may greatly alter HIF-1α expression in the kidneys. Additional studies taking into account the transcription regulation mechanisms of HIF-1α may better elucidate the specific association of HIF-1 with different kidney pathologies.

Recently, HIF-1α stabilizing as well as HIF-1 gene induction therapies have been utilized as therapeutic modalities for conditions such as anemia of kidney disease and wound healing. Given the growth of HIF-1-modulating therapies, noninvasive measures of HIF-1 expression and activity is warranted.

The field of nephrology is ever in search of noninvasive suitable biomarkers of kidney disease. Currently, the only standardized biomarker is serum creatinine, which is nonspecific, has a delayed rise compared with timeline of acute kidney injury, and is affected by many factors such as age, muscle mass, diet, and hydration status.

In this study we explored the relationship between HIF-1α expression and serum creatinine levels and found a weak correlation between urine HIF-1α mRNA and SrCr (Figure 5). This finding is not surprising, and further demonstrates that...
serum creatinine is a nonspecific indicator of renal function and does not correlate with specific kidney injury processes such as hypoxia. More specific urinary biomarkers of chronic kidney injury are needed to examine a correlation with HIF-1α mRNA levels.

Among renal pathologies are oxidative stress, inflammation, structural disease, and fibrosis. A number of molecules are activated in the kidneys under oxidative stress and inflammation. Among such are microRNA, which have recently received a great degree of attention as biomarkers and studies have shown upregulation and signaling via several microRNAs during various types of kidney pathologies. MicroRNA knockout models show protection against renal injury and inhibitors of microRNA show improvement of renal function. Therefore, urinary microRNA mRNA measurement has been suggested as a biomarker of kidney disease. In a similar fashion, HIF-1 upregulation and signaling has been noted in various kidney pathologies. HIF-1 is especially relevant to oxidative stress and inflammation. As mentioned before, reactive oxygen species and inflammatory mediators enhance HIF-1α gene expression. Interestingly enough, various microRNAs also regulate HIF-1α transcription and vice versa under hypoxia. Therefore, using HIF-1α mRNA as a biomarker in the subgroup of ischemia, oxidative stress, and inflammatory renal injury would be of significant value. HIF-1α mRNA can provide information regarding the degree of HIF-1 transcriptional activation under chronic and acute oxidative and inflammatory injury and can help select the use of HIF-1 targeted therapies. Additionally, according to our findings, HIF-1α mRNA is a suitable noninvasive biomarker, as it is readily obtained in urine samples, which clearly validates its advantage over more invasive diagnostic markers.

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Conflict of interest. The authors declared no conflict of interest.

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