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Urinary NGAL Marks Cystic Disease in HIV-Associated Nephropathy

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ABSTRACT

Nephrosis and a rapid decline in kidney function characterize HIV-associated nephropathy (HIVAN). Histologically, HIVAN is a collapsing focal segmental glomerulosclerosis with prominent tubular damage. We explored the expression of neutrophil gelatinase-associated lipocalin (NGAL), a marker of tubular injury, to determine whether this protein has the potential to aid in the noninvasive diagnosis of HIVAN. We found that expression of urinary NGAL was much higher in patients with biopsy-proven HIVAN than in HIV-positive and HIV-negative patients with other forms of chronic kidney disease. In the HIV-transgenic mouse model of HIVAN, NGAL mRNA was abundant in dilated, microcystic segments of the nephron. In contrast, urinary NGAL did not correlate with proteinuria in human or in mouse models. These data show that marked upregulation of NGAL accompanies HIVAN and support further study of uNGAL levels in large cohorts to aid in the noninvasive diagnosis of HIVAN and screen for HIVAN-related tubular damage.

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In 2007 alone, there were 2.7 million new infections with HIV and 2 million HIV-related deaths worldwide.[1] An important complication of HIV is a form of kidney disease called HIV-associated nephropathy (HIVAN), which occurs predominantly in patients of African descent.[2–4] The prevalence of HIVAN may be as high as 15% of HIV patients (based upon autopsy data), and 4232 new cases of HIVAN reached ESRD between 2002 and 2006 in the United States.[5]

HIVAN is a rapidly progressive form of chronic kidney disease (CKD) characterized by nephrotic range proteinuria. Kidney biopsies demonstrate histologic abnormalities in both glomeruli and tubules, including collapsing FSGS, podocyte proliferation and dedifferentiation, tubular dilation, microcyst formation, and tubulointerstitial inflammation.[6,7] The pathogenesis is believed to be due to dysregulation of podocytes and tubular epithelia by HIV-1 itself.[8,9] Early identification of HIVAN is important because highly active antiretroviral therapy (HAART), corticosteroids, and inhibition of renin–angiotensin may delay disease progression[6,10,11]. Nonetheless, because HIV infection may be associated with other glomerular diseases, definitive diagnosis of HIVAN requires a kidney biopsy. In fact, half of all patients with presumed HIVAN demonstrated different types of lesions once biopsied.[11,12]

Neutrophil gelatinase-associated lipocalin (NGAL) is a 22-kD protein that is markedly upregulated in renal tubules and urine (uNGAL) in response to epithelial damage.[13–15] Expression of NGAL peaks 12 h after acute injury[14,15] but remains elevated if injury is severe.[16] In our study of 650 patients presenting to an inner-city emergency department, we found that a single, spot uNGAL test could distinguish ongoing injury from physiologic changes in renal function found in prerenal azotemia and from periods of slow or limited progression found during the course of many types of CKD ("stable CKD").[13] However, in

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that study, we noted that one CKD patient later diagnosed with HIVAN (T. L. Nickolas, unpublished observations) had markedly elevated levels of uNGAL, suggesting that HIV-associated tubular disease regulated its expression.

To test the association of uNGAL and HIV, we examined a cohort of 13 patients with biopsy-proven HIVAN. Biopsied or autopsied kidneys showed global (41% of nephrons per section), collapsing (19%), and segmental (12%) glomerulosclerosis and tubular atrophy (48%) and microcysts (18%), consistent with HIVAN. This group was compared with 24 race-matched HIV-positive controls with normal kidney function, defined as an estimated GFR (eGFR) ≥60 ml/min and no evidence of proteinuria. Comparisons were also made to HIV-positive and HIV-negative cohorts with other forms of CKD.

Patients with HIVAN had significantly lower CD4 counts and higher viral loads, serum creatinine levels, and proteinuria compared with HIV-positive, race-matched controls (Table 1). In HIVAN, uNGAL was upregulated 11-fold in comparison with that of HIV-positive race-matched controls without kidney disease (mean values, 748 ± 1160 μg/g creatinine in HIVAN *versus* 68 ± 98 μg/g creatinine without HIVAN; *P* value 0.006). Furthermore, in comparison with HIV-positive patients with CKD of non-HIVAN etiology (CKD, HIV-positive), uNGAL was upregulated approximately fivefold regardless of race-matching (Table 2). Moreover, uNGAL was 34-fold higher in patients with HIVAN compared with HIV-negative patients with CKD secondary to membranous nephropathy (n = 16), non-HIVAN FSGS (n = 7), or diabetic and hypertensive kidney diseases (n = 12).[13] In fact, uNGAL levels in patients with HIVAN were more typical of patients presenting to an inner-city emergency department with acute kidney injury (AKI) (n = 30, Table 2) than those in the same emergency department with stable CKD (CKD, HIV-negative, n = 106).

The enhanced expression of uNGAL in HIVAN was not due to higher levels of serum creatinine, lower eGFR, or proteinuria. uNGAL showed no correlation with eGFR in HIVAN (r = 0.082, P = 0.8), whereas it was inversely related to eGFR in two other forms of CKD (Figure 1), membranous nephropathy (r = −0.665, P = 0.004), and FSGS (r = −0.753, P = 0.03). In fact, five HIVAN patients with relatively preserved kidney function (serum creatinine <2, mean eGFR 79.4, range 117.35 to 56.22) and limited proteinuria (0.68 g/L, range 0.0 to 3.0 mg/ml) demonstrated markedly elevated levels of uNGAL (mean 401 ng/ml, range 42 to 1285 ng/ml), suggesting that uNGAL can be expressed early in the course of progressive renal failure due to HIVAN. Consistently, there was no correlation between uNGAL and proteinuria (r = −0.28, P = 0.4). In contrast, uNGAL levels were significantly correlated with viral load (R = 0.469, P = 0.005), and there was a suggestion that uNGAL was suppressed by HAART (three of the patients with low levels of uNGAL were receiving HAART). In summary, rather than renal failure itself, characteristics of HIVAN appeared to accelerate uNGAL expression. The lack of association between uNGAL and glomerular functional markers (eGFR and proteinuria) implied that HIVAN stimulated uNGAL at a tubular site.

Because HIV-transgenic mice (TgFVB)[17] display a syndrome identical to HIVAN[18] and lack the confounds typically present in human cohorts, we measured NGAL expression in kidneys of TgFVB and wild-type (WT) littermates (Affymetrix, Mouse Genome 430 2.0 microarrays; Geo Accession Number Series GSE14221). NGAL was one of the most highly upregulated genes among 39,000 transcripts, demonstrating 62- and 109-fold increases at 6 and 8 wk, respectively (Figure 2A). After GC content-robust microarray averaging (GC-RMA) normalization of TgFVB and WT samples, NGAL was the most upregulated gene (TgFVB *versus* WT, $P = 1.4 \times 10^{-70}$). In fact, out of 23 proven AKI and 35 proven

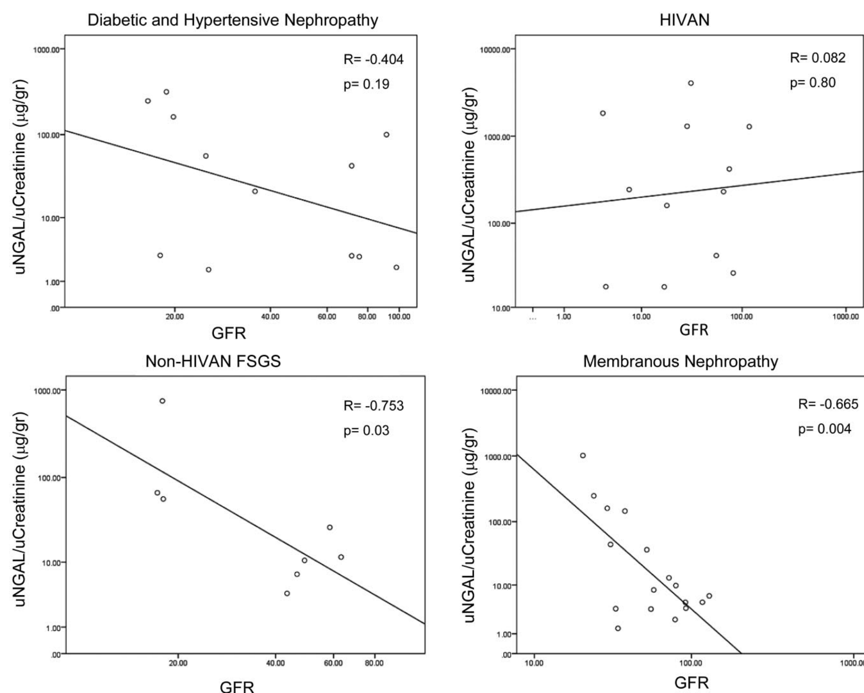

Figure 1. uNGAL levels displayed as a function of eGFR in patients with CKD due to HIVAN, non-HIVAN FSGS, and diabetic and membranous Nephropathies. There was no correlation between the GFR and uNGAL in the HIVAN population (r = 0.082, P = 0.8) or the patients with diabetic nephropathy (r = −0.348, P = 0.4), but a significant correlation existed between GFR and uNGAL for both non-HIVAN FSGS (r = −0.753, P = 0.03) and membranous nephropathy (r = −0.665, P = 0.00). All data were log-transformed.

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Table 1. Mean values and cohort characteristics for HIV-positive patients with HIVAN or race-matched controls[a]

	All Patients (n = 37)	HIVAN (n = 13)	HIV-Positive Race-Matched Controls (n = 24)
Age (yrs)	46 (10)	42 (9.6)	48 (10)
Male (%)	68	62	71
Hepatitis B (%)	13	10	14
Hepatitis C (%)	33	10	45
eGFR(ml/min)	99 (57)	42 (37)	128 (42)[b]
Creatinine (mg/dl)	2.3 (4.0)	5.3 (6.1)	0.8 (0.2)[b]
Proteinuria (mg/dl)	0.5 (1.3)	1.3 (1.9)	0.0 (0.0)
CD4 (cells/mm^3)	277 (324)	160 (258)	340 (342)[c]
Viral load (IU/ml)	121,755 (224,675)	232,535 (279,496)	73,591 (182,928)[b]
uNGAL (μg/g creatinine)	307 (750)	748 (1160)	68 (98)[b]

[a]Continuous data were log-transformed prior to statistical testing.
[b]P value <0.05 for comparison between HIVAN and HIV-positive race-matched controls.
[c]P value <0.01 for comparison between HIVAN and HIV-positive race-matched controls.

hypoxia-specific genes,[19] (Figure 2, A and B) NGAL was the most differentially expressed gene, fivefold higher than the next best gene and 100-fold higher than most others. We confirmed the expression of NGAL in HIVAN kidneys using real-time-PCR (Figure 3A) and the presence of NGAL in the urine by immunoblot (Figure 3B). NGAL mRNA and uNGAL protein increased over time with the progression of kidney disease (Figure 3, A and B).

TgFVB mice excrete as much as 10 g/L of high-molecular-mass proteins commonly seen in other glomerular diseases (Figure 3B).[17] In addition, as with other types of nephrosis, the proximal tubule demonstrated Prussian-blue-positive iron accumulation, presumably the result of the capture of iron-bearing proteins from the filtrate (Figure 3C). Given that both proteinuria and iron filtration have been implicated as agents that damage the proximal tubule,[20] we examined whether NGAL was expressed at this site. Surprisingly, *in situ* hybridization showed that NGAL mRNA was not expressed in the proximal tubule, where stainable iron was found, but rather in aquaporin-2-positive collecting ducts of TgFVB mice (Figure 3C). Although this may suggest that NGAL was activated downstream of the site of injury, NGAL was prominently expressed in dilated microcystic tubules rather than homogenously throughout a specific nephron segment, which would be typical of, for example, ischemia–reperfusion injury.[16] NGAL mRNA was detected in 39% of microcysts (n = 2698 microcysts), in both medulla and cortex. In contrast, NGAL mRNA was not detected in noncystic tubules. This unexpected finding suggested that NGAL was likely induced by a local stimulus associated with cystogenesis rather than a global stimulus such as proteinuria, luminal debris, urinary iron, or ischemic injury. In this regard, it was notable that, although proteinuria was constant between 6 and 8 wk of life, NGAL expression increased over time, consistent with an increase in cyst number per area of section as these mice age (Figure 3D).

In the present study, we found that uNGAL is highly activated in HIVAN. Yet unlike our previous findings in CKD, uNGAL achieved levels more typical of AKI. The elevated levels of uNGAL may originate from the filtrate, but in our study, there was no correlation between uNGAL and proteinuria in either human or mouse HIVAN. Similarly, in a larger cohort of patients with biopsy-proven CKD of various etiologies (M. Sise, L. Allegri, and T. L. Nickolas, unpublished observations), there was no correlation between proteinuria and uNGAL (Pearson correlation, $r = 0.085$, $P = 0.5$, $n = 88$ patients), suggesting that sources of NGAL other than the glomerular filtrate contribute to uNGAL. In HIVAN, we identified this source as cystic tubular epithelia. This finding is consistent with the observation that uNGAL is expressed in patients with poly-

Table 2. Median values of kidney injury biomarkers: HIV-positive patients with HIVAN and other cohorts[a]

	NGAL (μg/g creatinine)	Serum Creatinine (mg/dl)	GFR (ml/min)	Protein/Creatinine Ratio
Control, HIV-positive, race-matched (n = 24)	28 (4–481)[c]	0.9 (0.4–1.2)[c]	116 (83–251)[c]	0.0 (0.0–0.0)
Control, HIV-positive, non-race-matched (n = 14)	74 (5–361)	0.8 (0.4–1.0)[c]	114 (83–246)[c]	0.0 (0.0–0.3)[c]
CKD, HIV-positive, HIVAN (n = 13)	231 (18–4050)	2.6 (0.9–18.5)	30 (4–117)	0.6 (0.0–9.6)
CKD, HIV-positive, race-matched (n = 6)	88 (5–374)	2.1 (1.1–3.4)	37 (24–190)	0.2 (0.0–8.2)
CKD, HIV-positive, non-race-matched (n = 10)	51 (19–225)	1.5 (0.3–2.2)	57 (13–344)	0.5 (0.0–9.2)
CKD, HIV-negative (n = 106)	12 (1–344)[c]	1.5 (1–12.6)	43 (5–90)	N/A
CKD, HIV-negative, membranous (n = 16)	8 (1–248)[c]	1.3 (0.5–3.2)[b]	56 (21–128)	4.5 (1.0–15.3)[b]
CKD, HIV-negative, FSGS (n = 7)	19 (4–67)[b]	1.2 (1.1–3.7)	73 (17–63)	1.9 (1.0–2.4)
CKD, HIV-negative, diabetic and hypertensive kidney diseases (n = 12)	32 (2–316)[b]	2.7 (0.7–3.6)	26 (18–98)	2.3 (0.2–27.9)
AKI, all causes (n = 30)	296 (11–1833)	3.9 (0.7–28.6)	16 (2–53)	N/A

[a]All data presented as median (range). N/A, not available.
[b]P value <0.05 in comparison with the HIVAN cohort.
[c]P value <0.01 in comparison with the HIVAN cohort.

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Gene title	Gene Symbol	AffyProbeSet ID
Adrenomedullin	Adm	1416077_at
Bone morphogenetic protein 7	Bmp7	1418910_at
Clusterin	Clu	1454849_x_at
cAMP responsive element BP1	Creb1	1452901_at
Cysteine rich protein 61	Cyr61	1416039_x_at
Fibroblast growth factor 2	Fgf2	1449826_a_at
Glutathione S-transferase α1	Gsta1/2	1421041_s_at
Hepatitis A virus cellular receptor 1	Havcr1	1434716_at
Hypoxia inducible factor 1α	Hif1a	1427418_a_at
Heme oxygenase (decycling) 1	Hmox1	1448239_at
Insulin-like growth factor 1	Igf1	1452014_a_at
Insulin-like growth factor 1 receptor	Igf1r	1452982_at
Interleukin 18	Il18	1417932_at
Interleukin 6	Il6	1450297_at
Lipocalin 2	Lcn2	1427747_a_at
N-acetyl galactosaminidase α	Naga	1449043_at
Na+/H+ exchanger domain 1	Nhedc1	1432174_a_at
Phosphodiesterase 4B, cAMP	Pde4b	1422473_at
Selectin	Selp	1420558_at
Suppressor of cytokine signaling 3	Socs3	1455899_x_at
Secreted phosphoprotein 1	Spp1	1449254_at
Vimentin	Vim	1438118_x_at
Wingless-related integr.site 4	Wnt4	1441687_at
Aldolase 1, A isoform	Aldoa	1416921_x_at
BCL2/E1B interacting prt1 NIP3	Bnip3	1422470_at
Carbonic anhydrase 10	Car10	1431288_at
Carbonic anhydrase 11	Car11	1424379_at
Carbonic anyhydrase 12	Car12	1428485_at
Carbonic anhydrase 13	Car13	1421307_at
Carbonic anhydrase 14	Car14	1450725_s_at
Carbonic anhydrase 15	Car15	1449234_at
Carbonic anhydrase 2	Car2	1448752_at
Carbonic anhydrase 3	Car3	1449434_at
Carbonic anhydrase 4	Car4	1448949_at
Carbonic anhydrase 5a, mito	Car5a	1419525_at
Carbonic anhydrase 5b, mito	Car5b	1447928_at
Carbonic anhydrase 7	Car7	1443824_s_at
Carbonic anhydrase 8	Car8	1424958_at
Carbonic anhydrase 9	Car9	1427013_at
Connective tissue growth factor	Ctgf	1416953_at
Chemokine receptor 4	Cxcr4	1448710_at
Endothelin 1	Edn1	1451924_a_at
Enolase 1, alpha non-neuron	Eno1	1419023_x_at
Erythropoietin receptor	Epor	1423344_at
FMS-like tyrosine kinase 1	Flt1	1419300_at
Heme oxygenase (decycling) 1	Hmox1	1448239_at
Lactate dehydrogenase A	Ldha	1419737_a_at
Nitric oxide synthase 1 neuronal	Nos1	1422949_at
Nitric oxide synthase 1 adaptor	Nos1ap	1437485_at
Nitric oxide synthase 3 endo	Nos3	1422622_at
Nitric oxide synthase inter prot	Nosip	1418965_at
Solute carrier family 2 member1	Slc2a1	1434773_a_at
Transferrin receptor	Tfrc	1452661_at
Tissue inhibitor metalloproteinase1	Timp1	1460227_at
Transferrin	Trf	1425546_a_at
Transferrin receptor 2	Trfr2	1425381_a_at
Vascular endothelial growth factor a	Vegfa	1420909_at
Xanthine dehydrogenase	Xdh	1451006_at

Figure 2. (A) Acute kidney injury (blue), hypoxia-associated genes (green), and NGAL (red) in kidneys of 8-wk-old HIVAN and age- and sex-matched WT littermate mice. (B) Table of AKI and hypoxia genes.

COLOR

cystic kidney disease,[21] especially in those with rapid cyst enlargement.

We first identified NGAL as a tubulogenic factor,[22] a property supported by studies in cell lines.[23] Likewise, in adult kidney,[14] thyroid cells,[24] and gastric epithelia,[25] NGAL acts as a growth factor, whereas in other cells, such as polycystic-kidney-disease-related renal cysts, NGAL may serve as a proapoptotic factor.[26] A common growth mechanism may underlie these phenomena, perhaps with iron loaded NGAL,[22,24] but exact mechanisms are unclear. Nonetheless, it is striking that proliferation and apoptosis have been noted in HIVAN cysts,[27] and the intensive expression of NGAL at these sites implies that one or both of these activities might be modulated by NGAL. Further analysis must be based on mouse models that place the NGAL knockout allele on the HIVAN background.

In conclusion, uNGAL was markedly elevated in patients with biopsy-proven HIVAN and in a mouse model of HIVAN. NGAL was produced by tubular cysts and secreted into the urine. These data suggest the possibility that uNGAL may be useful to monitor the formation of renal tubular cysts and consequently distinguish HIVAN from common forms of CKD, such as diabetes or hypertension, or other glomerulopathies presenting in the HIV patient. In this light, the very high levels of uNGAL associated with HIVAN may provide a rationale for biopsy and aggressive HAART therapy to prevent the progression of HIVAN to ESRD.[28] However, this proposal requires further testing in a large cohort where we can determine the temporal relationships between NGAL expression and disease onset and between NGAL expression and HAART.

CONCISE METHODS

Patients

This protocol was approved by the Institutional Review Board of Columbia University. Deidentified urine samples from patients with biopsy-proven HIVAN were obtained from Mount Sinai School of Medicine ($n = 4$) and the Manhattan HIV Brain Bank ($n = 9$),[29]. Urine samples were collected at the time of kidney biopsy ($n = 2$), between 20 and 36 mo after kidney biopsy ($n = 2$), or between 0.3 and 37 mo before autopsy ($n = 9$) and then frozen at -80°C. HIV-positive controls and HIV-positive patients with other biopsy-proven CKD were derived from the same cohorts as HIVAN patients. HIV-positive controls divided into race and nonrace groups are presented separately in Table 2. HIV-positive controls had normal kidney function (defined by an estimated Modification of Diet in Renal Disease GFR of $\geq$60 ml/min),[30] lacked proteinuria, and had no clinical evidence of HIVAN. Investigators responsible for the laboratory and statistical analysis (C.S.F. and T.L.N.) were blinded to the clinical diagnosis of both patients and controls.

For comparison of uNGAL expression in HIVAN with other types of kidney disease, we used cohorts of HIV-negative patients with AKI or CKD. Patients with AKI and nonbiopsied, stable CKD were derived from our previously described investigation of the ability of uNGAL to detect AKI in patients presenting to an emergency department.[13] Patients with biopsy-proven glomerular or tubulointerstitial etiologies of CKD were obtained from the Kidney Biopsy Registry, University of Parma, Italy. These patients were >18-yr-old and underwent kidney biopsy as part of routine care (January 1, 2005, through April 1, 2008). Urine was collected at the time of biopsy. Formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin/eosin, silver methenamine, and periodic acid–Schiff stain. Patients with AKI or CKD were stratified by type of kidney disease.

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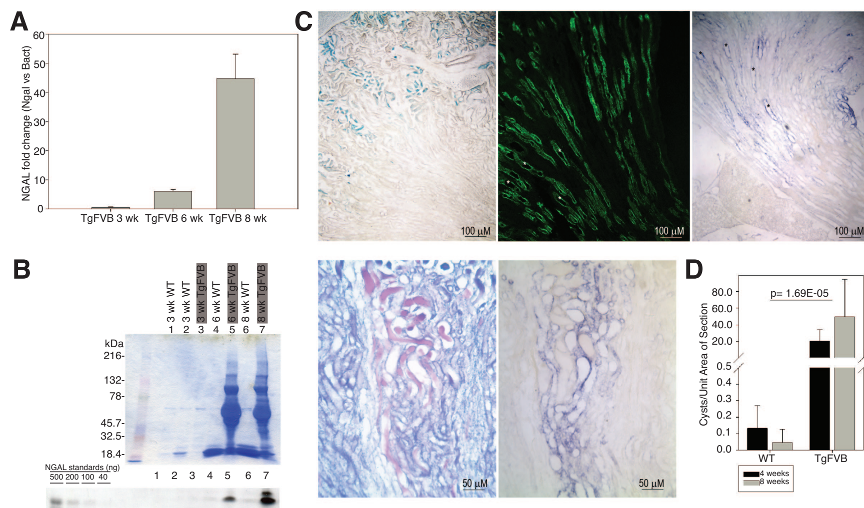

Figure 3. Induction of uNGAL. (A) NGAL real-time PCR in TgFVB kidneys from 3-, 6-, and 8-wk-old mice. Data were normalized for NGAL expression in age- and sex-matched WT FVB/N littermate mice. (B) Coomasie blue stained gels and immunoblots of uNGAL in 3-, 6-, and 8-wk-old TgFVB and WT littermate mice. uNGAL increased between 6 and 8 wk in TgFVB mice, whereas proteinuria remained constant. (C) Sections of 8-wk-old TgFVB mouse kidneys. From left to right: Prussian blue staining demonstrates iron accumulation in cortical proximal tubules. Aquaporin-2 immunocytochemistry marks medullary collecting ducts while *in situ* hybridization in an adjacent section reveals NGAL expression (blue) in dilated collecting ducts (asterisk represents dilated tubules). Higher power demonstrates cast formation (periodic acid—Schiff stain, purple) and NGAL expression (blue) in two adjacent sections. (D) Increasing number of cysts per unit area in TgFVB kidneys with aging. TgFVB differed significantly from WT kidneys ($n = 14$, $P = 0.00074$ at 4 wk; $n = 14$, $P = 0.001$ at 8 wk).

Statistical Analysis

SAS 9.1 (Cary, NC) was used for statistical analyses. All continuous data were log-transformed before analyses and are presented as non-log-transformed values. Pearson's correlation was used to determine relationships among uNGAL and other continuous variables, and a *t* test for unequal variances was used for comparisons. Fisher's exact test was used to compare categorical data among patients with and without HIVAN.

Animals

TgFVB mice[17] were bred with FVB/N mouse strain to produce heterozygotes. The HIV transgene correlated with the presence of cataracts.

Assays

The area of each kidney section was determined with Adobe Photoshop from 1× images, and total cysts per section were divided by this area to yield the cysts per unit area of each section.

NGAL was quantified by Western blots, using nonreducing 4 to 15% Tris-HCl gels (Bio-Rad Laboratories, Inc., Hercules, CA) and monoclonal (1:1000; AntibodyShop, Gentofte, Denmark) or rabbit polyclonal antibodies (RDSystems, Minneapolis, MN) together with standards (0.2 to 10 ng) of human or mouse recombinant NGAL protein. NGAL was reproducibly detected to 0.4 ng per lane. NGAL expression was quantified using ImageJ software (National Institute of Mental Health). NGAL mRNA was detected using digoxigenin-labeled antisense riboprobes generated from cDNAs encoding *Ngal* (exons 1 to 7, 566 bp) by linearization with XhoI followed by T7 RNA polymerase. Prussian blue staining in frozen sections used freshly prepared 2.5% potassium ferrocyanide and 2.5% hydrochloric acid for 20 min at room temperature.

Microarrays and real-time PCR utilized RNA isolated with the mirVANA RNA extraction kit (Ambion) and quantified by NanoDrop and gel electrophoresis. For real-time PCR analysis, samples were processed according to Bio-Rad SYBR GREEN, iCycler-MyiQ protocols. Target genes utilized respectively: Ngal 116 forward primer 5′-ctcagaact-tgatccctgcc-3′ and NGALa 593 reverse 5′-tccttgaggcccagacactt-3′; β-actin 415 forward primer 5′-ctaaggccaaccgtgaaaag-3′ and β-actin 696 reverse primer 5′-tctcagctgtggtggt-gaag-3′. The ΔΔCT method was used to calculated fold amplification of transcripts. For microarray analysis, double-stranded cDNA was synthesized from total RNA (7 μg) extracted from whole kidneys, and *in vitro* transcription biotin-labeled cRNA was generated for GeneChip hybridization (GeneChip One-Cycle Target Labeling Kit, Affymetrix). Fragmented biotin-labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips. The results of the Affymetrix chip experiments were normalized using GC-RMA (Bioinformatics Toolbox, Matlab R2008a, The Mathworks, Inc.). After standardization using WT information, the data were averaged. Given the distribution of expression profiles, the Bonferroni-corrected significance threshold was calculated to 3.17×10^{-6}.

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DISCLOSURES

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