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Glomerular *MYH9* expression is reduced by HIV-1

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Abstract

The continuing disease burden of HIV-associated nephropathy (HIVAN) warrants better elucidation of its pathogenic mechanisms. Given that loss of *MYH9* function causes a Mendelian renal disease, we hypothesized that renal expression of *MYH9* is downregulated by HIV-1 in HIVAN pathogenesis. Using immunofluorescence, we determined that glomerular expression of *MYH9* was reduced in the kidneys of HIV-1 transgenic mice. We further determined that *Myh9* expression was reduced in HIV-1 transgenic podocytes, statistically significantly at the protein level, and that *MYH9* expression was significantly reduced at protein and message level in human podocytes transduced with HIV-1. In analyzing expression in human tissue, we confirmed that *MYH9* is abundantly expressed in glomeruli, and podocytes specifically. Finally, we found that *MYH9* expression was significantly reduced in human glomeruli in the setting of HIVAN. We conclude that the podocyte host response to HIV-1 includes downregulation of *MYH9* expression, and hypothesize that this downregulation might play a role in the pathogenesis of HIVAN.

Keywords

HIVAN; FSGS; *MYH9*; Podocyte; Glomerulus; HIV-1

Introduction

People of African descent are three times more likely to suffer kidney disease, and no disease exemplifies this phenomenon better than HIV-associated nephropathy (HIVAN). HIVAN is limited virtually exclusively to individuals of African descent, and research into its pathogenesis has provided tremendous insight into the pathogenesis of kidney diseases and mechanisms of racial disparity in disease incidence.¹ HIVAN is mediated by podocyte injury leading to a rapidly progressive renal failure, histologically characterized by collapsing focal segmental glomerulosclerosis (FSGS) and proliferation and de-differentiation of podocytes.^{2, 3} Further elucidation of HIVAN's pathogenesis warrants attention as its prevalence remains high in the era of highly active anti-retroviral therapy (HAART). HIVAN particularly represents an epidemic threat to Sub Saharan Africa, as well as a potential crisis of secondary renal care in the HIV-positive population there.⁴

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Growing evidence indicates that the myosin, heavy chain 9 gene (*MYH9*) function plays an important role in healthy podocyte function. *MYH9* encodes non-muscle myosin heavy chain IIa (NMMHC-IIa or MYH9), which interacts with the actin cytoskeleton as the principal force generating ATPase. It is expressed ubiquitously, and has broad roles in maintaining cell architecture, contractility and motility.^{5, 6} Rare coding mutations in *MYH9* are responsible for a pleiotropic Mendelian disease, classified as MYH9-related disorders (MYH9RD), which include variably penetrant kidney disease characterized by glomerulosclerosis and podocyte effacement.^{5, 7} Additionally, *MYH9* expression *in vitro* is dysregulated following exposure of podocytes to plasma from FSGS patients.⁸ Johnstone, *et al.* demonstrated that podocyte-specific deletion of *Myh9* predisposed mice to renal injury.⁹ Several recent studies have shown that polymorphisms in *MYH9* are strongly associated with susceptibility to HIVAN and related forms of FSGS.¹⁰⁻¹² Genovese, *et al.* demonstrated that variation in the neighboring gene, *APOL1* is a stronger contributor to disease susceptibility,¹³ however recent evidence indicates that *MYH9* variants may predispose individuals to kidney disease as well.^{14, 15} Furthermore, Pattaro, *et al.* found that *MYH9* SNPs in genetically isolated European populations associated with elevations in serum creatinine, a marker of kidney disease.¹⁶

Given the link between MYH9 and kidney disease, we hypothesized that *MYH9* downregulation might be a host response of podocytes to HIV-1. We analyzed *MYH9* expression in the glomeruli of transgenic HIV-1 animals, transgenic HIV-1 podocytes, and WT podocytes transduced with HIV. In each of these settings, we found *MYH9* expression was reduced. Next, we examined MYH9 expression in healthy human kidneys, and from kidneys of patients diagnosed with HIVAN. We provided conclusive evidence that MYH9 is abundantly expressed in human glomeruli and podocytes, and here too found that glomerular MYH9 expression was reduced in the setting of HIV.

Methods

Mouse Histology

Mice on the FVB/N background heterozygously bearing a *gag/pol* deleted HIV-1 provirus as a transgene, known as the Tg26 line, were maintained as previously described.^{17, 18} Representative photomicrographs were obtained from tissue analyzed from four Tg26 mice and four WT littermates. Mice euthanized at 12 weeks of age, and kidneys were fixed in 10% neutral buffered formalin and paraffin embedded. Sections (4 μ m) were deparaffinized and rehydrated. Antigen retrieval was performed by incubation in 1X saline-sodium citrate (Acros) for 40 minutes at 95°C. Sections were then washed for 20 minutes in 1X TBS/0.06% Tween-20, blocked in 5% donkey serum for 1 hour, and incubated in 1:100 primary rabbit anti-myosin IIA (Sigma, M 8064) for 1 hour at room temperature. Slides were then washed three times in 1X TBS, and primary antibody was detected by incubating for 1 hour at room temperature with 1:100 Alexa Fluor 594 anti-rabbit antibody, with 1:40 Alexa Fluor 488 phalloidin and 1:1000 DAPI counterstains (Invitrogen, Cat# A21207, Cat# 12379 and Cat# D3571). Slides were then washed three times with 1X TBS. Serial sections were prepared with periodic acid-Schiff (PAS) stain to analyze tissue histology. Fluorescently-labeled sections were examined using wide field fluorescent microscopy. All animal work was approved the IACUC of Mount Sinai School of Medicine (protocol: 98-0953-00300-09).

Tissue Culture

Conditionally immortalized podocytes derived from Tg26 mice and WT mice on the FVB/N background were maintained as previously described.¹⁹ Cells expressing the temperature-sensitive SV-40 T antigen were shifted to 37°C to differentiate for 9-10 days. Transduction

experiments were performed using human conditionally immortalized podocytes,²⁰ which were shifted to 37°C to differentiate for 5 days. Cells were then transduced with VSV.G envelope pseudotyped pNL4-3 Δgag/pol-EGFP lentiviral particles, or pVVPW/BE-EGFP control as previously described.²¹ Cells were then lysed in T-PER Tissue Protein Extraction Reagent, supplemented with protease inhibitor (Thermo Scientific, Cat# 78510 and Cat# 78430).

Western Blotting

Protein was harvested six days after lentiviral transduction, or 9-10 days after differentiation in the case of transgenic podocytes. Individual 75 cm² tissue culture flasks containing approximately 1×10^6 cells constituted one data point, and the mean and standard deviation (SD) for each value were calculated from two replicate experiments. Protein was quantified by BCA assay, and SDS-PAGE was performed with 10 μg of each sample using Novex tris-glycine 4-20% gels under denaturing conditions (Invitrogen, Cat #EC6025). Protein was then transferred in 1X Novex tris-glycine transfer buffer (Invitrogen, Cat# LC3675) supplemented with 15% methanol and 0.01% SDS at 23 V overnight at 4°C onto PVDF membranes. Membranes were then blocked in PBS/0.1% Tween-20/5% powdered non-fat milk, and incubated in 1:1000 primary anti-myosin IIA antibody (Abcam, ab24762) overnight at 4°C. Following three washes in PBS/0.1% Tween-20, membranes were incubated for 2 hours at room temperature in 1:10,000 HRP-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, 711-036-152). Bands were detected using Lumi-Light Western blotting substrate (Roche, Cat# 12015200001). Membranes were re-blotted using 1:1000 HRP-conjugated anti-Actin goat IgG (Santa Cruz Biotechnology, Cat# sc-1615) to control for loading, in the case of transgenic cells, and with 1:5000 anti-GAPDH mouse IgG (Millipore, Cat# Mab374) followed by 1:200 HRP-conjugated anti-mouse IgG goat IgG (Zymax, Cat# 81-6520), in the case of transduced cells. Band intensity was quantified by densitometry using Image J, and values were expressed relative to housekeeper gene expression, and normalized to respective controls (WT podocytes in the case of transgenic podocytes and GFP transduced podocytes in the case of pNL4-3 Δgag/pol-EGFP transduced cells).

Real-time PCR

Samples for RNA analysis were collected 48 hours after transduction, or 9-10 days after differentiation, using RNeasy kits (Qiagen, Cat# 74104). Individual 75 cm² tissue culture flasks containing approximately 1×10^6 cells constituted one data point, and the mean and SD for each value were calculated from three replicate experiments. RNA was reverse transcribed using first-strand cDNA synthesis kit (USB, Cat# 75780). Real-time PCR analysis was performed using Quantitect SYBR green PCR kit (Qiagen, Cat# 204143) as previously described.²² The following oligonucleotides were used to detect human cyclophilin A: F: 5'-AAGGCTCTGGACAAGACCAA-3' R: 5'-TTGCCATCCAACCACTCAGTC-3'; human *MYH9*: F: 5'-AAGGCTCTGGACAAGACCAA-3' R: 5'-TCTCAATGAGGTTCGATGCAG-3'; mouse cyclophilin D: 5'-AACCCGCGAGTCTTCTTTGAC-3' R: 5'-TAATTCGGTGGAAAGGGCATC-3'; and mouse *Myh9*: F: 5'-GAGCGATACTACTCAGGGCTT-3' R: 5'-TCTTGCTCTTGTGTGAGGAGG-3'.

Human Histology

To localize MYH9 expression in normal human tissue, nephrectomy specimens were obtained from patients undergoing partial or whole nephrectomies for renal cell carcinoma (RCC). Cortical tissue unaffected by RCC was obtained, fixed in 10% neutral buffered formalin, and processed and stained for MYH9 immunofluorescence identically as in mouse

histology described above. Wide field immunofluorescent images of glomerular MYH9 expression were further processed by deconvolution using AutoQuant X2. These sections were then re-stained by PAS to outline glomerular basement membranes and positively identify podocytes. Specimens from two individuals were analyzed, and representative high power photomicrographs were obtained. To analyze MYH9 expression in the setting of HIVAN compared to in unaffected kidneys, renal biopsies were obtained from HIVAN patients or patients undergoing biopsy for hematuria and found to have normal appearing renal histology. Biopsies were fixed in 10% neutral buffered formalin and processed as described in mouse histology above. To quantify glomerular MYH9 expression, four to five (five when possible, but in one specimen, only four glomeruli were present) glomeruli were selected from each of two HIVAN and two normal biopsies. Glomeruli were selected randomly by visualization of nuclei by DAPI staining. Immunofluorescent staining of MYH9 from these glomeruli was then quantified using ImageJ by outlining Bowman's space, and quantifying mean density of signal within the glomeruli. All work with human tissue was approved by the IRBs of Mount Sinai School of Medicine and Columbia University (protocols: 98-953 0001 10ME and IRB-AAAE0986 respectively).

Statistical Analyses

Data means were compared using student's *t*-test. Statistical significance was defined at $p < 0.05$. Data are presented as means $\pm$ SD.

Results

Murine glomerular expression of MYH9

Glomeruli from 12 week-old Tg26 mice were observed to express less Myh9 when compared to WT controls (Fig. 1A, panels A and C). Serial sections from the same tissue were analyzed with PAS stain to assess the degree of glomerular injury (Fig. 1A panels B and D). These glomeruli were determined to not exhibit glomerular sclerosis or collapse suggesting that reduced expression of Myh9 precedes the visible pathologic changes characteristic of HIVAN.

Podocyte *in vitro* expression of MYH9

Western analysis confirmed that HIV-1 transgenic podocytes expressed significantly less MYH9 (Fig. 1B). Analysis of *Myh9* expression at the message level by real-time PCR detected a downward trend, which failed to reach statistical significance (Fig. 1D). Western analysis of human podocytes transduced with HIV-1 indicated a decrease in MYH9 expression (Fig. 1C). This reduction in *MYH9* expression was also detected at the message level when analyzed by real-time PCR, and determined to be statistically significant (Fig. 1E).

Human glomerular expression of MYH9

Immunofluorescence indicated that MYH9 was expressed predominantly in podocytes, though expression within mesangial and endothelial cells cannot be excluded (Fig. 2). Expression within podocytes was verified by detection of the glomerular basement membrane by PAS stain. Podocyte expression appeared limited to the cytoplasm. Immunofluorescent detection of MYH9 was used to compare expression in renal biopsies from histologically normal kidneys, and kidneys histologically diagnostic of HIVAN. The strong, glomerular expression of MYH9 was absent from HIVAN kidneys viewed at low power (Fig. 3A, B). And, this decrease in expression was found to be statistically significant when expression was quantified from multiple, randomly selected glomeruli from two normal kidney biopsies and two HIVAN biopsies (Fig. 3C).

Discussion

Elucidating the mechanistic role of *MYH9* in glomerular disease warrants attention, particularly to determine the relative contributions of *MYH9* and *APOL1* common variants to disease susceptibility. As Kopp, *et al* recently noted, *in vitro* investigations are required “to define the relative contributions of *APOL1*, *MYH9* and other near-by loci” in these processes.¹⁵ A growing body of evidence indicates that loss of *MYH9* function may be sufficient to cause kidney disease. In the setting of MYH9RD, patients experience a progressive renal disease, characterized by podocyte effacement, glomerular basement thickening, glomerulosclerosis and proteinuria.^{5, 23, 24} This kidney disease likely represents a loss of function, as the MYH9 mutation with the most severe renal phenotype has a dominant negative effect on MYH9 function,²⁵ and reduces glomerular expression of MYH9.²³ Furthermore, podocyte-specific ablation of *Myh9* predisposed mice to Adriamycin-induced glomerular injury.⁹ Interestingly, in that study mice exhibited many features of HIVAN, including podocyte effacement, glomerulosclerosis and proteinuria. These findings support the hypothesis that podocyte-specific loss of MYH9 function may contribute to the pathogenesis of HIVAN, and that HIV-1 might downregulate expression of MYH9. To test that hypothesis we examined *MYH9* expression in models of HIVAN, and in normal human glomeruli. Our analysis of normal glomeruli confirmed that MYH9 is indeed expressed in the cytoplasm of podocytes. Consistent with MYH9’s broad expression analysis also detected MYH9 in parietal epithelium of the glomerulus, mesangial cells and endothelium, however expression appeared greatest in podocytes.

We demonstrated that glomerular expression of MYH9 is reduced in the glomeruli of HIV-1 transgenic animals, and in patients with HIVAN. In both settings, the decrease in expression appeared in sclerotic glomeruli as well as in normal appearing glomeruli, which may indicate a loss of MYH9 expression preceding irreversible podocyte injury. These finding represent important *in vivo* descriptions of reduced MYH9 expression secondary to HIV-1. Interestingly, this downregulation mimics the reduced expression of MYH9 observed in rapidly progressing proteinuria secondary to MYH9RD.²³

To address whether reduction of expression occurred in podocytes, we analyzed *Myh9* expression at the message and protein level in HIV-1 transgenic conditionally immortalized podocytes. We demonstrated a reduction in message level *Myh9*, which failed to reach significance, and a statistically significant reduction of protein level expression.

To analyze whether this downregulation of MYH9 was a sequela of HIV-1 gene expression, rather than an artifact of the transgenic models, we examined *MYH9* expression in human podocytes transduced with HIV-1. We found that MYH9 expression was reduced at the protein level, and that this reduction involved reduced mRNA expression. Interestingly, both transduced human podocytes and transgenic mouse podocytes demonstrated similarly reduced levels of *MYH9* mRNA expression. However, there was greater variability in the expression of HIV-1 transgenic podocytes. Additionally, the loss of expression of MYH9 protein was far greater in both models than the loss of message level expression indicating posttranslational regulation may be reducing expression. These findings represent the first, detailed descriptions of *MYH9* regulation in the setting of HIV-1 gene expression. They indicate that *MYH9* downregulation is likely a component of the host response in the pathogenesis of HIVAN.

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Figure 1. *MYH9* expression in the setting of HIV-1

(A) Staining of MYH9 was dramatically reduced in the glomeruli of 12 week-old Tg26 mice (c) compared to WT (a). MYH9 was stained in fuchsia, with phalloidin in green and DAPI in blue. PAS staining indicated that neither WT (b) nor Tg26 (d) glomeruli had sustained sclerosis or collapse. Scale bar in the lower left indicates 100 μ m. Photomicrographs are representative of tissue analyzed from four WT and four Tg26 littermates. (B) Western blotting indicated that significantly reduced MYH9 was expressed in mouse Tg26 podocytes compared to WT controls. Bands were quantified as a ratio of actin expression, and normalized to WT MYH9 expression (n = 2). (C) Western blotting of human podocytes transduced with GFP or HIV-1 indicated that significantly less MYH9 was expressed in the setting of HIV-1. Bands were quantified as a ratio of GAPDH expression, and normalized MYH9 expression in GFP transduced control cells (n = 2). (D) Real-time PCR analysis of *Myh9* mRNA expression in mouse Tg26 and WT podocytes indicated a non-significant decrease in expression, with values expressed as a ratio of cyclophilin expression, and normalized to WT gene expression (n = 3). (E) Real-time PCR analysis of *MYH9* mRNA expression in human podocytes transduced with GFP or HIV-1 indicated that significantly less *MYH9* was expressed. Values were quantified as a ratio of cyclophilin expression and normalized to GFP transduced control cells (n = 3). All values are expressed as mean + SD of normalized ratios with * indicating $P < 0.05$ and ** indicating $P < 0.01$. Western bands and associated means represent data from two replicate tissue cultures. Quantification of RNA expression represents data from three replicate tissue cultures.

Figure 2. MYH9 is expressed *in vivo* in the podocytes of human glomeruli

MYH9 expression in human nephrectomies was visualized by immunofluorescence, in which MYH9 is stained red, and DAPI appears blue (B, D). The same sections were then re-stained by PAS to identify podocytes (A, C). Several podocytes are indicated by arrows, and are indicative of the cytoplasmic expression pattern of MYH9. Scale bar in the lower left indicates 40 μm. Photomicrographs are representative of tissue analyzed from nephrectomy specimens from two individuals.

Figure 3. Glomerular expression of MYH9 is reduced in HIVAN

(A) Biopsy specimen from histologically normal renal cortex was stained by immunofluorescence to detect MYH9 expression, in red. Expression was highest in glomeruli (arrows). Scale bar in lower left indicates 100 μm . (B) An identically obtained and processed renal biopsy from a HIVAN case was similarly stained for MYH9 expression. Here glomerular expression was reduced. (C) Expression of MYH9 in four to five glomeruli from each of two normal renal biopsies ($n = 10$), and two HIVAN biopsies ($n = 9$) was quantified and compared. MYH9 expression was significantly reduced in the setting of HIVAN. Values are expressed as mean fluorescent intensity within Bowman's capsule with ** indicating $P < 0.01$. Bars represent group mean $\pm$ SD.