Identification of the Nephropathy-Susceptibility Locus HIVAN4

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ABSTRACT

HIVAN1, HIVAN2, and HIVAN3 are nephropathy-susceptibility loci previously identified in the HIV-1 transgenic mouse, a model of collapsing glomerulopathy. The HIVAN1 and HIVAN2 loci modulate expression of Nphs2, which encodes podocin and several other podocyte-expressed genes. To identify additional loci predisposing to nephropathy, we performed a genome-wide scan in 165 backcross mice generated between the nephropathy-sensitive HIV-1-transgenic FVB/NJ (TgFVB) strain and the resistant Balb/cJ (BALB) strain. We identified a major susceptibility locus (HIVAN4) on chromosome 6 G3-F3, with BALB alleles conferring a twofold reduction in severity (peak LOD score = 4.0). Similar to HIVAN1 and HIVAN2, HIVAN4 modulated expression of Nphs2, indicating a common pathway underlying these loci. We independently confirmed the HIVAN4 locus in a sister TgFVB colony that experienced a dramatic loss of nephropathy subsequent to a breeding bottleneck. In this low-penetration line, 3% of the genome was admixed with BALB alleles, suggesting a remote contamination event. The admixture localized to discrete segments on chromosome 2 and at the HIVAN4 locus. HIVAN4 candidate genes include killer lectin-like receptor genes as well as A2m and Ptpro, whose gene products are enriched in the glomerulus and interact with HIV-1 proteins. In summary, these data identify HIVAN4 as a major quantitative trait locus for nephropathy and a transregulator of Nphs2. Furthermore, similar selective breeding strategies may help identify further susceptibility loci.


HIV-1 associated nephropathy (HIVAN) is an AIDS-defining complication of HIV-1 infection. HIVAN is diagnosed based on kidney biopsy findings of collapsing focal segmental glomerulosclerosis with microcystic tubular dilation.1 In HIVAN, the normally terminally differentiated glomerular podocytes display dedifferentiation, proliferation, and apoptosis; without antiretroviral therapy, this disorder frequently results in end-stage kidney failure.1 Patients with HIVAN are almost always of African ancestry and frequently have a strong family history of kidney failure, indicating an important role for genetic susceptibility in the pathogenesis of disease.2 Consistent with this epidemiology, recent genome-wide association studies in African Americans have identified a major susceptibility locus for HIVAN, focal segmental glomerulosclerosis, and nondiabetic kidney failure on chromosome 22, within the APOL1 gene.3,4 It is hypothesized that the nephropathy risk alleles propagated in West Africa be-

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cause the heterozygous genotype confers resistance to trypanosomiasis, it is not yet clear how homozygosity for these same APOL1 alleles produces susceptibility to histologically diverse nephropathies.5

Heterozygous HIV-1 transgenic mice on the FVB/NJ genetic background (TgFVB) display virtually all of the clinical and molecular features of HIVAN, enabling dissection of molecular pathways leading to disease.6–9 Studies in the murine model have indicated that HIVAN is caused directly by HIV-1 products, resulting in immune activation in the kidney and a hyperplastic response in epithelial cells.6–9 These studies have identified a large number of dysregulated pathways (e.g., NF-κB, MAPK1,2,12,13 Stat312,13) and pathogenic mediators (bFGF, VEGF, TNF16) that link epithelial proliferation to glomerulosclerosis and are similarly activated in human disease (reviewed recently in references 7 through 9). The nature of the pathogenic process that encompasses these dysregulated pathways, ultimately leading to proliferation and scarring of the kidney, is not yet known. However, in conjunction with observations in humans, where very few HIV-infected individuals develop nephropathy, understanding the unique host response to the virus likely holds the key. In this regard and similar to humans, genetic susceptibility has a profound effect on the development of nephropathy in the HIV-1 transgenic mouse model: the FVB/NJ strain is very susceptible, but F1 hybrids with BALB/cJ, C57BL/6J and CAST/EiJ are protected.17,18 Taking advantage of this strain dependence, we previously generated mapping cohorts between TgFVB and two counterstrains (C57BL/6) and CAST/EiJ), leading to identification of nephropathy quantitative trait loci (QTLs) on chromosomes 3, 13, and 4 (HIVAN1, HIVAN2, and HIVAN3 loci, respectively).17,18 Further examination of these loci with expression QTL analysis (expression profiling combined with linkage analysis) showed that the HIVAN1 and HIVAN2 loci regulate expression of Nphs2 (encoding Podocin) and several other podocyte-expressed genes.18 These data indicated the HIVAN1 and HIVAN2 susceptibility genes belong to a common regulatory pathway, and they likely introduce genetic lesions in an expression network involving multiple human nephropathy genes.

To identify additional loci predisposing to HIVAN and determine whether Nphs2 transregulation is a common molecular feature of HIVAN loci, we conducted a new mapping study with the third resistant counterstrain, Balb/cJ (BALB), leading to the identification of a new HIVAN QTL. Furthermore, we obtained confirmation and higher resolution of this locus in an independent TgFVB colony selectively admixed with BALB alleles.

RESULTS

Mapping a New HIVAN Locus to Chromosome 6 F3-G3

We had previously shown that TgFVBxBALB F1 hybrids do not develop nephropathy, suggesting that the BALB genome confers resistance to this trait.8 To identify nephropathy susceptibility loci, we generated an HIV1-transgenic backcross by breeding TgFVB with FVBxBALB F1 mice (52% male). Nearly one-quarter of the 165 transgenic backcross mice were free of nephropathy (total renal injury scores ≤3), while the remainder had varying severity of kidney disease, indicating segregation of nephropathy susceptibility alleles in this cross (Supplementary Figure 1a). Consistent with prior descriptions of the TgFVB model and previous mapping studies, there were no differences in severity of kidney disease based on gender.6,17,18

A genome-wide scan and analysis of linkage with 548 informative markers identified a major locus on chromosome 6 F3-G3, with a peak lod score of 4.0 for the total renal injury score (peak at rs6329892, 143 Mb, Supplementary Figure 1b). This locus, which we named HIVAN4, achieved genome-wide significance based on accepted threshold (≥3.3) and by 10,000 permutations of phenotype on genotype (genome-wide empirical p-value = 0.004, Figure 1a). All of the subcomponents of the histology score yielded peak lod scores of similar magnitude (lod range = 3.7 to 4.9, Figure 1a). This interval was also significant for BUN (lod = 3.0, empirical P = 0.05) and suggestive for serum albumin level (lod = 2.4). The lod scores for BUN and albumin diminished significantly (lod <0.5) after accounting for the histologic injury, indicating that these linkages do not represent a direct effect of HIVAN4 on BUN and albumin but are mediated by renal injury. Homozygosity for the FVB allele at HIVAN4 conferred a twofold increase in the histologic injury score compared with the heterozygote FVB/BALB genotype (Figure 1b). Finally, two intervals on chromosome 14 (peak lod 1.8 between rs13459144 and rs6256423) and chromosome 15 (peak lod = 1.9 at rs13482585) demonstrated suggestive linkage for the histologic injury score (Supplementary Figure 1b).

HIVAN4 Transregulates Nphs2 Transcript Abundance

Previous studies have shown that HIVAN1 and HIVAN2 loci regulate transcript levels of Nphs2. To determine if HIVAN4 also transregulates Nphs2 expression, we measured Nphs2 transcripts abundance in 137 transgenic backcross mice with available kidney tissue. Nphs2 expression was inversely correlated with renal histology score (r = −0.62, P = 0.0001), and genome-wide analysis of Nphs2 transcripts abundance revealed suggestive linkage to the HIVAN4 locus (LOD score 2.1, pointwise empirical p-value for linkage to HIVAN4 = 0.006). This result was obtained after adjustment for the severity of renal injury, indicating that this linkage is not a secondary effect of kidney injury but truly represents transregulation.

In contrast, there was no evidence of an eQTL for Ptpro (Protein tyrosine phosphatase, receptor type, O, also known as GLEPP1), which is located within the HIVAN4 locus and encodes a podocyte expressed protein. While Ptpro transcript levels was highly correlated with histology injury score (r = −0.46, p-value = 3.6 × 10−05) and Nphs2 transcript levels (r = 0.67, P = 2 × 10−10), there were no differences in transcript levels between mice with FVB/FVB and FVB/BALB genotypes, after correction for glomerulosclerosis (Lod <0.5). These find-
ings suggest that HIVAN4 alleles do not directly influence Ptpro transcript level and that the inverse correlation of Ptpro transcript level with glomerulosclerosis occurs secondary to kidney injury.

There were no other loci influencing Nphs2 or Ptpro transcript abundance across the genome; particularly, there was no evidence of an Nphs2 cis-eQTL in this cross, which is consistent with FVB and BALB sharing the same haplotype at the Nphs2 locus.

Independent Localization of HIVAN4 in an Admixed Colony of TgFVB

Concurrently, in a sister colony of TgFVB located at the MetroHealth Medical Center (MHMC), investigators noted a dramatic reduction in the penetrance of renal disease after the colony was transferred to a new mouse facility in 2006; however, two generation of backcrossing to FVB/NJ completely restored the original nephropathy phenotype excluding transgene silencing as a mechanism (Figure 2A). Since the colony maintained the albino coat color of the FVB/N background strain, this suggested the occurrence of a spontaneous mutation(s) that conferred resistance to HIVAN or, alternatively, inadvertent introgression of resistance alleles from one or more albino strains. To study this further, the low penetrance mice were homozygous and concordant with FVB alleles at 1292/1305 (99%) of markers across the genome. The 13 SNPs harboring non-FVB alleles were clustered within two distinct intervals on chromosome 2 (85 to 152 Mb between rs3689658 and rs6247960) and chromosome 6 (121 to 139 Mb, between rs13479006 and rs13479071), and both of these intervals were fixed to homozygosity in all mice tested (Figure 2C and Supplemental Figure 2). Consistent with the expectation of admixture with an albino strain, comparison with reference strains revealed that all 13 non-FVB alleles were derived from the BALB genome. Most strikingly, the admixed interval on chromosome 6 coincided perfectly with the HIVAN4 locus (Figure 2C), strongly suggesting that introgression of BALB alleles in this region accounted for the loss of the nephropathy phenotype. The lifespan of the low-penetrance TgFVB with the protective BALB alleles was $11022^9$ mo, whereas inbred TgFVB mice have considerably shorter life spans and require breeding at 6 to 8 wk of age to sustain the colony (67% die or are moribund at a mean age of 71 d). Since there were no noticeable changes in the severity of nephropathy before transfer to the new facility, this suggested that the contamination existed at a low level, but BALB alleles rapidly propagated in the new colony because a survival constraint was placed on selection of breeders, increasing the use of TgFVB with the protective BALB alleles. Consistent with this prediction, review of breeding records showed that delays during transfer to a new facility in 2006 left older mice with minimal nephrop-
athy as founders of the new colony, effectively creating a genetic bottleneck.

**Annotation of Positional Candidates Within the HIVAN4 Locus**

The refined HIVAN4 95th percentile confidence interval spans approximately 29 Mb between rs3695724 and rs3711088 and contains 473 positional candidates. Importantly, the interval delineated by the low-penetrance MHMC-TgFVB colony was approximately 35% smaller, spanning a 19-Mb region containing 364 genes (Supplementary Table 1). Because a previous mapping study between TgFVB and C57BL/6J (B6) strains did not identify linkage to HIVAN4, this indicated that the FVB and B6 strains share the same susceptibility allele at this locus. Consequently, we performed a three-way comparison of haplotypes, searching for regions within the HIVAN4 interval that are polymorphic between the FVB and BALB strains but are
monomorphic between FVB and B6 strains. In total, 176/364 genes within the reduced HIVAN4 interval fulfilled these selection criteria. We further annotated these genes based on the presence of potentially deleterious amino-acid substitutions (16 genes), evidence for interaction of human orthologs with HIV-1 (7 genes), and transcript enrichment in the glomerulus (8 genes, Supplementary Table 1). Thus, in addition to Ptpro, which was studied further by expression analysis (as described above), this annotation prioritizes multiple candidates that can be pursued by sequencing, expression analysis, and functional studies. For example, the A2m gene, encoding Alpha-2 macroglobulin, harbors a potentially pathogenic variant, is enriched in the glomerulus and is predicted to interact with the HIV-1 protease.

**DISCUSSION**

In this study, we describe a novel susceptibility locus for nephropathy on mouse chromosome 6 F3-G3 using the HIV-1 transgenic mouse model. This finding was supported by a genome-wide significant detection of this interval in a backcross cohort derived from inbred strains, and examination of a low-penetrance colony of TgFVB that demonstrated selective preservation of the BALB genome at the HIVAN4 locus. Since the segments of admixture represented <3% of the mouse genome, it is highly unlikely that the overlap with the HIVAN4 locus is coincidental. The TgFVB mice usually have a limited lifespan because they develop nephropathy within 3 to 6 wk of age.5,19 This strongly suggests that the protective BALB alleles at the HIVAN4 locus were retained because they conferred a survival and reproductive advantage in the MHMC colony, owing to the absence of nephropathy. Moreover, this admixture remained undetected due to the albino coat color in the BALB and FVB strains. These findings illustrate the strong effect of selection on beneficial alleles even within artificial colonies, providing further evidence that HIVAN4 exerts a large effect on the nephropathy phenotype. A second admixed segment on chromosome 2 was also detected in the low penetrance MHMC-TgFVB colony and thus represents a candidate locus for nephropathy. The BALB alleles at this locus would be predicted to have a recessive protective effect because this interval was not detected in the backcross, which can only identify additive effects. Further cross breeding can separate this chromosome-2 interval from the HIVAN4 locus and formally determine whether this region also imparts an effect on nephropathy.

Our findings indicate that murine nephropathy susceptibility alleles are under significant selection pressure and, given the opportunity, will be rapidly eliminated from the gene pool. Many intricate breeding strategies, such as selective breeding or advanced intercross lines, aim to reproduce conditions of selection pressure to localize large-effect QTLs.20 Alternatively, investigators sequentially generate interval-specific congenic and subcongenic lines for identification of causal variants.20 These conditions were inadvertently reciprocated in the low-penetrance MHMC-TgFVB colony. The mapping resolution was comparable to studies of outbred populations (approximately 19 Mb at HIVAN4) and, simultaneously, this colony offered an incipient congenic strain for rapid generation of HIVAN4 congenic lines. Our data indicate that selective breeding approaches may prove very powerful for localizing and refining murine glomerulosclerosis susceptibility loci.

To date, three crosses with the TgFVB model have identified four susceptibility loci for HIVAN on chromosomes 3A1-A3, 13A3-C2, 4A1-A5, and now 6F3-G3 (HIVAN1 to 4, respectively17,18). We had previously shown that the HIVAN1 and HIVAN2 loci regulate the expression of multiple podocyte-expressed genes in the absence of HIV-1.18 We hypothesized that these gene expression changes were reactive to genetic lesions introduced by the HIVAN risk alleles, suggesting that the murine HIVAN susceptibility genes and podocyte genes belong to the same regulated network. Here we show that HIVAN4 also transregulates Nphs2 expression, thereby placing HIVAN1, 2, and 4 loci in the same pathway. Although these intervals are large, the availability of multiple crosses now enables significant refinement of positional candidates by comparison of haplotypes among inbred strains.21 Thus, there are several noteworthy genes among the refined list of candidates at the HIVAN4 locus: a cluster of natural killer cell lectin-like receptor genes, which encode proteins important for defense against pathogens,22–26 and several genes (Bid, A2m, Cd69, Grin2b, Pik3c2g, Pldc1, Itpr2) with potential interaction with HIV-1. Of note, variation in Klra8 gene, encoding a receptor important for natural killer cell cytolysis, is associated with differential susceptibility to CMV infection in the mouse but is unlikely to be causal for HIVAN because FVB and BALB have a susceptibility haplotype to CMV infection23 Another positional candidate, Ptpro, encodes a podocyte expressed protein and has dysregulated expression in HIVAN27,28 but did not demonstrate a cis-eQTL on additional analysis. Finally, A2m, encoding Alpha-2 Macroglobulin, enriched in glomeruli,29 is a target for HIV-1 protease30 and harbors a missense variant that is predicted to affect protein function. While, to our knowledge, none of the murine HIVAN susceptibility loci overlap with other QTLs for nephropathy or proteinuria in the mouse,31–34 the HIVAN4 locus coincides with a QTTL for GFR identified in American Indians.35 Analysis of congenic lines will enable dissection of specific genes and variants within the HIVAN4 interval, identify relationships to other QTLs, and delineate common dysregulated pathways downstream of susceptibility alleles.

These studies also offer an interesting parallel to recent findings demonstrating the effect of natural selection on nephropathy susceptibility genes in humans. Studies have shown that genetic variation at the APOL1 locus on chromosome 22 is a major risk factor for nephropathy among...
African Americans with focal segmental glomerulosclerosis and non-diabetic end-stage kidney disease.\textsuperscript{3,4} The APOL1 nephropathy risk alleles have rapidly propagated because they provide resistance to trypanosomiasis in heterozygotes, but the increased susceptibility to kidney failure in homozygotes exerts a balancing effect, maintaining the alternative alleles in the population.\textsuperscript{3,4} The mechanism of kidney injury for the APOL1 risk-variants is not yet understood. APOL1 is only present in hominoids and some Old World monkeys. About 10 to 15% of healthy African Americans are homozygous for the chromosome 22 risk alleles, and, conversely, 10 to 24% of African Americans with FSGS or hypertensive end-stage kidney failure do not carry any APOL1 risk alleles.\textsuperscript{3,4} These data imply additional genetic or environmental requirements for the development of nephropathy. The recapitulation of virtually all phenotypic and molecular features of human disease in the TgFVB model further suggests that alternative genetic lesions, in the absence of APOL1, can predispose to HIVAN. Thus, identification of the murine genetic variants may clarify additional risk factors and biologic pathways required for the development of nephropathy.

**CONCISE METHODS**

**Animal Breeding and Phenotyping**
All inbred strains were purchased from the Jackson Laboratories (Bar Harbor, ME). The HIV-1 transgenic mouse line TgN(pNL43d14)26Lom (TgFVB) was produced on the inbred FVB/N genetic background and has been extensively characterized.\textsuperscript{6,17} The TgFVB colonies at Columbia University and MetroHealth Medical Center were established from founders received from the Mount Sinai School of Medicine (courtesy of Dr. Paul Klotman). The TgFVB x (FVB x BALB) F1 backcross was generated at Columbia University, and 165 heterozygous HIV-1 transgenic progeny were phenotyped at 8 wk of age. Animals that were moribund were euthanized before this end point. Primary phenotypes included renal histology, serum albumin, and blood urea nitrogen (BUN). Renal histology was scored independently by an investigator (VDD) blinded to genetic background and other traits. Three traits related to tubulointerstitial disease (epithelial regeneration/ degeneration, tubular casts and dilation, and interstitial infiltrates) and one related to glomerular injury (glomerular sclerosis) were scored using a semiquantitative scale: 0 = no disease; 1 = 1 to 25% of tissue showing abnormalities; 2 = 26 to 50%; 3 = 51 to 75%; and 4 = >75% of tissue affected. A global histologic score was calculated as an average of four renal injury scores (range 0 to 16). The protocol was approved by the IACUC committees at the Columbia University Medical Center and Case Western Reserve University.

**Measurement of Podocyte Gene Expression**
Total RNA was extracted from mouse whole kidneys using TRIzol reagent (Invitrogen), followed by DNasel treatment and further purification using the RNeasy kit (QIAGEN). cDNA was generated with the Omni-Script kit (Qiagen). Gene expression was measured in duplicates by quantitative PCR (qPCR) using SYBR-Green mix and an iQ5 thermal Cycler (Bio-Rad). Expression values were standardized to an internal control reference sample (a male FVB/NJ) included in each run, and \(\beta\)-actin was used as housekeeping control (Pfaffl algorithm). Primers for Nphs2 and \(\beta\)-actin were previously described,\textsuperscript{18} and primers for Ptpro were Ptpro F, 5’ DTGTCTAGAAGGCCATCCTTA-3’, and Ptpro-R, 5’-ACTGCGAAGAGTGACCTCGGA-3’.

**Genotyping and Analysis of Linkage**
The backcross cohort was genotyped using Illumina medium-density SNP panel, which contains 1449 SNPs distributed across the genome (Center for Inherited Disease Research genotyping service, Baltimore, MD). Of these, 1305 were successfully genotyped and 548 SNPs were informative between FVB and BALB. Linkage scans for histology score, BUN, and serum albumin were performed using R/QTL program with the nonparametric method. Lod scores of \(\geq 3.3\) were considered significant.\textsuperscript{36} In addition, we conducted 10,000 permutations of phenotypes on genotype to determine the empirical significance of the linkage findings. Nphs2 transcript level was square-root transformed to achieve a normal distribution and analyzed using a parametric method (Haley-Knott algorithm) with adjustment for the degree of glomerulosclerosis. Pointwise \(p\)-value for linkage of the Nphs2 transcript level to the HIVAN4 locus was calculated based on permutations analysis limited to the HIVAN4 lod-2 interval.

**Gene Annotation in the HIVAN4 Locus**
SNP identification and annotation across the HIVAN4 interval were obtained from the Mouse Phenome Database (http://phenome.jax.org/). Segments that were not identical by descent were identified by filtering for SNPs that are polymorphic between FVB/NJ and BALB/cBy, but monomorphic between FVB/NJ and C57BL/6J strains. Gene annotation was performed using the PANTHER database (http://www.pantherdb.org/). Missense SNPs were evaluated for potential to affect protein function using SIFT (http://blocks.fhcrc.org/sift/SIFT.html). HIV-1 interactions were obtained from the HIV-1, Human Protein Interaction Database (http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/). Glomerular enriched genes were annotated based on a curated list compiled by Lindenmeyer et al.\textsuperscript{29}

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