An Ancestral Haplotype Defines Susceptibility to Doxorubicin Nephropathy in the Laboratory Mouse

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Haplotype analysis was used to refine of the DOXNPH locus, which harbors the susceptibility gene for doxorubicin (DOX; Adriamycin) nephropathy, a Mendelian form of selective podocyte injury. Analysis of haplotype structure in three strains with contrasting susceptibility (148 single-nucleotide polymorphisms at 101-kb spacing) was complementary to analysis of recombinants in 176 F2 mice. For example, haplotype analysis but not meiotic mapping could exclude the Abcc1 multidrug transporter, and this was confirmed further by phenotypic evaluation of Abcc1 null mice. Next, comparison of haplotype structure (55 single-nucleotide polymorphisms at 44-kb spacing) with phenotype in 15 inbred strains revealed a risk haplotype that was shared by susceptible strains ($P = 0.00017$), thereby reducing the DOXNPH region to a 1.3-Mb interval. These data demonstrate that susceptibility to DOX nephropathy represents a founder mutation in the laboratory mouse. Haplotype analysis can be used for identification of the DOXNPH gene and prediction of strain susceptibility pattern.

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gated whether susceptibility to DOX nephropathy represents a founder mutation and whether haplotype analysis can help to refine the DOXNPH locus.

Materials and Methods
Animal Breeding and Phenotyping
We tested eight to nine male and female mice (6 to 8 wk of age) from the various strains. The 129S1/SvImJ, AKR/J, BALB/cj, BALB/cByJ, C57BL/6j, C57BL/10j, C3H/HeJ, CBA/J, LP/J, SJL/J, and SWR/J were obtained from Jackson Laboratories (Bar Harbor, ME). The 129S6/SvEvTac mice and Abcc1 null mice on the FVB/N genetic background (FVB.129P2-Abcc1atm1Bor N12) were obtained from Taconic Labs (Hudson, NY). For meiotic mapping of the DOXNPH locus, we also produced an F2 intercross between BALB/cj (BALB) and C57BL/6j (B6). DOX nephropathy was produced by injecting 10 mg/kg DOX by tail vein at 8 wk of age (18). Fifteen days after DOX injection, mice were killed for histologic analysis of kidneys; spontaneously voided urine was collected for urinalysis. Proteinuria was measured by spot urine dipsticks (Roche, Indianapolis, IN). Periodic acid-Schiff–stained kidneys sections were scored independently by two investigators (A.G.G. and V.D.D.), who were blinded to genetic background and genotype using a validated semiquantitative scale (18). As before, we applied dichotomous criteria to define affection status: Mice with at least 3+ proteinuria and histologic evidence of 5% or greater glomerulosclerosis at the time of death were classified as affected (18). Mice with less than 3+ proteinuria and normal histology were classified as unaffected. The protocol was approved by the Institutional Animal Care and Use Committee at Columbia University.

Genotyping, Haplotype Analysis, and In Silico Mapping
Genotyping was performed using informative microsatellite markers that were distributed across the DOXNPH interval; fluorescence primers were used to direct PCR from genomic DNA, and products were analyzed on a capillary sequencer (Spectrumedix, State College, PA). For construction of our haplotype map at the DOXNPH locus, we obtained marker positions and genotypes from the National Center for Biotechnology Information web site and the Mouse Phenome database (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn = docs/home). When public data were not available for relevant strains, single-nucleotide polymorphisms (SNP) were genotyped by direct sequencing.

For haplotype mapping, we used the three-SNP sliding window algorithm that was used by Fletcher et al. (27) (algorithm provided by Phillip McClurg and Tim Wiltshire). This algorithm applies a binomial generalized linear model to calculate the significance of association of SNP haplotypes with binary traits. In this association analysis, we used 14 strains with known phenotype and genotype data (Figure 1); BALB/cj and BALB/cByj were considered as a single strain because they are nearly identical (27).

Results and Discussion
We initially constructed a haplotype map of the DOXNPH region for the three laboratory strains with the most public data available (C57BL/6j versus BALB/cj and 129S1X/svJ). Using public data (96 SNP) and additional resequencing (52 SNP), we generated a haplotype map with an average spacing of 101 kb (Figure 1A). Consistent with the mosaic structure that was described previously in the laboratory mouse (25), the distribution of SNP among these three strains was nonrandom, falling into blocks that range from 0.3 to 4.2 Mb in size. Several blocks are monomorphic (i.e., sequence did not vary) between the BALB/cj and C57BL/6j strains (e.g., the interval between rs4165284 and rs4165296). Such regions of identity between strains with contrasting susceptibility suggest inheritance by descent and therefore are unlikely to harbor the DOXNPH gene. In contrast, the centromeric portion of the interval contains a large (approxi-
mately 3.4 Mb) block that is polymorphic between the resistant C57BL/6J and the susceptible BALB/cJ and 129X1/SvJ strains (Figure 1A) and therefore is predicted to contain the DOXNPH gene.

To validate the use of haplotype analysis as a tool for fine mapping and evaluation of positional candidates, we generated 176 (BALB/cJ × C57BL/6J) F2 mice (352 recombination events) to fine-map the DOXNPH linkage interval. These mice were genotyped for markers at the DOXNPH locus, revealing six informative recombinant progenies, which subsequently were tested for susceptibility to DOX nephropathy. With additional genotyping to define the recombinant site in the informative F2 progeny, the DOXNPH locus was reduced to a 2.3-Mb interval between rs8260258 and rs4165049 (Figure 1B). This meiotic interval was in excellent agreement with our haplotype map (Figure 1).

The validity of haplotype mapping for gene localization was supported further by analysis of specific positional candidates. Three positional candidates in the initial 14-Mb linkage interval (Abcc1, Abcc5, and Abcf3) were attractive because they belong to the ATP-cassette gene family. This gene family encodes efflux pumps that mediate drug transport and are implicated in xenobiotic metabolism and multidrug resistance to chemotherapy (28). Haplotypes in Abcc1, Abcc5, and Abcf3 clearly did not correlate with susceptibility to the three strains tested (Figure 1A), suggesting that mutations in these genes are not responsible for the DOX nephropathy phenotype. Consistent with these data, Abcc5 and Abcf3 were outside our new meiotic interval. Because Abcc1 still remained within the meiotic interval, we studied Abcc1 null mice on the resistant FVB/N genetic background. Abcc1 null mice have increased susceptibility to etoposide toxicity (29), but to our knowledge, phenotypic response to anthracyclines has not been reported. If DOX nephropathy is due to a loss-of-function mutation in Abcc1, then we would expect that Abcc1 knockout mice on the resistant FVB/N background would manifest the phenotype. However, these Abcc1 null mice were completely resistant to DOX nephropathy (no proteinuria, normal histology), thereby excluding this gene (Figure 2).

We next tested 10 additional strains for susceptibility to DOX nephropathy using the same protocol used in our original mapping study. We found that AKR/J, C3H/HeJ, CBA/J, C57BL/10J, LP/J, SWR/J, SJL/J, and 129S6/SvEvTac mice are resistant to DOX nephropathy, whereas 129S1/SvImJ and BALB/cByJ mice are susceptible (Figure 1B). With the addition of strains that were characterized previously (18), this provided phenotype data for 15 strains.

We then defined the SNP haplotypes for these additional strains and generated a haplotype map of the refined meiotic interval at the DOXNPH locus (Figure 1B). Partitioning of strains on the basis of phenotype confirmed a risk haplotype that was common to BALB/cJ, 129S1/SvImJ, and 129X1/SvJ (Figure 1B). To detect previously unrecognized SNP blocks, we also resequenced 30 additional SNP within the new meiotic interval (44 kb average spacing between rs4164241 and rs4165049). Consistent with published reports, increased map density uncovered some additional complexity in the haplotype structure of the region (30,31), identifying some SNP that are private to the C57BL clade (e.g., rs4164875, rs4164885). However, these additional SNP did not partition the large polymorphic block further, suggesting that there are no other ancestral recombinations in the strains tested. To test the significance of the association between haplotype and strain susceptibility, we also computed an F statistic using a three-SNP haplotype window (27); this mapping statistic was highly significant across the region between rs4164770 and rs4164851 (lowest P = 0.00017; Figure 3). Combining the results of meiotic and haplotype mapping enabled refinement of the trait locus to a 1.3-Mb region between rs4164770 and rs4165049 (Figures 1B and 3).

The positional candidates that remained in our refined interval comprise 20 genes. Of these, 13 are presently unknown/predicted and therefore will require functional annotation. The other positional candidates encode structural proteins (Pkb2 and Fgd4) or proteins that are involved in mitochondrial homeostasis (Dnm1l), cellular stress signaling, and DNA repair (Ube2v2, Prkdc, Mcm4, and Cebpd). Defects in these pathways have been implicated either in the development of chemotherapy cytotoxicity or in the pathogenesis of glomerulosclerosis (3–15,32–34), making these genes plausible candidates as DOXNPH. We now can proceed with systematic sequence and functional analysis of these remaining positional candidates to identify the susceptibility variant. However, the common ancestral origin of laboratory mice, which facilitates fine mapping by haplotype analysis, also signifies that this population is unlikely to harbor independent susceptibility alleles for this trait. Hence, identification of the DOXNPH gene may require further fine mapping to achieve an interval that is sufficiently small to permit differentiation of the functional variant(s) from
linked polymorphisms and execution of bacterial artificial chromosome transgenic rescue experiments.

Identification of new meiotic recombinants offers a fail-safe but laborious route to achieve further reduction of the DOXNPH interval. However, our data suggest that analysis of additional laboratory strains also may identify recombinants within the ancestral haplotype. In particular, examination of wild derived strains may be very powerful for this purpose. For example, the wild derived CAST/EiJ strain shows additional recombinations within the DOXNPH risk haplotype (Figure 1B), but interpretation of these data is difficult because we do not know yet whether the DOXNPH susceptibility allele was introduced before the separation of the lineage that led to laboratory strains. Confirmation that the susceptibility allele also segregates among wild-derived strains would enable proper interpretation of the CAST/EiJ haplotype data and increase the opportunities for finding ancestral recombinants that can reduce the interval.

In rats, clipping of the renal artery during DOX infusion prevents the development of nephropathy, suggesting that this trait is independent of extrarenal drug metabolism and that direct exposure of the kidneys to anthracyclines is a requirement for the development of podocyte injury (35,36). These data predict that the DOXNPH gene should be expressed in the kidney and that an additional method for prioritizing positional candidates would be to determine whether they are expressed in renal tissue, particularly in glomerular podocytes.

In the meantime, our findings have practical implications for execution and interpretation of studies that involve DOX nephropathy. Our data define the susceptibility pattern for 15 strains, enabling investigators to select the correct strain for application of this model. Moreover, we prospectively tested the predictive value of haplotypes by typing six informative loci in two additional strains (CFW and 129P2/OlaHsd); these strains have the B6 haplotype at the DOXNPH locus, and, as predicted by their genotype data, they were resistant to DOX nephropathy. Therefore, for strains not studied here, investigators now can determine haplotype status at the DOXNPH locus to predict susceptibility or resistance to DOX nephropathy.

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