Understanding Our Drugs and Our Diseases

Yingying Guo, Paul Weller, John Allard, Jonathan Usuka, Mohammad Masjedizadeh, Shao-Yong Wu, Bill Fitch, Douglas Clark, J. David Clark, Steve Shafer, Jianmei Wang, Guochun Liao, and Gary Peltz

Departments of Genetics and Genomics, Drug Metabolism and Pharmacokinetics, and Chemical Services, Roche Palo Alto; Veterans Affairs Palo Alto Health Care System; and Department of Anesthesiology, Stanford University, Palo Alto, California

Analysis of mouse genetic models of human disease-associated traits has provided important insight into the pathogenesis of human disease. As one example, analysis of a murine genetic model of osteoporosis demonstrated that genetic variation within the 15-lipoxygenase (*Alox15*) gene affected peak bone mass, and that treatment with inhibitors of this enzyme improved bone mass and quality in rodent models. However, the method that has been used to analyze mouse genetic models is very time consuming, inefficient, and costly. To overcome these limitations, a computational method for analysis of mouse genetic models was developed that markedly accelerates the pace of genetic discovery. It was used to identify a genetic factor affecting the rate of metabolism of warfarin, an anticoagulant that is commonly used to treat clotting disorders. Computational analysis of a murine genetic model of narcotic drug withdrawal suggested a potential new approach for treatment of narcotic drug addiction. Thus, the results derived from computational mouse genetic analysis can suggest new treatment strategies, and can provide new information about currently available medicines.

Keywords: computational biology; genetics; pharmacogenetics

The ultimate challenge for genetic research is whether it can enable discoveries that impact human health. The genetic changes responsible for more than 1,500 monogenic human diseases (for listing see <http://www.ncbi.nlm.nih.gov/Omim/mimstats.html>) have been identified by genetic analysis of human cohorts (1). Nevertheless, relatively little information about diseases affecting a much larger percentage of the human population, such as diabetes mellitus, asthma, schizophrenia, and many cancers, has been obtained by genetic analysis. Despite considerable effort, the genetic susceptibility factors for diseases that are common in the human population, which usually have a complex genetic origin, have not been identified through genetic analysis in human disease populations (reviewed in Reference 2). It is far more difficult to identify the underlying genetic susceptibility factors for the common diseases; where multiple genetic loci are involved, and each genetic factor makes a small contribution to overall disease susceptibility. In an effort to overcome these difficulties, investigators have studied mouse models that mimic human disease to better understand the genetic factors associated with disease susceptibility. Analysis of experimental murine models has provided key support for many current concepts about disease pathogenesis and treatment strategies (3).

QUANTITATIVE TRAIT LOCUS METHODS FOR ANALYSIS OF MOUSE GENETIC MODELS

Since statistical methods for genetic mapping were introduced, many traits of biomedical importance have been analyzed using

murine experimental genetic models. Although it can provide important information, analysis of experimental murine genetic models is costly, time consuming, and frustrating for many scientists (4). In each murine disease model, two inbred strains that differ in a well-characterized property that mimics the pathophysiology of the disease were intercrossed to generate a large number of progeny. The intercross progeny are then genetically analyzed using Quantitative Trait Locus (QTL) analysis methodology. This analysis requires producing, genotyping, and phenotyping 300 to 1,000 intercross progeny derived from two parental inbred strains. Then, chromosomal regions located between genotyping markers are analyzed, and those regions that are linked with phenotypic trait differences between the two parental inbred strains are identified. Identification of the causative genetic loci within these linked chromosomal regions, which are usually large and contain many genes, is a difficult and often unproductive process that has been a source of frustration for many (4). To expedite the identification of the causative genetic loci, gene expression analysis of tissue obtained from the target organs affected in the disease model was performed using whole-genome microarrays. Differentially expressed genes within the target organs of the parental strains are identified, and those encoded within the genetically identified chromosomal regions are evaluated as potential candidate genetic factors. The coupled use of two orthogonal approaches, positional genetic information and gene expression analysis, accelerates the pace of analysis of mouse genetic models.

We have analyzed mouse genetic models of asthma (5), systemic lupus (6), and osteoporosis (7). In each case, novel insight into the pathogenesis of a disease was generated by identification of a genetic factor responsible for strain differences in the disease-related trait. Analysis of a murine model of osteoporosis illustrates this process (7). Bone mineral density achieved in early adulthood (peak bone mass) is a major determinant of osteoporotic fracture risk. Genetic analyses of the progeny derived from two inbred mouse strains identified several chromosomal regions regulating peak bone mineral density, but the identities of the causative genes within these regions were unknown. We investigated a 31-Mb region on mouse chromosome 11 that strongly influenced peak bone mineral density in the 1,000 intercross progeny analyzed for this project. Microarray analysis of kidney and cartilage tissue obtained from parental and congenic mice indicated that *Alox15* was the only differentially expressed gene within this region. *Alox15* codes for a murine 12/15 lipoxygenase. Although lipoxygenases have been implicated in the pathogenesis of several diseases, including atherosclerosis, asthma, cancer, and glomerulonephritis, the biological function of murine or human *Alox15* was not known. An *Alox15* knockout mouse was previously reported to not have any detectable difference from wild-type littermates (8). However, the genetic analysis indicated increased *Alox15* expression was correlated with reduced bone density and bone strength. The genetic hypothesis was experimentally tested using inhibitors of this enzyme and by analysis of mice with an *Alox15* gene knockout. When compared with wild-type mice, the *Alox15* gene knockout mice exhibited significantly higher whole-body bone

(Received in original form January 26, 2006; accepted in final form February 8, 2006)

Supported by grant 1 R01 GM068885-01A1 from the National Institute of General Medical Sciences (Y.G.).

Correspondence and requests for reprints should be addressed to Gary Peltz, M.D., Ph.D., Roche Palo Alto S3-1, 3431 Hillview Avenue, Palo Alto, CA 94304. E-mail: gary.peltz@roche.com

Proc Am Thorac Soc Vol 3, pp 409–412, 2006

DOI: 10.1513/pats.200601-014AW

Internet address: www.atsjournals.org

mineral density, and had improved femoral structural competence as evidenced by increased failure load and stiffness. Pharmacologic inhibition of *Alox15* in mice and rats significantly improved bone mass and strength, as well as offset the bone loss that accompanied experimentally induced estrogen deficiency (7). The results demonstrate that inhibition of *Alox15* may provide a novel method for prevention of osteoporosis.

HAPLOTYPE-BASED COMPUTATIONAL GENETIC MAPPING

However, identifying the causative genetic variant in a murine genetic model using QTL methodology requires at least 5 yr, and can involve at least five scientists. QTL mapping requires at least 2 yr for completion of the initial genetic analysis, and this identifies a rather large chromosomal region that may extend across one-half of an entire chromosome (9). Two to five additional years are then required to identify the causative genetic variant, which usually involves two additional steps. During this period, additional recombinant mice are generated to further narrow the causative interval, and all gene candidates within an identified chromosomal region are identified and analyzed. The primary limitation of the QTL method is the lack of resolution, which is the reason that it identifies large chromosomal intervals. This produces the prohibitively high cost and long time frames associated with QTL analysis. Only a small number of recombinations can occur between parental chromosomes after two generations of experimental intercrossing, and within key chromosomal regions the number of informative recombinations is even smaller. Therefore, a very large number of intercross progeny must be generated and analyzed to identify a causative genetic factor within a chromosomal interval (10).

To eliminate the high cost and long time frames associated with QTL methods for mouse genetic analysis, we developed a new genetic analysis method. This new method eliminates the need to generate intercross progeny, and has the precision to identify individual genes or subgenic regions. This new approach enables researchers to identify a causative genetic factor by correlating a pattern of observable physiologic or pathologic differences among selected inbred strains with a pattern of genetic variation. Genomic regions where the pattern of genetic variation has the strongest correlation with the trait distribution among the inbred strains analyzed are then identified (11–14). In contrast to the 5- to 7-yr time frame required for QTL analysis, a computational mapping project can be completed within a very short time frame. The major factor is the time required to measure a parameter across a panel of inbred strains. Depending on the type of trait analyzed, the phenotypic data are usually obtained within a day to a week after starting the experiment. Once the phenotypic data are obtained, the data can be computationally analyzed and the predictions evaluated by a single scientist within a single day.

For computational mapping, the pattern of genetic variation was characterized by identifying single nucleotide polymorphisms (SNPs) within a genomic region, and determining the alleles at each SNP position for 18 inbred strains analyzed (13). The extent of linkage disequilibrium among SNP alleles within a region was calculated, and a map of haplotype blocks within genomic regions with high linkage disequilibrium was produced. The SNPs and haplotype map of the inbred strain genome are displayed at <http://mouseSNP.roche.com>. The computational mapping program assesses the extent of correlation between the trait values and strain groupings within each haplotype block using analysis of variance-based statistical modeling (11, 14). To assess this correlation, a *p* value is calculated to determine if the data are consistent with the null hypothesis that the mean

trait values for inbred strains with the same genotypic haplotype are equal. This computational method correctly identified the genetic basis for phenotypic differences among the inbred strains for several biomedically important traits, and identified a novel cis-acting allelic enhancer element regulating H2-E α gene expression (11, 14). The limitations and factors affecting the precision of this method have been reviewed (12). The major limitation is that it can successfully analyze genetic models with relatively limited genetic complexity. Simulations have shown that an individual genetic factor must be responsible for at least 40% of the phenotypic variation for it to be identified by this computational method when 15 inbred strains are analyzed (12, 14). Two examples of biomedically important traits that were analyzed using this computational genetic method are described.

Computational Pharmacogenetics

Analysis of warfarin metabolism. It is widely anticipated that pharmacogenomic information will impact drug development, and subsequently clinical practice (15). Using pharmacogenomic information can increase efficacy, reduce side effects, and improve treatment outcome for patients. However, it is essential that efficient strategies are developed to identify genetic factors affecting the metabolism or response to current and future therapies (15). Because of this need, we wanted to determine if the murine haplotype-based computational genetic analysis method in mice could be used to quickly identify factors affecting the metabolism of commonly prescribed medications.

Warfarin metabolism in mice was selected as an initial model system for assessing the utility of the computational pharmacogenetic approach. Warfarin is a commonly prescribed anticoagulant that has a very narrow therapeutic index, and is a leading cause of iatrogenic complications. It inhibits a γ -carboxylation reaction required for the synthesis of several blood-clotting factors, and the dose can vary by as much as 120-fold among treated individuals. Warfarin is a racemic mixture of *R*- and *S*-enantiomers, which have very complex patterns of metabolism. It is differentially metabolized by a variety of cytochrome P450 enzymes into different hydroxylated metabolites, which then undergo phase II enzyme biotransformation to glucuronidated or sulfated metabolites prior to excretion. The complexity of warfarin metabolism precluded the use of a computational genetic method that is dependent on a single genetic change having a large impact on the phenotypic trait. Warfarin biotransformation to over nine different metabolites in rats and humans is mediated by many different enzymes. Each individual genetic difference could be responsible for only a small portion of the interstrain differences in warfarin pharmacokinetics. Therefore, an experimental strategy that reduced the complexity of this metabolic process was developed.

To do this, the rate of clearance of *R*-warfarin and each of nine different metabolites produced after administration of ^{14}C -labeled *R*-warfarin to 13 inbred strains was characterized in detail (16). The amount of warfarin and nine identified metabolites in plasma was quantitated after dosing 13 inbred mouse strains. Among all of the metabolites produced, the inbred strains had the largest difference in the rate of production of 7-hydroxylated metabolites of warfarin. The murine haplotype-based computational method was used to identify genetic factors regulating the metabolism of warfarin to 7-hydroxylated metabolites. Strain-specific differences in the generation of 7-hydroxywarfarin metabolites were computationally correlated with genetic variation within a chromosomal region encoding cytochrome P450 2C enzymes. This computational prediction was experimentally confirmed by showing that the rate-limiting step in biotransformation of warfarin to its 7-hydroxylated metabolite was inhibited by an *Cyp2c* isoform-specific substrate

(tolbutamide) and was mediated by expressed recombinant *Cyp2c29* (16).

Although other genetic variables also contribute to the interstrain differences in *R*-warfarin pharmacokinetics, *Cyp2c29* was computationally identified because it is the rate-limiting enzyme within a major elimination pathway for this drug. This example indicates how genetic variants responsible for interindividual pharmacokinetic differences for clinically important drugs can be identified by computational genetic analysis in mice. Of broader significance, this example demonstrated how genetic changes in a rate-limiting component of a metabolic pathway can be successfully identified using murine haplotype-based computational genetic analysis. This required experimental dissection of a very complex multicomponent drug disposition process into its component parts. Warfarin metabolism generates at least nine different intermediate metabolites, and several different enzymes may be involved in the production of each metabolite. Genetic variation within each of these enzymes could contribute to the strain-specific differences in *R*-warfarin metabolism. It is therefore not surprising that the computational method could not analyze the overall rate of *R*-warfarin disappearance across the inbred strains. However, the rate of production of an individual metabolite is regulated by a much more limited set of genetic variables. Because of the reduced genetic complexity, the haplotype-based computational method could successfully analyze the strain-specific pattern of warfarin elimination through a single metabolic pathway.

Analysis of a murine model of narcotic drug withdrawal. Narcotics are commonly used to treat moderate to severe pain. Although they cause acutely reduced pain sensation, they also cause a longer lived paradoxical sensitization to painful stimuli. Narcotic withdrawal is characterized by diminished pain thresholds (hyperalgesia) and exacerbation of preexisting pain. The increased sensitivity to pain that occurs during withdrawal or in between scheduled doses of narcotics is an aversive experience that is believed to reinforce drug dependence, and also limits the clinical utility of these drugs.

A murine model that measures the effect of narcotic administration on pain sensitivity (17) was analyzed. In this model, the pain (nociceptive) response threshold to a mechanical stimulus was measured at baseline and after 4 d of morphine treatment in 15 inbred mouse strains (18). There were significant differences among the inbred strains in the extent of the pain sensitization acquired after morphine treatment and withdrawal. Haplotype-based computational genetic analysis of the inbred strain data was performed to identify the genetic factor(s) responsible for these differences. The haplotype block with the strongest correlation was within a segment of the *Adrb2* (β_2 -adrenergic receptor) gene on chromosome 18 (18). This computational prediction was then experimentally tested. Administration of a selective β_2 -adrenergic receptor (Butaxamine) antagonist caused a dose-dependent reversal of the narcotic-induced effect on pain sensitivity, at a dose range that was well within that considered to be selective for an *Adrb2* effect. Additional experimental confirmation was obtained by analysis of the response of β_2 -adrenergic receptor-null mutant mice (*Adrb2*^{-/-}) and wild-type littermates. In contrast to its effect on wild-type mice, withdrawal after morphine treatment had an insignificant effect on the mechanical pain threshold in mice with an *Adrb2* gene knockout. Thus, pharmacologic evidence and data obtained from *Adrb2* gene knockout mice supported the genetically derived hypothesis that β_2 -adrenergic function modulated the morphine withdrawal response.

CONCLUSIONS AND FUTURE DIRECTIONS

On a broader level, the pharmacogenetic example demonstrates how genetic analysis can be facilitated by resolution of complex

phenotypes into more discrete processes. The metabolism of warfarin, like that of many other drugs, involves many different genes and pathways. Analysis of the intermediate metabolites enabled examination of individual steps that contribute to its metabolism. Because each individual step is controlled by a smaller number of genes than are involved in the overall process, this enabled identification of a genetic factor regulating warfarin metabolism among the inbred mouse strains. It is important to emphasize that this murine experimental approach and genetic analysis tool may not always generate results that directly translate to human drug metabolism. For example, *R*- and *S*-warfarin are metabolized by different cytochrome P450 enzymes in different species. CYP1A2 and CYP3A4 are the major contributors to *R*-warfarin metabolism in humans (19), which differs from our findings in mice. Despite these differences, this computational mouse genetic approach can quickly identify polymorphisms in drug-metabolizing enzymes that contribute to differential drug responses among a panel of inbred mouse strains. It is highly likely that identification of the genetic basis for many common human diseases would be facilitated by careful dissection of the underlying physiologic alterations that contribute to the disease.

It is hoped that results generated from the murine genetic model of narcotic withdrawal will catalyze the initiation of translational studies in humans. Despite the fact that selective β_2 -adrenergic receptor antagonists with improved formulations are currently available, these agents have not been used to alleviate narcotic withdrawal symptoms. Clinical studies that use available β_2 -adrenergic receptor antagonists and existing clinical trial paradigms can now be performed to determine whether these drugs reduce withdrawal symptoms in narcotic-treated patients. Beyond its immediate potential application for treatment of narcotic dependence, this analysis demonstrates that haplotype-based computational genetic mapping in mice can be used to formulate novel and accurate hypotheses about traits of biomedical importance. Although there are ways to improve the function of this emerging computational genetic analysis method (12), it can generate genetic hypotheses that lead to discoveries of biomedical importance.

Conflict of Interest Statement: Y.G. is an employee (postdoctoral fellow) of Roche Palo Alto. P.W. is an employee of Roche Palo Alto since 1989. J.A. is an employee of Roche Palo Alto. J.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.-Y.W. is an employee of Roche Palo Alto. B.F. is an employee of Roche. D.C. is an employee of Roche Palo Alto. J.D.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.W. is an employee of Roche Palo Alto. G.L. is a full-time employee of Roche Palo Alto. G.P. is an employee of Roche Palo Alto. A patent on the computational analysis method has been applied for by Roche Palo Alto.

References

1. Peltonen L, McKusick VA. Genomics and medicine: dissecting human disease in the postgenomic era. *Science* 2001;291:1224-1229.
2. Risch NJ. Searching for genetic determinants in the new millennium. *Nature* 2000;405:847-856.
3. Usuka J, Peltz G. From mouse genetics to human therapeutics. *Curr Opin Drug Discov Devel* 2005;8:253-261.
4. Nadeau JH, Frankel WN. The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. *Nat Genet* 2000;25:381-384.
5. Karp CL, Grupe A, Schadt E, Ewart S, Keane-Moore M, Cuomo PJ, Kohl J, Wahl L, Kuperman D, Germer S, *et al.* Identification of complement factor 5 (C5) as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000;1:221-226.
6. Rozzo SJ, Allard J, Choubey D, Vyse T, Izui S, Peltz G, Kotzin BL. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. *Immunity* 2001;15:435-443.

7. Klein RF, Allard J, Avnur Z, Nikolcheva T, Rotstein D, Carlos AS, Shea M, Waters RV, Belknap JK, Peltz G, *et al.* Regulation of bone mass in mice by the lipoxygenase gene *Alox15*. *Science* 2004;303:229–232.
8. Sun D, Funk CD. Disruption of 12/15-lipoxygenase expression in peritoneal macrophages. *J Biol Chem* 1996;271:24055–24062.
9. Darvasi A, Soller M. A simple method to calculate resolving power and confidence interval of QTL map location. *Behav Genet* 1997;27:125–132.
10. Darvasi A. Experimental strategies for the genetic dissection of complex traits in animal models. *Nat Genet* 1998;18:19–24.
11. Liao G, Wang J, Guo J, Allard J, Chang J, Nguyen A, Shafer S, Puech A, McPherson JD, Foernzler D, *et al.* In silico genetics: identification of a novel functional element regulating H2-Ea gene expression. *Science* 2004;306:690–695.
12. Wang J, Liao G, Usuka J, Peltz G. Computational genetics: from mouse to man? *Trends Genet* 2005;21:526–532.
13. Wang J, Liao G, Chang J, Nguyen A, Guo J, Chou S, Hu S, Bach C, Allard J, Shafer S, *et al.* Haplotypic structure of the mouse genome. In: Peltz G, editor. Computational genetics and genomics: new tools for understanding disease. Totowa, NJ: Humana Press; 2005. pp. 71–81.
14. Wang J, Peltz G. Haplotype-based computational genetic analysis in mice. In: Peltz G, editor. Computational genetics and genomics: new tools for understanding disease. Totowa, NJ: Humana Press; 2005. pp. 51–70.
15. Guo Y, Shafer S, Weller P, Usuka J, Peltz G. Pharmacogenomics and drug development. *Pharmacogenomics* 2005;6:857–864.
16. Guo YY, Weller PF, Farrell E, Cheung P, Fitch B, Clark D, Wu S-Y, Wang J, Liao G, Zhang Z, *et al.* In silico pharmacogenetics: warfarin metabolism. *Nat Biotechnol* (In press)
17. Liang D, Li X, Lighthall G, Clark JD. Heme oxygenase type 2 modulates behavioral and molecular changes during chronic exposure to morphine. *Neuroscience* 2003;121:999–1005.
18. Liang D, Liao G, Wang J, Usuka J, Guo YY, Peltz G, Clark JD. In silico genetic analysis of opioid-induced pain sensitization. *Anesthesiology* (In press)
19. Kaminsky LS, Zhang ZY. Human P450 metabolism of warfarin. *Pharmacol Ther* 1997;73:67–74.