Mouse Genome-Wide Association Mapping Needs Linkage Analysis to Avoid False-Positive Loci

Giacomo Manenti¹, Antonella Galvan¹, Angela Pettinicchio¹, Gaia Trincucci¹, Elena Spada², Anna Zolin², Silvano Milani², Anna Gonzalez-Neira³, Tommaso A. Dragani¹*

1 Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy, 2 Istituto di Statistica Medica e Biometria “GAMaccaro”, Università di Milano, Milan, Italy, 3 Genotyping Unit (CeGen), CNIO, Madrid, Spain

Abstract

We carried out genome-wide association (GWA) studies in inbred mouse strains characterized for their lung tumor susceptibility phenotypes (spontaneous or urethane-induced) with panels of 12,959 (13K) or 138,793 (140K) single-nucleotide polymorphisms (SNPs). Above the statistical thresholds, we detected only SNP rs3681853 on Chromosome 5, two SNPs in the pulmonary adenoma susceptibility 1 (Pas1) locus, and SNP rs4174648 on Chromosome 16 for spontaneous tumor incidence, urethane-induced tumor incidence, and urethane-induced tumor multiplicity, respectively, with the 13K SNP panel, but only the Pas1 locus with the 140K SNP panel. Haplotype analysis carried out in the latter panel detected four additional loci. Loci reported in previous GWA studies failed to replicate. Genome-wide genetic linkage analysis in urethane-treated (BALB/c×C3H/He)F2, (BALB/c×SWR/J)F2, and (A/J×C3H/He)F2 mice showed that Pas1, but none of the other loci detected previously or herein by GWA, had a significant effect. The Lasc1 gene, identified by GWA as a functional element (Nat. Genet., 38:888–95, 2006), showed no genetic effects in the two independent intercross mouse populations containing both alleles, nor was it expressed in mouse normal lung or lung tumors. Our results indicate that GWA studies in mouse inbred strains can suffer a high rate of false-positive results and that such an approach should be used in conjunction with classical linkage mapping in genetic crosses.

Introduction

Association studies are based on linkage disequilibrium (LD) between genetic markers and a disease locus affecting a particular phenotype [1,2]. Such studies may allow the fine mapping of loci affecting monogenic diseases, as well as loci affecting complex diseases if the relevant alleles are common in the general population under investigation. This approach can be quite powerful when disease chromosomes are descended from a single founder mutation and the markers analyzed are tightly linked to the disease locus. In these cases, the LD approach has proven successful not only in humans for the fine mapping of rare diseases in isolated populations, but also in experimental animals. Indeed, our LD analysis in mouse inbred strains served in refining the mapping region of Pas1, the major locus affecting susceptibility to mouse lung tumorigenesis [3]. Subsequently, we found that the Pas1 locus consists of a conserved haplotype spanning ~400 kb and including 6 genes, whose polymorphisms define a susceptibility allele that is frequent in laboratory mouse inbred strains and apparently derived from an ancestral progenitor [4]. More recently, we used LD analysis to show that the Pas1 locus affects not only carcinogen-induced lung tumor multiplicity, but also spontaneous lung tumor incidence [5].

The availability of single nucleotide polymorphism (SNP) data for many inbred strains has led to the proposal of in silico genome-wide mapping of mouse quantitative trait loci (QTLs) [6]. In three genome-wide association (GWA) studies aimed at detecting lung tumor modifier loci, 5 loci (named Slt loci) affecting incidence of spontaneous lung tumors [7], 4 loci (named Clas loci) affecting incidence of urethane-induced lung tumors [8], and 5 loci affecting incidence of N-nitroso-N-ethylurea-induced lung adenomas or adenocarcinomas [9] were identified. Each of these three reports detected a unique, non-overlapping set of loci, despite analysis of similar populations of mouse strains and highly correlated tumor phenotypes, i.e., spontaneous, urethane-, or N-nitroso-N-ethylurea-induced lung tumor incidences [5].

The identification of authentic lung tumor modifier loci in the population of mouse inbred strains is fundamental to revealing the genetic elements that control tumor susceptibility in this mammalian model. To assess the reproducibility and functional relevance of GWA results, we carried out this analysis using a larger number of mouse strains and more phenotypes describing the lung tumor susceptibility trait than in previous studies and compared the results with those obtained from standard genetic linkage analyses in intercross populations.

Results

GWAs Detect Putative Lung Tumor Modifier Loci

Strain susceptibility to lung tumorigenesis can be described using different phenotypes, such as tumor incidence, tumor multiplicity, and tumor volume. Mouse inbred strains show a

Citation: Manenti G, Galvan A, Pettinicchio A, Trincucci G, Spada E, et al. (2009) Mouse Genome-Wide Association Mapping Needs Linkage Analysis to Avoid False-Positive Loci. PLoS Genet 5(1): e1000331. doi:10.1371/journal.pgen.1000331

Editor: Wayne N. Frankel, The Jackson Laboratory, United States of America

Received June 5, 2008; Accepted December 9, 2008; Published January 9, 2009

Copyright: © 2009 Manenti et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was funded in part by grants from Associazione and Fondazione Italiana Ricerca Cancro (AIRC and FIRC).

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: tommaso.dragani@istitutotumori.mi.it
wide range of lung tumor phenotypes, with mean spontaneous tumor incidence ranging from 0 to 82%, mean urethane-induced lung tumor incidence from 0 to 100%, and mean urethane-induced lung tumor multiplicity ranging from 0 to 28.5 tumors/mouse (Table 1). A highly significant correlation was found between the spontaneous tumor incidence and urethane-induced tumor multiplicity phenotypes ($r = 0.94$, $-\log P = 8.9$), whereas correlation between the spontaneous tumor incidence and urethane-induced tumor incidence phenotypes was weaker ($r = 0.76$, $-\log P = 5.8$) (Figure 1).

GWA using the WTCGH 13K SNP panel was carried out in 20 to 27 strains for which phenotype-genotype data were available (Table 1). For any of the lung tumor phenotypes, the Bonferroni’s statistical threshold ($\alpha = 0.1$ significance level) accounting for the number of statistical tests (i.e., 12,959) would result in a $-\log P$ value $= 5.1$. GWA analyses revealed that only rs3681853 on Chromosome 5 reached this statistical threshold for spontaneous tumor incidence, whereas no SNPs were associated with urethane-induced tumor incidence and only one SNP (rs1746448) on Chromosome 16 was associated with urethane-induced tumor multiplicity. Surprisingly, the known Pas1 locus was not detected. Using the suggestive thresholds obtained by permutation tests ($\alpha = 0.10$, Table 2), which also account for the correlation structure of the data, we detected only SNP rs3681853 on Chromosome 5, $-\log P = 5.3$, spontaneous incidence, SLT6, SNPs rs13459098 and rs13479086 in the Pas1 region (both SNPs at $-\log P = 4.7$, urethane-induced tumor incidence, Clas1) and SNP rs1746448 on Chromosome 16 ($-\log P = 6.5$, urethane-induced tumor multiplicity, Clas5) (Table 3). When Mus spraguei was excluded from the analysis, rs3681853 did not reach any statistical thresholds and the other results remained the same.

Due to the low sensitivity of detection of putative loci based on statistical thresholds, as demonstrated by the significant association of the known Pas1 locus with only one of three lung tumor phenotypes, we also examined putative loci whose associations were below the statistical thresholds. For example, at $-\log P \geq 4$, 10 and 11 SNPs showed significant association with spontaneous incidence (SLT loci) and urethane-induced multiplicity (Cas loci), respectively, of lung tumors. No other SNPs above $-\log P = 4$, except the two Pas1-associated SNPs at $-\log P = 4.7$, were detected for the phenotype “incidence of urethane-induced lung tumors.” By attributing a locus definition to chromosomal regions spanning less than 1 Mb in length and containing one or more SNPs associated with lung tumor phenotypes, these associations identified 8 new SLT loci that included Pas1, the previous Clas1 (Pas1), and 5 new Clas loci (not shown).

We then replicated the GWA using the BROAD SNP panel, which provided a higher SNP density (140K) but a lower number of strains (i.e., 20 to 29) (Table 1). To reduce the risk of false-positives due to the inclusion of genetically distant strains, we excluded the Mus spraguei strain (SPERET/Eij). Above the suggestive thresholds ($\alpha = 0.10$, Table 2), we detected only SNPs rs30118733 and rs30752783 ($-\log P = 7.5$ and 6.9, respectively, urethane-induced incidence), both of which were located in the Pas1 region.

SLT1 to SLT6 and Clas2 to Clas5 were not confirmed by analysis using the BROAD SNP panel, although SLT6 and Clas5 were detected by the WTCGH SNP panel (Table 3).

Finally, our haplotype-based GWA analysis using the BROAD dataset and a three-SNP sliding window revealed haplotype-associated lung tumor (Hal1 loci) (Table 4, Figure 2). For spontaneous lung tumor incidence, no haplotype reached statistical threshold ($\alpha = 0.10$, Table 2), whereas for urethane-induced lung tumor incidence, two associated haplotypes were detected: Hal1 in the Pas1 region and Hal2 on Chromosome 14 (Table 4). Associated to the urethane-induced tumor multiplicity phenotype, we detected five statistically significant haplotypes (Hal3- Hel7), two of which mapped in the Pas1 locus (Hal5) or in its flanking region (Hal6) (Table 4).

Results of GWA Studies Fail to Replicate

A previous GWA study in 13 inbred strains detected 5 loci, named SLT1 to SLT5, associated with spontaneous incidence of lung tumors [7]. Our analysis in 27 strains contained 12, 4, 10, 11, and 19 SNPs in the SLT1 to SLT5 regions, respectively. However, none of the SLT loci were confirmed in our GWA analysis (Table 3). To rule out the possibility that the non-replication of previous results [7] was due to the lack of inclusion of relevant SNPs in the SNP database used, we identified and selected SNPs in the same SLT regions showing exactly the same 13 strain distribution pattern reported [7] and genotyped the selected SNPs in the 27 strains of our study (Table 1) plus the O20/A strain [5]. However, none of the SNPs located in SLT1 (rs13478866), SLT2 (rs13479117), SLT3 (Gabat2 JC10664_20, Agt JC10667_5, Agt JC10669_3), SLT4 (rs13483600), and SLT5 (rs3667513) confirmed an association with spontaneous lung tumor incidence (not shown) and none of the SLT loci showed an association with urethane-induced lung tumor incidence or multiplicity (Table 3).

We also tested whether previous GWA results on lung tumor multiplicity [8] might be confirmed in our study. Unlike the spontaneous incidence data, the sizes of the two datasets on lung tumor multiplicity are almost identical (n = 21–22) [8], with very similar strain composition (Table 1 and [8]). Differences included two BALB/c substrains and the O20/A strain analyzed in [8], whereas we analyzed only one BALB/c strain and did not include the O20/A strain but did include the C58/J and the NZB/BIGd strains not analyzed in [8]. Overall, we expected to observe essentially overlapping results. Using the WTCGH 13K SNP panel, we confirmed the association at the Pas1 locus, with 2 SNPs showing $P$ values above the statistical threshold for the tumor incidence phenotype (Table 3). At the Clas2 locus on Chromosome 4, (CEL-4_30653207, alias rs27801920), [8], we found a $-\log P = 2.8$. Genotyping at this locus in the strains of the genome-wide scan plus the NGP/N and O20/A strains for the functional Lasc1 SNP D102E (rs32396036) revealed no significant associations with lung tumor phenotypes ($-\log P = 1.8$ to 2.4).
Furthermore, we detected no significant associations at the Clas3 and Clas4 regions (Table 3), despite the inclusion of 6 and 9 SNPs in the Clas3 and Clas4 regions, respectively. Even the higher SNP density offered by the BROAD SNP panel failed to reveal any Clas loci except Pas1 (Clas1).

Another recent GWA study conducted in 20 inbred strains treated with N-nitroso-N-ethylurea and scanned with the WTCHG SNP database detected several putative lung tumor susceptibility loci on Chromosomes 3, 6, 9, and 15 [9]; these loci did not correspond to Clas loci or to regions detected in the present study. Authors of [9] did not detect the Pas1 locus, which has been implicated in lung tumorigenesis independently of the type of chemical carcinogen [3]. In contrast with the GWA results, the Pas1 locus but none of the GWA loci was detected in a genetic linkage study that used the same carcinogen as in the GWA study, i.e., N-nitroso-N-ethylurea [10]. We observed no association with any lung tumor phenotype at any of the loci reported in [9], using either the WTCHG or the BROAD SNP panel.

### Loci Detected by Strain Survey Are Not Confirmed by Genetic Linkage Studies

Genetic linkage analysis of mouse crosses represents a formal approach to demonstrating functional activity of genetic loci on given phenotypes. In a single cross, not all loci affecting a specific complex phenotype in a given species are expected to be detected, since a locus can exert allele-specific effects only in crosses

### Table 1. Lung tumor phenotypes (spontaneous and urethane-induced incidence and urethane-induced tumor multiplicity) of mouse inbred strains used in this study.

| Strain         | Spontaneous Incidence (mean %) | Urethane-induced Incidence (mean %) | Urethane-induced Multiplicity (no. of tumors/mouse) | SNP Database |
|----------------|--------------------------------|-------------------------------------|------------------------------------------------------|--------------|
| 129X1/SvJ      | 1                              | 89                                  | 2.1                                                  | W, B         |
| A/J           | 82                             | 100                                 | 27.5                                                 | W, B         |
| AKR/J            | 0                              | 9                                   | 0.1                                                  | W, B         |
| BALB/c        | 33                             | 87                                  | 3.3                                                  | W, B         |
| C3H/HeJ       | 9                              | 7                                   | 0.2                                                  | W, B         |
| CS7BL/10J     | 3                              | 26                                  | 0.3                                                  | W, B         |
| CS7BL/6J     | 3                              | 13                                  | 0.5                                                  | W, B         |
| CS7BR/cdJ    | 3                              | 0                                   | 0                                                   | W, B         |
| CS7L/J         | 0                              | 10                                  | 0.1                                                  | W, B         |
| C58/J         | 0.5                            |                                     |                                                      | W, B         |
| CAST/Ei       | 0                              |                                     |                                                      | W, B         |
| CBA/J         | 17                             | 79                                  | 1.7                                                  | W, B         |
| CE/J          | 0                              |                                     |                                                      | W, B         |
| DBA/1J        | 3                              |                                     |                                                      | W, B         |
| DBA/2J        | 0                              | 25                                  | 0.4                                                  | W, B         |
| FVB/NJ        | 36                             |                                     |                                                      | W, B         |
| LP/J          | 5                              | 63                                  | 1.1                                                  | W, B         |
| MA/MyJ        | 42                             | 100                                 | 8.9                                                  | W, B         |
| NGP            |                                |                                     | 28.3                                                 |              |
| NZB/BINJ      | 0                              | 25                                  | 0.3                                                  | W, B         |
| NZW/LacJ      | 24                             |                                     |                                                      | W, B         |
| O20            | 41                             | 100                                 | 12.1                                                 | B            |
| P/J            | 3                              |                                     |                                                      | W            |
| PL/J          | 67                             | 2                                   |                                                      | W, B         |
| RF/J          | 26                             |                                     |                                                      | W            |
| RIIIS/J       | 34                             | 70                                  | 6.7                                                  | W, B         |
| SIL/J         | 6                              | 29                                  | 0.3                                                  | W, B         |
| SM/J          | 0                              | 50                                  | 0.5                                                  | W, B         |
| SPRET/EU      | 0                              |                                     | 0.5                                                  | W, B         |
| ST/bJ         | 86                             |                                     | 3.2                                                  | W, B         |
| STS/A         | 24                             |                                     |                                                      | W            |
| SWR/J         | 47                             | 96                                  | 20.1                                                 | W, B         |

1 Mean incidence of spontaneous lung tumors; data from [5], except for CAST/Ei, NZW/LacJ, SM/J, and SPRET/EU, which derived from [7].
2 Mean incidence of urethane-induced lung tumors; data from [5] or [22].
3 Mean lung tumor multiplicity after urethane treatment; data from [5], except for SPRET/EU, which derived from [23].
4 W, WTCHG; B, BROAD.

doi:10.1371/journal.pgen.1000331.t001
originating from two strains carrying different alleles and cannot be detected if the functional element(s) is non-polymorphic in the two parental strains. Accordingly, the \( \text{Pas1} \) locus is easily detected in crosses between strains carrying either of the two \( \text{Pas1} \) alleles (or haplotypes) but is not detectable in crosses between strains carrying the same haplotype [11,12].

To test whether loci detected by genome-wide strain survey may be involved in modulating lung tumorigenesis, we carried out genome-wide genetic linkage analyses of three intercross populations previously analyzed for urethane-induced lung tumor multiplicity [11–13]. In our population of \((\text{BALB/c} \times \text{C3H/He})\text{F2} \) mice, genotyping by SNP array detected a total of 383 non-redundant informative SNPs widely dispersed over the whole mouse genome. There was complete coverage of all chromosomes, with a range of 12–43 non-redundant SNPs genotyped for each chromosome, except for Chromosome 9 which contained only 4 SNPs. Above the R/qtl threshold, only the effect of the \( \text{Pas1} \) locus was observed, with LOD scores of 18.4 (Figure 3A, red line). By conditioning on the \( \text{Pas1} \) genotype, no additional QTLs were detected (Figure 3A, black line). In \((\text{A/J} \times \text{C3H/He})\text{F2} \) mice, 192 markers ensured genome-wide coverage, and composite interval mapping scan detected the known \( \text{Pas1} \) locus and no other locus (Figure 3B, red line), even by a

---

**Figure 1.** Spontaneous lung tumor incidence correlates with both urethane-induced lung tumor multiplicity (green) and incidence (red) in mouse inbred strains. Incidence is given as mean percentages, whereas multiplicity is mean number of tumors/mouse. See Table 1 for phenotype values. doi:10.1371/journal.pgen.1000331.g001

**Table 2.** Threshold probabilities (expressed as \(-\log P\)) corresponding to experiment-wide type I risk errors \( \alpha = 0.05 \) and \( \alpha = 0.10 \).

| Lung tumor phenotype | WTCGH (12,059 SNP) | BROAD (138,793 SNP) |
|----------------------|-------------------|---------------------|
|                      | \( \alpha = 0.05 \) | \( \alpha = 0.10 \) |
|                      | \( \alpha = 0.05 \) | \( \alpha = 0.10 \) |
| Permutation test     |                   |                     |
| Spontaneous incidence| 5.6               | 5.2                 |
| Urethane-induced incidence | 5.1               | 4.7                 |
| Urethane-induced multiplicity | 6.8               | 6.1                 |
| t-test (with Bonferroni's adjustment) | 5.4               | 5.1                 |
|                      | 6.4               | 6.1                 |

---

**Table 3.** Putative lung tumor modifier loci identified by previous genome-wide association studies or by the present study using the WTCGH SNP panel.

| Locus a | SNP b | Chromosome | Position (Mb) c | Spontaneous incidence d | Urethane-induced incidence d | Urethane-induced multiplicity d |
|---------|-------|------------|----------------|--------------------------|-----------------------------|-------------------------------|
| SLT1    | rs29883445 | 6 | 84.19 | 2.2 | 0.2 | 0.7 |
| SLT2    | rs31152907 | 7 | 10.75 | 1.5 | 0.5 | 0.8 |
| SLT3    | rs13480027 | 8 | 126.47 | 1.0 | 0.9 | 1.4 |
| SLT4    | rs13483600 | 19 | 34.75 | 3.7 | 1.0 | 3.6 |
| SLT5    | rs3667513 | X | 139.92 | 3.1 | 1.6 | 2.1 |
| SLT6    | rs3681853 | 5 | 150.21 | 5.3 | 0.8 | 3.9 |
| Class 1 (Pas1) | rs13459098 | 6 | 145.12 | 3.8 | 4.7 | 3.8 |
| Class 2 (Lasc1) | rs32396036 (D102E) | 4 | 30.36 | 1.8 | 2.4 | 1.9 |
| Class 3 | rs3690198 | 13 | 60.82 | 0.6 | 2.6 | 2.5 |
| Class 4 | rs13482741 | 15 | 100.95 | 0.5 | 2.4 | 2.5 |
| Class 5 | rs4174648 | 16 | 35.59 | 2.8 | 1.5 | 6.5 |

---

aSLT1 to SLT5 loci, reported to affect spontaneous lung tumor incidence in 13 strains [7]; Class 1 to Class 4 loci, reported to affect lung tumor multiplicity in 21 strains [8]. Additional SLT and Class loci derive from the present study; for each locus region (1 Mb size), the SNP showing the best statistical association is shown.
bSelected SNPs mapping in the reported SLT1 to SLT5 or Class 1 to Class 4 regions were genotyped in all strains for which phenotypes were available (Table 1); the SNPs showing the highest association with the phenotype are shown.
cPosition (in Mb) based on Ensembl release 49.
dThe association between each SNP and lung tumor phenotypes (expressed as \(-\log P\) value) was tested by t-test. Minus \( \log P \) values reported in bold type indicate the associations above the statistical threshold obtained by permutation (at \( \alpha = 0.10 \) significance level).
separate conditioning for the *Pas1* genotype (Figure 3B, black line). Analysis of the (BALB/c × SWR/J)F2 population by composite interval mapping scan confirmed the reported chromosomes 4 (*Pagp1*), 6 (*Par4*), and 18 (*Par2*) loci and detected an additional locus (LOD score = 5.3) on chromosome 1 between *D1Mit18* and *D1Mit22* markers (not shown).

Thus, none of the loci except *Pas1* identified by previous or the present (Table 3) genome-wide strain survey were confirmed by

---

**Table 4.** Haplotype-associated lung tumor modifier (*Halt*) loci identified by haplotype analysis, using the 140K BROAD SNP panel.

| Locus     | Markers                          | Chromosome | Position (Mb) | Global -log P | Lung tumor phenotype | Intercross |
|-----------|----------------------------------|------------|---------------|---------------|----------------------|------------|
| *Halt1*   | rs30907104–rs30843330–rs30118733 | 6          | 144.90        | 6.4           | UI                   | AHF2, CHF2 |
| *Halt2*   | rs31236017–rs31387917–rs31237574 | 14         | 100.68        | 6.7           | UI                   | -          |
| *Halt3*   | rs33459720–rs33172833–rs33360453 | 2          | 147.65        | 6.2           | UM                   | AHF2, CWF2 |
| *Halt4*   | rs29683518–rs32191266–rs29505322 | 5          | 121.63        | 6.2           | UM                   | AHF2, CHF2 |
| *Halt5*   | rs30913614–rs30514918–rs29924904 | 6          | 145.13        | 7.5           | UM                   | AHF2, CHF2 |
| *Halt6*   | rs3694732–rs30366423–rs52246948  | 6          | 147.51        | 6.2           | UM                   | AHF2, CHF2 |
| *Halt7*   | rs29724114–rs30023351–rs29867331 | 18         | 59.45         | 6.7           | UM                   | AHF2, CHF2, CWF2 |

*Position of the central SNP, in mega bases (Mb) based on NCBI m37 mouse assembly. *Global -log P is the minus logarithm of the p-value for the haplotype sliding window (window size: 3-SNPs). *UI, urethane-induced lung tumor incidence, UM, urethane-induced lung tumor multiplicity. *At each *Halt* locus, the intercrosses whose parental strains carry different alleles, and that have herein been analyzed, are indicated. AHF2, (A/J × C3H/He)F2; CHF2, (BALB/c × C3H/He)F2; CWF2, (BALB/c × SWR/J)F2. doi:10.1371/journal.pgen.1000331.t004

---

**Figure 2.** Genome wide scans for haplotype association with urethane-induced lung tumor multiplicity in mouse inbred strains using F-test for window size of 3 SNPs against the marker map plot. Threshold (in dotted green line) p value (α = 0.10) was calculated according to Bonferroni's criterion. doi:10.1371/journal.pgen.1000331.g002
Figure 3. Genome-wide genetic linkage analysis of loci affecting urethane-induced lung tumor multiplicity. (A) (BALB/c × C3H/He)F2 cross detects the Pas1 locus at LOD score = 18.4. (B) (A/J × C3H/He)F2 cross detected the Pas1 locus at LOD score = 18.7. Red curves indicate the results of the composite interval mapping, whereas black curves indicate the results of genome scan using the Kras genotype as covariate (conditioning on the Pas1 alleles). Horizontal lines indicate the threshold values (α = 0.05) of the LOD score. The Clas2 locus (Chromosome 4) showed no significant linkage, despite the presence of the claimed functional polymorphism (D102E) in both crosses. No other locus detected by whole-genome strain survey showed significant linkage.

doi:10.1371/journal.pgen.1000331.g003
genetic linkage analysis (Figure 3), although either the same or flanking SNPs detected by GWA at SLT1 to SLT6 loci and at Clas1 (Pas1) to Clas5 loci were, in fact, polymorphic in at least one of our three intercross populations.

Note that the Clas2 locus showed no effect in the (BALB/c × C3H/He)F2 cross, as indicated by the absence of any significant linkage of the whole Chromosome 4 (covered by 19 SNPs) to any lung tumor phenotype (Figure 3 and not shown). Moreover, since the claimed functional D10E2 polymorphism of the Lasc1 gene defining the Clas2 locus [8] was present in the (BALB/c × C3H/He)F2 cross, we genotyped that polymorphism; no significant linkage with lung tumor multiplicity was found at \( \alpha = 0.10 \) significance level.

Further testing of the D10E2 polymorphism by genotyping in the (A/J × C57BL/6J)F1 cross (total of 163 mice) [13], which also carries the polymorphism, again revealed no significant associations with either lung tumor multiplicity or volume (Figure 3B).

With regard to the loci detected by haplotype analysis, none but those linked with the Pas1 locus were confirmed by genetic linkage analysis. Except for the Halt2 locus on Chromosome 14, which could not be detected because all four parental strains of our genetic crosses carried the same haplotype, all other Halt loci could be detected in at least one of the intercrosses that displayed informative haplotypes (Table 4). In the (BALB/c × SWR/J)F2 intercross, a significant linkage was found in the pulmonary adenoma resistance 2 (Pas2) locus [12], with peak LOD score = 15.5 at D18Mit33, located at about 10 Mb distal from the Halt7 locus; no linkage near the Halt7 locus was found in the two other informative crosses (Table 4).

Absence of Lasc1 mRNA Expression in Mouse Lung

Notwithstanding the lack of significant linkage of the Lasc1 gene in two independent crosses, we examined Lasc1 mRNA expression in mouse normal lung and lung tumors. RT-PCR analysis of normal lung tissue from A/J and C57BL/6J mice, which carry different alleles of this gene, and of normal lung and tumor tissue from (A/J × C57BL/6J)F1 mice revealed no Lasc1-specific transcript fragments in either normal or tumor lung tissue, whereas genomic DNA was clearly amplified (Figure 4, top) and the Itpr2 or Gapdh positive controls were readily detected in cDNA samples (Figure 4, bottom and data not shown). In light of the reported widespread expression of Lasc1 [8], we examined Lasc1 mRNA expression in brain, liver, kidney, spleen, and testis of adult mice; only testis from which the AK076999 clone was originally derived revealed detectable Lasc1 mRNA (not shown).

Discussion

We found a highly significant correlation (\( r = 0.94 \)) between the phenotypes of spontaneous incidence and carcinogen-induced multiplicity of mouse lung tumors (Figure 1). Although we cannot exclude the possibility that part of this correlation resides in population structure, the result suggests that the genetic control of both phenotypes resides mainly in the same genetic loci. The high correlation between the two phenotypes may be explained by the Pas1 locus and its strong effects on both phenotypes, i.e., odds ratios of \(~12\) and \(~15\) with spontaneous and chemically induced lung tumorigenesis, respectively [5]. At present, it is not known whether additional lung tumor modifier loci can control both phenotypes. Proof that the same genetic elements control both spontaneous and chemically induced lung tumorigenesis in the mouse model could have important implications for other species, including humans, and therefore warrants further study.

The design of the present GWA study was similar to that of two previous studies carried out for either spontaneous [7] or urethane-induced incidence of lung tumors [8], although we had the opportunity to analyze a larger number of mouse strains. Indeed, in the spontaneous tumorigenesis association, we analyzed 27 strains with 13K SNPs (WTCHG) and 23 strains with 140K SNPs (BROAD) versus 13 strains with \(~135,900\) SNPs [7]. In the analysis of urethane-induced lung tumorigenesis, we analyzed 20–22 (WTCHG and BROAD) strains for tumor multiplicity and incidence (Table 1), respectively, versus an effective number of 19 strains with \(~125,000\) SNPs in [8], where the two BALB/c substrains should count as a single strain because of their overlapping phenotypes and genotypes, and where lack of available genotype data from the Broad Institute for the C57BL/10J strain allowed analysis only with the 13K WTCHG panel of SNPs.

The power to detect genotype-phenotype associations depends on the genomic length over which LD between functional and marker polymorphisms extends. Since LD decays with distance, a high-density map provides better resolution power than a low-density map. However, the haplotype structure of mouse inbred strains shows a mosaic pattern [14], and haplotype segments ranging from 12 to 608 kb in length have been reported [15]. Those findings suggest that even a medium-density map is sufficient to detect QTLs, especially considering the limited pool of founder genomes of the mouse laboratory strains and their consequent relatedness [16]. Indeed, our use of the BROAD high-density SNP panel (\(~20\) kb per SNP) confirmed the Pas1 detection but not the SLT6 and Clas5 loci detected by the medium-density WTCHG SNP panel (average density of \(~160\) kb per SNP). On the other hand, the power of QTL detection decreases as strain number decreases, and it has been proposed that there is little rationale supporting analysis of complex traits using less than 30 strains [17]. For comparisons, population-based association studies in humans require several hundreds or thousands of individuals, and confirmation of the results is also required [18].

We confirmed none of the 5 SLT loci detected in previous GWA studies. The previous association study on the urethane-induced lung tumor incidence phenotype detected 4 loci (Clas1 to Clas4) [8], none of which overlap with any of the SLT loci identified by...
the same group in another study [7]. Using the same phenotype, we confirmed the *Pas1* locus (also called *Clas1* in [8]), with 2 SNPs in both the WTCHG (rs13459690 in the *Clas1* gene and rs13479086 in the genomic region between *Iosas* and *full1* genes) and the BROAD panel (rs30118773 at 5′-end of the *Pas1* haplotype and rs30732783 near the *full1* gene) showing statistical associations. However, the *Clas2* to *Clas4* loci were not confirmed; genotyping of the functional *Clas2* element (*D102E*, rs3296092) in all strains for which phenotype data were available revealed a −log P = 2.4 for urethane-induced lung tumor incidence and a lower statistical association for the other tumor phenotypes (Table 3).

The discrepancy regarding *Clas2* to *Clas4* detection in our study and that of [8] may rest in small differences in strain composition. The reported functional *D102E* polymorphism at the *Lasc1* locus (69.83 Mb) [12] and its flanking SNP were detected in either (BALB/c×C3H/He)F2 or (A/J×C3H/He)F2 intercrosses carrying that polymorphism, and no *Halt7* transcript was detectable in normal lung tissue, in lung tumors, or in several mouse organs, except testis, despite its reported widespread expression [8]. Thus, the reported allele-specific effects by *in vitro*-transfected expression vectors containing either the 102D or 102E *Lasc1* allele (whose cDNA is contained in a single exon; Vega gene OTTMUSG000000004898, http://vega.sanger.ac.uk/index.html) cannot constitute evidence of a locus effect in the absence of such evidence by genetic linkage studies.

Haplotype analysis did not increase the reliability of GWA in comparison with single-point analysis, since the *Halt7* loci detected by haplotype analysis, with the exception of those linked to *Pas1*, were not confirmed by genetic linkage studies despite the haplotype differences between the parental strains originating the crosses. The *Halt7* locus might also represent a false-positive association, since significant genetic linkage near the *Halt7* locus position was detected in only one of three informative intercrosses, and since the mapping position of *Halt7* is ~10 Mb apart from the LOD score peak defining the *Pas2* locus (69.83 Mb) [12] and its candidate gene *Poli* (70.67 Mb) [19].

Overall, our comparison of the results of GWA studies in inbred strains with the genetic linkage analysis results confirmed none of the putative loci identified by strain survey, except the *Pas1* locus (Figure 3), notwithstanding the polymorphism of several loci in the genetic cross examined. Comparison of our GWA results with those of previous studies indicates a high variability of the statistical thresholds obtained by permutation. This is expected, since the thresholds are influenced by the number of SNPs and of strains, by the correlation structure of the data, and by the distribution of the phenotypes under study. Thus, inclusion or exclusion of even a single strain in a 20-strain study would strongly affect the statistical thresholds and the loci detected. Our study raises concern about the ability of GWA studies to detect authentic QTL loci and provides a note of caution to the mouse genetics field, where GWAs are seeing wide application across many phenotypes. In agreement with previous studies [17,20], our results support the notion that association mapping in the population of inbred mouse strains is characterized by a high false-positive rate and that such a method must be carried out with a large number of strains (i.e., 40 to 150). Accordingly, extensive computer simulation analyses have shown that the power of GWAs studies is low for phenotypes controlled by polygenic traits and that spurious associations are expected [21]. Thus, GWA studies should be carried out in conjunction with genetic linkage analysis to detect relevant loci.

**Materials and Methods**

**Mouse Phenotypes, DNAs, and RNAs**

Table 1 lists the data for 32 mouse inbred strains on spontaneous lung tumor incidence (n = 28), urethane-induced lung tumor multiplicity, i.e., number of tumors/mouse (n = 24), and urethane-induced lung tumor incidence (n = 21) derived from [5,7,22,23]. Genomic DNAs from the same inbred strains were obtained from The Jackson Laboratory Mouse DNA Resource (Bar Harbor, ME, USA). Intercross populations consisted of (BALB/c×C3H/He)F2 mice (n = 182 males) [11], (A/J×C3H/He)F2 (n = 87 males and 87 females) [13], and (BALB/c×SWR/J)F2 mice (n = 106 males and 112 females) [12]; all three populations had been treated with a single dose of urethane, observed without any further treatment, and evaluated quantitatively for lung tumor multiplicity phenotype. RNA was extracted from normal lung of adult male A/J, C57BL/6J, and (A/J×C57BL/6J)F1 mice, from urethane-induced lung tumors of (A/J×C57BL/6J)F1 mice [4], and from brain, liver, kidney, spleen, and testis of a male SM/J adult mouse, using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA, USA).

**SNP Genotype Extraction, Genome-Wide Scan, and SNP Genotyping**

Genotypes of 12,959 and 138,793 SNPs publicly available at Wellcome Trust Centre for Human Genetics (WTCHG) (http://www.well.ox.ac.uk/mouse/INBREDS/) and at Broad Institute (http://www.broad.mit.edu/), respectively, were extracted. Table 1 lists the strains for which WTCHG or BROAD genotypes are available. Genomic DNAs of (BALB/c×C3H/He)F2 mice were genotyped using Illumina SNP genotyping technology which allows the simultaneous analysis of 1536 SNPs [24]. Genomic DNAs of (A/J×C3H/He)F2 mice were genotyped using MassARRAY (Sequenom, Inc., San Diego, CA) with a multiplex PCR assays (iPLEX) designed by Sequenom SpectroDESIGNER software. The extension products were spotted onto a 384-well spectropCHIP before analysis by MALDI-TOF mass spectrometry. Selected SNPs were genotyped in mouse inbred strains by pyrosequencing on a PSQ96MA system (Biotage AB, Uppsala, Sweden). A short fragment containing the SNP was PCR-amplified using a biotinylated primer as one of the two PCR primers and pyrosequenced according to the manufacturer’s instructions.

**Lasc1 Expression Analysis**

*Lasc1* mRNA was searched by RT-PCR using primers: 5′-tatactctagtgttctactagctg-3′ and 5′-aggaatagccctcctc-3′; which flank the reported D102E polymorphism of the AK076999 cDNA sequence, and, according to the *Lasc1* gene structure (Vega gene OTTMUSG000000004898, http://vega.sanger.ac.uk/index.html), located in the same exon. The *Itpr2* (5′-tgagacctcggtctagcctggaggtcct-3′ and 5′-cggaggtctagcctggaggtcct-3′) genes served as positive controls. Normal lung tissue from 3 A/J, 3 C57BL/6J, and 3 (A/J×C57BL/6J)F1 mice and lung tumors from 3 urethane-treated (A/J×C57BL/6J)F1 mice were used, as well as brain, liver, kidney, spleen, and testis of a male SM/J mouse.

**Statistical Analysis**

The association between spontaneous and urethane-induced lung tumor phenotypes (mean percentages or mean multiplicities) was expressed as a correlation coefficient. The association between each SNP and lung tumor phenotypes (expressed as log10 of phenotype value) was tested by t-test. Haplotype analysis was
carried out according to [25], using a sliding window approach (window size of 3 SNPs) and the BROAD dataset. The association between each haplotype and lung tumor phenotypes (expressed as log+1 of phenotype value) was tested by F-test. To control the genome-wide false-positive fraction (12,959 and 138,793 t-tests for data on WT/PC and B6/129, respectively) statistical thresholds were computed both in accordance with the Bonferroni principle and with a permutation test (20,000 permutations). In particular, the distribution of the 20,000 smallest p-values among the 12,959 (or 138,793) p-values under the null hypothesis was obtained. This approach implicitly uses the correlation structure of the data [26]. The 5th and 10th centile of the reference distribution of the smallest genome scans were carried out using the ‘scanone’ function of R/using the MAPMAKER/EXP program [28]. Genetic distances chromosomes were established by multipoint analysis of the data mapping using R/qtl [27]. Marker order and position on

References

1. Chapman NH, Thompson EA (2001) Linkage disequilibrium mapping: the role of population history, size, and structure. Adv Genet 42: 413–437.
2. Abecasis GR, Doherty D, Nichols TE (2005) Linkage disequilibrium: ancient history drives the new genetics. Hum Hered 59: 118–124.
3. Manenti G, Stafford A, De Gregorio L, Gariboldi M, Falvello FS, et al. (1999) Linkage disequilibrium and physical mapping of Pas1 in mice. Genome Res 9: 639-646.
4. Manenti G, Galbiati F, Gianni-Barerra R, Pettinichio A, Acevedo A, et al. (2004) Haplootype sharing suggests that a genomic segment containing six genes accounts for the pulmonary adenoma susceptibility 1 (Pas1) locus activity in mice. Oncogene 23: 4495–4504.
5. Manenti G, Dragani TA (2003) Pas1 haplo-identive genetic predisposition to lung tumorigenesis in rodents: a meta-analysis. Carcinogenesis 26: 875–882.
6. Grupa A, Germer S, Usaka J, Aud D, Belknap JK, et al. (2001) In silico mapping of complex disease-related traits in mice. Science 292: 1915–1918.
7. Wang D, You M (2005) Five loci, SLT1 to SLT5, controlling the susceptibility to a common lung cancer susceptibility locus in the mouse. Nat Genet 38: 888–895.
8. Fenske TS, McMahon C, Edwin D, Jarvis JC, Cheverud JM, et al. (2006) Identification of candidate alkylation-induced cancer susceptibility genes by whole genome scanning in mice. Cancer Res 66: 5029–5038.
9. Devereux TR, Wiseman RW, Kaplan N, Garrett S, Foley JF, et al. (1994) Assignment of a locus for mouse lung tumor susceptibility to proximal chromosome 19. Mammm Genome 5: 749–755.
10. Manenti G, Gariboldi M, Fiorino A, Zedda AI, Pierotti MA, et al. (1997) Pas1 is a common lung cancer susceptibility locus in three mouse strains. Mammm Genome 8: 801–809.
11. Manenti G, Gariboldi M, Fiorino A, Zanesi N, Pierotti MA, et al. (1997) Genetic mapping of lung cancer modifier loci specifically affecting tumor initiation and progression. Cancer Res 57: 4164–4166.
12. Gariboldi M, Manenti G, Czarny F, Falvello FS, Radice MT, et al. (1993) A major susceptibility locus for murine lung carcinogenesis maps on chromosome 6. Nature Genet 3: 132–136.
13. Wade CM, Kulbokas EJ III, Kirby AW, Zody MC, Mullikin JC, et al. (2002) The mosaic structure of variation in the laboratory mouse genome. Nature 420: 574–578.
14. Frazer KA, Wade CM, Hind DA, Patil N, Cox DR, et al. (2004) Segmental phylogenetic relationships of inbred mouse strains revealed by fine-scale analysis of sequence variation across 4.6 mb of mouse genome. Genome Res 14: 1493–1500.
15. Yang H, Bell TA, Churchill GA, Pardo-Manuel d. V (2007) On the subspecific origin of the laboratory mouse. Nat Genet 39: 1100–1107.
16. Cervino AC, Darvazi A, Fallahi M, Mader CC, Tsioneremos NF (2007) An integrated in silico gene mapping strategy in inbred mice. Genetics 175: 321–333.
17. Vieland VJ (2001) The replication requirement. Nat Genet 29: 244–245.
18. Lee GH, Nishimori H, Saaski Y, Matsushita H, Kitagawa T, et al. (2003) Analysis of lung tumorigenesis in chimeric mice indicates the Pulmonary adenoma resistance 2 (Par2) locus to operate in the tumor-initiation stage in a cell-autonomous manner: detection of polymorphisms in the Poli gene as a candidate for Par2. Oncogene 22: 2374–2382.
19. Darvazi A (2001) In silico mapping of mouse quantitative trait loci. Science 294: 2423.
20. Payseur BA, Place M (2007) Prospects for association mapping in classical inbred mouse strains. Genetics 175: 1999–2008.
21. Malkinson AM (1989) The genetic basis of susceptibility to lung tumors in mice. Toxicology 54: 241–271.
22. To MD, Perez-Leoseda J, Mao JH, Hus J, Jacks T, et al. (2006) A functional switch from lung cancer resistance to susceptibility at the Pas1 locus in Kras2Al2 mice. Nat Genet 38: 926–930.
23. Shen R, Fan JB, Campbell D, Chang W, Chen J, et al. (2005) High-throughput SNP genotyping on universal bead arrays. Mutat Res 573: 70–82.
24. Zaykin DV, Westfall PH, Young SS, Karnoub MA, Wagner MJ, et al. (2002) Testing association of statistically inferred haplotypes with discrete and continuous traits in samples of unrelated individuals. Hum Hered 55: 78–91.
25. Simon RM, Korn EL, McShane LM, Radmacher MD, Wright GM, et al. (2004) Design and analysis of DNA microarray investigations. New York: Springer-Verlag. pp 80.
26. Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19: 889–890.
27. Lincoln SE, Daly M, Lander ES (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report.
28. Zeng Z-B (1994) Precision mapping of quantitative trait loci. Genetics 138: 1457–1468.
29. Sen S, Churchill GA (2001) A statistical framework for quantitative trait mapping. Genetics 159: 371–387.
30. Frazer KA, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19: 889–890.
31. Lincoln SE, Daly M, Lander ES (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report.
32. Zeng Z-B (1994) Precision mapping of quantitative trait loci. Genetics 138: 1457–1468.
33. Sen S, Churchill GA (2001) A statistical framework for quantitative trait mapping. Genetics 159: 371–387.