

Complex genetic control of lung tumorigenesis in resistant mice strains

Alice Dassano,¹ Giulia Pintarelli,¹ Chiara E. Cotroneo,¹ Angela Pettinicchio,¹ Elena Forcati,¹ Loris De Cecco,^{1,2} Andrea Borrego,³ Francesca Colombo,¹ Tommaso A. Dragani¹  and Giacomo Manenti¹

¹Department of Predictive and Preventive Medicine; ²Department of Experimental Oncology and Molecular medicine, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy; ³Laboratory of Immunogenetics, Instituto Butantan, São Paulo, Brazil

Key words

Genetic linkage, genetic loci, lung cancer, *Pas1*, quantitative trait loci

Correspondence

Tommaso A. Dragani, Fondazione IRCCS, Istituto Nazionale dei Tumori, Via G. A. Amadeo 42, 20133 Milan, Italy. Tel: +39-0223902642; Fax: +39-0223902764; E-mail: tommaso.dragani@istitutotumori.mi.it

Funding Information

This work was funded in part by a grant from Associazione Italiana Ricerca Cancro (AIRC) to GM (IG no. 15797). AD was recipient of an AIRC/FIRC fellowship no. 16353. The funders had no role in the design and conduct of the study, in the collection, analysis and interpretation of the data, and in the preparation, review or approval of the manuscript.

Received April 19, 2017; Revised July 27, 2017; Accepted August 5, 2017

Cancer Sci 108 (2017) 2281–2286

doi: 10.1111/cas.13349

The SM/J mouse strain is resistant to chemically-induced lung tumorigenesis despite having a haplotype, in the pulmonary adenoma susceptibility locus (*Pas1*) locus, that confers tumor susceptibility in other strains. To clarify this inconsistent genotype-phenotype correlation, we crossed SM/J mice with another resistant strain and conducted genome-wide linkage analysis in the (C57BL/6J × SM/J)F₂ progeny exposed to urethane to induce lung tumors. Overall, >80% of F₂ mice of both sexes developed from 1 to 20 lung tumors. Genotyping of 372 F₂ mice for 744 informative non-redundant SNPs dispersed over all autosomal chromosomes revealed four quantitative trait loci (QTLs) affecting lung tumor multiplicity, on chromosomes 3 (near rs13477379), 15 (rs6285067), 17 (rs33373629) and 18 (rs3706601), all with logarithm of the odds (LOD) scores >5. Four QTLs modulated total lung tumor volume, on chromosome 3 (rs13477379), 10 (rs13480702), 15 (rs6285067) and 17 (rs3682923), all with LOD scores >4. No QTL modulating lung tumor multiplicity or total volume was detected in *Pas1* on chromosome 6. The present study demonstrates that the SM/J strain carries, at the *Pas1* locus, the resistance allele: a finding that will facilitate identification of the *Pas1* causal element. More generally, it demonstrates that lung tumorigenesis is under complex polygenic control even in a pedigree with low susceptibility to this neoplasia, suggesting that the genetics of lung tumorigenesis is much more complex than evidenced by the pulmonary adenoma susceptibility and resistance loci that have, so far, been mapped in a small number of crosses between a few inbred strains.

Many mouse models of the inherited predisposition to disease are available today because, starting from the first decades of last century, researchers have created hundreds of inbred strains with high or low susceptibility to different diseases, including cancer.⁽¹⁾ Genetic linkage analysis of mouse populations obtained by crossing parental strains with high and low genetic predisposition to cancer, together with genotyping of genetic markers, has allowed the mapping of many loci modulating the susceptibility to a given tumor (reviewed in Dragani, 2003).⁽²⁾

Regarding lung cancer, various inbred mouse strains differ in their susceptibility to spontaneous and carcinogen-induced lung tumors. For example, the A/J strain is highly susceptible to both forms of lung tumorigenesis,⁽³⁾ and its tumors have a high frequency of somatic *Kras* mutations at codons 12 and 61 that vary depending on whether the tumors are spontaneous or not and on the carcinogen used to induce them.^(4,5) By crossing the A/J strain with the lung-tumor-resistant C57BL/6J strain (low susceptibility), we previously mapped a region of chromosome 6, called the pulmonary adenoma susceptibility 1 (*Pas1*) locus, which is the major determinant of lung tumor susceptibility in mice.^(6,7) This locus contains six genes, including *Kras*,⁽⁸⁾ but so far there is no consensus on which gene is responsible for modulating lung tumor susceptibility, warranting further study.

On the basis of 54 genetic markers in the *Pas1* locus, we previously observed that 29 inbred mice strains clustered into an A/J-like group (highly susceptible to lung tumors) and a C57BL/6J (B6)-like group (resistant to lung tumors), with some exceptions, such as the BALB/c strain with its intermediate susceptibility despite having an A/J-like haplotype.^(7,8) When we crossed the BALB/c strain with the highly susceptible strain SWR/J, we identified several chromosomal loci that had singular effects on either lung tumor multiplicity or total tumor volume.⁽⁹⁾ Tumor modifier loci were also identified when BALB/c mice were crossed with A/J mice or with a resistant strain.^(10,11) Another exception to the A/J versus B6 clustering of mice strains according to *Pas1* markers is the SM/J strain. SM/J mice are resistant to lung tumorigenesis despite the fact that they share 46 of 54 alleles in the *Pas1* locus with A/J mice.^(7,8) This observation prompted us to investigate whether the resistance of the SM/J strain to lung tumorigenesis is due to the suppression of the *Pas1* susceptibility allele by dominant lung tumor resistance alleles⁽¹²⁾ or to subtle genetic variations in the *Pas1* locus (not studied with the 54 markers) leading to a resistant phenotype.

To test these two possibilities, we carried out a genetic study by crossing the SM/J and C57BL/6J strains (both are resistant to spontaneous and chemically-induced lung tumorigenesis)^(7,13) and carrying out genome-wide linkage analysis in the

resulting F2 intercross. Linkage analysis enables the detection of quantitative trait loci (QTLs) that affect a studied phenotype only when the parental strains carry different alleles. Therefore, if the *Pas1* locus in SM/J mice contains the susceptibility allele but this is suppressed by modifier loci, then the genetic recombination in the (C57BL/6J × SM/J)F2 intercross should unmask the *Pas1* locus, allowing us to detect a QTL therein. In contrast, if the *Pas1* locus in SM/J mice carries fine genetic variants leading to resistance, we should not detect any QTL within this locus. This novel study design should clarify why SM/J mice are resistant to lung tumorigenesis and provide further insight into the genetic control of lung cancer in mice.

Materials and Methods

Ethics statement. All animals received humane care according to the criteria outlined in a protocol approved on 30 April 2013 with the internal reference INT_03_2013 by the institutional ethical committee for animal research (CESA) at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, and sent to the Ministry of Health as required by the Italian regulation (d.lgs. 116/92) in force at the time.

Mouse crosses, tissues and DNA. A pedigree of intercross mice was generated by crossbreeding two lung-tumor-resistant inbred strains, C57BL/6J and SM/J. First, 20 female C57BL/6J mice were crossed with 10 male SM/J mice in two rounds, resulting in 196 F1 mice. Of these, 20 males and 22 females were treated with a single intraperitoneal injection of urethane (1 g/kg body weight) at 4 weeks of age, according to the standard procedure for inducing the development of lung tumors.⁽³⁾ The remaining F1 hybrid mice were mated together to obtain an F2 intercross generation of 372 mice that, after weaning, were also treated with urethane in the same manner as the treated F1 mice. Animals were kept without any further treatment until 40 weeks of age, when they were killed.

Spleens of F2 mice were excised and frozen, and subsequently used for SNP genotyping. Lungs of F1 and F2 mice were filled with 0.5-mL RNALater solution (Thermo Fisher Scientific, Waltham, USA), removed, and placed in RNALater solution for tumor counting and sizing, as previously described.⁽¹⁴⁾ Briefly, the number of lung tumors per mouse (Nlung, lung tumor multiplicity) was recorded and the diameter of each tumor was measured with a micrometer (graduation = 0.1 mm) under a stereomicroscope. Tumor volume (mm³) was calculated assuming a spherical shape, and Vlung, the total tumor volume for each mouse, was calculated by summing the volumes of individual tumors.

For the subset of male F2 mice with lung tumors suitable for excision (approximately 1.7–2.0 mm in diameter), we resected one such tumor per animal under a stereomicroscope. These tumors were stored at –80°C until analysis of somatic mutations at the *Kras* gene.

Genomic DNA was extracted from spleens and lungs using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, USA), and quantified using the Picogreen dsDNA Quantitation Kit (Invitrogen, Life Technologies, Paisley, UK).

Genome-wide SNP and *Pas1* locus genotyping. Germline variants were assessed using genomic DNA from spleens of F2 mice. As a control for genotyping and to determine the derivation of specific alleles in F2 mice, we also genotyped splenic DNA from parental C57BL/6J and SM/J mice and their untreated F1 progeny (2 animals per group). First, genome-wide SNP genotyping was carried out at the Functional Genomics and Bioinformatics Core Facility (INT, Milan) with the

GoldenGate Genotyping Assay according to the manufacturer's protocol (Illumina, San Diego, CA, USA), using the Mouse MD Linkage Panel representing 1449 mouse loci. Positions of genetic markers were based on the mouse genome assembly GRCm38.p5.

In addition, the allele status of the *Pas1* locus was determined according to the presence or absence of a 37-bp tandem repeat polymorphism in *Kras*, which maps in the second intron of the *Kras* gene and discriminates between the A/J-type and C57BL/6J-type alleles.⁽¹⁵⁾ This analysis was done by PCR and gel electrophoresis, as previously described.⁽¹⁴⁾

Analysis of somatic mutations in *Kras*. Genomic DNA from lung tumors of male F2 mice was assessed for the presence of mutations in *Kras* codons 12–13 (exon 1) and 61 (exon 2) by pyrosequencing on a PSQ HS 96A instrument (Biotage, Uppsala, Sweden), using the oligonucleotides reported in Table S1. Nucleotide sequences were determined by automated calling procedure of the instrument and checked by visual inspection of the pyrograms, which, in the case of mutations, show an additional peak at any given nucleotide of the wild-type *Kras* sequence.

Statistical analyses. Nlung and Vlung values were square-root transformed to improve the normality of distribution. Sex differences in transformed Nlung and Vlung were tested by ANOVA.

To identify QTLs, transformed values of Nlung and Vlung were analyzed together with SNP genotype data by parametric simple interval mapping using R/qrtl,⁽¹⁶⁾ adjusting by sex. The resulting logarithm of the odds (LOD) scores were considered significant if greater than the 95% LOD threshold ($\alpha = 0.05$), calculated by 10 000 permutations. The percent phenotypic variance explained by a given QTL was calculated from the LOD score using the following formula: $R^2 = 1 - 10^{(-2LOD/n)}$,⁽¹⁷⁾ where n is the sample size. Similarly, the total variance using an additive model was calculated by the same formula using the total LOD score obtained by the “fitqtl” command in the R/qrtl.

Associations between *Kras* mutations (a categorical phenotype: present or absent) and SNP genotypes were tested by single-point analysis using the “assoc” command of the PLINK program,⁽¹⁸⁾ which runs a χ^2 allelic test. For statistical significance, 10 000 permutations were carried out to obtain, at individual SNP, both the nominal P -value estimate and the corrected P -value for multiple testing.

Results

After urethane treatment, most (C57BL/6J × SM/J)F1 mice (75% of males and 95% of females) developed one or a few lung tumors (range, 0–6); lung tumor multiplicity (Nlung) was 2.6 tumors/mouse in both sexes (Table 1). Compared to their F1 parents, (C57BL/6J × SM/J)F2 intercross animals had similarly high values of lung tumor incidence (88 and 82% in males and females, respectively) but higher values of Nlung. Indeed, in F2 mice, we observed a widening of the range of Nlung values, with some mice developing up to 20 lung nodules. Moreover, the total tumor volume (Vlung) in F2 mice was larger than in F1 animals, especially in male mice. In the F2 cross, male mice had significantly higher values of both Nlung ($P < 0.05$, ANOVA) and Vlung ($P < 0.01$, ANOVA) than their female counterparts. In contrast, in the F1 cross, there was no statistically significant sex difference in Nlung or Vlung.

Quantitative trait loci modulating Nlung and Vlung. To identify alleles that influence the two lung tumor phenotypes

Table 1. Lung tumor incidence, lung tumor multiplicity (Nlung), and total lung tumor volume (Vlung) in (C57BL/6J × SM/J) F1 and F2 mouse crosses

Cross	Sex	n	Incidence, n (%)	Nlung†	Vlung, mm ³ ‡
(C57BL/6J × SM/J)F1	M	20	15 (75)	2.6 ± 0.4 (0–6)	1.5 ± 0.4
	F	22	21 (95)	2.6 ± 0.4 (0–6)	1.4 ± 0.3
(C57BL/6J × SM/J)F2	M	208	182 (88)	3.8 ± 0.2 (0–20)	4.5 ± 0.4
	F	164	135 (82)	3.1 ± 0.2 (0–16)**	2.5 ± 0.5***

Animals were treated with a single intraperitoneal injection of urethane (1 g/kg body weight) at 4 weeks of age and then kept without any further treatment until 40 weeks of age. †Mean ± SE (range).

‡Mean ± SE. Comparison of male and female mice of the same cross: ** $P < 0.05$, *** $P < 0.01$; ANOVA on square-root transformed values.

(Nlung and Vlung), we performed genome-wide linkage analysis using genomic DNA from the 372 F2 mice and Illumina SNP arrays. Of the 1449 single nucleotide polymorphisms (SNPs) assessed, we identified 744 informative (polymorphic) non-redundant SNPs dispersed over the 19 autosomal chromosomes. These genotype data were analyzed by simple interval mapping to detect QTLs associated with squared root-transformed values of Nlung and Vlung.

For Nlung, we identified four QTLs (Fig. 1). In particular, we found a QTL on chromosome 3 (LOD score = 6.74, centered around SNP rs13477379), chromosome 15 (LOD score = 5.82, centered around SNP rs6285067), chromosome 17 (LOD score = 6.74, centered around SNP UT_17_3.221024) and chromosome 18 (LOD score = 5.03, centered around SNP rs3706601). No locus modulating Nlung was detected in the *Pas1* region on chromosome 6 (Fig. 1).

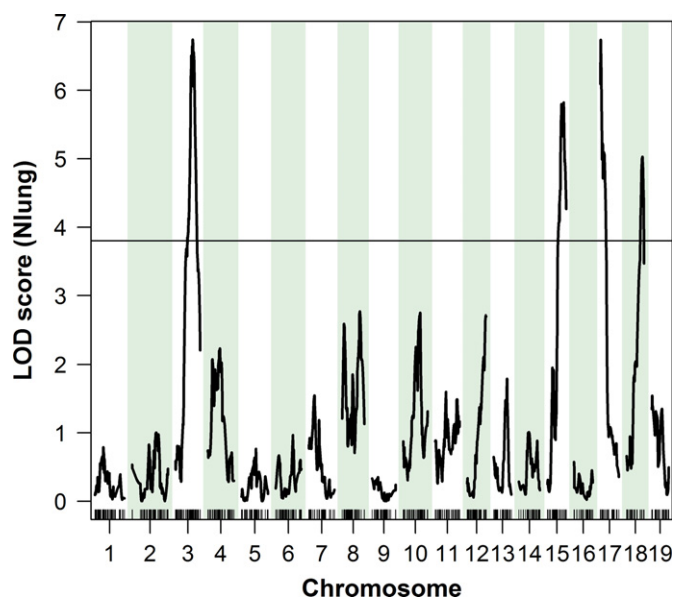


Fig. 1. Genome-wide interval mapping of urethane-induced lung tumor multiplicity (Nlung) in a (C57BL/6J × SM/J)F2 cross. Four quantitative trait loci (QTLs), on chromosomes 3, 15, 17 and 18, were detected on the basis of a peak logarithm of the odds (LOD) score above the genome-wide statistical threshold (LOD score = 3.80, $\alpha = 0.05$, horizontal line). Tick marks show the position of the genotyped markers in a recombinational map. Curves were generated by R/qtl on square-root-transformed values, adjusting by sex as a covariate.

When the mice were grouped according to genotype at the QTL peak marker SNP, two opposing patterns in Nlung values were observed (Table 2). For the QTL on chromosomes 3 and 18, Nlung was highest in mice that were homozygous C57BL/6J-derived allele (B6/B6 genotype) and lowest for those animals homozygous for the SM/J-derived allele (SM/SM genotype). In contrast, for chromosomes 15 and 17, the susceptibility allele modulating Nlung derived from the SM/J strain, as shown by the highest Nlung values in SM/SM mice. All QTLs had allele dosage effects, as heterozygous mice had an intermediate phenotype compared with homozygous mice. Percentages of variance explained by the various loci (R^2) ranged from 5.5% (chromosome 18) to 8.2% (chromosome 3). Together, under an additive model, the QTLs accounted for 27% of the total phenotypic variance of Nlung in the F2 population.

For Vlung, we also identified four QTLs (Fig. 2), including two of those already found for Nlung. The QTLs were found on chromosome 3 (LOD score = 4.24, centered around SNP rs13477379), chromosome 10 (LOD score = 7.12, centered around SNP rs13480702), chromosome 15 (LOD score = 4.36, centered around SNP rs6285067) and chromosome 17 (LOD score = 4.35, centered around SNP rs3682923). As for Nlung, the *Pas1* locus on chromosome 6 had no effect on Vlung (Fig. 2). The correspondence between QTLs for Vlung and Nlung is shown in Figure 3.

For Vlung, the susceptibility allele for the QTL on chromosomes 3 and 10 derived from the C57BL/6J strain, whereas for the QTLs on chromosomes 15 and 17, the susceptibility allele derived from the SM/J allele (Table 3). All four QTLs showed allele dosage effects, with heterozygous mice having an intermediate phenotype compared with homozygous mice. Percentages of variance explained by the various loci (R^2) ranged from 4.3% (chromosome 17) to 7.4% (chromosome 10). Together, under an additive model, the QTLs accounted for 26% of the total phenotypic variance of Vlung in the F2 population.

Kras mutations in lung tumors of the F2 population. Because both parental strains of the C57BL/6J × SM/J intercross are genetically resistant to lung tumorigenesis but urethane treatment, nonetheless, induced lung tumors in the offspring, we investigated whether these tumors carried somatic mutations in *Kras*. A total of 33 male F2 mice had a lung tumor accessible for excision, and pyrosequencing of genomic DNA from these tumors showed that 29 (88%) of these tumors harbored a *Kras* mutation in codon 61 (Table 4). The most frequent mutation was a CAA → CGA change, seen in 23 cases. In addition, a CAA → CTA change was seen in 6 cases. No *Kras* mutation was detected in codons 12–13.

Single-point analysis to scan the genome for loci modulating the frequency of *Kras* mutational status (wild type versus any mutation in codon 61) did not reveal any significant association with the QTLs linked to Nlung or Vlung, but did reveal an association with a locus on mid-chromosome 7, with peak LOD score at rs3714908, at 64.513 Mb (nominal $P = 0.003$, multiple testing corrected $P = 0.398$). When the analysis was repeated considering only the presence or absence of the most common mutated sequence at codon 61 (i.e. CGA), a nominal statistical association was found with a locus on chromosome 10 with peak LOD score at rs13480684 (85.873 Mb; nominal $P = 0.005$, multiple testing corrected $P = 0.524$); this locus is in the same position where we mapped a QTL modulating Vlung. There was no association between *Kras* mutations (any or CGA) and the *Pas1* locus ($P > 0.05$).

QTL				NLung [†]			R^2 [‡]
	Chromosome (Mb)	QTL peak marker	Closest gene	B6/B6	B6/SM	SM/SM	
3 (122.838)	rs13477379	<i>Pde5a</i>	4.8 ± 0.3	3.3 ± 0.2	2.4 ± 0.2	8.2	
15 (95.314)	rs6285067	<i>Nell2</i>	2.5 ± 0.3	3.5 ± 0.2	4.6 ± 0.4	6.6	
17 (6.039)	UT_17_3.221024	<i>Synj2</i>	2.4 ± 0.3	3.5 ± 0.2	4.4 ± 0.3	7.4	
18 (77.470)	rs3706601	<i>Rnf165</i>	4.5 ± 0.4	3.6 ± 0.2	2.7 ± 0.3	5.5	

[†]Mean ± SE. B6, C57BL/6J-derived allele; SM, SM/J-derived allele. [‡]R², percentage of variance explained. QTL, quantitative trait loci.

Table 2. QTLs modulating lung tumor multiplicity (NLung) in 372 (C57BL/6J × SM/J)F2 mice, and values of NLung according to the genotype at the peak marker

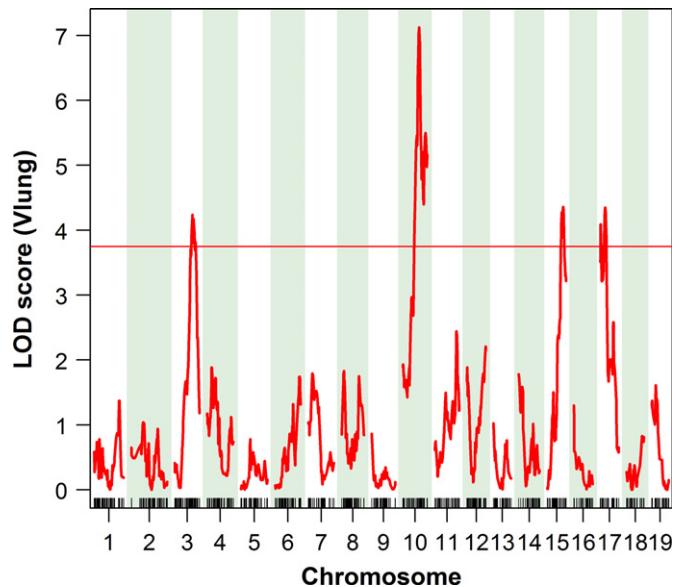


Fig. 2. Genome-wide interval mapping of urethane-induced total lung tumor volume (Vlung) in a (C57BL/6J × SM/J)F2 cross. Four quantitative trait loci (QTLs), on chromosomes 3, 10, 15 and 17, were detected on the basis of a peak logarithm of the odds (LOD) score above the genome-wide statistical threshold (LOD score = 3.75, $\alpha = 0.05$, horizontal line). Tick marks show the position of the genotyped markers in a recombinational map. Curves were generated by R/qtl on square-root-transformed values, adjusting by sex as a covariate.

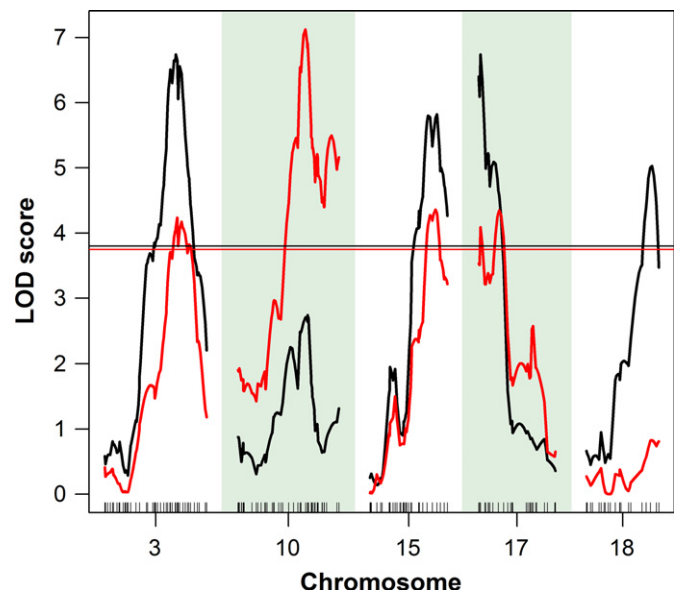


Fig. 3. High-resolution interval map comparing quantitative trait loci (QTLs) modulating NLung (black line) and Vlung (red line) in the (C57BL/6J × SM/J)F2 cross. Tick marks show the position of the genotyped markers in a recombinational map. Horizontal lines at LOD = 3.80 and LOD = 3.75 mark the corresponding 95% thresholds for significance. Curves were generated by R/qtl on square-root-transformed values, adjusting by sex as a covariate. LOD, logarithm of the odds.

Discussion

The present study aimed to dissect the mechanism by which SM/J mice remain resistant to lung tumors, notwithstanding the fact that their *Pas1* locus on chromosome 6 is genetically similar to that of the highly susceptible A/J strain. In particular, we asked whether, in SM/J mice, the *Pas1* locus contains: (i) a tumor susceptibility allele that is suppressed by other, dominant tumor resistance loci (whose effects would be removed by genetic recombination) or, alternatively, (ii) a tumor resistance allele despite its similarities to the A/J haplotype. Through genome-wide linkage analysis by interval mapping of the (C57BL/6J × SM/J)F2 intercross, we found no evidence for a QTL modulating lung tumor phenotype in the *Pas1* locus. This finding, therefore, excludes that the SM/J strain carries the susceptibility allele in the *Pas1* locus and demonstrates instead that it has a resistance allele, as do C57BL/6J mice.^(7,8)

These results should be considered conclusive, as we and others have previously shown that genetic linkage analysis is able to detect the *Pas1* susceptibility allele carried by strains with low or intermediate susceptibility to lung tumorigenesis.

For example, in crosses between the BALB/c and C3H strains, the *Pas1* locus was clearly detected with LOD scores of 22.6⁽¹⁰⁾ and 18.4,⁽¹⁹⁾ despite the fact that the chemically-induced NLung value in the BALB/c strain is approximately 10-fold less than that in the A/J strain.⁽⁷⁾

The (C57BL/6J × SM/J)F2 intercross mice had higher lung tumor multiplicity (NLung) and greater total lung tumor volume (Vlung) than the parental F1 animals, suggesting that the SM/J-derived lung tumor susceptibility alleles at loci on chromosomes 15 and 17 (influencing both NLung and Vlung; Tables 2 and 3) were unmasked by genetic recombination in the intercross. Regarding NLung, the four QTLs identified in the present study mapped on chromosomal regions where no lung tumor modifier loci have been previously mapped. On chromosome 3, a QTL centered around the SNP rs13477379, which maps in an intronic region of the phosphodiesterase 5A (*Pde5a*) gene, at 122.838 Mb. On chromosome 15, the peak marker for a QTL was rs6285067, which maps in the intronic region of the NEL-like 2 (*Nell2*) gene, at 95.314 Mb. On chromosome 17, the QTL marker SNP was UT_17_3.221024, which, based on sequence alignment, corresponds to rs33373629, mapping in

Table 3. QTLs modulating total lung tumor volume (VLung) in 372 (C57BL/6J × SM/J)F2 mice, and values of VLung according to the genotype at the peak marker

QTL				VLung [†]			<i>R</i> [‡]
	Chromosome (Mb)	Peak marker	Closest gene	B6/B6	B6/SM	SM/SM	
3 (122.838)	rs13477379	<i>Pde5a</i>	5.2 ± 0.7	3.2 ± 0.4	2.5 ± 0.5	5.2	
10 (91.060)	rs13480702	<i>Apaf1</i>	6.1 ± 0.9	3.3 ± 0.4	1.8 ± 0.3	7.4	
15 (95.314)	rs6285067	<i>Nell2</i>	2.3 ± 0.4	3.5 ± 0.4	5.2 ± 1.0	4.6	
17 (35.834)	rs3682923	<i>Tubb5</i>	2.2 ± 0.4	3.7 ± 0.5	4.6 ± 0.7	4.3	

[†]Mean ± SE. B6, C57BL/6J-derived allele; SM, SM/J-derived allele. [‡]*R*², percentage of variance explained. QTL, quantitative trait loci.

Table 4. Frequency of *Kras* codon 61 mutations in lung tumors of 1.7–2.0-mm diameter, induced by urethane in male (C57BL/6J × SM/J)F2 mice, by genotype at the *Pas1* locus (37-bp repeat in *Kras*)

Genotype	Sequence of <i>Kras</i> codon 61*		
	CAA (wt)	CGA	CTA
B6/B6	0	8	1
B6/SM	3	9	2
SM/SM	1	6	3
Total	4	23	6

B6, C57BL/6J-derived allele; SM, SM/J-derived allele. **P* = 0.548, Fisher's exact test.

the intronic region of the synaptojanin 2 (*Synj2*) gene, at 6.039 Mb. Finally, on chromosome 18, a QTL was marked by rs3706601, which maps in the intronic region of the ring finger protein 165 (*Rnfl65*) gene, at 77.470 Mb.

Among the four QTLs modulating VLung, two of them, mapping on chromosomes 3 and 15, are most likely the same QTLs linked to Nlung, as their LOD score peaks overlap (Fig. 3) and their strain-dependent allele effects were the same. Therefore, these two QTLs apparently modulate both Nlung and VLung, as does the *Pas1* locus.⁽⁶⁾ Another QTL modulating VLung was found on chromosome 10, where no QTL associated with Nlung was detected. This QTL had the highest LOD score and was centered around rs13480702, which is a synonymous variant of the apoptotic peptidase activating factor 1 (*Apaf1*) gene, mapping at 91.060 Mb. This QTL is the second example of a locus affecting lung tumor volume but not multiplicity, the first one being the *Papgl* locus mapping on chromosome 4.^(9,20) Finally, a QTL modulating VLung was found on chromosome 17, which also was found to contain a QTL modulating Nlung. Although these two QTLs showed the same strain derivation of the susceptibility alleles, their marker SNP are separated by almost 30 Mb; for this reason, these QTLs probably represent different genetic entities.

In (C57BL/6J × SM/J)F2 animals, a high percentage of analyzed lung tumors (29 of 33, 88%) carried somatic mutations in the *Kras* gene. Because these mutations occurred in lung tumors of small size (≤2 mm in diameter) in mice whose parents were genetically resistant to lung tumorigenesis, this finding confirms the driving role of *Kras* mutations in mouse lung tumorigenesis.^(5,14,21,22) In crosses between strains carrying the A/J-type susceptibility allele of the *Pas1* locus, we and others previously reported the presence of allele-specific *Kras* mutations.^(23,24) However, in this study, we found no association between the *Pas1* genotype and *Kras* mutation frequency, in agreement with the absence of genetic linkage between the *Pas1* locus and either Nlung or VLung in the (C57BL/6J × SM/J)F2 intercross. We found a nominal statistical

association between codon 61 CGA mutations at *Kras* and the QTL on chromosome 10 linked to VLung, suggesting that this locus may modulate both phenotypes. However, because of the low number of tumors (*n* = 33) with mutational analysis, the *P*-value did not pass the threshold for multiple corrections and, therefore, these results should be considered preliminary and deserving of validation in larger samples crosses.

Our study demonstrates that lung tumorigenesis is under polygenic control even in a population like the (C57BL/6J × SM/J)F2 intercross with low susceptibility to this disease. The novel loci identified in the present study were not previously identified in a (C57BL/6J × A/J) backcross or intercross,⁽²⁵⁾ nor in recombinant inbred strains from SM/J and A/J mice,⁽¹³⁾ although both studies did identify other loci modulating lung tumorigenesis. This is most likely because the loci identified here cannot reach the statistical threshold for detection in crosses in which one of the two parental strains is a highly susceptible strain (e.g. A/J). It is conceivable, therefore, that the genetics of lung tumorigenesis in mice is much more complex than evidenced by the pulmonary adenoma susceptibility and resistance loci that have, so far, been mapped in a relatively small number of crosses that originated from a small number of inbred strains.

Considering the enormous genetic heterogeneity of the human population,⁽²⁶⁾ it is surprising that genome-wide association studies in humans have so far detected only three loci (5p15, 6p21 and 15q25) associated with lung cancer risk in general^(27,28) and, more recently, three other loci (4p15, 11q21 and 18q12) associated with the risk of particular lung cancer histotypes.⁽²⁹⁾ A complication in detecting genetic effects in human studies is the dose-dependent, causative role of tobacco smoking in lung cancer,^(30,31) which is not modeled by the controlled exposure to chemical carcinogens in mouse studies.

Considering the experimental evidence from the simpler mouse genetic models, we predict that an individual's risk and outcome of lung cancer is under complex polygenic control, modulated by heterogeneous and redundant gene networks. Reasons for the failure to dissect such complex networks may be the confounding effects of genetic heterogeneity and the interactions between genetic and environmental risk factors. Indeed, GWAS have detected dozens of risk loci for other types of common cancer, such as non-familial colorectal carcinoma^(32,33) and non-familial breast cancer.⁽³⁴⁾ Moreover, retinitis pigmentosa, one of the most genetically complex diseases known today, has approximately 4500 causative mutations in more than 250 genes.⁽³⁵⁾ Because we have no reason to believe that lung cancer is a simpler disease than other common cancers or non-cancer diseases such as retinitis pigmentosa, we hope that new developments in artificial intelligence and in methods to detect complex interactions in association studies will shed light on the genetic factors modulating individual risk and outcome in lung cancer.

Acknowledgments

The authors acknowledge Valerie Matarese, for scientific editing.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

- 1 Stevens JC, Banks GT, Festing MF, Fisher EM. Quiet mutations in inbred strains of mice. *Trends Mol Med* 2007; **13**: 512–9.
- 2 Dragani TA. 10 years of mouse cancer modifier loci: human relevance. *Cancer Res* 2003; **63**: 3011–8.
- 3 Shimkin MB, Stoner GD. Lung tumors in mice: application to carcinogenesis bioassay. *Adv Cancer Res* 1975; **21**: 1–58.
- 4 You M, Candrian U, Maronpot RR, Stoner GD, Anderson MW. Activation of the ki-ras protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. *Proc Natl Acad Sci USA* 1989; **86**: 3070–4.
- 5 Dragani TA, Manenti G, Pierotti MA. Genetics of murine lung tumors. *Adv Cancer Res* 1995; **67**: 83–112.
- 6 Gariboldi M, Manenti G, Canzian F *et al*. A major susceptibility locus to murine lung carcinogenesis maps on chromosome 6. *Nature Genet* 1993; **3**: 132–6.
- 7 Manenti G, Dragani TA. Pas1 haplotype-dependent genetic predisposition to lung tumorigenesis in rodents: a meta-analysis. *Carcinogenesis* 2005; **26**: 875–82.
- 8 Manenti G, Galbiati F, Gianni Barrera R, Pettinicchio A, Acevedo A, Dragani TA. Haplotype sharing suggests that a genomic segment containing six genes accounts for the pulmonary adenoma susceptibility 1 (Pas1) locus activity in mice. *Oncogene* 2004; **23**: 4495–504.
- 9 Manenti G, Gariboldi M, Fiorino A, Zanasi N, Pierotti MA, Dragani TA. Genetic mapping of lung cancer modifier loci specifically affecting tumor initiation and progression. *Cancer Res* 1997; **57**: 4164–6.
- 10 Karasaki H, Obata M, Ogawa K, Lee GH. Roles of the Pas1 and Par2 genes in determination of the unique, intermediate susceptibility of BALB/cByJ mice to urethane-induction of lung carcinogenesis: differential effects on tumor multiplicity, size and Kras2 mutations. *Oncogene* 1997; **15**: 1833–40.
- 11 Obata M, Nishimori H, Ogawa K, Lee GH. Identification of the Par2 (pulmonary adenoma resistance) locus on mouse chromosome 18, a major genetic determinant for lung carcinogen resistance in BALB/cByJ mice. *Oncogene* 1996; **13**: 1599–604.
- 12 Manenti G, Acevedo A, Galbiati F *et al*. Cancer modifier alleles inhibiting lung tumorigenesis are common in inbred mouse strains. *Int J Cancer* 2002; **99**: 555–9.
- 13 Pataer A, Nishimura M, Kamoto T, Ichioka K, Sato M, Hiai H. Genetic resistance to urethane-induced pulmonary adenomas in SMXA recombinant inbred mouse strains. *Cancer Res* 1997; **57**: 2904–8.
- 14 Dassano A, Colombo F, Trincucci G *et al*. Mouse pulmonary adenoma susceptibility 1 locus is an expression QTL modulating kras-4A. *PLoS Genet* 2014; **10**: e1004307.
- 15 Chen B, Johanson L, Wiest JS, Anderson MW, You M. The second intron of the K-ras gene contains regulatory elements associated with mouse lung tumor susceptibility. *Proc Natl Acad Sci USA* 1994; **91**: 1589–93.
- 16 Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 2003; **19**: 889–90.
- 17 Nagelkerke NJD. A note on a general definition of the coefficient of determination. *Biometrika* 1991; **78**: 691–2.
- 18 Purcell S, Neale B, Todd-Brown K *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**: 559–75.
- 19 Manenti G, Galvan A, Pettinicchio A *et al*. Mouse genome-wide association mapping needs linkage analysis to avoid false-positive loci. *PLoS Genet* 2009; **5**: e1000331.
- 20 Dassano A, Noci S, Galbiati F *et al*. Multigenic nature of the mouse pulmonary adenoma progression 1 locus. *BMC Genom* 2013; **14**: 152.
- 21 Re FC, Manenti G, Borrello MG *et al*. Multiple molecular alterations in mouse lung tumors. *Mol Carcinog* 1992; **5**: 155–60.
- 22 Meuwissen R, Linn SC, van der Valk M, Mooi WJ, Berns A. Mouse model for lung tumorigenesis through Cre/lox controlled sporadic activation of the K-ras oncogene. *Oncogene* 2001; **20**: 6551–8.
- 23 Manenti G, Trincucci G, Pettinicchio A, Amendola E, Scarfo M, Dragani TA. Cis-acting genomic elements of the Pas1 locus control kras mutability in lung tumors. *Oncogene* 2008; **27**: 5753–8.
- 24 Chen B, You L, Wang Y, Stoner GD, You M. Allele-specific activation and expression of the K-ras gene in hybrid mouse lung tumors induced by chemical carcinogens. *Carcinogenesis* 1994; **15**: 2031–5.
- 25 Festing MFW, Yang A, Malkinson AM. At least four genes and sex are associated with susceptibility to urethane-induced pulmonary adenomas in mice. *Genet Res* 1994; **64**: 99–106.
- 26 Galvan A, Ioannidis JP, Dragani TA. Beyond genome-wide association studies: genetic heterogeneity and individual predisposition to cancer. *Trends Genet* 2010; **26**: 132–41.
- 27 Wang Y, Broderick P, Webb E *et al*. Common 5p15.33 and 6p21.33 variants influence lung cancer risk. *Nat Genet* 2008; **40**: 1407–9.
- 28 Hung RJ, McKay JD, Gaborieau V *et al*. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature* 2008; **452**: 633–7.
- 29 Brenner DR, Amos CI, Brhane Y *et al*. Identification of lung cancer histology-specific variants applying bayesian framework variant prioritization approaches within the TRICL and ILCCO consortia. *Carcinogenesis* 2015; **36**: 1314–26.
- 30 Pirie K, Peto R, Reeves GK, Green J, Beral V; Million Women Study Collaborators. The 21st century hazards of smoking and benefits of stopping: a prospective study of one million women in the UK. *Lancet* 2013; **381**: 133–41.
- 31 Jha P, Ramasundaramhettige C, Landsman V *et al*. 21st-century hazards of smoking and benefits of cessation in the united states. *N Engl J Med* 2013; **368**: 341–50.
- 32 Noci S, Dugo M, Bertola F *et al*. A subset of genetic susceptibility variants for colorectal cancer also has prognostic value. *Pharmacogenomics J* 2016; **16**: 173–9.
- 33 Peters U, Jiao S, Schumacher FR *et al*. Identification of genetic susceptibility loci for colorectal tumors in a genome-wide meta-analysis. *Gastroenterology* 2013; **144**: 799–807. e24.
- 34 Peng S, Lu B, Ruan W, Zhu Y, Sheng H, Lai M. Genetic polymorphisms and breast cancer risk: evidence from meta-analyses, pooled analyses, and genome-wide association studies. *Breast Cancer Res Treat* 2011; **127**: 309–24.
- 35 Sorrentino FS, Gallenga CE, Bonifazzi C, Perri P. A challenge to the striking genotypic heterogeneity of retinitis pigmentosa: a better understanding of the pathophysiology using the newest genetic strategies. *Eye (Lond)* 2016; **30**: 1542–8.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Oligonucleotide primers used to sequence Kras exons 1 and 2 by pyrosequencing, for mutation detection in lung tumors.