Complex genetic control of lung tumorigenesis in resistant mice strains

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Abstract
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 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. Moreover, 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,8,9 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.9,10,11 Tumor modifier loci were also identified when BALB/c mice were crossed with A/J mice or with a resistant strain.10,11,12 Another exception to the A/J versus B6 clustering of mouse 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 alleles12 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 \textit{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 a QTL therein. In contrast, if the \textit{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 outcrossing 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.\(^{(15)}\)

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 until 40 weeks of age, when they were killed.

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 \textit{Pas1} locus genotyping.** Germ line 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 \textit{Pas1} locus was determined according to the presence or absence of a 37-bp tandem repeat polymorphism in \textit{Kras}, which maps in the second intron of the \textit{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 \textit{Kras}.** Genomic DNA from lung tumors of male F2 mice was assessed for the presence of mutations in \textit{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 \textit{Kras} sequence.

**Statistical analyses.** \textit{Nlung} and \textit{Vlung} values were square-root transformed to improve the normality of distribution. Sex differences in transformed \textit{Nlung} and \textit{Vlung} were tested by ANOVA. To identify QTLs, transformed values of \textit{Nlung} and \textit{Vlung} were analyzed together with SNP genotype data by parametric simple interval mapping using R/qtl\(^{(16)}\), adjusting by sex. The resulting logarithm of the odds (LOD) scores were considered significant if greater than the 95% LOD threshold (\(z = 0.05\)), calculated by 10,000 permutations. The percent phenotypic variance explained by a given QTL was calculated from the LOD score using the following formula: \(R^2 = 1 - 10^{-2(LOD/n)}\)\(^{(17)}\) 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/qtl.

Associations between \textit{Kras} mutations (a categorical phenogenotype: present or absent) and SNP genotypes were tested by single-point analysis using the “assoc” command of the PLINK program,\(^{(18)}\) which runs a \(x^2\) 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 (\textit{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 \textit{Nlung}. Indeed, in F2 mice, we observed a widening of the range of \textit{Nlung} values, with some mice developing up to 20 lung nodules. Moreover, the total tumor volume (\textit{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 \textit{Nlung} (\(P < 0.05\), ANOVA) and \textit{Vlung} (\(P < 0.01\), ANOVA) than their female counterparts. In contrast, in the F1 cross, there was no statistically significant sex difference in \textit{Nlung} or \textit{Vlung}.

**Quantitative trait loci modulating \textit{Nlung} and \textit{Vlung}.** To identify alleles that influence the two lung tumor phenotypes...
(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 rs5706601). No locus modulating Nlung was detected in the Pas1 region on chromosome 6 (Fig. 1).

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 homogeneous C57BL/6J-derived allele (B6/B6 genotype) and lowest for those animals homogeneous 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 (R²) 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 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 (R²) 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 rs13408684 (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).

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, Nlung (%) | Nlung | Vlung, mm³ |
|-------|-----|----|-------------------|-------|---------|
| (C57BL/6J × SM/J) F1 | M 20 | 15 (75) | 2.6 ± 0.4 (0–6) | 1.5 ± 0.4 |
| (C57BL/6J × SM/J) F2 | F 22 | 21 (95) | 2.6 ± 0.4 (0–6) | 1.4 ± 0.3 |
| SM/J F1 | M 208 | 182 (88) | 3.8 ± 0.2 (0–20) | 4.5 ± 0.4 |
| SM/J F2 | 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 < 0.005, ****P < 0.01; ANOVA on square-root transformed values.
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 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
F2 mice, by genotype at the 

tions.(23,24) However, in this study, we found no association 
previously reported the presence of allele-specific 
A/J-type susceptibility allele of the 

the peak marker 

and values of Vlung according to the genotype at 

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Pas1 

does the 

these two QTLs apparently modulate both Nlung and Vlung, as 

their strain-dependent allele effects were the same. Therefore, 

ping on chromosomes 3 and 15, are most likely the same QTLs 

was detected. This QTL had the highest LOD score and was cen-

thesized lung tumors (29 of 33, 88%) carried somatic mutations 

Indeed, GWAS have detected dozens of risk loci for other 

interactions between genetic and environmental risk factors. 

Reasons for the failure to dissect such complex networks may 

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mouse genetic models, we predict that an individual’s risk and 

association between codon 61 CGA mutations at Kras and the 

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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 poly-
genetic 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 recom-

binant 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 
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 associ-
ation 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 carcino-
ma(32,33) and non-familial breast cancer.(34) Moreover, retini-
tis 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 can-
cers or non-cancer diseases such as retinitis pigmentosa, we 

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 Kras codon 61* |
|------------|----------------------------|
|            | CAA (wt) | CGA | CTA |
| B6/B6      | 0        | 8   | 1   |
| B6/SM      | 3        | 2   | 6   |
| SM/SM      | 1        | 3   | 6   |
| Total      | 4        | 23  | 6   |

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

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, 

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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 | Chromosome (Mb) | Peak marker | Closest gene | B6/B6 | B6/SM | SM/SM | R²‡ |
|-----|-----------------|-------------|--------------|-------|-------|-------|-----|
|     | 3 (122.838)     | rs13477379  | Pde5a        | 5.2 ± 0.7 | 3.2 ± 0.4 | 2.5 ± 0.5 | 5.2 |
|     | 10 (91.060)     | rs13480702  | Apaf1        | 6.1 ± 0.9 | 3.3 ± 0.4 | 1.8 ± 0.3 | 7.4 |
|     | 15 (95.314)     | rs6285067   | Neil2        | 2.3 ± 0.4 | 3.5 ± 0.4 | 5.2 ± 1.0 | 4.6 |
|     | 17 (35.834)     | rs3682923   | Tubb5        | 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. †R², percentage of variance explained. QTL, quantitative trait loci.
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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.

Disclosure Statement

The authors have no conflicts of interest to declare.