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A Functional Switch From Lung Cancer Resistance to Susceptibility at the *Pas1* Locus in *Kras2*^{LA2} Mice

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Abstract

Pulmonary adenoma susceptibility 1 (Pas1) is the major mouse lung cancer susceptibility locus on chromosome 6 (ref. 1), spanning a region containing six genes², one of which is *Kras2*. *Kras2* is a common target of somatic mutation in chemically induced mouse lung tumors^{3,4}, and is a candidate *Pas1* gene⁵. *M. spretus* mice (*SPRET*) carry a *Pas1* resistance haplotype for chemically induced lung tumors⁶. We demonstrate here that the *SPRET/Ei Pas1* allele is switched from resistance to susceptibility by fixation of the parental origin of the mutant *Kras2* allele in *Kras2*^{LA2} mice. This switch correlates with low expression of endogenous *Kras2* in *SPRET/Ei*. We propose that the *Pas1* modifier effect is due to *Kras2*, and that a sensitive balance between the expression levels of wild-type and mutant alleles determines lung tumor susceptibility. These data demonstrate that cancer predisposition should also be considered in the context of somatic events, and could have major implications for the design of human association studies to identify cancer susceptibility genes.

Keywords

Lung Cancer; *Pas-1*; Susceptibility; *Kras2*

The critical *Pas1* interval encompasses *Bcat1*, *Lrmp*, *Las1*, *Ghiso*, *Kras2*, and *Lmna-rs1* (ref. 2). Of these, only *Kras2* is a known target of mutation in lung tumors^{3,4}, and mice expressing oncogenic *Kras2* have a high incidence of early onset lung cancer^{7,8}. Lung tumors from F1-hybrid mice showed a strong bias for *Kras2* mutation involving the allele inherited from the more susceptible parent⁹, rendering *Kras2* a candidate for *Pas1*. However, no coding sequence differences in *Kras2* have been detected among all mouse strains studied, and the possible mechanisms by which *Kras2* may contribute to the effect of *Pas1* remain unclear, causing some investigators to focus on other genes in the *Pas1* region

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COMPETING INTERESTS STATEMENT

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as primary candidates^{10,11}. Moreover, *Pas1* was recently associated with susceptibility to lung inflammation¹², raising the possibility that the major effect of *Pas1* might be to control responses to the carcinogens used to induce lung tumorigenesis.

We decided to address these issues using the *Kras2*^{LA2} model⁷, in which mice carry a knock-in *Kras2* allele with an extra, but mutant, copy of exon 1. The *Kras2*^{LA2} allele is inherited in a dormant form, but becomes functionally activated by somatic recombination. Mice carrying *Kras2*^{LA2} show complete penetrance for lung cancer, with tumors in other tissues occurring at very low frequencies⁷. The use of these mice removes the need for a chemical carcinogen, and also fixes the origin of the mutant parental allele of *Kras2*. In interspecific crosses involving these mice, the mutant *Kras2* allele is always present on the *M. musculus* parental chromosome, in contrast to crosses involving mice with two wild-type alleles, which are both potentially susceptible to carcinogen-induced mutation. We bred the *Kras2*^{LA2} allele to a pure *FVB/N* strain background prior to carrying out crosses with *SPRET/Ei* resistant mice (see Methods). *FVB/N* mice are generally highly susceptible to epithelial tumor development¹³, and carry the same *Pas1* susceptibility haplotype as the *129/Sv* strain². *SPRET/Ei* mice carry dominant resistant alleles for tumor development in multiple tissues¹⁴, and in particular carry a *Pas1* resistance haplotype⁶. As expected, all *Kras2*^{LA2}*FVB/N* mice developed multiple lung adenomas by 6 months of age. In contrast, F1 hybrids generated between *Kras2*^{LA2}*FVB/N* and *SPRET/Ei* had relatively low tumor incidence and number at 6 months (**Fig. 1a**), and some animals survived well over one year without becoming moribund.

We mated female *FVBSPRET**F1* hybrids with male *Kras2*^{LA2}*FVB/N* mice to generate a large population of interspecific backcross mice. A total of 224 (*FVBSPRET**F1*)*FVBKras2*^{LA2} backcross mice were aged to 6 months, and sacrificed for assessment of tumor number. The distribution of tumor number was very wide (**Fig. 1a**), in agreement with the observation that there are many lung tumor susceptibility/resistance loci^{15,16}. These animals were genotyped using markers on chromosome 6 to test for linkage to *Pas1*. We initially envisaged two possible outcomes: (i) If *Kras2* is the *Pas1* gene, there should be no linkage to the region, as every mouse carries the same parental mutant allele. This would be compatible with a mechanism in which selection for a more highly active or highly expressed *Kras2* mutant allele in chemical carcinogenesis studies accounts completely for the effect of *Pas1* (ref. 9). (ii) If *Pas1* is a different gene(s) in the region, there should be linkage to distal chromosome 6, and the resistance allele should be inherited from the *SPRET/Ei* parent. This would be in agreement with the recent proposals that the major effect of *Pas1* is mediated by *Las1* (ref. 10) and/or *Lmna*-rs1 (ref. 11). In fact, the results showed that neither of these scenarios was correct. As shown in **Table 1**, we detected strong linkage to the *Pas1* region, but mice homozygous for the *FVB/N* haplotype developed significantly fewer tumors than the *FVB/SPRET* heterozygotes. The most susceptible mice carried one *SPRET/Ei* allele, rather than two *FVB/N* alleles as would have been expected from previous work¹⁷.

To confirm previous results that *SPRET/Ei* mice are resistant at the *Pas1* locus, 97 wild-type interspecific backcross mice [(*MUSSPRET**F1*)*MUS*; see Methods] involving *SPRET/Ei* was subjected to chemical carcinogenesis using urethane. Similar to the results with the

Kras2^{LA2} model, *MUSSPRETF1* mice were clearly resistant to urethane induced lung cancer (Fig. 1b), and backcross mice treated with urethane showed a wide distribution of tumor number. Since mice of the *FVB/N* or *NIH/Ola* strains have not previously been used for lung chemical carcinogenesis, we carried out molecular analysis of tumors to identify *Kras2* mutations. Activating point mutations were found in 46 of 61 cases, and most of these were at codon 61, as previously seen (Supplementary Table1). *Kras2* mutation frequency was similar in backcross animals derived from the *FVB* (30 of 38) or *NIH* (16 of 23) parents, in agreement with the observation that these closely related strains have the same haplotype at the *Pas1* locus (see Methods). Significant linkage was detected in the *Pas1* region, with mice homozygous for *M. musculus* alleles developing significantly more tumors than heterozygous *MUS/SPRET* mice, confirming that the *FVB/NIH Pas1* allele confers susceptibility and that of *SPRET/Ei* confers resistance to chemically induced lung tumors (Table 1). We also detected suggestive linkage at proximal chromosome 6, in the region of the pulmonary adenoma resistance 4 (*Par4*) locus¹⁸. Significant linkage at *Par4* was also detected in the (*FVBSPRETF1*)*FVBKras2^{LA2}* mice, and as with the chemical carcinogenesis approach, the *FVB/N* allele conferred relative resistance (Table 1).

These data demonstrate that fixation of the parental origin of the mutant *Kras2* allele leads to reversal of the resistance that is normally conferred by the *SPRET/Ei* allele at *Pas1*. The maintenance of directionality at *Par4* in both crosses rules out the possibility of unforeseen artifacts in the model systems used here. The variance in lung tumor numbers accounted for by *Pas1* in the chemical carcinogenesis ($R^2 = 10.2$) and the *Kras2^{LA2}* model ($R^2 = 11.7$) was very similar, suggesting that most or all of the effect of *Pas1* was reversed in these genetic crosses. While specific cancer QTLs can confer either resistance or susceptibility depending on the tissue¹⁹, to our knowledge, this is the first report of a status switch involving the same allele at a cancer susceptibility locus by genetic manipulation of the target gene. The results demonstrate a highly context-dependent mechanism associated with *Pas1* regulation of lung cancer susceptibility.

The most plausible explanation for the above results is that *Pas1* is in fact *Kras2*, and that a delicate balance between the expression levels of the wild-type and oncogenic alleles controls predisposition to lung cancer development. The ratio of wild-type to mutant *ras* gene copy number has been shown to be an important factor in tumor initiation and progression²⁰⁻²². Functional studies suggested that wild-type Ras can inhibit the effect of the corresponding mutant proteins^{23,24}, causing selection for loss of wild-type alleles during tumor development^{21,22}. Recent work demonstrated the ability of wild-type *Kras2* to efficiently suppress the oncogenic activity of mutant *Kras2* in a gene dosage-dependent manner²⁴. These studies collectively suggested that susceptibility to lung carcinogenesis could be associated with the expression level of endogenous *Kras2* alleles. Some limited data suggested higher levels of *Kras2* expression in susceptible strains of mice²⁵. We confirmed and extended this association using quantitative real-time PCR to accurately measure *Kras2* expression in normal lungs of 7 different strains of mice. Strains with a susceptible *Pas1* haplotype had higher levels of *Kras2* than those with a resistant haplotype (Fig. 2a). The most susceptible *A/J* mice have the highest level of *Kras2* expression, while other susceptible strains, including *FVB/N*, *NIH/Ola* and *129/Sv*, also express *Kras2* at levels

significantly higher than resistant strains (*C57Bl/6*, *SPRET/Ei* and *SPRET/Glasgow*). The levels of *Bcat1*, *Lmna-rs1*, *Las1*, *Ghiso* and *Lrmp* also vary (**Fig. 2b-f**), but without any apparent correlation with lung cancer susceptibility.

The model that we believe can best explain results from both chemical carcinogenesis studies and the data from the *Kras2*^{LA2} mouse is shown in **Figure 3**. In traditional chemical carcinogenesis (**Fig. 3a**), mice with 2 copies of the *Pas1* susceptibility allele developed more tumors than those with the other possible genotypes, presumably because of the presence of 2 highly expressed *Kras2* alleles, each of which is a target for mutation. When the initiating oncogenic mutation can occur on either of two alternative *Kras2* alleles in the heterozygotes, fewer lung tumors develop because mutations involving the poorly expressed *Kras2* allele (i.e. *SPRET/Ei*) are selected against by the strong inhibitory activity of the highly expressed wild-type *Kras2* (i.e. *FVB/N* or *NIH/Ola*). Conversely, in the *Kras2*^{LA2} model where the mutation is fixed on the parental *FVB/N* allele (**Fig. 3b**), mice heterozygous at *Pas1* are more susceptible to tumor formation because the poorly expressed wild-type *Kras2* allele of *SPRET/Ei* is less efficient in suppression of the activity of the highly expressed mutant allele. While we cannot rule out a role for coding polymorphisms in *Lrmp*, *Ghiso*, *Las1*, or *Lmna-rs1* at the *Pas1* locus, it is difficult to reconcile the unexpected reversal of the effect on cancer susceptibility at *Pas1* that would involve any of these genes. Notably, *SPRET/Ei* carries the “resistance” polymorphism in the *Las1* gene¹⁰, in addition to a *Kras2* allele that is poorly expressed. It can be concluded that the proposed resistance polymorphism in the *Las1* gene, and indeed candidate *Pas1* resistance alleles of the other genes within the locus, are outweighed by the effects of the reduced expression level of the *Kras2* gene from the *SPRET/Ei* parent, thus rendering *Kras2* responsible for most, if not all, of the effect of *Pas1* on lung cancer susceptibility.

A prerequisite for the proposed model of *Kras2* expression as a major determinant of lung cancer susceptibility is that one or more cis-acting polymorphisms should be responsible for the effect observed. If the expression of *Kras2* was solely under the control of trans-acting factors (trans-QTL)^{26,27} at different genomic locations, this would not be compatible with the model proposed. To examine these alternative possibilities, we generated an additional independent population of untreated, wild-type (*FVBSPRETF1*)/*FVB* backcross mice, and measured the expression levels of *Kras2* in the normal lungs of a total of 47 animals. Genotyping of the same mice at the *Kras2* locus showed an association between *Kras2* expression levels and *Kras2/Pas1* genomic status. Mice homozygous for the *FVB* allele at *Kras2/Pas1* had significantly higher average levels of *Kras2* mRNA than *FVB/SPRET* heterozygous mice ($p = 0.033$; **Supplementary Fig. 1**), confirming the presence of a cis-QTL that controls *Kras2* expression levels. The mouse *Kras2* genomic locus (www.genenetwork.org/cgi-bin/beta/snpBrowser.py) contains multiple polymorphisms among inbred mouse strains, and further detailed *in vitro* studies will be necessary identify the specific polymorphism(s) responsible for cis-control of *Kras2* expression. Nevertheless, the identification of a cis-QTL for *Kras2* expression provides strong support for the model presented in **Figure 3**.

Association studies of human lung cancer identified suggestive linkage to chromosome 12p^{28,29}, in the region of the human *Kras2* gene, but these findings remain controversial.

Our results suggest the importance of simultaneous analysis of the somatic events in tumors in association studies of this kind. Only about 15-30% of human lung tumors carry *Kras2* mutations, and while a highly expressed allele may confer susceptibility to tumors in which that particular allele is mutated, the same allele may confer resistance if the alternative allele undergoes mutation, or if the tumor carries no *ras* mutation at all. It will therefore be important to carry out similar association studies specifically on cases with tumors that have acquired somatic *Kras2* mutations.

METHODS

Mouse Breeding and Lung Tumorigenesis

The original *Kras2*^{LA2} strain (a mixed *C57Bl/6* and *129/Sv* background) was bred with *FVB/N* mice, carrying the *Kras2*^{LA2} allele through 13 generations. Interspecific backcross mice were generated by first breeding *FVB/N* with *SPRET/Ei* mice, and then using the wild-type female *FVBSPRETF1* animals to breed with male *FVBKras2*^{LA2} mice. All backcross animals positive for the *Kras2*^{LA2} gene were maintained until 6 months of age, at which time they were sacrificed and their lung tissues fixed in 70% ethanol to facilitate tumor counting under a dissecting microscope.

Wild-type (*FVBSPRETF1*)/*FVB* and (*NIHSPRETF1*)/*NIH* backcross mice were generated similarly to above. *FVB/N* and *NIH/Ola* strains are very closely related³⁰ and have very similar levels of *Kras2* expression (**Fig. 2a**). Using a number of sequencing length polymorphisms (*D6Int27*, *D6Int17*, *D6Mit109*, *D6Mit209* and *D6Int14.1*) around the *Pas1* region and single nucleotide polymorphisms in *Kras2* intronic sequences, we found *FVB/N* and *NIH/Ola* to be identical at these markers, indicating that they carry the same susceptible *Pas1* haplotype. (*FVBSPRETF1*)/*FVB* and (*NIHSPRETF1*)/*NIH* mice were therefore treated in the analysis as a single population, referred to as (*MUSSPRETF1*)/*MUS* mice. These mice were treated with a single dose of urethane (in PBS; at 1 g/1 kg of body mass) by intraperitoneal injection, and sacrificed 20 weeks later for analysis of lung tissues as above.

Genotyping

Genomic DNA was extracted from tail clippings by conventional methods. We genotyped backcross mice using markers on chromosome 6 (**Table 1**) using conventional PCR. *FVB/N* and *SPRET/Ei* alleles of *Kras2* were distinguished by a *FokI* digestion assay that targets the second intron of *Kras2* (ref. 17). Additional genotyping was performed by CIDR. A total of 224 (*FVBSPRETF1*)/*FVBKras2*^{LA2} were used in the analysis, but the genotyping assays failed in a very small number of cases. Statistical analyses were performed using the Mann-Whitney test as part of the SPSS package.

Gene Expression Studies

Lungs from untreated mice were immediately frozen in liquid nitrogen after sacrifice by cervical dislocation. RNA was extracted using Trizol Reagent following the instructions of the manufacturer. Residual contaminating genomic DNA was removed by Dnase treatment. Taqman assays for gene expression analysis of *Kras2* (Mm00517491_m1), *Bcat1* (Mm00500289_m1), *Las1* (Mm01259185_m1), *Ghiso* (Mm01259203_m1), *Lrmp*

(Mm00493168), and *Lmna-rs1* (Mm00511959_m1) were purchased from Applied Biosystems. The *Gadph* house-keeping gene was used as an internal control (see **Supplementary Table 2** for oligonucleotide sequences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Expression of the *Pas1* candidate genes in normal lungs of different strains of mice. The levels of (a) *Kras2*, (b) *Bcat1*, (c) *Lrmp*, (d) *Las1*, (e) *Ghiso* and (f) *Lmna-rs1* were measured in normal lungs of independent mice of strains with susceptible (A/J, FVB/N, NIH/Ola and 129/Sv) and resistant (C57Bl/6, SPRET/Ei and SPRET/Glasgow) haplotypes at the *Pas1* locus using real-time quantitative RT-PCR.

Figure 3.

Proposed model for the role of *Kras2* in lung tumor susceptibility. (a) Chemical carcinogenesis. The possible chromosome 6 genotypes resulting from an intercross between mice that are resistant (green chromosomes) or susceptible (red chromosomes) are shown. Progeny with two *FVB/N* highly expressed *Kras2* alleles (top) have the highest number of adenomas, as each allele can be independently mutated to initiate tumorigenesis. Mice that are heterozygous and carry only one highly expressed *Kras2* allele inherited from the *FVB/N* parent (middle) have a lower tumor number, as mutation of the *SPRET/Ei* allele is less likely to give rise to an actively growing tumor. Mice with two poorly expressed *SPRET/Ei* alleles (bottom) have the lowest tumor number, and the *FVB/N Pas1* haplotype is strongly linked to susceptibility, while the *SPRET/Ei Pas1* haplotype confers relative resistance. (b) *Kras2*^{LA2} model. The possible chromosome 6 genotypes resulting from the interspecific backcross are shown, with the color coding as described above. All backcross mice were selected to have the *Kras2*^{LA2} allele, and either inherited the *FVB/N* wild-type *Kras2* allele (bottom) or the wild-type *SPRET/Ei* allele (top). Since the poorly expressed *SPRET/Ei* allele is a poor suppressor of the activity of the mutant *Kras2*^{LA2}, initiated cells carrying the recombined mutant allele are more likely to develop into visible tumors. In this case, inheritance of the

SPRET/Ei allele effectively leads to tumor susceptibility and *SPRET/Ei Pas1* confers susceptibility rather than resistance as in scenario (a).

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Table 1

Average lung tumor number and correlation with genotypes

Markers (cM)	<i>Kras2^{LA2}</i>			Chemical Carcinogenesis		
	F ^{LA2} /F	F ^{LA2} /S	p-value	M/M	M/S	p-value
D6Mit116 (5.5) [<i>Par4</i>]	61.8 (n=113)	80.8 (n=105)	0.003	2.1 (n=48)	3.9 (n=49)	0.08
D6Mit284 (37.0)	64.1 (n=112)	77.5 (n=106)	0.02	2.5 (n=45)	3.5 (n=52)	0.5
D6Mit328 (49.0)	58.7 (n=115)	85.1 (n=102)	7.9×10 ⁻⁶	3.1 (n=45)	2.9 (n=52)	0.4
<i>Kras-2</i> (67.0) [<i>Pas1</i>]	56.9 (n=126)	88.8 (n=98)	1.4×10⁻⁷	4.0 (n=45)	2.1 (n=52)	0.004

Abbreviation for *FVB/N* (F) and *SPRET/Ei* (S).