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Cancer Genes and Genomics

Molecular Cancer Research

Progressive Genomic Instability in the *FVB/Kras^{LA2}* Mouse Model of Lung Cancer

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Abstract

Alterations in DNA copy number contribute to the development and progression of cancers and are common in epithelial tumors. We have used array Comparative Genomic Hybridization (aCGH) to visualize DNA copy number alterations across the genomes of lung tumors in the *Kras^{LA2}* model of lung cancer. Copy number gain involving the *Kras* locus, as focal amplification or whole chromosome gain, is the most common alteration in these tumors and with a prevalence that increased significantly with increasing tumor size. Furthermore, *Kras* amplification was the only major genomic event among the smallest lung tumors, suggesting that this alteration occurs early during the development of mutant *Kras*-driven lung cancers. Recurring gains and deletions of other chromosomes occur progressively more frequently among larger tumors. These results are in contrast to a previous aCGH analysis of lung tumors from *Kras^{LA2}* mice on a mixed genetic background, in which relatively few DNA copy number alterations were observed regardless of tumor size. Our model features the *Kras^{LA2}* allele on the inbred *FVB/N* mouse strain, and in this genetic background, there is a highly statistically significant increase in level of genomic instability with increasing tumor size. These data suggest that recurring DNA copy alterations are important for tumor progression in the *Kras^{LA2}* model of lung cancer and that the requirement for these alterations may be dependent on the genetic background of the mouse strain. *Mol Cancer Res; ©2011 AACR.*

Introduction

The development of cancer is largely driven by progressive accumulation of genetic and epigenetic alterations, resulting in concerted deregulation of biological functions that together contribute to the cancer phenotypes. Alterations in DNA copy number are common in cancers and especially in epithelial tumors. The gain or loss of DNA can occur focally and more often involves whole chromosomes or chromosomal arms (1, 2). DNA amplification commonly occurs in regions containing proto-oncogenes whereas deletion frequently targets regions with tumor suppressor genes. In consequence, genomic regions with recurring focal alterations have emerged as genomic landmarks for discovery of genes with important functions in tumorigenesis and that are commonly altered in tumors (1). The relevance of genomic imbalance is further underscored by the association between aneuploidy with disease aggressiveness in cancers of many tissues. Because molecular analyses have mostly focused on tumor tissues at relatively advanced stages, it has been difficult to formally study the evolution of genomic alterations during the natural course of tumor development.

Lung cancer is the second most common cancer in both men and women and is responsible for the highest number of cancer-related deaths in the United States. Adenocarcinoma is the most common subtype of lung cancer, accounting for 30% to 40% of the cases. Activating mutations in *KRAS* occur in approximately 30% of human lung adenocarcinomas and are associated with poor clinical outcome (3). Studies in the mouse have showed that oncogenic activation of *Kras* alone is sufficient for the initiation of lung tumors (4, 5). In the *Kras^{LA2}* model, spontaneous somatic activation of the latent *Kras^{LA2}* allele initiates lung tumor development with complete penetrance (5). The majority of these tumors are benign lung adenomas, some of which progress to invasive adenocarcinomas in a process that mirrors lung tumor development and progression in humans. Adult *Kras^{LA2}* mice develop multiple lung adenomas with a wide range in size that likely reflects the variable timing of the *Kras* activation and tumor initiation.

Previous analysis of genomic changes in tumors from the *Kras^{LA2}* mice were carried out on tumors from animals of

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mixed *C57BL6* and *129/SvJae* backgrounds (6). These studies showed some whole chromosome changes in a relatively small proportion of the tumors (27%) and noted the absence of any focal amplicons or deletions. We have transferred the $Kras^{LA2}$ allele onto the *FVB/N* background and noted that on average, tumor development was delayed relative to the previous data on the mixed background. Using array Comparative Genomic Hybridization (aCGH), we show that on the *FVB/N* background, there is a significant progressive increase in the degree of genomic instability associated with increasing tumor size. Some of the smallest tumors show focal amplification of *Kras* as the only detectable change, suggesting that this may be the earliest genetic event during lung tumor development in the $Kras^{LA2}$ model.

Materials and Methods

Animals

$Kras^{LA2}$ mice on a *C57BL6/129/SvJae* background were backcrossed with *FVB/N* mice to create an $Kras^{LA2}$ inbred line on the *FVB/N* strain background. The mice used in this study are in between generation N11 and N15. Animals were sacrificed at 6 months of age, and their lung tissues were collected for molecular studies.

Tumor genomic DNA isolation

We isolated 83 lung tumors of different sizes from $Kras^{LA2}$ mice. To avoid normal cell contamination, only the tumor mass inside the tumor capsule was used for DNA isolation. Genomic DNA was extracted using the Dneasy Tissue Kit (Qiagen) and further purified by phenol/chloroform extraction. DNA concentrations were measured using a Biorad fluorometer.

Array comparative hybridization and data processing

aCGH arrays were prepared and printed as reported previously (7, 8). Each array consisted 1,056 BAC/P1 clones printed in quadruplicate (2 spatially separated duplicates) onto 3D-Link activated slides (Motorola Life Sciences) using a custom DNA arraying device developed at the University of California San Francisco Cancer Center. Two arrays, each 12 mm × 12 mm in size, were printed per slide. The identities and genomic coordinates of the BAC/P1 clones were based on the February 2002 freeze of the assembled mouse genome through the UCSC Genome Browser at http://genome.ucsc.edu. A detailed list of the clones including their associated markers and genomic coordinates were previously published online (7). We labeled 1 μg of test (tumor) DNA and reference genomic DNA (normal lung) with CY3 and CY5 (Amersham), respectively. DNA labeling, hybridization, slide imaging, and data analyses were conducted essentially as previously described (8). The 16-bit TIFF images were collected using a custom-built charge-coupled device camera through CY3, CY5, and 4′,6-diamidino-2-phenylindole filters (9). Images were segmented and quantified using custom software (10). Data were normalized to the median $\log_2$ ratio of Cy3/Cy5. All clones except those mapping to chromosome 6, known to be frequently altered in $Kras^{LA2}$ lung tumors, were used for normalization. The mean and SD of the normalized $\log_2$ ratios of the quadruplicate spots were calculated. Clones were declared missing if their ratio was based only on a single spot or their SD exceeded 0.2. Clones were called amplified or deleted if their $\log_2$ ratio exceeded $\pm$ 0.3. Percentage of the genome altered was calculated by dividing each chromosome into 1,000,000 equally spaced bins and calling each bin amplified or deleted depending on the status of the most physically proximal probe. Association between tumor size category and percentage of altered loci was assessed with the Kruskal–Wallis rank-sum test. Statistical analysis was conducted with the R package (10). CGH data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus archive under accession GSE29230.

Results

The $Kras^{LA2}$ allele, originally on a *C57BL6/129/SvJae* mixed background, was backcrossed into the *FVB/N* background for more than 10 generations to minimize the effect of genetic heterogeneity on lung tumor development. Mice were sacrificed at 6 months of age, and a total of 83 surface lung tumors from more than 25 animals were obtained for molecular analyses. The size of the tumors was estimated on the basis of their surface area measured at the time of resection. We categorized the tumors into 4 groups on the basis of size (Table 1). Genomic DNA was isolated from the tumors, and genome-wide DNA copy number alterations were quantified using aCGH. Histologic analysis was

Table 1. Tumor categories and chromosome 6 alteration

Tumor category	Surface area, mm^2	No. of tumors	Focal amplification of distal chromosome 6	Amplification of chromosome 6[a]
1	<6	15	2/15 (13%)	3/15 (20%)
2	6–20	34	0/34 (0%)	20/34 (59%)
3	20–42	17	0/17 (0%)	12/17 (70%)
4	>42	17	0/17 (0%)	14/17 (82%)

[a]Tumors with greater than 30% of probes on chromosome 6 showing alterations.

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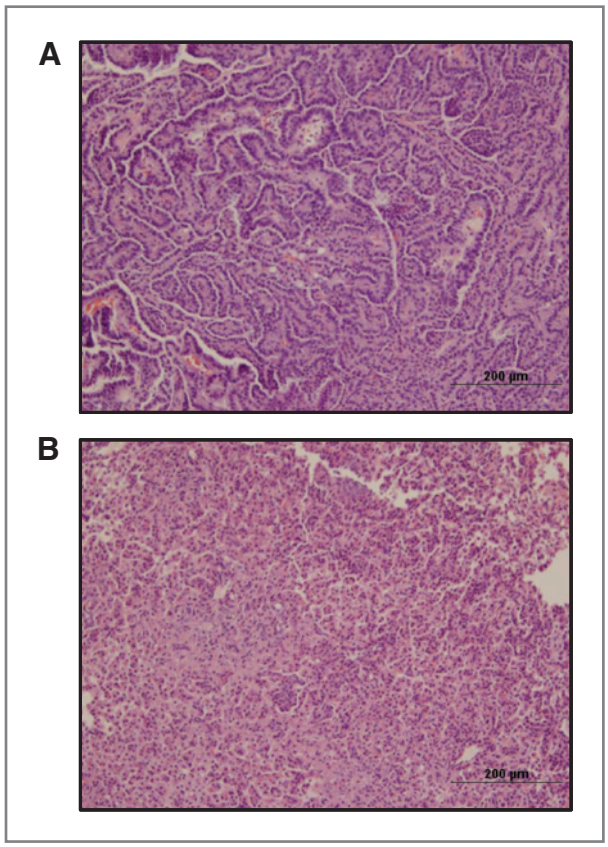

Figure 1. Histologic characteristics of lung tumors in the *FVB/Kras*^{LA2} model of lung cancer. A, the majority of lung tumors have a papillary architecture with features of glandular differentiation. B, a small number of lung tumors displayed a more solid pattern of growth. Tumor sections are stained with hematoxylin and eosin.

conducted on 27 of the tumors. The majority of these tumors (22 of 27) had a papillary histology (Fig. 1A), as has been previously described (5). Four tumors had a solid histology (Fig. 1B), but these were relatively small (categories 1 and 2) and we could not ascertain whether this pattern of growth was uniform throughout the tumor. Interestingly, one tumor had a mixture of both papillary and solid components.

Kras amplification is an early and common event in lung tumorigenesis

Somatic oncogenic activation of *Kras* initiates tumor formation in the *Kras*^{LA2} mouse model of lung cancer (5). We and others have showed that oncogenic *ras* mutations are often accompanied by DNA copy number alterations that result in extra copies of the mutant allele in tumors, often through gain of the whole chromosome (11, 12). The mouse *Kras* gene is located on distal chromosome 6, and gain of this region through focal amplification or trisomy of chromosome 6 was the most frequent alteration in the lung tumors analyzed. Focal amplification involving the *Kras* locus was observed in only 2 cases (Fig. 2A), but both were among tumors of the smallest size (category 1; <6 mm^2). The level of focal amplification in these tumors was as high as 15-fold, and with very few, if any, copy number alteration in other parts of the genome. In 3 additional tumors in category 1, more than 30% of the probes on chromosome 6, including those overlapping with the *Kras* locus, showed a gain of a single copy that we interpreted to reflect a gain of the entire chromosome (Fig. 2B). We conclude that gain of DNA copy number involving the *Kras* locus, either through focal amplification or whole chromosome gain, is the earliest detectable somatic alteration in oncogenic *Kras*-driven lung tumors.

Trisomy of chromosome 6 is the most common DNA copy number alteration across the 4 size categories of lung tumors. Of the 83 lung tumors analyzed, 49 (59%) had amplification of more than 30% of chromosome 6 probes (Fig. 3A and Table 1). Importantly, the prevalence of chromosome 6 gain increased with increasing tumor size. The frequencies of trisomy of chromosome 6 ranged from 20% of category 1 tumors to more than 80% of category 4 tumors ($P = 0.003$, χ^2 trend test; Table 1). When the threshold for calling trisomy was increased to 50% of probes, the number of tumors in each of the categories meeting this criterion decreased, but the trend between increasing tumor size and increasing incidence of trisomy 6 was maintained ($P = 0.07$, χ^2 trend test). Probes overlapping the *Kras* locus were amplified in all tumors classified as having trisomy of chromosome 6 at both levels of stringency. These data suggest that gain of chromosome 6, likely as a mechanism to upregulate the level of mutant *Kras*, is an important event in the development and progression of lung tumors driven by oncogenic *Kras*.

Lung tumor progression is accompanied by increased genomic alterations

While the genomes of the smallest tumors (category 1) were relatively stable apart from changes on chromosome 6 (Figs. 2 and 3B), frequent gains and losses of DNA copy number were evident among larger tumors (Fig. 3C–E). Considering all tumors together, we observed gains on chromosomes 1, 2, 8, 15, 17, and 19 as well as focal deletions or whole chromosomal loss on chromosomes 4, 5, 9, and 11 (Fig. 3A). Because these events commonly involve whole chromosomes or relatively large chromosomal regions, it was not possible to determine with confidence the targeted gene(s). However, there was a clear correlation between tumor size and level of genomic alterations as indicated by the progressive increase in percentage of aCGH probes altered across categories 1 to 4 tumors ($P = 8.3 - 10^{-7}$, Kruskal–Wallis rank-sum test for association between group rank and amount of variation; Fig. 4A). This strong correlation was not driven exclusively by the alterations on chromosome 6 because the relationship was still statistically significant when chromosome 6 probes were excluded from the analysis ($P = 1 \times 10^{-5}$, Kruskal–Wallis rank-sum test; Fig. 4B). These data showed the contribution of accumulating genomic alterations to the growth of otherwise histologically similar lung tumors.

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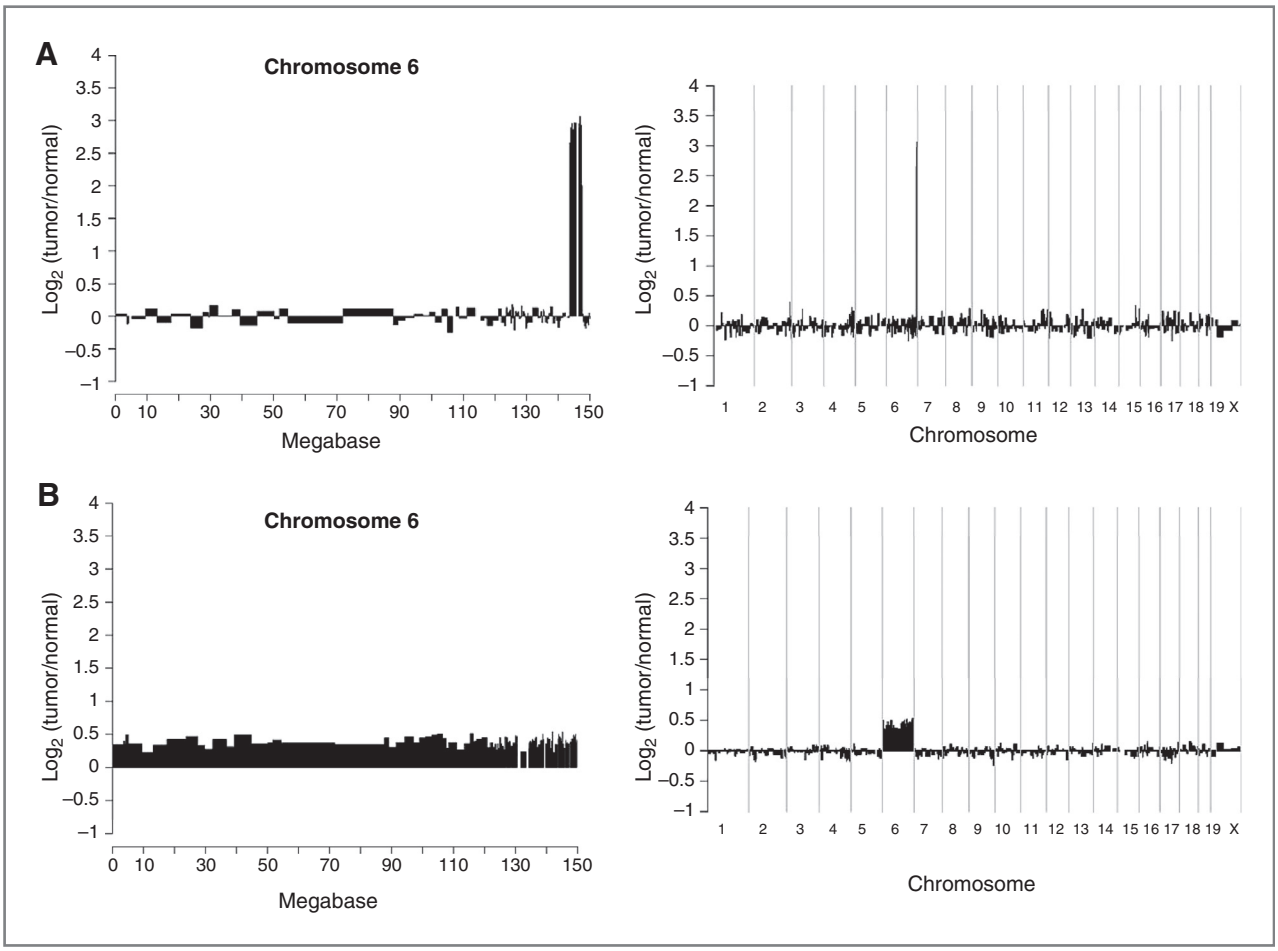

Figure 2. Alterations of chromosome 6 involving *Kras* occur in the smallest $Kras^{LA2}$ tumors. DNA copy number was measured by aCGH and copy number (*y*-axis) is expressed as $\log_2$ ratio of tumor to normal DNA. $\log_2$ ratios greater than 0.3 represent amplification. A, category 1 tumor with focal amplification of the *Kras* locus on distal chromosome 6. Left shows only chromosome 6. A $\log_2$ ratio of 3 represents an estimate of 16 copies of the *Kras* locus in the tumor. Right shows the whole genome of the tumor with little to no DNA copy number alterations apart from amplification of the *Kras* locus. B, a representative category 1 tumor with trisomy of chromosome 6. More than 30% of the aCGH probes on chromosome 6 had a $\log_2$ (tumor/normal) value of approximately 0.5 (left), consistent with the gain of a single copy of the chromosome. No copy alterations were evident on other chromosomes (right).

Discussion

We have used aCGH to generate a genome-wide view of DNA copy number alterations during the development and progression of mouse lung tumors in the $Kras^{LA2}$ model. We dissected surface lung tumors from multiple $Kras^{LA2}$ mice and used the surface area of the tumors as an approximate measurement of overall size. Somatic activation of the $Kras^{LA2}$ allele can occur throughout the lifetime of the mouse, and we reason that this variable timing in tumor initiation accounted for the differences in size of lung tumors within the same animal. We therefore used tumor size as an indicator of tumor progression, with larger tumors being further along the tumorigenic process. However, we cannot rule out the possibility that acquisition of particular alteration(s) during the early stage of tumor formation could have blunted or rapidly accelerated the progression of some tumors.

The major DNA copy number alterations in lung tumors from $Kras^{LA2}$ mice involved large chromosomal regions and whole chromosomes. Duplication of chromosome 6 is the most frequent event, but recurring gains of chromosomes 1, 2, 8, 12, 14, 15, 17, 18, and 19 as well as recurring loss of on chromosomes 4, 5, 9, and 11 were also observed. Recurring regions of focal deletion (e.g., proximal chromosome 4) were detected in a significant proportion of the largest tumors (category 4; Fig. 3E), but even here, the size of the region limited our ability to pinpoint the driver gene(s). A previous study also showed that lung tumors from the $Kras^{LA2}$ mice have frequent trisomy of chromosome 6, but the overall proportion of tumors with evidence of DNA copy number change was relatively small (6). Importantly, the authors reported a lack of association between tumor size and genomic alterations (6). In contrast, we observed a clear and significant increase in DNA copy number alterations with increasing tumor size (Fig. 4A). This correlation

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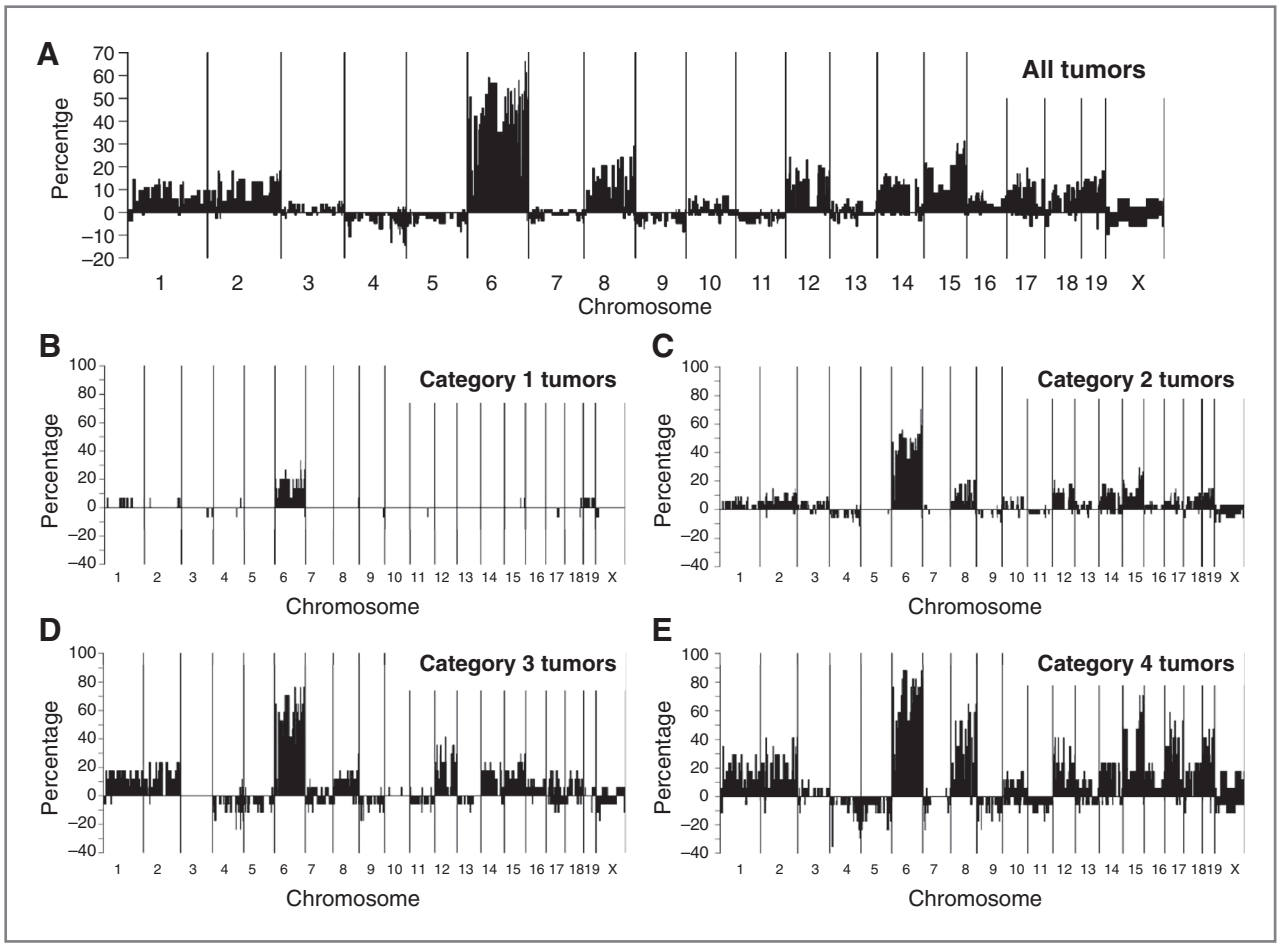

Figure 3. DNA copy number changes in *Kras*^{LA2} lung tumors occur first on chromosome 6 and increase in frequency as tumors progress. We use $\log_2$ ratios of tumor to normal measurements greater or less than 0.3 as thresholds for amplification and deletion, respectively. A, percentage of tumors with DNA copy number alterations at each locus across the genome using all 83 tumors. B–E, quantification of percentage of tumors with copy number alterations (*y*-axis) sampled across the genome (*x*-axis) of tumors in size categories 1 to 4.

remained highly statistically significant even after chromosome 6 was removed from the analysis (Fig. 4B). These data show the association of DNA copy number alterations with lung tumor progression in this model.

A likely explanation for the difference between our results and those of the prior study is that we have backcrossed the *Kras*^{LA2} allele into the *FVB/N* background for more than 10 generations whereas the allele was originally carried on a *C57BL6/129/SvJae* background (6). On a *C57BL6/129/SvJae* background, approximately 30% of animals carrying the *Kras*^{LA2} allele developed skin papillomas or carcinomas and thymic lymphoma (5), but the incidence of these tumors on a *FVB/N* background is dramatically reduced (data not shown). This result was surprising, as *FVB/N* mice are known to be highly susceptible to development and progression of skin tumors initiated by activating mutations in the *Hras* gene (13, 14). These observations suggest that although *FVB/N* mice are susceptible to *Hras*-driven skin tumors, they may be more resistant generally to activating mutations in *Kras* including *Kras* mutant lung tumors. Consequently, more genomic or genetic alterations may be required to drive *Kras* mutant lung tumor formation in this background. The relationship between frequency of genomic alterations and genetic background has also been observed in other murine models of cancer (15). Data from analyses of mouse (16) and human (17) squamous cell carcinomas also support the notion of genetic background affecting the pattern of somatic alterations in tumors. The potential interactions between germ line variants and somatic alterations could influence not only cancer susceptibility (18) but can also affect tumor progression and disease prognosis.

Activating mutations in the *ras* family of genes, particularly *Kras*, are capable of initiating and driving tumorigenesis in many tissues. Despite its oncogenic potency, tumors containing *ras* mutations have often been observed to have DNA copy alterations at the *ras* locus that result in a genomic imbalance in favor of the mutant allele. Mouse skin tumors harboring an activating *Hras* mutation commonly have gross chromosomal alterations that result in an

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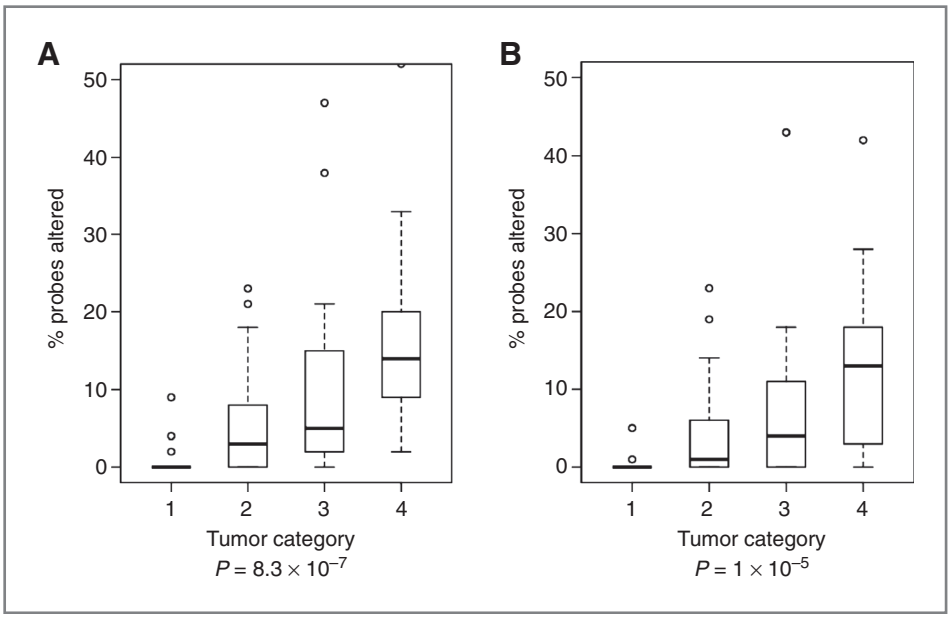

Figure 4. Increasing tumor size is significantly associated with a larger proportion of DNA copy number changes. A, percentage of all loci with DNA copy alterations in each tumor of size categories 1 to 4. B, same analysis as in (A) but with chromosome 6 probes excluded. Statistics were calculated using the Kruskal–Wallis rank-sum test.

increase in copy number of mutant *Hras* or loss of the wild-type (WT) *Hras* allele (11, 12). Furthermore, the imbalance at the *Hras* locus contributes to the progression of squamous carcinomas to more invasive tumors (12). We identified trisomy of chromosome 6 as the most common genomic alteration in lung adenomas from the $Kras^{LA2}$ mice, particularly in the largest tumors, suggesting that genomic imbalance on this chromosome plays an important role in tumor progression. Interestingly, LOH spanning the *KRAS* locus has been reported in human lung cancer that correlates with *KRAS* mutation and preferentially targets the WT *KRAS* allele (19). Genetic and *in vitro* studies have compellingly showed that WT *Kras* can effectively attenuate the oncogenic effect of mutant *Kras* through mechanisms that remain to be elucidated (18, 20, 21). We have further shown that the balance in expression levels of mutant and WT *Kras* is a major determinant of susceptibility to lung cancer development in the mouse (18). Therefore, the imbalance in favor of mutant *Kras* in lung tumors, through gain of the mutant allele or loss of the WT allele, is compatible with the requirement of tumor cells to overcome the tumor suppressor effect of WT *Kras*.

The occurrence of focal amplification involving the *Kras* locus supports the notion that *Kras* is the major driver of the genomic imbalance on chromosome 6. The level of focal amplification was as high as 15-fold. However, focal amplification and especially high level of focal amplification of distal chromosome 6 was rare and occurred only in the smallest tumors (category 1). Instead, the majority of tumors including many of the small tumors (categories 1 and 2) have gained only a single copy of chromosome 6. These data suggest that while *Kras* may be the main driver for genomic imbalance on chromosome 6, the accompanying dosage increase of other chromosome 6 gene(s) may also contribute to lung tumor progression. Interestingly, both *Braf* and *Raf1* (*cRaf*), encoding well-known downstream effectors of Ras signaling (22), are also located on chromosome 6 and must therefore undergo copy number gains in lung tumors. Alternatively, a subtle increase in copy number, rather than high levels of amplification, is more compatible with the growth and progression of the tumors. Cellular senescence is an important barrier against tumorigenesis, and high levels of oncogenic Ras (23) or Braf (24) can trigger this process. In addition, the cyclin-dependent kinase inhibitor *p27/Kip1* is located near *Kras*, and high levels of p27/Kip1 as a result of coamplification with *Kras* would potently inhibit tumor growth. Therefore, tumor cells must strike a balance between overcoming the suppression effect of WT *Kras* and avoiding the triggering of intrinsic mechanisms that have evolved to protect cells against conditions of high oncogenic activity. Further analysis of these tumors on the *FVB/N* background by high resolution CGH, gene expression, and tumor genome sequencing may reveal additional genetic targets that contribute to progressive genomic instability and tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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