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Advances in Brief

Carcinogenic Induction Directs the Selection of Allelic Losses in Mouse Lung Tumorigenesis[1]

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Abstract

Recent evidence suggests that genome-wide allelic imbalances are inducible by carcinogens and may occur as cells adapt to carcinogenic exposure during tumorigenesis. We investigated the role of carcinogenic exposure on global and selected loss of heterozygosity (LOH) during mouse lung adenocarcinogenesis. Tumor induction by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or vinyl carbamate (VC) resulted in a significant overall increase in the number of chromosomes affected by LOH per tumor when compared with spontaneous lung tumors. Allelic loss on chromosome 12 occurred at a frequency of 35% and 40% in NNK- and VC-induced tumors, respectively, compared with 8.3% in spontaneous tumors ($P < 0.01$). In contrast, spontaneous lung adenocarcinomas displayed LOH on chromosome 4 at a frequency of 77%, whereas a frequency of only 36% ($P < 0.001$) was observed in tumors induced by NNK. Sixty-four percent of the VC-induced tumors displayed LOH on chromosome 4. In addition, allelic losses on chromosomes 12 and 14 were significantly associated with an increase in chromosomal instability, suggesting that genes inactivated on these chromosomes may contribute to this effect. The results from this study demonstrate that genotoxic carcinogens increase chromosome instability, as evidenced by a significant increase in global LOH frequency, and significantly alter the selection of chromosomal alterations during lung tumor development.

Introduction

Human lung tumors are typically characterized by chromosomal structural instability, a specific type of genomic instability that produces chromosomal abnormalities including deletions, duplications, rearrangements, and unbalanced translocations (1–4). These alterations give rise to numerical changes in the copy number of oncogenes and tumor suppressor genes that are subjected to selective pressures as the tumor evolves. As a result, there is an emergence in tumors of regions of frequent losses or gains due to the dose effects of genes important for tumor growth or survival.

Many of the frequent chromosomal changes that occur during mouse lung adenocarcinogenesis have been shown to closely resemble those in human lung adenocarcinogenesis (1–8). Similar regions of frequent allele loss include two regions of mouse chromosome 4, one that targets the *Ink4a/Arf* locus (human 9p21) and the other in a region homologous to human chromosome 1p36 (6). Deletions of this chromosome have occurred at a frequency of ~50% during lung tumor progression (5–7). In addition, LOH[3] on chromosomes 12 and 14 each occurred at frequencies of 28% in lung adenocarcinomas (8). Affected by deletions on chromosome 14 were the retinoblastoma tumor suppressor gene and a region homologous to human chromosome 3p, which contains tumor suppressor genes frequently targeted for LOH in human lung adenocarcinomas (8, 9). Mouse chromosome 11 harbors the *p53* tumor suppressor gene, which was affected by allele loss in 26% of the tumors examined (8). Karyotype analysis of lung tumors and derived cell lines has revealed evidence of intrachromosomal instability affecting many of those regions observed to undergo allelic imbalances by other methods (1, 2, 10). Thus, multiple chromosomal alterations occur in the genesis of human and mouse lung adenocarcinomas, suggesting that chromosomal structural instability may influence the development of these tumors.

Environmental exposure plays a significant role in the development of many common cancers that exhibit chromosomal instability, such as lung cancer, colon cancer, head and neck cancer, and others including breast cancer where carcinogen exposure has been implicated as a causative factor (11–16). In fact, nearly all lung cancers are associated with smoking and exposure to environmental carcinogens (17, 18). Recent evidence has shown that carcinogens can induce specific types of genomic instability in a manner related to the type of DNA damage that they cause and may occur in tumorigenesis as an evolutionary adaptation to the tumor's environment (19–21). Bulky DNA adduct-forming carcinogens typically induce chromosomal instability (19). Potent lung carcinogens present in cigarette smoke, including benzo(*a*)pyrene and NNK, form bulky adducts with DNA and cause DNA strand breaks, suggesting that they could contribute to the chromosomal instability observed in lung cancer (22–25). The present study is designed to test the hypothesis that lung tumor induction by carcinogens, including the tobacco-specific nitrosamine NNK, plays an important role in directing the selection of chromosomal alterations that underlie lung tumorigenesis.

Table 1 *Polymorphic genetic markers used for PCR-LOH analysis*

Chromosome	Marker
1	D1MIT45, D1MIT148, D1MIT90, D1Nds2
2	D2MIT82, D2MIT128
3	D3MIT64, D3MIT110
4	D4MIT84, D4MIT18, D4MIT77, D4MIT27, D4MIT54, D4MIT68, D4MIT251, D4MIT13, D4MIT158
5	D5MIT31, D5MIT115
6	D6MIT54, D6MIT14, K-ras, D6MIT26
7	D7Nds1, D7MIT126
8	D8MIT18, D8MIT68, D8MIT65
9	D9MIT159, D9MIT24, D9MIT125
10	D10MIT3, D10MIT96, D10MIT170
11	D11MIT4, D11MIT67, Atb1b2
12	D12MIT8, D12MIT56, D4MIT58, D4MIT170
13	D13MIT16, D13MIT67
14	D14MIT78, D14MIT98, D14MIT34, D14MIT41, D14MIT207, D14MIT134
15	D15MIT63, D15MIT58
16	D16MIT4, D16MIT5
17	D17MIT34, D17MIT11, D17MIT96
18	D18MIT55, D18MIT60, D18MIT36, D18MIT68
19	D19MIT10, D19MIT45

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[3] The abbreviations used are: LOH, loss of heterozygosity; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, VC, vinyl carbamate.

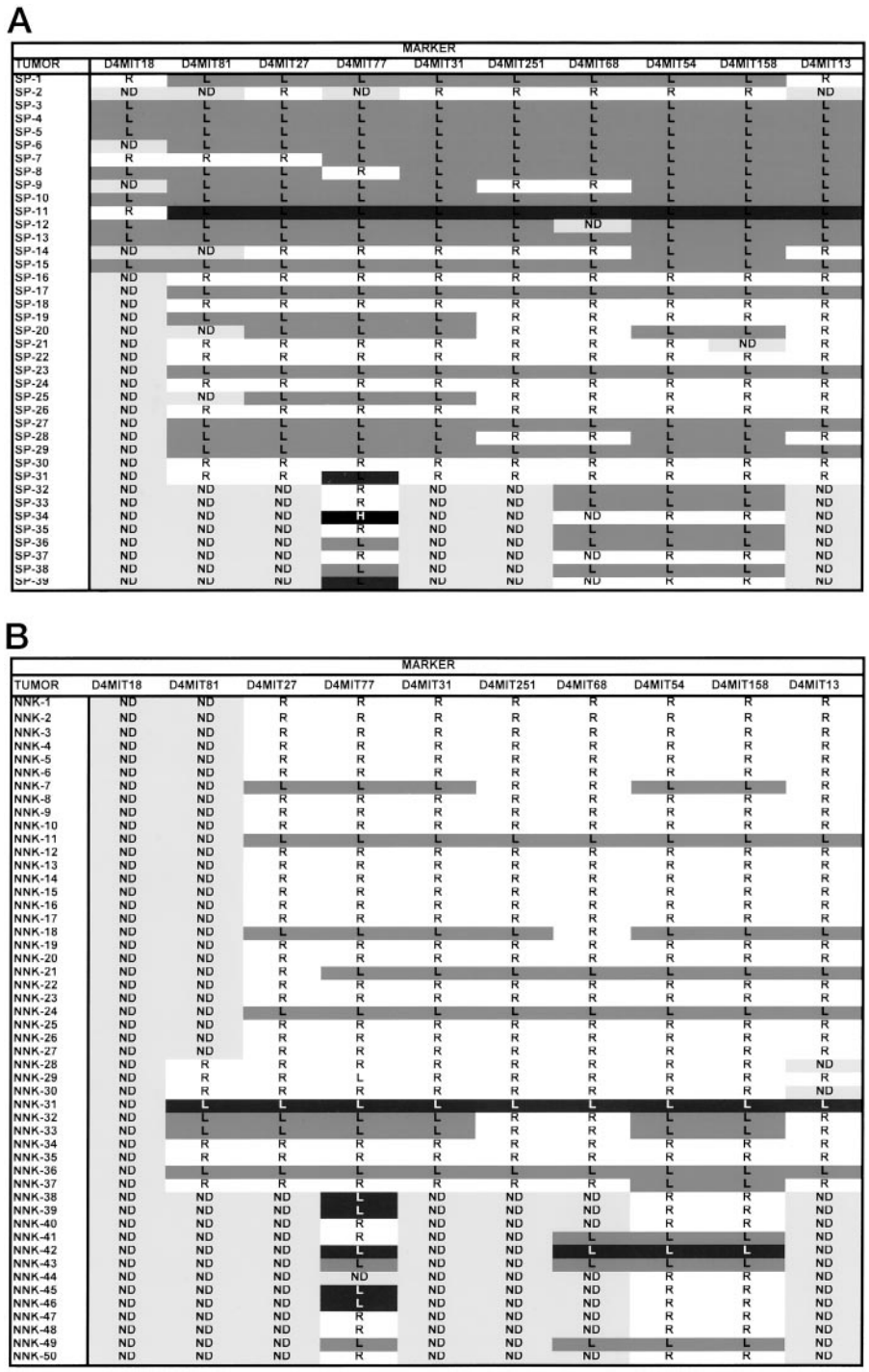

Fig. 1. LOH on chromosome 4 in mouse lung carcinomas. *Dark gray*, loss of C3H allele; *light gray*, loss of A/J allele; *black*, loss of both alleles. *A*, Spontaneous tumors (*SP*). *B*, NNK-induced tumors. *C*, VC-induced tumor. *L*, LOH; *R*, retained heterozygosity; *H*, homozygous deletion; *ND*, not done. *D*, representative LOH analysis. *N*, normal AC3 lung DNA; *T1*, tumor NNK-43; *T2*, tumor VC-39. Relative distances between markers are: centromeric D4MIT18 − 30 cM D4MIT77 − 30 cM − D4MIT54 telomeric.

Materials and Methods

Tumors. C3H/HeJ × A/J (C3A) and A/J × C3H/HeJ (AC3) F_1 hybrid lung carcinomas either developed in the absence of carcinogenic induction (spontaneous) or were induced by either VC or NNK. Tumors were induced with 20 or 60 mg VC/kg body weight by a single i.p. injection at 7 weeks of age. Tumors were induced by i.p injection of 50 mg NNK/kg body weight 3 times/week for 8 weeks. All tumors were obtained from 6–14-month-old mice with the latency period for the spontaneous tumors approximately two times that of the carcinogen-induced tumors. Genomic DNA was isolated from tissues as described previously (6).

LOH Analysis. Informative DNA markers on each of the 19 mouse chromosome pairs were used to determine LOH frequencies in spontaneous and NNK- and VC-induced AC3 F_1 and C3A F_1 lung tumors by PCR as described previously (Table 1; Ref. 8). For global LOH analysis, markers were selected to provide sufficient coverage of each chromosome. Typically, 100 ng of tumor or normal lung DNA were PCR amplified as follows: 1 min at 95°C, 1 min at 58°C, and 30 s at 72°C for 25 cycles. One PCR primer of each pair was labeled with [^{32}P]ATP before PCR. PCR products were then electrophoresed in 8% denaturing polyacrylamide and autoradiographed. LOH was scored as a reduction of one of the alleles by 50% relative to the other and normalized against the allelic ratios determined from normal lung DNA.

Homozygous Deletion Analysis. Homozygous deletion of the Ink4a/Arf tumor suppressor gene was examined by multiplex PCR using one intragenic primer pair together with the D10MIT3 primer pair (26). The latter is infrequently deleted in hybrid mouse lung tumors, as we showed previously (8). Simultaneous PCR amplification of both products was carried out essentially

C

	MARKER									
TUMOR	D4MIT18	D4MIT81	D4MIT27	D4MIT77	D4MIT31	D4MIT251	D4MIT68	D4MIT54	D4MIT158	D4MIT13
VC-1	ND	R	R	R	R	R	R	R	R	R
VC-2	ND	R	R	R	R	R	R	R	R	R
VC-3	L	L	L	L	L	L	L	L	L	L
VC-4	L	L	H	H	L	L	L	L	L	L
VC-5	ND	ND	R	R	R	R	R	R	R	R
VC-6	ND	ND	R	R	R	R	R	R	R	R
VC-7	ND	ND	R	R	R	R	R	R	R	R
VC-8	R	L	H	H	L	L	L	L	L	R
VC-9	R	ND	L	L	L	L	L	L	L	R
VC-10	ND	R	R	R	R	R	R	R	R	R
VC-11	R	L	L	L	R	L	L	L	L	L
VC-12	L	L	R	L	R	L	L	L	L	R
VC-13	L	L	L	H	L	L	L	L	L	L
VC-14	ND	L	L	L	L	L	L	L	L	L
VC-15	ND	ND	R	R	R	R	R	R	R	R
VC-16	ND	ND	L	L	R	R	R	R	R	R
VC-17	ND	ND	L	L	R	R	R	R	R	R
VC-18	ND	ND	R	R	R	R	R	R	R	R
VC-19	ND	ND	R	R	R	R	R	R	R	R
VC-20	ND	ND	R	L	L	L	L	L	L	ND
VC-21	ND	ND	L	L	L	L	L	L	ND	L
VC-22	ND	ND	R	R	R	R	L	L	L	L
VC-23	ND	R	R	R	R	R	R	R	R	ND
VC-24	ND	R	R	L	R	R	R	R	R	ND
VC-25	ND	R	R	R	R	R	R	R	R	ND
VC-26	ND	R	R	R	R	R	R	R	R	ND
VC-27	ND	R	R	R	R	R	R	R	R	ND
VC-28	ND	ND	R	R	R	R	R	R	R	R
VC-29	ND	L	L	L	L	L	L	L	L	L
VC-30	ND	L	L	L	L	L	L	L	L	L
VC-31	ND	R	R	R	ND	R	R	R	R	R
VC-32	ND	L	L	L	L	L	L	L	L	L
VC-33	ND	R	R	R	ND	R	R	R	R	R
VC-34	ND	ND	ND	L	ND	ND	L	L	L	ND
VC-35	ND	ND	ND	L	ND	ND	ND	R	R	ND
VC-36	ND	ND	ND	R	ND	ND	ND	R	R	ND
VC-37	ND	ND	ND	R	ND	ND	L	L	L	ND
VC-38	ND	ND	ND	L	ND	ND	L	L	L	ND
VC-39	ND	ND	ND	L	ND	ND	L	L	L	ND
VC-40	ND	ND	ND	R	ND	ND	R	L	L	ND
VC-41	ND	ND	ND	L	ND	ND	R	R	R	ND
VC-42	ND	ND	ND	L	ND	ND	R	R	R	ND
VC-43	ND	ND	ND	L	ND	ND	L	L	L	ND
VC-44	ND	ND	ND	R	ND	ND	R	L	L	ND
VC-45	ND	ND	ND	R	ND	ND	ND	R	R	ND
VC-46	ND	ND	ND	R	ND	ND	L	L	L	ND
VC-47	ND	ND	ND	R	ND	ND	R	L	L	ND
VC-48	ND	ND	ND	L	ND	ND	L	L	L	ND

D

Fig. 1. C–D, Continued.

as described above. Homozygous deletion was scored as a reduction of >50% of the target (p16) fragment relative to the internal control (D10MIT3). The p16INK4a primers are as follows: exon 1 (forward), 5′-TCCGCGAGGAAAGCGAACTCGA-3′; exon 1 (reverse), 5′-GAATCGGGGTACGACCGAAAG-3′; exon 2 F, 5′-GATGATGGGCAACGTTCAC-3′; and exon 2 R, 5′-AGGCGACCCCAGGCATCGC-3′.

Results

We examined the effect of carcinogen induction on the selection of LOH during mouse lung adenocarcinogenesis. Chromosome 4 is most frequently affected by LOH in lung adenocarcinomas of AC3 and C3A F_1 mouse hybrids (5–7). Detailed LOH analysis of this chromosome showed that there are at least two targeted tumor suppressor loci spanning ~30 cM (5, 6). Whereas 30 of 39 spontaneous tumors (77%) displayed losses, only 18 of 50 (36%) NNK-induced tumors ($P < 0.001$) and 30 of 48 (62%) VC-induced tumors had losses on this chromosome. Most of the tumors with LOH on chromosome 4 incurred large deletions encompassing both of the mapped tumor suppressor loci, which are localized near markers D4MIT77 and D4MIT54, respectively (Fig. 1). In all groups, there was a strong bias for retention of the defective *Ink4a* allele near D4MIT77 donated by the C3H parent, as described previously (27).

Chromosomes 12 and 14 were also examined for differences in LOH frequency between the treatment groups. Both chromosomes were shown to undergo LOH in mouse lung adenocarcinomas at a frequency of 28% (9). Further analysis of chromosome 12 showed LOH in only 2 of 24 (8.3%) spontaneous tumors compared with 7 of 20 (35%) NNK-induced tumors ($P < 0.01$) and 10 of 25 (40%) VC-induced tumors ($P < 0.01$). In contrast, a similar frequency of LOH was observed on chromosome 14 for all treatment groups, in which 4 of 16 (25%) spontaneous, 7 of 22 (32%) NNK-induced, and 4 of 10 (40%) VC-induced tumors displayed losses (Table 2).

Some tumors from each treatment group (16 spontaneous, 10 NNK-induced, and 10 VC-induced tumors) were selected on the basis of DNA availability and subsequently analyzed for genome-wide LOH using polymorphic genetic markers on each of the 19 autosomes (Table 1). The number of chromosomes harboring LOH per tumor was determined to be significantly greater in tumors induced by either NNK or VC compared with the spontaneous tumors (Fig. 2). In the spontaneous group, 1.7 chromosomes incurred LOH compared with 3.2 ($P < 0.05$) and 2.7 ($P < 0.05$) in the NNK- and VC-induced groups, respectively (Fig. 3).

LOH on chromosomes 4, 12, and 14 was examined for association with differences in global LOH frequency (Table 3). There was a significant association between allelic loss on chromosome 12 and increased genome-wide LOH frequency in the carcinogen induced-tumors. Tumors with losses on this chromosome displayed a mean of 4.13 chromosomes with LOH compared with a mean of 2.16 in tumors without chromosome 12 LOH ($P < 0.05$). Allelic losses on chromosome 14 also were significantly associated with a higher global LOH frequency. Tumors with losses on this chromosome had a mean of 3.5 chromosomes with LOH compared with 1.83 in tumors without chromosome 14 LOH. As shown in Table 2, losses on chromosome 14 were independent of carcinogenic induction. By comparison, LOH on chromosome 4 was not associated with a trend in global LOH frequency (Table 3).

Table 2 *Comparison of LOH frequencies between treatment groups on commonly targeted chromosomes*

Tumor	Chromosome 4	Chromosome 12	Chromosome 14
SP[a]	30/39 (77%)	2/24 (8.3%)	4/16 (25%)
NNK	18/50 (36%) $P < 0.001$[b]	8/20 (40%) $P < 0.01$	7/22 (32%)
VC	30/48 (62%)	10/25 (40%) $P < 0.01$	4/10 (40%)

[a] SP, spontaneous tumors.
[b] χ^2 compares the frequencies in carcinogen-induced tumors (NNK or VC) with the spontaneous tumors.

	CHROMOSOME																		
TUMOR	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	TOTAL
SP-16														■						1
SP-17				■																1
SP-18																				0
SP-19				■																1
SP-20				■																1
SP-21																			■	1
SP-22	■	■																■	■	4
SP-23				■											■		■			3
SP-24													■							1
SP-25												■								1
SP-26				■																1
SP-27												■	■							4
SP-28				■										■						2
SP-29	■		■	■										■						2
SP-30																				1
SP-31		■		■									■							3
NNK-28			■																	1
NNK-29				■				ND	ND			■								2
NNK-30												■		■						2
NNK-31		■		■																2
NNK-32				■																1
NNK-33			■	■			■	■	■		■	■		■						8
NNK-34	■															■	■			3
NNK-35			■		■		■	■	■					■				■		7
NNK-36				■							■	■		■					■	5
NNK-37																				1
VC-23												■		■						2
VC-24				■																1
VC-25										■		■								2
VC-26	■						■			■		■					■		■	6
VC-27			■							■		■	■	■	■					6
VC-28				■						■										2
VC-29				■										■						2
VC-30															■					1
VC-31				■										■						2
VC-32	■								■									■		3

Fig. 2. Global LOH in mouse lung carcinomas. *Black boxes*, LOH of at least one chromosomal marker; *white boxes*, retained heterozygosity at all markers tested.

Discussion

The present study demonstrates that the genotoxic lung carcinogens NNK and VC cause a significant decrease in genomic integrity and directly alter the selection of frequent chromosomal alterations during lung tumorigenesis in mice. In the absence of carcinogenic induction, lung tumors evolve via a consistent pattern of genetic changes with strong selection for deletions on chromosome 4, but with a minimal number of losses on other chromosomes. LOH on chromosome 4 has occurred frequently during malignant conversion of mouse lung tumors (6). The targets of these deletions were mapped to two independent loci: (*a*) *Ink4a/Arf* near marker D4MIT77; and (*b*) an unknown gene ~30 cM distal to *Ink4a/Arf*. AC3 and C3A F_1 mice are heterozygous at the *Ink4a/Arf* locus, and LOH in lung tumors is biased for the preferential deletion of a more potent allelic variant of *Ink4a* with retention of a functionally diminished allele (27). The presence of at least two important tumor suppressor loci together with functional variation of p16 on chromosome 4 therefore renders this chromosome prone to allele loss during lung tumorigenesis. Our results suggest that this indeed is manifested in spontaneous lung tumors in which large deletions occurred at a very high frequency (77%). However, aside from frequent large deletions on chromosome 4, spontaneous lung tumors retained genome-wide chromosomal integrity, incurring significantly fewer global losses compared with tumors induced by either NNK or VC.

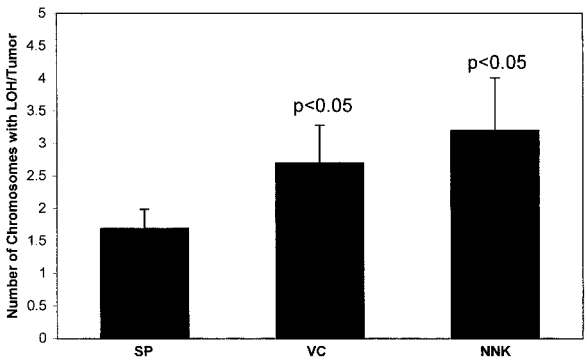

Fig. 3. Mean number of chromosomes affected by LOH in lung tumors. *SP*, spontaneous tumors ($n = 16$); *VC*, VC-induced tumors ($n = 10$); *NNK*, NNK-induced tumors ($n = 10$). One-tailed student's t-test comparing spontaneous and carcinogen-induced tumors.

Table 3 *Association between chromosome-specific and genome-wide LOH*[a]

	+ LOH		− LOH	
Chromosome	Mean[b] (*n*)	SD	Mean (*n*)	SD
12[c]	4.13[d] (8)	2.42	2.16 (12)	1.70
4	2.33 (18)	1.81	2.44 (18)	2.04
14	3.50[d] (12)	2.43	1.83 (24)	1.31

[a] Student's *t* test (two-tailed) comparing frequencies of global LOH in tumors with and without LOH on specified chromosomes.
[b] Mean number of chromosomes with LOH per tumor.
[c] Analysis of chromosome 12 used carcinogen-induced tumors only. Analyses of chromosomes 14 and 4 used tumors from all treatment groups. Data are taken from Fig. 2.
[d] $P < 0.05$.

LOH on chromosome 12 and LOH on chromosome 14 also are frequent events in mouse tumorigenesis and occurred significantly more often in tumors with higher global LOH frequencies. Unlike LOH on chromosome 12, the losses on chromosome 14 were independent of carcinogen exposure. Chromosome 14 harbors the retinoblastoma gene (*Rb*), which was recently shown to be haploinsufficient for maintaining chromosomal stability in mouse embryonic stem cells (29). LOH affecting this locus may then be a contributing factor leading to increased chromosomal stability during mouse lung tumorigenesis.

Carcinogenic induction significantly increased the frequency of global LOH and altered the frequency of LOH on chromosomes 12 and 4 in lung tumors. Whereas NNK induction produced lung adenocarcinomas with a reduced frequency of LOH on chromosome 4 ($P < 0.001$), tumors induced by NNK or VC had a higher frequency of LOH on chromosome 12 ($P < 0.01$) when compared with spontaneous adenocarcinomas. Of note, no mutations of *Ink4a/Arf* were detected upon examination of 22 NNK-induced tumors with retained

heterozygosity on chromosome 4, suggesting that inactivation of this principle target gene did not occur by this alternative mechanism (data not shown). These findings suggest that NNK induction of lung tumors significantly reduced the selective pressure for deletional inactivation of tumor suppressor genes on chromosome 4 during tumor progression. This may be attributable to the observed increase in carcinogen-induced global mutation frequency, from which evolutionary changes for continued tumor growth and survival would be drawn. In this regard, LOH on chromosome 12 might represent a selected change emerging from cells harboring an increase in the number of carcinogen-induced mutational events subjected to evolutionary selection. The specificity of these events to carcinogen-induced tumors suggests further that the inactivation of a gene on chromosome 12 may confer a selective adaptation to carcinogen exposure or carcinogen-induced DNA damage during carcinogenesis.

The increase in global LOH frequency induced by NNK and VC in lung tumorigenesis is consistent with current evidence suggesting that carcinogens may force genetic instability in cells as an evolutionary adaptation during carcinogenesis (13, 19–21). Carcinogens that form bulky DNA adducts are thought to force chromosomal instability presumably as an adaptive response to their propensity for causing DNA damage that leads to erroneous repair-recombination events (13, 19). The reactive intermediate of NNK (oxobutyl diazohydroxide) that is purported to account for its carcinogenicity affects DNA in this way (23). Both NNK and VC are genotoxic carcinogens that cause DNA strand breaks as a consequence of DNA-adduct formation, which is also likely to compromise chromosomal integrity (22, 28). These carcinogens similarly affected chromosomal stability and LOH on chromosome 12, but they induced very different frequencies of LOH on chromosome 4 in the lung tumors examined. Differences in genotoxity or in the treatment protocols used in this study for NNK and VC (see "Materials and Methods") are likely to have contributed to the carcinogen-specific differences in LOH patterns reported here. Further study on the mechanisms underlying these differences is warranted.

Our results implicate a gene on mouse chromosome 12 as playing a significant role in protecting against carcinogen-induced chromosomal instability and lung carcinogenesis. Although the identity of this gene is presently unknown, its location is homologous with human chromosome 14q, which is a frequent target of LOH in several cancer types including non-small cell lung cancer (30, 31). Recent studies have implicated genes that are important in the repair of bulky carcinogen-DNA adducts and in mitotic checkpoint control, which maintains cellular control of chromosome stability, as potential targets for inactivation during carcinogenesis (19, 32–35). Such a gene may be targeted for inactivation on mouse chromosome 12 during lung carcinogenesis, the identification of which requires further study.

p53 also is important in protecting genetic integrity, and evidence has shown that its inactivation in cancer can give rise to genomic instability (35). We analyzed 17 NNK-induced lung tumors for abnormal accumulation of p53 by immunohistochemical staining as described previously (36), but we found that only 3 of these tumors showed p53 abnormalities (data not shown). One of these (NNK-33) clearly displayed evidence of chromosomal instability, and another (NNK-36), which also had chromosomal instability, displayed LOH at the p53 locus. These results suggest that p53 inactivation may have played a minor role in the elevation of carcinogen-induced global LOH observed in this study.

The current study shows that the likely human lung carcinogen NNK directs the selection of chromosomal changes underlying lung tumorigenesis in mice. This is the first demonstration *in vivo* that carcinogens not only accelerate tumorigenesis but they also directly influence the evolutionary process of tumorigenesis through their deleterious effects on the genome. A similar effect on the evolution of lung tumors in humans would be expected, particularly in smokers, who are chronically exposed to NNK and other potent carcinogens over the course of several years. Further study on the genomic consequences of DNA-carcinogen interactions is needed for the development of more informative biomarkers of carcinogen-induced cancer risk and ultimately for more effective cancer treatment and preventive measures.

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