

# Differential Expression of the Normal and Mutated *K-ras* Alleles in Chemically Induced Thymic Lymphomas

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## ABSTRACT

The presence of point mutations in the *K-ras* gene was examined in murine thymic lymphomas induced by a single dose of *N*-methylnitrosourea by the RNase A mismatch cleavage method and by allelic-specific oligonucleotide hybridization of *in vitro* amplified DNA by polymerase chain reaction. The results show that the frequency of mutations is lower than that of tumors induced by multiple *N*-methylnitrosourea treatments. Four mutations identified were the aspartic acid at codon 12, a G:C to A:T transition in its second position. A G:C to T:A transversion in codon 146 was also found in one thymic lymphoma, changing the amino acid alanine to serine. The use of the RNase A assay allowed an estimation of the relative expression levels of both normal and mutant *K-ras* alleles. The results show that in approximately one half of the tumors the mutant allele is predominantly expressed, suggesting that the normal allele has been lost or that the mutant allele has been amplified relative to the normal. Altogether, these findings are consistent with *ras* mutations occurring in some instances during tumor development and with a *ras* effect being not strictly dominant but favoring selection for increasing levels of expression from the oncogenic allele.

## INTRODUCTION

Many human cancers are thought to result from accumulation of genetic alterations either spontaneously or due to exposure to radiation or chemical carcinogens occurring several years prior to onset of tumor growth. The genetic alterations that are frequently associated with neoplasia alter dominant oncogenes and/or suppressor genes and contribute to the manifestations of the malignant phenotype (1). The oncogenes most often associated with human neoplasia are the members of the *ras* family (2). These highly conserved genes (*K-ras*, *H-ras*, and *N-ras*) code for similar membrane-bound proteins of *M<sub>r</sub>* 21,000. *ras* proteins bind GTP, possess intrinsic GTPase activity, and are thought to be involved in the process of signal transduction across the cell membrane (3). Different protocols involving radiation or chemical carcinogens are known to induce tumorigenesis in animals. Skin papillomas and carcinomas induced in mice (4), as well as keratoacanthomas induced in rabbits (5) by treatment with dimethylbenzanthracene, mammary carcinomas induced in rats with NMU<sup>3</sup> (6), and liver carcinomas induced in mice by the chemicals furan and furfural (7), all show a high incidence of *ras* oncogene activation.

A murine model of thymic lymphoma is the system used here to study the contribution of *ras* gene point mutations to tumorigenesis. Previously, we reported activation of *ras* oncogenes in thymic lymphomas induced in mice by  $\gamma$ -irradiation or by 5 weekly i.p. injections of NMU (8) as well as neutron irradiation

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<sup>3</sup> The abbreviations used are: NMU, *N*-methylnitrosourea; PCR, polymerase chain reaction.

(9). Only two members of the *ras* gene family, the *N-ras* and *K-ras* genes, have been found activated so far in this mouse system.

Methods for induction of tumors in experimental animals fall into two categories: fractionated exposure to a carcinogen and a single dose. Our aim in this study was to compare these two types of protocols. Therefore, we induced thymic lymphomas in mice after one single injection of NMU in an attempt to understand whether the frequency and spectrum of *ras* mutations correlate with the carcinogen dose by comparing these results with our previous ones using multiple doses (8). The methods we used for determining the presence of mutations in the *K-ras* gene as well as their nature and position were gene amplification by PCR and the RNase A protection assay. The particular advantage of the RNase A protection assay is that it permits the separate detection of the message for each allele. This method allows for an estimation of the relative expression of both normal and mutant *ras* alleles in the same tumor cells (10).

## MATERIALS AND METHODS

### Induction of Tumors

C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and were 6–10 weeks old at the time of carcinogen administration. Thymic lymphomas were induced in mice by one single i.p. injection of 80 mg/kg body weight of the chemical carcinogen NMU (11). Tumors were immediately frozen in liquid N<sub>2</sub> after removal from the animal. Histological analysis of these tumors has previously shown that they are predominantly composed of neoplastic cells, with little contamination by normal stromal tissue (12).

### Nucleic Acid Extraction, Electrophoresis, and Hybridization

Frozen tumors were divided into two parts. DNA and RNA were extracted according to the methods of Maniatis *et al.* (13) and Chomczynski and Sacchi (14), respectively. For Northern blot analysis, total RNA was electrophoresed on 1.2% agarose-formaldehyde. The gels were blotted onto nitrocellulose filters (13) and hybridized to random primer <sup>32</sup>P-labeled probes (15). The probes used were inserts purified from the following plasmids: BS9 (Viral *H-ras*) (16), *K-ras* (17), *N-ras* (18), and  $\alpha$ -tubulin (from the laboratory of Frank Solomon, Massachusetts Institute of Technology, Cambridge, MA).

### Detection of Mutant *K-ras* Genes

In the DNA. Sequences spanning first, second, and third exons of the *K-ras* gene were amplified *in vitro* using specific primers (9). One  $\mu$ g of genomic DNA and 0.2  $\mu$ M of each of the two primers were added to a reaction mixture containing 1  $\mu$ M (each) deoxynucleotide phosphate and 1 unit of *Thermus aquaticus* (Taq) polymerase (19). Thirty cycles of denaturation (94°C, 30 s), annealing (60°C, 1 min), and extension (72°C, 1 min) were done with an automated DNA thermal cycler (Perkin-Elmer/Cetus). Direct sequencing of the PCR products was done as described previously (20). Amplified DNA was also analyzed by oligonucleotide mismatch hybridization using end-labeled allelic-specific oligonucleotide probes containing all possible activating changes in codons 12, 13, 61, and 146. Prehybridization, hybridization, and washing of filters with 3 M tetramethylammonium chloride were done as described before (21).

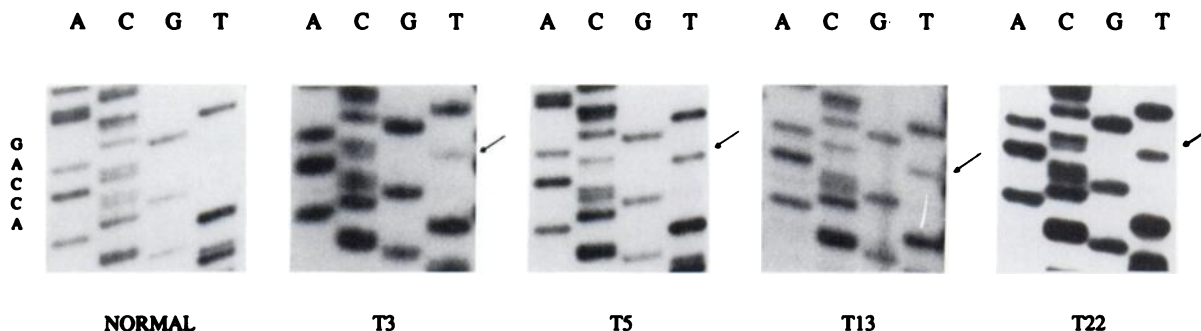


Fig. 1. Direct sequencing of amplified K-*ras* first exon from NMU-induced thymic lymphomas. The region around codon 12 from normal thymus as well as tumors T3, T5, T13, and T22 is shown. The antisense primer used for the amplification *in vitro* was the sequencing primer. Arrows, bands corresponding to mutated base pairs.

In the RNA. The expression levels and the presence of point mutations within the first coding exon of the K-*ras* gene were analyzed by the RNase A mismatch cleavage method (10). Uniformly labeled RNA probes corresponding to the first coding exon of the K-*ras* gene were synthesized with the SP6 polymerase *in vitro* transcription system (22) using pAK1MSer (23), except that 100  $\mu$ M unlabeled CTP was used. The conditions for hybridization, RNase A digestion, and analysis by denaturing polyacrylamide gels and autoradiography were reported previously (12). The use of this probe generates a G:U mismatch at codon 12 when hybridized with normal transcripts, but this mismatch is not recognized by the enzyme. Transcripts with mutations at the second position of codon 12, on the other hand, generate RNA hybrids with double mismatches which are very efficiently cleaved by RNase A (23).

## RESULTS

**K-*ras* Mutations in Thymic Lymphomas Induced by a Single Dose of NMU.** One single dose of NMU was injected into C57BL/6 mice. A total of 46% of the animals (28 of 61) developed thymic lymphomas with a latency period of 3–6 months (mean latency, 4.4 months).

Previously, we showed that 80% of the tumors induced in C57BL/6 mice treated with 5 injections of NMU contained activated K-*ras* genes (12). Sixty % of the K-*ras* mutations occurred in codon 12, resulting in a G:C to A:T transition in the second position, changing the amino acid glycine to aspartic acid. To determine whether the tumors induced in C57BL/6 mice treated with a single injection of NMU also contained activated K-*ras* genes, DNA from the tumors was amplified *in vitro* by PCR for the first exon of the K-*ras* gene. The amplified DNA was analyzed by direct sequencing of the PCR products. Four of 28 tumors (T3, T5, T13, and T22) contained mutations in the second position of codon 12, a G:C to A:T transition (Fig. 1). No other mutations in codon 12 or codon 13 were observed. Two uncommon mutations have been previously identified in the second and third exons of the K-*ras* gene in neutron- and NMU-induced thymic lymphomas<sup>4</sup> (9). The mutations involve codons 61 and 146. To determine whether the K-*ras* gene from the thymic lymphomas contained mutations other than in the first exon, we next amplified the second and third exons of the gene and analyzed the PCR products with different oligonucleotides containing all the possible activating mutations in codons 61 and 146. A mutation in codon 146 was identified in one thymic lymphoma, T14 (Fig. 2). It consists of a G:C to T:A transversion in the first position, changing the amino acid alanine to serine. This mutation in codon 146 has not been described previously. No other mutations were found

(data not shown). Altogether, our results show that 5 of 28 (17%) thymic lymphomas induced with a single dose of carcinogen contained a mutated K-*ras* gene. This result is a significant decrease when compared to our previous observation in the same strain of mouse in which 8 of 10 tumors induced by multiple doses of NMU contained mutated K-*ras* genes (12).

**Expression of the K-*ras* Gene in NMU-induced Thymic Lymphomas.** To determine whether the level of expression of the K-*ras* gene differed in thymocytes obtained from age-matched control animals or from animals with tumors, Northern blot analysis was performed. This analysis indicated that expression of the K-*ras* gene was not increased in the tumors with respect to normal thymus (Fig. 3). In addition, the expression of the K-*ras* gene was not increased in tumors containing an activated *ras* gene (lanes 3 and 5) when compared with tumors in which activated *ras* genes were not detected (lanes 1, 2, 4, and 6–9). Hybridization to the  $\alpha$ -tubulin probe was performed to monitor the amount of RNA loaded in the gel (Fig. 3). Densitometric analysis indicates that the ratios between K-*ras* and  $\alpha$ -tubulin signals were very similar for all the samples. Similar results were obtained using the H- and N-*ras* genes (data not shown).

The relative expression of the normal *versus* mutated K-*ras* allele was examined in the tumors containing activated K-*ras* genes using the RNase A mismatch assay. The tumors not harboring activated K-*ras* alleles were also included in this analysis in an attempt to verify the expression data using an alternative method. The results are shown in Fig. 4. Single nucleotide substitutions within the first coding exon of the K-*ras* gene were detected by RNase A cleavage at mismatches in RNA:RNA hybrids and polyacrylamide gel electrophoresis of the RNase A-resistant products. The *in vitro* synthesized radioactively labeled antisense RNA molecules corresponding to the human K-*ras* gene were hybridized to total cellular RNA prepared from normal thymus (lane N) and to RNA from 22 of 28 thymic lymphomas (lanes 1–22). The mismatch-specific bands indicated by arrows are diagnostic of the presence of a mutation in codon 12 (lanes 3, 5, 13, and 22). These bands are detected neither in the RNA used as a negative control (lane N) nor in the RNA from any other tumor sample (other lanes). Other bands present in the gels originated by cleavage of some of the mismatches present in RNA hybrids between the mouse K-*ras* transcripts and the human antisense riboprobe. In total, 4 of the 22 tumors contained a mutation in codon 12 of K-*ras*, confirming the results obtained by oligonucleotide mismatch hybridization.

Because of the efficient cleavage by the enzyme of the double mismatch present in the RNA hybrids, the RNase protection assay allows an estimation of the relative expression of both

<sup>4</sup> W. Bayona, O. Brathwaite, and E. W. Newcomb, manuscript in preparation.

A)

B)

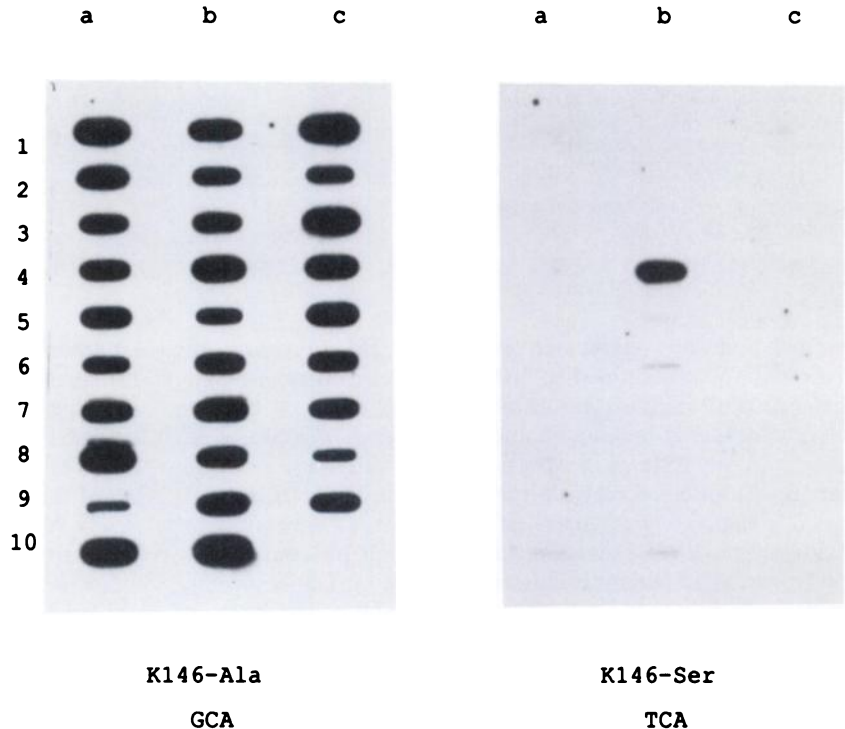


Fig. 2. Analysis of codon 146 *ras* mutation. Slot blot of K-*ras* exon 3-amplified DNA from thymic lymphomas and hybridized to a  $^{32}\text{P}$ -labeled specific probe for the normal GCA codon (A) and for the G:C to T:A mutation in codon 146 (B). Slot c9 corresponds to normal thymus used as a negative control. The only positive sample is the DNA from tumor T14 (slot b4).

normal and mutant alleles. Thus, thymic lymphomas T5 and T13 contained predominantly mutant codon 12 K-*ras* transcripts (compare the relative intensity of the bands in Fig. 4, lanes N, 5, and 13). In the case of tumors T3 and T22, the mutant transcripts are as abundant as the normal (Fig. 4, lanes N, 3, and 22).

## DISCUSSION

The first aim of this study was to compare the frequency and spectrum of *ras* mutations in C57BL/6 mice given a single dose of NMU versus those given multiple doses. In these studies we observed a lower percentage of *ras* activation with a single dose of carcinogen (17%, 5 of 28 tumors) compared with a much higher incidence of *ras* activation with multiple doses (80%, 8 of 10 tumors) (12). This result shows that K-*ras* is involved in only one of several pathways for thymic lymphoma development and that with only one carcinogenic treatment is activated at low frequency. On the other hand, if multiple doses of NMU are administered, the frequency of hits in many more genes increases, and since K-*ras* is able to contribute to lymphomagenesis, its activation seems to be selected during tumor development, therefore appearing in a larger fraction of tumors.

The spectrum of mutations with the single-dose protocol is similar to that observed with multiple doses of carcinogen featuring the G:C to A:T transition in the second base of codon 12 as the most prevalent. However, in these experiments we detected a G:C to T:A transversion in codon 146 which has not been identified before. A G:C to A:T transition in 146 codon has been previously reported by our group in one neutron-induced tumor (9) and has been detected in 2 of 14 NMU-induced tumors.<sup>4</sup> The presence of the G-T transversion at codon 146 is not consonant with the mutation being the direct result of the NMU effect and, therefore, suggests that it occurred as an indirect result of the carcinogenic treatment, later during tumor progression.

This study also addresses the question of the contribution of the relative expression of normal and activated *ras* alleles in tumorigenesis. It has been reported that *ras* expression as detected by immunohistochemistry is increased in a variety of

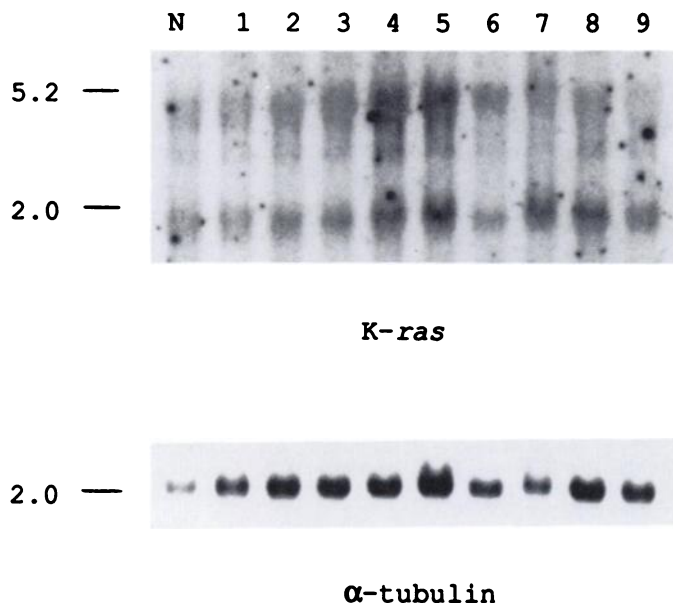
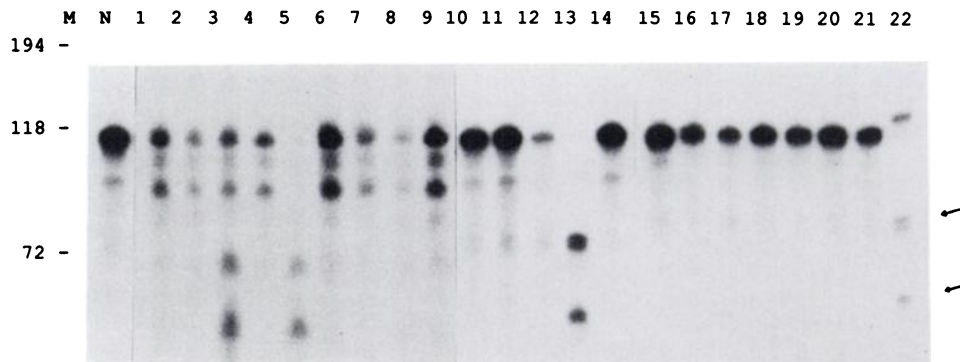


Fig. 3. Northern blot analysis of RNA from NMU-induced thymic lymphomas. Total RNA (15  $\mu\text{g}$ ) was run on an agarose gel, transferred to a nitrocellulose filter, and hybridized with the  $^{32}\text{P}$ -labeled probe corresponding to the K-*ras* gene or the  $\alpha$ -tubulin gene. Lane N, normal thymus; lanes 1-9, thymic lymphomas T1-T9, respectively. Ordinate, kilobases.

Fig. 4. RNA:RNA mismatch analysis of the first exon of the K-ras gene from NMU-induced thymic lymphomas. RNA extracted from the tumors was hybridized with <sup>32</sup>P-labeled antisense-RNA directed by the specific plasmid, digested for 30 min with RNase A, and electrophoresed through a denaturing acrylamide gel. Lane M, molecular weight marker (in thousands); lane N, normal thymus used as a negative control; lanes 1-22, thymic lymphomas T1-T22, respectively; arrows, positions of the codon 12-specific mismatch bands.



pre-malignant and malignant tumors (24, 25). Previously *ras* expression was examined by immunohistochemistry in NMU-induced tumors. No clear correlation was observed between elevated levels of *ras* protein and the presence of activated *ras* genes or with stage of the disease (26). In this study no correlation was observed between the expression levels of the activated allele and the latency of the tumor as a measure of aggressiveness. Analysis of steady state levels of K-ras mRNA on Northern blot analysis indicates that the *ras* family member most frequently found activated in this model system does not display any significant change among the tumors whether or not they contained mutated K-ras genes or compared to a thymus from an age-matched control. Therefore, it appears that in NMU-induced lymphomagenesis in mice the main alteration in *ras* activation is qualitative rather than quantitative.

In order to analyze in greater detail the issue of dominance for the activated *ras* gene, it was necessary to determine the expression of the two alleles independently. Differential expression of the K-ras alleles was determined by the RNase A protection assay. The analysis of RNase A mismatch digestion revealed that, while two tumors containing mismatches in the first exon of K-ras expressed similar levels of mutant versus normal K-ras transcripts, two other tumors expressed significantly higher levels of the mutated K-ras allele with respect to the normal.

Because these lymphomas are essentially homogeneous populations of neoplastic cells (12), it appears that some tumors can be functionally heterozygous at the expression level for the activated K-ras allele, in support of the dominant role of the mutant p21 *ras* (3). The activating mutation in the positive tumor samples was evaluated by densitometry comparing the signals obtained for each allele in an oligonucleotide mismatch hybridization (data not shown). The results of this quantitation indicated that, in those tumors in which the expression of the activated allele was predominant in the RNA, an increase in copy number was seen at the genomic level. From the intensity of the signals it can be inferred that the imbalance is mostly caused by an increase in the number of copies for the activated allele and a decrease for those of the normal as previously described in this system (18, 27) and in skin tumorigenesis (28) as well as in some human tumor cell lines (10, 29). These observations favor the hypothesis of a reduplication of the chromosome containing the activated allele, and subsequent increasing loss of the chromosome harboring the normal K-ras gene, as a mechanism to account for the unbalanced allelic expression. These findings are consistent with the notion that as a dominant oncogene, *ras* acts through an altered product whose presence, even in the presence of the normal counterpart, is able to subvert the cellular machinery. Nevertheless, these

experiments also show that during tumor development there is a frequent selection for increased copies of the activated allele and an added pressure to express additional amounts of the activated allele.

The molecular explanation is an imbalance in the copy number of the different alleles with a concomitant increase in the steady state levels of the message for the activated product. These results support the notion that *ras* genes contribute to tumorigenesis in a dominant but dose-dependent fashion in which a high ratio of activated to normal message is favored in tumor development.

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