Environmental Health Perspectives
Vol. 93, 125–131, 1991

Frequency and Spectrum of Mutations at Codons 12 and 13 of the C-K-*ras* Gene in Human Tumors

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The frequency of point mutations at codons 12 and 13 of the c-K-*ras* gene has been determined in a panel of more than 400 human tumors. Mutant c-K-*ras* genes were detected in about 75% of adenocarcinomas of the pancreas (n = 84); 40% of adenomas (n = 72) and carcinomas (n = 244) of the colon and rectum; 30% of carcinomas of the bile duct (n = 19); 25% of carcinomas of the lung (n = 92), and in lower frequency in other carcinomas, including liver, stomach, and kidney. No mutations were found in carcinomas of the breast, prostate, esophagus, and gall bladder, among others. Comparative analysis of the spectrum of mutations show that while G to A transitions were the most frequent mutations in pancreatic and colo-rectal tumors, G to T transversions were more prevalent in lung carcinomas. The aspartic acid mutation at codon 13 (GGC → GAC) was relatively frequent in colo-rectal tumors but rare in pancreatic and lung carcinomas. The differences in the mutation spectrum of the c-K-*ras* gene in cancers of the gastrointestinal and respiratory tracts are suggestive of differential exposure to genotoxic agents.

Introduction

The theory that neoplastic transformation is the result of combinations of genetic alterations that occur in a single cell during the life of an individual and that accumulate in a sequential manner is becoming generally accepted as the experimental evidence is increasing rapidly (*1*). According to this theory, the balanced interplay between proto-oncogenes and tumor-suppressor genes that control cell growth and differentiation is disrupted by accumulative genetic damage and, despite the fail-safe mechanisms provided by the evolution by natural selection, neoplastic transformation occurs. Oncogenes are the activated forms of cellular proto-oncogenes, whose products are involved in cell growth or differentiation (*2,3*). Tumor-suppressor genes are those playing a negative regulatory role in these processes (*4,5*). The recent development of methods for the detection and characterization of genetic alterations responsible for oncogene activation and tumor-suppressor gene inactivation is providing new insights in the understanding of the molecular genetics of malignancy and is finding applications for cancer diagnosis at the molecular genetic level (*6,7*). Single base substitutions are the most frequent of these genetic alterations, both activating the malignant potential of proto-oncogenes and disrupting the inhibitory function of tumor-suppressor genes (*2–5,8*).

The oncogenes most often associated with human neoplasia are three members of the *ras* gene superfamily: c-H-*ras*, c-K-*ras*, and N-*ras*. The *ras* gene products, p21*ras*, are membrane-bound proteins which possess guanine nucleotide binding and hydrolytic activities and appear to be involved in the signal transduction pathway of cell growth and differentiation of mammalian cells (*9*). The discovery of a cellular protein, termed GAP (GTPase activating protein), which stimulates the intrinsic GTPase activity of *ras* proteins (*10*), is substantially contributing to our understanding of the mechanisms of *ras* function and activation. GAP interacts with *ras*, stimulating its GTPase activity and inhibiting *ras* function (*11*). Phosphorylation by tyrosine protein kinases, such as membrane receptors (i.e., epidermal growth factor and platelet-derived growth factor [PDGF]) and other oncogene proteins like p60 *src* (*12,13*) may in turn regulate GAP. Whether or not GAP is also the effector for *ras* or only acts upstream in the *ras* pathway is not clear. In any case, these studies may provide a link in the signal transduction pathway between *ras* proteins and extracellular signals via their receptors and GAP. How the signal is finally transmitted to the nucleus, leading to DNA replication, remains to be determined. However, it is clear that other key

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components in the control of cell replication and therefore in the potential for malignant transformation are nuclear transcriptional factors with oncogenic activities (i.e., *myc, fos, jun*) and also the products of the recently characterized tumor-suppressor genes, the retinoblastoma susceptibility (*Rb*) and the p53 genes. The importance of the last class of genes is clearly demonstrated by the fact that DNA tumor viruses (SV40, adeno, and papilloma) exert their oncogenic effects via interaction (and presumably inactivation) of these two tumor-suppressor gene products with their corresponding oncogenic proteins (T antigen, E1A and E1B, and E6 and E7 gene products) (*4*).

The oncogenic potential of *ras* genes is activated by single base substitutions, usually in codons 12, 13, or 61 (*14*). The mechanism by which single amino acid substitutions in critical domains result in dramatic increases in the oncogenic potential of *ras* proteins involve diverse types of disruption of protein structure, all leading to an increase in their active, GTP-bound state. In animal model systems, tumors induced by a variety of carcinogens reproducibly contain single point mutations in the *ras* oncogenes (*9,15*). These results provide persuasive evidence to the notion that the carcinogen mutates its target, the *ras* proto-oncogene, activating its oncogenic potential and initiating the tumorigenesis process. In contrast, examples of comparable high incidence of *ras* mutations associated with a specific type of human cancer had not been reported. Consequently, the etiology of these mutations and their role in human carcinogenesis have remained uncertain. We will summarize here the results of our analysis on the frequency of point mutations at codons 12 and 13 of the c-K-*ras* gene in human tumors and the comparative analysis of the spectrum of mutations at these positions in pancreatic and lung carcinomas and in colo-rectal adenomas and carcinomas.

Methods

The recent development of the polymerase chain reaction (PCR), a simple technique for the *in vitro* amplification of DNA sequences (*16,17*), has facilitated the detection of *ras* mutations and other genetic alterations and the analysis of their role in tumorigenesis (*7*). Single base substitutions can be readily detected by a number of methods, including allelic-specific oligonucleotide (ASO) hybridization (*18,19*), RNAse A mismatch cleavage of the DNA-RNA hybrids (*20,21*), or DNA sequencing, either directly (*22,23*) or after cloning the PCR products in plasmids (*24,25*). The generation or destruction of restriction endonuclease sites (RFLP) by point mutations also allows their rapid detection after the genomic sequences have been amplified by the PCR (*26,27*).

The great sensitivity of the PCR allows the amplification of DNA sequences from very small amounts of tissue such as tumor biopsies. In addition, the technique can use formalin-fixed, paraffin-embedded tissue specimens (*28*) obtained after surgical resections and also from autopsies (*29*). This facilitates the analysis of some types of tumors for which surgical specimens are difficult to obtain due to their low incidence or to their poor prognosis. For instance, fresh specimens of pancreatic adenocarcinoma are difficult to collect, as surgical resection is often not attempted due to the morbidity of the procedure and its poor therapeutic outcome. Formalin-fixed specimens of pancreatic adenocarcinoma (or any tumor) are more readily available, since they are stored for years in paraffin blocks by most pathology departments. We developed a procedure that allows the detection of single point mutations in the c-K-*ras* gene present in a single 5 to 10 μm section of the formalin-fixed, paraffin-embedded tumor tissue (*20*).

To detect mutations in the c-K-*ras* gene, we initially used the RNAse A mismatach cleavage method either alone or in combination with the PCR (*20,21,30*) because the method permits the simultaneous analysis of multiple samples and is highly sensitive. However, RNAse A mismatch cleavages does not characterize the molecular nature of the mutation. More recently, we have used the artificial RFLP approach originally described by Kumar and Barbacid (*26*), as modified by Jiang et al. (*27*) to detect and characterize point mutations at codons 12 and 13 of the c-K-*ras* gene. We will describe here our procedure to detect any mutation at codon 12 (GGT) as an example of the strategy (Fig. 1). Replacement of the second G at codon 13 (GGC) by an A, by using a single base mismatched primer generates an HphI site (GGTGA) that is destroyed by any mutation at any of the two Gs of codon 12. Mutations of the following T and G are not considered, because they would be erased by the PCR primers. Mutations are detected by the presence of DNA fragments not digested by the enzyme. Another HphI site introduced by the upstream primer is used as internal control to test the completion of digestion and the efficiency of incorporation of the mutant nucleotide.

By this approach, mutant alleles can be detected when present in about 5 and 1% of the total by staining with ethidium bromide and by Southern blots, respectively. The sensitivity of this method is initially high because the mutation is detected by the presence of nondigested DNA fragments, which includes not only mutant sequences, but also hybrid molecules between wild-type and mutant DNA strands. This is likely to occur during the melting and annealing steps of the last cycles, especially if their number is high in the PCR experiment. Conversely, the sensitivity of mutation detection is considerably decreased when the mutant allele is digested with an endonuclease, but the wild-type allele is not. By using other RFLPs at codon 12, either natural or artificially introduced by the use of other mutant primers, all six different amino acid substitutions at this position can be readily characterized without the use of radioactive isotopes. Mutations can be also detected and characterized in DNA sequences PCR-amplified from single 5 to 10 μm sections of paraffin blocks by the artificial RFLP method as described before (Fig. 1).

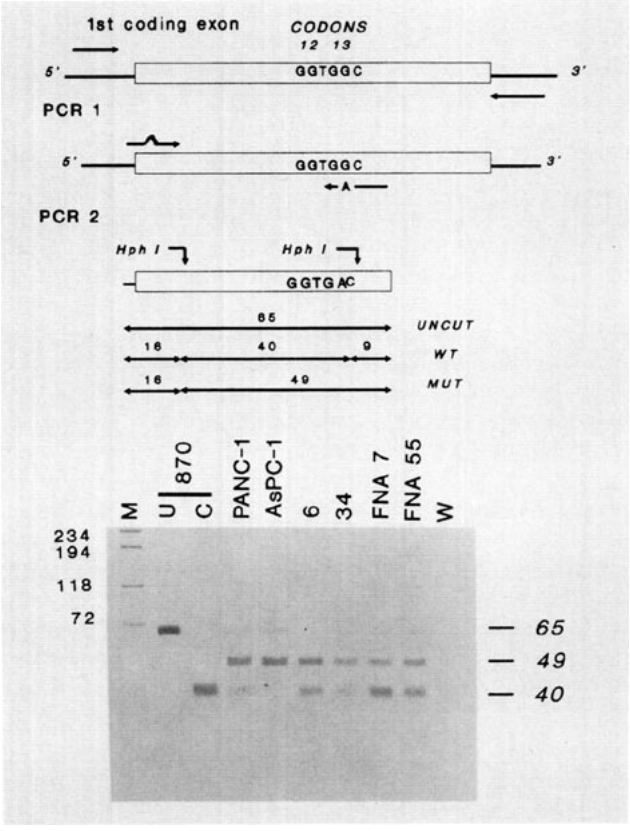

FIGURE 1. Detection of c-K-*ras* mutations in human tumors by RFLP of PCR amplified DNA. DNA sequences of the first coding exon of the c-K-*ras* gene were amplified using the primers 5′ GAGAAGCTTATGTGTGACATGTCTA 3′ and 5′ GAAGGATCCTGCACCAGTAATATGCA 3′ for 25 cycles (50°C for 35 sec; 72°C for 1 min and 25 sec; 94°C for 1 min) (PCR 1). One microliter of the PCR product was reamplified (PCR 2) with nested, mutant amplimers 5′ CCTGGTGAAAATGACTGAAT 3′ and 5′ AGGCACTCTTGCCTACGTCA 3′ for other 35 cycles (50°C for 15 sec; 72°C for 30 sec; 94°C for 15 sec), yielding a 65 bp DNA fragment. After digestion with HphI following manufacturer's directions, the samples were analyzed in a 12% acrylamide native gel and stained with ethydium bromide. (M) Hae III-digested OX179 fragments; (870) DNA obtained from normal colonic mucosa; (U) undigested PCR product; and (C) DNA sample after digestion. (PANC-1 and AsPC-1) human pancreatic carcinoma cell lines used as positive controls for c-K-*ras* codon 12 aspartic acid mutations. PANC-1 is heterozygous for the mutation, while AsPC-1 contains only the mutant c-K-ras allele. (6 and 34) Colorectal adenoma and carcinoma, respectively. (FNA 7 and 55) Fine needle aspirates from pancreatic adenocarcinomas. In these four cases DNA was amplified from formalin-fixed paraffin-embedded tissues.

The technical details of this methodology are described in Shibata et al. (*31*).

Results

Frequency of c-K-*ras* Mutations in Human Tumors

The results of our studies on the involvement of point mutations in the c-K-*ras* gene in various types of human tumors, mainly carcinomas, are summarized in Table 1.

Table 1. Frequency of point mutations at codons 12 and 13 of the c-K-*ras* gene in human tumors.

Tumor type	Positive[a]	Total	Percent
Pancreas[b]	63	84	75.0
Colon and rectum			
Adenomas	28	72	38.8
Carcinomas[c]	98	244	40.1
Bile duct[d]	6	19	31.5
Lung[e]	23	92	25.0
Liver	1	14	7.1
Stomach	1	14	7.1
Kidney	1	14	7.1
Other[f]	0	57	0.0

[a]All at codon 12 but one pancreatic carcinoma, 1 extrahepatic bile duct carcinoma, 1 lung carcinoma, and 26 colo-rectal tumors (12 adenomas and 14 carcinomas) with mutations at codon 13.

[b]Primary and metastatic (55 positive of 72 analyzed) and tumor cell lines (8/12).

[c]Primary and metastatic carcinomas (89/232) and tumor cell lines (9/12).

[d]Cholangiocarcinomas (2/5), periampullary (3/12), and extrahepatic bile duct (1/2) carcinomas.

[e]Primary carcinomas (17/85) and tumor cell lines (6/7).

[f]Carcinomas of the breast (12), prostate (10), thyroid (8), esophagus (5), gallbladder (5), endometrium (4), and others (13). In addition, 20 melanomas and 5 tumors of the endocrine pancreas were found negative.

The frequency of c-K-*ras* genes with mutations at codon 12 or 13 fluctuates considerably depending on the type of tumor. The most striking observation is the high incidence of c-K-*ras* mutations in pancreatic carcinomas. This high frequency appears to be specific for this tumor because it is significantly lower in cholangiocarcinomas, extrahepatic bile duct carcinomas, and carcinomas of the ampulla of Vater and absent or much lower in gall bladder, hepatocellular, gastric and esophageal carcinomas, and tumors of the endocrine pancreas (*21,31*). Because the pathway for malignancy for the majority of human pancreatic adenocarcinomas involves the mutation at codon 12 of the c-K-*ras* gene, the presence of the mutation can serve as a molecular genetic marker for this malignancy. Aspirates of pancreatic adenocarcinoma, obtained by fine needle (FNA), were analyzed for c-K-*ras* mutations (*32*). The results of this combined molecular genetic and cytologic approach indicate that in selected cases, the detection of mutated c-K-*ras* genes allowed a correct diagnosis of malignant disease, although this diagnosis was suspected but not established by cytology.

We have also analyzed about 300 colo-rectal tumors for the presence of mutations in the first coding exon of the c-K-*ras* gene. In our initial studies using the RNAse A mismatch cleavage method (*33*), we detected about 40% of colo-rectal adenomas and carcinomas positive for mutations at codon 12 (*21,30*). However, mutations at codon 13 of the c-K-*ras* gene, exclusively the aspartic acid (ASP13) substitution (GGC → GAC), were reported by Vogelstein et al. (*19*). We know now that the ASP13 mutations in the c-K-*ras* sequences generate single base mismatches in the RNA:RNA hybrids that are not recognized by the enzyme. Using RFLP analysis with c-K-*ras* sequences amplified by the PCR, we have

confirmed these findings (*19*) and have found that about 10% of colo-rectal tumors contain c-K-*ras* genes with the ASP13 mutation.

Our results show that the incidence of c-K-*ras* mutations increases during colo-rectal tumorigenesis and correlates with tumors conserving a well-differentiated phenotype (*21,30*). On the other hand, no correlations were found with the sex, race, or anatomical localization of the tumors (*21,30,34*). Follow-up analysis of the colo-rectal cancer patients is underway to determine the possible value for cancer prognosis of the presence or absence of mutated c-K *ras* genes (*35*) or differences in the amino acid substitutions in the positive cases (see below).

Mutation Spectrum of c-K-*ras* in Human Carcinomas

Analysis of the specific nucleotide changes at codons 12 and 13 of the c-K-*ras* gene in human carcinomas reveals a marked heterogeneity, although some mutations are more frequent than others. Differences in the spectrum of mutations can be also observed depending on the type of tumor (Fig. 2). In both colo-rectal and pancreatic carcinomas, the most prevalent mutations involve G to A transitions. The most characteristic difference between the mutations occurring in these two different tumors is the aspartic acid substitution at codon 13 (ASP13). This mutation is relatively frequent in colo-rectal tumors, but very rare in pancreatic adenocarcinomas and also in lung carcinomas. In this context, it is remarkable that c-K-*ras* genes with the ASP13 mutation are found predominantly in benign tumors (adenomas) and in carcinomas of older colo-rectal cancer patients compared with those containing other mutations or with tumors without mutant c-K-*ras* genes (M. Perucho et al., manuscript in preparation).

In contrast with the prevalence of G to A transitions in these gastrointestinal tumors, in carcinomas of the lung, the G to T transversions are more frequent. This

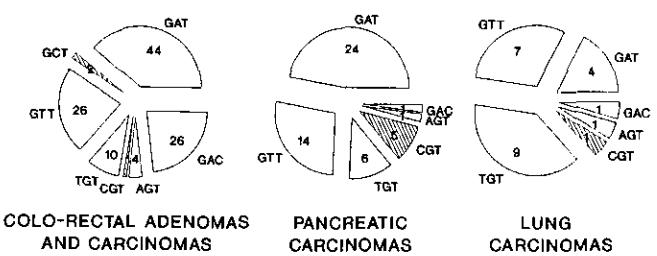

FIGURE 2. Spectrum of c-K-*ras* mutations at codon 12 and 13 in human carcinomas. The total number of mutations characterized is indicated inside the sectors. One hundred thirteen mutations have been characterized in colo-rectal tumors (27 in adenomas and 86 in adenocarcinomas). No tumor cell lines have been included. Fifty-one mutations have been characterized in human pancreatic carcinomas (44 in tumors and 7 in cell lines); 23 mutations have been characterized in human lung carcinomas (17 in tumors and 6 in tumor cell lines). Only the aspartic acid mutation (GAC) was found at codon 13.

observation is in agreement with the results of Rodenhuis et al. (*36*). This observation is also in consonance with other studies of lung adenomas and carcinomas induced by some chemical carcinogens in animal model systems (*23*).

Discussion

Our finding that a vast majority of human pancreatic carcinomas contain point mutations at codon 12 of the c-K-*ras* gene suggests that these mutations are important events in the genesis of cancer of the pancreas. However, the timing of activation of the c-K-*ras* gene in pancreatic carcinoma, as well as the role it plays in the tumorigenesis process, remains to be determined. Because there is a significant proportion of these tumors that do not harbor *ras* mutations (*21*), pancreatic adenocarcinoma might also develop through a pathway of genetic alterations that does not involve *ras* somatic mutational activation. Moreover, because the point mutation in the c-K-*ras* gene cannot be sufficient to account for this aggressive neoplasia, other genetic alterations must be also involved in pancreatic oncogenesis. Preliminary results indicate that inactivation of the p53 gene is involved in the genesis of pancreatic cancer. We have detected hemizygous mutations in conserved positions of the gene coding region in most of the tumor cell lines examined and in some primary tumors (J. Schaeffer et al., manuscript in preparation).

The disruption of the balanced interplay between oncogenes and tumor-suppressor genes is clearly exemplified in colo-rectal tumorigenesis. It is believed that a majority of carcinomas from the colon and rectum develop through a previous benign stage, the adenoma or adenomatous polyp (*37,38*). Work by Vogelstein's group has convincingly shown that colo-rectal tumorigenesis is a complex and accumulative process involving the inactivation of at least two tumor-suppressor genes, p53 and DCC (deleted in colon cancer). These genes encode a nucleoprotein and a membrane protein with similarities to the adhesins and other membrane glycoproteins, respectively (*8,39,40*). Together with the gene located in chromosome 5 (*41*), segregating with the syndrome of familial polyposis, the process of colo-rectal oncogenesis may involve inactivation of at least three loci with up to six total genetic alterations.

The finding that nearly one-half of colo-rectal tumors contain mutated c-K-*ras* genes (*18,19,21,30*) also indicates that activation of this dominant-acting oncogene must be also an important event in the process. Moreover, accumulative alterations leading to an increase in the ratio of mutant/normal *ras* allele also occur often during tumor progression (*21*). We have also shown that moderate increases in the copy number and transcript levels of a mutant human c-K-*ras* gene have profound consequences in the manifestation of the malignant phenotype both *in vitro* and *in vivo* (*42*). Therefore, the accumulative genetic alterations occurring in the same single locus, the c-K-*ras* gene, add another level of com-

Table 2. Relative frequency of G to A transitions at codons 12 and 13 of *ras* genes in human tumors.[a]

Gene	Sequence 12	Sequence 13	Codon 12 AGT (SER)	Codon 12 GAT (ASP)	Codon 13 AGT (SER)	Codon 13 GAT (ASP)	Rest[b]	Total (%)
K-*ras*	GGT	GGC	16	154	0	45	228	443 (71.3)
N-*ras*	GGT	GGT	10	33	1[c]	11	114	167 (26.9)
H-*ras*	GGC	GGT	0	6	0	0	5	11 (1.8)
Total			26	193	1	56	347	621
%			4.1	31.0	0.1	9.0	55.8	

[a]Compiled from Ellis et al. (*13*), Shibata et al. (*31*), Nagata et al. (*50*), Tada et al. (*51*), and our unpublished results..
[b]Rest of mutations at codons 12 and 13 and all mutations at codon 61.
[c]Detected in a multiple myeloma that also contained a GCT(ALA) mutation at codon 12 (*49*).

Table 3. Aspartic acid mutations at codon 12 of *ras* genes in carcinogen-induced tumors.

Tumor[a]	Carcinogen[b]	Gene	Incidence	(%)	Reference
Lung adenoma	Spontaneous	K	1/5	(20)	(*23*)
Lung carcinoma	Spontaneous	K	2/5	(40)	(*25*)
Lung adenoma	MNU	K	15/15	(100)	(*25*)
Lung adenoma	BP	K	4/14	(28)	(*23*)
Thymoma	MNU	K	22/55	(40)	(*46*)
Thymoma	X-rays	K	7/37	(19)	(*46*)
Thymoma	MNU	N	1/55	(2)	(*46*)
Thymoma	X-rays	N	1/37	(3)	(*46*)
Skin papilloma	MNU	H	5/12	(41)	(*52*)
Skin papilloma	MNNG	H	11/15	(73)	(*52*)
Skin carcinoma	MNNG	H	2/13	(15)	(*56*)
Lung carcinoma	TNM	K	9/9	(100)	(*53*)
Lung carcinoma	TNM	K	14/14	(100)	(*53*)
Mammary carcinoma	MNU	H	61/61	(100)	(*54*)
Kidney mesenchimal	MNU	K	31/35	(88)	(*54*)

[a]All tumors are mouse tumors but the last three, which are in rats.
[b]MNU, methylnitrosourea; BP, benzo[*a*]pyrene; MNNG, methylnitrosoguanidine; TNM, tetranitromethane.

plexity to the spectrum of genetic alterations involved in human colo-rectal tumorigenesis.

Moreover, the diversity of point mutations occurring both in the *ras* oncogenes and in the p53 tumor-suppressor gene (*43,44*) could have influence on tumor behavior. Of the mutations occurring at codons 12 and 13 of *ras* genes, the G to A transitions resulting in the replacement of glycine by aspartic acid are the most remarkable because of their relatively high incidence. Table 2 summarizes the frequency of point mutations in the three *ras* genes found in human tumors. Although this is not a comprehensive list of all mutations described in the literature, it is sufficient to give a panoramic view of the relative frequencies of different *ras* mutations. It is clear that the most prevalent activated *ras* gene is the c-K-*ras* and that nearly one-half of all *ras* mutations are G to A transitions at codons 12 and 13. However, while the serine mutation at codon 13 (SER13) has been described only once (and in a multiple myeloma containing an alanine at codon 12 [ALA12] mutation), the aspartic acid mutation at codon 12 (ASP12) is more than twice as frequent as the two remaining mutations together: the serine mutation at codon 12 (SER12, AGT) and the aspartic acid mutation at codon 13 (ASP13, GAC). This makes the G to A transition at the second position of codon 12 the single most frequent *ras* mutation found in human tumors, composing about 30% of the total. The same G to A mutation at codon 13, although significant in its overall prevalence, is relatively much less frequent.

A similar situation is probably also true in the field of animal carcinogen-induced tumor model systems. Despite evident specificities in the association between some carcinogens and the type of induced mutations (*45*), the same G to A transition at the second position of codon 12 of each of the three *ras* genes is also predominantly found in tumors, spontaneous or induced, in rats and mice with a wide sample of carcinogens and carcinogenic treatments. In contrast, we have found only one report of ASP13 mutations, in a thymoma induced in mice with methylnitrosourea (*46*). Table 3 summarizes an incomplete survey of the animal tumor model system literature.

The heterogeneity in the spectrum of c-K-*ras* mutations found in human carcinomas is not consonant with the interpretation that these mutations are caused by specific, defined carcinogens. It seems more likely that they are caused by multiple and diverse mutagens, or by spontaneous errors of the DNA replicative machinery, or both. However, the differences in the mutation spectrum between carcinomas of the gastrointestinal and respiratory tracts point to broad but significant etiological differences, perhaps due to differential exposure to genotoxic agents: dietary in the first and airborne in the second. The characterization of the *ras* mutation spectrum in different human carcinomas pro-

vides a molecular epidemiological approach that could shed some light on their etiologies. For instance, it could direct the search for carcinogens present in the diet or in cigarette smoke. The similarities in the spectra of *ras* mutations found in lung tumors and those induced by aromatic hydrocarbons such as benzopyrene (*47*) and in gastrointestinal tumors with those induced by alkylating agents such as methylnitrosourea (*48*) are certainly very suggestive.

This work was supported by grant CA38579 from the National Cancer Institute. G.C. is a fellow from the Spanish Ministry of Sciences (MEC).

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