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Incidence and Possible Clinical Significance of K-*ras* Oncogene Activation in Adenocarcinoma of the Human Lung[1]

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ABSTRACT

47 tumor samples, 45 of which were obtained at thoracotomy for non-small cell lung cancer were examined for mutational activation of the oncogenes H-*ras*, K-*ras*, and N-*ras*. A novel, highly sensitive assay based on oligonucleotide hybridization following an *in vitro* amplification step was employed. *ras* gene mutations were present in nine of 35 adenocarcinomas of the lung (all K-*ras*), in two of two lung metastases of colorectal adenocarcinomas (1 × K-*ras*, 1 × N-*ras*) and in one adenocarcinoma sample obtained at autopsy (H-*ras*). All K-*ras* and H-*ras* mutations were in either position 1 or 2 of codon 12, while the N-*ras* mutation was in position 2 of codon 61.

The potential clinical significance of K-*ras* activation was analyzed using the combined results of this and of our earlier study (S. Rodenhuis *et al.*, New Engl. J. Med., *317*: 929–935, 1987). Lung adenocarcinomas with K-*ras* mutations tended to be smaller and were less likely to have spread to regional lymph nodes at presentation. With a median follow up of 10 months, survival data are still immature. None of six adenocarcinomas of nonsmokers had a K-*ras* mutation and only one of four who had stopped smoking more than 5 years before. We conclude that mutational K-*ras* activation is present in about a third of adenocarcinomas of the lung and that the mutational event may be a direct result of one or more carcinogenic ingredients of tobacco smoke. Studies involving larger numbers of patients are required to confirm the association of K-*ras* activation with smoking and the inverse relation with tumor progression.

INTRODUCTION

Despite intensive clinical research efforts of the past 15 years, little real progress has been made in the management of lung cancer and the outlook for patients whose tumors cannot be completely resected remains grim. Simultaneously, important advances have been made in the basic science of cancer and a concept of the molecular mechanisms that give rise to malignant transformation is beginning to emerge. Most of these data, however, are derived from *in vitro* studies or from animal systems and the role of the known oncogenes in the pathogenesis of human cancer is largely conjectural (1, 2).

At least two major families of oncogenes, *myc* and *ras*, play a part in the pathogenesis of human lung cancer (3). While *myc* genes appear to be primarily important in small-cell lung cancer, we have recently shown that activation of the K-*ras* oncogene is specifically associated with the histological features of adenocarcinoma and does not or only very infrequently occur in other types of NSCLC[3] (4).

The family of *ras* genes includes three well-characterized genes, H-*ras*, K-*ras*, and N-*ras*. All three genes are being expressed in a wide variety of normal cells. They code for closely related M_r 21,000 guanosine triphosphate-binding proteins (p21) that are localized at the inner surface of the cell membrane and that are thought to be involved in growth signal transduction (2). The *ras* proteins acquire transforming potential when a single amino acid located at one of the critical positions 12, 13, or 61 is replaced as a result of a point mutation in the gene (5). Such mutations may represent direct effects of exposure to carcinogenic agents or radiation (6–11).

The methodological problems to reliably detect mutationally activated *ras* genes in uncultured tumor samples have recently been solved by the introduction of a novel, highly specific assay based on oligonucleotide hybridization (12). This test has been further improved by incorporation of an *in vitro* method by which the relative proportion of the DNA sequences of interest can be increased more than 10,000-fold (13, 14). Using this technique, we have shown previously that K-*ras* was activated in five of 10 adenocarcinomas of the lung, while no H-*ras* and N-*ras* activations were found in a total of 35 nonadenocarcinoma NSCLC specimens (4). We also speculated that the activating point mutation in codon 12 might be a result of a carcinogenic ingredient of tobacco smoke. To determine the incidence of K-*ras*-12 mutation more accurately and to confirm the association between smoking and K-*ras* activation, we now report on a second series of tumors which includes 35 samples of primary adenocarcinomas removed at thoracotomy.

MATERIALS AND METHODS

Tumor Specimens. All specimens were obtained at thoracotomy with curative intent. The resected material was transported on ice to the pathology department and after examination by a pathologist, a representative part of the tumor was snap frozen and stored at −70°C until analysis. Prior to the isolation of the DNA, cryostat sections of the frozen materials were obtained and stained, and admixture of nonmalignant tissue or cells was identified. Nonneoplastic parts of the specimen, necrotic areas, and areas with dense inflammatory infiltrates were removed as completely as possible and additional cryostat sections were obtained to estimate the percentage of tumor cells in the final specimen. This percentage was usually 50% or higher. Specimens judged to contain less than 25% malignant cells were discarded.

Paraffin sections of all tumors were routinely stained with hematoxylin & eosin and additional mucin stains, and were independently classified according to the WHO classification (15) by two histopathologists (W. J. M. and Sj. Sc. W.). DNA was isolated from the mechanically disrupted tumor specimens (wet weight between 100 and 300 mg) using standard techniques (16).

Dot Blot Procedure for Detection of Activating Point Mutations in H-*ras*, K-*ras*, or N-*ras* Genes. This method allows identification of point mutations in codons 12, 13, and 61 of the three *ras* genes employing hybridizations to panels of oligonucleotides that correspond to the possible mutations in these sites. To increase the sensitivity of the method, the six regions of interest (centering around either codons 12 and 13 or around codon 61 in each of the three genes) were selectively amplified *in vitro* according to a primer-mediated, DNA polymerase I-catalyzed method.

The experimental details of the method have recently been published (14). Briefly, 0.25 μg of a DNA sample was heat denatured and allowed

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[3] The abbreviation used is: NSCLC, non-small cell lung cancer.

Table 1 *Histological classification of tumors*

WHO classification system

	Previous study (3) (N = 39)	This study (N = 47)	Total (N = 86)
Epidermoid carcinoma	15	5	20
Small cell carcinoma	1	1	2
Adenocarcinoma	10	35	45
Large cell carcinoma	10	0	10
Adenosquamous carcinoma	0	2	2
Carcinoid	1	1	2
Thymoma	0	1	1
Lung metastases			
Breast	1	0	1
Colorectal	1	2	3

to hybridize to two synthetic oligonucleotides of 20 residues, that were complementary to sequences flanking one of the six target sequences. One of the 20-mers hybridized to the coding strand, the other to the noncoding strand. After annealing, the oligonucleotides were used as primers for the heat-stable DNA polymerase I from *Thermus aquaticus*, which subsequently copied the adjacent stretches of DNA containing the target sequence. Such a round of amplification was repeated 20–25 times, leading to a more than 10,000-fold increase of the sequence of interest. Separate *in vitro* amplifications were done for each of the six target sequences, each generating amplified stretches of 65–130 base pairs.

The *in vitro* amplified DNAs were subsequently spotted on nylon filters (Gene Screen Plus, New England Nuclear), using approximately 10 ng per spot. Separate filters were prepared for each of the six target sequences. Each of the filters was then hybridized to a ^{32}P end-labeled oligonucleotide probe corresponding to the "wild-type" (nonmutated) sequence of the *ras* gene for which it had been amplified *in vitro*. One-basepair mismatched probes were prevented from hybridization by incubation in either 3.0 M tetramethylammonium chloride or 5 × SSPE (1 × SSPE: 10 mM sodium phosphate pH 7.0, 0.18 M NaCl, 1 mM EDTA) under temperature conditions at which only fully matched hybrids were stable (14). Thus, any signal at autoradiography corresponded to the wild-type sequence in the DNA.

Similar hybridizations were done employing full panels of oligonucleotide probes for each of the possible point mutations in codons 12, 13, and 61 of the *ras* genes that would lead to an amino acid substitution in the encoded protein (12, 17). This procedure does not only allow detection of mutations but also identifies the exact sequence of the mutated codon.

RESULTS

DNA could be extracted from a total of 47 tumor samples, 46 of which had been obtained at thoracotomy and one at autopsy. 35 samples were classified as adenocarcinoma, the histology of the remaining tumors is indicated in Table 1. The

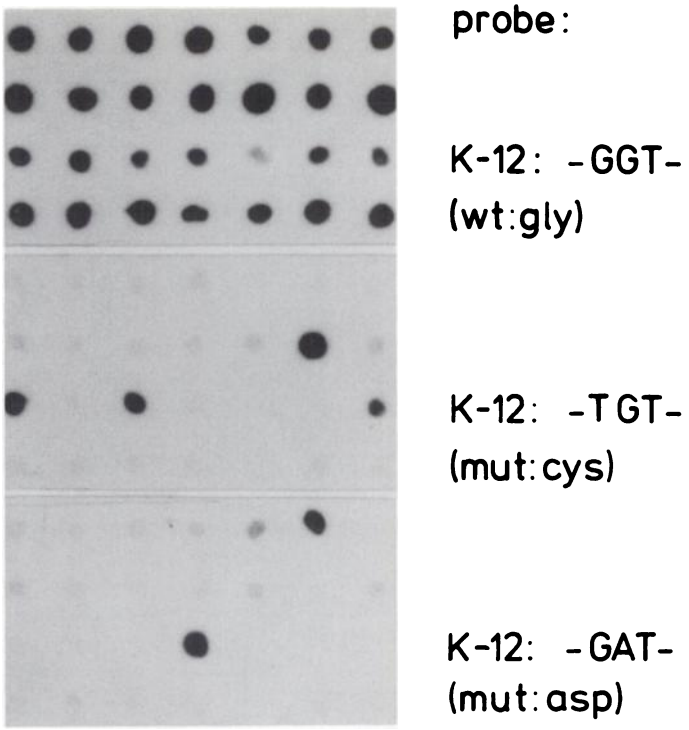

Fig. 1. Characterization of point mutations in codon 12 of the K-*ras* gene. The three panels show examples of hybridizations of the dot-blots to three different oligonucleotide probes. *Top*, probe for the normal codon 12 of K-*ras*. *Middle*, hybridization of a duplicate filter to a probe specific for a G-T mutation in position 1 of K-*ras* codon 12. This mutation is detected in four of the samples shown. *Bottom*, hybridization of a duplicate filter to a probe specific for a G-A mutation in position 2 of K-*ras* codon 12. Two samples are positive.

percentage of tumor cells per sample ranged between 25 and 90 (median, 50).

10 K-*ras*, one N-*ras*, and one H-*ras* mutations were detected (Fig. 1). All mutations were present in positions 1 or 2 of codon 12, except for the N-*ras* mutation, which was in position 2 of codon 61 (Table 2). All *ras* mutations were found in adenocarcinomas, although in two cases (including the N-*ras*-61 mutation) a solitary metastasis of a colorectal carcinoma was involved instead of a lung primary. The single H-*ras* mutation was found in the sample obtained at autopsy.

To determine the clinical significance of *ras* activation in untreated adenocarcinoma, the presenting features and course of disease were compared between lung adenocarcinoma patients with and without K-*ras* mutations. Particular attention was paid to the smoking histories, which included the number of pack-years smoked, the total number of years smoked, and

Table 2 *Sequence of mutated ras genes in DNA isolated from adenocarcinomas*

The normal codon 12 of K-*ras*: GGT, codes for gly; H-*ras*: GGC, codes for gly. The normal codon 61 of N-*ras*: CAA, codes for gyn. gly, glycine; cys, cysteine; asp, aspartic acid; val, valine; ala, alanine; gln, glutamine.

Pat. no.	*ras* gene	Codon	Sequence/amino acid	Histopathological type of adenocarcinoma	TNM category
50	K	12	TGT cys	Well differentiated	$T_2N_0M_0$
61	K	12	GAT asp	Well differentiated	$T_2N_0M_0$
62	K	12	TGT cys	Moderately differentiated	$T_2N_0M_0$
80	K	12	TGT cys	Moderately differentiated	$T_1N_0M_0$
47	K	12	GAT asp	Poorly differentiated	$T_1N_0M_0$
55	K	12	GAT asp	Poorly differentiated	$T_2N_2M_0$
66	K	12	GCT ala	Poorly differentiated	$T_1N_0M_0$
68	K	12	GTT val	Poorly differentiated	$T_1N_0M_0$
83	K	12	GTT val	Poorly differentiated	$T_2N_0M_0$
54	K	12	TGT cys	Metastasis colorectal well differentiated	
74	N	61	CTA leu	Metastasis colorectal, moderately diff.	
89	H	12	GTC val	Well differentiated (autopsy sample)	$T_3N_2M_1$

Table 3 *Differences between K-ras-positive and K-ras-negative carcinomas at presentation*

		K-*ras* 12 mutation		Normal K-*ras*
Tumor size[a]	T_1	6		7
	T_2	7	$P = 0.055$[b]	18
	T_3	0		5
Nodal status[a]	N_0	10		12
	N_1	2	$P = 0.029$[b]	9
	N_2	1		9
Degree of differentiation				
Well		2		7
Moderately		4		16
Poorly		7		8
Bronchioloalveolar		1		0
Smoking histories				
Years smoked		38 (15–66)[c]		40 (0–61)
Pack-years		36 (5–80)		38 (0–88)

[a] TNM category system of the American Joint Committee for Cancer Staging and End Results Reporting, 1979. In two cases no full surgical staging was performed.
[b] χ^2 test for trend.
[c] Median (range).

Table 4 *Mutational activation of K-ras in adenocarcinoma of the lung: Relation to smoking*

$P = 0.050$ (χ^2 test for trend).

	K-*ras* mutation			K-*ras* normal		
	Non-smokers			Nonsmokers		
	Never smoked	Stopped > 5 yrs	Smokers	Never smoked	Stopped > 5 yrs	Smokers
Previous study (3)	0	0	5	2	1	2
Present study	0	1	8	4	2	17
Both studies	0	1	13	6	3	19

the dates at which smoking was stopped completely. From three patients no detailed smoking histories could be obtained. These were excluded from the analysis of the smoking histories. Since the number of nonsmokers in this study was small (4), the results were combined with the results from our previous study (4), which employed essentially identical methods as the present one.

Adenocarcinomas with K-*ras* mutations tended to be smaller ($P = 0.055$) and were less likely to have spread to the regional lymph nodes at operation ($P = 0.029$), as shown in Table 3. The degrees of differentiation of the tumors were not significantly different. No other significant differences in either presenting features, survival, or relapse-free survival could be found between K-*ras*-positive and -negative patients. The median follow up of 10 months is, however, too short to exclude later survival differences. The total number of years or pack-years smoked was very similar between the groups (Table 3). As in our previous study, however, none of the nonsmokers had a K-*ras* mutation (Table 4). The association between smoking and K-*ras* appears to be confirmed and, combined with the findings of our earlier study, now reaches borderline statistical significance (Table 4).

DISCUSSION

A total of 86 lung tumor samples, the large majority of which were removed at potentially curative thoracotomy, have now been examined in our two studies for mutational *ras* gene activations (Table 1). In the 77 samples from NSCLC, a total of 14 K-*ras* mutations were detected, and a single H-*ras* mutation. All of these were encountered in the 45 adenocarcinomas, establishing the high degree of specificity of this oncogene activation for this lung cancer subtype. Thus, K-*ras* activation is present in about one third of all adenocarcinomas of the lung at thoracotomy but not in other types of non-small cell lung cancer. The H-*ras* activation was found in the only sample obtained at autopsy in this study.

All point mutations in the K-*ras* genes were found to be present in the 12th codon, leading to five different amino acid substitutions of the normal glycine at that position in the protein. All of these have been described as having transforming activity in *in vitro* assays (18). Adenocarcinomas with K-*ras* activation could not be distinguished microscopically from those without mutations and the type of amino acid substitution did not correlate with the degree of differentiation or with other morphological features.

Analysis of the presenting features of the patients with adenocarcinomas revealed some differences between K-*ras*-positive and -negative patients that approached statistical significance. K-*ras*-positive adenocarcinomas tended to be smaller and had less often spread to the regional lymph nodes than K-*ras*-negative ones (Table 3). These differences were, however, only gradual and whether they reflect real differences in biological behavior remains to be determined in prospective studies. At present, with a median follow up of 10 months (range, 1–34 months) no conclusion can be drawn from the survival data. More meaningful survival analysis will be possible in another 2 or 3 years.

We have recently speculated that mutational activation of the K-*ras* oncogene might be a direct effect of one or more carcinogenic ingredients of tobacco smoke (4). Several lines of evidence suggest that this is not an unlikely scenario. In fact, *ras* oncogenes provide a direct link between carcinogen exposure and the pathogenesis of tumors (6, 11). K-*ras* itself can be activated by chemical carcinogens (7, 9, 10) and exposure of dogs to plutonium caused lung cancers containing mutationally activated K-*ras* genes in all of eight cases (8). To analyze this relationship in our patients, the smoking histories of the K-*ras*-positive cases were compared to those of the K-*ras*-negative ones.

Only a small number of the patients had never smoked (6/42) and only a few (4/42) had stopped smoking for at least 5 years before diagnosis. The large majority of these patients (9/10) did not have a K-*ras* mutation (Table 4), suggesting that tobacco smoking is the major causative factor in the induction of K-*ras* point mutations. This relationship is, however, not a simple one. If the total exposure to tobacco smoke between the K-*ras*-positive and -negative adenocarcinoma patients is compared, either in terms of total number of years of exposure or number of pack-years, the groups are very similar (Table 4). Even of the heavy smokers with adenocarcinomas, less than 50% have a K-*ras* mutation in their tumors. Carcinogenic ingredients of tobacco smoke may thus induce additional, still unidentified genetic alterations that can contribute to the pathogenesis of adenocarcinomas and which apparently bypass the need for a K-*ras* gene activation.

The fact that the adenocarcinomas with K-*ras* mutations tended to be smaller and had less often metastasized at diagnosis may suggest that K-*ras*-positive tumors either give rise to earlier symptoms leading to earlier detection, or that they actually grow somewhat slower than the K-*ras*-negative ones. The latter explanation would obviously be in conflict with the hypothesis that K-*ras* mutations render a selective growth advantage to already malignant cells, because in that case rapidly growing tumors of a higher degree of malignancy would be expected. Thus, we favor the hypothesis that K-*ras* mutation does represent an essential event in the pathogenesis of adeno-

carcinoma of the lung but that alternative and still unknown pathways exist that lead to microscopically similar tumors with possibly more aggressive behavior. This concept can now be tested in prospective clinical studies.

A potential clue for the biological role of *ras* oncogene activations in human malignancies is provided by our findings in the three solitary lung metastases of colorectal primaries that were removed at thoracotomy. In all three of these, a *ras* activation was found: a K-*ras* amplification in our previous series (4), a K-*ras* 12 and a N-*ras* 61 mutation in our present series (Table 4). We (19) and others (20) have previously shown that K-*ras* mutations are frequent in colorectal adenocarcinomas, while N-*ras* activations may occur occasionally. In view of animal data suggesting that *ras* activation may confer metastatic ability to cancer cells (21), it may be interesting to evaluate a potential role of *ras* activation in the metastatic process of colorectal tumor cells. Obviously, larger numbers of these metastases, and preferably also the primary tumors from which they are derived, must be studied to examine this question.

The first practical goal of studying the activation of cellular oncogenes in clinical tumor specimens is to refine the classification of these tumors. It is likely that K-*ras* activation status will eventually contribute to such a molecular genetic classification system, but several additional genetic events may be required for the development of adenocarcinoma of the lung. The identification and characterization of these alterations remains an exciting challenge for the years to come.

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