Clinical Significance of ras Oncogene Activation in Human Lung Cancer

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Abstract

Activation of ras oncogenes is commonly found in human neoplasms. We have investigated 280 human lung cancer specimens for ras activation, including 38 that have not been reported previously, using an oligonucleotide detection assay. From a total of 141 adenocarcinoma samples from smokers, 41 tested positive for a point mutation in codon 12 of K-ras (30%), while three tumors had another type of ras activation. Only two of 40 cases from nonsmokers had a K-ras mutation (5%), suggesting that K-ras mutations may be directly caused by exposure to carcinogens in tobacco smoke. The majority of the point mutations in adenocarcinomas were guanine to thymine transversions in codon 12 of the K-ras oncogene. Occasional point mutations in ras oncogenes were detected in adenosquamous carcinomas (one of five cases) and large cell carcinoma (one of 24 cases), but no ras activations were found in small cell carcinomas (six cases), squamous carcinomas (48 cases), carcinoid carcinomas (15 cases), or thymoma (one case).

Analysis of the clinical and pathological features of the adenocarcinoma cases showed no apparent associations between the K-ras activation and age at diagnosis, sex, disease stage, and the occurrence of other neoplasms. K-ras-positive adenocarcinomas tended to be less differentiated than the K-ras-negative ones (P = 0.044, χ² test for trend). K-ras mutations identify a subgroup of patients with adenocarcinoma of the lung who have a very poor prognosis despite radical resection of their tumor. Although K-ras has been proposed as a target for antitumor therapy, its major clinical significance could be to aid in the selection of patients for specific therapeutic interventions, such as adjuvant chemotherapy.

Introduction

The remarkable progress in molecular biology in the past decade has led to the elucidation of many events involved in malignant transformation. Central among these appear to be a limited set of genetic alterations that are able to disrupt strictly controlled cellular processes such as differentiation and growth, by interfering with normal programs of gene expression. The pivotal genes involved include the dominant oncogenes and the tumor-suppressor genes (antioncogenes) (1).

The development of a human cancer probably requires at least 5 to 10 different genetic alterations, each assumed to consist of the activation of an oncogene or the inactivation of a tumor-suppressor gene. It is likely that only certain combinations of these genes (in)activations give rise to malignancies when superimposed on the genetic activity pattern of the cell type in question. It also seems clear that several different combinations can lead to similar types of tumors, as data from colon cancer indicate (2).

If one accepts the view that all properties of both normal and malignant cells must ultimately reflect the repertoire of genes being expressed, then the vast variability in phenotype and in clinical behavior of human cancers would suggest that a useful molecular classification of a given tumor would require assessment of the activities of an unpractically large number of genes. It is immediately clear, however, that at least one major reduction in complexity can be achieved by taking the cell type of origin of the tumor into consideration, as has been successfully done by pathologists for many years. But even then, a remarkable degree of heterogeneity remains between tumors of identical origin, and a molecular characterization focused on these differences might still be expected to require data on countless combinations and permutations of active genes.

It is at present still uncertain whether a second major reduction in complexity will prove valid, the consideration of the key genetic events, i.e., the specific oncogenes activated in a given tumor. In other words, does the specific pattern of oncogene activations largely account for the variability in clinical course and response to therapy in patients with similar tumors? If so, the activity of certain activated oncogenes should correlate with important clinical features of the disease, such as pace of progression, pattern of spread, degree of invasiveness in surrounding tissues, and other parameters.

Numerous studies have attempted to correlate (onco)gene alterations in specific neoplasms with clinical features, but nevertheless, only a few clinical applications have emerged so far. This may result from technical problems involved in the detection of activated dominant oncogenes in human tumor tissues, but also from the fact that only a few tumor-suppressor genes have been characterized, whereas it is certain that many more exist and are important in the frequently encountered human tumor types (3).

The methodological problems to reliably detect mutationaly activated ras genes in uncultured tumor samples have recently been overcome by the introduction of a novel, highly specific assay based on oligonucleotide hybridization (4). This test has been further improved by incorporation of the polymerase chain reaction (5), and very small tissue samples, even when routinely fixed in formalin and embedded in paraffin, are now sufficient for analysis (Fig. 1). As a result, the ras oncogenes are among the first genes of which the clinical significance can be studied routinely in clinical oncology.

The ras Oncogenes

The three well-characterized ras genes, H-ras, K-ras, and N-ras, belong to a superfamiliy of genes coding for small monomeric GTP-binding proteins (see, for review, Ref. 6). The three ras genes code for highly homologous Mr 21,000 proteins p21ras that are localized at the inner side of the cell membrane. Their homology with G-proteins strongly suggests a function in signal transduction (7). The ras gene proteins can exist in two states: an "active state" in which GTP is bound to the molecule and in which presumably a signal is relayed into an as yet unidentified secondary messenger pathway; and an "inactive state" in which the GTP has been hydrolyzed to GDP (7). The ras proteins possess intrinsic GTPase activity which eventually leads to their inactivation, but this inactivation is greatly enhanced by a second protein, called the GAP (8). GAP has been shown to bind to the domain that is involved in the transduction of the ras signal, the "effector domain" of p21ras (9). It is currently unclear whether GAP is only involved in enhancing ras GTPase activity, or whether it is also the effector molecule.
Activations are predominantly found in myeloproliferative disorders and in lymphomas (16, 18). For unknown reasons, K-ras is particularly associated with adenocarcinomas and has been reported to be activated in the majority of pancreatic cancers (19, 20), in about half of all colorectal cancers (21, 22), and in about one third of lung adenocarcinomas (see below). In ovarian cancer or breast cancer, ras point mutations are very exceptional (23, 24).

Activation of ras genes as a result of gene amplification has also been described, and very high levels of p21ras are able to transform NIH/3T3 cells in vitro (25). Most data indicate, however, that ras gene amplification is a very infrequent mechanism of activation in human tumors (7, 26).

For p21ras (10, 11). The discovery that GAP can be phosphorylated by activated platelet-derived growth factor receptor has provided evidence for a direct biochemical link between tyrosine kinases and the ras signalling pathway (12, 13).

The ras genes have been highly conserved in evolution, and expression of one or more of the three genes can be demonstrated in most or even all mammalian cells (14). Thus, the presence of ras proteins must be essential for the normal physiology of the cell. The event that turns a normal ras gene into an oncogene is a single point mutation which leads to a single amino acid substitution in the encoded protein (7). Only point mutations resulting in loss of the intrinsic GTPase activity (thus causing inability of p21ras to switch back to the inactive state) appear to be associated with transforming activity of the protein. Thus, activating mutations are invariably found in the GTP-binding regions of p21ras (15). In practice, virtually all relevant mutations are found in codons 12, 13, and 61 of H-, K-, and N-ras (16).

Mutational activation of ras genes have been found in a wide range of human tumors (16). H-ras is only infrequently found to be activated, mainly in thyroid carcinomas (17), and N-ras activations are predominantly found in myeloproliferative disorders and in lymphomas (16, 18). For unknown reasons, K-ras point mutations resulting in loss of the intrinsic GTPase activity (thus causing inability of p21ras to switch back to the inactive state) appear to be associated with transforming activity of the protein. Thus, activating mutations are invariably found in the GTP-binding regions of p21ras (15). In practice, virtually all relevant mutations are found in codons 12, 13, and 61 of H-, K-, and N-ras (16).

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**Table 1** Findings in 280 lung cancer specimens, tested for mutations in codons 12, 13, and 61 of K-ras, N-ras, and H-ras

<table>
<thead>
<tr>
<th>Histology</th>
<th>K-ras, codons 12 and 61</th>
<th>K-ras, codons 13 and 61</th>
<th>N-ras, codons 12, 13, and 61</th>
<th>H-ras, codons 12 and 61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cell</td>
<td>0/6</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Epidermoid</td>
<td>0/48</td>
<td>0/29</td>
<td>0/22</td>
<td>0/23</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>43/181</td>
<td>1/131b</td>
<td>1/124c</td>
<td>1/50d</td>
</tr>
<tr>
<td>Large cell</td>
<td>1/24</td>
<td>0/18</td>
<td>1/13c</td>
<td>0/16</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>1/5</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Carcinoid</td>
<td>0/15</td>
<td>0/14</td>
<td>0/12</td>
<td>0/14</td>
</tr>
<tr>
<td>Thymoma</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Total investigated</td>
<td>280</td>
<td>200</td>
<td>177</td>
<td>115</td>
</tr>
</tbody>
</table>

*a* See Table 2 for spectrum of mutations in codon 12 of K-ras.

*b* K-ras codon 61, position 1.

*c* N-ras codon 12, position 1.

*d* H-ras codon 12, GTC (valine).

*f* N-ras codon 61, position 2.

Unpublished observation.
negative.\(^5\) We (28) and others (31) have shown that ras mutations other than those in the 12th codon of K-ras are infrequent in uncultured lung cancer specimens, although somewhat more frequent in Japan (30). In our series, most were encountered in either nonadenocarcinomas or in samples obtained at autopsy from patients with advanced disease [one case with a H-ras codon 12 mutation (28)]. It is possible that these "uncommon" ras mutations represent late events in the natural history of the disease, but the number of cases investigated is too low to conclude this with any degree of certainty.

The relatively low frequency of ras mutations in lung adenocarcinomas raises the question of whether other types of ras activation, such as overexpression of the nonmutated gene, could be present in some of the mutation-negative tumors. We have examined this by studying both gene copy numbers and expression levels in a series of lung tumors (26, 32), and we found no evidence of gene amplification or high-level overexpression. The findings with regard to gene amplification are consistent with those reported in the literature (33).

Another possibility, although remote, could be that the ras mutation-positive adenocarcinomas are, in fact, not primary lung carcinomas but, rather, metastases from occult gastrointestinal carcinomas, in which the frequency of ras mutations is known to be high (see above). In addition to the clinical and pathological data arguing against this explanation, we were recently able to show that the spectrum of K-ras codon 12 mutations encountered in lung adenocarcinomas (Table 2) is quite different from that in colon cancers (21, 34, 35). While guanine to thymine transversions (mainly at position 1 of K-ras codon 12) are predominant in lung adenocarcinomas, the predominant mutation in colon carcinoma is a guanine to adenine transition (at position 2) and, if a guanine to thymine transversion does occur in colon cancer, it is usually at position 2.

### Clinical Significance of K-ras Activation

Since mutational activations of ras in lung cancer are largely confined to adenocarcinomas and almost always involve codon 12 of the K-ras gene, our analyses focused on potential differences between adenocarcinomas with or without this mutation (Table 3). We have previously reported associations of K-ras mutations with tumor stage (28), smoking history (36), and prognosis (37). In the following, an overview is given of the available clinical data of the patients in these studies, combined with those of an additional 38 patients not reported on before.

### Tumor Stage

Retrospective analysis of the clinical data of all 181 patients (Table 3) with adenocarcinoma does not confirm the trend for smaller tumors and lower nodal status in patients with K-ras-positive tumors which was present in earlier series, which were mainly limited to operable patients (28). The reason for this discrepancy is at present not entirely clear. The relation between K-ras activation and stage of disease requires evaluation in a prospective study.

### Tumor Differentiation

K-ras point mutation-positive tumors appear to be less differentiated than those without; in this series, a statistically significant trend becomes apparent (\(P = 0.044, \chi^2\) test for trend). However, the oncogene activation could also be detected in several cases with well-differentiated, or bronchioalveolar, lung carcinomas. It is of interest that, in experimental systems, activated ras is associated with adenocarcinoma differentiation. Introduction of an activated ras oncogene in immortalized bronchial epithelial cells (38) or in small cell lung cancer cells (39) can induce a phenotype consistent with adenodifferentiation.

### Smoking History

Chemical carcinogens are well-documented causes of ras gene mutations in laboratory animals. In fact, ras gene mutations have provided the first link between chemical carcinogenesis and oncocenes, as has been elegantly demonstrated in the experiments of Barbadic (7). Single injections of nitrosomethylurea in rats were able to induce breast cancers with specific H-ras mutations. A significant number of other experimental systems in which carcinogenic agents were able to induce tumors with ras mutations have been reported since (7, 40).

We investigated the relationship between smoking and ras mutations in two independent series, one retrospectively (28) and one prospectively (36). Both series have now shown that K-ras mutations are significantly less frequent in patients who have never smoked than in those with a smoking history. Summarizing all data from our laboratory, K-ras mutations were found in 41 of 141 adenocarcinoma samples from smokers or exsmokers (30%), but in only 2 of 40 tumors of patients who had never smoked (5%) (Table 3). These data indicate that smoking and K-ras mutations are indeed linked. In a recent Japanese study a similar association was reported, except for

| Table 2 Spectrum of K-ras codon 12 mutations in lung cancer |
|-----------------|----------------|----------------|----------------|----------------|
| Histology       | TGT cys        | AGT ser         | CGT arg         | GAT val         |
| Adenocarcinoma  | 25 0 1 7 8 2 43| 0 0 0 0 1 0 1   |                |
| Large cell      | 0 0 0 0 0 0 1   |                |                |
| Adenosquamous   | 0 0 0 0 0 0 1   |                |                |

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\(^a\) The normal sequence of codon 12 of the K-ras gene is GGT, coding for glycine. cys, cysteine; ser, serine; arg, arginine; val, valine; asp, aspartic acid; ala, alanine.

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### Comparison of K-ras mutation-positive and K-ras mutation-negative lung adenocarcinomas

<table>
<thead>
<tr>
<th>K-ras normal</th>
<th>K-ras mutated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (yr)</td>
<td>66 (40-87)(^a)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>70/68</td>
</tr>
<tr>
<td>Stage (%)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>67 (48)(^a)</td>
</tr>
<tr>
<td>II</td>
<td>22 (16)</td>
</tr>
<tr>
<td>III</td>
<td>27 (20)</td>
</tr>
<tr>
<td>IV</td>
<td>14 (10)</td>
</tr>
<tr>
<td>Unstaged</td>
<td>8 (6)</td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
</tr>
<tr>
<td>Well(^d)</td>
<td>40 (30)</td>
</tr>
<tr>
<td>Moderately(^d)</td>
<td>55 (40)</td>
</tr>
<tr>
<td>Poorly(^d)</td>
<td>38 (27)</td>
</tr>
<tr>
<td>Not determined</td>
<td>5 (3)</td>
</tr>
<tr>
<td>Other neoplasms</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>115 (83)</td>
</tr>
<tr>
<td>Benign</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Epidermoid</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Hematological</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Not determined</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>100 (72)</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>38 (28)</td>
</tr>
</tbody>
</table>

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\(^a\) Numbers in brackets, range.

\(^b\) Numbers in parentheses, percentage.

\(^c\) Three samples were obtained from autopsies.

\(^d\) \(P = 0.044, \chi^2\) test for trend.
ras mutation-negative adenocarcinomas.

Patients after radical surgery for adenocarcinoma of the lung (37). P = 0.021, log-rank test. In Fig. 2, an updated survival curve is shown, now with actually slightly more favorable than in the K-ras-negative group. In view of these findings it is reasonable to assume that the K-ras mutations observed in lung adenocarcinomas are a direct result of exposure to one or more carcinogenic ingredients of tobacco smoke.

**Prognosis.** We have reported on 69 patients whose lung adenocarcinomas had been completely resected and who were believed to be free of tumor after the operation (37). Forty-eight patients had Stage I, 14 had Stage II, and 7 had Stage IIIA disease. Nineteen tumors contained a K-ras codon 12 point mutation. The K-ras point mutation-positive group did significantly worse than the K-ras-negative one, with poorer disease-free interval and overall survival (log-rank tests: P = 0.038 and P = 0.002, respectively). This difference was readily detectable despite the fact that the stages of disease of the K-ras-positive group were actually slightly more favorable than in the K-ras-negative group. In Fig. 2, an updated survival curve is shown, now with a median follow-up of 47 mo.

In conclusion, K-ras mutation is a strongly unfavorable prognostic factor that identifies a subgroup of patients with a very poor prognosis despite apparently successful surgery for Stage I or Stage II disease. A recent study of the National Cancer Institute essentially confirmed these results by analyzing ras genes in 52 cell lines derived from patients with lung adenocarcinomas (42). Patients from whom ras mutation-positive cell lines had been established had significantly shorter survival than those from whom ras mutation-negative cell lines were obtained. ras mutations adversely affected patient survival irrespective of disease extent.

**Possible Clinical Applications.**

The now detailed knowledge of the structure and biochemistry of the ras proteins renders relevance to the question of whether direct inhibition of the activity of these proteins could represent effective antitumor therapy. Several pharmacological approaches to this end have been proposed, most of which interfere with the posttranslational modifications of the protein required for membrane localization (43, 44). It is, however, uncertain what toxicity to normal tissues might ensue from inhibition of this unquestionably important signal transduction system. Furthermore, the results of inhibiting the action of mutated ras proteins in human lung cancer cells in vivo are difficult to anticipate. Data from in vitro studies using plasmids containing anti-sense K-ras constructs suggest that this may result in inhibition of growth in culture and in decreased tumorigenicity (45). But obviously, a mere decrease in degree of malignancy is likely to be of little clinical benefit to patients with lung cancer as long as a lethal disease persists.

Determination of ras point mutations may also aid in distinguishing neoplasms of different origin. In our series, one patient developed a primary lung adenocarcinoma within a field that had been irradiated because of treatment for breast cancer (46). In this case, the breast tumor contained a c-myc amplification, while the primary lung adenocarcinoma harbored a point mutation in codon 12 of K-ras. In another patient, a point mutation in K-ras was found in a primary lung adenocarcinoma from a patient who had previously been treated for an ethmoid sinus carcinoma. Two years after the thoracotomy, a lobectomy was performed for a large cell carcinoma, which microscopically resembled the ethmoid sinus tumor. This tumor did not harbor a K-ras point mutation, providing additional evidence that this tumor was a metastasis from the ethmoid sinus tumor rather than from the pulmonary adenocarcinoma.

As mentioned above, patients with K-ras-positive lung adenocarcinoma, despite having been radically operated for Stage I or Stage II disease, have only a 35% chance to survive 2 yr without relapse (37) and might thus be regarded as potential candidates for an adjuvant treatment strategy. Adjuvant radiotherapy has been shown to be disappointing (47) in non-small cell lung cancer, but a prospective randomized trial of adjuvant chemotherapy in non-small cell lung cancer has shown a significant survival advantage in the chemotherapy group (48). A study of adjuvant chemotherapy in K-ras-positive lung cancer patients must now be considered.

In addition to K-ras, several other prognostic markers have recently been reported to be potentially useful, such as erbB-2 (neu) (49), neural cell adhesion molecule (50), and blood-group antigen A expression (51). It is likely that the combination of these and other markers will eventually allow further refinement of the classification of lung cancer, thereby approaching the ideal of a “molecular” classification that may provide a firm basis for therapeutic decisions in the clinic.

**References.**


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