Molecular Staging of Non-Small Cell Lung Cancer According to K-ras Genotypes

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ABSTRACT

We have previously demonstrated a strong association between K-ras gene mutations, as determined by PCR followed by allele-specific oligonucleotide hybridization (ASO-h), and survival in non-small cell lung cancer patients. The purpose of this study was to determine the relationship between tumor aggressiveness and specific-type K-ras point mutations in non-small cell lung cancer. We developed procedures to examine the status of the K-ras gene by ASO-h and by single-strand conformation polymorphism assay of DNA obtained from formalin-fixed paraffin-embedded tumors. K-ras point mutations at codons 12 and 61 were assessed in 275 consecutively treated stage I-IV non-small cell lung cancers. Among patients with stage I disease, median survival time was 41.5 months in those whose tumors had no evidence of K-ras mutations and 27 months in those with K-ras 12 mutations; among patients with stage II disease, median survival time was 7 months in those with K-ras codon 12 aspartic and serine mutations and 15 months in those with other K-ras mutations (P = 0.01). In a multivariate analysis, specific-type K-ras codon 12 point mutation remained a strong predictive factor (hazard ratio for death, 2.06; 95% confidence interval, 1.11–3.81; P = 0.02) after adjustment for other evaluated factors, including TNM stage and histology. Thus, we concluded that in patients with non-small cell lung cancer, specific K-ras 12 point mutations detected by DNA amplification and either ASO-h or single-strand conformation polymorphism methods predicted a significantly increased risk of recurrence and death, independently of stage and histology.

INTRODUCTION

Mutations of the ras genes are the most frequently detected oncogenes in human and rodent tumors (1). ras genes encode proteins that bind guanine nucleotides, exhibit a slow intrinsic GTPase activity, and are localized at the inner face of the cytoplasmic membrane. The highly mutable sequences include those at codons 12, 13, and 59–61 and are typically activated by single-point mutation. Diverse amino acid substitutions at codon 12 activate the K-ras gene except proline, which has no transforming properties (1). Furthermore, in both in vitro and in vivo transfection and tumorigenicity assays, it has been demonstrated that aspartic 12 mutations are more oncogenic than other ras mutations such as cysteine 12 and valine (2, 3). Mutations of the K-ras gene were primarily observed to occur in a third of lung adenocarcinoma and considered a potential prognostic marker (4).

Currently, determining prognosis and selecting patients for therapy rely mainly on clinical and pathological staging. Patients with stage I lung cancer (T1,N0,M0) commonly have an acceptable life span, whereas patients with stage IIIB and IV have curtailed survival. However, predicting outcome in patients with intermediate stages is rather complex. Those with resectable stage IIIA (mediastinal lymph node involvement) non-small cell lung cancer have a 5-year survival rate of about 15% or higher when preoperative chemotherapy is given (5, 6). Better means of formulating a prognosis in non-small cell lung cancer could improve patient selection for new chemoradiotherapy strategies in the stage III setting.

In the present study, we sought to determine whether the presence of specific-type K-ras mutations is a relevant factor in forecasting the clinical behavior of lung cancer. Our previous work has suggested that K-ras gene alterations can predict survival in resected non-small cell lung cancer (7). However, a Japanese study pointed out that K-ras mutations have prognostic value only in resected stage I lung adenocarcinoma (8). We demonstrate in 275 patients with either adenocarcinoma, squamous cell carcinoma, or large cell undifferentiated lung cancer that the presence of specific-type K-ras point mutations identifies patients with aggressive lung cancer that is independent of stage and histology.

MATERIALS AND METHODS

Clinical Specimens. We studied 275 patients with biopsy-proven non-small cell lung cancer (stages I-IV, according to the TNM classification (9)). All patients included were treated consecutively at the Germans Trias i Pujol Hospital between January 1988 and October 1992 for whom follow-up data and tumor samples from the surgery specimens or bronchoscopy (preserved in archival paraffin-embedded tissue blocks) were available. The study also included 66 patients described previously whose tumors underwent molecular analysis for K-ras (7). Thus, of the group of 275 patients, 209 had not been previously evaluated for K-ras point mutations.

Received 12/29/95; revised 2/20/96; accepted 3/11/96.

1 Supported by Grant 95/0177 from the Fondo de Investigaciones Sanitarias de la Seguridad Social, Madrid, Spain.

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Patients with small cell carcinoma and carcinoid tumors of the lung were excluded. Of the 275 patients eligible for the study, surgical staging was carried out in 212 who had undergone complete surgical resection of T1-3, N0-2 lesions (stage I-IIIA), whereas clinical staging was performed in the remaining 63 patients classified as stage IIIB or IV. The median age of all patients was 62 (range, 37 to 77) years; 95% were men. The patients' main characteristics studied are summarized in Table 1. All tumors were reevaluated for histology by two of the investigators. The median follow-up for the 275 patients was 6.0 years, with 85% having at least 3 years of follow-up. No patient was lost to follow-up. Patients whose tumors were resected were seen at 3-month intervals during the first postoperative year, every 4 months during the second and third year, and every 6 months thereafter. Follow-up consisted of biochemical profile, chest radiograph and computed tomographic scan, and physical examination. Data on recurrences of lung cancer and causes of death were obtained.

**Testing for K-ras Mutations.** Extraction of cellular DNA and PCR were done as described previously (6). DNA integrity was checked with agarose-gel electrophoresis and ethidium bromide staining as well as with a spectrophotometric technique. K-ras coding sequences flanking codons 12 and 61 of the K-ras gene were amplified by PCR using two sets of primers. Template DNA was obtained from tumor tissues that were microdissected from three to five 5-μm-thick unstained paraffin slides. Thirty-five cycles of PCR amplification were required to generate sufficient template for the allele-specific oligonucleotide hybridization and SSCP\(^3\) analysis. Specific hybridization was detected by exposing the membranes to autoradiographic film (Kodak OAR, Rochester, NY). All mutations were confirmed by DNA from a second independent PCR-SSCP assay.

**Statistical Analysis.** The data were computed according to the BMDP program package. The association of the K-ras oncogene with other clinicopathological factors was assessed using the \(\chi^2\) test. Survival curves were drawn for each group of different variables using the Kaplan-Meier method (10), and differences among the curves were computed using the log rank test (11). The most significant prognostic factors were identified using the Cox proportional hazard method (12).

**RESULTS**

Of the 275 non-small cell lung cancer tumors examined for specific-type K-ras point mutations, 57 had K-ras mutations. To detect K-ras mutations at codon 12 and 61, the PCR technique followed by allele-specific oligonucleotide hybridization was used. Mutated codon 12 sequences were confirmed by the SSCP

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\(^3\) The abbreviations used are: SSCP, single-strand conformation polymorphism; RER, replication error.
K-ras mutations were more commonly found at codon 12 with wild-type glycine (GGT) changing to aspartic acid (GAT) in 13 individual tumors, serine (AGT) in 8, valine (GTT) in 14, cysteine (TGT) in 10, arginine (CGT) in 6, and alanine (GCT) in 1. K-ras mutations at codon 61 were: leucine (CTA) in one case, glutamine (GAA) in three cases, and histidine (CAT) in one case.

The population included 262 men and 13 women. Seventy-nine patients were classified as stage I, of whom 70% underwent lobectomy. All of them had hilar and mediastinal lymph node dissection. The same surgical procedure was performed in 31 stage II patients and in 102 stage IIIA patients. Patients with unresectable disease included 33 in stage IIIB and 30 in stage IV. The overall survival of the 275 patients analyzed according to the Cox proportional hazard model was as follows: 36 months in stage I, 16 months in stage II, 14 months in stage IIIA, 10 months in stage IIIB, and 5 months in stage IV. Mutations were present in 20 (26%) of 78 adenocarcinomas as well as in other histological types: 29 (18%) of 163 squamous cell carcinomas and 8 (23.5%) of 34 large cell undifferentiated carcinomas. There was a trend toward being K-ras-negative tumors in lower stages, e.g., in 65 (82%) of 79 stage I patients, 28 (91%) of 31 stage II, 80 (78%) of 102 stage IIIA, and 25 (76%) of 33 stage IIIB as opposed to 20 (67%) of 30 stage IV patients (P < 0.05). In the 102 stage IIIA patients, median survival time was 15 months (95% confidence interval, 12-20) for the negative K-ras mutation group, only 7 months (95% confidence interval, 5-10) for the aspartic and serine codon 12 K-ras mutation group, and 15 months (95% confidence interval, 12-24) for other ras mutations. When survival of the K-ras-positive group in stage IIIA patients is broken down by K-ras genotypes, the difference between 7 months and 15 months median survival time for aspartic and serine mutations and other mutations, respectively, is significant (P = 0.01; Fig. 1).

To identify the most powerful prognostic factors, we performed multivariate analyses with the Cox proportional hazards model. The hazard ratios were calculated with two models using clinical variables interrelated with K-ras gene mutations. The first model combined tumor stage, histology, and K-ras gene status. The second model combined tumor stage and K-ras genotypes because the combination gave the best fit attainable with any combination of the three prognostic factors (Table 2). In the K-ras mutant group, further correcting for specific K-ras mutations (aspartic and serine codon 12 mutations) yielded a hazard ratio of 2.06 (95% confidence interval, 1.11-3.81).

### DISCUSSION

In this study of non-small cell lung cancer, K-ras mutations affected 21% of 275 cases. Our results support previous findings which suggested that K-ras mutations in non-small cell lung cancer were not restricted to adenocarcinoma type (7, 13) and that K-ras mutations may well be an independent prognostic marker mainly in early stages of non-small cell lung cancer. We found that K-ras mutation detection can provide prognostic information in patients with non-small cell lung cancer. The subgroup of stage IIIA patients whose tumors had K-ras codon 12 aspartic or serine mutations (8%) had a worse outcome (7 months median survival time), whereas survival in patients whose tumors had no K-ras mutations was substantially better (15 months). Mounting evidence indicates that K-ras gene mutations could be a notable prognostic marker in non-small cell lung cancer (Table 3). K-ras mutations were detected in 19 of 69 adenocarcinomas in a Dutch study (14), mostly in stage I, and those patients whose tumors had mutated K-ras had poorer survival. This finding was confirmed in a series of 66 non-small cell lung cancer cell lines in the stage I-III A setting but not in more advanced disease (15). When survival in advanced disease was broken down into those tumors with K-ras codon 12 mutations as compared with the remainder (ras negative plus mutations at codon 61), the difference turned out to be significant (15). In our previous experience, we detected 13 tumors with K-ras mutations among 66 resected non-small cell lung cancers of any histological type with significant differences in survival (7). More recently, Kern et al. (16) observed 16 of 44 stage I-IIIA lung adenocarcinoma patients harboring K-ras mutations; the difference in survival approached significance, although in the subset of 6 patients (14%) whose tumors contained K-ras mutations and c-erbB-2 overexpression there was a much worse outcome.
In addition to K-ras status, other genomic perturbations have been singled out although their prognostic value is still controversial. For example, in a recent study, c-erbB-2 was overexpressed in 93% of lung adenocarcinoma (17) as compared with 34% in the above-mentioned study (16). These differences are likely to be related to immunohistochemistry-related conditions. Furthermore, overexpression of p53 protein (a surrogate for missense type p53 gene mutations) has been reported to be associated with decreased survival of non-small cell lung cancer patients but not with p53 gene mutations (18), whereas a mutation both in the p53 and K-ras genes has been detected very rarely in the same lung tumor (4–7%: Refs. 17–19). It seems that the presence of mutations in one gene did not correlate with the presence of mutations in the other gene.

It is important to make two caveats to this analysis: there may be geographical differences (in the frequency, type, and prognostic significance of K-ras mutations) and a large European study involving a number of countries is desirable to substantiate the hypothesis that the presence of specific K-ras genotypes may be an independent prognostic factor.

Some K-ras codon 12 genotypes may help to define tumor aggressiveness and could serve to select resected non-small cell lung cancer patients as candidates for adjuvant chemotherapy, whereas in the more advanced disease setting, we hypothesize that K-ras mutations could help to identify patients who exhibit chemoresistance. A current limitation of the technique we have described is that K-ras mutations are present in only one-fifth of non-small cell lung cancers. However, other genetic aberrations in non-small cell lung cancer may provide additional information, and RERs as indicated by microsatellite instability represent a novel mechanism of carcinogenesis in different human cancers, primarily highlighted in hereditary nonpolyposis colorectal cancer (20). Furthermore, unpublished data from our laboratory indicate that p53 mutations and RER changes are rare in the same DNA samples from non-small cell lung cancer patients, whereas other K-ras genotypes tend to be linked to RER abnormalities. For example, microsatellite instability with specific dinucleotide markers of chromosomes 2p and 3p may serve as a second molecular marker for non-small cell lung cancer (21). A second limitation is that this technically challenging molecular analysis takes 3 days to perform. At the present time, since prognosis and therapeutic strategy relies on TNM staging, the addition of molecular staging may avoid overtreatment and undertreatment of large patient cohorts.

ACKNOWLEDGMENTS

We thank Maura O’Sullivan for secretarial assistance.

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