Implications and Prognostic Value of K-ras Mutation for Early-Stage Lung Cancer in Women

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Background: Because there is no clear consensus as to the predictive value of K-ras gene mutation for survival in patients with lung cancer, we examined the occurrence of K-ras mutations in a large, prospective case series of non-small-cell lung cancer (NSCLC). Our goals were to define the patient characteristics associated with K-ras mutation and to determine whether mutation of this gene might be a biomarker of patient prognosis.

Methods: Consecutive, newly diagnosed patients with lung cancer treated with potentially curative resection over a 4-year period were recruited for study. The mutation status of K-ras codon 12 in each patient’s tumor DNA was determined by means of polymerase chain reaction–restriction fragment length polymorphism analysis of archived pathology specimens. Analyses were restricted to adenocarcinoma.

Results: There was a statistically significant association between female sex and K-ras mutation after adjustment for carcinogen exposures (odds ratio = 3.3; 95% confidence interval [CI] = 1.3–7.9); mutations were found only in smokers. Comparison of Kaplan–Meier curves indicated a strong association between K-ras mutation and decreased patient survival (two-sided P = .009); analysis stratified by pathologic staging groups revealed that this association was statistically significant only for stage I tumors (two-sided P = .002). Cox proportional hazards modeling indicated that K-ras codon 12 mutation was a statistically significant predictor of patient survival, after adjustment for the effects of age, sex, and stage (risk ratio = 1.8; 95% CI = 1.1–3.1). Conclusions: After adjustment for environmental exposures, non-small-cell lung tumors in women appear to be more likely than those in men to harbor K-ras mutations, suggesting a possible role of estrogen exposure in either the initiation or the selection of K-ras mutant clones in adenocarcinoma. In addition, our data suggest that K-ras codon 12 mutation is a marker of aggressive NSCLC, as evidenced by its association with decreased patient survival, particularly for early-stage disease. [J Natl Cancer Inst 1999;91:2032–8]

The characteristics of the continuing lung cancer epidemic in the United States have shifted in recent years, and newer data show a dramatic rise in the incidence of adenocarcinoma (1–5). At the same time, the incidence of lung cancer has increased sharply among women (1–13). It is interesting that adenocarcinoma also occurs much more frequently in women than in men. In addition, women cigarette smokers have been found to have substantially elevated risks of lung cancer compared with men, given similar amounts of tar exposure (8,12). Zang and Wynder (8) reported that the higher risk for women could not be explained by differences in baseline exposure, smoking history, or body size. Several investigators (1,5,8,11) have reasoned that shifts in the histology of lung cancer are a reflection of changes in the composition of cigarettes, as well as alterations in smoking behavior (e.g., deeper inhalation of larger volumes of smoke). However, to date there are few clues to explain the sex-specific differences in lung cancer. The nature of mutations in tumors that are induced by tobacco carcinogen exposures should reflect the critical mechanistic steps responsible for generating the epidemiologic trends in this important public health epidemic. In addition, understand-
ing these changes may help improve the therapeutic approach to this disease. Here, we have concentrated on mutations within the K-ras gene in non-small-cell lung cancer (NSCLC).

The K-ras gene encodes a protein that is known to be oncogenic when mutated or overexpressed [reviewed in (14)]. Mutation at codon 12 of K-ras is very specific for adenocarcinoma, occurring rarely in squamous cell or small-cell lung cancer. Ras proteins are known to act as signaling switches that relay growth signals from the cell surface to the MAP kinase cascade [reviewed in (15)]. As such, they are believed to contribute to hyperproliferative growth when they are mutated or overexpressed and may potentiate growth factor and hormone signaling. These proteins, however, are also involved in cell functions other than the MAP kinase cascade. Examples include interaction with the rho pathway (16,17) and integrin activation (18), suggesting that altered ras protein may affect the early metastatic potential of a neoplastic cell. Indeed, patients whose tumors have K-ras mutations have been observed to have poorer survival than patients whose tumors have no such mutations in some studies (19–28) but not in other studies (29–35). Differences in the demographic and clinical characteristics of these populations may also have an impact on these results. For example, some groups have reported that the occurrence of K-ras mutation in lung adenocarcinoma is associated with male sex (22,26–28,36), heavy smoking (26,27,37–39), and asbestos exposure (39–41).

Lung cancer is the leading cause of cancer mortality in the United States (42), and surgical resection for cure is often only applicable to early-stage NSCLC. Recent studies [reviewed in (43)] have suggested that combined modality treatment of NSCLC may improve survival in selected patients (43). One of the most critical questions in choosing the appropriate use of combined therapy is determining which patients might benefit from the more aggressive treatment. Several approaches have been suggested, including treatment based on markers of poor prognosis, such as the K-ras gene mutation in adenocarcinoma.

While the precise explanation for the lack of consensus among studies that have asked whether the K-ras mutation is associated with survival of patients with NSCLC is not known, the mutation-screening method and individual study design may be critical for observing an association between K-ras mutation status and patient outcome. In an effort to reduce misclassification attributable to tumor heterogeneity, we used a relatively nonsensitive mutation-screening method. This was done to bias the detection of mutations toward those tumors that were homogeneous for this alteration. It may be that ras mutations are important for prognosis only in tumors that are homogeneous for such alterations. Finally, we also used a prospective study design to assess the association of K-ras mutation with survival. Retrospective studies are problematic if selection criteria are biased by mutation status. That is, if K-ras mutation status is related to survival of disease, it is plausible that patient follow-up may be biased. Because surgery alone is often the treatment of choice for stage I NSCLC, patients without mutations might be significantly more likely to do well after surgery and, therefore, selectively not return for follow-up. These patients then may not appear in retrospectively designed studies, thus introducing significant bias toward a null result. Here, we report findings from a large, prospective study of patients with resected NSCLC in the New England area and examine the association of K-ras mutation status with sex, carcinoembryonic antigen, and patient survival.

**SUBJECTS AND METHODS**

**Study Population**

Case subjects consisted of all newly diagnosed patients with resectable lung cancer who received treatment at the Massachusetts General Hospital (MGH) Thoracic Surgery, Oncology, and Pulmonary Services from November 1992 through December 1996. There were 461 case patients enrolled during that time period; this number represents approximately 90% of all eligible patients. Information regarding patient demographics and exposure histories was obtained with the use of an interviewer-administered questionnaire at the time of hospitalization. Written informed consent was obtained from all subjects.

The protocol for staging and surgical intervention has been described elsewhere (44). Briefly, all patients underwent mediastinoscopy in addition to radiographic evaluation for preoperative staging. Patients who were judged to be candidates for complete extirpation of primary and nodal disease underwent thoracotomy. Postoperative staging was then done, and these data were added to the database maintained by the MGH Cancer Registry.

Tumor tissue was obtained from archived pathology specimens, and information describing the tumors (i.e., histology, size, and degree of differentiation) was available from clinical pathologic evaluation. The MGH Cancer Registry was used to obtain information on staging (clinical and pathologic, with the stage variable being designated as the higher of the two), as well as patient follow-up.

Of the 461 case patients enrolled, 21 were excluded because the resected tumor was determined to be either a recurrence of lung cancer or a metastatic lesion. For 69 case patients, a tissue specimen was not retrieved (i.e., an appropriate specimen was not available, or there was insufficient material for DNA extraction). Determination of K-ras mutation status was not possible for six case patients. Thus, a total of 365 case patients were included in the study of K-ras mutation (Table 1, A). There were 355 case patients within the cohort with available follow-up information to examine patient survival as a function of K-ras mutation.

**Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) Detection of K-ras Mutation**

DNA was extracted with the use of a method modified from Banerjee et al. (45). From five to 10 adjacent 10-μm sections were cut from paraffin-embedded tumor blocks. Excess wax and normal tissue were removed, and the remaining material was placed in a 1.5-ml Eppendorf tube. Then 500 μL of digestion buffer (50 mM Tris–HCl, 1 mM EDTA, 0.5% Tween, and distilled deionized water) was added to the tissue. Samples were microwaved at high power for 30–60 seconds in 10-second intervals, after which they were centrifuged at 9000g for 10 minutes at room temperature, and the paraffin layer was removed manually. The remaining material was resuspended in digestion buffer and digested with 5 μL (10 mg/mL) of Proteinase K (PKA) for 3 hours at 65°C (or overnight at 56°C). Samples were then centrifuged at 5000g at room temperature for 5 minutes, and the supernatant was removed and PKA inactivated by heat treatment (100°C for 10 minutes).

Mutation screening was done with the use of a modified method of Mitsudomi et al. (46). PCR amplification of the region surrounding codon 12 was performed under standard PCR conditions for 15 cycles with the use of primers 5’-CATTTCTGTTATTAGCTACA and 5’-AACAGATTACTGTTATTTG. The PCR product was then diluted 1:100 for a second round of PCR that used a mismatched upstream primer that introduces a restriction site. Again, standard PCR reaction conditions were used, and primers were 5’-ACTGAATATAACTTGGTGGATGTGGACCT and 5’-TCAAGAATAAGTTGCTGACC for 40 rounds. The PCR product was then digested with BstNI restriction endonuclease enzyme, and the products were analyzed on 4% MetaPhor® agarose gels (FMC BioProducts, Rockland, ME). Proteinase K digestion was performed under standard conditions for 15 minutes at 56°C. Proteinase K was removed by phenol–chloroform extraction, and DNA was precipitated with ethanol and re-suspended in diethylpyrocarbonate-treated water.

Statistical Analyses

Statistical analyses of mutation status and tumor traits included χ², Wilcoxon rank-sum of means, and unconditional logistic regression. Survival time was
defined as the time from surgery to the patient’s death, known recurrence, or the last time that the patient was known to be alive. Survival probability curves were constructed for various groupings of patients with the use of the Kaplan–Meier method, and differences between groups were tested with the use of the logrank method. Cox’s proportional hazards modeling was used to examine the simultaneous effects of several variables on patient’s outcome. The data were consistent with the assumptions of Cox’s proportional modeling. All P values represent two-sided statistical tests and are considered to be statistically significant for \( P < 0.05 \).

**RESULTS**

The case patients for whom tumor tissue was not available did not differ in age, sex, histology, or smoking history from those studied. Among the 365 case patients studied for K-ras status, we observed a strong association of K-ras mutation with adenocarcinoma histology: 22.1% (95% confidence interval [CI] = 16.5%–28.5%) versus 4.8% (95% CI = 2.1%–9.3%); \( P < 0.001 \) for other histologies. There was a low prevalence of mutation in squamous cell carcinomas (2.8% [95% CI = 0.06%–7.8%]) as well as an intermediate prevalence of mutation in both large-cell carcinoma and adenocarcinoma (8.3% [95% CI = 1.0%–27.0%] and 7.7% [95% CI = 0.2%–36.0%], respectively). There were no mutations detected in the 10 bronchioloalveolar carcinomas.

Given the very strong association between mutation and adenocarcinoma histology, all subsequent analyses were restricted to the patients with adenocarcinoma. A comparison of adenocarcinoma case patients with and without K-ras mutations in their tumors revealed a tendency for mutations to occur in former smokers and for those with mutations to have smoked for more pack-years and to have quit smoking for less time, although none of these associations were statistically significant (Table 1, B). The distribution of K-ras mutations by measures of smoking intensity and duration is shown in Fig. 1 and Table 1, B. Among the patients with adenocarcinoma, 53.8% (95% CI = 46.8%–61.0%) were female. The prevalence of K-ras mutation was higher among women (26.2% [95% CI = 18.1%–35.6%]) than among men (17.4% [95% CI = 10.3%–26.7%]). The spectrum of mutations in codon 12 of K-ras, including the prevalence of specific amino acid changes, is presented in Table 2.

Because we have previously established that occupational asbestos exposure was significantly associated with K-ras mutation status (41), it was necessary to control for this in the analysis. Given the strong association between asbestos exposure and male sex, there was a potential confounding effect of asbestos exposure on the association of sex and K-ras mutation. This potential confounding effect was handled in the analysis by the use of two methods. First, we employed a logistic regression model to adjust for asbestos exposure and pack-years of smoking. This method showed a statistically significant association between K-ras mutation and female sex (odds ratio [OR] = 3.3; 95% CI = 1.3–7.9). An unconditional logistic regression analysis adjusting for asbestos exposure alone produced an OR of 2.8 (95% CI = 1.2–6.6). We next stratified the analysis by asbestos exposure. That is, those patients with a known history of occupational asbestos exposure, regardless of sex, were removed from the analysis. In this case, the
Fig. 1. Comparison of the median values of cigarette smoking duration and intensity by K-ras mutation status for all case patients. K-ras status was determined by polymerase chain reaction–restriction fragment length polymorphism analysis. Bars give median values with 95% confidence intervals (C.I.) for patients with wild-type (black) or mutant (gray) K-ras genes.

Table 2. Prevalence of specific amino acid substitutions in patients with adenocarcinoma and K-ras mutations

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Prevalence* (%) of all adenocarcinoma mutations</th>
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</thead>
<tbody>
<tr>
<td>GGT→AGT, serine</td>
<td>4/44 (9.1)</td>
</tr>
<tr>
<td>GGT→GAT, aspartate</td>
<td>4/44 (9.1)</td>
</tr>
<tr>
<td>GGT→GCT, alanine</td>
<td>4/44 (9.1)</td>
</tr>
<tr>
<td>GGT→GTT, valine</td>
<td>9/44 (20.5)</td>
</tr>
<tr>
<td>GGT→TGT, cysteine</td>
<td>23/44 (52.3)</td>
</tr>
</tbody>
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*Number of patients with specific amino acid substitutions/total number of patients with mutation.

The crude OR for the association between sex and K-ras mutation was 3.1 (95% CI = 1.3–7.6), with an adjusted OR of 4.4 (95% CI = 1.6–11.7)—adjusted for pack-years of smoking with the use of unconditional logistic regression. Using either method, we observed a statistically significant association between female sex and the presence of a K-ras mutation in the resected tumor.

When the spectrum of mutations in the K-ras gene at codon 12 was examined by sex, we observed that the majority of mutations were G→T and occurred at the second guanine, giving rise to a cysteine. When this change was compared with all other amino acid substitutions combined, there was an excess of cysteine mutations among women, which did not reach statistical significance (OR = 2.6; 95% CI = 0.73–9.1).

To examine K-ras mutation as a marker of patient outcome, we calculated Kaplan–Meier survival probability curves for groups of patients and examined the differences by using the logrank test. Considering all adenocarcinoma case patients for whom we had survival data (n = 195), there was a statistically significant association between K-ras mutation and decreased survival time (Fig. 2, A; P = .009, logrank test). When the K-ras survival association was examined in a stage-specific manner, it was observed that the only stratum where there was a statistically significant association between K-ras mutation and worse outcome was among stage I cases (Fig. 2, B; P = .002, logrank test).

Multivariate analysis was used to determine if the association between K-ras mutation and patient survival was independent of other outcome predictors. Variables included in the analysis were K-ras mutation status, stage, sex, and age. With the use of Cox’s proportional hazards model, K-ras mutation remained a statistically significant independent predictor of poor outcome, with a risk ratio of 1.8 (95% CI = 1.1–3.1). A similar model, restricted to stage I tumors, retained K-ras mutation as having a statistically significant association with outcome, with a risk ratio of 3.7 (95% CI = 1.6–8.6). Neither tumor size nor asbestos exposure history was a statistically significant or confounding parameter in the Cox model.

**DISCUSSION**

In this large, prospective study of resectable lung cancer, we found that codon 12 K-ras mutations occurred predominantly in adenocarcinomas and only among those subjects with a history of cigarette smoking; however, there was no association of mutation with duration of smoking or intensity of smoking. It is likely, therefore, that tobacco carcinogens induce essentially all K-ras mutations and that they occur quite early in the clonal evolution of lung cancers. If this were true, it would explain why there is a requirement for tobacco carcinogen exposure but no association with measures of lifelong cigarette consumption. It is interesting that, in experimental studies, the overexpression of ras genes has been shown to lead to cell senescence (47). The clonal expansion of ras mutant cells requires a second event to abrogate this normally lethal cellular event. We, therefore, hypothesize that the initiated cells containing K-ras mutations undergo clonal expansion in vivo through the action of secondary events, such as enhanced expression of growth factors or alterations in genes such as those controlling cell cycle checkpoints.

We also observed that women were statistically significantly more likely to have mutations in codon 12 of the K-ras oncogene. There have been reports of a preponderance of K-ras mutations in males, but these were small studies, which primarily included only nonsmoking women, and none of the studies were prospective in design (as is the current study). Hence, the results of these other investigations either are not comparable or were subject to various selection biases (22,26,27,36). At the same time, the in-
crease in lung cancer incidence among women that has been consistently reported in recent years is clearly associated with an increase in adenocarcinoma incidence (1–13). Our data suggest that one mechanism for this increase might involve hormonally mediated selection of K-ras mutant clones. The second event that is needed to negate the ras-induced senescence experimentally observed by Serrano et al. (47) might be related to hormonal effects on cells of an adenocarcinoma histology. It is known that the adenocarcinoma precursor cell can exhibit estrogen binding and express estrogen receptors (48). Furthermore, Kato et al. (49) have shown that K-ras mutation can enhance the expression of estrogen receptors and induce cell transformation in NIH3T3 mouse embryo fibroblast cells. Finally, Caracta et al. (50) have observed that adenocarcinoma cell lines, unlike other NSCLC cell lines, express both estrogen receptor α and estrogen receptor β. Consequently, our data are consistent with the following sequence: Cigarette smoking induces K-ras mutation. The resultant clones are further expanded by a second event that may involve the growth-promoting effects of hormones (like estrogen) that may be specific for the adenocarcinoma histology. This model would account for the increased occurrence of K-ras mutations that we observed in women.

We also investigated the outcome of surgical therapy in the patients studied and found a statistically significant association between the presence of a K-ras mutation and aggressive disease. The proportion of tumors with K-ras mutation was slightly higher with increasing stage, and those tumors with mutation tended to be larger and to have associated lymph node metastasis. Associations of K-ras mutation with worsened patient survival are consistent with many previous studies (19–28). In addition, this study demonstrates that K-ras mutation has potential predictive value in a U.S. population that includes both men and women.

Of particular import is the very strong association between K-ras mutation and poorer survival among patients with stage I tumors, supported by results from other groups (19,20,22,25,27). In addition, our data suggest that the K-ras—survival association may be specific to stage I disease. One hypothesis consistent with these data is that K-ras mutation confers a metastatic advantage. That is, stage I K-ras mutant tumors may be more likely to have micrometastases undetected at diagnosis. Once clinically evident metastases have occurred, there is no difference in survival based on ras mutation status. Consistent with this hypothesis is the expectation that K-ras mutations are overrepresented among patients with metastasis at the time of diagnosis, as is true in this study. It must be noted, however, that the design of this study is insufficient for determining if K-ras mutation status is associated with patient outcome in advanced disease because the cohort was restricted to patients treated with surgical resection.

Data from in vitro studies are congruent with the hypothesis that K-ras mutant tumors have increased metastatic potential. Ras has repeatedly been shown to transform fibroblasts. In addition, bronchial epithelial cells transformed with the Ki-ras virus no longer arrest cell growth in response to transforming growth factor β (51). Other work has shown that mutant ras transfected into epithelial cells weakens their adhesion to the substratum (52). More recently, it has been demonstrated that the ras protein is involved in integrin activation (18). The implication from this work is that ras mutation might alter integrin functioning and, thus, change cell morphology and behavior. Since the ras protein is also linked to the rho-signaling pathway (involved in membrane ruffling), it is possible that mutation might affect the ability of the cell to crawl, which also might make ras mutants more likely to metastasize.
The strong association of K-ras mutation with patient outcome, particularly in patients with stage I adenocarcinoma, has important clinical implications that should be investigated further.

REFERENCES


**NOTES**

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