K-ras Oncogene Activation in Atypical Alveolar Hyperplasias of the Human Lung

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ABSTRACT

Atypical alveolar hyperplasia (AAH) is a potential precursor lesion from which lung adenocarcinomas arise and may be a good target for studying the early events of lung tumorigenesis. A common genetic alteration in lung adenocarcinoma is mutational activation of K-ras. To determine the timing of K-ras activation, we evaluated formalin-fixed and paraffin-embedded tissue samples of 41 AAHs and their paired lung neoplasms from 28 patients for codon 12 point mutations of the K-ras oncogene. K-ras codon 12 mutations were detected using PCR followed by allele-specific oligonucleotide hybridization. Mutations were found in 16 (39%) of the 41 AAHs, 8 (42%) of the 18 adenocarcinomas, and none (0%) of the 5 lung neoplasms that were not adenocarcinomas. Of the 18 patients with both an AAH and a synchronous lung adenocarcinoma, 6 had K-ras mutations in the adenocarcinoma but not in the AAH, 6 had mutations in the AAH but not in the adenocarcinoma, 4 did not harbor mutations in either the AAH or the adenocarcinoma, and 2 had mutations in both their AAH and their synchronous adenocarcinoma. In just 1 of the 18 patients was the same K-ras mutation present in the AAHs and adenocarcinomas of the patient. The detection of independent activating point mutations in a cancer-causing gene points to the neoplastic nature of AAH and suggests that glandular neoplasms of the lung arise from a background of field cancerization.

INTRODUCTION

The ability to study various stages of human neoplasms has been instrumental in defining the sequence of molecular events underlying tumorigenesis. For example, in the colorectum, where carcinomas develop through a well-defined sequence of distinct histological stages, early genetic alterations can be distinguished from late events (1). In the lung, the inability to define the precursor lesion of glandular neoplasms has precluded direct evaluation of the timing and sequence of molecular alterations in the development of adenocarcinomas. Indeed, this inability to appreciate a premalignant lesion has perpetuated the vague concept that lung adenocarcinomas arise de novo (2), a notion that is not compatible with current models of molecular tumorigenesis.

Mutated ras genes are the most commonly encountered activated oncogenes in human malignancies (3, 4). In the lung, activating point mutations involving codon 12 of the K-ras oncogene are detected in 24–50% of all primary adenocarcinomas, but they are only rarely observed in other types of lung carcinomas (5, 6). The timing of K-ras activation in lung tumorigenesis, however, is unresolved. Taking into account the uniform distribution of K-ras mutations in small lung adenocarcinomas, Li et al. (7) speculated that K-ras activation occurs during the earliest stages of tumorigenesis, preceding clinically evident tumor growth. Conversely, Sugio et al. (8) have argued that K-ras activation is a late event based on observations that mutations are rarely detected in preneoplastic epithelium adjacent to invasive lung carcinomas (8). Among the various preneoplastic lesions that these investigators examined, the only K-ras mutation identified was found in an area of alveolar type II dysplasia (8). Thus, although K-ras activation appears to be an uncommon event in preneoplastic lesions of the bronchioles and more central airways, further work is needed to determine its frequency in dysplasias of the alveolar epithelium, sites where some peripheral lung adenocarcinomas are believed to arise (9).

AAHs3 are focal and microscopic proliferations of epithelial cells lining the alveoli. They are usually noted as incidental microscopic findings in lungs resected for primary adenocarcinomas (10–12). The close association between AAH and adenocarcinomas suggests that AAHs may represent a precursor from which some lung adenocarcinomas arise, a proposal that has been supported by several cytomorphic studies (13, 14). The development of microdissection techniques coupled with PCR amplification has now made it possible to study molecular alterations in AAHs. Our purposes in evaluating AAHs for activating point mutations of the K-ras oncogene were a) to more fully evaluate the nature of AAH as a potential neoplasm, and b) to establish the timing of K-ras activation in lung tumorigenesis.

MATERIALS AND METHODS

Patients and Tissues. We evaluated 93 formalin-fixed and paraffin-embedded tissue samples obtained from 28 patients for point mutations at codon 12 of the K-ras oncogene. These samples included 41 AAHs, 24 synchronous primary lung neoplasms, and 28 histologically normal lung samples taken from the lung parenchyma adjacent to the AAHs. These 93 samples were obtained from 28 lung resections identified through a review of the histological slides of all lung resections performed at The Johns Hopkins Hospital from 1984 to 1995. The AAHs were identified using the criteria of Nakanishi (10). Specifically, AAHs were identified as a growth of cytologically atypical cuboidal to columnar cells along the alveolar septa in the absence of significant inflammation and fibrosis of the surrounding lung parenchyma (Fig. 1). Furthermore, AAHs were not included if they were contiguous with or directly adjacent to a primary lung tumor. AAHs tend to be microscopic lesions, and only those cases in which sufficient cells remained for DNA isolation were included in this study. Slides of the corresponding synchronous primary lung neoplasm were reviewed, and when available, an appropriate tissue block of the primary lung neoplasm was also microdissected for K-ras evaluation. Smoking histories were obtained from a review of the medical records of the patient.

Whole Genome Amplification and K-ras Mutation Detection. All analyses of AAHs were performed in duplicate on cells microdissected from consecutive glass slides in independent experiments. Cells from histologically normal lung parenchyma were microdissected from the same slides and also analyzed using whole genome amplification followed by K-ras detection. These normal lung samples served as a control for procedure-induced mutations. The synchronous primary lung neoplasms were similarly microdissected, but whole genome amplification was not performed, given the abundance of neoplastic cells in these larger neoplasms.

DNA from AAHs and normal tissue was isolated by incubation of the microdissected cells for 16–18 h at 56°C in 25 μl of a buffer containing 0.01% Tween and 100 μg/μl proteinase K. Proteinase K inactivation and DNA denaturation was accomplished by incubation for 10 min at 95°C. Whole genome amplification was performed by adding a mixture containing each

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1 The abbreviation used is: AAH, atypical alveolar hyperplasia.
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RESULTS

We identified 41 lesions that met histological criteria for AAH (Fig. 1) and that had sufficient cells for analysis. The mean size of the AAHs was 3 mm (median size, 2 mm). These AAHs were all incidental histological findings seen in the lung resection specimens from 28 patients. Three of the patients had lung resections for nonneoplastic disease (e.g., trauma, patient 7; hyalinized granuloma, patient 17; interstitial lung disease, patient 10). None of the AAHs in these 3 patients harbored K-ras mutations. Twenty-five patients had lung resections for primary lung neoplasms. In two cases (patients 21 and 24), tissue blocks of the primary neoplasms (both adenocarcinomas) were not available for analysis. One patient (patient 1) had two separate primary lung adenocarcinomas. Of the 24 primary lung neoplasms that were available for analysis, 19 (83%) were adenocarcinomas, whereas the other primary lung neoplasms included large cell carcinoma (3 cases), a small cell carcinoma (1 case), and a carcinoid tumor (1 case). This strong association between AAH and lung adenocarcinoma is consistent with the observations of others (10).

Of the 41 AAHs, 16 (39%) harbored an activating point mutation in codon 12 of the K-ras oncogene (Fig. 2). Similarly, point mutations were detected in 8 (42%) of the 19 adenocarcinomas. Mutations were not detected in any of the 5 primary lung neoplasms that were not adenocarcinomas or in any of the 28 samples of histologically normal lung parenchyma adjacent to the AAHs. A G—»T transversion at position 1 or 2 of codon 12 was the predominant type of mutation seen in both the AAHs (63%) and the primary adenocarcinomas (88%). In the AAHs, the normal GGT sequence (glycine) was mutated to TGT (cysteine) in six cases, to GTT (valine) in four cases, to GAT (aspartic acid) in four cases, and to GCT (alanine) in two cases. Seven of the primary adenocarcinomas harboring codon 12 K-ras mutations had the TGT sequence (cysteine), and one had the GAT (aspartic acid) sequence.

It is unlikely that these mutations represent DNA replication errors introduced by the pfu DNA polymerase. First, duplicate samples from the AAHs were analyzed independently for K-ras mutations, and in every instance these duplicate samples yielded concordant results. Second, no mutations were detected when small portions of histologically normal lung parenchyma from the same patients were similarly subjected to whole genome PCR amplification and K-ras mutation detection.

To better understand the relationship of AAHs to adenocarcinomas, we compared base sequences at codon 12 of K-ras in the synchronous lung neoplasms (Fig. 2). Others have found that a comparison of specific gene alterations is useful in differentiating primary neoplasms from metastases and in confirming the independent origin of multifocal neoplasms of the aerodigestive tract (16–19). Point mutations in the AAHs were found to occur independently of K-ras mutations in the adenocarcinomas. Of the 18 patients with AAHs and synchronous lung adenocarcinomas, only 2 harbored mutations in both the AAHs and the paired adenocarcinomas, and in just 1 of these 2 cases were the mutations identical. Of the remaining 16 patients, 6 had K-ras mutations in the primary adenocarcinoma but not in the AAH, 6 had mutations in the AAH but not in the adenocarcinoma, and 4 did not harbor a mutation in either the AAH or the adenocarcinoma.

Patient 1 provided the most striking example of the independent nature of K-ras activation in multifocal glandular lesions of the lung (Figs. 3 and 4). This patient had two noncontiguous primary adenocarcinomas, one arising in the left lingual and the other in the left upper lobe. In addition, 6 separate foci of AAH were dispersed throughout the parenchyma of the left lung. Comparison of this group of lesions demonstrated that the pattern of base sequences at codon 12 of K-ras was diverse. One of the adenocarcinomas and 2 of the AAHs showed the normal (wild-type) GGT sequence. Of the 5 lesions that harbored a K-ras mutation, the second adenocarcinoma showed a TGT sequence, 3 of the AAHs showed a GTT sequence, and 1 of the AAHs showed a GCT sequence.

All patients were found to be smokers (mean, 58 pack-years; median, 50 pack-years; range, 15–160 pack-years). We have shown previously that mutations in codon 12 of K-ras are much more common in lung adenocarcinomas from patients who smoke than they are in adenocarcinomas from patients who never smoked (20). Such a direct comparison between smoking and nonsmoking patient groups was not possible in the present study because all 28 patients were found to be smokers.
Fig. 2. Distribution of codon 12 K-ras mutations in atypical alveolar hyperplasias and corresponding lung neoplasms.

**DISCUSSION**

On purely morphological grounds, the nature of AAH is not clear. Whether these lesions represent early glandular neoplasia, metastases from a fully malignant bronchogenic carcinoma, or merely reactive hyperplasia is an unresolved question that has awaited molecular genetic analysis. Using a molecular approach whereby point mutations can be detected in small clonal populations of cells, we found that over one-third of AAHs harbor activating mutations of the K-ras oncogene. Clonal expansion of cells carrying activating alterations to oncogenes and tumor suppressor genes defines neoplasia at a fundamental genetic level. Accordingly, our findings suggest that AAH represents a neoplastic process rather than a reactive proliferation of nonneoplastic cells.

The detection of activating K-ras mutations points to the neoplastic nature of AAH, but it does not establish the independent origin of these lesions in early tumorigenesis. AAHs possibly could represent direct extension along the alveolar septa from a nearby lung carcinoma or metastatic implantation from a noncontiguous carcinoma. If this were the case, AAHs should consistently harbor the same K-ras codon 12 base sequences carried by the synchronous primary carcinoma. When we compared AAHs and their synchronous lung carcinomas, however, the paired lesions often did not share the same pattern of base changes at codon 12 of K-ras. These discordancies suggest that AAHs arising in patients with primary lung carcinomas are distinct lesions.

The independent nature of K-ras activation points to field cancerization as the molecular basis underlying the multifocality of lung cancer. Patients with primary lung adenocarcinomas often have additional glandular neoplasms. In a careful evaluation of resection specimens from patients with primary lung adenocarcinomas, in which the specimens were thinly sectioned and thoroughly sampled, 19% were found to have additional adenocarcinomas (11), and 25% were found to have foci of AAH (12). The genetic mechanisms underlying the multifocality of lung cancer are not well understood. On the basis of the observation that allele-specific chromosome 3 deletions may be widespread throughout the respiratory epithelium of patients with lung cancers, some investigators have raised the possibility of single-clone expansion (21, 22). According to this theory, multicentric lung neoplasms arise from the progeny of a single cell. Our results show that multicentric glandular neoplasms of the lung often harbor independent K-ras mutations. This finding is consistent with the original concept of "field cancerization"; namely, that multiple cell groups undergo neoplastic transformation under the stress of regional carcinogenic activity (23). At present, we have not evaluated AAHs for allelic loss on chromosome 3 or other presumed early alterations that may precede K-ras activation. Conceivably, AAHs could represent distinct subclones arising from a single progenitor clone.

Given the independent nature of K-ras activation in the setting of multifocal glandular neoplasia of the lung, it is difficult to draw definite conclusions regarding the evolution of AAH to adenocarcin-
Early stage in the development of lung adenocarcinomas. First, we provide further circumstantial evidence that AAH may represent an malignant progression. On the other hand, some of our findings represent harmless neoplastic proliferations without the potential for malignant progression. For example, in the absence of resection specimens from heavy smokers (13). Epidemiological studies have convincingly implicated the constituents of tobacco smoke as important initiators and promoters of lung carcinogenesis (24, 25). Third, we found that AAHs and lung adenocarcinomas share a similar mutational profile at codon 12 of the K-ras oncogene. The frequency of codon 12 K-ras mutations in AAHs and lung adenocarcinomas is comparable (39% versus 41%, respectively), and the predominant type of mutation is a G→T transversion at position 1 or 2 for both AAHs and lung adenocarcinomas (63% versus 88%, respectively).

The above findings have several relevant implications. From a pathological perspective, the finding of a clonal alteration to a recognized oncogene confirms the neoplastic nature of AAH. From a molecular biology perspective, the identification of a microscopic neoplasm provides a potential target for studying the early events of lung tumorigenesis. Further studies are needed to address the potential of these small lesions to progress to malignant cancers and to identify other genetic alterations that may participate in this progression. From a clinical point of view, the identification of a marker for early glandular neoplasia may be an initial step toward the development of molecular screening strategies for the early detection of lung adenocarcinomas. Other studies have shown that molecular analysis of exfoliated cells is potentially feasible in detecting clinically silent human neoplasms (26-30). Of particular note, K-ras mutations harbored in primary carcinomas have been recovered and identified in clinical samples, including sputum (27, 30). At the same time, optimism regarding the molecular screening of exfoliated cells in the early detection of glandular neoplasms of the lung must be tempered by our other findings. A significant proportion of AAHs, like many lung adenocarcinomas, do not harbor codon 12 K-ras mutations. Thus, lung tumorigenesis can progress through other genetic pathways that do not require activation of the K-ras oncogene. Furthermore, until the natural history of AAH is better understood, it remains uncertain whether K-ras analysis will be relevant in predicting the biological and clinical progression of early glandular neoplasia.

REFERENCES

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