LKB1 Protein Expression in the Evolution of Glandular Neoplasia of the Lung

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ABSTRACT

Purpose: About one-third of sporadic lung adenocarcinomas demonstrates biallelic inactivation of the LKB1 gene, but the timing of this event is not known.

Design: We performed LKB1 immunohistochemistry on 35 primary lung adenocarcinomas and 96 atypical adenomatous hyperplasias (AAH), a form of early glandular neoplasia from which some lung adenocarcinomas arise.

Results: In all cases, strong cytoplasmic staining was noted in the non-neoplastic epithelium lining the airways from the bronchi to the terminal bronchioles. There was a marked reduction in LKB1 staining in 9 of 35 (26%) adenocarcinomas and in 10 of 96 (10%) AAHs. When the AAHs were subclassified on the basis of cytoarchitectural atypia, loss of LKB1 expression was more frequent in the high-grade lesions (7 of 33, 21%) than low-grade lesions (3 of 63, 5%; P = 0.021). For the 21 adenocarcinomas where the genetic status was known, immunohistochemistry staining reliably reflected the activational state of the LKB1 gene (95% concordancy).

Conclusions: In AAH, loss of LKB1 expression is strongly associated with severe dysplasia, suggesting that LKB1 inactivation may play a role in the critical transition from premalignant to malignant tumor growth.

INTRODUCTION

Lung cancer remains the leading cause of cancer-related mortality in the United States for both men and women. Approximately 172,000 new cases of lung cancer will be diagnosed this year. Of those affected, 85% will not survive 5 years (1).

Adenocarcinomas now represents the most frequent subtype of lung cancer (2), and they are usually discovered late in the course of the disease even in the setting of vigilant radiographic and cytologic screening (3). Novel strategies based on the detection of genetic markers offer new hope for improved risk assessment, early cancer detection, and tumor surveillance, but the impact of these strategies has been limited by an incomplete understanding of the biology of lung cancer. Disappointedly, only a few genetic alterations critical to the development of lung adenocarcinomas are currently recognized, and the timing of these alterations during glandular neoplasia remains to be delineated.

LKB1, also known as STK11, is a serine/threonine kinase that functions as a suppressor of tumor growth. Germ-line mutations of LKB1 cause Peutz-Jeghers syndrome, an autosomal-dominant disorder characterized by gastrointestinal hamartomatous polyposis, mucocutaneous pigmentation, and an increased risk of intestinal malignancies (4–6). Importantly, patients with germ-line mutations are also at an increased risk of developing extra-intestinal malignancies, including occasional lung adenocarcinomas (7–9). As a tumor suppressor gene, LKB1 may also be a target of inactivation in the evolution of certain sporadic malignancies.

Indeed, we recently reported mutations in about one-third of sporadic lung adenocarcinomas, implicating LKB1 inactivation as one of the more frequent and important genetic events in the development of lung adenocarcinomas (10).

AAH is a precursor lesion from which some lung adenocarcinomas arise (11, 12). They represent localized proliferations of enlarged and atypical epithelial cells lining intact alveolar septae and are usually noted as incidental microscopic findings in lungs resected for primary adenocarcinomas. Morphological and genetic evidence suggests that AAH encompasses a heterogeneous population of lesions representing different points along the progression toward overt lung adenocarcinoma (13, 14). Under the light microscope, the cytological and architectural atypia that characterize AAH falls along a spectrum ranging from mild to severe (13–15). At the genetic level, the frequency of p53 alterations and loss of heterozygosity at 3p, 9p, and 17p tend to increase in proportion to the severity of atypia (13–17). As such, AAH is now valued as a good target for studying the timing and sequence of the genetic alterations driving multistep lung tumorigenesis, and it represents a useful lesion for addressing the timing of LKB1 inactivation in the development of lung adenocarcinomas.

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2 The abbreviations used are: AAH, atypical adenomatous hyperplasia; IHC, immunohistochemistry; BAC, bronchioloalveolar carcinoma.
MATERIALS AND METHODS

Patients and Lesion Classification. One component of the study group consisted of 15 patients identified through a review of the histopathologic slides of all lung resections performed at The Johns Hopkins Hospital from 1995 to 2002. From the lung resections of these 15 patients, 96 AAHs and 14 paired primary lung cancers were available for LKB1 IHC. The other component of the study group consisted of 21 patients with primary lung adenocarcinomas that were already well characterized with respect to LKB1 gene as reported previously (10). Briefly, 30 primary lung carcinomas had been screened using an extensive panel of highly polymorphic microsatellite markers that map to the LKB1 region of chromosome 19p. Of these, 21 carcinomas (all adenocarcinomas) showed loss of heterozygosity at 19p and on this basis were selected for complete analysis of the LKB1 gene to search for: (a) point mutations in all exons and intron–exon boundaries; (b) partial and complete deletions and insertions; and (c) gene promoter hypermethylation using methylation sensitive PCR. The medical records and histopathologic slides were reviewed to obtain information regarding stage, grade, and type of the lung carcinomas.

The AAHs were identified using the criteria of Nakanishi (18). Specifically, AAHs were identified as a growth of cytotopically atypical cuboidal to columnar cells along the alveolar septa. Lesions were not included that showed frank invasion of the lung parenchyma or if they showed direct contiguous extension from a primary lung carcinoma. The AAHs were graded by one of us (W. H. W.) without knowledge of LKB1 staining, using criteria established previously (13–15). AAHs classified as low grade were characterized by a single layer of round to cuboidal cells with low to moderate cellular density; small cell size; minimal variation in cell size, shape, and chromaticity; and minimal thickening of the alveolar septa (Fig. 1A). By comparison, AAHs classified as high grade had increased cellular density, larger cell size, greater variation in cell size and shape, and thickening of the alveolar septa (Fig. 1C). Although some of these high-grade lesions showed striking cytoarchitectural atypia, they were very small and did not show invasion of the lung parenchyma.

LKB1 IHC. Sections (5 μm) were deparaffinized, treated with sodium citrate epitope retrieval buffer (Ventana-Bio Tek Solutions; Ventana Medical Systems, Tucson, AZ), and then steamed for 20 min at 90°C. After cooling for 5 min, the slides were labeled with a rabbit polyclonal anti-LKB1 antibody (Cell Signaling Technology, Beverly, MA) using the Bio Tek-Mate 1000 automated stainer (Ventana Medical Systems). This anti-LKB1 antibody recognizes the COOH terminus of human LKB1. Labeling with the primary antibody was carried out at a

Fig. 1  Histological progression and LKB1 staining of AAH. Low-grade AAH characterized by small atypical cells lining a delicate intersitium (A). In high-grade AAH, there is cell crowding, increased cytologic atypia, and more prominent thickening of the septal walls (C). LKB1 staining is limited to the atypical cells and not seen in the adjacent normal lung parenchyma in both the low-grade (B) and high-grade (D) lesions.
1:20 dilution. The LKB1 antibody was visualized using the avidin-biotin technique (DAKO LSAB Kit; DAKO Cytomation, Carpinteria, CA).

The non-neoplastic airway epithelium served as an internal positive control. For the AAHs and adenocarcinomas, the following criteria were used to guide measurement of LKB1 staining: (a) interpretation was limited to areas of the neoplasm adjacent to normal airways, such that staining intensity could be interpreted relative to internal control; (b) LKB1 staining was classified as positive if the staining intensity in the neoplasms matched or exceeded the staining intensity of the normal airway epithelium; and (c) conversely, LKB1 staining was classified as negative if the staining intensity in the neoplasms was reduced relative to the normal airway epithelium. A pancreatic adenocarcinoma with a known homozygous deletion of chromosome 19p encompassing the LKB1 gene served as a negative control. Staining was interpreted as positive or negative without knowledge of the genetic status of the LKB1 gene.

RESULTS

We tested 96 AAHs and 35 primary lung adenocarcinomas for LKB1 inactivation by IHC. The 96 AAHs included 63 low-grade and 33 high-grade lesions. An example of each AAH category is shown in Fig. 1. The AAHs ranged in size from 0.1 to 7 mm. The mean size of the low-grade lesions was 1.5 mm. The mean size of the high-grade lesions was 3.3 mm. All of the AAHs were incidental histological findings in lung resection specimens from 15 patients. One of these patients had a lung resection for non-neoplastic disease, and the other 14 had lung resections for primary lung carcinomas. All of the lung carcinomas were adenocarcinomas, including 2 nonmucinous BACs and 12 non-BACs. Of the 21 lung adenocarcinomas that were not associated with AAH, 4 were BACs, and the other 17 were non-BACs. The pathologic stage of the lung carcinomas ranged from T1 \( (n = 28) \) to T2 \( (n = 5) \) to T3 \( (n = 1) \) to T4 \( (n = 1) \), and the histological grade ranged from well \( (n = 13) \) to moderate \( (n = 16) \) to poor \( (n = 6) \).

In the non-neoplastic lung tissue, strong LKB1 staining was limited to the epithelium lining the airways from the large central bronchi to the terminal bronchioles (Fig. 2). Staining tended to be diffuse throughout the cytoplasm, but there was occasional accentuation of staining in the apical cytoplasm of the ciliated columnar cells. The only other cell type that demonstrated strong LKB1 staining was the chromogranin-positive neuroendocrine cell (i.e., Kulchitsky cells). LKB1 staining was observed in the normal epithelium lining the pancreatic ducts, but it was not observed in the control pancreatic adenocarcinoma that harbored a homozygous deletion of chromosome 19p encompassing the LKB1 gene.

LKB1 staining of the epithelium lining the alveolar septa was weak or entirely absent. In striking contrast, 86 of the 96 (90%) AAHs demonstrated strong LKB1 staining (Fig. 1, B and D). Although negative LKB1 staining was not common in AAH overall, negative staining was much more frequent in high-grade lesions. Loss of LKB1 expression was noted in only 3 of the 63 (5%) low-grade lesions but in 7 of 33 (21%) high-grade lesions. In the overly malignant lung adenocarcinomas, negative LKB1 staining was noted in 9 of 35 (26%) tumors (Fig. 3). LKB1 protein inactivation increased in frequency with progression from low-grade AAH to high-grade AAH to overt adenocarcinoma (Fig. 4). The differences in LKB1 staining in this progression were significant \( (P = 0.005, \text{Cochran-Armitage test for trend}) \). By logistic regression estimates, differences in staining were significant when the low-grade AAH was compared with high-grade AAH \( (P = 0.021) \) and adenocarcinoma \( (P = 0.006) \). The differences in LKB1 staining were not significant between high-grade AAH and lung adenocarcinoma \( (P = 0.662) \). There was no relationship between LKB1 staining in an AAH and its
paired lung carcinoma. In other words, LKB1 expression in the primary lung carcinoma was not predictive of LKB1 staining in the paired AAHs. For the lung adenocarcinoma, there was no correlation between LKB1 staining and tumor subtype (BAC versus non-BAC), grade, or stage.

For the 21 adenocarcinomas where the genetic status was known, LKB1 IHC staining reliably reflected the activational state of the LKB1 gene (Table 1). Fifteen of the lung adenocarcinomas did not show biallelic inactivation, and all of these tumors exhibited LKB1 staining. Conversely, negative LKB1 staining was noted in 5 of the 6 of the lung adenocarcinomas that demonstrated biallelic gene inactivation.

DISCUSSION

Lung cancer doggedly persists as the leading cause of cancer-related mortality (1). Novel strategies based on the molecular genetic profiling of lung cancer offer renewed hope for early cancer detection and effective therapy, but recognition of the genes responsible for lung tumorigenesis remains a daunting challenge. LKB1 is a tumor suppressor gene that encodes a serine/threonine kinase (4–6). Germ-line mutations of LKB1 typically give rise to oral mucocutaneous hyperpigmentation, intestinal hamartomas, and gastrointestinal malignancies, but there is also an increased risk of extraintestinal malignancies, including occasional lung adenocarcinomas (7–9). Inactivation of LKB1 is also observed in some sporadic lung adenocarcinomas (19). We have shown previously that about one-third of lung adenocarcinomas harbor inactivating alterations of the LKB1 gene, resulting in complete loss of protein expression or the production of a truncated protein (10). Thus, alterations of the LKB1 tumor suppressor gene appear to be one of the more common and relevant genetic events in the development of lung adenocarcinoma. In the present study, we used an immunohistochemical approach to determine the distribution of LKB1 protein expression in the lung and various stages of glandular neoplasia of the lung.

The expression of LKB1 in human adult tissues remains to be more fully characterized. Previous studies using Northern
blot and reverse transcription-PCR analyses indicate that LKB1 is widely expressed in most adult tissue types (5, 6), but the cell-specific patterns of LKB1 expression are difficult to discern from studies of gross tissue extracts. In situ and immunohistochemical methods for LKB1 expression permit greater resolution of LKB1 localization within adult tissues. Using an in situ hybridization approach, Rowan et al. (20) confirmed widespread expression in adult tissues, including the lung. In addition, they observed that LKB1 mRNA expression was: (a) strictly confined to the epithelia; (b) increased in cells undergoing continuous or rapid division; and (c) lost in a subset of carcinomas. We were able to validate these findings using an immunohistochemical approach. In the non-neoplastic adult lung, LKB1 protein expression was confined to the epithelium lining the airways. Cytoplasmic staining tended to be strong and intense from the large bronchi to the terminal bronchioles. LKB1 protein expression was considerably attenuated in the more static epithelial cells lining the alveoli (i.e., pneumocytes and Clara cells).

Just as the immunohistochemical observations support some widespread functional role for LKB1 protein expression in epithelial tissues of the lung, they also suggest that loss of this functional activity may play some role in the pathogenesis of a subset of sporadic lung adenocarcinomas. A portion (26%) of the lung adenocarcinomas demonstrated absent or reduced LKB1 protein expression by IHC, a frequency predicted by the known prevalence of biallelic gene inactivation in lung adenocarcinomas (10). Indeed, there was strong correlation between status of the LKB1 gene and predicted immunohistochemical staining. Of the 15 lung adenocarcinomas that did not show biallelic inactivation of the LKB1 gene, all demonstrated strong LKB1 staining. Conversely, of the 6 lung adenocarcinomas with biallelic inactivation of the LKB1 gene, 5 showed negative LKB1 staining. Disparity between LKB1 gene status and protein expression was noted in only a single case (case 4), where strong staining was noted in a carcinoma that demonstrated loss of heterozygosity in the region of LKB1 and a point mutation giving rise to a stop codon.

For some critical tumor suppressor genes, such as p53, inherent sensitivity and specificity problems render IHC an unreliable indicator of true gene status (21). This does not appear to be the case for LKB1 IHC. Although LKB1 IHC may underestimate the true incidence of biallelic inactivation in lung adenocarcinomas (as a result of tumor heterogeneity, binding of a polyclonal antibody to truncated protein, or some other mechanism), overall, it is a reliable method for assessing LKB1 gene status. As IHC is rapid, inexpensive, and widely available to most diagnostic laboratories, this method may circumvent the need for more cumbersome genetic evaluation of the LKB1, particularly when sufficient quantities of tumor-rich DNA are available. As an early stage of glandular neoplasia, AAH is valued as a good target for delineating the timing and sequence of genetic alterations in the development of lung adenocarcinomas. In turn, the study of AAHs has been helpful in formulating a molecular genetic progression model for certain lung cancers (11). According to the current understanding of glandular neoplasia of the lung, activation of the K-ras oncogene seems to be an early event involved in the initiation of AAH (12, 22). Progression of AAH through increasing degrees of morphological dysplasia requires the silencing of key tumor suppressor genes, such as p16 (17). Ultimately, activation of telomerase (23) and inactivation of the p53 tumor suppressor gene (13–16) appear to be important in triggering invasive tumor growth. Like p53, inac-

\begin{table}
\centering
\caption{Correlation between biallelic inactivation of the LKB1 gene and LKB1 protein expression as determined by immunohistochemistry$^a$}
\begin{tabular}{ll}
\hline
Case & Biallelic gene inactivation & Protein expression \\
\hline
1 & Yes & Negative \\
2 & Yes & Negative \\
3 & Yes & Negative \\
4 & Yes & Positive \\
5 & Yes & Negative \\
6 & Yes & Negative \\
7 & No & Positive \\
8 & No & Positive \\
9 & No & Positive \\
10 & No & Positive \\
11 & No & Positive \\
12 & No & Positive \\
13 & No & Positive \\
14 & No & Positive \\
15 & No & Positive \\
16 & No & Positive \\
17 & No & Positive \\
18 & No & Positive \\
19 & No & Positive \\
20 & No & Positive \\
21 & No & Positive \\
\hline
\end{tabular}
\end{table}

$^a$ Biallelic inactivation as evidenced by loss of heterozygosity for microsatellite markers near the LKB1 locus, coupled with an LKB1 non-sense mutation or promoter hypermethylation as reported previously.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Loss of LKB1 expression across various stages of glandular neoplasia of the lung. Loss of LKB1 expression increases in frequency from low-grade AAH to adenocarcinoma. The difference in frequency of LKB1 expression was statistically significant ($P = 0.005$).}
\end{figure}
activation of LKB1 likely participates in the critical transition from premalignant to malignant tumor growth. LKB1 expression is generally very high in AAh relative to its expression in normal epithelial cells lining the alveoli. Negative LKB1 staining was detected in only a small percentage of AAh. However, when AAh is subclassified on the basis of its morphological features, negative staining increases with the severity of cytoarchitectural atypia. Negative staining was observed in only 5% of the low-grade lesions but in ≤21% of the high-grade lesions.

The natural history of early glandular neoplasia is not well understood. In particular, the frequency and pace at which AAh progresses to overtly malignant lung adenocarcinoma are not known. Longitudinal observation of AAh now permitted by high-resolution imaging techniques indicates that the mere presence of AAh does not necessarily indicate sure and unremitting progression to adenocarcinoma (24). Indeed, AAh appears to represent a morphologically and genetically diverse group of lesions with varying potentials for malignant transformation (13, 25). Unraveling the molecular genetic alterations that initiate the development and drive the progression of these lesions might provide new insight into the biology of lung cancer and facilitate the design of novel strategies for early detection of lung cancer. Alterations of LKB1 function may represent one of the critical steps in the transition from a benign to a potentially malignant proliferation of pneumocytes.

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