Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma

Marie Wislez, MD,*† David G. Beer, PhD,‡ Ignacio Wistuba, MD,*, Jacques Cadran, MD, PhD,* Nagahiro Saijo, MD,§ and Bruce E. Johnson, MD||

Abstract: The charge of the Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma Committee was to evaluate the molecular biology, genomic changes, and proteomic findings in patients with bronchioloalveolar carcinoma compared with other types of lung cancer. The literature was reviewed and unpublished information was presented by the committee members at the session. The molecular biology studies have included findings on epidermal growth factor receptor (EGFR) mutations, p53 mutations, K-ras mutations, and loss of heterozygosity. The genomic changes have mostly focused on the miRNA expression arrays as well as protein studies. The current state of knowledge was reviewed, the missing information was acknowledged, and proposals for future research were identified.

Key Words: Lung neoplasm, Adenocarcinoma, Bronchioloalveolar, Adenocarcinoma, Carcinoma, Non-small cell lung cancer.

(J Thorac Oncol. 2006;1: S8–S12)

Little information is available about p53 mutations and p53 protein overexpression detected by immunohistochemistry, microsatellite loss of heterozygosity (LOH), and K-ras mutations in adenocarcinoma of the bronchioloalveolar subtype, according to the last World Health Organization (WHO) pathological classification proposed in 1999. However, the frequency of these molecular abnormalities seems to increase during the multistep process of carcinogenesis of peripheral adenocarcinoma going from atypical alveolar hyperplasia adenocarcinoma to bronchioloalveolar carcinoma (BAC) and to invasive adenocarcinoma.

ATYPICAL ADENOMATOUS HYPERPLASIA

There is an increasing body of evidence to support the concept of atypical adenomatous hyperplasia (AAH) as the precursor of at least a subset of adenocarcinomas.1 AAH is most frequently detected in lungs from patients bearing lung cancers (9–20%), especially adenocarcinomas (up to 40%) compared with squamous cell carcinomas (11%).2 Several molecular changes frequently present in lung adenocarcinomas are also present in AAH lesions, and there is further evidence that AAH may represent true preneoplastic lesions.1 The most important findings are the presence in AAHs of K-ras (codon 12) mutations (40%),3 loss of LKB1 function (20%),4 allelic losses in chromosomes 3p (20%), 9p (p16INK4a, 10%), 9q (50%), 17q, and 17p (TP53, 5%),5,6 and overexpression of cyclin D1 (70%), p53 (ranging from 10 to 60%),7 and survivin (50%).8 Despite the evidence that AAH is a precursor lesion for a subset of lung adenocarcinomas, there is general consensus that the pathogenesis of most adenocarcinomas is still unknown. The findings of relatively infrequent tyrosine kinase domain epidermal growth factor receptor (EGFR) mutations in AAH lesions (three out of 40 examined)9,10 and no EGFR mutation11,12 or relatively low frequency in true BACs of the lung9 support the concept that genetic abnormalities of EGFR are not relevant in the pathogenesis of alveolar types of lung neoplasia. In addition, Tang et al.13 recently reported that EGFR mutation is an early event in the pathogenesis of lung cancer, being identified in histologically normal epithelium of small bronchi and bronchioles adjacent to EGFR mutant lung adenocarcinomas in nine out of 21 (43%) patients examined, but in none of the patients without mutation in the tumor. These data further support the notion than AAH lesions are not involved in the pathogenesis of EGFR mutant lung adenocarcinomas.

BAC, ADENOCARCINOMA WITH BRONCHIOALVEOLAR FEATURES, AND ADENOCARCINOMA OF THE LUNG

The frequency of EGFR mutations has also been studied in patients with BAC, adenocarcinoma with BAC features, and adenocarcinomas of the lung. Although responses to EGFR tyrosine kinase inhibitors have been reported to be higher14 and EGFR mutations were preferentially observed in tumors having BAC features,12,15 we did not find association with the BAC subtype of adenocarcinoma in 97 cases from...
the United States\(^\text{11}\) using the criteria stated by the 1999 WHO classification of lung tumors.\(^\text{16,17}\)

In addition to the WHO system, Noguchi et al.\(^\text{18,19}\) have classified adenocarcinomas into different categories that have different frequencies of genetic changes. Koga et al.\(^\text{20}\) reported that p53 mutations were present in approximately 0\% of 17 pure BAC, 11\% of 27 mixed adenocarcinoma with BAC features, and 48\% of 101 invasive adenocarcinomas. Similar to the frequency of mutations, the frequency of p53 protein overexpression detected by immunohistochemistry increased from 6\% (2/32 tumors) in pure BAC to 28\% (27/133) in BAC with foci of active fibroblastic proliferation (Noguchi type C) and to 40\% (14/35) in adenocarcinoma.\(^\text{21}\) p53 mutation and protein overexpression were also correlated with the size and invasive component of small peripheral adenocarcinomas (\(\geq 5\) mm: 41\%; <5 mm: 20\%).\(^\text{22,23}\)

The frequency and type of K-\textit{ras} mutation in BAC are related to the cytological features (mucinous versus nonmucinous). This raises the question of whether the mucinous form might represent a biological entity separate from the nonmucinous form. Small series of tumors (all <50) from patients with adenocarcinoma of the lung show that the K-\textit{ras} mutation is present in 73 to 100\% of the mucinous types and that the type of the mutation was usually G to A (codon 12), whereas it was seen in 10 to 43\% in the nonmucinous types, usually in G to T transversions.\(^\text{25-27}\) Mutations at codon 12 of the K-\textit{ras} oncogene were found in 39\% of 41 AAH, 42\% of 18 adenocarcinomas, and none of five lung neoplasms that were not adenocarcinomas. Of the patients with both an AAH and a synchronous adenocarcinoma, more than half did not have the mutation in both the AAH and the synchronous lung adenocarcinoma, suggesting that peripheral adenocarcinomas arise not always from AAH but sometimes directly from a background of field cancerization.\(^\text{27}\)

Adenocarcinomas with BAC features are also characterized by an intense inflammatory reaction especially containing alveolar neutrophils and macrophages. Increased numbers of tumor-infiltrating neutrophils are linked to poorer outcomes in these patients.\(^\text{28}\) Tumor environment drives local neutrophil recruitment and activation via C-X-C chemokine release such as interleukin-8 and epithelial cell–derived neutrophil activating protein 78 but also prolongs alveolar neutrophil recruitment and activation via C-X-C chemokine receptor 4 (Noguchi type C) and to 40\% (14/35) in adenocarcinoma.\(^\text{21}\) p53 mutation and protein overexpression were also correlated with the size and invasive component of small peripheral adenocarcinomas (\(\geq 5\) mm: 41\%; <5 mm: 20\%).\(^\text{22,23}\)

The frequency and type of K-\textit{ras} mutation in BAC are related to the cytological features (mucinous versus nonmucinous). This raises the question of whether the mucinous form might represent a biological entity separate from the nonmucinous form. Small series of tumors (all <50) from patients with adenocarcinoma of the lung show that the K-\textit{ras} mutation is present in 73 to 100\% of the mucinous types and that the type of the mutation was usually G to A (codon 12), whereas it was seen in 10 to 43\% in the nonmucinous types, usually in G to T transversions.\(^\text{25-27}\) Mutations at codon 12 of the K-\textit{ras} oncogene were found in 39\% of 41 AAH, 42\% of 18 adenocarcinomas, and none of five lung neoplasms that were not adenocarcinomas. Of the patients with both an AAH and a synchronous adenocarcinoma, more than half did not have the mutation in both the AAH and the synchronous lung adenocarcinoma, suggesting that peripheral adenocarcinomas arise not always from AAH but sometimes directly from a background of field cancerization.\(^\text{27}\)

Adenocarcinomas with BAC features are also characterized by an intense inflammatory reaction especially containing alveolar neutrophils and macrophages. Increased numbers of tumor-infiltrating neutrophils are linked to poorer outcomes in these patients.\(^\text{28}\) Tumor environment drives local neutrophil recruitment and activation via C-X-C chemokine release such as interleukin-8 and epithelial cell–derived neutrophil activating protein 78 but also prolongs alveolar neutrophil survival through the production of soluble antiapoptotic factors (granulocyte–macrophage colony–stimulating factor and granulocyte colony–stimulating factor).\(^\text{29,30}\) The mechanisms by which neutrophils influence the prognosis of these types of tumors, especially through its mitogenic and scattering properties, favoring c-Met expressing tumor-cell migration along the alveolar basal membrane.\(^\text{33}\) Lastly, neutrophils might be involved in luminal tumor spread by promoting tumor-cell shedding (M. Wislez, AACR 2004), described pathologically as the presence of micropapillary clusters that are also involved in the mechanism of aerogenous progression.\(^\text{34}\)

### GENOMIC AND PROTEOMIC STUDIES OF BAC

As mentioned before, BAC is thought to arise from AAH and is potentially an intermediate to invasive adenocarcinoma. Extensive analyses of BAC using gene-expression profiling and proteomic-based studies have not yet been performed and are only available for limited numbers of these cancers. These types of studies may have the potential to define similarity or differences in the observed types of adenocarcinoma of the lung. Of particular interest is the potential regulatory pathway involved in the lepidic growth patterns of BAC, which is different from most other adenocarcinomas of the lung. The observation that some adenocarcinomas can exhibit regions of BAC provides complexity and has resulted in multiple pathological-based classifications.\(^\text{14,16-19}\) Genomic studies have the potential to define the similarities as well as key differences between BAC, adenocarcinomas with BAC features, and adenocarcinomas of the lung.

Recent studies examining individual genes have hinted at differences between BAC and adenocarcinomas. The tumor suppressor in the lung cancer-1 gene encodes an adhesion molecule and is frequently associated with LOH at that locus in non–small-cell lung cancer. Both normal lung cells and BAC retain expression of tumor suppressor in lung cancer-1, whereas 63\% of adenocarcinomas demonstrated decreased expression detected by immunohistochemistry.\(^\text{35}\) BACs have very low p53 DNA mutation frequencies compared with adenocarcinomas of the lung.\(^\text{20}\) LOH at the 3p FHIT loci was observed in 43\% of BAC, and 12th codon K-\textit{ras} mutations are detected in the mucinous form of BAC.\(^\text{36}\) A comparative LOH study between 14 BAC and 20 stage I lung adenocarcinomas using nine chromosomal regions revealed that the most frequently affected chromosomal regions in BAC were 8q and 17p.\(^\text{37}\) In adenocarcinomas of the lung, LOH at 1p, 3p, 7q, and 18q was more frequent than in BAC, and fractional allele loss was greater in adenocarcinomas of the lung than BAC.

Using immunocytochemistry to examine protein expression, detection of the thyroid transcription factor-1 (TTF-1), cytokeratin 7, and cytokeratin 20 were measured in both mucinous and nonmucinous BAC.\(^\text{38}\) TTF-1 was detected in 17\% of mucinous and 94\% of nonmucinous BAC, cytokeratin 7 was detected in 100\% of mucinous and 23\% of nonmucinous BAC, and cytokeratin 20 was detected in 60\% of mucinous and 0\% of nonmucinous BAC.\(^\text{38}\) In a study that examined MUC protein expression in AAH, BAC, and adenocarcinomas with BAC features, MUC1 decreased from

Copyright © 2006 by the International Association for the Study of Lung Cancer

S9
AAH to BAC and from BAC to adenocarcinoma, whereas MUC2, MUC5AC, MUC6, and depolarized MUC6 increased. Alterations in p53 and the increased expression of MUC1, MUC5AC, and MUC6 were noted.

ADDITIONAL GENOMIC AND PROTEOMIC STUDIES

A comparison of normal lung tissue and BAC using oligonucleotide arrays was reported by Goodwin et al. and identified 12 up-regulated and six down-regulated genes in the BAC tumors. Although this analysis provides some information, a comparison of BAC and adenocarcinomas was not included, which may be most relevant in defining critical genes involved in the development of these cancers. We used oligonucleotide arrays to examine gene expression in 14 BAC and 73 adenocarcinomas. The most highly expressed genes that were significantly different between the BAC tumors and adenocarcinomas and higher in BAC included the surfactant pulmonary-associated proteins A1, A2, C and D, MUC1, TTF-1 and TTF-3, villin 2, and prostat gland syntheatin D2 synthetase. Interestingly, higher mRNA expression for both fos and jun B were detected in BAC, which may reflect an elevated AP-1 activity and upstream signaling events in these tumors. The higher level of expression of surfactant genes is consistent with the well-differentiated phenotypic characteristics of BAC. TTF-1 was the most differentially expressed gene between BAC and adenocarcinomas, consistent with the high TTF-1 protein expression reported in BAC. Because of the small numbers of tumors for our analyses, it was not possible to divide the BAC tumors into separate categories such as mucinous, nonmucinous, and mixed histology. Although we found MUC1 mRNA present in both BAC and adenocarcinomas of the lung, the significantly increased expression in BAC is consistent with the higher MUC1 protein levels that have been reported in these tumors.

Analysis of survival-related genes revealed prostaglandin D2 synthetase and neutrophil elastase 2 to be more highly expressed in BAC than the other adenocarcinomas. In contrast, much lower levels of vascular endothelial growth factor were detected in the BAC, possibly reflecting a lesser level of angiogenesis and hypoxia in these tumors relative to the adenocarcinomas. Adenocarcinomas also expressed increased levels of metallothionein 2A and thioredoxin reductase mRNA. We speculate that these genes may correspond to smoking-related alterations because these genes may change in response to reactive oxygen species originating from tobacco smoking or in response to inflammatory cells. Alternately, the expression of thioredoxin reductase and metallothionein 2 may reflect the higher rates of cell proliferation in the lung adenocarcinomas relative to BAC.

Few, if any, large-scale proteomic analyses of BAC have been reported. We examined the same BAC and lung adenocarcinomas for mRNA using oligonucleotide arrays and also at the protein level with two-dimensional gel electrophoresis and mass spectrometry. A total of 682 protein spots were quantified, and 75 proteins were found to differ significantly (p < 0.05) between BAC and lung adenocarcinomas. Thirty-eight protein spots were successfully identified using mass spectrometry. Of interest were the relatively higher expression of the ras-related protein RAB-14, glutathione-s-transferase-pi, cytokeratin 7, and three isoforms of the selenium-binding protein 1 in BAC compared with adenocarcinomas of the lung. Adenocarcinomas expressed higher levels of phosphoglycerate kinase 1, pyruvate kinase M1/M2, and stathmin (OP-18) compared with BACs. Increased phosphoglycerate kinase 1 is consistent with higher hypoxia-induced glycolysis in the adenocarcinomas of the lung relative to BAC.

Future studies that include sufficient numbers of the various histological subtypes of BAC are needed to provide insight into the similarities and differences among these tumors and as compared with lung adenocarcinomas. The NCI Director’s Challenge: Validation Study of Lung Adenocarcinomas will examine gene expression using Affymetrix 133A oligonucleotide arrays among approximately 500 tumors. Thus, a relatively large number of BACs will be included in this study, allowing potential gene pathways to be defined that may be relevant to our understanding of the growth- and cell-signaling systems in BAC. These analyses will also incorporate detailed pathologic assessment of each tumor so that the subtypes of each BAC can be compared. It is expected that these data, made available to the research community, will then stimulate further research into potential new markers for early diagnosis and possible therapeutic intervention strategies that may be effective for BAC.

FUTURE DIRECTIONS

The Committee responsible for Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma outlined studies that will provide further insights into BAC. The most important part of the meeting was partial agreement and understanding about the interpretation of the pathological classification. The participants in the meeting agreed on a common set of descriptors for the pathological interpretation of BAC that will be used more consistently in the future.

| TABLE 1. Different Biological Properties in Atypical Adenomatous Hyperplasia, Pure Bronchioloalveolar Cancer, Adenocarcinoma with Bronchioloalveolar Cancer Features, and Adenocarcinoma of the Lung |
|----------------------------------|------------------|----------------|------------------|----------------|
| EGFR mutation                   | ↓ <5%            | 10%            | ↓ 0%             | ↑ 40%          |
| TP53 mutations                  | Not reported     | ↓ 0%           | ↓ 10%            | ↑ 50%          |
| p53 by immunohistochemistry     | Not reported     | ↓ 5%           | ↑ 30%            | ↑ 50%          |

Copyright © 2006 by the International Association for the Study of Lung Cancer
Upcoming technological improvements will provide additional insights into the biology of BAC. These will include the increasing ability to detect genetic changes in BAC and adenocarcinomas including, but not be limited to, EGFR, HER-2/neu, B-raf, K-ras, and TP53. In addition, there is the ability to detect genetic loss in the whole genome using studies with single-polynucleotide polymorphisms or array chromosomal genomic hybridization. There is increasing ability to use small and smaller amounts of DNA and DNA from paraffin-embedded tissues. Future studies will provide information on the degree of genetic changes seen in early lesions (<1cm) that are being detected more often as computerized tomographic scanning of the chest is becoming more widely used. These findings can be compared with the more advanced lesions. The genetic changes can also provide insights into the clonality of the BACs to determine whether the multiple lesions in the lungs arise from single or multiple clones. Table 1

REFERENCES


