Prognostic Significance of Mucin and p53 Expression in Stage IB Non-small Cell Lung Cancer

A Laboratory Companion Study to CALGB 9633

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Introduction: Cancer and Leukemia Group B 9633 was a phase III trial that randomized patients with stage IB non-small cell lung cancer to observation or four cycles of carboplatin and paclitaxel. A statistically significant effect in favor of adjuvant chemotherapy was seen for disease-free survival (DFS) and overall survival (OS) in the subgroup of patients with tumors ≥4 cm. A laboratory companion study was conducted to see whether molecular and clinical factors could provide additional prognostic information.

Methods: Formalin-fixed, paraffin-embedded blocks were obtained for 250 of the 344 patients enrolled. Immunohistochemical staining for bcl-2, p53, blood group antigen A, and mucin was correlated with DFS and OS.

Results: The prevalence of the markers was bcl-2, 17%; p53, 47%; blood group antigen A, 25%; and mucin, 45%. Univariate analysis for DFS showed a statistically significant effect for the presence of mucin ($p = 0.0005$) and p53 ($p = 0.05$) and for OS showed a significant effect for mucin ($p = 0.0005$). In the multivariate Cox model, there was a statistically significant association between shorter DFS and presence of mucin ($p = 0.002$; hazard ratio [HR] 2.05) and p53 ($p = 0.003$; HR 1.95) and between shorter OS and presence of mucin ($p = 0.004$; HR 2.03) and p53 ($p = 0.0005$; HR 2.30). Of the clinical factors, male gender and larger tumor volume were also significant adverse prognostic factors ($p < 0.05$).

Conclusions: A statistically significant association between shorter DFS and OS was seen for the patients with p53 protein expression, mucin expression, male gender, and larger tumors in this cohort of patients with stage IB non-small cell lung cancer treated on Cancer and Leukemia Group B 9633.

Key Words: Non-small cell lung cancer, prognostic factors, p53, mucin.

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Lung cancer is the leading cause of cancer-related mortality for both men and women in the United States. In 2008, it is estimated that there will be 215,000 new cases and 162,000 deaths from lung cancer.[1] The World Health Organization estimates 1.2 million new cases annually, making it the most common cancer worldwide.[2] Non-small cell lung cancer (NSCLC) makes up approximately 85% of all newly diagnosed cases of lung cancer.

Recently reported randomized clinical trials have reported the benefit of cisplatin-based adjuvant chemotherapy for the patients with early stage NSCLC.[3–5] These trials have suggested survival benefit for stages II and IIIA, but the benefit for those with stage IB disease has been questioned.[6] The randomized phase III trial, Cancer and Leukemia Group B (CALGB) 9633, is the only study that studied the patients with stage IB disease exclusively.[7] In this study, after surgical resection, patients were randomized to observation or four cycles of carboplatin or paclitaxel. The final results have been reported recently, and although there were trends for superior disease-free and overall survival (OS) for the chemotherapy group, these did not reach statistical significance. A retrospective subset analysis did show a statistically significant survival advantage for patients with tumors ≥4 cm.

As part of this clinical trial, formalin-fixed, paraffin-embedded tissue blocks were collected for a planned laboratory companion study to evaluate the ability of selected clinical and biologic markers to add to the well-established

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prognostic value of tumor stage. At the time, several large single institution studies had suggested the potential prognostic value of a number of molecular and biologic factors including microvessel density, tumor size, K-*ras* mutation, and expression of p53, blood group antigen A, bcl-2, and mucin.[8–10]

The purpose of this study was to investigate the prognostic significance of selected markers, including p53 expression, loss of blood group antigen A, bcl-2, and mucin as determined at the time of protocol initiation.

MATERIALS AND METHODS

CALGB 9633 was a randomized phase III trial of observation versus four cycles of carboplatin or paclitaxel after surgery for patients with stage IB NSCLC. Eligibility criteria included histologically proven NSCLC, T2 with pathologically negative lymph nodes at mediastinoscopy or at the time of thoracotomy. Patients were required to undergo at least a lobectomy. Patients were randomized 4 to 8 weeks after surgery, and chemotherapy was given every 3 weeks. Treatment and collection of paraffin-embedded blocks was approved by all involved institutional review boards.

Formalin-fixed, paraffin-embedded tumor tissue blocks were obtained prospectively and sent to the CALGB Pathology Coordinating Office. Four-micron sections were cut and placed on the glass slides. One slide was stained with hematoxylin and eosin to facilitate interpretation. All studies were performed without knowledge of clinical outcome.

A modification of the avidin-biotin-peroxidase complex technique was used for immunohistochemical (IHC) studies.[11,12] For p53, the sections were treated with enzyme digestion and a high temperature antigen unmasking technique to facilitate labeling with p53 clones 240 and 1801 (Labvision Corporation, Fremont, CA) antibody cocktail. This antibody cocktail produces nuclear staining and detects p53 overexpression. Positive (strongly reactive colon cancer) and negative controls (normal colon) were used. For bcl-2, the monoclonal antibody clone 124 (Dako, Carpinteria, CA) was used, and for blood group antigen A, the monoclonal antibody clone 81FR 2.2 was used (Biogenex Laboratories, San Ramon, CA). Details of the procedures used are described in detail elsewhere.[13,14] The results for p53, bcl-2, and blood group antigen A were graded from 0 to 4 according to the intensity of the staining and from 0 to 4 by the percentage of positive cells: 0; 1, $<$25%; 2, 25 to $<$50%; 3, 50 to $<$75%; and 4, 75–100%. Positives were considered $\geq$2 nuclear (p53 and bcl-2) or membrane (blood group antigen A) staining in any percentage of cells, and the slides were read by one pathologist (A.H.T.).

The general histochemical technique to detect mucin was used, and the details are published elsewhere.[15] Mucicarmine that detects all mucins was used. Briefly, the slides were deparaffinized and hydrated in distilled water, exposed to Mayer's hematoxylin for 10 minutes, washed in running tap water for 5 minutes, and then exposed to Southgate's Mucicarmine solution at room temperature for 1 hour. The slides were then rinsed quickly in distilled water and exposed to Metanil yellow stain for 30 to 60 seconds. The slides were then dehydrated quickly in three changes of absolute alcohol until clear and then coverslipped in Permount (The Science Company, Denver, CO). The mucin stain was a deep rose color. A positive result was the presence of mucicarmine positive secretory vacuoles within tumor cells or within the lumens of tumor glands. In general, however, the mucicarmine result was kept independent from the subclassification of the tumor, which was determined by just the routine hematoxylin and eosin stain. In other words, positive mucicarmine results were not used to reclassify tumors as adenocarcinomas, and negative mucicarmine results were not used to reclassify tumors as nonadenocarcinomas. The slides were interpreted by one pathologist (R.T.V.).

The primary objective of CALGB 9633 was to determine whether adjuvant chemotherapy improved OS after resection of stage IB NSCLC. The major endpoint of this laboratory study was to determine the prognostic importance of the specific markers on disease-free survival (DFS) and OS. In addition, complete clinical information was available on each patient including sex, age, performance status (PS), surgical procedure, tumor size (from pathology report), tumor node metastasis staging, presence of symptoms (chest pain, cough, and hemoptysis), and smoking history (ever smoker versus nonsmoker).

OS was defined as a time from random assignment to death from any cause. DFS was defined as a time from random assignment to recurrence or death, whichever comes first. The Kaplan-Meier product-limit estimator was used to estimate DFS and OS for subgroups of patients with stage IB NSCLC as defined by the presence or absence of the various markers.[16] The log-rank test was used to compare subgroups.[17] Cox proportional hazards model was used to examine the joint effect of markers on DFS and OS. Additional analyses using the Cox model were conducted to further examine the effect of markers on DFS and OS after adjusting for the effects of chemotherapy and other baseline prognostic factors, and whether there was an interaction between treatment and the presence of a marker.[18] Statistical analyses were performed by CALGB statisticians (L.G. and X.W.) using SAS 9.1. All p values reported are two sided.

Patient registration and clinical data collection were managed by the CALGB Statistical Center. As a part of the quality assurance program of the CALGB, members of the Audit Committee visit all participating institutions at least once every 3 years to review source documents. The auditors verify compliance with federal regulations and protocol requirements, including those pertaining to eligibility, treatment, adverse events, tumor response, and outcome in a sample of protocols at each institution. Such on-site review of medical records was performed for a subgroup of 136 patients (40%) of the 344 patients under this study.

RESULTS

CALGB 9633 was activated October 15, 1996 and closed on January 6, 2004. During that time period, 344 subjects were registered to this protocol and randomly assigned to one of the two arms—observation or adjuvant chemotherapy. Six patients were canceled or never treated (one on observation and five on chemotherapy). Seven pa-

