BCL-2 Expression is Prognostic for Improved Survival in Non-small Cell Lung Cancer

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Objective: We used a large patient population to identify immunohistochemical biomarkers to enable improved prognostication in patients with non-small cell lung carcinoma (NSCLC).

Methods: A tissue microarray was constructed using duplicate 0.6 mm cores of formalin-fixed paraffin-embedded tissue blocks from 609 patients with NSCLC. Immunohistochemical was used to detect 11 biomarkers including epidermal growth factor receptor, Her2, Her3, p53, p63, bcl-1, bcl-2, Thyroid transcription factor, carcinoembryonic antigen, chromogranin, and synaptophysin. A clinical database was generated prospectively at the time of tissue collection. Survival outcomes were obtained from a Provincial Cancer Registry database. Univariate and multivariate analyses were performed to look for a relationship between biomarker expression, smoking history, and survival.

Results: Survival data for 535 cases were available. As of June 2005, 429 patients (80%) had died; of these 286 (54%) died of lung cancer, 117 (22%) died of other known causes, and for 26 (5%) the cause of death was not available. Univariate analysis revealed that bcl-2 (p = 0.007) was the only biomarker prognostic for improved overall survival (OS). bcl-2 (p = 0.021) and p63 (p = 0.025) were both found to be prognostic for improved disease-specific survival (DSS). Multivariate analysis (using age and biomarker expression) revealed that bcl-2 expression is prognostic for improved OS (p = 0.005) and DSS (p = 0.021).

Conclusions: Our results suggest that bcl-2 expression is prognostic for improved OS and DSS in NSCLC. Testing for bcl-2 expression in a prospective study will help to determine its clinical relevance in prognostication.

Key Words: Non-small cell lung cancer, Prognostic markers, Immunohistochemistry, bcl-2, EGFR.

Lung cancer is the most prevalent of all cancers in North America. Non-small cell lung cancer (NSCLC) comprises the majority of lung cancer and there are a number of factors known to affect prognosis. The most useful prognostic factor is stage of the disease. Other prognostic factors include surgically resectability of the tumor and pathologic features such as tumor size, histologic subtype, lymphatic and blood vessel invasion, and tumor differentiation. Useful clinical prognostic factors include performance status and weight loss. Recently, there has been a great deal of interest in using molecular markers to refine prognosis.

Immunohistochemical (IHC) markers in NSCLC have been the subject of a recent review that divided them into several groups based on the proposed function of the molecule. One such group is molecules responsible for the self-sufficiency of growth pathway, and includes epidermal growth factor receptor (EGFR), Her 2, and cyclin D1. Studies of the prognostic value of EGFR expression reported variable results, some suggesting a negative association, and other studies failing to find any prognostic significance. Studies of Her 2 have also revealed conflicting results. Cyclin D1, which causes rearrangement of bcl-1, has been found to be associated with a negative prognosis. A second group of markers studied are those involved in resistance to apoptosis, particularly p53 and bcl-2. Studies of p53 have found conflicting results regarding prognosis, whereas its analogue p63 has been suggested to have a positive prognostic value. The prognostic utility of bcl-2 was recently reviewed in a meta-analysis that concluded that bcl-2 expression is associated with a positive prognosis.

Other markers that have been examined include chromogranin (Ch) and synaptophysin (SNP), both neuroendocrine markers with unclear prognostic significance. Thyroid transcription factor (TTF-1), a lung cancer tumor marker, and carcinoembryonic antigen (CEA) are also potentially useful markers with uncertain prognostic significance.

In a review of IHC markers of prognosis in NSCLC, Zhu et al. described the inconsistent results found with most markers. These findings may be explained by the markers studied thus far having limited prognostic utility, or possibly because of variable methodologies and populations used. Added to these issues are the small sizes of many of the studies, which have been combined in meta-analyses that are difficult to interpret because of methodological variability.
To address these issues, we took a large patient population and used tissue microarray technology to enable staining of numerous tissue samples at once, thereby minimizing variability associated with multiple staining techniques, to examine 11 biomarkers for their association with prognosis.

METHODS

Case Selection

Cases of primary NSCLC from patients with early stage (stage I and II) disease, diagnosed between 1978 and 2002, were identified from the archives of St. Paul’s hospital (A large tertiary center in Vancouver, British Columbia). Other than resectable lung cancer, no other specific selection criteria were used. Tissue blocks were used to construct a duplicate core tissue microarray. Carcinoids, atypical carcinoids, large cell neuroendocrine carcinomas, and metastatic tumors were all excluded.

Tissue Microarray Construction

Tissue microarrays were constructed as previously described. Briefly, areas containing tumor were marked on the paraffin tissue blocks. Sector maps were designed using Microsoft Excel (Redmond, WA) spreadsheets to identify the location of each specimen on the array blocks. Duplicate 0.6-mm tissue cores were used to construct the tissue microarrays with an arraying machine (Beecher Instruments Sun Prairie, WI). Array blocks were sectioned to produce serial 4-μm sections, and the first section was stained with hematoxylin and eosin to assess adequacy. The remaining sections were stored at room temperature for 3 weeks before immunostaining.

Biomarker Selection

Eleven biomarkers were chosen for assessment. The markers were selected based on previous data suggesting possible prognostic utility outlined in the introduction, and antibody availability. The methods and results of the staining for the neuroendocrine markers (Ch and SNP) have been previously reported.26 Score results for the duplicate cores were consolidated into 1 score with higher positive staining results always superseding weaker positive, negative, or uninterpretable staining results.

Immunohistochemistry and Scoring

Staining for each marker was conducted using standard antibodies (Table 1). The staining techniques varied according to the manufactures recommendations. All samples were evaluated and scored by two pathologists blinded to outcome information. The scoring was performed independently and the discrepant scores were resolved by double-scoping and discussion of cases in question. A third pathologist was used as an arbitrator for cases in which a consensus could not be reached. The scoring system for the 11 biomarkers is summarized in Table 2. The final results were reported as negative (score = 0), positive (score = 1, 2, or 3), or uninterpretable, with the exception of Her2 staining in which 0 and 1+ are considered negative and 2+ and 3+ positive. Details of Her2 scoring are reported elsewhere.27 Staining in nontumor cells was not considered. The scores were entered into blank sector maps of the corresponding array at the time of scoring, and uninterpretable results were eliminated from further consideration.

Data Analysis and Statistics

Data on the score sheets were converted into an Excel spreadsheet format using the TMA-Deconvoluter program as previously described.28 Score results for the duplicate cores were consolidated into 1 score with higher positive staining results always superseding weaker positive, negative, or uninterpretable staining results.

Survival estimates were calculated for each outcome (overall survival (OS) and disease-specific survival (DSS)), and a log-rank statistic was used to test for differences between groups. Log-rank statistics were used to look for any relationships between biomarker expression and survival. A significant difference was declared if the p value from a two-tailed test was less than 0.05. Multivariate analysis using age, and biomarker expression were tested using Cox regression analysis. Statistical calculations were performed using SPSS 13.0 software (SPSS, Chicago, IL).

RESULTS

The tissue microarray was constructed from 609 patients with early stage (stage I and II) NSCLC who had their tumors resected between 1978 and 2002 (Table 3). A total of 588 of 609 cases were available for inclusion in the analysis. Hematoxylin–eosin stained sections were reviewed and subclassified as follows: 243 adenocarcinoma (ACA), 272 squamous cell carcinoma (SCC), 35 large cell carcinoma, 32 non-small cell carcinoma NOS, and 6 other (carcinoma, giant cell carcinoma). Twenty-one cases were excluded because on review, they were not considered to represent primary lung tumor (12 cases), exhibited neuroendocrine differentiation (three cases) or no pathologic diagnosis was available (six cases). Table 4 provides a summary of the scoring results for each biomarker. The results for Ch and SNP have been previously reported.26 No prognostic significance was noted for these two neuroendocrine markers.

Outcome data were available for 535 patients up to 26.5 years (median 3.52 years; range 34–9696 days) and these were used for survival analysis. As of June 2005, 429 patients (80%) had died; of these 286 (54%) died of lung cancer, 117 (22%) died of other known causes, and for 26 (5%) the cause of death was not available (Table 3). The latter cases were excluded from the DSS analysis.
Ever smokers (#; percent) 438; 95%
Age of diagnosis (mean; range) 63; 35–82

Biomarker Expression Scoring Results (See Table 2 for a Description of the Scoring System)

<table>
<thead>
<tr>
<th>Stain Name</th>
<th>Staining Pattern</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Nuclear</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>p63</td>
<td>Nuclear</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>Bcl-1</td>
<td>Cytoplasmic</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Cytoplasmic</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>TTF</td>
<td>Nuclear</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>CEA</td>
<td>Cytoplasmic</td>
<td>&lt;5</td>
<td>5–50 weak/moderate</td>
<td>&gt;5 strong OR</td>
<td>&gt;50 weak/moderate</td>
</tr>
<tr>
<td>EGFR</td>
<td>Nuclear/cytoplasmic</td>
<td>&lt;5</td>
<td>5–25</td>
<td>25–75</td>
<td>&gt;75</td>
</tr>
<tr>
<td>Her2</td>
<td>Membranous (see methods)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Her3</td>
<td>Nuclear/cytoplasmic</td>
<td>0</td>
<td>Any cells, weak</td>
<td>Any cells, strong</td>
<td>Any cells, strong</td>
</tr>
</tbody>
</table>

Number (#) of male patients; Number of female patients 400; 209
Age of diagnosis (mean; range) 63; 35–82
Ever smokers (#; percent) 438; 95%

<table>
<thead>
<tr>
<th>n</th>
<th>No. of years of smoking (mean; range)</th>
<th>Total # of patients with outcome data</th>
<th>Median survival (yr)</th>
<th>Range of survival (d)</th>
<th>Follow up (mean, range of yr)</th>
<th>No. of living</th>
<th>No. of deceased</th>
<th>No. of deceased from lung cancer</th>
<th>No. of death from other known causes</th>
<th>No. of with cause of death unavailable</th>
</tr>
</thead>
<tbody>
<tr>
<td>459</td>
<td>40; 0–69</td>
<td>535</td>
<td>3.5 yr</td>
<td>34–9673 d</td>
<td>5.8; 4–26.5</td>
<td>106 (20%)</td>
<td>429 (80%)</td>
<td>286 (54%)</td>
<td>117 (22%)</td>
<td>26 (5%)</td>
</tr>
</tbody>
</table>

There were no significant associations between biomarker expression and survival (OS and DSS) noted within histologic subtypes (other than what would be expected by chance alone), (Tables 6 and 7). For bcl-2, the relationship between expression and OS was not significant within histologic subtypes, other than in the small group of NSCLC not otherwise specified adenocarcinoma (p = 0.087), squamous cell carcinoma (p = 0.14), NSCLC not otherwise specified (p = 0.035), large cell carcinoma (p = 0.162). These findings may be related to the smaller sample size within the individual histologic subgroups.

A multivariate analysis model using age and biomarker staining pattern was used to look for an association between biomarker expression and OS. The only biomarker found to have a significant association with survival was bcl-2. After allowing for age, bcl-2 expression remained significantly associated with increased survival, both OS (p = 0.005) and DSS (p = 0.021) for all NSCLC. A further multivariate analysis was conducted including age, gender, biomarker expression, and histologic subtype. Stage was not included in this analysis, because although all patients were early stage, detailed staging information (stage I versus II) was not available for most patients. The results again confirmed that bcl-2 expression was associated with OS (p = 0.004) and DSS (p = 0.018).
DISCUSSION

A NSCLC tissue microarray was used to investigate the associations between IHC markers and survival. Univariate analyses demonstrated significant associations for bcl-2 with OS, and both bcl-2 and p63 with DSS. After allowing for the effect of age, only the association with bcl-2 expression remained.

There has been extensive research on IHC biomarkers in NSCLC with limited success in identifying markers that have clinical utility. To date, none of the markers tested have significant prognostic utility, which may reflect heterogeneity in the methods used in these studies. Specifically, this relates to variability in antibody use, scoring methods, and the different disease stages of patients included. This heterogeneity is especially important when interpreting the numerous meta-analyses that have been performed in this area. Through the use of tissue microarray technology, we were able to overcome many of these issues and study a large population to identify several biomarkers that seem to have prognostic utility, the most compelling being bcl-2.

bcl-2 is a proto-oncogene involved in cellular apoptosis. A meta-analysis performed in 2003 suggested that overexpression of bcl-2 in NSCLC was associated with improved survival.22 Using univariate analysis, we confirmed this, finding bcl-2 expression to be associated with both OS (p = 0.007) and DSS (p = 0.021). Multivariate analysis using age and marker expression also revealed an association between bcl-2 expression and both OS (p = 0.012) and DSS (p = 0.029). These results suggest that bcl-2 expression is prognostic for improved survival. In contrast, none of the other biomarkers we examined demonstrated a strong association with survival. Overexpression of p63, an analogue of p53, has

TABLE 5. Univariate Analysis of the Prognostic Significance of Biomarkers on Overall Survival (OS) and Disease-Specific Survival (DSS)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Association with OS (All Non-small Cell Lung Cancer) (P)</th>
<th>Association with DSS (All Non-small Cell Lung Cancer) (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (n = 467)</td>
<td>0.65</td>
<td>0.137</td>
</tr>
<tr>
<td>p63 (n = 457)</td>
<td>0.29</td>
<td>0.025 (longer DSS)</td>
</tr>
<tr>
<td>Bcl-1 (n = 452)</td>
<td>0.054</td>
<td>0.434</td>
</tr>
<tr>
<td>Bcl-2 (n = 451)</td>
<td>0.007 (longer OS)</td>
<td>0.021 (longer DSS)</td>
</tr>
<tr>
<td>TTF (n = 463)</td>
<td>0.188</td>
<td>0.799</td>
</tr>
<tr>
<td>CEA (n = 454)</td>
<td>0.2</td>
<td>0.443</td>
</tr>
<tr>
<td>EGFR (n = 459)</td>
<td>0.233</td>
<td>0.809</td>
</tr>
<tr>
<td>Her 2 (n = 455)</td>
<td>0.611</td>
<td>0.635</td>
</tr>
<tr>
<td>Her 3 (n = 457)</td>
<td>0.83</td>
<td>0.214</td>
</tr>
</tbody>
</table>

OS, overall survival; DSS, disease-specific survival.
previously been demonstrated to be associated with improved survival.\textsuperscript{21} We also found p63 expression to be associated with DSS in all NSCLC ($p = 0.025$) using univariate analysis, but this association was no longer present after allowing for the effect of age. We did not find any association of EGFR expression and survival, in keeping with the findings of a previous meta-analysis.\textsuperscript{12}

It is unclear why expression of bcl-2 is associated with improved prognosis. On the basis of the current understanding of bcl-2 function, one would expect that bcl-2 expression would enable cells to escape apoptosis and therefore would be associated with a worse prognosis. One possible explanation for why bcl-2 expression may be associated with a good prognosis comes from the breast cancer literature. In breast cancer, bcl-2 expression has also been associated with improved prognosis. Martinez-Arribas et al.\textsuperscript{29} recently published a study demonstrating that bcl-2 expression is associated with biologic features of tumors, which define a better prognosis. Therefore, although bcl-2 may function to promote tumor growth, its expression may be associated with other features of tumors that define a more favorable prognosis.

There is interest in the targeted therapies against bcl-2, and there is ongoing research regarding this therapeutic modality in various tumor sites.\textsuperscript{30} In the future, bcl-2 may be found to be a useful predictive marker to predict response to these therapies. There is also a chance that bcl-2 expression may have a predictive role regarding response to nontargeted therapies. There is preliminary evidence\textsuperscript{31} that p53 expression predicts response to vinorelbine and cisplatin. Further studies are required to demonstrate whether bcl-2 expression has any predictive role.

The major strength of this study is the use of a large patient population to investigate biomarkers that may have prognostic utility. Because of the large size, we are able to avoid the heterogeneity that has made it difficult to interpret meta-analyses of smaller studies. Our study provides compelling evidence that bcl-2 is an important prognostic marker in NSCLC, as it is associated with improved OS and DSS. p63 may also have a prognostic role although our results are less definitive. This study was limited in that no postsurgical treatment information was available for the patients included in the database, which may have contributed to differences in survival. In addition, detailed staging information (stage I versus II) was not available for most patients. A prospective study is required to determine the clinical relevance of these biomarkers, and our results suggest that bcl-2 is worthy of such a trial. The hope for the future is that biomarkers such as bcl-2 can be used to individualize prognosis and potentially guide therapeutic decisions.

### REFERENCES


