Carcinoids account for no more than 2% of all primary lung tumors (1, 2). According to their histologic phenotype, bronchopulmonary carcinoids have been further classified as typical (TC) and atypical (AC), the latter being associated with a worse prognosis than the former. Quite recently, the WHO typified these tumors (1, 2). According to their histologic phenotype, bronchopulmonary carcinoids were correlated with the tumor phenotype assessed by combining conventional histology with a panel of immunohistochemical markers exploring cell differentiation (chromogranin, NSE, TTF1), cell turnover (Mib1), and apoptosis (Bcl2, Bax). The histologic distinction between low-grade typical and intermediate-grade atypical bronchopulmonary carcinoids basically lies on cellular differentiation, mitotic activity, and presence of “neoplastic” necrosis; at single patient level, however, none of these features enables a reliable prediction of the clinicopathologic outcome.

Experimental Design: The long-term postsurgical outcome of a single-institution series of 67 radically treated bronchopulmonary carcinoids was correlated with the tumor phenotype assessed by combining conventional histology with a panel of immunohistochemical markers exploring cell differentiation (chromogranin, NSE, TTF1), cell turnover (Mib1), and apoptosis (Bcl2, Bax).

Results: Fifty-eight (86.6%) carcinoids were assessed as low-grade typical and nine (13.4%) were assessed as intermediate-grade atypical. The mean follow-up was of 85.13 months (range, 28-168; median, 82.0). All cases expressed neuroendocrine markers, whereas TTF1 was never expressed. At univariate analysis, tumor recurrence (n = 6) correlated significantly with the carcinoid histotype (P = 0.002) and with each of the following variables: tumor location (P = 0.01), mitotic index (P = 0.003), necrosis (P = 0.002), tumor vascular invasion (P = 0.0001), Mib1 expression (P = 0.005), Bcl2 expression (P = 0.024), and synchronous node metastasis (P = 0.028). The best cutoffs for Mib1 and Bcl2 expression (calculated by receiver operating characteristic curves) discriminating recurrent versus nonrecurrent tumors were 5.4% for Mib1 and 2.0% for Bcl2 (Mib1: sensitivity, 83%; specificity, 97%; area under curve, 0.844 ± 0.14; Bcl2: sensitivity, 83%; specificity, 65%; area under curve, 0.769 ± 0.12). By stratifying the patients according to the obtained cutoffs, significant differences emerged in the patients’ disease-free survival (log-rank test: Mib1, P = 0.0001; Bcl2, P = 0.01).

Conclusions: Mib1 and Bcl2 significantly discriminate between recurrent versus nonrecurrent tumors, producing a biologically plausible, diagnostically suitable immunohistochemical pattern.
immunohistochemical evaluation of proliferative activity has been suggested (10). Ki67 (Mib1) is a nuclear antigen expressed in proliferating cells, yielding positive staining in 0.2% to 1.1% of LG-TC and in 0.3% to 20.3% of IG-AC (11–16); in addition, strong Mib1 expression has been associated with unfavorable clinical outcome (17, 18).

Apoptosis deregulation is crucial to tumorigenesis: Bcl2 suppresses apoptosis and is counteracted by Bax, which heterodimerizes with Bcl2, thereby promoting programmed cell death. A few long-term follow-up studies have considered Bcl2/Bax expression in bronchopulmonary carcinoids: Bcl2-positive nuclei are more frequently detected in IG-AC than in LG-TC (11, 19–23) and a shorter survival has been reported in association with both Bcl2 overexpression (18) and Bax down-regulation (20).

In this retrospective study, the long-term postsurgical outcome of a single-institution series of radically treated bronchopulmonary carcinoids was correlated with tumor phenotype using conventional histology combined with a panel of immunohistochemical markers exploring cell differentiation, cell turnover, and apoptosis.

Materials and Methods

Patients. All cases of bronchopulmonary carcinoid (and cases termed as well/moderately differentiated neuroendocrine tumor/carcinoma) reported between 1993 and 2003 were retrieved from the archives of the Department of Pathology of Padova University. Of the 191 cases found initially, 124 were subsequently excluded for the following reasons: (a) shortage of clinically relevant information (demographics, tumor location and/or size, and/or patients lost to follow-up or followed up for <36 months), 59 cases; (b) prior surgical or endoscopy treatments, incomplete surgical resection, or inadequate sampling of mediastinal nodes, 11 cases; (c) archival tumor sample inconsistent with the study aims (due to tumor size or tissue samples being unsuitable for immunohistochemical tests), 46 cases; (d) modification of the original histologic diagnosis, 3 cases. Informed consent from involved patients was obtained in all but five cases (which were ruled out). Thus, 67 patients were considered, who had all been treated surgically at the same institution (Thoracic Surgery Unit of Padova University) where the bronchopulmonary neoplasia had all been completely resected (as confirmed, where necessary, by frozen section histology) and the mediastinal nodes had been sampled. Postsurgical antineoplastic therapy was never provided unless the tumor recurred.

Pathology. The gross features of the tumor were obtained from both the gross description of the specimen as recorded at the time of surgery and/or from the original histology report.

Serial sections (4-6 μm thick) were obtained from archival paraffin-embedded tissue samples and were stained with Hematoxylin & Eosin for histologic reassessment or used for immunophenotyping.

In each case, the following histologic features were jointly assessed by two pathologists (M.F. and R.C.) unaware of any clinical details: (a) growth pattern (infiltrative i.e., tumor growth in form of isolated/small clusters of cells) versus expanding (i.e., tumor with well defined margins); ref. 24; (b) nuclei/cytoplasm ratio (classified as follows: Cn, nucleus predominant over cytoplasm in two thirds or more of the cell population; cn, cytoplasm predominant over nucleus in two thirds or more of the cell population; cn, cell population consisting of cells polymorphic in size and shape, with no consistent Nc or Cn features); (c) mitotic index, number of mitoses per 10 high-power fields; (d) necrosis defined as present or absent (only necrosis consisting of either punctate foci or larger areas of so-called “neoplastic” necrosis were considered); (e) vascular invasion was recorded as present or absent; lymphatic and blood vessels were grouped together because of the difficulty in distinguishing between them.

Based on H&E staining alone, cases were ultimately classified as LG-TC or IG-AC according to both Travis’s (4) and the WHO criteria (3). Immunophenotyping. A panel of commercial antisera against the following antigens was applied in all cases, including the following:

- NSE (clone BBS/NC/VI-H14; DAKO; working dilution 1:300; incubation time, 32 min at 37°C).
- Chromogranin A (clone DAK-A3; DAKO; working dilution 1:100; incubation time, 32 min at 37°C).
- TTF1 (clone 8G7G3/1; DAKO; working dilution 1:100; incubation time, 32 min at 37°C).
- Mib1 for human ki-67 antigen (clone code no. M7240; DAKO; working dilution 1:100; incubation time, 32 min at 37°C).
- Bcl-2 oncoprotein (clone 124; DAKO; working dilution 1:50; incubation time, 32 min at 37°C).
- Bax (polyclonal antibody; Assay Designs; working dilution 1:100; incubation time, 60 min at room temperature).

Using the standard avidin-biotin-peroxidase complex method, staining was done automatically (Ventana Benchmark XT system) with the Ventana ES secondary detection kit peroxidase/3,3′-diaminobenzidine. Sections were then lightly counterstained with hematoxylin.

### Table 1. Tumor location, and histologic and immunohistochemical variables in all cases considered

<table>
<thead>
<tr>
<th>Location</th>
<th>Growth</th>
<th>Mitotic rate</th>
<th>NC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central/peripheral</td>
<td>Expanding/infiltrative</td>
<td>Number x 10 hpf</td>
<td>Cn</td>
</tr>
<tr>
<td>All cases, 67</td>
<td>47/20</td>
<td>0.69 ± 1.62 (M = 0.0)</td>
<td>59</td>
</tr>
<tr>
<td>LG-TC, 58</td>
<td>44/14</td>
<td>0.34 ± 0.60 (M = 0.0)</td>
<td>54</td>
</tr>
<tr>
<td>IG-AC, 9</td>
<td>8/1</td>
<td>4.33 ± 4.16 (M = 3.0)</td>
<td>5</td>
</tr>
</tbody>
</table>

NOTE: Necrosis, vascular invasion, and synchronous nodal metastases are reported as present (+) or absent (-); Mib1, Bcl2 and Bax are reported as a percentage of positive nuclear immunoreaction; tumors are distinguished as recurrent or nonrecurrent. All values are also shown for LG-TC and IG-AC. The results of the statistical analysis are given on the differences between LG-TC and IG-AC. Tumor recurrence is also reported.

Abbreviations: M, median; NC ratio, nucleus/cytoplasm ratio (distinguished according to predominant tumor cytology, see text); N, necrosis; VI, vascular invasion; LN, synchronous nodal metastases; R+ve, recurrent; R-ve, nonrecurrent; F, Fisher’s exact test; MW, Mann-Whitney test; ns, nonsignificant.
Appropriate positive and negative controls were run concurrently for all the applied antisera.

The expression of each immunohistochemical marker was jointly scored by two of the authors (M.F. and R.C.). Chromogranin A and NSE expression was dichotomized as absent or present (a definite immunoreaction was always detected in the tumor cells).

Nuclear TTF1 immunoreaction was categorized as positive or negative (alveolar epithilia were used as a positive internal control).

Mib1, Bcl2, and Bax expression was scored in 2,000 tumor cells and expressed as a percentage of positive nuclei.

Statistical analysis. Univariate analysis was done on each variable. The Mann-Whitney, Fisher’s exact, and Spearman’s ρ tests were used to analyze the differences. The odds ratio (odds ratio of 1 is not significant) was calculated, when appropriate.

The primary outcome considered for the purposes of this study was disease-free survival, defined as the time between surgery and first tumor recurrence expressed in months; Kaplan-Meier product limit survival curves were also calculated. Patients with no recurrent disease were censored at the time of their last follow-up or death (unrelated to their carcinoid).

Overall disease-free survival was compared across the levels of prognostic factors using the log-rank test.

To identify variables independently associated with patient outcome, backward selection multivariate analysis was done using Cox’s regression model; interaction between significant variable was also tested (25).

The nonparametric analysis of the receiver operating characteristics was used to identify the most suitable cutoffs for the nuclear expression of MiB1 and Bcl2 for stratifying cases according to their clinical outcome (recurrence versus nonrecurrence).

P values <0.05 were considered as significant.

All the statistical assessments were done with STATA software (Stata Corporation).

Results

Patient demographics and clinical outcome. Among the 67 patients, the male to female ratio was 30:37, with no differences between LG-TC and IG-AC (24:34 and 6:3, respectively). Overall, the patients’ mean age was 45.9 ± 19.1 (range 8-78; median 49). No significant difference emerged between the mean ages of LG-TC (45.9 ± 18.3; median 49) versus IG-AC patients (45.7 ± 25.4; median 55).

After surgery, all patients were followed up clinically for at least 36 months or until death (mean follow-up, 85.13 months; range, 28-168; median, 82.0). The tumor recurred in six patients, and four of them died of disease (median interval, 1.5-55.9; Fisher’s exact test, P = 0.017), a higher nucleus/cytoplasm ratio (odds ratio, 10.8; 95% confidence interval, 2.1-56.9; Fisher’s exact test, P = 0.008), and a greater prevalence of both vascular invasion (odds ratio, 108.0; 95% confidence interval, 10.7-109.1; Fisher’s exact test, P = 0.000) and synchronous node metastases (odds ratio, 9.2; 95% confidence interval, 1.5-55.9; Fisher’s exact test, P = 0.026).

Immunohistochemical phenotyping. All cases unequivocally featured cyttoplasmic expression of both chromogranin A and NSE, with no difference between LG-TC and IG-AC.

Even in the presence of the internal positive control, carcinoid cells never expressed TTF1.

Overall, the percentage of MiB1-positive nuclei was 2.56% (SD ± 4.23; median 1.2%) and MiB1 expression correlated significantly with the mitotic rate (Spearman’s ρ, P = 0.0001). MiB1 expression was significantly higher in IG-AC than in the typical variant (Mann-Whitney, P = 0.001; Table 1).

Overall, the mean prevalence of Bcl2 and Bax expression was 2.54% and 49.91%, respectively. Bcl2 expression was significantly lower in LG-TC than in IG-AC (Mann-Whitney, P < 0.002; Table 1).

Table 1. Tumor location, and histologic and immunohistochemical variables in all cases considered (Cont’d)

<table>
<thead>
<tr>
<th>N</th>
<th>VI</th>
<th>LN</th>
<th>MiB1 % Positive nuclei</th>
<th>Bcl2 % Positive nuclei</th>
<th>Bax % Positive nuclei</th>
<th>Tumor outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>+/-</td>
<td>+/-</td>
<td>+/−</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>4/63</td>
<td>12/55</td>
<td>6/61</td>
<td>2.56 ± 4.2 (M = 1.2)</td>
<td>2.54 ± 2.7 (M = 2.0)</td>
<td>49.91 ± 76.3 (M = 26.0)</td>
<td>6/61</td>
</tr>
<tr>
<td>0/58</td>
<td>4/58</td>
<td>3/55</td>
<td>1.59 ± 1.5 (M = 1.0)</td>
<td>2.13 ± 2.2 (M = 1.0)</td>
<td>53.97 ± 80.4 (M = 30.5)</td>
<td>2/56</td>
</tr>
<tr>
<td>4/5</td>
<td>8/9</td>
<td>3/6</td>
<td>8.84 ± 9.0 (M = 5.1)</td>
<td>4.63 ± 4.00 (M = 3.0)</td>
<td>23.67 ± 33.7 (M = 10.0)</td>
<td>4/5</td>
</tr>
</tbody>
</table>

F: P = 0.0001 F: P = 0.0001 F: P = 0.028 MW: P = 0.005 MW: P = 0.002 F: P = ns F: P = 0.002
An explicative example of the phenotype and the immunophenotype of a nonrecurrent tumor (case 33) is shown in Fig. 1.

**Pathology versus tumor outcome.** At univariate analysis, tumor recurrence correlated significantly with the carcinoid histotype (Fisher’s exact test, $P = 0.002$) and with each of the following variables: (a) tumor location (Fisher’s exact test, $P = 0.01$); (b) mitotic index (Mann-Whitney, $P = 0.003$); (c) necrosis (Fisher’s exact test, $P = 0.002$); (d) tumor vascular invasion (Fisher’s exact test, $P = 0.0001$); (e) Mib1 expression (Mann-Whitney, $P = 0.005$); (f) Bcl2 expression (Mann-Whitney, $P = 0.024$); (g) synchronous node metastasis (Fisher’s exact test, $P = 0.028$; Table 1). No significant relationship emerged between clinical outcome and the patients’ gender or age, tumor size, pathologic staging (pathologic tumor-node-metastasis), or Bax expression.

All the potential prognostic variables emerging from the univariate analysis were tested in the Cox multiple stepwise regression model. The multivariate model only confirmed Mib1 expression (risk ratio, 1.29; 95% confidence interval, 1.14-1.15; $P = 0.0001$) and Bcl2 expression (risk ratio, 1.21; 95% confidence interval, 1.03-1.40; $P = 0.024$) as independent predictors of disease recurrence. No statistical interaction was shown between the two variables ($P = 0.110$).

The best cutoffs for Mib1 and Bcl2 nuclear expression were calculated (using the receiver operating characteristic curves) to discriminate between recurrent and nonrecurrent tumors: They were 5.4% for Mib1 and 2.0% for Bcl2 (Mib1: sensitivity, 83%; specificity, 97%; area under curve, 0.844 ± 0.14; Bcl2: sensitivity, 83%; specificity, 65%; area under curve, 0.769 ± 0.12). By stratifying the patients according to the cutoffs obtained, significant differences emerged in the patients' disease-free survival (log-rank test: Mib1, $P = 0.0001$; Bcl2, $P = 0.01$; Fig. 2B).

**Discussion**

The histologic phenotype enables bronchopulmonary carcinoids to be divided into LG-TC and IG-AC types. LG-TC is associated with 5- and 10-year survival rates of 87% to 100% and 82% to 92% (26, 27), respectively, whereas the atypical variant coincides with a consistently worse, but more variable, outcome (the 5- and 10-year survival rates being 56-78% and 35-67%, respectively; refs. 2, 4, 5, 11, 27-29). It is worth noting here that the largest reported series refers mainly to cases collected at different centers, which carries the risk of a different presurgical evaluation, inconsistent surgical management, different handling of the gross specimen for the pathologic assessment, and different adjuvant therapies (1, 27, 28, 30). All such inconsistencies may help to explain the reported variability in patient outcome, particularly for the IG-AC variant. The present single-institution study minimizes intercenter variability at any stage in the diagnostic/therapeutic process and also rules out the confounding effect of adjuvant chemotherapy in the assessment of outcome after surgery.

The LG-TC/IG-AC ratio (58:9) and the prevalence of the tumor location (central/peripheral, 47:20) in our series were basically consistent with those in the literature (1, 28, 30, 31). As reported elsewhere, IG-ACs were significantly associated with a higher mitotic count, neoplastic necrosis, vascular invasion, and nodal metastases. Univariate analysis significantly associated each variable with tumor recurrence, but their prognostic effect disappeared when the expression of Mib1 and Bcl2 immunohistochemical was added in Cox’s multivariate regression model.

Bronchial carcinoids stain positive for neuroendocrine markers and only a minority of IG-AC reportedly exhibit faint

![Fig. 1. Case 33 (LG-TC; the tumor did not recur after 87 mo from the surgical treatment). The H&E stain shows the organoid pattern of a typical carcinoid tumor (A). The chromogranin antigen is strongly expressed by the tumor (B), whereas no positive nuclei are detected by applying TTF1 immunoreaction (C). Positive nuclei are shown by staining for MIB1 antigen (D). H&E original magnification, ×20.](image-url)
or patchy immunostaining (chromogranin A in 100% of TC versus 77-100% of AC; NSE in 92-100% of TC versus 83-100% of AC; refs. 5, 32–34). In our series, all tumors featured unequivocal immunohistochemical expression of neuroendocrine commitment.

TTF1 expression in carcinoids is a matter of debate. TTF1 expression was never detected in a pilot immunohistochemical study using rabbit polyclonal antibody in eight TCs, and this result was confirmed by RNA protection assay (35). More recently, Folpe et al. (36) applied 8G7G3/1 monoclonal antibody, demonstrating TTF1-positive tumor cells in 35% and 100% of TCs and ACs, respectively; similar results were achieved by Kauffmann et al. (37) and Oliveira et al. (38). No TTF1 expression was detected in LG-TC or IG-AC in our series, in line with data recently published by Sturm et al. (39), who suggest that the previously reported TTF1 expression may be due to neuroendocrine carcinomas (mostly of large cell type, whose histologic phenotype may overlap with AC) being misinterpreted as AC (39–41).

Carcinoids frequently feature low-grade expression of Bcl2 antiapoptotic protein and Bax proapoptotic protein expression accordingly tends to prevail over that of Bcl-2 (21–23). The correlation between low Bcl2 expression and unfavorable outcome has repeatedly been claimed, but has yet to be codified in terms of a cutoff suitable for use in routine diagnostic assessment (11, 18, 21–23, 42). Comparing TC versus AC, a lower Bax/Bcl2 ratio has been associated with the typical variant; such an immunohistochemical pattern is consistent with the biological profile of a tumor more prone to apoptosis than its atypical counterpart (42). The greater aggressiveness associated with Bcl2 overexpression is further supported by its correlation with lymph node metastases and recurrence (18). In our series, the Bax/Bcl2 ratio differed significantly in LG-TC (25.33) and IG-AC (5.11). Multivariate analysis disclosed antiapoptotic activity (i.e., Bcl2 expression; cutoff value, 2%) correlating more closely with patient outcome than the traditional histologic criteria and significantly discriminated between recurrent and nonrecurrent disease (11).

The relationship between carcinoid proliferative activity and tumor outcome is unquestioned; less consistent information is available on (a) the best way to assess cell proliferation reliably; (b) the cutoff for the proliferative cell population that predicts a “benign” as opposed to an “aggressive” behavior; and (c) the independent value of the proliferative rate in discriminating different tumor outcomes. In his seminal study, Travis (4) revised Arrigoni’s criteria (43, 44) and distinguished TC from AC by applying a cutoff of “2 to 10 mitoses/2 mm² (10 high-power fields) . . . ”; such a value has been substantially adopted in subsequent publications and, most recently, adopted among the WHO recommendations (3).

When the proliferative activity of the tumor is reportedly Mib1-positive nuclei, there are some notable differences in the criteria to adopt in distinguishing LG-TC from IG-AC. In a well-conducted study on 47 carcinoids (TC, 31; AC, 16; mean follow-up, 59 months; range, 12-134), Costes et al. (17) found less than 1% of Mib1-positive nuclei in the TC, whereas the atypical variants featured a significantly higher proportion of cells (2.43%) with a positive immunoreaction. In 2001, Arbiser et al. evaluated tumor proliferative activity as “Mib1 nuclear-positive cells in a 1,000 cell count’’: The absolute count of Mib1-positive nuclei was lower in TC than in AC, but the difference was not significant (45). In another 31 carcinoids (TC, 21; AC, 10), Laitinen et al. (11) found fewer than 1% of Ki67-positive nuclei in 28 of 31 cases and a significant association between higher percentages of positive nuclei (10-20%) and AC. In our cases, multivariate analysis significantly correlated Mib1 expression with long-term outcome. The cutoff of 5.4% (receiver operating characteristic analysis) distinguished recurrent from nonrecurrent tumors with a high sensitivity and specificity (83% and 97%, respectively), proving a solid variable for use in carcinoid prognostic stratification (14, 17, 18).

In conclusion, this single-institution study excluded tumors failing to express neuroendocrine markers (chromogranin A, NSE) and/or featuring TTF1 expression from the spectrum of bronchopulmonary carcinoids. Concerning the histologic prediction of long-term clinical outcome, Mib1 and Bcl2 expression proved to be the only independent variables associated with tumor prognosis (with no statistical interaction between the two). Such a biologically plausible immunohistochemical pattern can be also suitable in the routine histologic assessment of bronchopulmonary carcinoids.
References


