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Distinction of Pulmonary Large Cell Neuroendocrine Carcinoma from Small Cell Lung Carcinoma Using a Panel of Bcl-2, p63, and 34βE12

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Background: Making the distinction between large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) is difficult in some samples of biopsy tissues, but we have to separate LCNEC from SCLC because the two types of cancer may need different therapy and they have different prognostic implications. Thus far, there are no specific immunohistochemical markers that allow distinguishing these two kinds of tumors. **Methods:** We performed an immunohistochemical analysis to study the expressions of p63, Bcl-2, and 34βE12 and to investigate whether these 3 molecules have correlations in LCNEC and SCLC. We also evaluated the expression of the neuroendocrine markers chromogranin, synaptophysin and CD56. **Results:** A statistical analysis was performed for p63, Bcl-2, and 34βE12 in separate and combined panels. According to the combinations of p63, Bcl-2, and 34βE12, there were frequent expressions of p63-/Bcl-2+ or Bcl-2+/34βE12- in the SCLC, and there was a superior proportion of them in the SCLC rather than that in the LCNEC. The p63-/Bcl-2+ and Bcl-2+/34βE12- antibody combinations showed higher specificities compared to any single antibody for diagnosing SCLC. **Conclusions:** Bcl-2 and selective p63 or 34βE12 made up a most useful panel of markers for making the differential diagnosis of LCNEC and SCLC.

Key Words: Large cell neuroendocrine carcinoma; Small cell lung carcinoma; p63; Bcl-2; 34βE12

Tumors of the lung with a neuroendocrine (NE) morphology, according to their features on light microscopy, comprise a spectrum of tumor types with different biology and clinical features. The morphologic types include low-grade malignant "typical" carcinoid (TC), intermediate-grade malignant "atypical" carcinoid (AC) and 2 high-grade tumors called large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC).[1] Light microscopy is sufficient to diagnose SCLC, TC, and AC in most cases without the need for special tests, but distinguishing LCNEC from SCLC could be difficult in some biopsy tissues.[2,3] However, we have to separate LCNEC from SCLC because they may need different therapy and they have different prognostic implications. In addition, the optimal therapy for LCNEC has not been established, so once a diagnosis is established, clinicians are often unsure how to treat patients.[4-6]

p63 is a p53-related nuclear protein, and p63 is structurally similar to p53. p63 is a well known marker of squamous differentiation and the reported sensitivity ranges from 78% to 100%.[1,7,8] Previous studies have reported that there was a greater positive expression of p63 in LCNEC than that in SCLC and a strong expression of p63 have been suggested to herald a poor outcome in patients with NE tumors.[2,7,9] Bcl-2 is one of several antiapoptotic proteins, and it has been shown that Bcl-2 is expressed in 83% to 90% of SCLCs, and this makes Bcl-2 a potential therapeutic target for patients with this disease. A set of high-molecular-weight cytokeratins (CK1, 5, 10, and 14 of Moll's catalogue) are characteristic of the complex and stratified epithelia and these cytokeratins are particularly expressed in the bronchi, and they are recognized by the antibody 34βE12.[10-12] These cytokeratins have been used as a marker of the basal cell layer for distinguishing benign from malignant processes in the prostate and breast.[13,14] Shy *et al.*[15] found that 25.7% of SCLC cases were immunostained with 34βE12, but many studies reported a consistent absence of a 34βE12 expression in lung NE tumors.[10,13,14]

The purpose of this study was to determine the utility of p63, Bcl-2, and 34βE12 in the distinguishing LCNEC from SCLC.

Thus far, the expressions of p63, Bcl-2 and 34βE12 in NE tumors have only been independently examined. In this study, we decided to investigate whether these 3 molecules have correlations in LCNEC and SCLC.

MATERIALS AND METHODS

Clinical data acquisition

From 2006 to December 2009, 117 NE lung tumor patients who underwent bronchial biopsy or surgery at Chonnam National University Hwasun Hospital were compiled. The cases diagnosed as being other than LCNEC or SCLC were excluded from this study. The patients' clinical records and histopathological diagnoses were fully documented.

Immunohistochemical assays

Four-μm sections from the tissue microarray blocks were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Immunohistochemistry staining of the tissue microarray samples was performed using the antibodies listed in Table 1 and according to the manufacturer's instructions. The slides were counterstained with hematoxylin.

Two pathologists independently performed semiquantitative evaluation of the staining, without knowledge of the patients' information. When disagreement arose, the slides were reviewed together and a consensus view was obtained. The specimens were scored for the intensity of reactivity (0, none; 1+, weak; 2+, moderate; 3+, strong) and the percentage of positive tumor cells. Positive immunostaining for all antibodies required 10% or more cells with an intensity of at least 2+ on the relevant subcellular localization.

Statistical analysis

The analyses were performed using the SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was used to evaluate the association between the NE markers and each of the clinicopathologic characteristics. p-values less than 0.05 were considered statistically significant.

Table 1. The antibodies used in the study

Antibody	Clone	Dilution	Source
Chromogranin	DAK-A3	1 : 100	Mouse
Synaptophysin	SY38	1 : 20	Mouse
CD56	123C3	1 : 50	Mouse
p63	4A4	1 : 100	Mouse
Bcl-2	124	1 : 100	Mouse
34βE12	34βE12	1 : 50	Mouse

RESULTS

Clinicopathologic features of the NE tumors

The hematoxylin and eosin-stained slides were independently reviewed by each of the two pathologists to confirm the original diagnosis, which was based on the World Health Organization criteria. Discordant independent readings were eliminated in this study. Among the 117 patients, 25 (21%) were classified as having LCNEC and 92 (79%) were classified as having SCLC. The mean age of the patients was 66 years (range, 39 to 81 years) and 100 subjects (86%) were men. The 27 LCNEC patients were classified as follows: stage I, 11 (41%) patients; stage II, 5 (19%) patients; stage III, 3 (11%) patients; stage IV, 6 (22%) patients. Forty-seven patients received surgical resection, while 70 samples were biopsy tissues. There are 8 biopsy tissues and 17 surgical tissues for the LCNECs, while there were 62 biopsy tissues and 30 surgical tissues for the SCLCs. The 92 SCLC patients were classified as 62 (67%) with extensive stage disease and 30 (33%) with limited stage disease.

Immunohistochemical findings

All the NE tumors in this study showed a variable degree of NE morphology by light microscopy and they were at least positive for 1 of 3 NE markers (synaptophysin, chromogranin, and CD56).

p63 was positive in the nuclei of the basal cells of the bronchial epithelium and the tumor cells. The tumor cells were stained focally with anti-p63 antibody in three SCLCs among 92 (3%). This pattern was in contrast with the tumor cells that were stained in LCNEC (8/25, 32%). The ratio of p63-positive tumor cells was significantly higher in the LCNEC ($p < 0.001$) (Table 2). The expression of Bcl-2 protein was found in 13 (52%) of the 25 LCNEC cases and in 84 (91%) of the 92 SCLC cases. The ratio of Bcl-2-positive tumor cells was significantly higher in the SCLC ($p < 0.001$) (Table 2). The 34βE12 protein was essentially undetectable in the SCLC except for 7 (9%) cases, while 12 (48%) of 25 LCNECs showed strong positivity. The ratio of the 34βE12-positive tumor cells was significantly higher in the

Table 2. Summary of the immunohistochemical findings

		Total cases studied			Resected tissues		
		LCNEC (n=25)	SCLC (n=92)	p-value	LCNEC (n=17)	SCLC (n=30)	p-value
p63	+	8 (32)	3 (3)	<0.001	7 (35.3)	1 (3.3)	<0.05
	-	17 (68)	89 (97)		11 (64.7)	29 (96.7)	
Bcl-2	+	13 (52)	84 (91)	<0.001	8 (47.1)	27 (90)	<0.001
	-	12 (48)	8 (9)		9 (53.9)	3 (10)	
34βE12	+	12 (48)	7 (8)	<0.001	10 (58.8)	2 (6.7)	<0.001
	-	13 (52)	85 (92)		7 (41.2)	28 (93.3)	

Values are presented as number (%).
LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

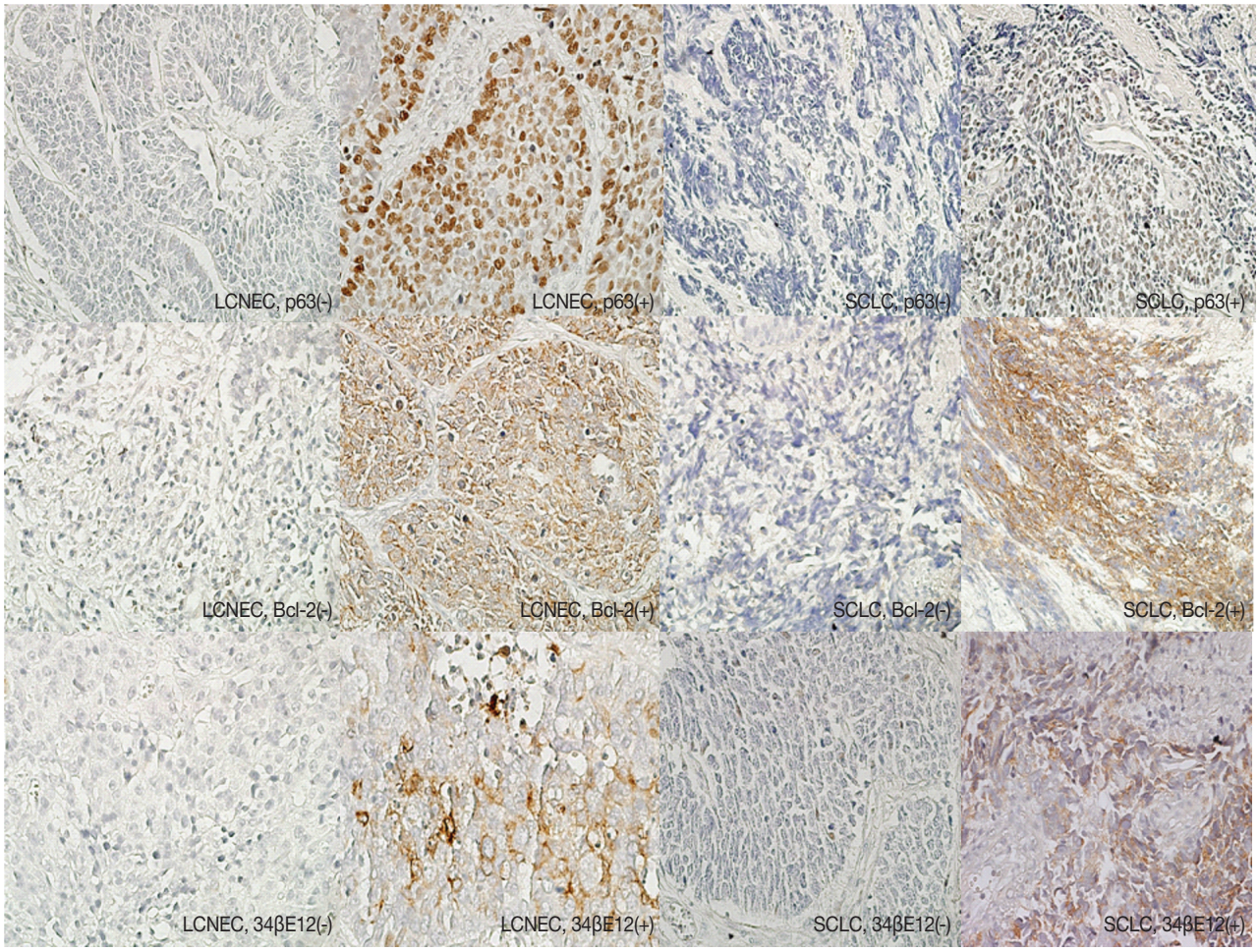

Fig. 1. Immunohistochemical staning of p63, Bcl-2, and 34βE12 in large cell neuroendocrine carcinoma and small cell lung carcinoma. LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

LCNEC ($p<0.001$) (Table 2). There was no significant difference between the biopsy tissues and the surgically resected tissues for the expression of these 3 markers (Table 2). The positive and negative expressions of these 3 antibodies are summarized in Fig. 1.

Antibody combinations

A statistical analysis was performed for p63, Bcl-2, and 34βE12 in the combined panels. According to the combinations of p63, Bcl-2, and 34βE12, 81 (88%) of 92 SCLCs were p63-/Bcl-2+, while 8 (32%) of 25 LCNECs were p63-/Bcl-2+, and

Table 3. Immunohistochemical findings by the antibody combinations

	Total cases studied			Resected tissues		
	LCNEC (n=25)	SCLC (n=92)	p-value	LCNEC (n=17)	SCLC (n=30)	p-value
p63-/Bcl-2+	8 (32)	81 (88)	<0.001	4 (24)	24 (80)	<0.001
Others	17 (68)	11 (12)		13 (76)	3 (11)	
Bcl-2+/34βE12-	7 (28)	77 (84)	<0.001	3 (18)	25 (83)	<0.001
Others	18 (72)	15 (16)		14 (82)	5 (17)	
p63-/34βE12-	13 (56)	82 (88)	<0.001	7 (41)	26 (87)	<0.001
Others	12 (44)	10 (12)		10 (59)	4 (13)	
p63-/Bcl-2+/34βE12-	7 (28)	74 (80)	<0.001	3 (18)	22 (73)	<0.001
Others	18 (72)	18 (20)		14 (82)	8 (27)	

Values are presented as number (%).
LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

Table 4. Sensitivity and specificity of immunohistochemical markers for 92 small cell lung carcinomas

	Single antibody			Antibody combinations		
	p63(-)	Bcl-2(+)	34βE12(-)	p63-/Bcl-2+	Bcl-2+/34βE12-	p63-/Bcl-2+/34βE12-
Sensitivity	89/92 (97)	84/92 (91)	85/92 (92)	81/92 (88)	77/92 (84)	74/92 (80)
Specificity	8/25 (32)	13/25 (52)	12/25 (48)	17/25 (68)	18/25 (72)	18/25 (72)
Positive predictive value	89/106 (84)	84/96 (88)	85/98 (87)	81/89 (91)	77/84 (92)	74/81 (91)
Negative predictive value	8/11 (73)	13/21 (62)	12/19 (63)	17/28 (61)	18/33 (55)	18/36 (50)

Values are presented as positive/total (%). Sensitivity, TP/TP+FN; Specificity, TN/TN+FP; Positive predictive value, TP/TP+FP; Negative predictive value, TN/TN+FN.
TP, true positives; FN, false negatives; TN, true negatives; FP, false positives.

77 (84%) of 92 SCLCs were Bcl-2+/34βE12-, while 7 (28%) of 25 LCNECs were Bcl-2+/34βE12- ($p<0.001$). There were more frequent expressions of p63-/Bcl-2+ or Bcl-2+/34βE12- in the SCLC than that in the LCNEC (Table 3).

When evaluating p63, Bcl-2, and 34βE12 separately, their sensitivities in SCLC were more than 90%, while the specificities were less than 52%. However, the specificities of p63-/Bcl-2+ and Bcl-2+/34βE12- were 68% and 72% to predict SCLC. Although the p63-/Bcl-2+ and Bcl-2+/34βE12- antibody combination groups showed lower sensitivity than did the single antibody groups, the specificity was significantly superior in these combined antibody groups (Table 4). The combination of p63 and 34βE12 also showed statistical significance between SCLC and LCNEC, but the sensitivity and specificity were not superior to the combination of Bcl-2/p63 or Bcl-2/34βE12, as well as the combination of all of them (Tables 3, 4).

DISCUSSION

LCNEC accounts for 9% of all the malignant neoplasms of the lung, and LCNEC is defined as a carcinoma that lacks the typical features of SCLC, squamous cell carcinoma and adenocarcinoma by light microscopic examination.[1] There are no specific immunohistochemical or molecular markers that allow separating these tumors. From a clinical standpoint, appropriate management of pulmonary neoplasm is based on the proper histological classification, and so the simple distinction between LCNEC and SCLC is still useful and appropriate.[16]

In this study, we selected a 3-stain immunopanel of commercially available antibodies with good specificity and sensitivity for distinguishing LCNEC from SCLC, and the antibodies included p63, Bcl-2, and 34βE12. We applied them in a relatively large series of LCNECs and SCLCs to clarify their differences. The expression of SCLC was p63-negative, Bcl-2-positive and 34βE12-negative, and it was statistically different from LCNEC. It is unclear why the expressions of p63, Bcl-2, and 34βE12 are different between SCLC and LCNEC and few studies have evaluated the expressions of these 3 markers to distinguish LCNEC from SCLC. However, our results demonstrated that the expressions of these 3 markers are more valuable than the highly specific NE markers such as synaptophysin, chromogranin A and CD56/NCAM for making the distinction of LCNEC from SCLC.

Moreover, our study showed that the combination of Bcl-2 and p63 or 34βE12 showed superiority when compared with each protein separately, and the combination of p63 and 34βE12 as well as the combination of all of them. We demonstrated that Bcl-2 was a powerful marker to distinguish LCNEC from SCLC.

Our aim is to perform fewer immunohistochemistry examinations in the clinical setting, but to still diagnose lung tumors correctly. A panel of many markers is recommended, but there is limited tissue to do all the immunohistochemistry examinations since the diagnosis of LCNEC is based on small biopsies and the cytology. We found that Bcl-2 and selective p63 or 34β-E12 made up a most useful panel of immunohistochemical markers for making the differential diagnosis of LCNEC and SCLC. The sensitivity of p63-/Bcl-2+ was 88% and the specificity of 34βE12-/Bcl-2+ was up to 72% for diagnosing SCLC. The p63-/Bcl-2+ and the 34βE12-/Bcl-2+ antibody combinations showed higher specificities compared to any single antibody. Additional large series studies are needed to analyze the biological behavior of LCNEC and SCLC, including their sensitivity to chemotherapeutic agents.

The limitations of this study must also be addressed. 1) Only biopsy samples in the nonsurgical patients were collected in our study since most patients with SCLC are not candidates for resection, and so it was difficult to obtain enough specimens or to perform an immunohistochemical study. 2) The cases that a consensus could not be reached were excluded in this study. 3) Although there were no standards or universally accepted criteria to evaluate the Bcl-2, p63, and 34βE12 expressions by immunohistochemistry, we tried to reach a consensus between two pathologists. 4) The number of LCNEC cases was too small and so further studies should be performed.

In conclusion, a panel of Bcl-2 and selective p63 or 34βE12 is very useful for making the distinction between the morphologically overlapping subclasses of LCNEC and SCLC.

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