TTF-1, Cytokeratin 7, 34βE12, and CD56/NCAM Immunostaining in the Subclassification of Large Cell Carcinomas of the Lung

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Abstract

We selected a 4-stain immunopanel including thyroid transcription factor (TTF)-1, cytokeratin (CK) 7, 34βE12, and CD56/neural cell adhesion molecule (NCAM) to subclassify a series of 45 pulmonary large cell carcinomas (LCCs) on bronchial biopsy. All cases consisted of a large tumor cell proliferation with abundant cytoplasm, vesicular nuclei, and prominent nucleoli. Immunohistochemically, 27 tumors (60%) were subclassified as adenocarcinoma (TTF-1+/CK7+, 24; CK7+ only, 3), 10 (22%) as squamous cell carcinoma (34βE12+ only), and 4 (9%) as LCC with neuroendocrine differentiation (CD56+, variably stained with TTF-1 and CK7, 34βE12–). In 4 cases, the tumors coexpressed CK7 and 34βE12 (3 cases) or were completely unstained (1 case). Surgically resected tumors matched exactly with the corresponding original biopsy specimens in 21 of 23 cases; consistent CD56 expression was a reliable marker in confirming a diagnosis of large cell neuroendocrine carcinoma even on biopsy. Our results suggest that the proposed 4-stain set of commercially available markers might help subclassify LCC even in small biopsy material, validating expression-profiling studies aimed at lung cancer classification and permitting more consistent patient enrollment for trials with targeted treatments.

For clinical purposes, lung cancers are subdivided into 2 main groups: small cell carcinoma (SCLC) and non–small cell carcinoma (NSCLC).[1,2] So far, this simple dichotomous classification coupled with the tumor stage provides the essential data to establish the appropriate management of lung cancer.[3] However, the advent of novel therapies based on the discovery of molecules targeted against specific neoplastic markers seems to provide an additional role for the exact definition of tumor histotype.[4] For example, the response rate for patients with pulmonary adenocarcinoma treated with epidermal-growth factor receptor inhibitors is significantly higher than that for other tumor histotypes.[5,6]

Up to two thirds of malignant neoplasms of the lung are inoperable, and the pathologic diagnosis is established by cytologic examination or small biopsy specimens.[2,3] Even in these cases, SCLC is distinguished by practicing pathologists from NSCLC with a high degree of accuracy, mainly based on the nuclear characteristics of the tumor cells,[7] and the great majority of lung cancers can be classified by light microscopic examination alone into 1 of 4 major histologic types: squamous cell carcinoma (SCC), adenocarcinoma, SCLC, and large cell carcinoma (LCC).[2,8] While the first 3 lesions can be considered real entities, the latter is a diagnosis of exclusion, representing the endpoint of differentiation of various lung tumors.[1,2,8-10] In fact, LCC consists of sheets and nests of large polygonal cells with vesicular nuclei and prominent nucleoli lacking squamous or glandular differentiation in light microscopic examination.[2,8-10] Defined as such, it represents the "wastebasket" of the World Health Organization (WHO) classification.[2] Confirming this, extensively sampled lung tumors obtained from surgical excisions revealed the presence of a definitive differentiation adjacent to undifferentiated areas in almost all cases.[8]

After a careful review of previous immunohistochemical and gene expression profiling studies aimed at lung cancer classification,[11-24] we selected a panel of 4 commercially available antibodies, including thyroid transcription factor (TTF)-1, cytokeratin (CK) 7, CD56/neural cell adhesion molecule (NCAM), and high-molecular-weight CKs 1, 5, 10, and 14 (34βE12), and we evaluated their role in subclassifying a series of 45 primary pulmonary LCCs. Because the majority of cases of lung cancer are inoperable and a biopsy specimen represents the only histologic documentation, we limited the study to bronchial biopsy specimens. When possible, we compared the original diagnosis with the diagnosis from the corresponding surgically resected specimens.

Materials and Methods

Among 618 bronchial biopsies performed for lung cancer between July 2001 and March 2004, 45 consecutive cases (7.3%) of primary LCC of the lung were obtained from the files of the Section of Pathology, University of Modena and Reggio Emilia, Modena, Italy. All samples consisted of bronchial biopsy specimens and were fixed in 10% buffered formalin and embedded in paraffin. In each case, H&E-stained sections were reviewed, and alcian blue–periodic acid–Schiff and/or Kreyberg staining was performed to highlight the presence of mucin deposition. None of the patients in this series underwent preoperative neoadjuvant chemotherapy or radiotherapy, and none had a history of cancer elsewhere in the body.

All cases were reviewed at a multiheaded microscope by 3 pathologists (G.R., M.M., A.C.), and a diagnosis of LCC was established according to the histopathologic criteria of the 1999 WHO classification of lung tumors.[2] Briefly, LCC was defined as "an undifferentiated malignant epithelial tumour that lacks the cytologic features of small cell carcinoma and glandular or squamous differentiation. The cells typically have large nuclei, prominent nucleoli and a moderate amount of cytoplasm."[2]

For immunohistochemical analysis, 4-μm-thick paraffin sections were obtained from the representative block, incubated overnight at 37°C, and stained with a streptavidin-biotin complex method using an automated immunostainer (Benchmark, Ventana, Tucson, AZ). The following antibodies were applied in each case: TTF-1 (clone 8G7G3/1, dilution 1:100; Dakopatts, Glostrup, Denmark); high-molecular-weight CKs 1, 5, 10, and 14 (clone 34βE12, dilution 1:200; Dakopatts); CK7 (clone OV-TL 12/30, dilution 1:800; Dakopatts); and CD56/NCAM (clone 123C3, dilution 1:100; NeoMarkers, Fremont, CA). We used 3-3'-diaminobenzidine and Harris hematoxylin as the chromogen and counterstain, respectively. Normal bronchial epithelium served as the appropriate positive internal control samples for CK7 and 34βE12, whereas prepared sections of an SCLC known to express TTF-1 and CD56/NCAM were included in each batch as positive external control samples. Negative control samples were prepared by replacing the primary antibodies with nonimmune mouse IgG.

All immunostains were recorded for intensity of reactivity (0, none; 1+, weak; 2+, moderate; 3+ strong) and percentage of positive neoplastic cells. Positive immunostaining for all antibodies required 10% or more cells with an intensity of at least 2+ on the relevant subcellular localization (nuclear for TTF-1, cytoplasmic for CK7 and 34βE12, cytoplasmic and membranous for CD56/NCAM).

A tumor showing any combination of immunostaining with TTF-1 and/or CK7 only was considered as having an adenocarcinomatous differentiation, whereas a diagnosis of SCC was performed when tumor cells expressed only 34βE12. A tumor was considered LCC with neuroendocrine differentiation (LCCND) when the cells expressed CD56 but not 34βE12, with or without immunopositivity for TTF-1 and/or CK7. No clear-cut differentiation was made for any other immunostaining combination. When possible, a comparison between the phenotype of LCC on biopsy and the corresponding surgical resection was performed. This comparison was based mainly on morphologic features and immunohistochemical studies using neuroendocrine markers (chromogranin, synaptophysin, and CD56) to further confirm a diagnosis of large cell neuroendocrine carcinoma (LCNEC).

The correlation between immunohistochemical results was determined by using contingency table methods and tested for significance using the Pearson χ^2 test. A difference with probability (P) values .05 or less was considered significant.

Results

Among the 45 patients, there were 31 men and 14 women with a mean age of 66.6 years (range, 45-86 years). All patients except 2 were cigarette smokers. In all cases, a diagnosis of LCC was established by examination of bronchial biopsy specimens. Of the patients, 23 (51%) underwent a subsequent surgical resection, whereas the remaining patients had inoperable tumors and were treated by chemotherapy or a multimodal approach (chemotherapy plus radiotherapy).

Light microscopic examination revealed that the biopsy material in all cases consisted of malignant cells larger than those seen in SCLC, growing in sheets without clear-cut differentiation (keratinized cells, squamous pearls, or intercellular bridges for SCC; gland formation coupled with mucin-producing cells for adenocarcinoma). The tumor cells had a moderate to abundant amount of amphophilic to eosinophilic cytoplasm and large vesicular nuclei with prominent nucleoli **Image 1A**. In all cases, histochemical stains (alcian blue–periodic acid–Schiff and Kreyberg) failed to highlight

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mucin production. We observed that 43 cases had a solid growth pattern **Image 1B**, and the other 2 had a looser structure **Image 1C**. Overall, in immunohistochemical analysis 33 cases (73%) were strongly stained for CK7, 13 (29%) for 34βE12, 27 (60%) for TTF-1, and 4 (9%) for CD56 **Table 1**.

Based on the immunostaining patterns, 27 tumors (60%) were subclassified as adenocarcinoma, with 24 coexpressing TTF-1 and CK7 **Image 2A**, **Image 2B**, and **Image 2C** and 3 positive only for CK7. Of the 45 cases, 10 (22%) were immunoreactive with 34βE12 only and were classified as SCC **Image 2D** and **Image 2E**. Four cases (9%) showed strong immunostaining for CD56 in the absence of any staining with 34βE12 and were classified as LCCND **Image 2F**, **Image 2G**, and **Image 2H**. In particular, 2 cases coexpressed CD56, CK7, and TTF-1; 1 coexpressed CD56 and TTF-1; and 1 coexpressed CD56 and CK7. In 3 cases (7%), tumor cells displayed conflicting immunostaining for CK7 and 34βE12, revealing a double line of differentiation (glandular and squamous). Finally, 1 case was completely unstained with all the tested markers: positive staining for broad-spectrum CKs (AE1/AE3) and the presence of scattered pleomorphic, bizarre tumor cells led us to consider a diagnosis of pleomorphic carcinoma in this case.

In 23 cases, the corresponding surgical specimen was available for review. The biopsy diagnosis was confirmed in every case. However, minor discrepancies were observed in 2 cases that showed a combined component on surgical specimens. In particular, an LCC (case 16) coexpressing 34βE12 and CK7 **Image 3A** and **Image 3B** was diagnosed definitively as adenosquamous carcinoma **Image 3C**, while the other case (case 18) originally considered to be LCCND (TTF-1+, CK7+, and CD56+) **Image 3D** **Image 3E**, and **Image 3F** displayed an LCNEC associated with an adenocarcinomatous component on lobectomy (combined LCNEC and adenocarcinoma) **Image 3G**.

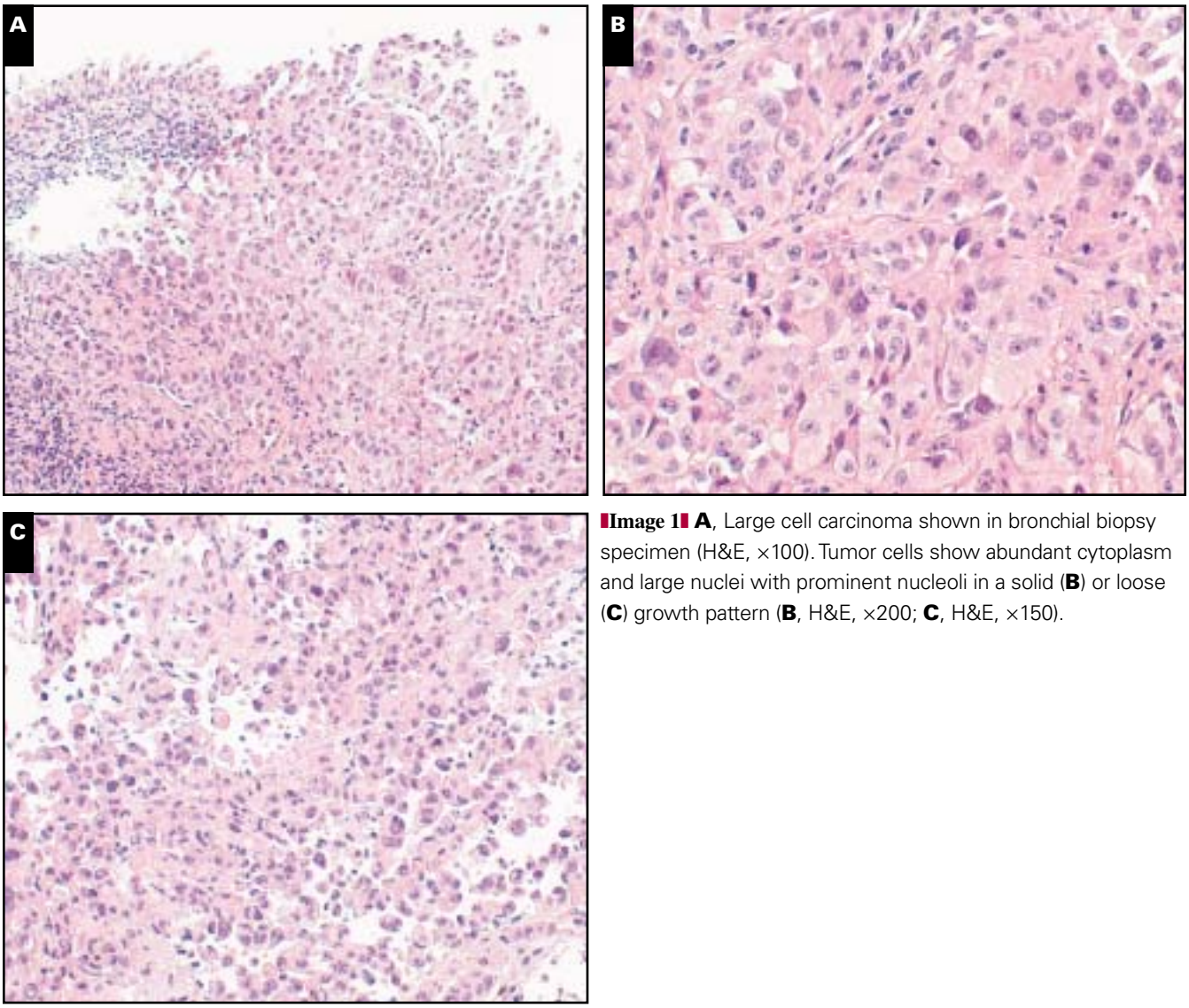

Image 1 **A**, Large cell carcinoma shown in bronchial biopsy specimen (H&E, ×100). Tumor cells show abundant cytoplasm and large nuclei with prominent nucleoli in a solid (**B**) or loose (**C**) growth pattern (**B**, H&E, ×200; **C**, H&E, ×150).

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Statistical analysis revealed a significant direct correlation between TTF-1 and CK7 immunostaining ($P < .0001$), while both of these markers showed a significantly inverse correlation with 34βE12 expression ($P < .0001$). Finally, no tumor showed coexpression of 34βE12 and CD56, and no statistically significant association was noted when we matched expression patterns between the other tested markers.

Discussion

LCC accounts for 9% of all malignant neoplasms of the lung.[25] It is defined as a carcinoma that lacks the typical features of SCLC, SCC, and adenocarcinoma by light microscopic examination.[2] The absence of a clear-cut differentiation is related mainly to sampling, given that extensively sampled surgical specimens generally reveal foci of squamous or glandular formations.[8] Thus, we agree with Yesner[9,10] that the term LCC has 2 meanings: (1) to distinguish a lung tumor from SCLC (in this case LCC is a synonym for NSCLC); (2) to define a group of lung carcinomas that are undifferentiated on morphologic grounds, thus representing a final stage of differentiation.

Several works have documented well that LCC reveals a line of differentiation when studied at the ultrastructural level or by immunohistochemical analysis.[26-34] However, while electron microscopy cannot be performed routinely on small specimens, the immunohistochemical stains previously used

Table 1
Immunohistochemical Reactivity of Large Cell Carcinomas of the Lung

Case No./Sex/ Age (y)	CK7	TTF-1	34βE12	CD56/NCAM	Carcinoma Diagnosis	
					On Biopsy Specimen	On Surgical Resection Specimen
1/M/69	–	–	+	–	Squamous cell	Squamous cell
2/F/58	+	+	–	–	Adenocarcinoma	Adenocarcinoma
3/M/69	+	+	–	–	Adenocarcinoma	Adenocarcinoma
4/M/71	–	–	+	–	Squamous cell	NA
5/M/65	+	+	–	–	Adenocarcinoma	Adenocarcinoma
6/F/67	+	+	–	–	Adenocarcinoma	Adenocarcinoma
7/F/64	+	+	–	–	Adenocarcinoma	NA
8/M/74	+	+	–	–	Adenocarcinoma	NA
9/M/73	+	+	–	–	Adenocarcinoma	NA
10/M/67	+	–	–	–	Adenocarcinoma	Adenocarcinoma
11/F/76	+	–	–	+	LCCND	LCNEC
12/M/53	–	–	+	–	Squamous cell	NA
13/M/66	+	+	–	–	Adenocarcinoma	NA
14/M/65	+	+	–	–	Adenocarcinoma	NA
15/M/73	+	+	–	–	Adenocarcinoma	NA
16/M/65	+	–	+	–	Large cell	Adenosquamous
17/F/66	+	+	–	–	Adenocarcinoma	NA
18/F/70	+	+	–	+	LCCND	Adenocarcinoma; LCNEC
19/M/78	+	–	+	–	Large cell	NA
20/M/49	+	+	–	+	LCCND	LCNEC
21/F/71	+	+	–	–	Adenocarcinoma	NA
22/M/70	+	+	–	–	Adenocarcinoma	Adenocarcinoma
23/M/76	–	–	–	–	Pleomorphic	NA
24/M/73	+	–	–	–	Adenocarcinoma	NA
25/M/57	+	+	–	–	Adenocarcinoma	Adenocarcinoma
26/F/70	+	+	–	–	Adenocarcinoma	NA
27/M/67	–	+	–	+	LCCND	LCNEC
28/F/63	–	–	+	–	Squamous cell	Squamous cell
29/M/62	–	–	+	–	Squamous cell	NA
30/M/72	+	–	–	–	Adenocarcinoma	NA
31/M/52	+	+	–	–	Adenocarcinoma	Adenocarcinoma
32/M/68	+	–	+	–	Large cell	NA
33/F/64	–	–	+	–	Squamous cell	NA
34/F/45	+	+	–	–	Adenocarcinoma	Adenocarcinoma
35/M/74	+	+	–	–	Adenocarcinoma	NA
36/F/68	+	+	–	–	Adenocarcinoma	Adenocarcinoma
37/M/70	+	+	–	–	Adenocarcinoma	NA
38/M/53	–	–	+	–	Squamous cell	NA
39/M/72	+	+	–	–	Adenocarcinoma	NA
40/F/68	–	–	+	–	Squamous cell	Squamous cell
41/M/70	+	+	–	–	Adenocarcinoma	Adenocarcinoma
42/M/70	–	–	+	–	Squamous cell	Squamous cell
43/M/86	–	–	+	–	Squamous cell	Squamous cell
44/M/57	+	+	–	–	Adenocarcinoma	Adenocarcinoma
45/F/63	+	+	–	–	Adenocarcinoma	Adenocarcinoma

CK, cytokeratin; LCCND, large cell carcinoma with neuroendocrine differentiation; LCNEC, large cell neuroendocrine carcinoma; NA, not available; NCAM, neural cell adhesion molecule; TTF, thyroid transcription factor; +, positive; –, negative.

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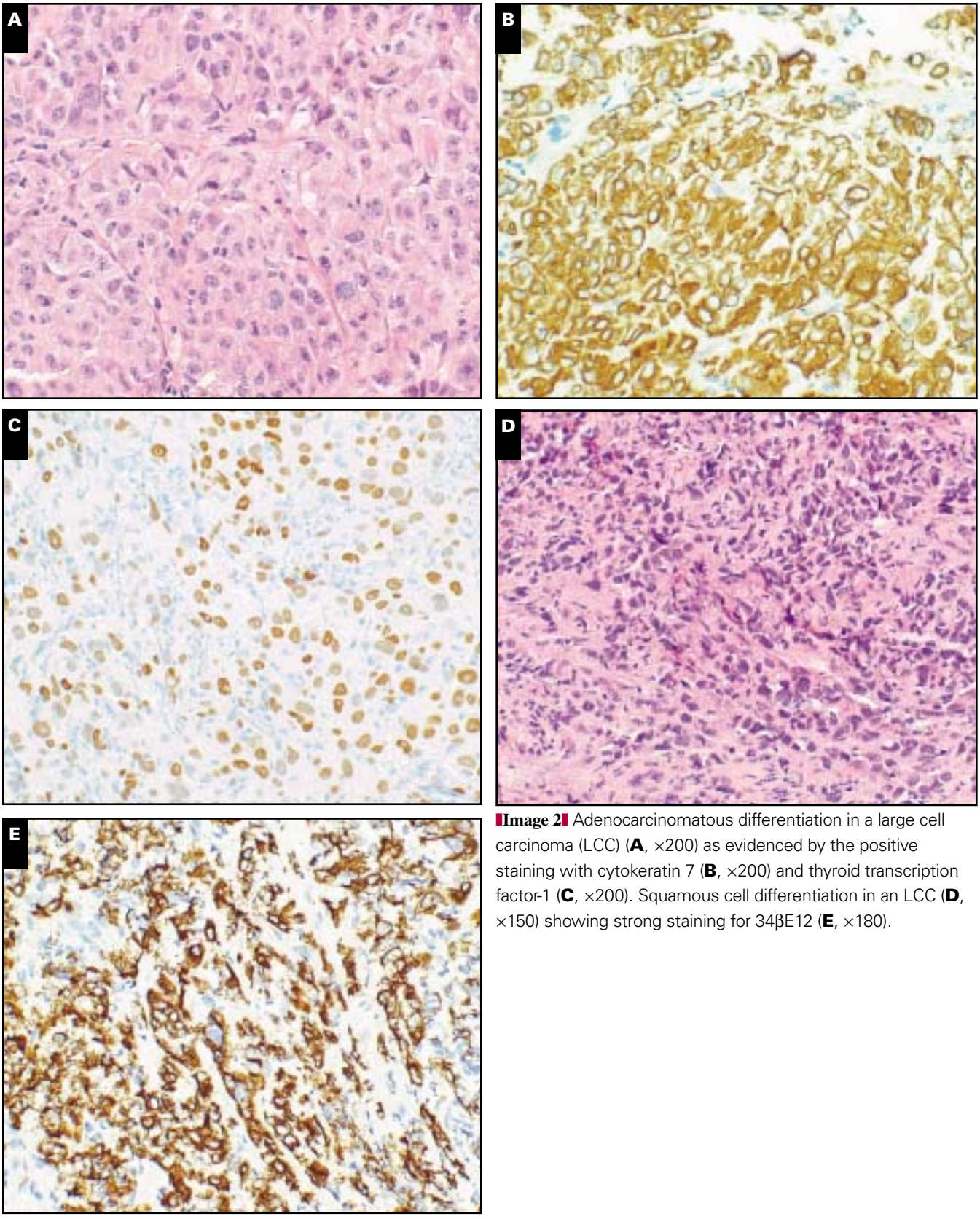

Image 2 Adenocarcinomatous differentiation in a large cell carcinoma (LCC) (**A**, ×200) as evidenced by the positive staining with cytokeratin 7 (**B**, ×200) and thyroid transcription factor-1 (**C**, ×200). Squamous cell differentiation in an LCC (**D**, ×150) showing strong staining for 34βE12 (**E**, ×180).

to address this issue, such as neuron-specific enolase, epithelial membrane antigen, and carcinoembryonic antigen, actually have a low specificity.

From a clinical standpoint, the simple distinction between SCLC and NSCLC is still useful and appropriate for tumor management.[2,3,35] However, the new therapeutic options

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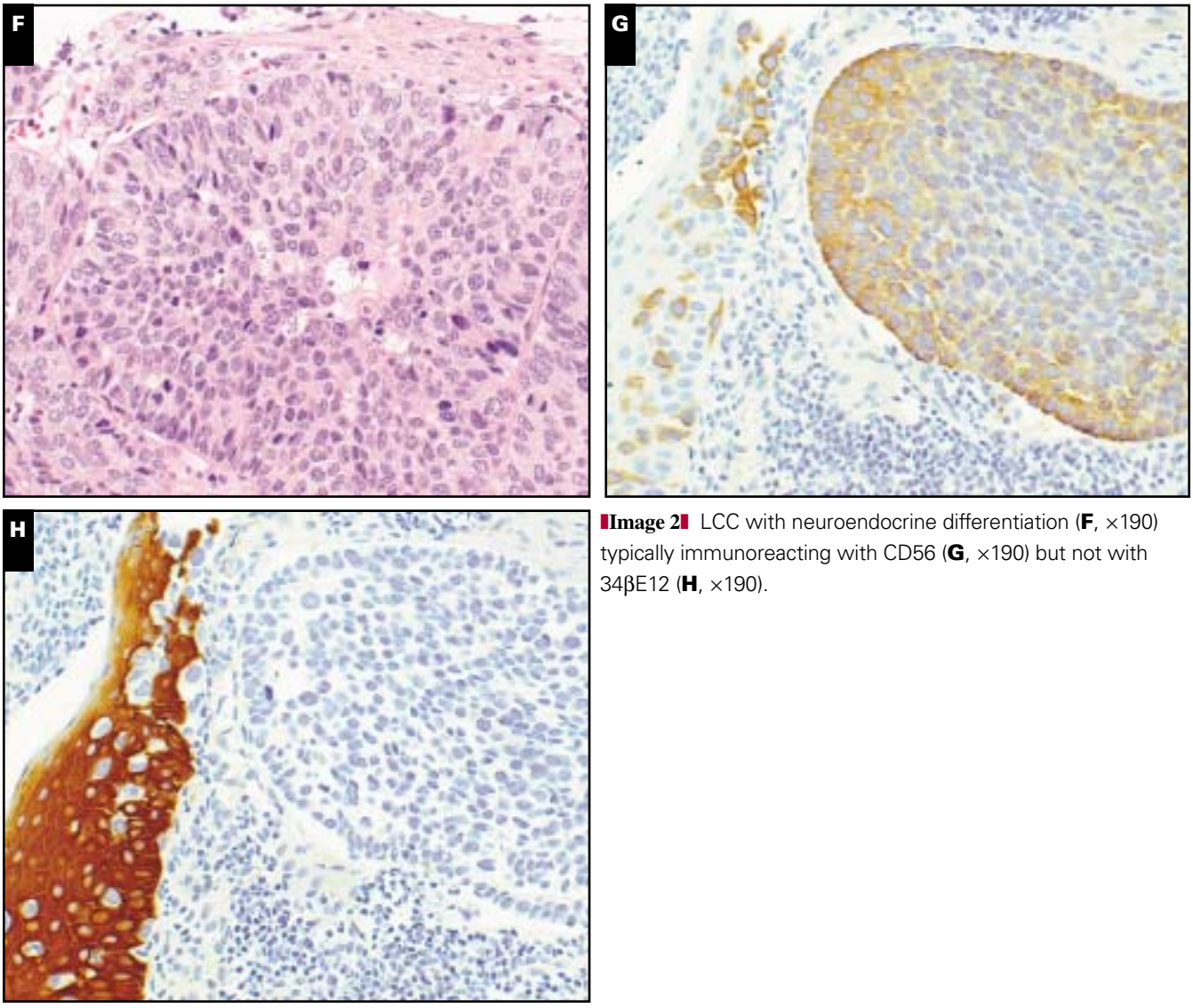

Image 2 LCC with neuroendocrine differentiation (**F**, ×190) typically immunoreacting with CD56 (**G**, ×190) but not with 34βE12 (**H**, ×190).

adopting alternative, promising targeted therapies against specific tumor markers seem to provide a more important value to knowing the tumor histotype.[4] In this regard, recent and ongoing clinical trials targeting epidermal-growth factor receptors in locally advanced or metastatic NSCLC demonstrated that the response rate for patients with adenocarcinoma was significantly higher than for other tumor histotypes,[5,6] providing a major therapeutic implication for a better tumor definition in the future enrollment of patients with NSCLC in clinical trials. Moreover, the exact definition of NSCLC seems to correlate significantly with survival when different chemotherapy protocols are used.[36]

In this setting, independent gene expression profiling studies aimed at lung tumor classification have identified several genes significantly expressed in different classes of lung cancer.[21-24] From among these markers, we selected a 4-stain immunopanel of commercially available antibodies with good specificity and sensitivity in lung tumor pathology, including TTF-1, CK7, 34βE12, and CD56, and applied them in a relatively large series of LCCs to clarify their differentiation. The expression of all these markers also has been reported to be associated with the lung cancer phenotype on immunohistochemical grounds,[11-20] confirming a good correlation between genes and protein-related expression.

In particular, TTF-1 is a homeodomain-containing nuclear transcription protein of the NKx2 family regulating development, cell growth, and differentiation in thyroid, lung, and selected areas of brain.[37] In lung tumors, TTF-1 is mainly expressed in adenocarcinoma and high-grade neuroendocrine tumors (SCLC and LCNEC), whereas it is not found in SCC.[11-13,16] These findings have been confirmed not only by immunohistochemical analysis but also by several microarray-based gene expression profiling studies.[22-24,37-39]

CD56 (also known as NCAM) is a cell-surface sialoglycoprotein of the immunoglobulin family involved in cell-to-cell interactions during neural development.[40] CD56 is expressed

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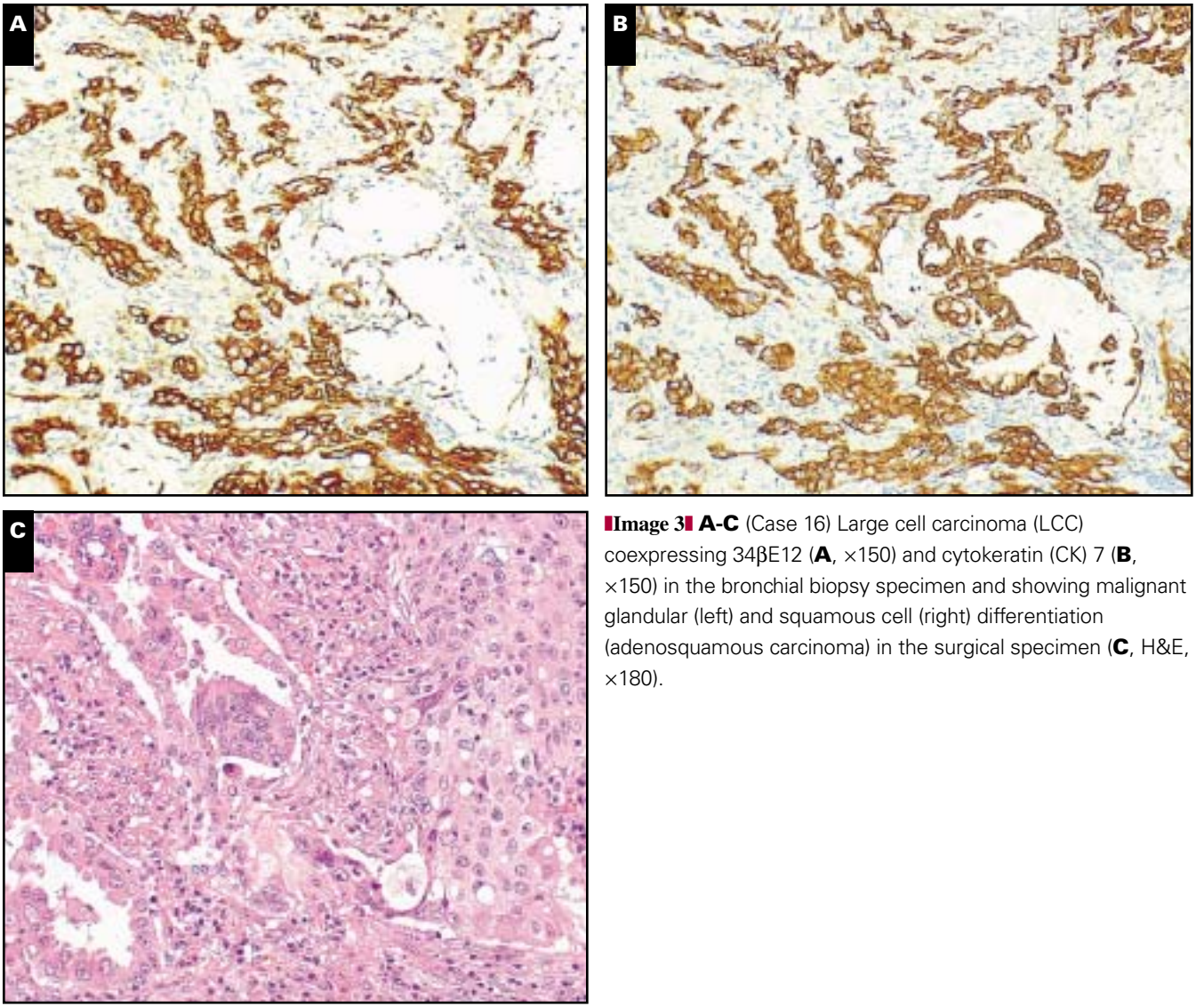

Image 3 **A-C** (Case 16) Large cell carcinoma (LCC) coexpressing 34βE12 (**A**, ×150) and cytokeratin (CK) 7 (**B**, ×150) in the bronchial biopsy specimen and showing malignant glandular (left) and squamous cell (right) differentiation (adenosquamous carcinoma) in the surgical specimen (**C**, H&E, ×180).

in natural killer (NK) cells and NK-derived malignant neoplasms, but it also is found in many neural and neuroendocrine tissues and in several neuroendocrine tumors (carcinoid tumors, paragangliomas, small cell carcinomas, LCNECs).[19,20,41] In addition, CD56 seems to be one of the most sensitive and specific markers in confirming neuroendocrine differentiation in malignant neoplasms of various sites and is extremely helpful in discriminating LCNEC from other mimickers.[19,41] Profiling studies of molecular expression revealed that CD56/NCAM and human achaete-scute homologue-1 (hASH1) probably are the most specific genes associated with SCLC.[21-24,42,43] In addition, clinical trials using a humanized monoclonal antibody (BB-10901) against CD56 in patients with relapsed or chemotherapy-refractory SCLC are ongoing.[44]

CK7 is an intermediate-weight CK expressed exclusively in simple epithelia and in a subset of adenocarcinomas arising in various organs, including lung.[17] Pulmonary SCCs usually are unstained with CK7.[14,17,45]

The antibody 34βE12 recognizes a set of high-molecular-weight CKs (CKs 1, 5, 10, and 14) that are expressed mainly in complex and stratified epithelia.[17] In the lung, this marker identifies the basal and parabasal cells of the normal bronchi, and in tumors, its expression is restricted mainly to SCCs and basaloid carcinomas,[14-18] whereas adenocarcinomas and high-grade neuroendocrine carcinomas typically are negative.[14-18] Together with p63,[46-48] high-molecular-weight CKs are the most specific antibodies for SCC. As found by Lyda and Weiss,[14] 34βE12 expression is restricted mainly to SCC, while strong staining for CK7 characterizes adenocarcinoma. Similar data also were reported in the review article on keratin expression by Chu and Weiss.[17] Of note, the *CK5* gene is one of the most specific markers of SCC at complementary DNA microarray analysis.[21-24]

Classification of lung cancers based on gene expression profiling studies essentially matches the WHO nomenclature, which is based on routine morphologic examination.[49]

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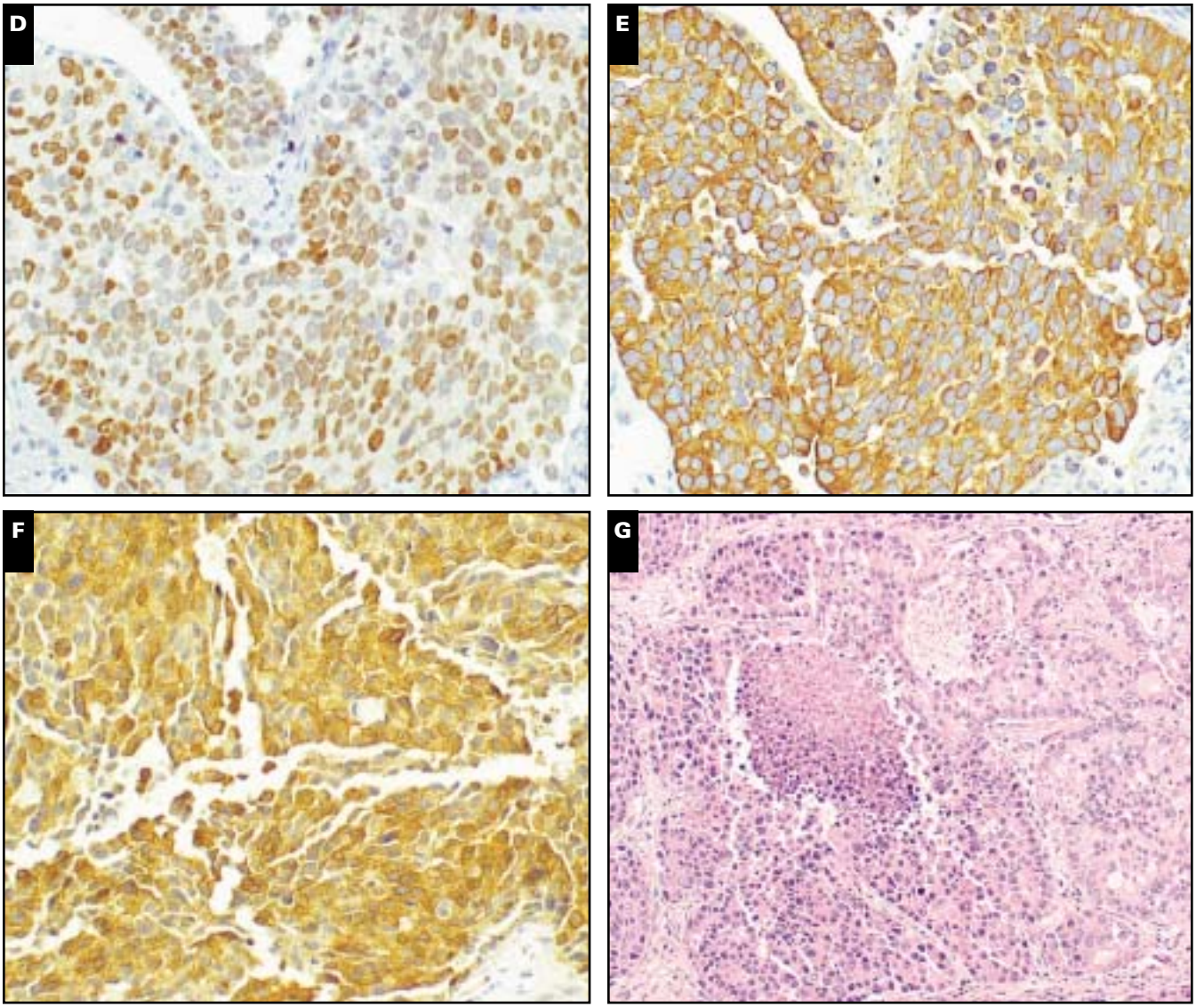

Image 3 **D-G** (Case 18), LCC coexpressing thyroid transcription factor-1 (**D**, ×200), CD56 (**E**, ×200), and CK7 (**F**, ×200) in the biopsy specimen and appearing as combined large cell neuroendocrine carcinoma–adenocarcinoma in the lobectomy specimen (**G**, H&E, ×100).

However, LCCs appear as a genetically heterogeneous group of tumors and are subclassified by expression profile analysis as adenocarcinomas or SCCs in the great majority of cases.[21-24,49] In particular, clinicopathologic and ultrastructural features suggest a closer relationship to adenocarcinoma than to other histotypes.[32,50] Although not further validated by outcome data, our results seem to agree with the reported molecular observations, confirming that it is possible to subclassify LCC even by immunohistochemical analysis using a small panel of antibodies. Moreover, the present immunopanel was successful in discriminating nonoverlapping subsets of LCC, reflecting the morphologic phenotype of LCCs. As expected and previously observed by Hammar[32] in ultrastructural studies, the majority (27 [60%]) of LCCs in our series had a phenotype similar to that commonly observed in adenocarcinomas, coexpressing TTF-1 and CK7. Ten cases (22%) had the immunophenotype of SCC, expressing 34βE12 only, whereas 4 cases (9%) of the LCCs strongly stained with CD56, equally coexpressed TTF-1 and/or CK7, but did not stain with 34βE12. Of these 4 cases, 2 coexpressed CD56 and TTF-1 and CK7, 1 was TTF-1+/CD56+, and 1 was CD7+/CD56+. These latter cases were subclassified as LCCND, and all were diagnosed as LCNEC on surgical specimens (3 pure and 1 combined with an adenocarcinoma component), confirming the value of CD56 as a marker of neuroendocrine differentiation in recognizing LCNEC even on small biopsy specimens.

In this regard, by using a combination of hierarchical clustering, Bhattacharjee et al[22] found that a subgroup of adenocarcinomas expressed different neuroendocrine markers and were associated with a significantly dismal outcome. In

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our view, this subset of lung tumors could include cases of LCNEC, a controversial variant of LCC showing clear-cut neuroendocrine features in morphologic examination and immunostaining,[51,52] usually displaying TTF-1 immunoreactivity[11,16,52] but behaving more closely to SCLC than to NSCLC.[53,54] As evidenced by ultrastructural examination,[26-32] a small number of LCCs failed to disclose differentiation when we applied immunostaining. Accordingly, in our series, 3 LCCs showed an unusual coexpression for CK7 and 34βE12. One of these cases revealed a combination of foci of glandular and squamous differentiation at subsequent examination of the surgical excision specimen and then was classified as adenosquamous carcinoma, whereas the remaining 2 tumors consisted only of a biopsy specimen, and a definitive diagnosis could not be made (LCC, not otherwise specified). Nevertheless, coexpression of CK7 and 34βE12 does not authorize a diagnosis of adenosquamous carcinoma on bronchial biopsy specimens, because this diagnosis should be made only on surgical specimens and requires both SCC and adenocarcinoma "with each comprising at least 10% of the whole tumour."[2] These problematic cases further reflect the extreme heterogeneity of lung cancer as suggested in the classic article by Roggli et al.[55]

Only 1 case did not display any positivity at immunostaining, but the presence of scattered bizarre neoplastic cells and the immunoreactivity for pan-CK led us to consider a pleomorphic carcinoma. This is not surprising because pleomorphic carcinoma represents an extremely undifferentiated epithelial malignant neoplasm that morphologically looks like sarcoma, might show true divergent sarcomatous areas (also known as carcinosarcoma), and is less frequently positively stained with TTF-1 and CK7 than other conventional lung carcinomas.[56]

We propose a limited immunopanel that can be helpful to subclassify pulmonary LCC, translating at the immunohistochemical level the new insights in lung cancer classification provided by gene expression profiling studies. At present, our attempt can be considered academic, given that treatment decisions in NSCLC are made on the basis of clinicopathologic staging rather than on exact histologic features.[3] However, in the near future, subclassifying LCC might become an important issue for at least the following reasons: (1) to have a more reliable selection of lung tumor specimens to be analyzed with molecular studies (ie, gene expression profiling) for assessing identification of novel drug targets or prognostic and predictive gene markers, and (2) to permit more consistent patient recruitment in ongoing and future clinical trials of alternative treatments.

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