Journal of Cellular Biochemistry Supplement 24:92–106 (1996)

Spectrum of Neuroendocrine Differentiation in Lung Cancer Cell Lines Featured by Cytomorphology, Markers, and Their Corresponding Tumors

R. Ilona Linnoila

Biomarkers and Prevention Research Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Rockville, Maryland 20850

Abstract Lung cancer cell lines which show features of neuroendocrine (NE) differentiation can be divided into 4 types which have distinct clinicopathologic correlates: classic small cell lung cancer (SCLC), variant SCLC, pulmonary carcinoid, and non-small cell lung cancer with NE features (NSCLC-NE). These cell lines form a spectrum regarding their degree of NE differentiation which ranges from high levels seen in carcinoid cell lines to very low which is typical of the variant SCLC. A careful comparison of the properties of tumors and their cell lines and correlating these data with the clinical history of the patient has markedly enhanced the relevance of cell lines as models for NE biology and lung carcinogenesis. © 1996 Wiley-Liss, Inc.*

Key words: lung cancer cell lines, neuroendocrine differentiation, cytomorphology, markers, tumors

For a number of clinical and biological reasons lung cancers can be divided in two main categories: small cell lung cancer (SCLC) comprising 20–25%, and non-small cell lung cancer (NSCLC), comprising 75% of all lung cancers [1]. In 1959, Azzopardi described the uniform composition and behavior of SCLC [2]. Subsequently the unique nature of SCLC with its neuroendocrine (NE) features [3,4], early dissemination [5], and initial responsiveness to cytotoxic therapy [6] prompted extensive research efforts, including the establishment of large panels of continuous SCLC tumor cell lines.

The first continuous SCLC cell line was described by Oboshi et al. in 1971 [7]. Interestingly, this cell line was a floater, unlike most human epithelial tumor cultures which grew as attached monolayers of cells with characteristic "epithelial" appearance. Few reports on occasional SCLC cell lines were published until the 1980s when several independent groups presented panels of SCLC cell lines, and by now hundreds of cell lines have been characterized in the world literature [8–11].

In contrast to SCLC which is the most common cancer with NE differentiation, bronchial carcinoids are rare but unique NE tumors with distinct clinicopathologic features [12]. Pulmonary carcinoid tumors are characterized by organoid growth pattern, well-differentiated NE phenotype with indolent growth, and infrequent metastases. Typically these cancers are initially managed by surgery which often leads to cure. In 1967, Bensch et al. demonstrated the presence of neurosecretory, dense core granules in this tumor, confirming the NE nature of this neoplasm [13]. While Bensch et al. [1976] succeeded in growing explants of bronchial carcinoids in organ culture for as long as 5 months without the cells losing their ability to produce large numbers of neurosecretory granules, permanent cell lines were not established [14]. The first permanent human carcinoid cell lines, growing as monolayers at a slow rate but fully retaining their unique NE features, were established from intestinal tumors in the early 1980s [15,16]. In 1972, Arrigoni and colleagues reported a group of lung tumors that they designated as atypical carcinoids characterized by increased numbers of mitotic figures, disorganization of architec-

Received January 24, 1996.

Address reprint requests to R. Ilona Linnoila, Biomarkers and Prevention Research Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, 9610 Medical Center Drive, Suite 300, Rockville, MD, 20850.

ture, or tumor necrosis [17]. Perhaps most markedly, metastases were present in 70% of cases, and 30% of patients had died, on average, at 27 months following the diagnosis. Since then, multiple reports have confirmed the clinicopathologic entity of atypical carcinoids. A number of NE cell lines established from this lung cancer type are featured in the current manuscript.

The expression of NE properties is not limited to SCLC or bronchopulmonary carcinoids [18]. Approximately 10–20% of histologically defined NSCLCs also demonstrate NE features, and some studies suggest that clinically and biologically these tumors may demonstrate features in between SCLCs and NSCLCs with initial responsiveness to cytotoxic treatment and early metastases [19].

In 1985, Luster et al. reported a group of 25 lung cancer cell lines of four histologic types (SCLC, squamous cell, adenocarcinoma, and large cell carcinoma) all of which were capable of synthesizing a broad spectrum of immunoreactive peptide hormones including bombesin/gastrin releasing peptide (GRP), calcitonin, and ACTH and possessed characteristic dense core vesicles by electron microscope (EM) [20]. We have previously shown that by routine histology NSCLC tumors with NE features which we have designated as NSCLC-NE are indistinguishable from other NSCLCs [21]. A group of such cell lines is included in the current paper.

The objective of this manuscript is to illustrate the characteristic patterns of NE differentiation in human lung cancer cell lines that have been established at the NCI-Navy Branch. Cell culture routines, tissue procurement, and their relationship to clinical protocols will be reviewed, in a complementary fashion to the article by Oie et al. in this supplement [22]. Features of SCLC cell lines will be summarized. Subsequently the characteristics of cell lines obtained from pulmonary carcinoids and NSCLC-NE, and how they reflect the pathobiology and range of NE differentiation of their original tumors will be presented. Finally we will discuss the expression of NE spectrum in vivo and in vitro, and its clinical implications.

MARKERS OF NEUROENDOCRINE DIFFERENTIATION

The distinctive function of normal as well as neoplastic NE cells is the formation, packaging, and secretion of specific peptide and amine products. Pulmonary NE tumors such as SCLCs and carcinoids as well as their normal counterpart pulmonary endocrine cells (PECs) share characteristics of neural and endocrine cells, collectively referred to as NE features, and they are part of the diffuse endocrine system (DES) distributed throughout the body [23]. Other components of DES include paraganglia, pancreatic islets, thyroid C cells, endocrine cells of the breast, gastrointestinal and genitourinary tracts, and Merkel cells of the skin. The markers for NE properties can be divided into general or "pan" NE markers and specific products.

The expression of specific products is highly variable, is limited to subgroups of NE cells and their tumors, and can be species dependent [24] such as calcitonin (CT) in thyroid C cells and in subsets of human PECs and insulin or glucagon in pancreatic islet cells. One of the most characteristic peptide products of human lung is gastrin releasing peptide (GRP) or the mammalian analog of bombesin [25]. In addition to the specific products, PECs and pulmonary NE tumors share characteristics common to the entire DES. These general markers include: dense-core granules (the storage site of the amine and peptide products), high levels of the key amine-handling enzyme L-dopa decarboxylase (DDC) [26], and certain surface markers, other enzymes, and proteins (Table I). The advantage of using general NE markers is that it allows investigators to study the entire population of NE cells, normal or neoplastic, at one time. Moreover, using lung cancer cell lines and tumor specimens we have demonstrated excellent concordance between such markers as dense-core granules by electron microscopy, high DDC levels, and chromogranin A expression both at the mRNA and protein levels [27].

ESTABLISHMENT OF CELL LINES AND TISSUE PROCUREMENT

Initially, using a few established SCLC cell lines, a number of different hormones and growth factors were tested for their mitogenic effects on growth [28], which led to the development of the serum-free HITES medium [22]. Subsequent studies showed that this medium was useful also in selectively supporting the growth of clinical (fresh) specimens of SCLC, but not of stromal cells or tumor cells obtained from specimens of different histologic tumor type [29]. This work provided the basis for the oldest protocol at the NCI-Navy Medical Oncology Branch on *in vitro* growth and drug testing

TABLE I. Markers of Neuroendocrine Differentiation in Lung Cancer Cell Lines

Marker	Common techniques	Comments
A. General or "pan" markers		Common to all or most neuroendocrine cells
Dense-core granules[a]	Electron microscopy	Storage site of specific products visualized as ultrastructural organels
	Histochemistry	Indirect detection of dense-core granules at light microscopic level using argyrophilic, masked metachromasia, or lead hematoxylin reactions
L-dopa decarboxylase (DDC)[a]	Bioassay	One of key amine-producing enzymes
Chromogranin A	Immunohistochemistry, radioimmunoassay, Northern blot	Matrix protein of granules
Synaptophysin	Immunohistochemistry	Structural protein of cytoplasmic clear vesicles of neurons and neuroendocrine cells
Cluster I SCLC antigens	Immunohistochemistry	Antigens/compounds related to neural cell adhesion molecules (n-CAM) such as Leu-7, NKH-1, and MOC-1; shared by neurons and neuroendocrine cells
PGP9.5	Immunohistochemistry	Neural protein
Neuron-specific enolase	Immunohistochemistry, radioimmunoassay	Glycolytic isoenzyme found in neurons and neuroendocrine cells
Brain isoenzyme	Bioassay, immunohistochemistry	High levels detected in brain and endocrine tissues
B. Specific products		
Peptides and amines	Radioimmunoassay, immunohistochemistry, Northern blot	More than 35 specific peptide and amine products identified, including gastrin releasing peptide (GRP), calcitonin, adrenocorticotropin, atrial natruretic peptide, serotonin, etc.

[a]Markers consistently applied to classify the cell lines at NCI-Navy Medical Branch.

of human tumors which is still running in 1996. The goal of this protocol is to create a ready source of replicating well-characterized tumor cell lines for a variety of biological studies, and also to provide information on initial histology and patient history as well as fresh tumor and non-neoplastic tissue, as previously described [30–32].

By 1984, the establishment of permanent cell lines from SCLC had become part of Branch routine, and clinical protocols for SCLC and NSCLC were devised to prospectively select individualized chemotherapy based on *in vitro* drug sensitivity testing of established cell lines derived from the patient's SCLC tumor or short-term cultures of fresh SCLC or NSCLC tumors [33,34]. Figure 1 illustrates the establishment of new cell lines as the function of specimens which were received in the laboratory from November 1983 till December 1990. Approximately half of the cell lines were derived from the 430 patients in the 4 NCI-Navy clinical protocols which have been previously described [34–38].

Specimens were obtained in sterile RPMI growth media or physiological saline from an anatomic pathology laboratory following mostly routine diagnostic, surgical, or staging procedures, and transported to the laboratory as quickly as possible. The specimens were then prepared for culture as described in detail elsewhere [22,39]. At this time, a representative

Fig. 1. Establishment of lung cancer cell lines and tissue procurement at the NCI-Navy Medical Oncology Branch. These diagrams illustrate the number of specimens (continuous black line corresponding to the scale on the right) that were received at the laboratory during November 1983 through December 1990, the number of specimens containing tumor cells as identified by cytospin preparations (positive specimens; discontinuous gray line corresponding to the scale on the right) and number of established cell lines (solid bars corresponding to the scale on the left). **A:** Small cell lung cancer (SCLC). Note the relatively small fraction of positive specimens due to the standardized pathologic staging procedures which were part of the clinical protocols producing multiple negative samples from the same patient. **B:** Non-small cell lung cancer (NSCLC). Note that most specimens contained tumor cells, because a requirement in the main clinical protocol [34] included the procurement of a positive tumor specimen for *in vitro* studies. **C:** A small number of selected other tumor specimens were received in the laboratory leading to the establishment of cell lines.

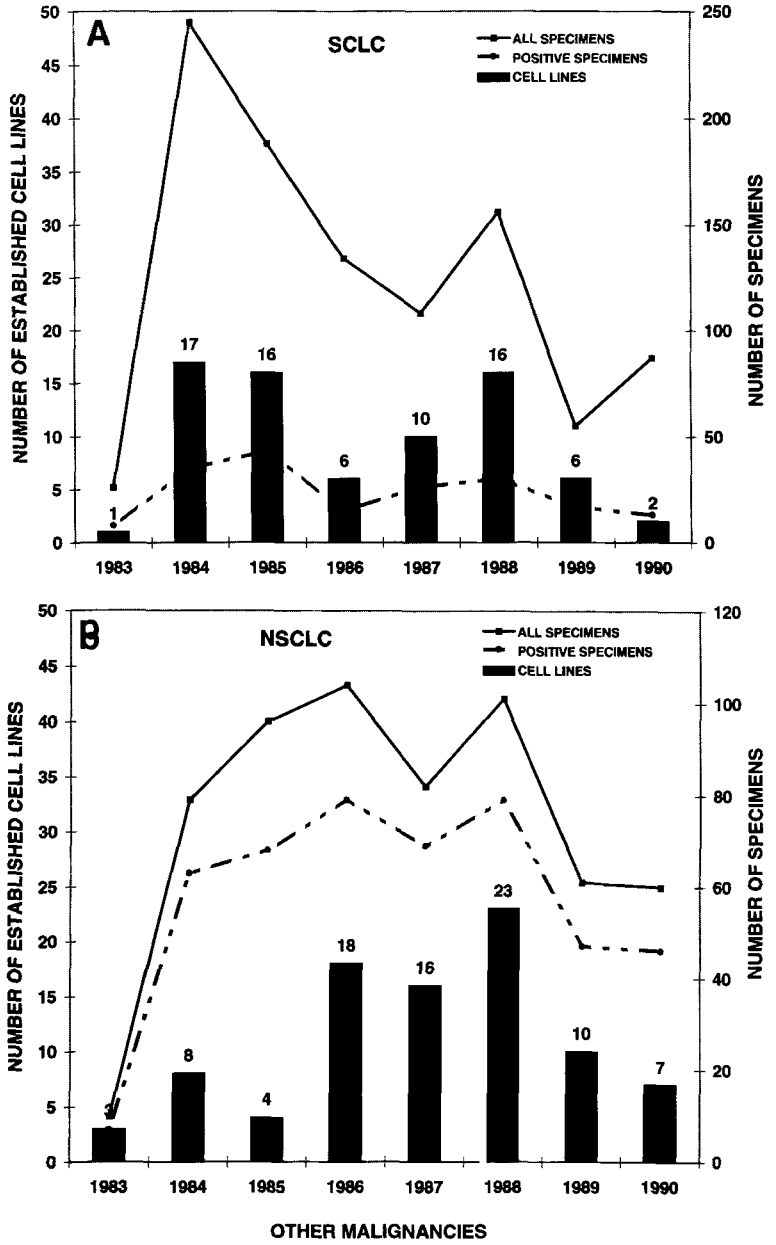
A
SCLC
ALL SPECIMENS
POSITIVE SPECIMENS
CELL LINES
NUMBER OF ESTABLISHED CELL LINES
NUMBER OF SPECIMENS
1
17
16
6
10
16
6
2
1983
1984
1985
1986
1987
1988
1989
1990
B
NSCLC
ALL SPECIMENS
POSITIVE SPECIMENS
CELL LINES
NUMBER OF ESTABLISHED CELL LINES
NUMBER OF SPECIMENS
3
8
4
18
16
23
10
7
OTHER MALIGNANCIES
C
NUMBER OF ESTABLISHED CELL LINES
NUMBER OF SPECIMENS
0
7
2
4
3
6
0
5

Figure 1

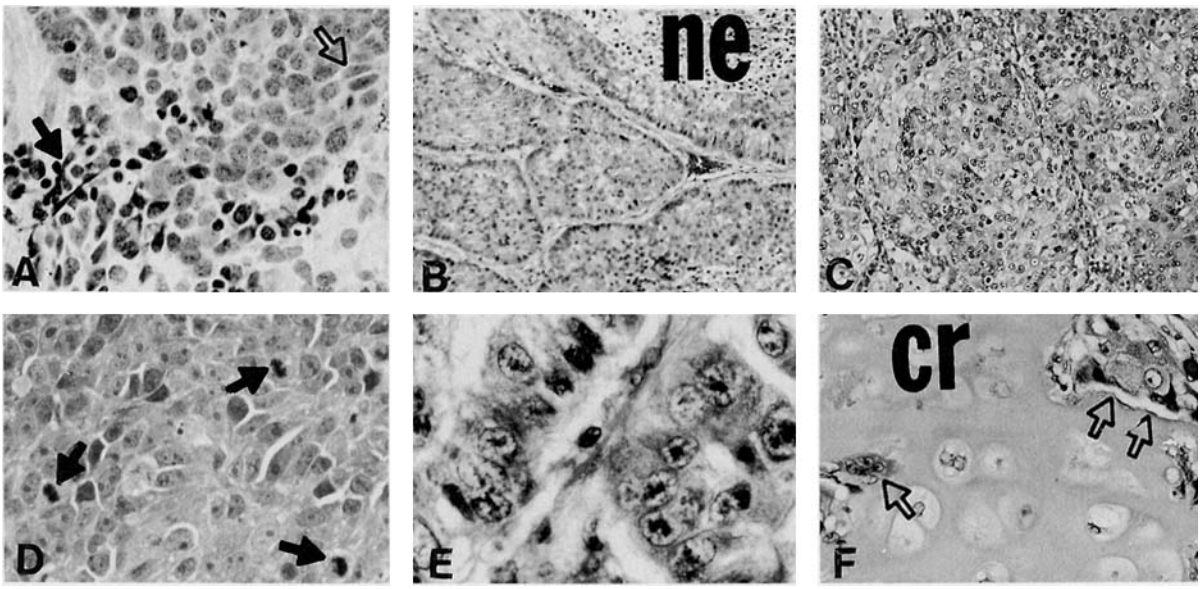

Fig. 2. Photomicrographs of characteristic tumor morphology in pulmonary neuroendocrine neoplasms which correspond to cell lines. **A:** The mediastinal lymph node of a 59-year-old man with extensive stage disease which gave rise to the classic SCLC cell line NCI-H1769. Normal architecture was effaced by sheets of tumor cells with salt-and-pepper chromatin and scanty cytoplasm with nuclear molding (open arrow). Note areas of necrosis and DNA streaking (solid arrow; hematoxylin and eosin stain, ×330). **B:** Low power view of an atypical carcinoid in the right lung of a 41-year-old female leading to the establishment of NCI-H1435 cell line reveals an organoid pattern with palisading tumor cells and areas of necrosis (ne); (hematoxylin and eosin stain, ×80). **C:** The original tumor of NCI-H1570, an NSCLC-NE, obtained from the right lung of a 48-year-old man, shows poorly differentiated adenocarcinoma (hematoxylin and eosin stain, ×80). **D:** A nude mouse tumor of the variant SCLC line NCI-Nut 417 shows a diffuse growth pattern by larger cells than in A with prominent nucleoli and multiple mitoses (solid arrows; hematoxylin and eosin stain, ×300). **E:** At higher power the original atypical carcinoid tumor for NCI-H1435 revealed large atypical cells with abundant cytoplasm and palisading on the left and efforts to form gland-like structures on the right (hematoxylin and eosin stain, ×500). **F:** The aggressive nature of the original NSCLC-NE tumor of NCI-H1570 was evidenced by the invasion of cartilage (cr) by tumor cells (open arrows) in the lung specimen (hematoxylin and eosin stain, ×170).

cytological sample from each specimen was fixed in Saccomanno's fixative [40], and H&E stained cytospin slides were prepared in duplicate. These slides constituted a permanent record at the NCI-Navy Medical Branch, and were evaluated for the presence and histology of malignancy by the pathologist. This simple procedure was highly accurate, and results correlated well with the routine histopathology specimens and *in vitro* ("flask") morphology and growth. Consequently, as has been also noted by other investigators, it may be unnecessary to culture the samples determined to lack tumor cells, because the yield of cell lines is negligible [9]. Based on the NCI-Navy Medical Oncology Branch experience in 1983–1991, the establishment of one cell line would have been potentially missed (0.5%) if all the samples called negative by cytospin would have been discarded immediately following the assessment.

It was not uncommon to obtain multiple specimens from the same patient. This is particularly true for SCLC (Fig. 1A) because repeated, often negative specimens such as bilateral bone marrow aspirates were procured as part of routine staging and follow-up procedures. For example, in 1988, out of the 156 specimens obtained from SCLC patients and brought to the laboratory, 30 (19%) were positive for tumor and gave rise to 16 (53%) SCLC cell lines. In NSCLC (Fig. 1B) the goal was more focused on procuring a tumor containing specimen to the laboratory. Moreover, the formative years of 1984 and 1985 showed success rates (percentage of NSCLC cell lines established from histologically positive samples) of only 13 and 6%, respectively. However, in 1988 a total of 101 specimens from NSCLC patients were received in the laboratory of which 78 contained tumor cells leading to the establishment of 23 (29%) continuous lung cancer cell lines.

SMALL CELL LUNG CANCER

SCLC accounts for 20–25% of lung cancers, and over 100 SCLC cell lines have been established at the NCI-Navy Medical Oncology

Branch. Because SCLC has a tendency to metastasize early and resection specimens are rarely available, most cell lines have been established from metastatic sites such as a lymph node, bone marrow, pleural effusion, liver, mediastinum, or subcutaneous nodule [41]. No differences have been observed in the biological properties of SCLC lines established from these different metastatic sites. Since the development of the serum-free defined HITES medium, it has been possible to propagate tumor cell growth in up to 74% of SCLC specimens containing tumor cells [42]. With HITES medium, cell

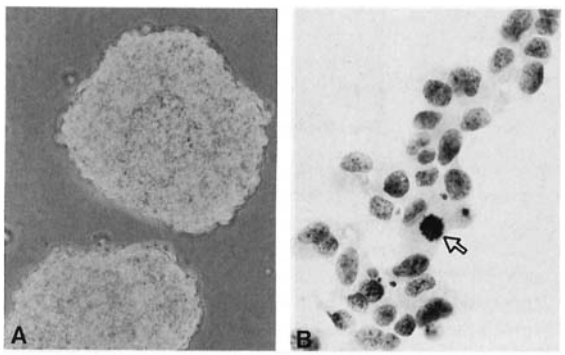

Fig. 3. Photomicrographs of *in vitro* growth pattern and cytology of classic SCLC. **A:** Classic SCLC cell lines characteristically grow in floating aggregates or tight spherules without recognizable cellular details (phase contrast microscopy, ×100). **B:** A cytospin of the classic SCLC line NCI-H128 passage 36 reveals fine salt-and-pepper chromatin pattern. Note the presence of a mitotic figure (open arrow; hematoxylin and eosin stain, ×460).

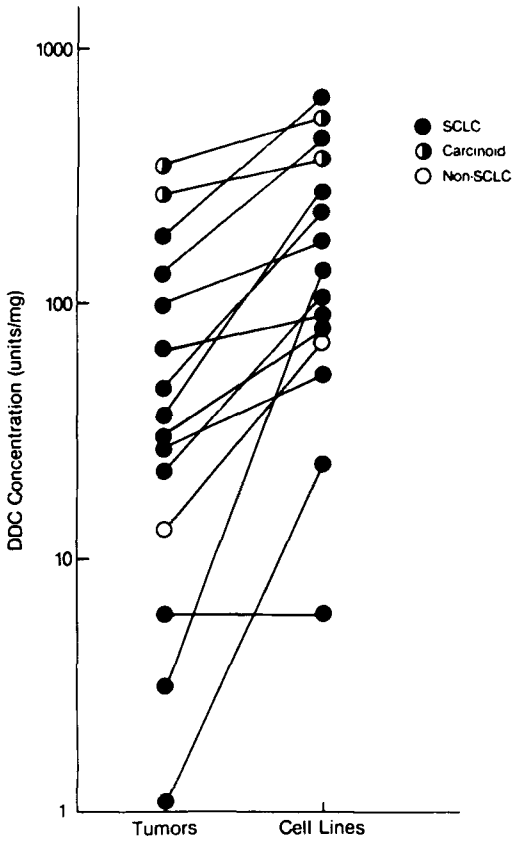

Fig. 5. Comparison of the concentrations of DDC levels in cell lines and the corresponding, original tumors. DDC activity was measured in freshly frozen tumor specimens (**left**) of 12 SCLCs, 2 carcinoids, and 1 NSCLC-NE and the cell lines (**right**) established from the same tumors. (Illustration courtesy of Dr. Adi Gazdar.)

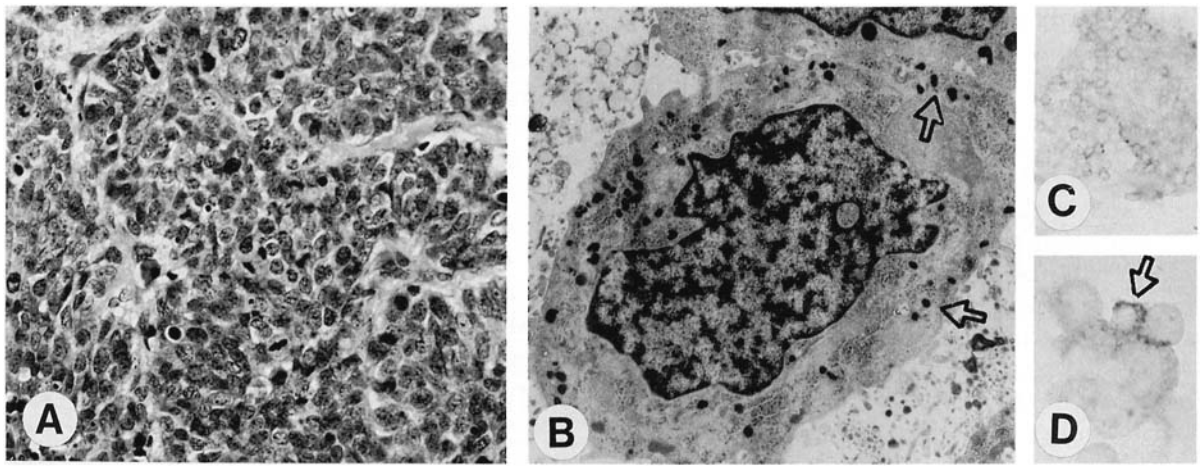

Fig. 4. Neuroendocrine characteristics of the classic SCLC line NCI-H774. **A:** Original tumor was a soft tissue mass composed of sheets of tumor cells obtained from a 42-year-old man (hematoxylin and eosin stain, ×300). **B:** Electron micrograph of the established cell line demonstrates several small clusters of dense-core endocrine-type granules (open arrows; lead-uranyl-acetate stain, magnification 3,200). **C:** The cytospin preparation of the cell line at passage 25 reveals immunoreactivity for synaptophysin in the majority of the cells (immunoperoxidase, ×210). **D:** Scattered cells also show granular immunoprecipitate for chromogranin A (open arrow; immunoperoxidase, ×500).

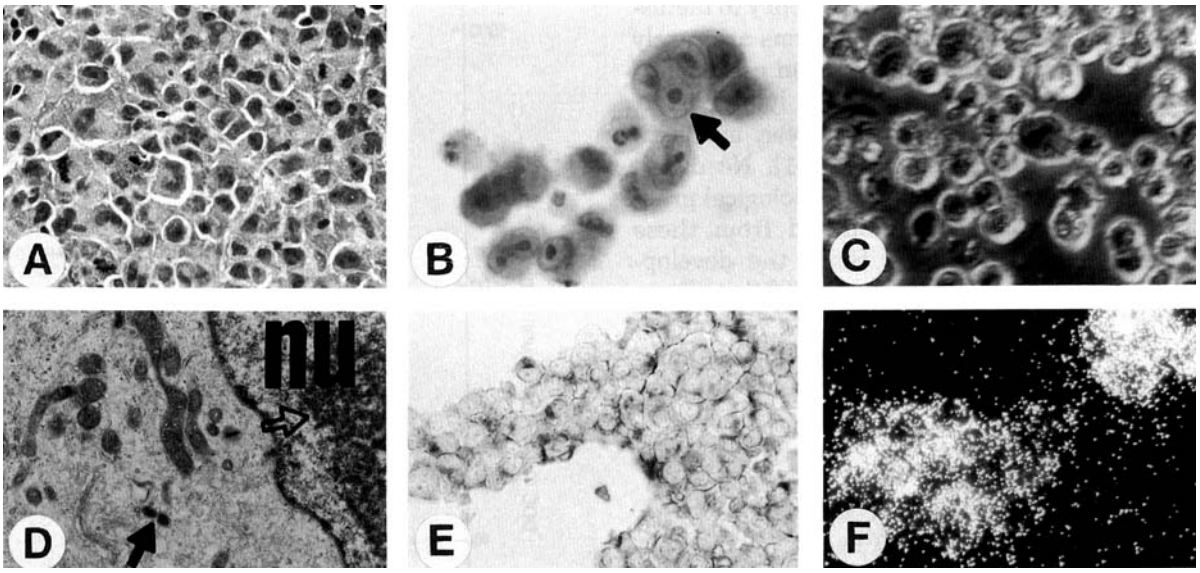

Fig. 6. Characteristics of variant SCLC lines. **A:** Original tumor of the variant SCLC line NCI-H841 was a supraclavicular lymph node from a 51-year-old man revealing sheets of tumor cells with moderate amounts cytoplasm and prominent nucleoli. Mitoses were frequent (hematoxylin and eosin stain, ×310). **B:** A cytospin of passage 14 of the established cell line also demonstrated conspicuous nuclei (solid arrow; Papanicolaou stain, ×550). **C:** In vitro growth was characterized by floating cells in very loose clusters (passage 16, phase contrast micrograph, ×340). **D:** Electron micrograph of passage 10 revealed rare dense-core granules (solid arrow). Note the presence of a nucleolus (open arrow; lead-uranylacetate stain, ×8,200). nu = nucleus. **E:** A cytospin of the variant SCLC line NCI-N417 at passage 28 with intense synaptophysin immunoreactivity (immunoperoxidase, ×170). **F:** Intense labelling for *c-myc* mRNA [49] in the variant SCLC line NCI-H82 at passage 30, a reflection of the *myc* gene amplification (RNA-RNA in situ hybridization, dark field illumination, ×340).

lines have been established with equal efficiency from treated and untreated patients.

By histologic appearance SCLC is a poorly differentiated neoplasm composed of sheets of cells and areas of necrosis. Typical features include "crush artifact" especially in small biopsies when tumor cells exhibit marked artifactual distortion and nuclear encrustation or DNA streaking when basophilic nuclear material is deposited on the blood vessel walls and adjacent connective tissues (Fig. 2A). In the oat-cell subtype cells appear small, round, and hyperchromatic and in the intermediate type individual cells are small with finely dispersed "salt and pepper" chromatin pattern, and because of their scanty cytoplasm, they may display nuclear molding. Most of the established, classic SCLC cell lines recapitulate the intermediate features. They grow in spherules or floating aggregates without substrate adherence, and cytospin preparations reveal the characteristic fine chromatin pattern (Fig. 3). The consistent display of this appearance by both the cell lines as well as most nude mouse xenografts suggests that the intermediate subtype of SCLC, irrespective of the subtype of the original tumor, is the true morphologic appearance of the original tumor, while the oat-cell subtype is an ischemic artifact [43] (Fig 4A).

Classic SCLC cell lines express the entire range of NE markers [30]. By electron microscopy small clusters of small, dense core (endocrine-type) granules are present (Fig. 4B). Accordingly, by immunohistochemistry, scattered cells demonstrate reactivity for chromogranin (Fig. 4D), which is a structural protein of the storage granules, in 57% of cell lines [44,45]. Interestingly, while only scanty immunoprecipitates were visualized in individual cells, relatively high levels of chromogranin mRNA can be detected in classic SCLC cell lines, suggesting that these cells are able to manufacture it while there may be deficiencies in the storage of the peptide product [46,47].

The presence of dense core granules and chromogranin in classic SCLC cell lines is also associated with high levels of the DDC activity. In fact, the cultures frequently express DDC and other NE products at higher concentrations and at higher frequencies than the tumors from which they originated (Fig. 5). This is due to the fact that cell lines represent pure tumor cell populations which lack stromal cells, and contain higher percentages of cycling, active cells than do tu-

mors, which may often have extensive areas of necrosis.

In addition to general markers, classic SCLC cell lines express a variety of NE peptides characteristic of SCLC. Individual lines may express as many as 10 peptides. One of the most commonly produced peptides by classic SCLC cell lines has been GRP which is also the major product in non-neoplastic human pulmonary endocrine cells. The concentrations of bombesin/GRP-like immunoreactivity have been 1–2 logs higher than other peptides and appear to be clonally and temporally retained [48].

A subset of SCLC cell lines called variant SCLC cell lines [30,31] differs from classic SCLC lines by a number of morphologic and biologic characteristics (Fig. 6). They compose approximately 5% of all the established SCLC cell lines. Original tumors typically contain larger cells with prominent nucleoli than do classic SCLCs, and mitoses are more conspicuous (Figs. 2D, 6A). Variant SCLC cell lines grow as loose, floating aggregates which occasionally develop ribbon-like projections or sometimes adhere loosely to the bottom of tissue culture dishes, and cytospin preparations of established cell lines again display prominent nucleoli (Fig. 6B, C).

Variant SCLC cell lines express only a partial spectrum of NE markers [31] (Fig. 6D, E). By electron microscopy, only rare solitary or no dense core granules are detected, which is associated with low DDC levels or the lack of chromogranin expression in these cell lines. The expression of other general or "pan" NE markers such as neuron specific enolase, synaptophysin, and Leu-7 is retained (Fig. 7). In contrast to classic lines, variant SCLC cell lines do not produce neuropeptides such as GRP or calcitonin. Moreover, variant SCLC cell lines are associated with shorter doubling time, increased cloning efficiency, and frequently with c-myc amplification [31,49] (Fig. 6F). As discussed elsewhere in this issue by Johnson et al., it appears that *MYC* family DNA amplification in SCLC is more frequently present following treatment with chemotherapy regimens [50]. Other laboratories have also established variant lines [42,51–53].

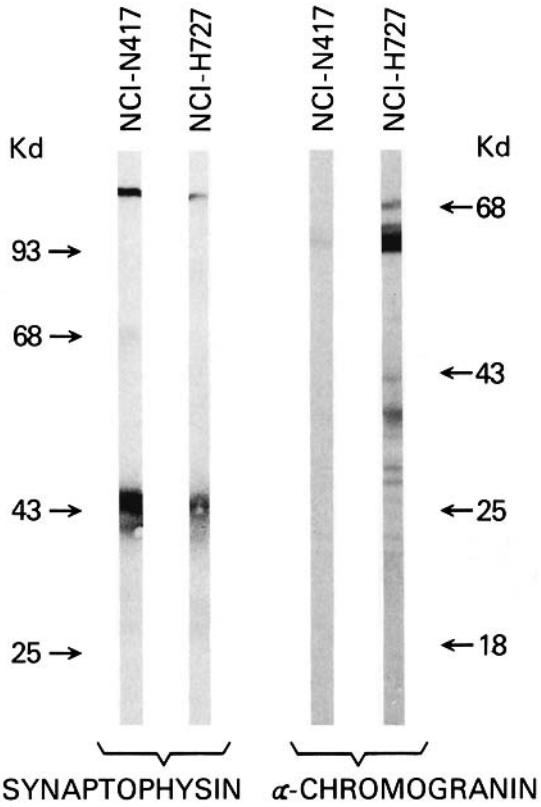

Fig. 7. *Western blot analysis of the general neuroendocrine markers synaptophysin and chromogranin A in a variant SCLC (NCI-N417) and carcinoid (NCI-H727) cell lines. Whole cell lysates were subjected to SDS-PAGE fractionation under reducing conditions and electroblotted to nitrocellulose membranes [68]. The membranes were treated with anti-synaptophysin (α-synaptohysin, SY38, **left**) or anti-chromogranin A (α-chromogranin, LK2H10, **right**) antisera, respectively, as previously described [68]. Both cell lines contained peptides with synaptophysin-like immunoreactivity at M_r 43,000, while only the carcinoid line NCI-H727 contained immunoreactivity characteristic for chromogranin A with a major band at the range of M_r 64,000 to 66,000. When membranes were treated with undiluted indifferent monoclonal antibody MOPC21 (data not shown), no bands were seen, verifying the specificity of the immune reaction.*

PULMONARY CARCINOID

In contrast to SCLCs, carcinoids are rare tumors that constitute approximately 2% of lung tumors. They are well-differentiated NE neoplasms with distinct clinicopathologic features [12]. Six out of the 160 lung cancer cell lines (4%) established at the NCI-Navy Medical Oncology Branch between 1984–1991 that were examined fulfill these criteria. Pulmonary carcinoids are slow growing tumors, and not surprisingly, all six carcinoid lines were established from lung primary tumors obtained from lung resection specimens. Original tumors demonstrated characteristic organoid growth pattern with fibrovascular stroma and islands of palisading tumor cells (Figs. 2, 8A). Cells had moderate amounts of cytoplasm, and prominent nucleoli. Due to the areas of necrosis, cellular atypia, and frequent mitoses, these tumors were classified as atypical carcinoids [12,17,54].

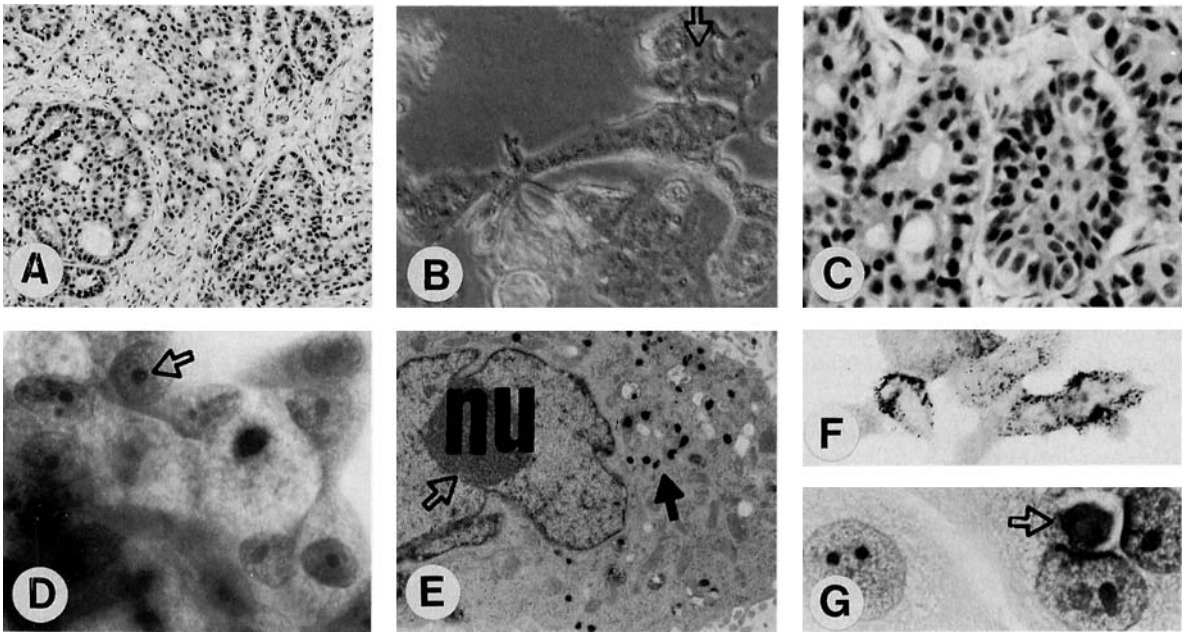

Fig. 8. Morphologic and ultrastructural characteristic of a bronchopulmonary carcinoid cell line (NCI-H727). **A:** In the original resection specimen from a 65-year-old woman, organoid growth pattern and fibrovascular stroma typical for a carcinoid tumor were present (hematoxylin and eosin stain, ×90). **B:** In vitro growth at passage 12 in RPMI 1640 with 10% calf serum was characterized by substrate attachment, large epithelioid cells with conspicuous nucleolus (arrow; phase contrast, ×330). **C:** Nude mouse xenograft revealed organoid growth pattern, similar to the original tumor (hematoxylin and eosin stain, ×330). **D:** A cytological examination of cultured tumor cells at passage 8 in R10 revealed conspicuous nucleolus (arrow) and abundant cytoplasm (Papanicolaou stain, ×790). **E:** Ultrastructurally, these cells contained numerous relatively large dense core granules (solid arrow) throughout the cytoplasm. Note the conspicuous nucleolus (open arrow). nu = nucleus. (Electron micrograph, ×4,700.) **F:** Intense granular chromogranin-like immunoreactivity in the cytoplasm of the NCI-H727 cells grown on a slide in R10 (immunoperoxidase, ×300). **G:** A subculture of the NCI-H 727 original tumor during early passages in LA3 medium revealed intracellular mucin droplets (arrow; mucicarmine, ×790).

TABLE II. Characteristics of Pulmonary Carcinoid Cell Lines*

Cell line (NCI)	Date	Specimen	Growth pattern/ media	Electron microscopy	DDC (tumor) (U/mg protein)
H679	1/84	Lung	S/F H5	Clusters of DCGs	580 (129)
H720	4/84	Lung	F H5	Clusters of DCGs of various sizes	100 (9.4)
H727	4/84	Lung	S R10	Abundant DCGs of various sizes	174 (15.6)
H835	10/84	Lung	S/F A4	Small DCGs scattered and clusters	3.1 (1.9)
H1435	6/86	Lung	S A4	Clusters of DCGs with large halos	52.2 (31.5)
H1734	9/87	Lung	S SCRRL	Clusters of DCGs of various sizes	54.1 (7.0)

*S = sticker, growing with substrate adherence; F = floater, growing in floating aggregates without substrate adherence; H5 = HITES with 5% serum; R10 = RPMI with 10% serum; A4 = adenocarcinoma medium [22]; SCRRL = modified squamous cell medium [22]; DCG = dense core granule; DDC = L-Dopa decarboxylase; Units = nmol CO_2 per hour.

In vitro, four of the carcinoid cell lines grew as stickers, while two of them lacked the substrate adherence and grew as floating aggregates similar to SCLC lines. Nude mouse xenografts of the cell lines demonstrated the characteristic organoid growth pattern. Carcinoid cell lines expressed the entire range of NE markers (Table II; Figs. 7, 8). In fact, established cell lines demonstrated 2–11-fold increase in the DDC activity when compared with the activity measured in the original tumors. Ultrastructurally, all carcinoid cell lines showed abundant dense core, endocrine-type granules which appeared larger in size and more variable in appearance than the same granules in SCLC lines. By immuno-electronmicroscopy, granules were positive for chromogranin A, confirming their endocrine nature (Fig. 9). In addition to the general

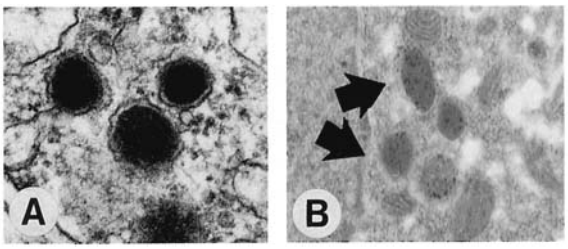

Fig. 9. Dense core granules with chromogranin-like immunoreactivity in a carcinoid cell line (NCI-H727). **A:** Transmission electron microscopy revealed characteristic dense core granules surrounded by a unit membrane (lead, uranylacetate stain, ×26,000). **B:** Dense core granules labeled with chromogranin A-like immunoreactivity from the same cell line. Note numerous fine spheres over four granules (arrows; immunogold, ×13,000).

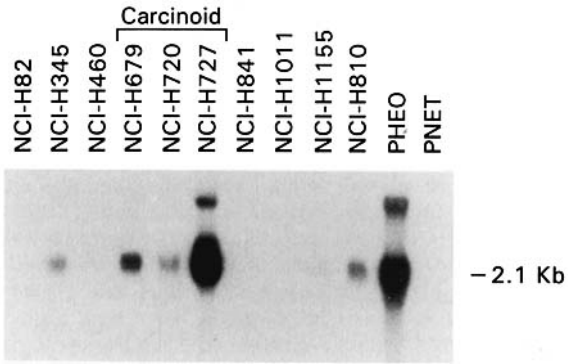

Fig. 10. Expression of chromogranin A mRNA in lung cancer cell lines. Northern blot analysis was performed using 10 μg of total RNA per lane from the specimens listed. The pulmonary carcinoid cell lines NCI-H679 (passage 26), NCI-H720 (passage 22), and NCI-H727 (passage 16) all showed consistent expression of chromogranin mRNA at 2.1 Kb which is the expected size as shown in the lane for the positive control tissue derived from human pheochromocytoma tumor (pheo). The NSCLC-NE lines NCI-H1155 (passage 13) and NCI-H810 (passage 20) reveal variable amounts of chromogranin mRNA, comparable to the amount obtained for the classic SCLC line NCI-H345 (passage 68). No mRNA was detected in the lanes of the variant SCLC lines NCI-H 82 (passage 30) and NCI-H841 (passage 22). Negative controls included an esthesioneuroblastoma cell line (NCI-H1011) and peripheral neuroectodermal tumor (PNET; Northern blot a courtesy of Dr. Lee Helman).

NE markers, synaptophysin, neuron specific enolase, and chromogranin, many carcinoid lines produced several neuropeptides.

By multiple criteria of morphology and markers, the carcinoid line NCI-H727 appeared most differentiated (Fig. 10), also producing large amounts of immunoreactive calcitonin at 21,600 pg/mg and neurotensin at 3,000 pg/mg of protein by radioimmunoassay [55]. Subsequently this cell line has been used in a variety of studies. Interestingly, culturing this cell line in a media defined for adenocarcinomas [22] leads to a phenotype characterized by low DDC activity, and no chromogranin but, in contrast, abundant mucin expression (Fig. 8G).

NON-SMALL CELL LUNG CANCERS WITH NEUROENDOCRINE FEATURES

NSCLC-NE by definition are tumors which by routine hematoxylin-eosin stain are morphologically indistinguishable from other NSCLCs, but by special techniques demonstrate NE features (Figs. 2C, F, 10). The potential significance of this subgroup is that clinicopathologically it may behave like SCLCs with early dissemination and initial responsiveness to chemotherapy [19,56]. Among the 86 NSCLC cell lines established at the NCI-Navy Medical Oncology Branch from 1984 through 1991, which were reviewed for NE differentiation, 6 cell lines (7%) fulfilled the criteria of NSCLC-NE of two (high DDC level and the presence of dense core granules) or more NE markers positive.

In accordance with the potentially aggressive nature of this neoplasm, 4 out of the 6 cell lines were established from metastatic lesions and 2 from the primary lung tumors. Figure 2F illustrates the original tumor of NCI-H1570 infiltrating a bronchial cartilage. With the exception of NCI-H820, which originated from a papillary adenocarcinoma, all the other tumors were poorly differentiated, large cell carcinomas composed of cells with abundant cytoplasm and prominent nucleoli, and similar histologies were seen both in metastases and nude mouse tumors (Figs. 2, 11). Differential diagnoses included the large cell-neuroendocrine tumors, but in spite of careful analysis, features of organoid pattern or endocrine appearance suggestive of this entity were not noted in the original tumors [1,57]. In the case of NCI-H1155 the original specimen consisted of a very small biopsy in which case the presence of areas with more differentiated patterns cannot be totally excluded.

All 6 NSCLC-NE cell lines grew as floating aggregates without substrate adherence (Fig. 11D). Cytospins of the established cell lines revealed the features of the original tumor cells, including abundant cytoplasm and prominent nucleoli (Fig. 11E). The NSCLC-NE cell lines demonstrated features of the entire NE program (Table III). By electron microscopy four cell lines contained clusters of dense core, endocrine-type granules, which were variable in size and shape, while 2 cell lines demonstrated only

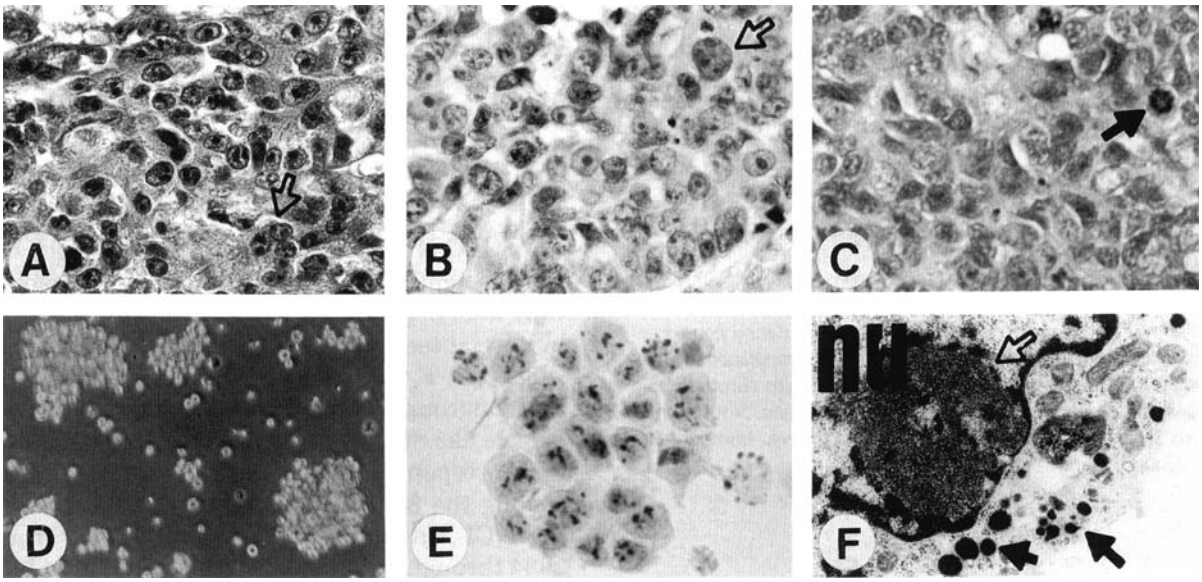

Fig. 11. Morphologic and ultrastructural characteristics of the cell line NCI-H810, a non-small cell lung cancer with neuroendocrine features (NSCLC-NE). **A:** Original lung tumor from which the cell line was established consisted of sheets of undifferentiated large cells with abundant cytoplasm and prominent nucleoli. Frequently multiple nucleoli were seen in the same nucleus (arrow; hematoxylin-eosin stain, ×380). **B:** One of the subcutaneous nodules that developed 10 months later was biopsied and revealed metastatic disease with similar morphology. An arrow points to a cell with multiple nucleoli (hematoxylin-eosin stain, ×380). Subsequently, this patient was treated by chemotherapy which led to complete remission, including the disappearance of his skin nodules [34,56]. **C:** A nude mouse xenograft of NCI-H810 at passage 29 also demonstrated similar morphology with frequent mitoses (arrow; hematoxylin-eosin stain, ×420). **D:** In vitro growth was characterized by floating cell aggregates (passage 14; phase contrast microscopy, ×80). **E:** Cytospin of the cell line revealed large cells with moderate amount of cytoplasm, and conspicuous, often multiple nucleoli (passage 14; Papanicolaou stain, ×420). **F:** Ultrastructural features at passage 20 included cytoplasmic clusters of dense core granules of variable sizes (solid arrows) and conspicuous nucleoli (open arrow). nu = nucleus. (Transmission electron micrograph, lead, uranylacetate stain, ×4,600.)

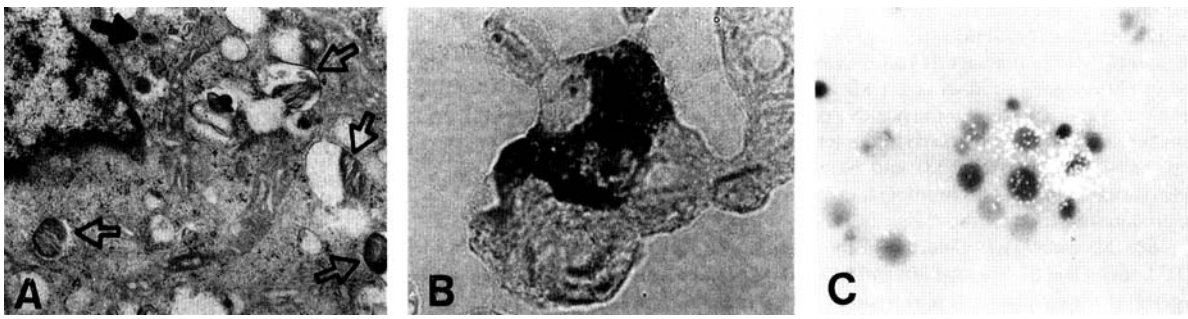

Fig. 12. Evidence for dual differentiation in a non-small cell lung cancer cell line with neuroendocrine features (NSCLC-NE; NCI-H1570). **A:** Ultrastructural analysis of the cell line at passage 12 demonstrated characteristic dense core granules (solid arrow) and lamellar bodies (open arrows) in the cytoplasm (transmission electron micrograph, lead, uranylacetate stain, ×6,600). **B:** In the cells cultured on chamber slides dark cytoplasmic precipitate indicated chromogranin A-like immunoreactivity (passage 70; immunoperoxidase, ×830). **C:** RNA in situ hybridization [58] revealed labelling for surfactant associated protein A (passage 12; dark field, ×330).

solitary small dense-core granules. Interestingly, it was these 2 cell lines which also contained lamellar bodies suggesting type 2 pneumocyte, non-endocrine differentiation [58]. This multidirectional differentiation was further confirmed by the expression of surfactant associated protein A (SPA) in both cell lines (Fig. 12C).

As expected, the presence of dense core granules correlated with high levels of DDC activity and the expression of chromogranin A (Table III). Selected NSCLC-NE cell lines also produced high levels of neuropeptides. Additional evidence for NE differentiation in these cell lines was provided recently by the detection of high

TABLE III. Characteristics of Non-Small Cell Lung Cancer Lines With Neuroendocrine Features (NSCLC-NE)*

Cell line	Date	Specimen	Growth pattern/media	Electron microscopy	DDC (tumor) (U/mg protein)
H810	9/84	Lung	F H2	Clusters of DCGs of variable size	406 (12.9)
H820	9/84	Lymph node	F A4	Rare DCGs	16 (neg)
H1155	9/85	Mediastinal biopsy	F A4	Clusters of small DCGs	20.4 (neg)
H1385	4/86	Hilar mass	F R10	Abundant DCGs of various size and shape	253 (not done)
H1570	12/86	Lung	F SCRRL	Scattered small DCGs	249 (142)
H1944	6/88	Subcutaneous nodule	S R10	Clusters of small DCGs	38.5 (1.7)

*S = sticker, growing with substrate adherence; F = floater, growing in floating aggregates without substrate adherence; DCG = dense core granule; DDC = L-Dopa decarboxylase; U = nmol CO_2 per h. For media see Table II and [22].

levels of the human homolog-1 of aschaete-scute basic helix-loop-helix transcription factor, which is essential for nervous system development in Drosophila [59,60].

IMPLICATIONS OF PULMONARY NEUROENDOCRINE CELL LINES

The establishment of continuous lung cancer cell lines which express NE differentiation has allowed extensive characterization of a plethora of features associated with this phenotype. These cell lines continue to provide an essential and renewable resource for the testing of novel NE markers or concepts [61,62].

The results, in return, have greatly advanced our knowledge of biology and molecular genetics of lung carcinogenesis in general and, more specifically, led to a better understanding of the implications of NE differentiation. Despite marked differences between individual cell lines, possible alterations during *in vitro* growth, and the well-documented heterogeneity of lung tumors in general, clear patterns of NE marker expression have emerged which appear to be relatively stable. As shown in the current article, using cytomorphology, ultrastructure, biochemical markers, and the phenotype of corresponding tumors, NE lung cancer cell lines can be divided into 4 distinct groups (Fig. 2, Table IV): classic and variant SCLC, pulmonary carcinoid, and NSCLC-NE.

The most common of the NE lung cancer cell lines is the classic SCLC which is a morphologically poorly differentiated tumor but expresses the entire spectrum of NE markers including the production of specific neuropeptides. *In vitro* drug sensitivity testing of large panels of SCLC cell lines has indicated that they are representative of the clinical sensitivity of this tumor type [33]. Pulmonary carcinoids are more differentiated, and express NE markers at a higher level. *In vitro* chemosensitivity testing of cell lines has

TABLE IV. Major Properties of Human Neuroendocrine Lung Cancer Cell Lines

Property	Classic SCLC	Variant SCLC	Carcinoid	NSCLC-NE
Original tumor histology	SCLC	Small cell/large cell	Atypical carcinoid	Adeno or large cell
Growth pattern and substrate adherence	Floating aggregates	Loose floating aggregates or semiadherent	Floating or adherent monolayer	Floating or adherent monolayer
Xenograft histology	SCLC	Large cell	Carcinoid or NSCLC	NSCLC
Doubling time	Long	Short	Long	Long
Degree of NE differentiation	Moderate	Low	High	Moderate, variable
General NE markers				
Dense-core granules	Present	Absent	Abundant	Present
Others	Present	Variable	Abundant	Present
Specific NE markers				
GRP	Present	Absent	Present	Present, variable
Other peptides	Present	Absent	Present	Present
Chemosensitivity	High	Low	Low	High
Sensitivity to radiation	High	Low	Low	Low

confirmed the clinically known fact that, in general, this tumor type is chemoresistant [63].

On the other hand, both variant SCLC as well as NSCLC-NE cell lines express some features which are more characteristic of NSCLCs such as morphology. The evidence for multidirectional differentiation which is seen in both SCLC and NSCLC-NE cell lines is in accordance with the current hypothesis that all lung cancer types, including those with NE differentiation, originate from one multipotent stem cell [64,65]. Variant SCLC cell lines express only some NE markers, and while NSCLC-NE cell lines express the entire program of NE markers, selected cell lines also express specific markers of peripheral airway cell differentiation. Moreover, there is now *in vitro* evidence that NSCLC-NE may be sensitive for chemotherapy, as suggested by some clinical studies [19,34,63]. The overall correlation of in vitro drug sensitivity testing results with response to chemotherapy and survival is further discussed by Shaw et al. in this supplement [66].

ACKNOWLEDGMENTS

I thank all the staff and associates of the NCI-Navy Medical Oncology Branch who have contributed to this research throughout the years. I specifically thank Drs. John Minna, Adi Gazdar, Des Carney, Dan Ihde, Bruce Johnson, and Jim Mulshine who allowed me to work with these cell lines. Moreover, I acknowledge Dr. Herb Oie, Mr. Edward Russell, and Ms. Sylvia Stephenson for their expertise in cell culture, and Mr. Pat Riordan and Dr. Yukio Kurita for ultrastructural analyses. Thanks are also due to Ms. Tess Bunnag for creating the computer data base for the cytospin records and to Drs. Adi Gazdar and Eva Szabo for their helpful comments on the manuscript.

REFERENCES

1. Linnoila RI, Aisner SC (1994): Pathology of lung cancer: Exercise in classification (101 Refs). In Johnson BE, Johnson DH (eds): "Lung Cancer. Current Clinical Oncology." New York: John Wiley & Sons, pp 73–95.
2. Azzopardi JG (1959): Oat-cell carcinoma of the bronchus. J Pathol Bacteriol 78:513–519.
3. Hattori S, Matsuda M, Tateishi R, Tatsumi N, Terazawa T (1968): Oat-cell carcinoma of the lung containing serotonin granules. GANN 59:123–129.
4. Bensch KG, Corrin B, Pariente R, Spencer H (1968): Oat-cell carcinoma of the lung: Its origin and relationship to bronchial carcinoid. Cancer 22:1163–1172.
5. Matthews MJ, Kanhouwa S, Pickren J, Robinette D (1973): Frequency of residual and metastatic tumor in patients undergoing curative surgical resection for lung cancer. Cancer Chem Rep (Part 3) 63–67.
6. Green RA, Humphrey E, Close H, Patno ME (1969): Alkylating agents in bronchogenic carcinoma. Am J Med 46:516–525.
7. Oboshi S, Tsugawa S, Seido T, Shimosato Y, Koide T, Ishikawa S (1971): A new floating cell line derived from pulmonary carcinoma of oat cell type. GANN 62 505–516.
8. Gazdar A (1994): Cell culture of lung cancers. In Hay RJ, Park JG, Gazdar A (eds): "Atlas of Human Tumor Cell Lines." Orlando: Academic Press, Inc., pp 121–160.
9. Baillie-Johnson H, Twentyman PR, Fox NE, Walls GA, Workman P, Watson JV, Johnson N, Reeve JG, Bleehen NM (1985): Establishment and characterization of cell lines from patients with lung cancer (predominantly small cell carcinoma). Br J Cancer 52:495–504.
10. Engelholm SAA, Spang-Thomsen M, Vindelov LL, Brunner N, Nielsen MH, Hirsch F, Nielsen A, Hansen HH (1986): Comparison of characteristics of human small cell carcinoma of the lung in patients, *in vitro* and transplanted into nude mice. Acta Pathol Microbiol (Scand) 94:325–336.
11. Sorenson GD, Pettengill OS (1980): Structural and functional characteristics of small cell carcinoma of the lung, *in vitro* and *in vivo*. Biol Cell 39:277–280.
12. Eggleston JC (1984): Bronchial carcinoids and their relationship to other pulmonary tumors with endocrine features. In Becker KL, Gazdar AF (eds): "The Endocrine Lung in Health and Diseases." Philadelphia: W.B. Saunders, pp 389–405.
13. Bensch KG, Gordon GB, Miller LR (1965): Electron microscopic and biochemical studies on the bronchial carcinoid tumor. Cancer 18:592–602.
14. Bensch KG, Bonikos DS, Hockberger PE (1976): Pulmonary carcinoid tumors in tissue and organ culture. Cancer 38:2006–2016.
15. Debons-Guillemin MC, Launay JM, Roseto A, Peries J (1982): Serotonin and histamine production by human carcinoid cells in culture. Cancer Res 42:1513–1516.
16. Pelletier G, Cortot A, Launay JM, Debons-Guillemain MC, Nemeth J, Le Charpentier Y, Celerier M, Modigliani R (1984): Serotonin-secreting and insulin-secreting ileal carcinoid tumor and the use of in vitro culture of tumoral cells. Cancer 54:319–322.
17. Arrigoni MG, Woolner LB, Bernatz PE (1972): Atypical carcinoid tumors of the lung. J Thorac Cardiovasc Surg 64:413–421.
18. Berger CL, Goodwin G, Mendelsohn G, Eggleston C, Abeloff MD, Aisner S, Baylin SB (1981): Endocrine-related biochemistry in the spectrum of human lung carcinoma. J Clin Endocrinol Metab 53:422–429.
19. Linnoila RI, Piantadosi S, Ruckdeschel JC (1994): Impact of neuroendocrine differentiation in non-small cell lung cancer. The LCSG experience. Chest 106:367S–371S.
20. Luster W, Gropp C, Kern HF, Haveman K (1985): Lung tumor cell lines synthesizing peptide hormones established from tumours of four histologic types: Characterization of the cell lines and analysis of their peptide hormone production. Br J Cancer 51:865–875.
21. Linnoila RI, Mulshine JL, Steinberg SM, Funa K, Matthews MJ, Cotelingam JD, Gazdar AF (1988): Neuroendocrine differentiation in endocrine and non-endocrine lung carcinomas. Am J Clin Pathol 90:641–652.

22. Oie HK, Russell EK, Carney DN, Gazdar AF (1996): Cell culture methods for the establishment of the NCI series of lung cancer cell lines. J Cell Biochem (Suppl).
23. Nylen ES, Becker KL (1990): The diffuse endocrine systems. In Becker KL (ed): "Principals and Practice of Endocrinology and Metabolism." Philadelphia: J.P. Lippincot, pp 1276–1283.
24. Polak JM (1993): Lung endocrine cell markers, peptides, and amines. Anat Rec 236:169–171.
25. Sunday ME, Kaplan LM, Motoyama E, Chin WW, Spindel ER (1988): Biology of disease: Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. Lab Invest 59:5–24.
26. Baylin SB, Abeloff MD, Goodwin G, Carney DN, Gazdar AF (1980): Activities of L-dopa decarboxylase and diamine oxidase (histaminase) in human lung cancers and decarboxylase as a marker for small (oat) cell cancer in cell culture. Cancer Res 40:1990–1994.
27. Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, Schuller HM, Park JG (1988): Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 48:4078–4082.
28. Simms E, Gazdar AF, Abrams PG, Minna JD (1980): Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor-supplemented medium. Cancer Res 40:4356–4363.
29. Carney DN, Bunn PA Jr, Gazdar AF, Pagan JA, Minna JD (1981): Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. Proc Natl Acad Sci USA 78:3185–3189.
30. Carney DN, Gazdar AF, Bepler G, Guccion J, Marangos PJ, Moody TW, Zweig MH, Minna JD (1985): Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Res 45:2913–2923.
31. Gazdar AF, Carney DN, Nau MM, Minna JD (1985): Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological and growth properties. Cancer Res 45:2924–2930.
32. Gazdar AF, Minna JD (1996): The NCI series of cell lines: An historical perspective. J Biol Chem (Suppl).
33. Gazdar AF, Steinberg SM, Russell EK, Linnoila RI, Oie HK, Ghosh BC, Cotelingam JD, Johnson BE, Minna JD, Ihde DC (1990): Correlation of *in vitro* drug-sensitivity testing results with response to chemotherapy and survival in extensive-stage small cell lung cancer: A prospective clinical trial. J Natl Cancer Inst 82:117–124.
34. Shaw GL, Gazdar AF, Phelps R, Linnoila RI, Ihde DC, Johnson BE, Oie HK, Pass HI, Steinberg SM, Ghosh BC, Minna JD, Mulshine JL (1993): Individualized chemotherapy for patients with non-small cell lung cancer determined by prospective identification of neuroendocrine markers and *in vitro* drug sensitivity testing. Cancer Res 53:5181–5187.
35. Johnson BE, Salem C, Nesbitt J, Gazdar AF, Lesar M, Phelps R, Edison M, Linnoila RI, Phares J, Pass H, Mulshine JL, Minna JD, Glatstein E, Ihde DC (1993): Limited stage small cell lung cancer treated with concurrent hyperfractionated chest radiotherapy and etoposide/cisplatin. Lung Cancer 9(Suppl 1):S21–S26.
36. Bunn PA, Lichter AS, Makuch RW, Cohen MH, Veach SR, Matthews MJ, Johnston-Anderson A, Edison M, Glatstein E, Minna JD, Ihde DC (1987): Chemotherapy alone or chemotherapy with chest radiation therapy in limited stage small cell lung cancer. A prospective, randomized trial. Ann Intern Med 106:655–662.
37. Ihde DC, Mulshine JL, Kramer BS, Steinberg SM, Linnoila RI, Gazdar AF, Edison M, Phelps RM, Lesar M, Phares JC, Grayson J, Minna JD, Johnson BE (1994): Prospective randomized comparison of high-dose and standard-dose etoposide and cisplatin chemotherapy in patients with extensive-stage small-cell lung cancer. J Clin Oncol 12:2022–2034.
38. Johnson BE, Bridges JD, Sobczeck M, Gray J, Linnoila R, Gazdar AF, Hankins L, Steinberg SM, Edison M, Frame JN, Pass H, Nesbitt J, Holden D, Mulshine J, Glatstein E, Ihde DC (1996): Patients with limited stage small cell lung cancer treated with concurrent twice daily chest radiotherapy and etoposide/cisplatin followed by cyclophosphamide, doxorubicin, and vincristine or in vitro selected therapy. J Clin Oncol 14:806–813.
39. Carney DN, Brower M, Bertness V, Oie HK (1984): Selective growth of human small cell lung cancer cell lines and clinical specimens in serum-free medium. In Barnes DW, Sirbasku DA, Sato G (eds): "Methods for Serum-Free Culture of Epithelial and Fibroblastic Cells." New York: Alan Liss, Inc., pp 57–71.
40. Saccomanno G, Saunders RP, Ellis H, Archer VE, Wood BG, Beckler PA (1963): Concentration of carcinoma or atypical cells in sputum. Acta Cytol 7:305–310.
41. Phelps RM, Johnson BE, Ihde DC, Gazdar AF, Linnoila RI, Matthews MJ, Bunn PA Jr, Carney D, Minna JD, Mulshine JL (1996): The NCI-Navy Medical Oncology Branch cell line data base. J Cell Biochem (Suppl).
42. Carney DN, Bepler G, Gazdar AF (1985): The serum-free establishment and *in vitro* growth properties of classic and variant small cell lung cancer cell lines. Rec Res Cancer Res 99:157–166.
43. Gazdar AF, Linnoila RI (1988): The pathology of lung cancer: Changing concepts and newer diagnostic techniques. Semin Oncol 15:215–225.
44. Jensen SM, Gazdar AF, Cuttitta F, Russell EK, Linnoila RI (1990): A comparison of synaptophysin, chromogranin, and L-dopa decarboxylase as markers for neuroendocrine differentiation in lung cancer cell lines. Cancer Res 50:6068–6074.
45. Deftos LJ, Linnoila RI, Burton DW, Leong SS, O'Connor DT, Murray SS, Gazdar AF (1988): Demonstration of chromogranin A in human neuroendocrine cell lines by immunohistology and immunoassay. Cancer 62:92–97.
46. Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, Schuller HM, Park JG (1988): Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 48:4078–4082.
47. Helman LJ, Gazdar AF, Park JG, Cohen PS, Cotelingam JD, Israel MA (1988): Chromogranin A expression in normal and malignant human tissues. J Clin Invest 82:686–690.
48. Gazdar AF, Carney DN, Becker KL, Deftos LJ, Liang V, Go W, Marangos PJ, Moody TW, Wolfsen AR, Zweig MH (1985): Expression of peptide and other markers in lung cancer cell lines. Rec Res Cancer Res 99:168–174.

49. Broers JLV, Viallet J, Jensen SM, Pass H, Travis WD, Minna JD, Linnoila RI (1993): Expression of c-myc in progenitor cells of the bronchopulmonary epithelium and in a large number of non-small cell lung cancers. Am J Respir Cell Mol Biol 9:33–43.
50. Johnson BE, Russell E, Simmons AM, Phelps R, Steinberg SM, Ihde DC, Gazdar AF (1996): MYC family DNA amplification in 124 tumor cell lines from patients with small cell lung cancer. J Cell Biochem (Suppl) (in press).
51. De Leij L, Postmus PE, Buys CHCM, Elema JD, Ramaekers F, Poppema S, Brouwer M, van der Veen AY, Mesander G, The, TH (1985): Characterization of three new variant type cell lines derived from small cell carcinoma of the lung. Cancer Res 45:6024–6033.
52. Bepler G, Jaques G, Haveman K, Koehler A, Johnson BE, Gazdar AF (1987): Characterization of two cell lines with distinct phenotypes established from a patient with small cell lung cancer. Cancer Res 47:1883–1891.
53. *Graziano SL*, Cowan BY, Carney DN, Bryke CR, Mitter NS, Johnson BE, Mark GE, Planas AT, Catino JJ, Comis RL, Poiesz BJ (1987): Small cell lung cancer cell line derived from a primary tumor with a characteristic deletion of 3p. Cancer Res 47:2148–2155.
54. Harpole DH Jr, Feldman JM, Buchanan S, Young WG, Wolfe WG (1992): Bronchial carcinoid tumors: A retrospective analysis of 126 patients. Ann Thorac Surg 54:50–55.
55. Nylen ES, Linnoila RI, Snider RH, Tabassian AR, Becker KL (1987): Comparative studies of hamster calcitonin from pulmonary endocrine cells *in vitro*. Peptides 8:977–982.
56. Linnoila RI, Gazdar AF (1990): Non-small cell lung carcinoma with neuroendocrine features. Anat Pathol 18:1–5.
57. Travis WD, Linnoila RI, Tsokos M, Hitchcock CL, Cutler GB, Nieman L, Chrousos G, Pass H, Doppman J (1991): Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. Am J Surg Pathol 15:529–533.
58. Broers JLV, Jensen SM, Travis WD, Pass H, Whitsett JA, Singh G, Katyal SL, Gazdar AF, Minna JD, Linnoila RI (1992): Expression of surfactant associated protein-A and clara cell 10 kilodalton mRNA in neoplastic and non-neoplastic human lung tissue as detected by *in situ* hybridization. Lab Invest 66:337–346.
59. Ball DW, Azzoli CG, Baylin SB, Chi D, Dou S, Donis-Keller H, Cumaraswmy A, Borges M, Nelkin BD (1993): Identification of a human achaete-scute homolog highly expressed in neuroendocrine tumors. Proc Natl Acad Sci USA 90:5648–5652.
60. Ball DW, Borges M, Linnoila RI, van de Veldo HJK, Mabry M, Nelkin BD, Baylin SB (1996): Achaete-scute homolog 1 is a determinant of the normal and neoplastic pulmonary neuroendocrine cell phenotype. Proc Am Assoc Cancer Res 37:533.
61. van de Velde HJK, Senden NHM, Roskams TAD, Broers JLV, Ramaerkers FCS, Roebroek AJM, van de Ven WJM (1994): NSP-encoded reticulons are neuroendocrine markers of a novel category in human lung cancer diagnosis. Cancer Res 54:4769–4776.
62. Lai S-L, Brauch H, Knutsen T, Johnson BE, Nau MM, Mitsudomi T, Tsai C-M, Whang-Peng J, Zbar B, Kaye FJ, Gazdar AF (1995): Molecular genetic characterization of neuroendocrine lung cancer cell lines. Anticancer Res 15:225–232.
63. Gazdar AF, Kadoyama C, Venzon D, Park J, Tsai C, Linnoila RI, Mulshine JL, Ihde DC, Giaccone G (1992): Association between histological type and neuroendocrine differentiation on drug sensitivity of lung cancer cell lines. J Natl Cancer Inst Monogr 13:191–196.
64. McDowell EM, Trump BF (1981): Pulmonary small cell carcinoma showing tripartite differentiation in individual cells. Hum Pathol 12:286–294.
65. Gazdar AF, Carney DN, Guccion JG, Baylin SB (1981): Small cell carcinoma of the lung: Cellular origin and relationship to other pulmonary tumors. In Greco FA, Oldham RK, Bunn PA Jr (eds): New York: Grune & Stratton, pp 145–175.
66. Shaw GL, Gazdar AF, Phelps R, Steinberg SM, Linnoila RI, Johnson BE, Oie HK, Russell EK, Ghosh BC, Pass HL, Minna JD, Mulshine JL, Ihde DC (1996): Correlation of *in vitro* drug sensitivity testing results with response to chemotherapy and survival: Comparison of non-small cell lung cancer and small cell lung cancer. J Cell Biochem (Suppl) (in press).