

# Establishment and Identification of Small Cell Lung Cancer Cell Lines Having Classic and Variant Features<sup>1</sup>

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## ABSTRACT

Using a chemically defined medium containing hydrocortisone, insulin, transferrin, 17 $\beta$ -estradiol and selenium, with or without serum supplementation (2.5% v/v), continuous cell lines can be established from 72% of all fresh biopsy specimens of small cell lung cancer (SCLC) containing tumor cells. No differences were observed in the rate of establishing cell lines from newly diagnosed untreated patients, or from patients who have relapsed from prior therapy, or from a variety of different organ sites. Biochemical characterization of 50 SCLC cell lines for the expression of L-dopa decarboxylase; bombesin-like immunoreactivity; neuron-specific enolase, and the brain isozyme of creatine kinase, revealed that SCLC cell lines can be subdivided into two distinct classes: classic SCLC cell lines (35 lines), which express elevated levels of all four biomarkers; and variant SCLC cell lines (15 lines) which have undetectable levels of L-dopacarbonylase and bombesin-like immunoreactivity, but continue to express neuron-specific enolase and the brain isozyme of creatine kinase. The presence of the latter two markers distinguishes variant lines from non-SCLC cell lines. In addition, four distinct classes were identified morphologically. The biomedical differences among established SCLC cell lines may account for the differences in response rates to cytotoxic therapy observed in newly diagnosed SCLC patients. A prospective study of biomarker characterization of SCLC tumors will determine if clinical differences exist between classic and variant SCLC tumors.

## INTRODUCTION

SCLC<sup>3</sup> accounts for 25% of all new cases of primary lung cancer. Although objective remissions can be achieved in 90% of cases treated with intense cytotoxic therapy, only 40% gain a "complete clinical remission," and less than 10% of patients will achieve long-term disease-free survival and potential cure (1, 2). While host factors (such as general physiological status) play a role in responses, it is reasonable to test the hypothesis that

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<sup>3</sup> The abbreviations used are: SCLC, small cell lung cancer; DDC, L-dopa decarboxylase; BLI, bombesin-like immunoreactivity; NSE, neuron-specific enolase; CK-BB, creatine kinase brain isozyme; DCG, dense-core granules; SSM, serum-supplemented medium; APUD, amine precursor uptake and decarboxylation; GIBCO, Grand Island Biological Co., Grand Island, NY; FBS, fetal bovine serum; EM, electron microscopy; HITES, RPMI 1640 supplemented with hydrocortisone (10<sup>-8</sup> M), insulin (5.0  $\mu$ g/ml), transferrin (10  $\mu$ g/ml), 17 $\beta$ -estradiol (10<sup>-8</sup> M), and sodium selenite (3  $\times$  10<sup>-8</sup> M).

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these differences in therapeutic responses depend on properties inherent in the tumor cells themselves. These differences could represent expression of drug or radiation resistance genes, or genes coding for more malignant behavior, such as the cellular oncogenes. In order to systematically evaluate tumors from individual patients exhibiting different clinical courses, it would be of great help to reproducibly grow tumor cells *in vitro* from potentially all SCLC patients.

We and others have reported on the establishment of continuous tissue culture cell lines of SCLC (3-9). Initially using non-selective culture conditions (RPMI 1640 supplemented with 10 to 20% heat-inactivated fetal bovine serum), cell lines could be established from 10% of clinical specimens containing large quantities of tumor cells. In addition, cell lines were more readily established from heavily pretreated patients who had relapsed from initial therapy, suggesting that culture conditions supported only the growth of the more "aggressive" population of SCLC cells. Next we found that the use of a serum-free, chemically defined medium (HITES) resulted in the initial selective isolation and growth of SCLC tumor cells compared to normal cells from patient biopsy specimens (10, 11). In these initial studies it appeared that the serum-free hormone-supplemented medium was superior to SSM in the initial growth of tumor cells from clinical specimens (10).

Following these observations, a new approach using both serum-free and SSM was developed to establish continuous cell lines from patients with SCLC. Using these techniques, we wanted to study prospectively our ability to establish cell lines from all tumor-containing specimens. In addition, we and others have reported on a panel of markers which characterize SCLC including DDC, NSE, CK-BB, and the peptide hormone bombesin or its mammalian homologue gastrin-releasing peptide (BLI) (5, 12-22). Thus, it was important both to assay for the expression of these markers in a large panel of SCLC lines, and to compare the expression of markers within individual lines to each other and to cell lines of non-SCLC. In this publication we report on the growth and detailed biological characterization of 50 independent continuous SCLC tumor cell lines established from SCLC patients over a 7-year period (1977 to 1984), and the results of a prospective study (1982 to 1984), using defined growth media.

The results of these studies show: (a) with improved and better-defined culture conditions, SCLC cell lines can now be established from 70% of all tumor-containing specimens, with no differences in success rate among specimens obtained from newly diagnosed untreated or previously treated patients. In addition, cell lines can be readily established from a wide range of metastatic sites. (b) We confirm that the biochemical markers

of SCLC cell lines can clearly distinguish SCLC lung cancer from non-SCLC and other human tumor cell lines. (c) Finally, we observed that the biochemical markers allow SCLC cell lines to be subgrouped into 2 major classes; classic SCLC cell lines which express elevated levels of all 4 biomarkers evaluated, and variant SCLC cell lines which fail to express one or more of the APUD markers evaluated (DDC and/or BLI), but continue to express elevated levels of NSE and CK-BB. In addition 4 morphological classes of SCLC cell lines were found. The variant SCLC cells may represent a clinically important subset of SCLC.

## MATERIALS AND METHODS

**Clinical Specimens.** Specimens for culture were obtained from patients with a confirmed histological diagnosis of SCLC at the time patients were undergoing approved protocol-staging procedures and included bone marrow aspirates and biopsies, lymph node aspirate and biopsies, pleural effusions, and surgically resected tumor masses.

**Specimen Collection and Preparation.** The procedures for collecting and processing specimens for culture were as previously described (5, 10, 23, 24). Bone marrow aspirate specimens were collected in syringes containing preservative-free heparin, and were then resuspended in RPMI-1640 (GIBCO). Mononuclear cells were then collected by Ficoll-Hypaque centrifugation and washed twice in culture medium. Lymph node aspirates and other solid tumor specimens obtained immediately after surgery were mechanically dissociated under aseptic conditions. Cell suspensions of these were obtained by mincing, passing through a 60-gauge steel mesh, and by pipeting in culture medium. Pleural effusions were collected in sterile heparinized vacuum bottles, and the cells were collected by centrifugation. Biopsy specimens and effusions that contained an excess of debris or nonviable cells were clarified by Ficoll-Hypaque centrifugation. After washing in phosphate-buffered saline, cells ( $1 \times 10^6$ /ml) were resuspended in culture medium. The viability of the cell preparations, as determined by trypan blue exclusion, ranged from 20 to 95%, with the lowest viability in the solid tumor specimens (usually less than 50%), and the highest in bone marrow specimens (>90%).

**Culture of Cell Lines.** The vast majority of cell lines (NCI series) used in this study were established in the laboratories of D. N. Carney or A. F. Gazdar. In our initial attempts to establish SCLC cell lines, specimens were plated in RPMI 1640 (GIBCO) supplemented with 10 to 20% heat-inactivated fetal bovine serum (FBS) (GIBCO). Using these media, cell lines were only established from a minority of specimens received (5).

However, in a preliminary study of 14 fresh tumor specimens, we demonstrated that growth of SCLC cells in a chemically defined serum-free medium (HITES) was superior to growth in non-hormone-supplemented medium containing serum (10). In addition, cloning studies in soft agarose (23, 24), using established SCLC cell lines, revealed that the colony-forming efficiency in HITES plus 2.5% FBS was equal to or superior to that observed in SSM alone.<sup>4</sup> Thus, in a prospective study from January 1982, all specimens were cultured in both HITES medium and HITES medium supplemented with 2.5% FBS (serum-supplemented HITES; SS-HITES). All factors in the HITES and SS-HITES media were obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions of these factors were prepared at concentration 100 to 2500 times the final concentration and stored at  $-20^\circ\text{C}$  until use (10, 11). Antibiotics were not routinely used.

For culture, approximately  $1 \times 10^6$  viable cells seeded into 25-sq cm flasks containing 7.0 ml of either (a) HITES medium, or (b) SS-HITES. Cells were then incubated in a humidified atmosphere of 5%  $\text{CO}_2$ :95% air at  $37^\circ\text{C}$ .

Following plating of cells in culture medium, flasks were examined twice weekly to determine both the presence and rate of tumor cell proliferation, as the SCLC cells are easily recognized in early cultures.

<sup>4</sup> Unpublished observation.

The outgrowth of SCLC tumor cells was usually detected in tumor-positive specimens within 3 to 5 days of culture. The subsequent handling and passaging of the tumor cells was determined in part by the density of the tumor cell population in the culture flasks, and by the culture media in which maximum proliferation and outgrowth of tumor cells was initially observed (HITES versus SS-HITES).

Of great importance, all fresh specimens and early-passage cultures (during the first 3 to 6 months of culture) were maintained at a heavy cell density ( $5 \times 10^5$  ml), and were routinely passaged (1:2 to 1:3 split) when such a density was reached, or when tumor cell viability within flasks appeared to decrease. At passage, cells were fed with 50% conditioned medium from the flasks in which cells had been growing. If the initial growth of tumor cells was superior in one culture condition compared to the other, all aliquots of the specimen were subsequently maintained in that culture medium.

After 3 to 6 months the continued growth and ability to passage the cells at a lower density indicated the cell lines were "established." Thereafter, the cell lines could be maintained in 75-sq cm flasks. Cell lines isolated in SS-HITES, once established, could be routinely maintained in RPMI 1640 plus 10% FBS. However, for most cell lines established in HITES medium, and which had demonstrated poor or no growth in SS-HITES, the addition of serum was usually associated with either cessation of growth or a decreased growth rate. Thus, these lines were maintained indefinitely in HITES medium.

The nature of culture cells was determined by gross morphological appearance *in vitro*, cytology examination, and nude mouse tumorigenicity (25), and by analysis of cell pellets for DDC, BLI, NSE, and CK-BB, using previously described assays (5, 12, 16-18). For comparative purposes, the expression of these 4 biomarkers was also evaluated in a panel of other types of lung cancer cell lines, and non-lung cancer lines. Specimens for biomarker determinations were collected when cells were in log-phase growth, 2 days following a medium change.

## RESULTS

Since 1977, 50 independent continuous cell lines have been established from 45 patients with a confirmed histological diagnosis of SCLC. From a total of 400 specimens obtained from SCLC patients, 280 were cytopathologically negative for tumor cells, including 250 bone marrow aspirates and 30 pleural effusions. When these cytopathological-negative specimens were plated in either SSM or serum-free HITES medium without added growth factors to support differentiated marrow cell proliferation, no growth of tumor cells was observed. In SSM, growth of adherent stromal cells was frequently observed, whereas in HITES medium, death and lysis of all cells was usual by 7 to 10 days after plating (10).

Visible tumor cells were present in 120 specimens (30% of total specimens). The fraction of tumor cells in each specimen, as determined by cytology examination of the specimen processed for culture, ranged from specimens composed predominantly of tumor cells (usually lymph node aspirates or biopsies) to those in which only rare clumps of tumor cells could be found. From these 120 tumor-positive specimens, 50 continuous SCLC cell lines were established. Although the overall success rate for the establishment of continuous SCLC cell lines was 42% (50 of 120) (1977 to 1984), for the 24-month period commencing January 1982, when culture conditions were optimized, and when both HITES serum-free defined medium, and SS-HITES medium were used to culture all fresh specimens, cell lines were successfully established from 72% (31 of 43) of all clinical tumor-containing specimens received.

**Culture Conditions and Cell Lines.** Among all 50 cell lines,

28 were established in SSM; 12 cell lines were established in serum-free HITES medium alone; and 10 specimens were established as continuous cell lines in both media. Since January 1982, when growth conditions were optimized, 31 cell lines were established from 43 specimens containing tumor cells (Table 1). Of these 31 lines, 12 were established only in HITES, 9 were established in SS-HITES, and 10 were established in both media in parallel. Thus, of these 31 lines, 22 were successfully established as continuous cell lines direct from patient biopsy specimens in serum-free chemically defined medium. However, as some cell lines could not be established without serum supplementation, it is clear that further growth factor(s) addition to HITES is required if all fresh SCLC specimens can be cultured in serum-free defined media.

The use of the improved growth media to support the growth of tumor cell lines from treated and untreated patients, and from a variety of different organ sites, is indicated in Table 2. With these media, cell lines were successfully established from 72% of tumor-containing specimens, and with equal efficiency from a variety of different sites, including bone marrow, pleural effusions lymph nodes, and other sites. No cell lines were established from specimens pathologically and cytologically negative for SCLC tumor cells (Table 2). In addition, lines were established with equal efficiency from both treated and untreated patients (Table 3).

**Origin of the 50 SCLC Tumor Cell Lines.** Forty-one cell lines were established directly from patient biopsy material, and 9 cell

lines from nude mouse xenografts of patients tumors (Table 2). Thirteen cell lines were established from females and the remainder from males. Cell lines were established from newly diagnosed, previously untreated patients (19 cell lines), and from heavily pretreated patients who had relapsed from prior therapy (31 cell lines). The cell lines were established from a variety of different organ sites, including bone marrow aspirates (14 cell lines), pleural effusion (14 lines), lymph node aspirates and biopsies (10 lines), lung biopsies (6 lines), s.c. nodules (3 lines), and one cell line each from a brain, adrenal, and liver metastases of SCLC. All lines have been maintained in continuous culture for periods ranging from 6 to 74+ months, have been successfully cryopreserved, and are free of *Mycoplasma* contamination (Microbiological Associates, Bethesda, MD). All 40 cell lines tested are of human origin as determined by isozyme analysis and/or cytogenetic studies (5, 26, 27).

**Properties of Established SCLC Cell Lines.** Although some cell lines have been established in SSM and others in HITES medium, we have observed no significant differences, morphologically or biochemically, among lines established either in different media, including cell lines cultured in parallel in both SSM and HITES. Thus, in the remainder of this paper the culture appearances and biochemical data presented will be on the 38 lines established and maintained in SSM, and those 12 cell lines which could only be established and continuously maintained in serum-free HITES medium.

**Culture Appearances of Cell Lines.** The culture appearance of the established cell lines as determined by phase contrast microscopy, demonstrated considerable heterogeneity (Fig. 1).

Based on their appearance, the lines could be subgrouped into 4 major categories, which we call types 1, 2, 3, and 4. Type 1 lines grew as tightly packed spherical aggregates of floating cells, which frequently demonstrated areas of central necrosis in the larger spheroids. Type 2 lines grew as relatively densely packed floating aggregates, amorphous and irregular in outline, and lacking central necrosis. Type 3 lines grew as very loosely adherent floating aggregates growing in small clumps and intertwined cords. Central necrosis was absent in these lines. Type 4 lines grew attached to substrate. These cells consisted of large overlapping polygonal cells lacking the epithelioid appearance of adherent non-SCLC lung cancer cultures (Fig. 2). The frequency of the different types of cell lines was: type 1 (20 lines, 40%); type 2 (17 lines, 34%); type 3 (9 lines, 18%); and type 4 (3 lines, 6%). One cell line, NCI-H446, grew as a mixed floating-attached cell culture (types 2 and 4, Fig. 2A). The characteristics of SCLC cell lines are distinctly different from those of non-SCLC cell lines (Fig. 2B).

Cytospin preparations of the cell lines in most instances revealed the typical cytopathological characteristics of SCLC, with appearances very similar to the tumor cells present in the original clinical specimen (See Ref. 25).

**Nude Mouse Tumorigenicity.** All 44 cell lines which were inoculated into athymic nude mice formed tumors which histologically resembled the tumor cells in the original biopsy specimen. The latent period to tumor formation ranged from 2 to 16 weeks after the s.c. inoculation of  $1 \times 10^6$  cells; s.c. tumors grew as expansile lesions at the site of inoculation and were noninvasive. These tumors grew progressively, reached a size of 1-3 g, and could be serially transplanted. Metastases were not detected grossly or microscopically.

Table 1

Prospective study (1982 to 1984) of the establishment of SCLC cell lines: effect of growth media

HITES	HITES + 2.5% FCS	No. of lines
+a	-	12
-	+	9
+	+	10
Total		31

<sup>a</sup> +, cell line established; -, no cell line established.

Table 2

Prospective study (1982 to 1984): overall success rate and effect of site on establishment of SCLC lines

Specimen source	No. of specimens	Cell lines established 1982-1984		1977-1984, all lines
		SCLC negative	SCLC positive	
Bone marrow	200	0/184	12/16 (75) <sup>a</sup>	14
Pleural effusion	28	0/17	7/11 (60)	14
Lymph node	12	0/0	8/12 (66)	10
Lung	1	0/0	1/1 (100)	6
Other	3	0/0	3/3 (100)	6
Total	244	0/201	31/43 (72)	50

<sup>a</sup> Numbers in parentheses, percentage.

Table 3

Prospective study (1982 to 1984): effect of therapy status on establishment of SCLC cell lines

Therapy status	Prospective study	All lines
Untreated	13/17 (76) <sup>a</sup>	19
Treated	18/26 (69)	31
Total	31/43	50

<sup>a</sup> Numbers in parentheses, percentage.

IDENTIFICATION OF SMALL CELL CANCER SUBCLASSES

**EM Studies.** EM studies were carried out on 25 cell lines. Dense core granules, occurring either as clusters, (Fig. 3A), or singly (Fig. 3B) were observed in 21 of 25 cell lines. None were evident in the remaining 4 cell lines studied (Fig. 3C).

**Biochemical Characterization of SCLC Cell Lines.** DDC expression was evaluated in cell pellets of all 50 cell lines. Elevated levels (>1.0 unit/mg soluble protein) were observed in 35 lines (70%) (mean DDC for all lines, 139 ± 26 (SE); range, <0.1 to 646 units/mg cellular protein). Of interest is the observation of elevated levels of DDC in all cell lines which demonstrated clusters of DCG by EM examination, and undetectable levels in those 4 lines in which DCG were not evident on EM (Table 4).

BLI was detected (>0.1 pmol/mg soluble protein) in 36 of 49 (75%) of cell lines evaluated. The mean BLI was 2.81 ± 0.63 pmol/mg soluble protein (range, <0.01 to 22 pmol/mg soluble

protein). An excellent association between DDC and BLI expression was observed. The only cell line (NCI-H620) showing expression of DDC but not BLI had relatively low levels of DDC.

NSE determinations were carried out in 43 SCLC cell lines. Elevated levels (>100 ng/mg soluble protein) were observed in 42 cell lines (mean, 1118 ± 176 ng/mg cellular protein; range, 49 to 5493 ng/mg protein).

CK-BB determinations were carried out in all 50 cell lines. Elevated levels (>400 ng/mg) were detected in 49. The mean CK-BB level of SCLC cell lines was 6210 ± 619 ng/mg protein (range, 245 to 17555).

**Influence of Therapy Status on the Expression of Biomarkers in SCLC Lines.** Because SCLC tumors in relapsed, previously treated patients behave more aggressively than do tumors in newly diagnosed patients, we examined the impact of prior therapy on the biological properties of SCLC lines by comparing

Table 4  
Characteristics of established human SCLC cell lines (1977 to 1984)

The NCI series of cell lines reported in this and subsequent tables was established in the laboratories of D. N. Carney or A. F. Gazdar.

Cell line	Sex	Prior treatment	Source	Growth media		Time in culture	Gross morphology	Nude mouse tumor	EM	DDC (units/mg)	BLI (pmol/mg)	NSE (ng/mg)	CK-BB (ng/mg)	Class
				SSM	HITES									
1. NCI-H60	F	Yes	PE <sup>a</sup>	+	NT	74	2	+	++	110	0.34	570	15,773	C
2. NCI-H64	M	Yes	LN	+	NT	72	1	+	++	44	0.36	5017	12,823	C
3. NCI-H69	M	Yes	PE	+	NT	71	2	+	++	240	1.7	817	15,921	C
4. NCI-H82	M	Yes	PE	+	NT	66	3	+	A	0.6	0.2	317	6,340	V
5. NCI-H123	M	No	LN	+	+	42	1	NT	++	517	3.6	751	5,993	C
6. NCI-H128	M	Yes	PE	+	NT	60	1	+	++	646	15	2045	11,768	C
7. NCI-H146	M	Yes	PE	+	NT	57	2	+	++	341	0.17	1365	1,207	C
8. NCI-N177	F	Yes	L	+	NT	47	3	+	S	<0.1	<0.01	660	2,158	V
9. NCI-N179	F	Yes	L	+	NT	47	1	+	S	<0.1	<0.01	555	8,964	V
10. NCI-H182	F	Yes	LN	+	NT	51	1	+	++	420	5.1	2432	3,078	C
11. NCI-H187	M	No	PE	+	NT	50	2	+	S	22	1.7	368	16,155	C
12. NCI-H196	M	Yes	PE	+	NT	50	4	+	NT	<0.1	<0.01	49	245	V
13. NCI-H209	M	No	BM	+	NT	48	2	+	++	223	22	967	3,303	C
14. NCI-H211	F	Yes	BM	+	NT	47	3	+	A	<0.1	<0.01	160	1,675	V
15. NCI-H220	M	No	PE	+	+	60	2	+	++	136	0.49	1260	3,963	C
16. NCI-N230	F	No	L	+	NT	60	2	+	++	31	6.7	NT	2,701	C
17. NCI-N231	F	No	L	+	NT	52	2	+	++	193	7.2	521	2,947	C
18. NCI-H249	M	Yes	BM	+	-	40	2	+	NT	493	0.31	811	2,704	C
19. NCI-H250	M	No	Brain	+	-	40	2	+	++	110	1.35	503	3,861	C
20. NCI-H285	M	No	LN	+	-	8X	1	+	NT	500	NT	703	10,733	C
21. NCI-H289	F	No	PE	-	-	8X	1	+	NT	<0.1	<0.01	280	12,740	V
22. NCI-H298	M	Yes	PE	+	-	36	1	+	++	40	9.7	1224	3,730	C
23. NCI-H345	M	Yes	BM	+	+	30	2	+	NT	98	4.7	4075	5,814	C
24. NCI-H360	M	Yes	SCN	+	-	30	4	+	NT	<0.1	<0.01	NT	NT	V
25. NCI-H369	M	Yes	BM	-	+	29	1	+	NT	12	0.3	3217	5,201	C
26. NCI-H372	M	Yes	BM	+	+	27	4	+	A	<0.1	<0.01	730	4,515	V
27. NCI-H378	F	Yes	PE	-	+	25	1	+	++	75	0.3	5493	4,281	C
28. NCI-N390	M	Yes	LN	+	NT	60	2	+	++	479	0.7	510	3,323	C
29. NCI-N408	M	No	Liver	+	NT	50	2	+	++	183	2.4	714	3,081	C
30. NCI-N417	F	No	L	+	NT	60	3	+	A	<0.1	<0.01	580	8,024	V
31. NCI-H432	M	Yes	SCN	+	-	19	2	+	NT	9	0.35	387	6,720	C
32. NCI-H433	M	Yes	SCN	+	+	19	3	+	NT	<0.1	<0.01	352	4,989	V
33. NCI-H446	M	Yes	PE	+	-	18	2 & 4	+	NT	<0.1	<0.01	1112	7,455	V
34. NCI-H449	F	Yes	BM	+	-	18	1	+	NT	98	0.8	1998	13,207	C
35. NCI-H450	F	Yes	LN	+	-	18	1	+	NT	657	1.4	1764	8,857	C
36. NCI-H462	M	No	BM	+	+	18	1	+	NT	52	0.66	2287	2,090	C
37. NCI-H463	M	No	BM	+	NT	18	1	+	NT	114	0.99	1209	2,025	C
38. NCI-H478	F	No	L	-	+	18	3	+	NT	71	3.0	401	3,152	C
39. NCI-H510	M	Yes	Adrenal	-	+	15	3	+	NT	214	7.4	491	2,732	C
40. NCI-H524	M	Yes	LN	+	+	13	2	+	NT	<0.1	<0.01	234	8,631	V
41. NCI-H526	M	No	BM	+	+	13	3	+	NT	<0.1	<0.01	742	9,133	V
42. NCI-H568	M	No	LN	+	+	10	1	+	NT	77	18	491	6,370	C
43. NCI-H578	M	Yes	LN	-	+	9	1	NT	NT	4.9	3.4	NT	2,635	C
44. NCI-H580	M	No	BM	-	+	8	1	+	NT	0.9	<0.03	NT	10,723	V
45. NCI-N592	M	Yes	BM	+	+	40	2	+	++	204	0.13	1285	17,555	C
46. NCI-H606	M	Yes	PE	-	+	7	1	NT	NT	77	7.3	NT	7,483	C
47. NCI-H618	M	No	BM	-	+	7	1	NT	NT	37	3.1	NT	4,197	C
48. NCI-H620	M	Yes	BM	-	+	7	1	NT	NT	2	<0.02	NT	1,554	V
49. NCI-N691	F	Yes	PE	-	+	30	2	+	++	320	0.83	1182	5,221	C
50. NCI-H660	M	No	LN	-	+	6	1	NT	NT	112	3.54	NT	NT	C

<sup>a</sup> PE, pleural effusion; LN, lymph node; L, lung; BM, bone marrow; SCN, s.c. nodules; NT, not tested; C, classic; V, variant; A, absent; S, single.

the expression of DDC, BLI, NSE, and CK-BB in cell lines established from newly diagnosed, previously untreated patients, and in lines established from patients who had relapsed from prior intensive combination chemotherapy. Results are indicated in Chart 1. As can be observed, considerable overlap exists in biomarker expression, with no significant differences observed among cell lines in either group.

**Heterogeneity of Biomarker Expression in SCLC Cell Lines: Classic and Variant SCLC Cell Lines.** In the 50 cell lines derived from patients with SCLC, elevated levels of DDC were observed in 35 (70%); BLI in 36 of 49 (75%); NSE in 42 of 43 lines (98%), and CK-BB in 49 lines (98%). Thus, based on their expression of these biochemical markers, cell lines of SCLC can be subdivided into 2 major categories: classic SCLC cell lines (35 lines, 70%) which express elevated levels of all 4 biomarkers evaluated, and variant SCLC cell lines, which lack either DDC or BLI expression (15 lines, 30%). With one exception, cell lines which lack DDC, also lack BLI expression (Table 4). In contrast to non-SCLC (see below), both classes of cell lines express considerably elevated levels of CK-BB and NSE, the latter indicating their APUD relationship. Of interest, the majority of the variant cell lines had either type 3 or type 4 morphology in culture: NCI-H82, N177, H211, N417, H437, H524, and H526 (all type 3); and NCI-H196, H372, H360, and H446 (type 4). Thus, of the 13 cell lines belonging to the morphological classes types 3 and 4 (including NCI-H446), 10 lines (77%) belonged to the variant class of SCLC. In contrast, of all SCLC cell lines with either type 1 or 2 morphology (37 cell lines), 33 cell lines (88%) were classic SCLC cell lines. Thus, based on gross morphological appearance, in most instances one can accurately define the phenotype (classic or variant) of the cell line in culture. As these appearances are observed within days of the onset of culture, it is possible to type the SCLC phenotype.

**Biomarker Expression of Non-SCLC and Non-Lung Cell Lines.** The expression of DDC, BLI, NSE, and CK-BB was evaluated in 17 non-SCLC cell lines, including 7 adenocarcinoma lines, 2 large cell lines, 3 squamous cell lines, 1 mixed squamous-adenocarcinoma cell line, and 3 mesothelioma cell lines (Table 5). All of these cell lines, in contrast to most SCLC cell lines,

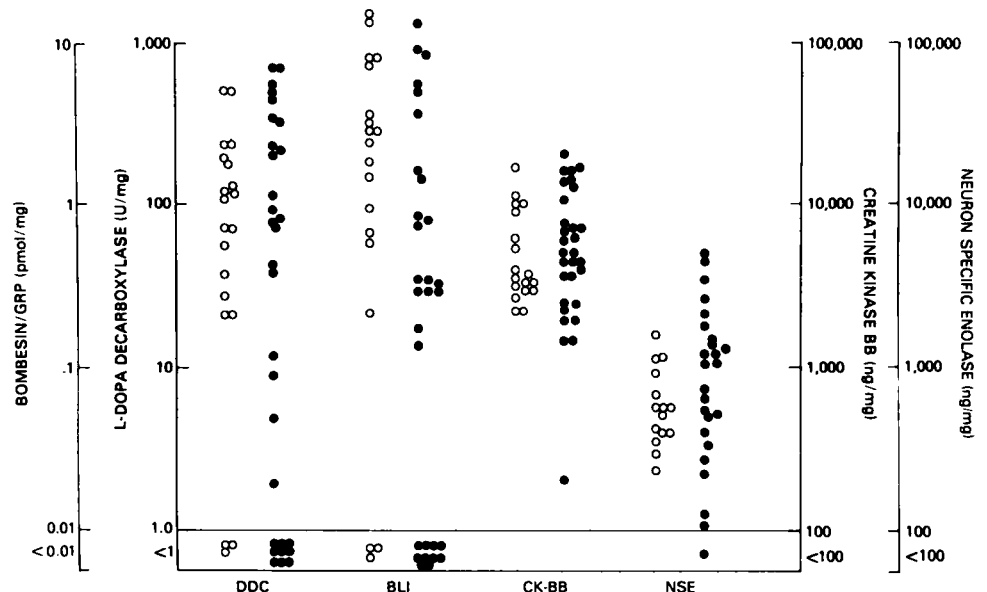
grow as adherent cell cultures. Sixteen of these 17 lines were established from patients who had not received cytotoxic therapy, and have been maintained in continuous culture in serum-supplemented medium (RPMI 1640 supplemented with 10% heat inactivated FBS) for periods ranging from 6 to 72+ months. DDC was detected in 2 of 15 lines; BLI was detected in 0 of 9 lines; NSE (>100 ng/mg protein) was detected in 1 of 9 lines, and high CK-BB (>1000 ng/mg) was detected in 2 of 12 cell lines. In addition to the non-SCLC lines, DDC, BLI, and CK-BB expression was evaluated in a panel of 18 other human cell lines, including breast cancer, malignant T-cell lymphoma lines, ovarian cancer cell lines, malignant melanoma lines, B-lymphoblastoid cell lines, and a renal cell carcinoma cell line (Table 6). As indicated, DDC or BLI was not detected in any of these lines. CK-BB was elevated in 2 of 14 lines, both breast cancer cell lines.

**Comparison of Biomarker Expression among SCLC, Non-SCLC, and Non-Lung Cancer Cell Lines.** The expression of DDC, BLI, NSE, and CK-BB among the groups of cell lines studied is shown in Table 7. As indicated, elevated levels of CK-BB and NSE are present in 95 to 98% of cell lines of SCLC lineage, while DDC and BLI activity is detected in 70 to 75% of SCLC cell lines. In contrast, among non-SCLC cell lines, and among non-lung cancer lines, detection of any of these biomarkers is uncommon, occurring in less than 15% of the cell lines evaluated.

DISCUSSION

In this paper, the isolation, establishment, continuous growth, and characterization of multiple cell lines from patients with SCLC are described. The results of this study show: (a) using improved culture techniques, including serum-free defined medium, cell lines can now be established from 70 to 75% of all tumor-containing specimens obtained from SCLC patients. These continuous cell lines can be established in serum-free defined HITES medium with an efficiency equal to or better than that observed using serum-supplemented medium. (b) Cell lines can be established from a variety of metastatic sites, including bone marrow, lymph node, pleural effusions, liver, brain, and adrenal, in addition

Chart 1. Comparison of the expression of biomarkers in cell lines started from SCLC tumors from newly diagnosed untreated patients (O), and from heavily pretreated patients (●).



**Table 5**  
*Expression of DDC, BLI, NSE, and CK-BB in non-SCLC cell lines*

Cell line	Type	DDC (units/mg)	BLI (pmol/mg)	NSE (ng/mg)	CK-BB (ng/mg)
1. NCI-H23	ACL <sup>a</sup>	<0.1	<0.01	<100	94
2. NCI-H28	Meso	0.6	<0.01	137	<5
3. NCI-H125	ACL	<0.1	<0.01	<100	68
4. NCI-H157a	LC	<0.1	<0.01	<100	420
5. NCI-H226	Sq	<0.1	<0.01	<100	14
6. NCI-H290	Meso	<0.1	<0.01	<100	7
7. NCI-H292	M/E	<0.1	<0.01	<100	213
8. NCI-H324	ACL	<0.1	<0.05	NT	2873
9. NCI-H358	ACL	<0.1	<0.01	NT	NT
10. NCI-H441	ACL	<0.1	<0.01	NT	5
11. NCI-H460	ACL	NT	NT	NT	NT
12. NCI-N495/H207	ACL	<0.1	<0.01	NT	NT
13. NCI-H513	Meso	<0.1	<0.1	NT	<5
14. NCI-H520	Sq	<0.1	<0.01	NT	5867
15. NCI-H522	A/Sq	<0.1	<0.01	NT	NT
16. NCI-N666	LC	<0.1	NT	NT	NT
17. A549	ACL	0.2	<0.01	<100	30
18. CALA6	LC	<0.1	<0.01	NT	NT
19. U1752	Sq	<0.1	<0.01	<100	NT

<sup>a</sup> ACL, adenocarcinoma; Meso, mesothelioma; LC, large cell; M/E, mucocypidermoid; A/Sq, adenocarcinoma-squamous; Sq, squamous; NT, not tested.

**Table 6**  
*Expression of DDC, BLI, and CK-BB in non-lung cancer cell lines*

Cell line	Type	DDC (units/mg)	BLI (pmol/mg)	CK-BB (ng/mg)
1. ZR75	Breast	<0.1	<0.01	1197
2. MDA231	Breast	<0.1	<0.01	<5
3. MCF7	Breast	<0.1	<0.01	345
4. T47D	Breast	<0.1	<0.01	5
5. BT20	Breast	<0.1	<0.01	25
6. NCI-H150BL	B-Lymphoblastoid	<0.1	<0.01	36
7. NCI-H128BL	B-Lymphoblastoid	<0.1	<0.01	2
8. NCI-H155BL	B-Lymphoblastoid	<0.1	<0.01	44
9. NCI-H209BL	B-Lymphoblastoid	<0.1	<0.01	11
10. HUT 78	T-Cell lymphoma	<0.1	<0.01	NT <sup>a</sup>
11. HUT 102	T-Cell lymphoma	<0.1	<0.01	NT
12. MOIT 4	T-Cell lymphoma	<0.1	<0.01	NT
13. NCI-H280	Ovarian	<0.1	<0.01	NT
14. NCI-H385	Ovarian	<0.1	<0.01	56
15. NCI-H502	Ovarian	<0.1	<0.01	NT
16. NCI-H234	Melanoma	<0.1	<0.01	29
17. NCI-N892/425	Melanoma	<0.1	<0.01	86
18. NCI-H201	Hypernephroma	<0.1	<0.01	>5

<sup>a</sup>NT, not tested.

**Table 7**  
*Comparison of biomarkers among SCLC, non-SCLC, and non-lung cancer cell lines*

Cell lines were scored as positive if they expressed detectable levels of DDC, BLI, or NSE, or if their concentrations of CK-BB were >400 ng/mg.

Biomarker	SCLC	Non-SCLC	Non-lung
DDC	35/50 (70) <sup>a</sup>	2/15 (13)	0/18 (0)
BLI	36/49 (75)	0/9 (0)	0/18 (0)
NSE	42/43 (96)	1/9 (11)	NT <sup>b</sup>
CK-BB	49/50 (98)	3/12 (25)	2/14 (13)

<sup>a</sup> Numbers in parentheses, percentage.

<sup>b</sup> NT, not tested.

to primary lung tumors, and from treated and untreated patients. (c) Morphologically there is considerable heterogeneity in the appearances of the established SCLC cell lines in culture. The majority of cell lines (46 of 50, 92%) grow as floating aggregates of tightly to loosely packed cells (types 1, 2, and 3), while 3 lines (6%) grow as attached monolayer cultures (type 4). One of the 50 SCLC lines grows as a mixed floating and/or attached culture (types 2 and 4). (d) Based on their expression of the 4 biomarkers evaluated, SCLC cell lines can be subgrouped into 2 major

categories, classic SCLC cell lines which express elevated levels of all 4 markers, including DDC, BLI, NSE, and CK-BB, and variant SCLC cell lines which lack either the key APUD enzyme, DDC, and, in addition, fail to express the peptide hormone BLI, but continue to express elevated levels of NSE and CK-BB. Among 25 cell lines evaluated, an excellent correlation between the presence of elevated DDC, and DCG on electron microscopy examination was observed. (e) The pattern of biomarker expression by SCLC cell lines clearly distinguishes them from those of non-SCLC cell lines. In particular, all SCLC cell lines express elevated levels of NSE, which is usually undetectable (<100 ng/mg protein) in non-SCLC lung cancer cell lines. In 18 other lines evaluated, expression of elevated levels of DDC, BLI, or CK-BB was rarely observed.

Among patients with lung cancer, SCLC accounts for 20 to 25% of the approximately 135,000 new cases which will be diagnosed this year in the United States (2). Because of differences both biologically (in particular, the propensity for early widespread metastases at diagnosis in most patients), and therapeutically (SCLC is highly sensitive to both chemotherapy and radiotherapy), the management of patients with SCLC is usually separated from patients with other forms of lung cancer (non-SCLC). The present work with our panel of SCLC cell lines confirms and extends the usefulness of the biomarkers DDC, BLI, NSE, and CK-BB in distinguishing SCLC from non-SCLC. This work presents for the first time, the simultaneous analysis of all 4 markers in SCLC, and indicates the interrelationship of all markers to each other. These data provide for the following prospective clinical trials using the markers to type lung cancer specimens. First, to correlate the markers in a coded fashion with light microscopy and response to chemotherapy, so that they can be used to distinguish SCLC from non-SCLC cell lines. Second, to test if these are clinical differences between "classic" tumors expressing all 4 markers, with "variant" tumors which express some but not all of the markers. Finally, do the occasional non-SCLC which express one or more of the markers have different clinical and therapy properties than non-SCLC without these markers? The ability to culture continuous SCLC cell lines from the majority of clinical specimens should greatly facilitate these studies. In addition, the improved growth rate of SCLC may now permit *in vitro* drug sensitivity studies to be determined on a much greater number of specimens, which, at the present time and using standard growth media, can only be carried out on 20 to 30% of tumor-containing specimens (24).

The differences in the *in vivo* behavior of SCLC has prompted many investigators to attempt to culture and establish continuous cell lines of this tumor *in vitro*. Oboshi *et al.* (7) first reported on the establishment of a cell line of SCLC (OAT) from a metastatic lymph node biopsy. These cells grew as floating aggregates, and exhibited prominent DCG on electron microscopy examination. The initial growth of the cells was slow, and the first subculture was done 99 days after the initial plating. Once established, the doubling time of the cells was approximately 24 h. Ohara and Okamoto (8), in 1977 reported on the cell lines of OAT-1975 which was established from a lung biopsy of a patient with SCLC. After a 5-month latent period, the line became attached as a monolayer culture with a doubling time of 24 h, and a plating efficiency of 60%. Although the cell line formed typical SCLC tumors in nude mice, DCG were not demonstrable on EM examination. *In vitro* radiation survival studies

of this line revealed a  $D_0$  of 88 rad, and an extrapolation value ( $n$ ) of 10.6.

Since these initial reports, several other investigators have reported on their success in the establishment of continuous SCLC cell lines in culture (3–6, 9). These reports, coupled with our initial reports of SCLC lines, demonstrate the considerable heterogeneity that exists among SCLC cell lines. In some reports, all SCLC lines grow as floating aggregates (3, 5, 6), while in others, cell lines grow as both floating and attached monolayer cultures (9).

Thus, while analysis of reports of previously described SCLC cell lines clearly indicates that considerable heterogeneity exists in lines of this tumor type, because of differences used in isolation techniques, culture conditions, and methods used to characterize lines, the frequency of the different phenotypes of SCLC cell lines cannot be determined from these studies.

In this study, a detailed analysis of 50 cell lines established in a single institution, from patients with histologically confirmed SCLC, and characterized in a uniform manner is presented. While the overall success rate in the establishment of SCLC cell lines has been greatly improved using defined media, with no major differences observed in either the rate of establishment or the characteristics of cell lines derived from different metastatic sites, it is clear that SCLC cell lines can exhibit major differences, both in their *in vitro* growth pattern and in their expression of a variety of biomarkers, including typical APUD cell markers.

While the majority of SCLC cell lines grow a floating aggregate of cells, in 3 instances (6%), cell lines were established as adherent monolayer culture, not unlike other non-SCLC lung cancer cell lines. Although it is possible that these 3 SCLC lines may represent the outgrowth from the initial biopsy of a second tumor type present, this appears unlikely. All 3 lines formed typical SCLC tumors of the intermediate cell type when inoculated into athymic mice. In addition, and in contrast to non-SCLC lines, all 3 lines expressed elevated levels of both NSE (>100 ng/mg) and 2 of 3 CK-BB (>1000 ng/mg), markers which are much lower or absent in non-SCLC cell lines.

Of major interest in the biological properties of SCLC cell lines is the recognition that not all SCLC cell lines express all of the APUD cell characteristics (e.g., cell line NCI-N417; Table 4). In prior studies of a limited number of SCLC cell lines, all lines expressed elevated levels of DDC, and had DCG on EM (5). In addition, all lines exhibited formaldehyde-induced fluorescence, another feature of APUD cells. In this study, 15 lines (30%) lacked DDC, and 14 of those 15 lacked BLI. In addition, in 4 of these 15 variant lines tested, DCG were not evident on EM examination. Although the APUD enzyme NSE was elevated in these variant lines, levels were significantly less than those observed in the typical SCLC cell lines [see Gazdar *et al.* (25)]. Most variant lines could be distinguished on their gross morphology from classic cell lines. In addition, we and others have shown that variant lines, in contrast to classic lines, are radioresistant *in vitro* (6, 28), are amplified for and have increased expression of the *c-myc* oncogene (25, 29). These variant cell lines may clearly represent an important biological subset of SCLC tumors.

The evaluation of the 4 biomarkers DDC, BLI, NSE, and CK-BB in all lines derived from patients with SCLC, and in a variety of other human tumors can distinguish, in most instances, cell lines of SCLC lineage from those of non-SCLC origin. In all SCLC

lines NSE was present and CK-BB concentrations were elevated. In most SCLC lines, in addition, both DDC and BLI expression were elevated. Elevation of one or more of these markers was rarely observed in other human tumor cell lines. Among non-SCLCs, elevated levels of CK-BB were detected in a few lines. In addition, low concentrations of DDC or NSE were present in some lines. Mixed tumors of SCLC and non-SCLC are observed occasionally in biopsy specimens of lung cancer, suggesting that in some instances, tumors may be derived from a "stem" cell with capabilities of differentiation along several pathological pathways. Therefore, it is not surprising to find some SCLC markers elevated in some lines of non-SCLC. Other studies have demonstrated the occasional occurrence of high concentrations of DDC in some non-SCLC lung tumors (12). In addition, non-SCLC tumors may express other neuroendocrine properties such as peptide production and DCGs. While DDC concentrations in the positive non-SCLC lines reported herein were very low, much higher concentrations were present in 2 recently established non-SCLC cell lines.<sup>5</sup>

In conclusion, we have shown that SCLC cell lines can now be readily established from the majority of clinical tumor-containing specimens received. Lines can be established from a variety of sites, primary and metastatic, and can be established and continuously maintained in both serum-supplemented medium and in serum-free chemically defined medium. Established lines demonstrate heterogeneity in both gross morphological appearance in culture, and in their expression of a panel of biomarkers. The presence or absence of these biomarkers in cells may imply important biological differences among these tumors. Because these differences, both morphologically and biochemically, exist among established SCLC lines, and because such differences may have important clinical implications, the complete biological characterization of established SCLC lines must be carried out before results of *in vitro* studies can be applied in clinical situations. The prospective evaluation of these biomarkers in fresh biopsy specimens of SCLC and their correlation with responses to cytotoxic therapy will indicate the chemical and prognostic implications of these markers.

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Fig. 1. *In vitro* morphological characteristics of cell lines of SCLC. Four different appearances can be observed: type 1 (A); type 2 (B); type 3 (C); and type 4 (D). (See text for description.)

Fig. 2. *In vitro* growth pattern of SCLC cell line NCI-H446. This cell line grows as a mixed adherent/floating cell line (A). The *in vitro* characteristics of SCLC cell lines are clearly different than cell lines of non-SCLC lung cancer (B), an adenocarcinoma cell line.

Fig. 3. A, electron micrograph of a tumor cell from classic cell line NCI-N408, showing a cluster of neurosecretory granules in a cytoplasmic process (uranyl acetate and lead citrate stain;  $\times 57,600$ ). Bar = 0.1  $\mu\text{m}$ . B, electron micrograph of a tumor cell from classic cell line NCI-H187 showing a single dense body consistent with a neurosecretory granule (uranyl acetate and lead citrate stain;  $\times 57,600$ ). Bar = 0.1  $\mu\text{m}$ . C, electron micrograph of a tumor cell from variant cell line NCI-N417 having no neurosecretory granules (uranyl acetate and lead citrate stain;  $\times 12,000$ ). Bar = 0.5  $\mu\text{m}$ .



