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Relation between Nucleolar Size and Growth Characteristics in Small Cell Lung Cancer Cell Lines

Takeo Matsumoto,[1,7] Takeo Terasaki,[1,8] Kiyoshi Mukai,[1] Makio Wada,[2] Aikou Okamoto,[2] Jun Yokota,[2] Ken Yamaguchi,[3] Kanefusa Kato,[4] Toshiharu Nagatsu[5] and Yukio Shimosato[6]

[1]*Pathology and* [3]*Endocrinology Division and* [2]*Section of Studies on Metastasis, National Cancer Center Research Institute,* [6]*Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104,* [4]*Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, 713-8 Kamiya-cho, Kasugai 480-03 and* [5]*Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466*

Relationships among cytological features, doubling time, S-phase percentage, expression of *myc*-family oncogenes, DNA ploidy and biochemical properties were studied in thirteen small cell lung cancer cell lines. Six cell lines that grew slowly (average doubling time 99 h) and had lower S-phase percentages (average 32%) showed inconspicuous nucleoli (average area of 1.5 μm^2), and the remaining seven cell lines that grew quickly (average doubling time 45 h) and had higher S-phase percentages (average 44%) showed large and prominent nucleoli (average area of 6.1 μm^2). DNA index value obtained from flow cytometric DNA histograms showed that all cell lines except for H-69 cell line displayed aneuploidy. Ribbon-like cell arrangements were observed in the 7 cell lines that grew quickly, and in 1 cell line that grew slowly. Biochemically, six slow-growing cell lines and four fast-growing cell lines showed high levels of aromatic L-amino acid decarboxylase activity, while in the remaining three fast-growing cell lines its level was low. A high level of c-*myc* or N-*myc* oncogene expression was observed in all 7 cell lines that grew quickly, but not in any of the 6 cell lines that grew slowly. It appears that small cell lung cancer cell lines that grow quickly can be expected to have large nucleoli and ribbon-like cell arrangements and to express high levels of *myc*-family oncogenes, and that nucleolar size is a good indicator for growth characteristics.

Key words: Morphology — Cell growth — *myc* oncogene family — Small cell lung cancer, *in vitro*

SCLC[9] accounts for 15–25% of all lung cancers and is one of the most malignant and well investigated tumors.[1-3] Many SCLC cell lines have recently been established *in vitro* and used for the elucidation of the biological properties of SCLC.[4-6] These cell lines were subgrouped into 2 major classes mainly on the basis of their biochemical properties.[4,7] Classic SCLC cell lines express elevated levels of biomarkers (AADC, NSE, CK-BB) and grow slowly, while variant cell lines fail to express AADC activity, grow quickly and frequently have amplified and/or expressed c-*myc* oncogene.[7] However, recent studies[8,9] have shown that amplification and/or expression of the c-*myc* oncogene occur not only in variant cell lines but also in classic cell lines. In order to determine the relationship among morphology and biochemical, biological or genetic properties, we studied the cytological features, doubling time, S-phase percentage, degree of expression of *myc*-family oncogenes, DNA ploidy and biochemical properties (AADC, NSE, CK-BB activity and GRP content) of 13 SCLC cell lines. Here we present the correlations among growth characteristics, expression of c-*myc* or N-*myc* oncogenes and nucleolar area in SCLC cell lines.

[7] Present address: Pathology Section, Clinical Laboratory, National Matsudo Hospital, Takatsukashinden 123-1, Matsudo, Chiba 271.

[8] To whom correspondence should be addressed.

[9] The abbreviations used are: SCLC, small cell lung cancer; AADC, aromatic L-amino acid decarboxylase; NSE, neuron-specific enolase; CK-BB, creatine kinase, brain isoenzyme; PBS, phosphate-buffered saline; GRP, gastrin-releasing peptide.

MATERIALS AND METHODS

Cell lines and culture media Thirteen SCLC cell lines were used. Eight of them, Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-135, Lu-139, Lu-140[10] and Lu-141 were established in our laboratory from tissues obtained at surgery or from xenotransplanted tumors in nude mice, and five of them, N-230, N-231, N-417, H-69 and H-82 were kindly supplied by Dr. A. F. Gazdar, Bethesda, Maryland, USA.[7] Culture medium used was serum-supplemented medium that contained RPMI-1640 and 10% fetal calf serum for all cell lines except for Lu-24, which was cultured in serum-free medium that contained RPMI-1640, 10 μM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM 17β-estradiol and 30 nM sodium selenite.

Growth patterns of cultured cells Growth patterns of cultured cells were observed under a phase contrast

microscope and were grouped into four types according to the classification of Carney *et al.*[4] Type 1 cells grew as floating, tightly packed, spherical aggregates, which frequently demonstrated central necrosis in the larger spheroids. Type 2 cells grew as relatively densely packed floating aggregates, irregular in outline, and lacking central necrosis. Type 3 cells grew as very loosely adherent floating aggregates in small clumps and intertwined cords. Central necrosis was absent in these lines. Type 4 cells grew attached to substrate.

Light and electron microscopy For cytological studies, cells were centrifuged at 40–150*g* for 5 min, resuspended in PBS and smeared onto glass slides using an autosmear apparatus. Cells were fixed in 95% ethanol for 60 min and stained according to Papanicolaou's method. For histological study, cell pellets obtained as above were fixed in 10% formalin and embedded in paraffin. Three μm sections were stained with hematoxylin and eosin. For electron microscopical studies, cell pellets were fixed in 2.5% glutaraldehyde solution for 60 min, then in 1% OsO_4 for 60 min, and processed routinely. Epon-embedded ultrathin sections were observed under a Hitachi H-600 electron microscope.

Measurement of nucleolar area Nucleolar areas of cultured cells were measured on microphotographs ($\times$1000) of Papanicolaou-stained cells using a semi-automatic morphometric apparatus (Kontron, MOP-AMO3, Munich). Nucleolar area of the largest nucleoli was measured when more than two nucleoli were found in a nucleus.

Cell growth study Growth curves were drawn after protein measurement according to the Oyama-Eagle method.[11] Cell suspensions of each cell line were seeded on ten 5-ml dishes and cultured in a CO_2 incubator. Cells from two dishes of each cell line were packed by centrifugation every other day for up to 10 days. Then they were washed twice with PBS, frozen and stored at $-20°C$ until measurement. Doubling times of each cell line were determined from the points of most rapid growth of their growth curves.

Flow cytometric analysis Cells were centrifuged at 20–40*g* for 5 min, resuspended in PBS and passed through a 26-gauge needle 2–4 times until almost all cells appeared as single cells under a phase contrast microscope. The cells were washed three times with PBS, fixed in 70% ethanol for 1 h, treated with 0.1% RNase at 37°C for 30 min, and stained with propidium iodide (0.2%) for 60 min. The cells were again passed through a 26-gauge needle 2–4 times and then passed through a 60 μm nylon mesh. DNA histograms of these single cell suspensions were obtained by flow cytometry (Becton-Dickinson FACS IV, USA). Peaks of G_0G_1 and G_2M were obtained from the DNA histogram and the percentage of cells in the S-phase was determined from the area between these two peaks.[12] DNA indices of the cell lines were determined by comparing the position of the first peak of the histogram with that of normal lymphocytes.[13]

RNA analysis Poly(A)$^+$ RNA (2 μg) prepared by guanidine thiocyanate/cesium chloride gradient centrifugation and oligo(dT)-cellulose affinity chromatography was denatured and electrophoresed on 1% agarose/formaldehyde gel and transferred to nitrocellulose filters.[14] The filters were then hybridized with probes. Probes used to evaluate the level of expression of the *myc*-family oncogenes included a 1.8 kbp *Sma*I-*Eco*RI for L-*myc*, a 1.0 kbp *Eco*RI-*Bam*HI fragment for N-*myc*, and a 1.5 kbp *Cla*I-*Eco*RI fragment for c-*myc*.[14] The probe also included a 2.0 kbp *Pst*I-*Pst*I fragment for β-actin.[15]

Biochemical analysis Cultured cells in the exponential growth phase were washed with PBS, and the washed pellet was frozen at $-80°C$ and stored until use. For AADC activity, dopamine was assayed by high-performance liquid chromatography with electrochemical detection.[16] NSE and CK-BB were determined by ultrasensitive enzyme immunoassay systems.[17, 18] The concentration of GRP in SCLC cells was determined by a radioimmunoassay method after extraction of GRP with boiling water.[19]

RESULTS

Growth pattern of cultured cells The growth patterns of cultured cells were subgrouped into 4 categories (Table I). The frequency of each type was: Type 1, 1 line, (8%);

Table I. Nucleoli and Growth Pattern of SCLC Cell Lines

Cell line	Nucleoli[a]	Cell arrangement[a]	Growth pattern[b] (type)
Lu-24	Inconspicuous	Ribbon-like	1
Lu-130	Inconspicuous	Sheet	2
Lu-134A	Inconspicuous	Sheet	2
Lu-134B	Inconspicuous	Sheet	2
Lu-140	Inconspicuous	Sheet	2
Lu-141	Inconspicuous	Sheet	2
Lu-135	Prominent	Ribbon-like	3
Lu-139	Prominent	Ribbon-like	2
N-230	Prominent	Ribbon-like	2
N-231	Prominent	Ribbon-like	2
N-417	Prominent	Ribbon-like	3
H-69	Prominent	Ribbon-like	2
H-82	Prominent	Ribbon-like	2

a) Washed cells were smeared onto glass slides using a autosmear apparatus, fixed in ethanol and stained by Papanicolaou's method.

b) Grouped as described in "Materials and Methods."

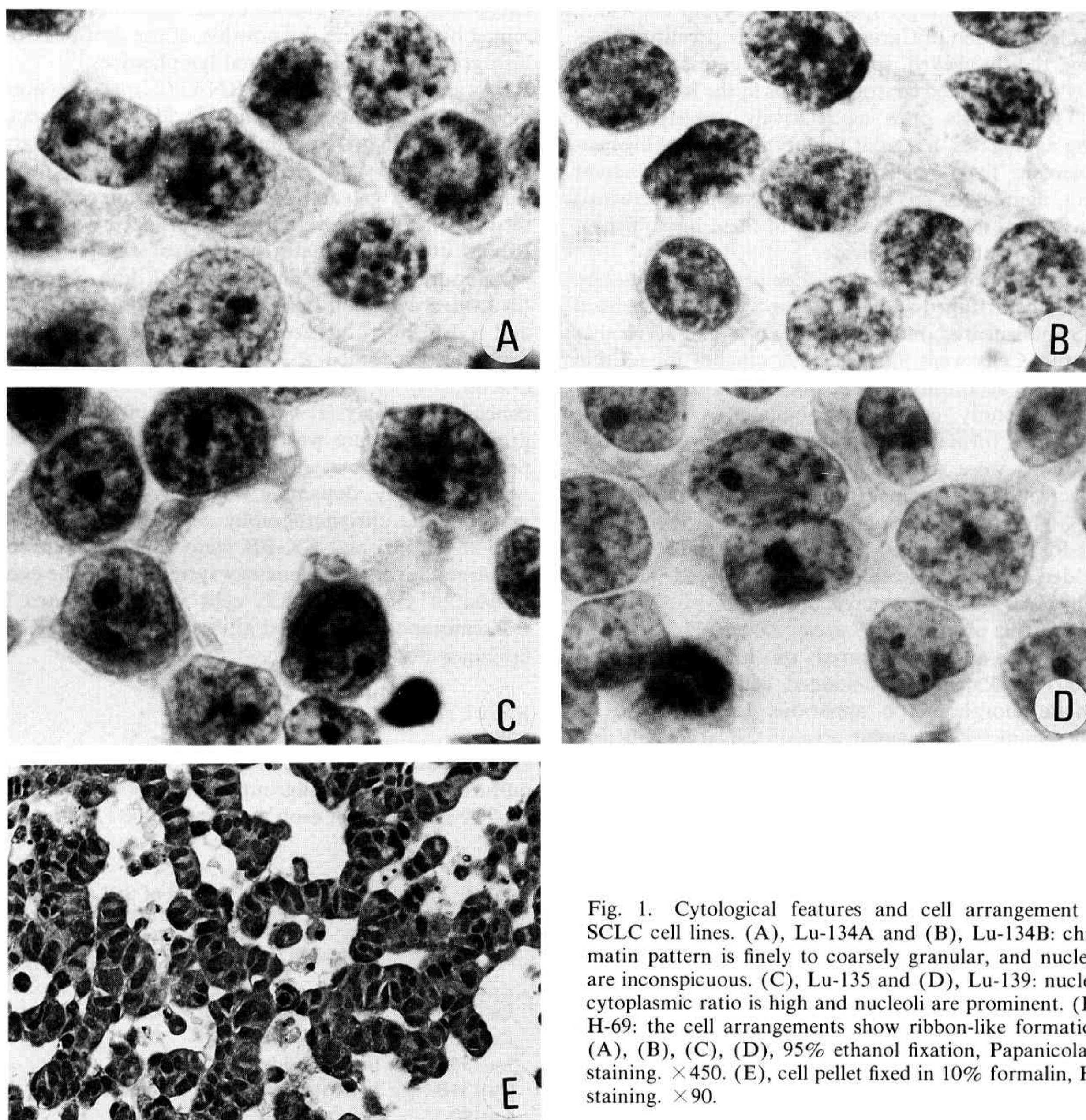

Fig. 1. Cytological features and cell arrangement of SCLC cell lines. (A), Lu-134A and (B), Lu-134B: chromatin pattern is finely to coarsely granular, and nucleoli are inconspicuous. (C), Lu-135 and (D), Lu-139: nuclear cytoplasmic ratio is high and nucleoli are prominent. (E), H-69: the cell arrangements show ribbon-like formation. (A), (B), (C), (D), 95% ethanol fixation, Papanicolaou staining. ×450. (E), cell pellet fixed in 10% formalin, HE staining. ×90.

Type 2, 10 lines, (77%); Type 3, 2 lines, (15%); Type 4, 0 line, (0%).

Cytological study All SCLC cells showed finely and densely granular nuclei and high nuclear/cytoplasmic ratio. Six cell lines (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140 and Lu-141) showed inconspicuous nucleoli (Fig. 1-A, -B), while the other seven cell lines (Lu-135, Lu-139, N-230, N-231, N-417, H-69 and H-82) showed prominent nucleoli (Fig. 1-C, -D). Ribbon-like cell arrangements (Fig. 1-E) were observed in the 7 cells lines that had prominent nucleoli and 1 cell line that had inconspicuous nucleoli (Table I). H-69 cell line contained prominent multinuclear giant cells. Cell pellets embedded in paraffin and stained with hematoxylin and eosin were composed of cells possessing round or spindle-shaped and finely granular nuclei in every cell line. Cytoplasmic organellae were poorly developed in general and a few neuroendocrine-type granules were observed

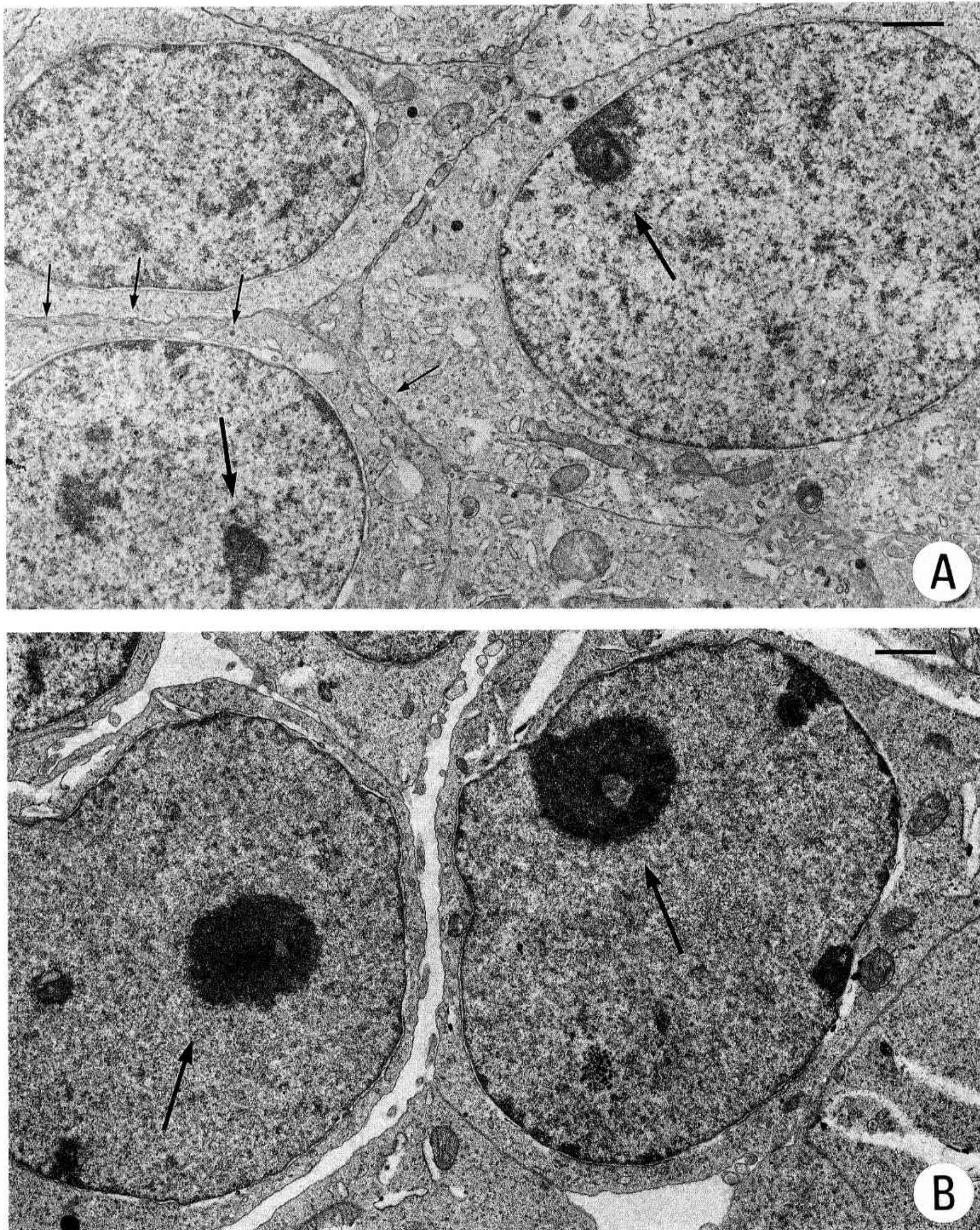

Fig. 2. Electron micrographs of SCLC cell lines. (A), Lu-134A, showing inconspicuous nucleoli (large arrows) with nucleoli organizing center. Small arrows indicate dense-cored granules. (B), Lu-135, showing prominent nucleoli (arrows) with nucleoli organizing center. Bars indicate 1 μm.

under an electron microscope. Inconspicuous nucleoli in six cell lines (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140, and Lu-141) and prominent nucleoli in seven cell lines (Lu-135, Lu-139, N-230, N-231, N-417, H-69 and H-82) with nucleoli organizing centers were also observed electron microscopically (Fig. 2).

Nucleolar area The difference of nucleolar size among cell lines observed by cytological study was evaluated quantitatively using a semi-automatic morphometric apparatus (Table II). Average nucleolar area of the six cell lines that had inconspicuous nucleoli was 1.5 μm^2, ranging from 1.3 to 1.9 μm^2, and that of the seven cell lines that had prominent nucleoli was 6.1 μm^2, ranging from 4.0 to 8.2 μm^2. A distinct difference in the nucleolar area was recognized statistically between the two groups ($P<0.001$).

Table II. Biological Characteristics of SCLC Cell Lines

Cell line	Nucleolar area[a] (μm^2)	Doubling time[b] (h)	Cells in S-phase[c] (%)	DNA index[c]
Lu-24	1.4	108	37	1.4
Lu-130	1.9	78	38	2.2
Lu-134A	1.3	70	39	1.6
Lu-134B	1.7	85	23	2.5
Lu-140	1.5	151	27	2.0
Lu-141	1.3	101	29	1.2
Lu-135	4.8	45	42	1.3
Lu-139	6.6	47	64	2.3
N-230	5.3	38	43	1.8
N-231	8.1	48	45	2.1
N-417	5.6	36	44	1.6
H-69	4.0	50	NT[d]	1.1
H-82	8.2	48	44	1.3

a) Measured on light microphotographs of Papanicolaou-stained cells using a semi-automatic morphometric apparatus.
b) Determined from the growth curve.
c) Determined from DNA histograms obtained by flow cytometry.
d) Not tested.

Doubling time Doubling times of SCLC cell lines are shown in Table II. Six cell lines (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140 and Lu-141) had long doubling times ranging from 70 to 151 h with an average of 99 h. On the other hand, the remaining seven cell lines (Lu-135, Lu-139, N-230, N-231, N-417, H-69 and H-82) had short doubling times ranging from 36 to 50 h with an average of 45 h. All 7 cell lines that showed shorter doubling times had large and prominent nucleoli, while the 6 other cell lines that showed longer doubling times revealed inconspicuous nucleoli.

Flow cytometric DNA histogram Percentages of cells in the S-phase are shown in Table II. The average percentage of cells in the S-phase for the six cell lines that showed long doubling times was 32% with the range of 23 to 39% (Fig. 3-A). On the other hand, that of the seven cell lines with short doubling times was 47%, ranging from 42 to 64% (Fig. 3-B). The difference of S-phase cell frequency between the two groups is significant.

DNA ploidy DNA index value obtained from the DNA histogram showed that all cell lines except for H-69 demonstrated aneuploidy (Table II).

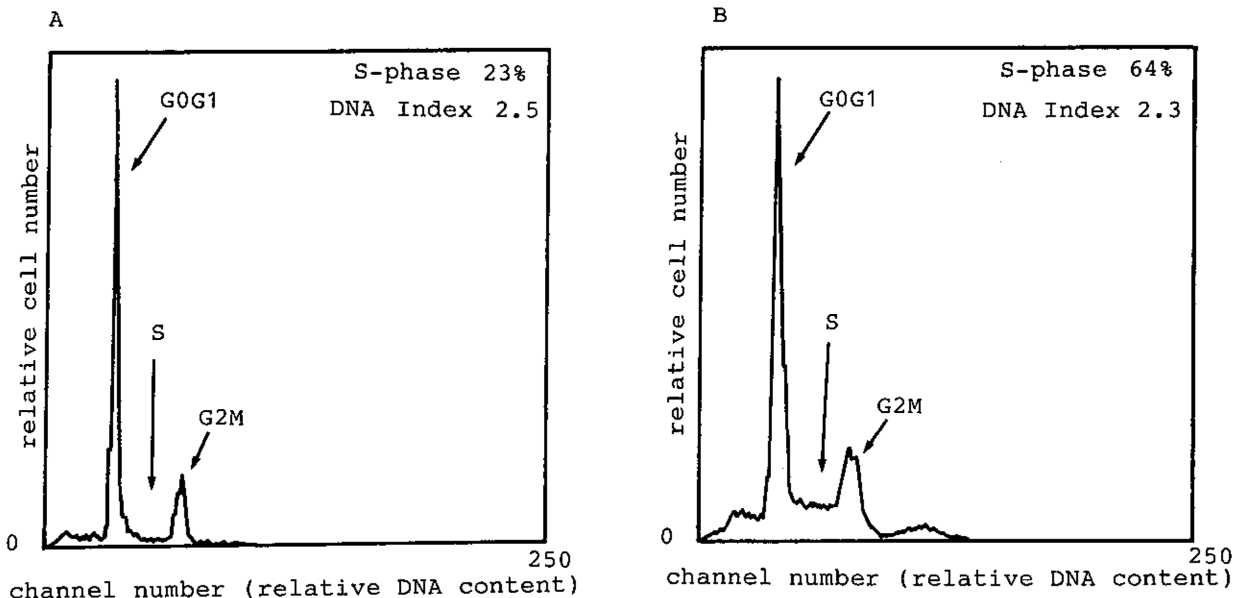

Fig. 3. DNA histograms of SCLC cell lines. (A), Lu-134B, showing a low S-phase percentage (23%). (B), Lu-139, showing a high S-phase percentage (64%).

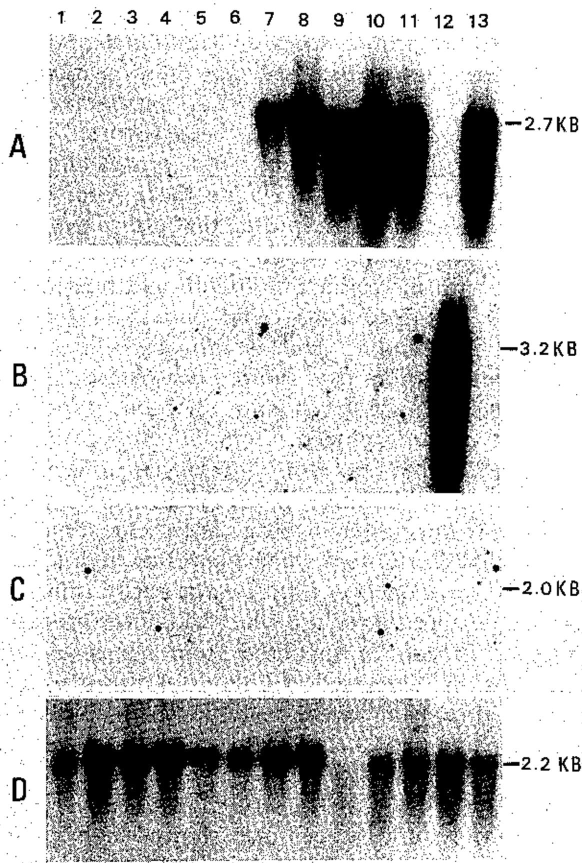

Fig. 4. m-RNA expression of c-*myc* (A), N-*myc* (B), L-*myc* (C) and β-actin (D) genes in 13 SCLC cell lines. The levels of c-*myc* mRNA expression relative to that of β-actin mRNA expression were extremely high in 6 cell lines, Lu-135 (lane A7), Lu-139 (lane A8), N-230 (lane A9), N-231 (lane A10), N-417 (lane A11) and H-82 (lane A13). The level of N-*myc* mRNA expression was extremely high in H-69 (lane B12) cell line. Neither of these *myc*-family oncogenes was expressed in 6 other cell lines, Lu-24 (lane 1), Lu-130 (lane 2), Lu-134A (lane 3), Lu-134B (lane 4), Lu-140 (lane 5) and Lu-141 (lane 6).

RNA analysis Expression of *myc*-family oncogenes in these cell lines was analyzed by Northern blot analysis (Fig. 4). High levels of c-*myc* oncogene expression were observed in 6 of the cell lines (Lu-135, Lu-139, N-230, N-231, N-417 and H-82) that had prominent nucleoli and grew quickly, and that of the N-*myc* oncogene was observed in the H-69 cell line that also had prominent nucleoli and grew quickly. On the other hand, expression of *myc*-family oncogenes was not detected by Northern blot analysis in the 6 cell lines that showed inconspicuous nucleoli and grew slowly (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140 and Lu-141). L-*myc* oncogene was not expressed in any of these cell lines.

Table III. Morphological and Biochemical[a] Characteristics of SCLC Cell Lines

Cell line	AADC[b] (pmol/min/mg)	NSE[c] (ng/mg protein)	CK-BB[c] (ng/mg protein)	GRP[d] (ng/mg protein)	Type
Lu-24	4170	5350	NT[e]	0.89	C[f]
Lu-130	4400	2270	4580	NT	C
Lu-134A	2477	1520	5590	1.30	C
Lu-134B	3639	604	4020	0.50	C
Lu-140	4200	3690	5520	0.59	C
Lu-141	29100	3030	2590	9.30	C
Lu-135	174	3180	5865	UD[g]	V[h]
Lu-139	9600	1875	6475	0.37	C
N-230	2930	1090	3185	NT	C
N-231	4020	867	2210	NT	C
N-417	6	495	6625	UD	V
H-69	4900	1120	5120	0.87	C
H-82	900	573	2255	NT	V

a) Cultured cells in the exponential growth phase were washed and the pellets were frozen at −80°C until use.
b) Dopamine was assayed by high-performance liquid chromatography.
c) Determined by ultrasensitive enzyme immunoassay methods.
d) Determined by a radioimmunoassay method after extraction of GRP with boiling water.
e) Not tested. *f*) Classic. *g*) Undetectable. *h*) Variant.

Biochemical analysis Results of biochemical analysis are shown in Table III. All 13 cell lines showed high levels of NSE and CK-BB. All six cell lines that grew slowly (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140 and Lu-141) and four cell lines that grew quickly (Lu-139, N-230, N-231 and H-69) showed high activity of AADC, while the other three fast-growing cell lines (Lu-135, N-417 and H-82) showed low activity of AADC. GRP was detected in cell lines that showed high AADC activity (Lu-24, Lu-134A, Lu-134B, Lu-140, Lu-141, Lu-139 and H-69) and was not detected in cell lines that expressed low AADC activity (Lu-135 and N-417).

DISCUSSION

We have examined the relationships among cytological features, biochemical properties, growth characteristics and degree of expression of *myc*-family oncogenes of 13 SCLC cell lines. The 6 cell lines that had inconspicuous nucleoli showed significantly longer doubling times, lower percentages of cells in the S-phase and no expression of *myc*-family oncogenes, as compared with the other 7 cell lines that had large nucleoli and showed shorter doubling times, higher percentages of cells in the S-phase and high levels of expression of *myc*-family onco-

genes. The findings strongly suggest close correlations among nucleolar area, growth speed and level of expression of *myc*-family oncogenes in SCLC cell lines. It has been recognized that there is a correlation between S-phase proportions obtained from the flow cytometric DNA histogram and by measuring incorporation of tritiated thymidine.[12, 13, 20] Cell lines that had a longer doubling time showed a smaller proportion of S-phase cells, and *vice versa* in our study. The relationship between growth speed and nucleolar area in SCLC cell lines was confirmed by this experiment.

Ribbon-like cell arrangements were found in the seven cell lines that grew quickly and in one cell line that grew slowly. Lu-24 cells, which grew slowly, showed ribbon-like cell arrangements, but the percentage of cell arrangements was low compared with that of the seven quickly-growing cell lines. Lu-24 cells changed from classic type to variant type when the culture medium was changed from serum-free medium to another kind of serum-free medium (unpublished data). It was assumed that the Lu-24 cell line has a few variant cells that might show a ribbon-like cell arrangement. The ribbon-like cell arrangement seems to be correlated with cell growth speed in cultured cells.

It has been reported that SCLC had more chromosomal aneuploidy than non-SCLC. Carney *et al.*[21] showed an aneuploidy proportion of 60% (9 of 15 cases) in SCLC cell lines using flow cytometry. Waters *et al.*[22] found an aneuploidy proportion of 100% (8 of 8 cases) in SCLC cell lines from a chromosomal study. Our results from DNA indices and chromosome numbers (data not shown) showed an aneuploidy proportion of 92% (12 of 13 cases), close to that of Waters *et al.*[22] No correlation between the degree of aneuploidy and the growth speed in SCLC cell lines was observed. Meyer and Coplin[12] reported no relationship between DNA index and growth speed in breast cancer, and this is supported by our results.

C-*myc* oncogene has been reported to be amplified and/or highly expressed in a variant type of SCLC, which is characterized by lack of AADC activity.[7] In this study, expression of c-*myc* oncogene was observed not only in variant-type cell lines (N-417, H-82 and Lu-135), but also in classic-type cell lines (Lu-139, N-230 and N-231). These cell lines with high levels of c-*myc* oncogene expression grew quickly, showing conspicuous nucleoli, and another fast-growing cell line (H-69) also expressed a high level of N-*myc* oncogene. On the other hand, none of the *myc*-family oncogenes was expressed in slow-growing, classic-type cell lines (Lu-24, Lu-130, Lu-134A, Lu-134B, Lu-140 and Lu-141). There are some reports that not only variant-type SCLC cell lines, but also classic-type cell lines showed elevated expression of the c-*myc* oncogene.[8, 9] It is suggested that expression of the *myc*-family oncogenes is not related to cell type, but is related to the growth speed and morphology of cells. Amplification and/or expression of the c-*myc* oncogen in N-231,[23] N-417[8, 23-25] and H-82,[23-25] and that of the N-*myc* oncogene in H-69 have been reported.[24] Our results concerning expressions of the *myc*-family oncogenes in these cells were similar to those reported previously. Johnson *et al.*[26] reported that c-*myc* expression in the c-*myc*-transfected classic-type SCLC cell line was associated with increased cell growth and altered morphology without decreasing AADC activity. This is consistent with our finding that fast-growing cells of either classic type or variant type showed conspicuous nucleoli and a high level of *myc*-family oncogene expression. Yokota *et al.*[14] concluded, from *in vivo* and *in vitro* studies, that amplification of c-*myc* oncogene would not have occurred at the time of malignant transformation but probably during tumor progression. Gemma *et al.*[27] reported that SCLC cells which expressed high levels of c-*myc* or L-*myc* oncogene were more easily transplantable to nude mice than those which did not express these oncogenes. It can be supposed from our results that SCLC cells that expressed high levels of c-*myc* or L-*myc* gene grew quickly and were easily transplantable to nude mice compared to SCLC cells that did not express these oncogenes and grew slowly.

AADC, NSE, CK-BB and GRP are biomarkers of SCLC. In our study, all cell lines showed high activities of NSE and CK-BB. GRP content and AADC activity are known to be found in high levels in the classic cell type and in low levels in the variant cell type.[4] In our study, three cell lines that grew quickly showed low activity of AADC and two of them showed undetectable GRP content, although 4 other fast-growing cell lines and 6 cell lines that grew slowly showed high activity of AADC and seven of them showed detectable GRP content. A low GRP content in H-82 cell line and high GRP contents in N-230 and N-231 have been reported by Carney *et al.*[4] The three fast-growing cell lines, Lu-135, N-417 and H-82, with low levels of AADC activity and amplification of c-*myc* oncogene,[23, 24] were classified as morphological variants according to the classification of Gazdar *et al.*[7] Four other fast-growing cell lines, Lu-139, N-230, N-231 and H-69, with high levels of AADC activity had prominent nucleoli. These 4 cell lines would not be classified as variant type because of their high level of AADC activity, or as classic type because of their rapid growth and prominent nucleoli. In this study, we showed clearly that cell growth of SCLC is not related to classic or variant cell type but to the nucleolar status and *myc*-family oncogene status. There was a distinct difference between slow-growing and fast-growing cell lines as to growth speed, nucleolar size, and *myc*-family oncogene expression without overlapping of the cases between

the groups. This indicates that a more reasonable subtyping of SCLC as to growth speed can be achieved not by using biochemical markers such as AADC but by using morphology or expression of *myc*-family oncogenes.

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