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Proto-Oncogene and Growth Factor/Receptor Expression in the Establishment of Primary Human Non-Small Cell Lung Carcinoma Cell Lines

Chi Liu and Ming-Sound Tsao

From the Department of Pathology, Montreal General Hospital and McGill University, Montreal, Quebec, H3G 1A4, Canada

In our effort to delineate factors that govern the ability of non-small cell lung carcinoma (NSCLC) to form monolayer cell lines, we have attempted to derive monolayer cell lines from the primary cultures of 29 unselected human NSCLCs. Eight new lines were obtained. Cell lines were easier to establish from poorly differentiated tumors, especially adenocarcinomas. One cell line was from a large cell neuroendocrine carcinoma. All cell lines were aneuploid, and they exhibited heterogeneous nutritional requirements for growth in vitro. Cell line-forming primary tumors demonstrated higher mean messenger RNA expression levels for transforming growth factor-α and c-met proto-oncogene than did tumors that failed to form cell lines. Although a high level of c-myc expression was correlated with the ability of NSCLC cell lines to form xenograft tumors in nude mice, it was not correlated with the ability of primary tumors to establish cell lines. The results suggest that autocrine growth loops play important roles in the ability of NSCLC cells to proliferate continuously in monolayer culture. The fact that the overexpression of transforming growth factor-α in NSCLCs has been negatively correlated with patient survival and that most cell lines can be established only from poorly differentiated carcinomas may provide the explanation for a previous report that the capability for cell line establishment constitutes a negative prognostic indicator for patient survival. However, when the genotype and phenotype of the cell lines were compared with those of their corresponding primary or xenograft tumors, the tumor cells that grew continuously as a cell line often represented a selective subpopulation of the heterogeneous neoplastic cells in the primary tumors. This finding should be taken into consideration when cell lines are used to evaluate the chemo- and radiosensitivity of tumor cells. (Am J Pathol 1993, 142:413–423)

Lung cancer is the leading cause of death from neoplasia in both men and women in the United States, and its incidence continues to increase steadily.[1] Human lung carcinomas are classified morphologically into two major groups, small cell carcinoma (SCLC) and non-small cell carcinoma (NSCLC). They show striking differences in morphology, biochemical and molecular properties, and clinical behavior and response to therapeutic modalities. The four major histological types of NSCLC are epidermoid (squamous cell) carcinoma, adenocarcinoma, large cell carcinoma, and adenosquamous carcinoma.[2] In spite of progress that has been made in the early diagnosis and treatment of lung cancers, the mortality remains very high. A more complete understanding of the molecular pathogenesis and biology of these tumors may contribute further to their accurate classification and therefore to the future design of effective therapies.

Tumor cell lines have been used extensively to study the molecular genetics and the biochemistry of human lung carcinomas. The establishment of cell lines from primary human NSCLC is unpredictable and the success rate has been low.[3–7] It is important to know the factors that govern the ability of NSCLC cells to grow and propagate *in vitro* as cell lines,

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Address reprint requests to Dr. Ming-Sound Tsao, Department of Pathology, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, H3G 1A4, Canada.

because this phenotype has been shown to be an independent prognostic factor for the survival of NSCLC patients treated both curatively and palliatively.[4] The reasons for the inability of most NSCLCs to grow continuously in monolayer culture have not been investigated systematically. It is possible that the continuous proliferation of these cells *in vitro* requires the expression of a specific set of growth factor/receptor genes and/or proto-oncogenes. Polypeptide growth factors, their receptors, and proto-oncogenes/oncogenes play important roles in the pathogenesis and biology of human malignancies.[8] Approximately two thirds of human NSCLCs demonstrate increased expression of transforming growth factor-α (TGF-α),[9,10] and TGF-α has been shown to function as an autocrine growth factor for the proliferation of some lung carcinoma cell lines.[11–13] Siegfried et al[5] reported that the conditioned medium from the A549 human adenocarcinoma (bronchioloalveolar type) cell line contained certain factors that promoted the proliferation of human lung cancer cells in primary culture and facilitated the establishment of cell lines in 40% of the primary NSCLCs studied.

Although several investigators have stated that the morphology and biochemical properties of lung cancer cell lines are similar to those of the primary tumors,[14,15] few studies have actually been performed to demonstrate that the phenotype and genotype of the neoplastic cells in the cell lines are indeed representative of those in the primary tumors. We have established eight new tumor cell lines from the primary cultures of 29 unselected, surgically removed, primary NSCLCs. The expression of several growth factor and receptor genes and proto-oncogenes has been compared in tumors that either succeeded or failed in yielding cell lines. Furthermore, we have compared the expression of these genes *in vitro* with their expression *in vivo* in the primary and xenograft tumors.

Materials and Methods

Twenty-nine NSCLCs resected at this hospital were obtained unselectively, usually within 30 min after their surgical resection. These tumors included 17 adenocarcinomas, three large cell carcinomas, one adenosquamous carcinoma, and eight epidermoid carcinomas. The pathological diagnosis of each tumor was based on light- and electron-microscopic studies.[16] Poorly differentiated epidermoid carcinomas and adenocarcinomas are usually dominated by sheets of anaplastic tumor cells with 1) focal areas showing either squamous or glandular differentiation or 2) the presence, as revealed by transmission electron-microscopic studies, of ultrastructural features of squamous or glandular cells. Adenosquamous carcinoma represents a tumor that shows both squamous and glandular differentiation. The large cell undifferentiated carcinomas consist of a sheet of anaplastic tumor cells with neither squamous nor glandular differentiation, at both light-microscopic and ultrastructural levels.

Approximately 0.5 cm^3 of tumor tissue from each specimen was used for the primary culture. Tumor tissues were also snap frozen in liquid nitrogen and stored at −80 C for subsequent isolation of the RNA and DNA.

Establishment of Cell Lines from Primary Tumors

Tumor tissue was gently teased and minced with a scalpel into approximately 1- to 2-mm fragments. The dissociated cells and tissue fragments were washed once with Mg^{++}/Ca^{++}-free Hanks' balanced salt solution (GIBCO, Grand Island, NY) and then were plated into six or eight 100-mm tissue culture dishes (Falcon) in R-10 medium composed of RPMI 1640 plus 10% fetal bovine serum (GIBCO). The cultures were incubated at 37 C in a humidified 5% CO_2 atmosphere. Three or 5 days later, when cell attachment was considered adequate and initial colony formation had commenced, the medium in half (three or four plates) of the culture dishes was changed to hormonally defined serum-free ACL-4 medium.[17] Thus, for each tumor specimen, cell line establishment was attempted in both serum-free and serum-containing media. The media were subsequently changed twice a week.

Subculture was performed initially by dissociating the colonizing cells with Hanks' balanced salt solution containing 0.05% trypsin and 0.53 mmol/l ethylenediamine tetraacetic acid (EDTA) (GIBCO), at 1:3 or 1:4 split ratios. Later-passage cultured cells were subcultured at seeding densities of 2 to 5 × 10^5 cells/100-mm dishes. Occasionally, maintenance of the primary cultures at confluence with frequent medium changes was used to enrich preferentially the population of neoplastic epithelial cells over their surrounding fibroblasts.

Pure epithelial cell cultures, as observed microscopically by the absence of large flattened or spindle-shaped fibroblasts, were usually obtained after three or four subcultures. All cell lines have been subcultured more than five times, and cells at passage 4 to 6 were used for further phenotypic and genotypic studies.

Xenografts of Tumor Cells in Nude Mice

Pure epithelial tumor cell lines were injected into the nude mice to study their tumorigenicity. CD1 athymic (*nu/nu*), 6-week-old, male nude mice were purchased from Charles River (Kingston, NY) and were maintained in a pathogen-free condition. About 1×10^6 trypsinized tumor cells were suspended in 0.5 ml of Hanks' balanced salt solution and were injected subcutaneously into the back of these mice (two to four mice/cell line). They were checked every 2 weeks for up to 3 months for the formation of tumors. Tumors that had reached 0.5 cm or more in diameter were removed and processed for routine histology, electron-microscopic study, and RNA/DNA isolation.

Growth Rate

The proliferation of each tumor cell line was studied in both serum-containing and serum-free media. Trypsin-dissociated cells were plated in six-well tissue culture dishes (Falcon), at a density of 2 or 5×10^4 cells/well, in R-10 or ACL-4 medium. After 3 to 5 days, the medium was changed with either fresh R-10 or ACL-4 medium. The number of cells in each of the triplicate wells was counted every 2 or 3 days, and the population doubling time was determined from the exponential phase of each growth curve.

Flow Cytometric DNA Content Analysis

Approximately 2×10^6 trypsin-dissociated cells from each cell line were processed according to the method of Lee et al.[18] In order to obtain single-cell suspension, cells were trypsinized, washed in a buffer containing 0.1 mol/l NaCl, 11 mmol/l glucose, 5.6 mmol/l Na_2HPO_4, 5.4 mmol/l KCl, 0.4 mmol/l $CaNO_3$, and 0.4 mmol/l $MgSO_4$, passed three times through a 16-gauge syringe needle, and fixed by the addition of cold 95% ethanol to a final concentration of 70%. Cultured normal lung fibroblasts, isolated from one of the NSCLC primary cultures that failed to grow epithelial tumor cells, were used as a reference for diploid DNA content. Cells were stained with propidium iodide and examined with an Epic Profile II (Coulter Electronics, Hialeah, FL) flow cytometer. The relative DNA content of the tumor cells was expressed as the ploidy index, which is the ratio between the modal value of the G_1/G_0 aneuploid peak of the tumor cells and that of the diploid peak of the normal fibroblasts.

Nucleic Acid Probes

Plasmids carrying complementary DNA (cDNA) for human TGF-α, epidermal growth factor receptor (EGFR), and c-*erb*B-2 genes were obtained from the American Type Culture Collection (Rockville, MD). The TGF-α cDNA probe was a 0.93-kilobase (kb) *Eco*RI insert of clone phTGF1:10–925. The EGFR messenger RNA (mRNA) was probed with a 2.4-kb *Cla*I EGFR cDNA insert of the pE7 plasmid. The c-*erb*B-2 (*neu*/HER-2) mRNA was probed with a 3.4-kb *Stu*I insert of pCER204. The c-*met* mRNA was probed with a 1.1-kb *Eco*RI fragment of the human c-*met* cDNA.[19] The c-*myc* and the human β-actin cDNA probes were purchased from Oncor (Gaithersburg, MD) and Clonotech (Palo Alto, CA), respectively.

RNA Isolation and Northern Blot Analysis

Total cellular RNA from cultured cells and from approximately 1 g of tissue was isolated in a 4 mol/l guanidine isothiocyanate solution, pH 7.0, containing 25 mmol/l sodium citrate, 0.1 mol/l β-mercaptoethanol, and 0.5% sarkosyl.[20] Thirty micrograms of RNA from each sample were separated electrophoretically and Northern blot analysis was performed as described.[20]

DNA Isolation

Trypsinized cells were digested overnight at 37 C with 100 mg/ml proteinase K in an aqueous solution containing 10 mmol/l TRIS-HCl, pH 7.4, 0.1 mol/l EDTA, and 0.5% dodecyl sodium sulfate (Anachemia, Rouses Point, NY). After two extractions with equal volumes of phenol and chloroform and one with chloroform alone, the aqueous phase containing DNA was precipitated overnight at –20 C with 3 volumes of 95% ethanol. The DNA was spooled and solubilized in 5 ml of TRIS-EDTA buffer containing 10 mmol/l TRIS-HCl, pH 7.4, and 0.1 mmol/l EDTA. After digestion with DNAse-free RNAse (Boehringer Mannheim, Dorval, Quebec, Canada), DNA was re-extracted with phenol and chloroform and precipitated in 95% ethanol. The recovered DNA was dissolved in TRIS-EDTA buffer. The amount of DNA was estimated by measuring the absorbance at 260 nm wavelength.

DNA was isolated similarly from the primary and xenograft tumors. Approximately 0.5 g of frozen tissue was pulverized in liquid nitrogen with a mortar and pestle and then digested overnight at 37 C with 1 mg/ml proteinase K in a buffer containing 10 mmol/l TRIS-HCl, pH 7.4, 10 mmol/l NaCl, 25 mmol/l EDTA, and 1% sodium dodecyl sulfate. The DNA was extracted as described above for cultured cells.

Endonuclease Digestion and Southern Blot Analysis

Ten micrograms of the DNA sample were digested overnight at 37 C with 10-fold excess units of *Eco*RI enzyme. Separation was then performed in a 0.8% agarose gel, in running buffer containing 40 mmol/l TRIS-acetate and 0.1 mmol/l EDTA, at 35 V for 16 hours. After depurination and denaturation by sequential washes in 0.25 mol/l HCl solution for 15 min, 0.2 mol/l NaOH solution containing 0.6 mol/l NaCl for 30 min, and 0.5 mol/l TRIS solution, pH 7.6, containing 1.5 mol/l NaCl for 30 min, the nucleic acids were transferred onto a Hybond-N membrane in 20× standard saline citrate solution and were cross-linked by exposure to ultraviolet light.

Nucleic Acid Hybridization

The membranes were probed sequentially with each of the six cDNAs as described previously.[20] Membranes were exposed to XAR-5 Kodak x-ray film, using an intensifying screen, for 3 to 5 days at −80 C. Densitometric measurements were performed by using a Hoefer GS-300 scanning densitometer. In order to compare quantitatively the autoradiographic signals from different membranes exposed to x-ray films for varying lengths of time, a reference sample consisting of 30 mg of total cellular RNA from A431 cells was co-electrophoresed in every gel. The A431 cell line was chosen arbitrarily and used strictly for analytical and not comparative purposes. The indexed mRNA signal for each sample was first normalized to the β-actin expression level of that sample and was then calculated as a ratio to its expression by the A431 cell line.

EGFR and c-*met* expressed multiple mRNA species, but only their full length transcripts (10.5 kb for EGFR and 8.5 kb for c-*met*) were measured. TGF-α and c-*erb*B-2 were expressed as single mRNA species.

Results

In all cases, tumor cells consistently attached well during the first 3 days of primary culture. The abundance of tumor cells in primary cultures was generally greater when the tumor was not necrotic or desmoplatic. In most cases these neoplastic epithelial cells started to form small colonies during the next 5 to 7 days, but in tumors that failed to form a cell line they ceased to expand, gradually died, and disappeared thereafter or they were overwhelmed by the growth of surrounding fibroblasts.

Only eight of these 29 tumors formed propagable cell lines. They included four adenocarcinomas (MGH-8, -13, -24, and -26), two large cell carcinomas (MGH-4 and -14), one epidermoid carcinoma (MGH-7), and one adenosquamous carcinoma (MGH-30) (Figure 1). The success rate varied among the different types of NSCLCs. In general, poorly differentiated carcinomas, especially adenocarcinoma were easier to grow *in vitro* (Table 1). The difference between the well or moderately differentiated tumors and the poorly differentiated tumors is statistically significant ($P < 0.05$, χ^2 test).

Tumor cell lines exhibit variable but specific nutritional requirements for their proliferation. MGH-4 and -14 lines were formed only in ACL-4 serum-free medium, whereas MGH-13 and -24 lines were established only in serum-containing medium. The remaining four cell lines (MGH-7, -8, -26, and -30) established in both media. When the proliferation of these cell lines in both the ACL-4 and R-10 media was studied, we found that cell lines that were established only in ACL-4 were unable to proliferate in serum-containing medium (Figure 2). Likewise, cell lines that established in R-10 medium grew better in this medium than in ACL-4 medium. In contrast, cell lines that established with equal facility in both types of medium demonstrated little preference for proliferation in either medium (Figure 3).

All cell lines demonstrated aneuploid DNA composition and long population doubling times, ranging from 53 to 204 hours (Table 2). Although these cell lines were not obtained clonally, the flow cytometric analyses demonstrated that most of them were composed of a relatively homogeneous population of cells with a single and relatively narrow aneuploid peak (Figure 4).

Tumor tissue from 22 of these primary NSCLCs was available for RNA extraction and a study of the expression of TGF-α, EGFR, and c-*erb*B-2, c-*met*, and c-*myc* proto-oncogenes. They included six cell-line-forming carcinomas and 16 carcinomas that failed to form cell line. The former expressed higher mean levels of TGF-α and c-*met* mRNAs than did the latter (Table 3); however, the differences are not statistically significant. One of the 16 carcinomas that did not form a cell line expressed unusually high c-*met* mRNA, and when this sample is excluded the mean c-*met* mRNA level of the cell-line-forming tumors is significantly higher than that of the non-cell-line-forming tumors ($P = 0.05$). There was no correlation between the expression levels of EGFR or c-*erb*B-2 and the ability of the NSCLCs to form a propagable cell line. In constrast, cell-line-

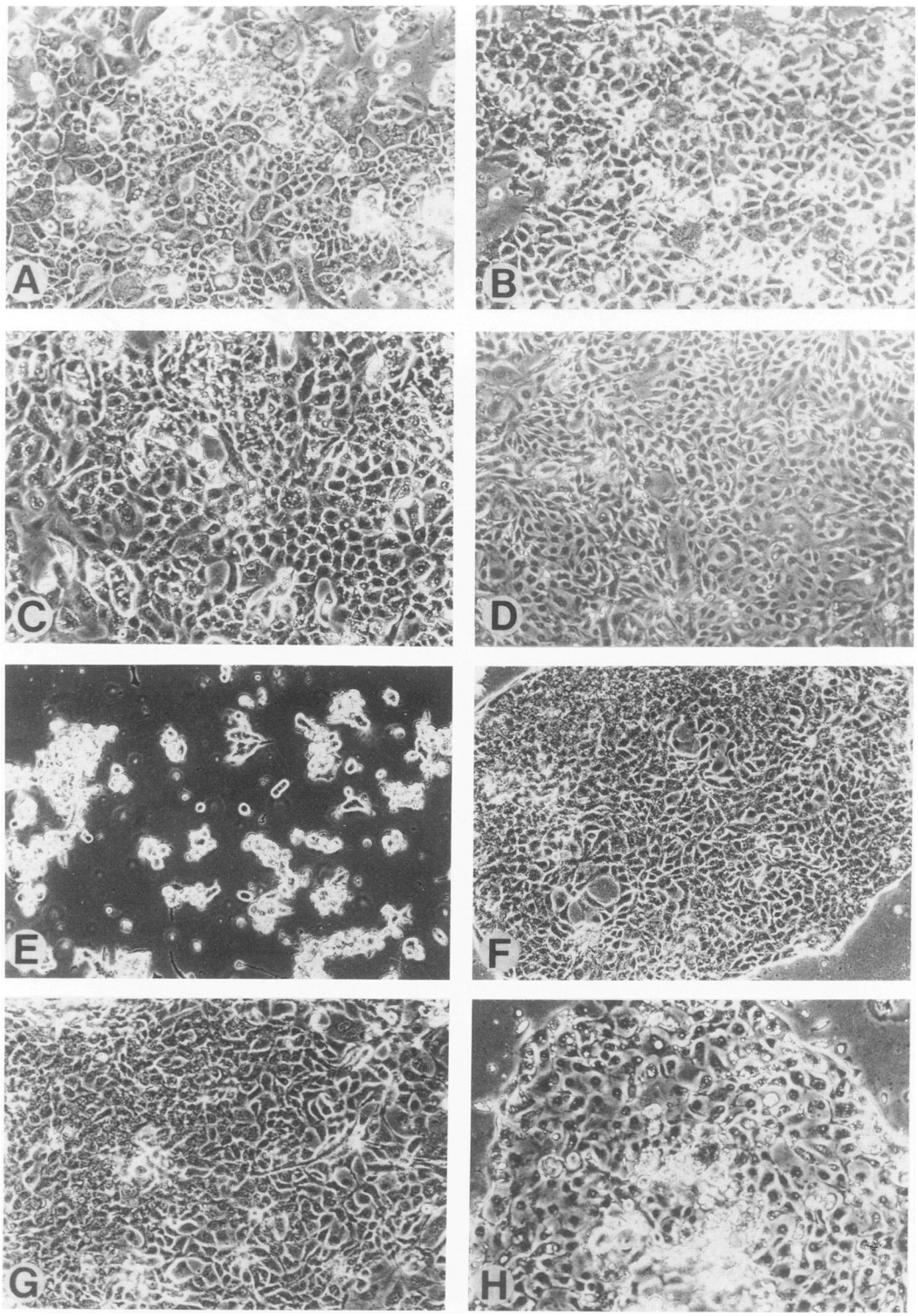

Figure 1. *Microphotographs of all eight newly established human NSCLC cell lines (phase contrast, ×200).* **A:** *MGH-4, a large cell undifferentiated carcinoma;* **B:** *MGH-7, a poorly differentiated epidermoid carcinoma;* **C, F,** *and* **G:** *MGH-8, -24, and -26, respectively, poorly differentiated adenocarcinomas;* **D:** *MGH-13, a moderately differentiated adenocarcinoma;* **E:** *MGH-14, a large cell neuroendocrine carcinoma;* **H:** *MGH-30, a poorly differentiated adenosquamous carcinoma.*

Table 1. *Success Rate of Establishing Cell Lines from the Various Histological Types and Grades of Primary Human NSCLC*

	No. of tumors	No. of cell lines	Success rate (%)
Epidermoid carcinoma	8	1	12.5
Adenocarcinoma	17	4	23.5
Large cell carcinoma	3	2	66.7
Adenosquamous carcinoma	1	1	
Well or moderately differentiated	15	1	7.0
Poorly differentiated*	14	7	50.0

* Including large cell carcinomas.

forming tumors demonstrated a lower mean c-*myc* mRNA level than did tumors that failed to form a cell line.

Five of the eight cell lines (MGH-4, -7, -14, -24, and -30) injected into nude mice formed tumors, and the histological appearances of the xenograft tumors were identical to those of their respective primary tumors. Four of the cell lines (except MGH-14) that formed tumors in nude mice expressed relatively higher levels of c-*myc* mRNA than did the cell lines that failed to form xenograft tumors, except MGH-13 (Figure 5).

When the levels of mRNA for TGF-α, EGFR, c-*erb*B-2, and c-*met* in four primary tumors and their corresponding cell lines were compared, expression was generally higher in cultured cells (Figure 6). A further comparative study with their xenograft tumors suggests that in some cases a selective proliferation

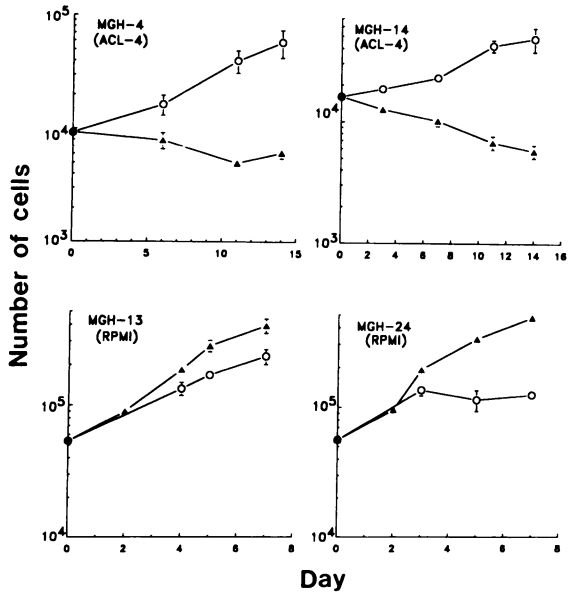

Figure 2. *Growth curve for MGH-4, -13, -14, and -24 cell lines in ACL-4 serum-free medium (○) or RPMI serum-containing medium (▲). The type of medium in which the cell line was initially established is indicated in parentheses.*

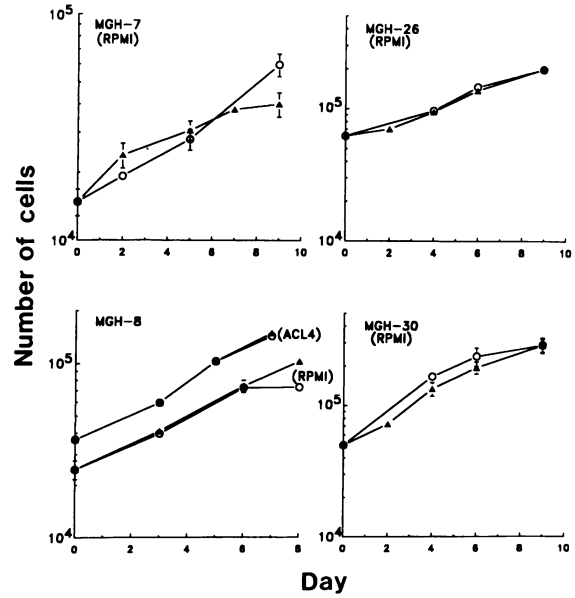

Figure 3. *Growth curve for MGH-7, -8, -26, and -30 cell lines in ACL-4 serum-free medium (○) or RPMI serum-containing medium (▲). The type of medium in which the cell line was initially established is indicated in parentheses.*

of a tumor cell subpopulation occurred during the cell line establishment. In MGH-4, amplification and overexpression of the EGFR gene were observed in the primary tumor but were absent or insignificant in the cell line and in the xenograft tumor (Figure 7). Furthermore, a marked amplification of the c-*myc* gene was primarily demonstrated in the cultured cells and the xenograft tumor, although a lower level amplification was also seen in the primary tumor. The MGH-7 cell line showed a marked overexpression without amplification of the EGFR and c-*met* genes (Figure 6). Although their expression in the xenograft tumor was down-regulated, it remained significantly higher than in the primary tumor. In MGH-14, the xenograft tumor showed markedly down-regulated c-*met* expression, compared with the primary tumor.

Table 2. *Population Doubling Time and Ploidy Index of Eight Newly Established Human Lung Carcinoma Cell Lines*

Cell line	Type*	Doubling time (hours)	Ploidy index
MGH-4	Large cell undifferentiated	125	1.40
MGH-7	Epidermoid (p.d.)	120	1.62
MGH-8	Adeno (p.d.)	80	1.74
MGH-13	Adeno (m.d.)	55	1.94
MGH-14	Large cell neuroendocrine	204	1.35
MGH-24	Adeno (p.d.)	53	1.77
MGH-26	Adeno (p.d.)	134	1.43
MGH-30	Adenosquamous (p.d.)	79	1.77

* p.d., poorly differentiated; m.d., moderately differentiated.

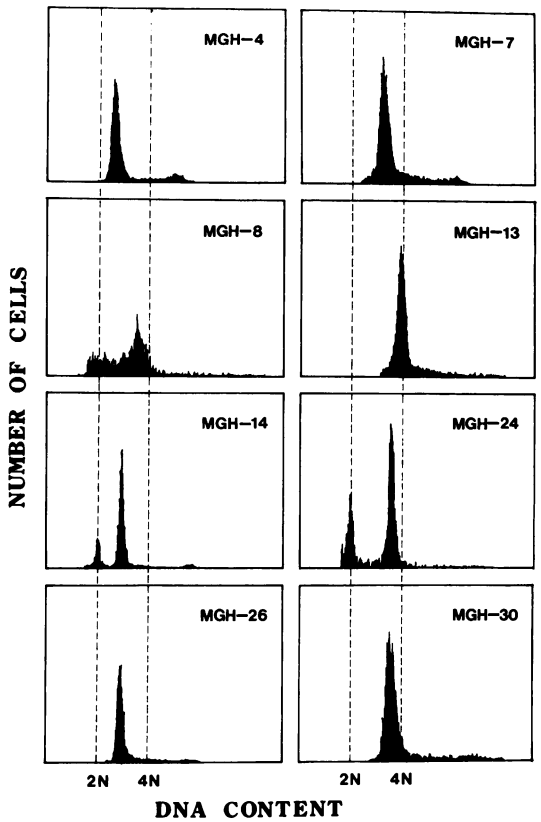

Figure 4. *Flow cytometric profile of the DNA content of these cell lines. Normal fibroblasts were added to the samples of MGH-14 and -24 to demonstrate the position of the diploid (2N) DNA content. 4N, normal tetraploid DNA content.*

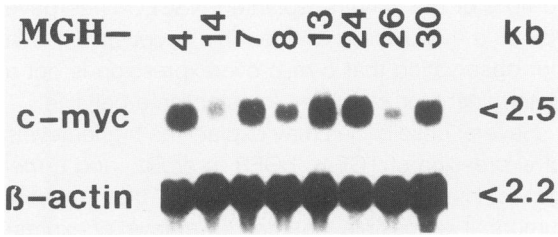

Figure 5. *Relative levels of c-myc and β-actin mRNA expression in confluent cultures of these cell lines.*

Only in MGH-24 were the phenotype and genotype of the cell line and its xenograft tumor similar to those of the primary tumor.

Discussion

The results of this study confirm that monolayer cell lines can be established from approximately one third of the primary tumors of NSCLCs; however, this remains significantly lower than the 75% success rate for SCLC cell lines.[15] Several investigators have reported the difficulty of establishing cell lines from primary NSCLCs.[4,6,7,21,22] Stevenson and Gazdar

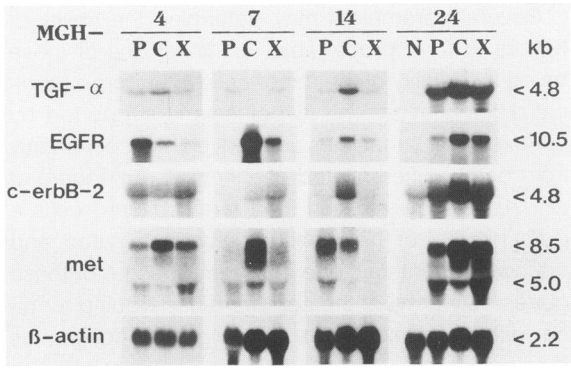

Figure 6. *Comparative mRNA expression of various growth factor and receptor genes in the primary tumor (P), cell line (C), and xenograft tumors (X) of four of the tumorigenic cell lines. N, corresponding normal lung tissue.*

and colleagues[4,22] reported previously that it is easier to establish a cell line from the metastatic deposit than from the primary lung carcinoma. Their reported success rate in establishing cell lines from NSCLCs metastatic to the mediastinal and peripheral lymph nodes was 44% and 42%, respectively. In contrast, cell lines were established successfully in only 8% (five of 63) of the primary tumors. Bepler et al[21] were able to derive only one line directly from 44 primary NSCLCs, although two other lines were subsequently established from tumors initially xenotransplanted in nude mice. Duchesne et al[7] also reported that xenotransplanted lines of NSCLCs were easier to establish than monolayer cultured cell lines. The highest success rate for the establishment of monolayer lines from primary NSCLCs was reported by Siegfried and Owens,[5] who utilized a serum-containing conditioned medium of A549 cells to obtain nine cell lines from 22 primary tumors. They suggested that the A549 cells elaborate certain factors that facilitate the propagation of NSCLC cells in monolayer culture.

Table 3. *Comparison of the mRNA Expression between the Primary Tumors that Succeeded or Failed in Forming Cell Lines**

	Cell line-forming	Non-cell line-forming	*P* value
TGF-α	2.11 ± 1.32	1.22 ± 1.44	0.06
EGFR	0.09 ± 0.07	0.12 ± 0.12	Not significant
c-*erb*B-2	0.45 ± 0.41	1.06 ± 1.91	Not significant
c-*met*	0.78 ± 0.76	0.67 ± 1.56 (16)	0.09
		0.29 ± 0.38 (15)†	0.05
c-*myc*	0.66 ± 0.18	1.22 ± 0.17	0.02

* All values represent the mean ± standard error of ratios of tumor RNA expression level to expression level of A431 cells. Six cell line-forming and 16 non-cell line-forming tumors were analyzed; *P* values are calculated using the nonparametric Wilcoxon rank sum test.

† The mean ± standard error after one sample with a very high c-*met* expression was excluded.

Several parameters may determine the ability of tumors to form propagable cell lines, one of these being the facility of the tumor cells to undergo terminal differentiation. This hypothesis is supported by our experience that lines are derived most commonly from poorly differentiated carcinomas. Antequera et al[23] have shown that in long term cultured cells a great number of the genes become methylated, and the consequent loss of expression of some of these genes may prevent the cells from undergoing terminal differentiation, hence allowing their continuous proliferation *in vitro*.

Another factor that may determine the ability of tumor cells to proliferate continuously *in vitro* is their ability to express, synthesize, or respond to a specific set of autocrine and paracrine growth factors or growth inhibitors. The fact that some tumors exhibit a specific medium requirement for their proliferation confirms that such a nutritional requirement is heterogeneous. Similar results have been reported by Brower *et al.*[3] Our investigation suggests further that the TGF-α/EGFR and hepatocyte growth factor (HGF)/c-*met* autocrine growth loops may play critical roles in the ability of NSCLC cells to grow continuously *in vitro*. Tumors that established cell lines demonstrated higher mean levels of expression for TGF-α and c-*met* than did tumors that failed to form a cell line. TGF-α was reported previously to function as an autocrine growth-stimulatory factor in A549 and PC-9 lung adenocarcinoma cell lines.[12,13] Monoclonal antibody to TGF-α inhibits the proliferation of these cells in a dose-dependent manner, and this inhibition can be neutralized by exogenous TGF-α. HGF was originally identified as a platelet-derived serum factor that stimulates DNA synthesis in hepatocytes during liver regeneration.[24–27] HGF was also shown recently to be identical to the scatter factor, a fibroblast-derived protein that disperses cohesive colonies of epithelial cells.[28–31] HGF/scatter factor has been established primarily as a paracrine factor, because it is expressed and synthesized in abundance by mesenchymal cells. In contrast, its receptor, the c-*met* proto-oncogene, is expressed by most epithelial cells.[32–36] We are currently investigating the role of HGF and c-*met* as an autocrine growth loop in the proliferation of cultured NSCLC cells.

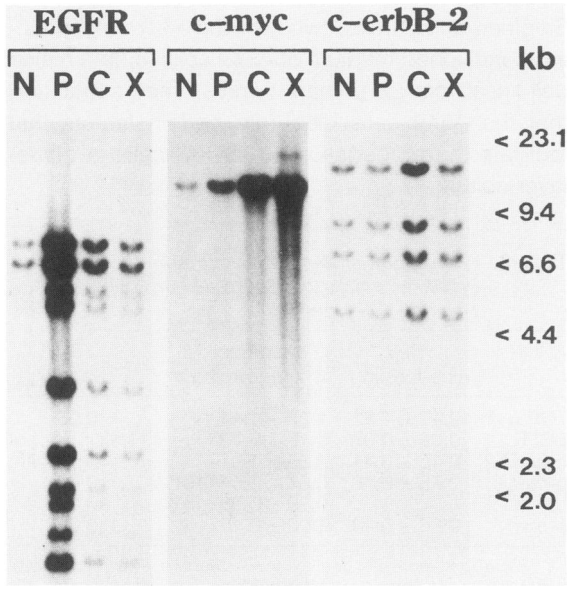

Figure 7. *Southern analysis of DNA from the primary tumor (P), cell line (C), normal lung tissue (N) and xenograft tumor (X) of MGH-4, showing amplification of the EGFR gene in the primary tumor but not in the cell line and amplification of the c-myc gene in the cell line but not in the primary tumor.*

A high level of c-*myc* expression does not correlate with the capability for cell line formation, but it appears to be a determining factor for the ability of these NSCLC lines to form xenograft tumors in nude mice. Gemma et al[37] have demonstrated that, whereas 11 of 13 primary lung carcinomas with c-*myc* gene amplification were able to form tumors in nude mice, only five of nine tumors without amplification demonstrated tumorigenicity in nude mice. However, they did not report the level of c-*myc* mRNA expression in their tumors. Gazzeri et al[38] reported c-*myc* overexpression in all five xenografted NSCLC established directly from fresh tumor specimens. It is important to note that only 20 to 40% of the xenotransplanted NSCLC lines gave rise to a monolayer cell line. This is consistent with our observation that c-*myc* overexpression is not a determinant for the ability to establish a cell line.

Several possibilities may explain the higher levels of expression of TGF-α, EGFR, c-*erb*B2, and c-*met* genes in cell lines, compared with their primary tumors. It is possible that their lower level of expression *in vivo* is caused by a dilution of the tumor cell population by the presence of non-neoplastic (host/stromal) cells that do not express these genes, in contrast to the pure population of tumor cells in the cell lines. It is also conceivable that the tumor cells in propagable culture actually show higher constitutive levels of expression of these genes than do the tumor cells *in vivo*. Such a situation may be brought about by 1) the monolayer culture condition preferentially allowing the proliferation of tumor cell subpopulations with relatively high expression levels of these genes or (b) the up-regulation of tumor cell expression of these growth factor and/or receptor genes during their adaptation to proliferation in a new *in vitro* environment. The results of our comparative study on the phenotype and genotype of the cell

lines and their respective primary and xenograft tumors, however, suggest strongly that cell lines may not always be representative of the primary tumor cell population from which they are derived.

All cell lines except MGH-14 grew as adherent monolayers on tissue culture dishes. MGH-14 was derived from a large cell neuroendocrine carcinoma. The primary tumor was diagnosed morphologically as an undifferentiated large cell carcinoma, but transmission electron-microscopic study showed the presence of neuroendocrine granules and the immunohistochemical studies revealed strong positive staining for chromogranin (Figure 8) and neuron-specific enolase. These tumor cells grow *in vitro* as loosely attached cell aggregates (Figure 2*E*), thus resembling the growth of SCLC cells in culture. However, in contrast to SCLCs, which usually lack the expression of EGFR and c-*erb*B-2 at both the mRNA and protein levels,[39,40] the MGH-14 cell line and its tumors expressed EGFR and c-*erb*B-2 mRNA. In contrast to the SCLC variants, MGH-14 cells showed neither amplification nor overexpression of the c-*myc* proto-oncogene.[41–43] Large cell neuroendocrine carcinomas,[44] or atypical endocrine tumors of the lung,[45–47] have been grouped as a specific type of lung tumor that falls into the "gray area" between the NSCLC and SCLC,[47] and their clinical behavior and response to chemotherapy are controversial. Some investigators reported that they behave like SCLC, with a rapid clinical course and a good initial responsiveness to chemotherapy.[44,47] Others have reported that they more closely resemble NSCLC in their clinical behavior.[45] In the context of these controversies, we prefer to classify MGH-14 as a large cell neuroendocrine carcinoma, which some investigators refer to as a "morphological or biochemical" variant of SCLC.[48,49]

In summary, the negative prognostic value of the capacity for cell line establishment in NSCLCs may be related to several parameters, including the poor differentiation and the comparatively higher levels of expression of growth factors and/or tyrosine kinase receptors, that correlate with the ability of these tumors to form a cell line. The use of cell lines for evaluating the chemosensitivity of NSCLCs, however, should take into consideration that some tumor

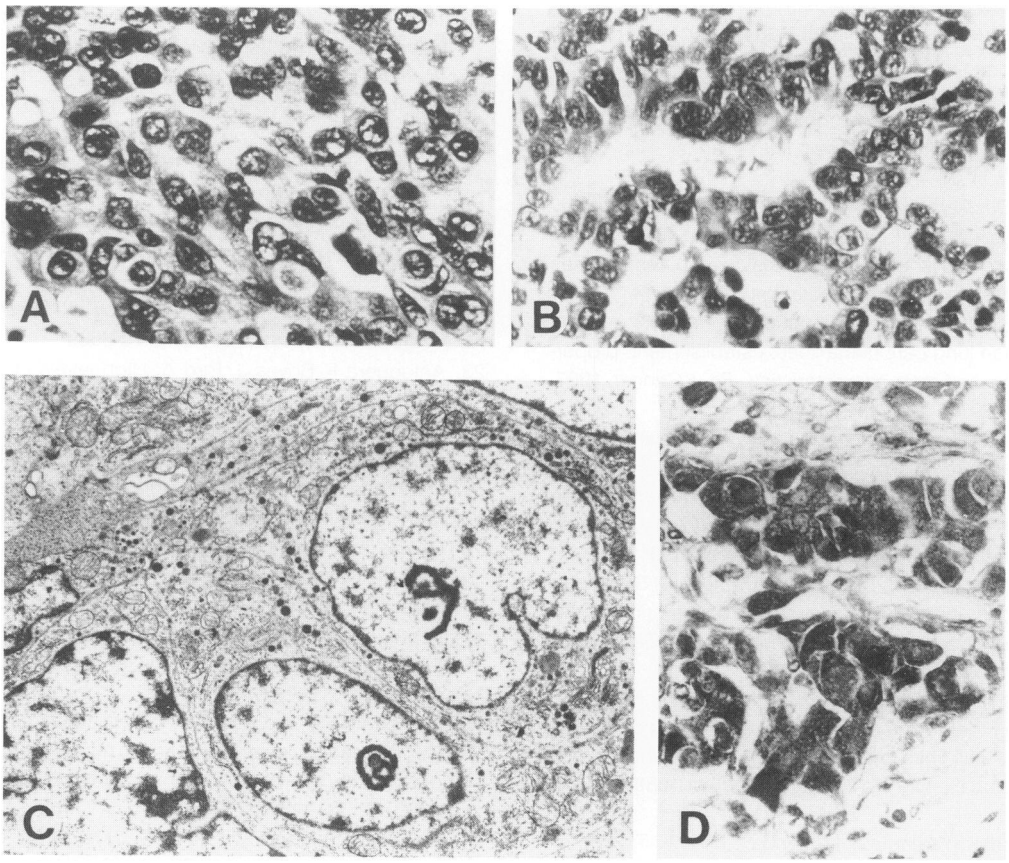

Figure 8. *Light and electron microscopic appearances of the MGH-14 tumor.* A: *Primary tumor;* B: *xenograft tumor in nude mouse;* C: *transmission electron micrograph of the primary tumor showing numerous dense-core endosecretory granules;* D: *strong positive immunohistochemical staining of tumor cells for chromogranin (*A, B, *and* D, *×400;* C, *×4000).*

cell lines are not entirely representative of all the neoplastic cells present in the primary tumor.

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