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Establishment of Cell Lines with High- and Low-metastatic Potential from PC-14 Human Lung Adenocarcinoma

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This article reports the establishment of variant cell lines with high and low metastatic potential by repeated selection and the dilution plating technique. Five clones with high metastatic potential, Lu-2, Lu-7, Lu-4, Lu-1 and Lu-5, and four clones with low metastatic potential, 3S, 7S, 8S and 13S, were established from PC-14 human lung adenocarcinoma. The high-metastatic cell lines produced enhanced lung metastases, but the low-metastatic cell lines did not produce lung metastasis by injection into the tail vein of 5-week-old BALB/c nude mice. The high-metastatic cell lines produced enhanced tumors on both visceral and parietal pleurae, and enhanced metastases to the mediastinum and contralateral pleural cavity. The low-metastatic cell lines produced reduced tumors on both visceral and parietal pleurae and reduced metastases to the mediastinum and contralateral pleural cavity after injection into the left preceral cavity of the nude mice. When the nine variant cell lines and original PC-14 cells were embedded in collagen gels, the PC-14 cells and the low-metastatic cell lines gave rise to colonies with a dendritic morphology, and cells were tightly associated. The high-metastatic cell lines were more loosely associated and scattered into three-dimensional colonies. These nine cloned cell lines originated from heterogeneous populations of the parental PC-14 cells should be useful tools for studying the process of metastasis of lung cancer.

Key words: Human lung adenocarcinoma PC-14 — Establishment of cell lines — Metastasis

Lung carcinoma is one of the most common human cancers and is a predominant cause of cancer-related death throughout the world.[1)] Despite advances in surgical techniques and the development of aggressive adjuvant therapies for treatment of primary neoplasms, many lung cancers are not curable due to metastases.[1, 2)] Cancerous tumors contain heterogeneous populations of cells with different biological properties that include invasion and metastasis.[2–4)] The process of metastasis consists of selection and sequential steps that include angiogenesis, detachment, motility, invasion of extracellular matrix, intravasation, circulation, adhesion, extravasation into the organ parenchyma and growth.[4)] The outcome of metastasis depends on the continuous interaction of metastatic cells with host factors, and the ability of tumor cells to produce metastasis is due to a unique set of properties. The multiple genes that regulate different steps in the lung metastatic process have been identified. Several oncogenes and tumor suppressor genes, such as *ras*, *myc*, *p53*, *RB* and allelic loss of chromosomes play very important roles.[5–8)] But, it is likely that other genes in addition to these genes are also involved in the process of metastasis of lung cancer. The identification of those unknown genes that relate to metastasis is important in order to understand the genetic and phenotypic characteristics of the metastatic lung cancer cells, and this requires a dependable model system. Namely, the cells should be stable with regard to their metastatic potential, and a number of clones with high and low metastatic potential from the same origin should be available. Variant cell lines with different metastatic potential from many human carcinoma, such as breast carcinoma,[9, 10)] colon carcinoma,[11–13)] pancreatic carcinoma,[14)] melanoma cell,[15–17)] bladder carcinoma[18)] and Lewis lung carcinoma[19–21)] have already been established. However, almost all of these cell lines are highly metastatic and there are not enough to compare the cellular and molecular basis of high- and low-metastatic characters. Therefore, we tried to establish variant cell lines with high and low metastatic potentials from PC-14 human lung adenocarcinoma. In this article we describe the establishment of five clones with high metastatic potential and four clones with low metastatic potential, and evaluate the metastatic and tumorigenic behavior and morphology of these cloned cell lines.

MATERIALS AND METHODS

Mice For all experiments, 5-week-old female athymic BALB/c nude mice were supplied by Charles River Laboratories (Tokyo). Sterile food and water were fed to the mice. The mice were maintained in sterile cages on sterile bedding and housed in rooms at constant temperature and humidity.

Tumor lines PC-14 human lung adenocarcinoma cells, derived from a previously untreated patient with pulmonary adenocarcinoma was kindly provided by Prof. Y. Hayato, Tokyo Medical College. PC-14 was used to estab-

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lish variant cell lines with high and low metastatic potential.

Conditions of culture PC-14 and variant cells with different metastatic potential from PC-14 cells were grown in RPMI 1640 medium (Nissei Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a highly humidified atmosphere of 5% CO_2 at 37°C. The cell lines were subcultured once or twice a week as necessitated by growth rate.

Selection of cell lines with high metastatic potential from PC-14 PC-14 cells were harvested by a brief incubation in 0.05% trypsin and 0.53 m*M* EDTA and washed by centrifugation and resuspended in serum-free RPMI 1640 medium. Cell number and viability were determined by staining a small volume of cell suspension with 0.2% trypan blue saline solution and counted with a culture counter. Cells were suspended in serum-free RPMI 1640 medium and 5×10^5 cells/0.2 ml were injected into the tail vein of the nude mice using 27 gauge needles as described by Fidler.[22] Mice were killed when signs of illness were noted. All experiment groups contained at least four mice. At autopsy, metastasized tissues were dissected from different organs (lungs and lymph nodes), minced finely, and plated on tissue culture dishes in culture medium. These procedures were repeated four times to select cell lines with high metastatic potential from PC-14.

Selection of cell lines with low metastatic potential from PC-14 Cells from parental PC-14 cells were cloned by a technique based on the dilution plating principle.[23] Single cell suspensions were seeded into the 96-well plates (Nippon Becton Dickinson Co., Ltd., Tokyo) tissue culture plate at 1 cell/well. Wells containing a single cell were identified under a light microscope (×100 magnification) and marked. Colonies were transferred after sufficient growth to vessels of increasing size to expand the cell population. The cloned subpopulations obtained 3 weeks after cloning were divided into multiple samples. The eighteen samples were injected into the tail vein of the mice at a density of 5×10^5 cells/0.2 ml. The mice were killed 98 days later and examined for the presence of lung metastases. All experimental groups contained at least four mice.

Experimental metastasis assay The metastatic potential of PC-14 cells and variant cell lines with high and low metastatic potential derived from the original PC-14 cells was evaluated by the quantitative lung colony assay, as described by Fidler *et al*.[24] Cells were injected into the tail vein of the mice at a density of 5×10^5 cells/0.2 ml (n=4 or 5). Mice were killed at 21–34 days and 21–35 days for the cell lines with high metastatic potential, and 130 days and 175 days for the cell lines with low metastatic potential after cell inoculation. The number of lung colonies was counted using a magnifying glass.

Growth and metastasis of cell lines injected into pleural cavity of nude mice Cells were injected into the left pleural cavity of the mice using a syringe with a 27 gauge needle. The needle was advanced approximately 5 mm through the chest wall into the cavity and cells were dispersed into the pleural cavity at a cell density of 2×10^6 cells/0.2 ml (n=5), based on the report by Wang *et al*.[25] Mice were killed when signs of illness were noted and malignant pleural effusion was observed.

Survival curve The length of survival of mice injected with PC-14 cells and cloned cell lines with different metastatic potential into the tail vein of the mice at a density of 5×10^5 cells/0.2 ml (n=5) and into the left pleural cavity of the nude mice at a density of 2×10^6 cells/0.2 ml (n=5) was evaluated with the death of the mice as the endpoint. When the last mouse injected with PC-14 into the tail vein died at 165 days, all remaining mice were killed and lung colonies were counted.

Tumorigenicity in nude mice and histopathological examination The tumorigenicity of PC-14 cells and cloned cell lines with different metastatic potential was examined by injection of 1×10^7 cells/0.2 ml into the subcutis of the mice (n=5). Tumor growth was monitored three times a week. Tumor volume (V) was calculated by using the formula $V=1/2\times\text{length}\times[\text{width}]^2$. For histopathological examination, when tumor volume exceeded 1 cm^3, tumors were dissected, fixed with 10% formalin, and stained with hematoxylin and eosin.[26]

Morphology The morphology of PC-14 cells and cloned cell lines with different metastatic potential was examined microscopically.

Collagen embedded culture and dispersion index Type I collagen stock solution (Cell Matrix Type IA, Nitta Gelatin, Osaka) was mixed with 5× concentrated RPMI 1640 and a reconstituting buffer (0.05 *N* NaOH solution containing 2.2% $NaHCO_3$ and 200 m*M* HEPES) to achieve 2 mg/ml final concentration of collagen, according to the manufacturer's instructions. Fetal bovine serum (FBS) was then added to this mixture to a final concentration of 10%, and 0.5 ml of this solution was plated on a 6 cm plate as the basal layer. After polymerization at 37°C for 30 min, 2 ml of the same collagen solution containing 5×10^3/ml was overlaid on the basal collagen layer, and again allowed to polymerize at 37°C for 30 min. Finally, 3 ml of RPMI 1640 supplemented with 10% FBS was added to the top of collagen gels, and the cells were cultured.[27] After 14 days, the number of dispersed colonies per total of 50 colonies was counted. For each cell line, four plates were used for the count and statistical analysis.[28] Statistical significance of differences was calculated by means of Student's t test.

Homozygous deletion High-molecular-weight DNA was obtained from PC-14 cells, cloned cell lines, and the human small cell lung carcinoma cell line N417 according to the previously described method.[29] Information on the

DNA markers *D9S162*, *p16/CDKN2A* and *D9S265* was obtained from the Genome Database (http://gdbwww.gdb.org/gdb/). The oligonucleotides used to detect the *D9S162* marker were 5′-GCAATGACCAGTTAAGGTTC and 5′-AATTCCCACAACAAATCTCC, and 5′-TGGTGAAGC-CTATTCTTGGT and 5′-CATTGGCAAAGTGTGCG were used for the *D9S265* marker. The oligonucleotide pairs used to detect the *p16/CDKN2A* gene were 5′-CTGC-GGAGAGGGGGAGAGCAG (sense) and 5′-TCCCCT-TTTTCCGGAGAATCG (antisense) for exon 1, and 5′-ACAAGCTTCCTTTCCGTCATGCCG (sense) and 5′-TGAGCTTTGGAAGCTCTCAG (antisense) for exon 2.[30] Homozygous deletions were detected using comparative multiplex polymerase chain reaction (PCR) assays.[31, 32] Each PCR assay was carried out in a 20 μl reaction mixture containing 50 ng of genomic DNA, 10 m*M* Tris HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 125 ng of each primer, 250 μM of each dNTP, and 0.1 unit of *Taq* DNA polymerase (TaKaRa, Tokyo). PCR conditions for the *D9S162* and *D9S265* markers were 30 cycles of 60 s at 94°C, 60 s at 55°C, and 90 s at 72°C. The *p16/CDKN2A* gene was amplified for 35 cycles of PCR at 94, 67 and 72°C for 50 s at each temperature in the presence of 5% dimethylsulfoxide (DMSO). PCR products were analyzed by electrophoresis on a 3% agarose gel.

Direct sequencing of PCR products The *p53* gene mutation was analyzed by direct sequencing of PCR products using 50 ng of genomic DNA obtained from either PC-14, cloned cells, or the human lung adenocarcinoma cell line A427. Genomic DNA was amplified by PCR using the *p53*-specific primer pairs, 5′-GCGCACTGGCCTCAT-CTTG (sense) and 5′-GCCAGTGTGCAGGGTGGC (antisense) for exon 7.[33, 34] The PCR conditions were 35 cycles at 95°C for 20 s, 58°C for 30 s, and 72°C for 30 s. The PCR products were purified using a QIA quick-spin PCR purification kit (QIAGEN, Tokyo) and directly sequenced in both directions with BigDye terminator cycle sequencing pre-mix kits (Amersham Pharmacia Biotech, Foster City, CA) and the ABI 310 DNA Sequence System (Perkin-Elmer, Tokyo).[33]

RESULTS

Isolation and selection of variant cell lines with high and low metastatic potential from PC-14 cells Five clonal sublines with high metastatic potential, Lu-2, Lu-7 and Lu-4, Lu-1 and Lu-5, and four clonal sublines with low metastatic potential, 3S, 7S, 8S and 13S, were obtained from the cloning of the parental cell population as described in "Materials and Methods."

Table I. Experimental Lung and Lymph Node Metastases of PC-14-derived High- and Low-metastatic Human Lung Adenocarcinoma Cell Lines

Experiment	Day of autopsy (range)	Cell line	Lung metastasis		No. of mice with lymph node metastasis/total mice
			No. of mice with lung metastasis/total mice	Median lung colonies/mouse (range)	
1	34 (21–34)	PC-14	3/4	11 (0–12)	0/4
	34 (31–34)	Lu-2	4/4	104 (68–149)	0/4
	34 (32–34)	Lu-7	5/5	152 (74–270)	0/5
	34	Lu-4	5/5	9 (3–20)	1/5
	34	Lu-1	4/4	18 (9–41)	0/4
	34	Lu-5	4/4	19 (3–20)	2/4
	130	3S	0/5		0/5
	130	7S	0/5		0/5
	130	8S	1/5		0/5
	130	13S	0/5		0/5
2	35 (31–35)	PC-14	4/5	2 (0–11)	0/5
	35 (32–35)	Lu-2	5/5	85 (36–187)	0/5
	35 (32–35)	Lu-7	5/5	188 (81–260)	0/5
	35 (34–35)	Lu-4	4/5	17 (0–38)	0/5
	35 (21–35)	Lu-1	4/5	4 (0–31)	0/5
	35	Lu-5	4/5	2 (0–26)	2/5
	175	3S	0/5		0/5
	175	7S	0/5		0/5
	175	8S	1/5		0/5
	175	13S	0/5		0/5

BALB/c nude mice were injected intravenously with 5×10^5 viable cells and killed at 21–130 days (Exp. 1) and 21–175 days (Exp. 2) after injection. The number of lung colonies of Lu-2 and Lu-7 were significantly different from that of parental PC-14 cells (Mann-Whitney *U* test, $P>0.01$).

Experimental metastatic ability of 9 cloned cells Collected results from representative individual experiments measuring the metastatic capacity of the nine cloned cells after injection of cells into the tail vein of the mice are presented in Table I. Lu-2 and Lu-7 had a higher lung colonization potential than that of the parental PC-14 cells. Lu-2 and Lu-7 caused metastases in all mice, with median numbers of lung metastases per mouse of 104 and 85, and 152 and 188, respectively (Table I, Exp. 1 and 2). Colonies produced by Lu-2 and Lu-7 became partly confluent on the surface of the lung (Fig. 1, B and C). Lu-4 developed large metastatic colonies in the lung in 9 of 10 mice (Table I, Exp. 1 and 2) and the sizes of a few colonies were more than 5 mm in diameter. The original PC-14 produced 0–12 lung colonies in 7 of 9 mice (Table I, Exp. 1 and 2). The sizes of the colonies produced by PC-14 cells were very small and most of the colonies were less than 1 mm in diameter (Fig. 1A). Both Lu-1 and Lu-5 produced 0–41 and 0–26 lung colonies in 8 of 9 mice (Table I, Exp. 1 and 2) and a few colonies were more than 1 mm in diameter. Mice given injections of 3S, 7S, 8S and 13S were sacrified at 130 days (Table I, Exp.1) and 175 days (Table I, Exp. 2) after the injection. None of these cell lines showed a marked ability to colonize the lungs and lymph nodes (Table I, Exp. 1 and 2), producing a few lung colonies in only 1 to 10 mice given an injection of 8S (Table I, Exp. 1 and 2).

Pathogenesis We sought to quantify both the pathogenic and metastatic potentials of the nine cloned PC-14-derived cell lines compared to the parental PC-14 cells. Therefore, either the tail vein or pleural cavity of mice was injected with each cell line. We found significant differences in the survival of mice injected with the cloned cells compared to those injected with PC-14 cells (Table II, Exp. 1 and 2). Mice that had either 3S or 13S cell lines injected into the tail vein had one death at 123 and 94 days post-injection, respectively, but the deaths were not due to lung metastasis. Furthermore, we could not detect any other metastatic colony. The two mice probably died of other diseases. Mice injected with PC-14 or the cloned cell lines in the pleural cavity had multiple tumors on both visceral and parietal pleurae. Metastases were observed within the mediastinum and contralateral pleural cavities (figure not shown). Strikingly, the increased or decreased incidence of metastatic lung colonies in mice injected with PC-14 or the cloned cell lines (Table I) was directly related to their survival times (Table II, Exp. 1 and 2).

Growth and tumorigenicity When PC-14 cells and the nine cloned cells were inoculated subcutaneously into the mice at a cell density of 1×10^7, PC-14 cells and the five high-metastatic cell lines were highly tumorigenic. The

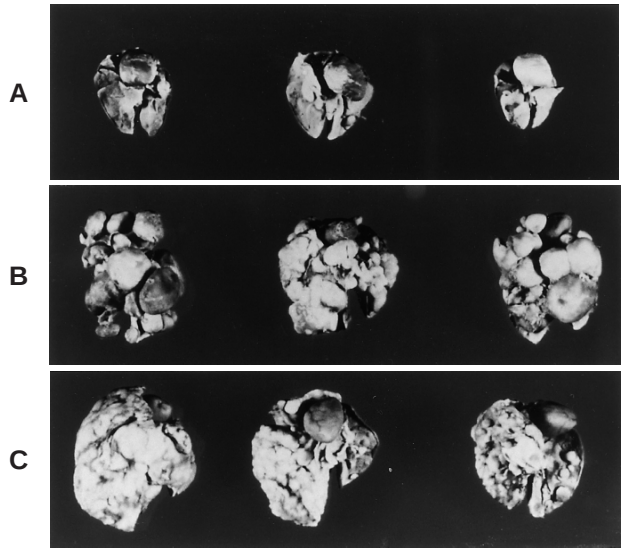

Fig. 1. Experimental lung metastasis of PC-14, Lu-2 and Lu-7. A, PC-14; B, Lu-2; C, Lu-7.

Table II. Length of Survival of Mice Injected with PC-14-derived High- and Low-metastatic Human Adenocarcinoma Cell Lines

Experiment	Cell line	Length of survival (Days)	Mean±SD
1	PC-14	80, 102, 123, 155, 165	125.0±31.81
	Lu-2	36, 37, 38, 38, 41	38.0±1.673[a)]
	Lu-7	25, 29, 36, 41, 53	36.8±9.806[a)]
	Lu-4	42, 51, 55, 60, 62	54.0±7.127[a)]
	Lu-1	36, 36, 45, 45, 48	42.0±5.020[a)]
	Lu-5	43, 55, 55, 57, 62	54.4±6.248[a)]
	3S	123, 165, 165, 165, 165	156.6±16.80
	7S	165, 165, 165, 165, 165	165.0±0.000
	8S	113, 165, 165, 165, 165	146.2±23.24
	13S	94, 165, 165, 165, 165	150.8±28.72
2	PC-14	28, 29, 29, 30, 31	29.4±1.020
	Lu-2	15, 16, 16, 17, 18	16.4±1.020[a)]
	Lu-7	17, 18, 18, 20, 21	18.8±1.470[a)]
	Lu-4	15, 16, 16, 17, 17	16.2±0.748[a)]
	Lu-1	19, 19, 22, 22, 25	21.4±2.244[a)]
	Lu-5	16, 17, 17, 18, 19	17.4±1.020[a)]
	3S	90, 95, 168, 168, 171	138.4±37.52[a)]
	7S	78, 84, 89, 89, 89	85.8±4.354[a)]
	8S	36, 46, 50, 59, 59	50.0±8.649[a)]
	13S	45, 48, 52, 54, 58	51.4±5.417[a)]

Exp. 1. Length of survival of mice injected with PC-14 and cloned cells in the tail vein.
Exp. 2. Length of survival of mice injected with PC-14 and cloned cells in the pleural cavity.
Statistical significance of differences was calculated by using Student's *t* test.
a) Different from PC-14 at $P<0.05$.

growth of the primary tumors produced by the five high-metastatic cell lines was faster than that of PC-14 cells (Fig. 2). The doubling times of tumor volume were 4.5 days for PC-14 cells, 4.0 days for Lu-2, 3.8 days for Lu-4, 4.0 days for Lu-7, 4.0 days for Lu-1 and 3.8 days for Lu-5. Subcutaneous tumors formed by the inoculation of PC-14 and the five high-metastatic cell lines did not produce lung metastasis or lymph node metastasis in any of the tumor-bearing mice. The four low-metastatic cells were not tumorigenic 100 days after inoculation into nude mice at a cell density of 1×10^7. The *in vitro* growth rate of the PC-14 cells and the nine cloned cells did not differ significantly (data not shown).

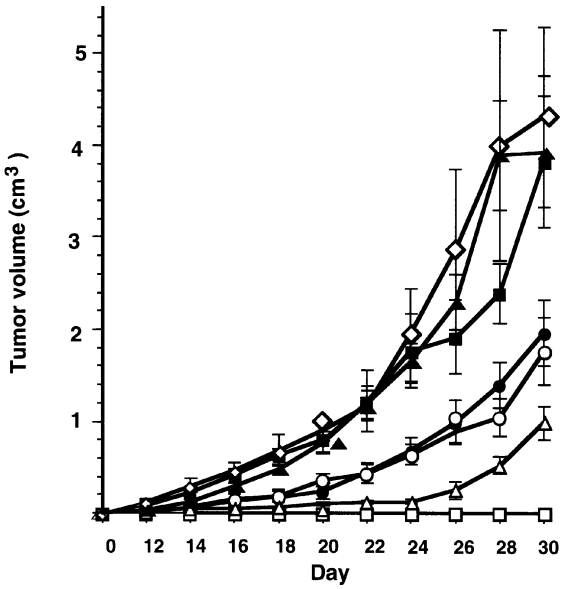

Fig. 2. Tumorigenicity of PC-14-derived high- and low-metastatic human lung adenocarcinoma. PC-14 (△), Lu-2 (■), Lu-7 (○), Lu-4 (▲), Lu-1 (●), Lu-5 (◇), 3S, 7S, 8S, 13S (□).

Morphology Cells were plated and allowed to grow to 60% confluency. Cell morphology was examined by phase-contrast microscopy. Morphology was obviously different among the five cell lines with high metastatic potential and the four cell lines with low metastatic potential. The five high-metastatic cell lines (Fig. 3, B–F) were round-shaped, formed clear multilayered growths, and were released into culture medium, while the four low-metastatic cell lines (Fig. 3, G–J) were spindle-shaped and showed little tendency to multilayered growth. The morphology of PC-14 (Fig. 3A) was heterogeneous.

Colony morphology in collagen gels Cloned cell lines and PC-14 were embedded in collagen gels. The morphology of colonies was observed and the number of dispersed colonies was counted after 14 days in culture. The morphology of colonies was different among the high-metastatic cell lines and the low-metastatic cell lines. The four low-metastatic cell lines gave rise to colonies with dendritic morphology and smooth surfaces, in which the cells were tightly associated with each other (Fig. 4, G–J). Cells rarely migrated out of the colonies. The five high-metastatic cells also formed dendritic colonies. In these colonies, however, the outlines of individual cells could be seen, suggesting that their association is loose. Moreover,

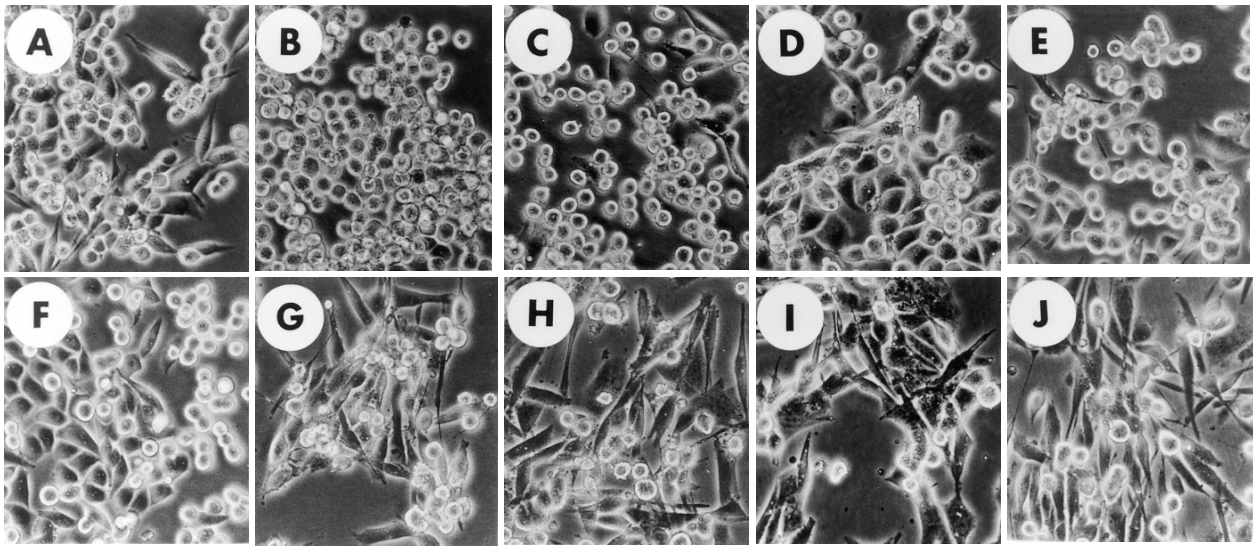

Fig. 3. Morphology of PC-14-derived high- and low-metastatic human lung adenocarcinoma. A, PC-14; B, Lu-2; C, Lu-7; D, Lu-4; E, Lu-1; F, Lu-5; G, 3S; H, 7S; I, 8S; J, 13S.

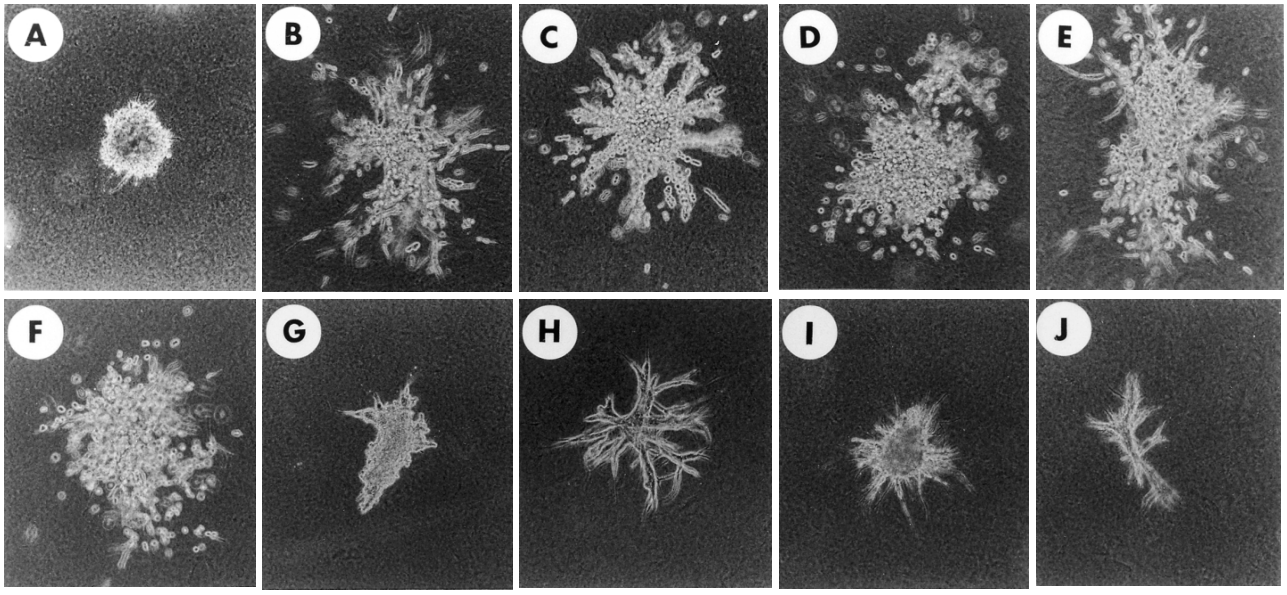

Fig. 4. Cell colonies in collagen gels. A, PC-14; B, Lu-2; C, Lu-7; D, Lu-4; E, Lu-1; F, Lu-5; G, 3S; H, 7S; I, 8S; J, 13S.

some cells were released from the main body of colonies and migrated into the matrix (Fig. 4, B–F). We examined the morphology of the colonies after 35 days in culture. Colonies formed by the low-metastatic cell lines had already died, whereas colonies formed by the high-metastatic cell lines grew actively, and cells released from the colonies formed new colonies. The five cell lines with high metastatic potential formed 20–35 clearly dispersed colonies per total of 50 colonies, and the four low-metastatic cell lines formed 5–15 weakly dispersed colonies (Table III).

Histology Histological examination of tumors formed in nude mice inoculated in the back with the PC-14-derived cell lines Lu-7, Lu-4 and Lu-1 all showed enhancement of angiogenesis comparable to that observed for PC-14 (Fig. 5, A–D). In addition, tumors formed by inoculation of the cell lines Lu-2 and Lu-5 developed blood vessels and were bloody in color (data not shown).

Genetic analysis of the 9p21 region and the *p53* gene Genomic DNA obtained from PC-14 and each of the nine cloned cell lines was subjected to PCR analysis of the homozygous deletions within the 9p21 region containing the *p16* gene. All cell lines had an identical homozygous 9p21 deletion. However, deletions were not detected by PCR within the 9p21 DNA markers *D9S162* and *D9S265* (Fig. 6). Finally, direct sequencing of PCR products showed that PC-14 and the two cloned cell lines, Lu-2 and 7S had the same arginine-to-tryptophan mutation (CGG→TGG) at amino acid position 248 in the *p53* gene (Fig. 7, B–D). The other seven cloned cell lines had the same point mutation in exon 7 of the *p53* gene (data not shown).

Table III. Dispersion of PC-14-derived High- and Low-metastatic Human Lung Adenocarcinoma in Collagen Gels

Cell line	Number of dispersive colonies / 50 colonies	Mean±SD
PC-14	7, 7, 6, 6	6.50±0.500
Lu-2	25, 20, 24, 24	23.3±1.92[a)]
Lu-7	30, 35, 31, 29	31.3±2.28[a)]
Lu-4	33, 35, 34, 35	34.3±0.829[a)]
Lu-1	32, 28, 31, 28	29.8±1.79[a)]
Lu-5	32, 29, 30, 32	30.8±1.30[a)]
3S	7, 7, 6, 5	6.25±0.830
7S	11, 11, 4, 8	8.50±2.87
8S	10, 15, 9, 15	12.3±2.77
13S	10, 13, 12, 11	11.5±1.12

Cells were cultured in collagen gels. After 14 days in culture, the number of invasive colonies per total of 50 colonies was counted. For each cell line, four plates were used for the count and statistical analysis.
Statistical significance of differences was calculated by using Student's *t* test.
a) Different from PC-14 at $P<0.05$.

DISCUSSION

Metastasis is a major factor that contributes to the high death rate of people with lung carcinoma. Metastasis is a

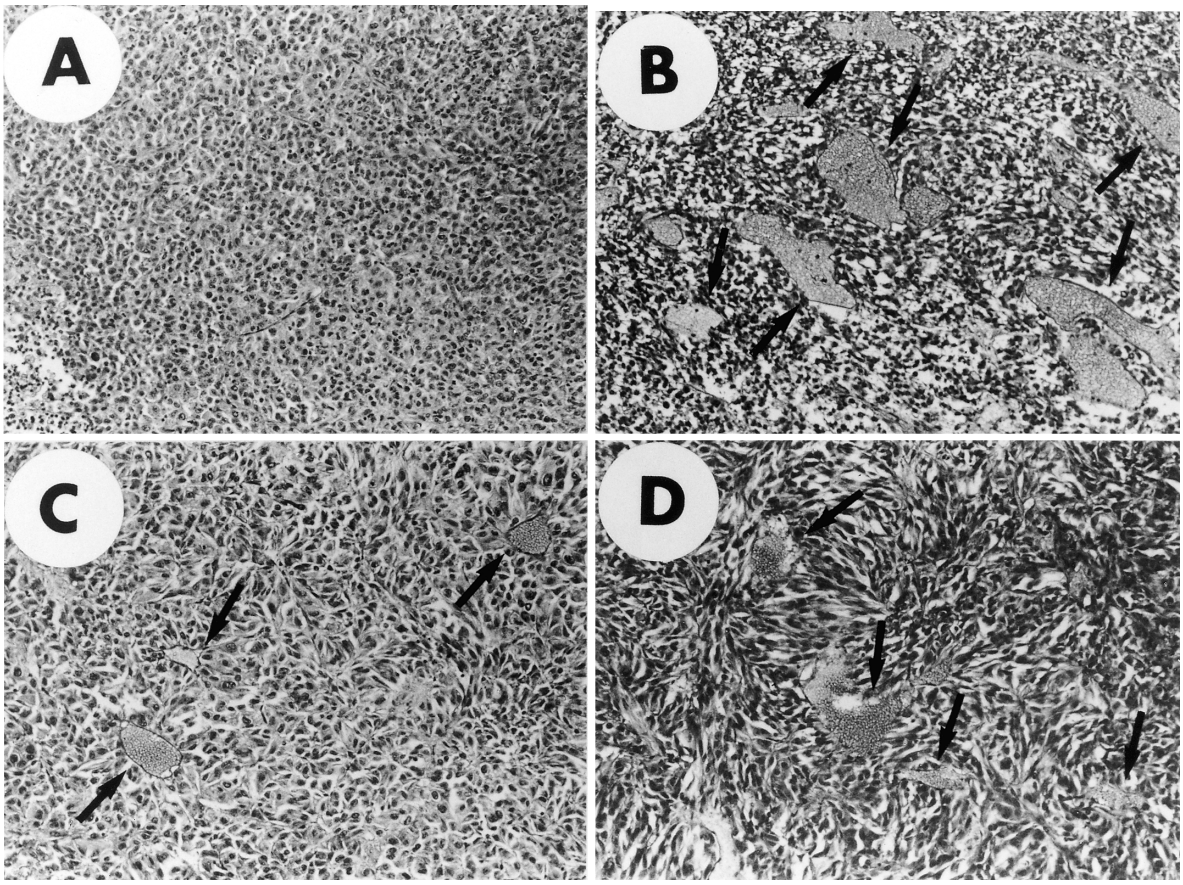

Fig. 5. Histology of tumor tissues. A, PC-14; B, Lu-7; C, Lu-4; D, Lu-1. Arrowheads show blood vessels.

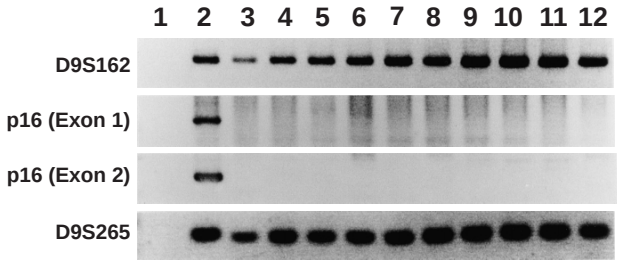

Fig. 6. PCR analysis of chromosome band 9p21 markers in PC-14 and cloned cell lines. Lane 1, minus template; lane 2, N417; lane 3, PC-14; lane 4, Lu-2; lane 5, Lu-7; lane 6, Lu-4; lane 7, Lu-1; lane 8, Lu-5; lane 9, 3S; lane 10, 7S; lane 11, 13S; lane 12, 8S. 9p markers are arranged from telomeric (*D9S162*) to centromeric (*D9S265*). N417 does not have a deletion of *p16* gene.

complex process that is dependent upon the fulfillment of a number of sequential steps. Many factors can influence the process of metastasis including detachment, motility, adhesion, invasion of the cellular matrix, extravasation into the organ parenchyma, angiogenesis, and tumor growth. In the present study, we isolated five cell lines with high metastatic potential by repeated selection, and four cell lines with low metastatic potential from the human lung adenocarcinoma cell line PC-14, using the dilution plating technique. The high-metastatic cell lines produced many rapidly growing metastatic colonies on the lung. In the highly metastatic cell lines Lu-5 and Lu-7, a distinct phenotypic difference was observed. Lu-7 produced more colonies on the lung than Lu-5, even though its growth rate was slower than that of Lu-5. This difference suggests that factors regulating motility and invasion were responsible for the high levels of metastases observed for Lu-7, while Lu-5's high metastatic potential was most likely due to its rapid growth rate.

The nine cloned cell lines were each cultured in collagen gels in order to assess their invasiveness. The high-metastatic cells formed dendric colonies that released cells from the main body, and scattering of these cells resulted in the formation of three-dimensional colonies. It was previously reported that the scattering phenotype of highly

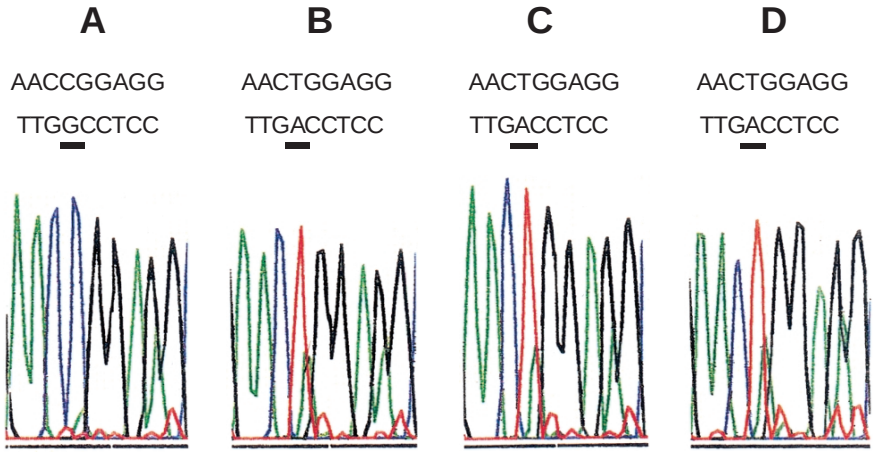

Fig. 7. Mutation of *p53* gene in PC-14 and the cloned cell lines. A, A427; B, PC-14; C, Lu-2; D, 7S. The mutated nucleotides are underlined. The *p53* gene of A427 is wild-type.

metastatic cell lines was due to increased migratory activity,[28] membrane-type matrix metalloproteinase (MMP) activity,[35, 36] and reduced levels of cell-adhesion molecules.[37] We attempted to identify factors that correlated with cell scattering activity. The cells were seeded on coverslips coated with colloidal gold particles and the migration of the seeded cells was measured at 12, 18 and 24 h post-plating. We observed that the four low-metastatic cell lines migrated faster than the five high-metastatic cell lines (data not shown). Thus, the migratory activity of these cell lines did not correlate with their scattering activity. We also assayed for secreted MMP activity by the nine cloned cell lines. Cells were cultured in serum-free RPMI for 72 h. Briefly, serum-free conditioned medium was applied to a gelatin-embedded polyacrylamide gel and stained using Coomassie blue. The MMP activity of the nine cell lines was very similar in these assays (data not shown). It has been demonstrated that an adequate blood supply is critical for tumor development and progression.[38–41] The initiation of angiogenesis within a metastatic tumor depends upon a critical balance between angiogenic activators and inhibitors that are up- or down-regulated at specific stages of tumor development. We observed that the tumors formed by inoculation of highly metastatic cells into the subcutis of nude mice developed blood vessels. This suggests that the metastatic potential of PC-14 is governed by, at least, an enhanced growth rate, and improved invasiveness and angiogenesis. A large number of genes must be involved in the metastasis of PC-14 cells. The nine cloned cell lines exhibited the same homozygous deletions in the 9p21 region containing the *p16* gene,[42–46] and the same arginine-to-tryptophan mutation (CGG→TGG) at amino acid position 248 in the *p53* gene of parental PC-14 cells.[34, 47] These results suggest that the nine cloned cell lines that originated from a heterogeneous population of PC-14 cells have unique characteristics. Thus, deletion within *p16* and mutation of the *p53* gene do not fully explain the metastatic ability of the five highly metastatic cell lines, suggesting that these cell lines may offer a means to identify novel factors that contribute to the mechanism of metastasis of human lung cancer. We had previously used mRNA differential display methods to detect genes associated with the metastatic potential of three high-metastatic cell lines[48, 49] and three low- or non-metastatic cell lines[50] derived from K-1735 mouse melanoma cells. The *elm*-1 gene[51, 52] was specifically expressed in the low-metastatic cell lines from K1735 and belongs to the CCN (connective tissue growth factor [CTGF], Cyr61/Cef10, neuroblastoma overexpressed gene [*Nov*]) family of proteins, which consist of secreted cysteine-rich proteins with growth-regulatory functions.[53–56] The metastatic potential of highly metastatic cells transfected with *elm*-1 was reduced in proportion to the level of *elm*-1 expression. Transfected cells that expressed the highest levels of *elm*-1 did not produce lung metastasis after injection into the tail vein of mice. We have isolated a novel actin-related gene, expressed in low-metastatic cell lines, 3S, 7S, 8S and 13S, but not expressed by the highly metastatic cell lines Lu-2, Lu-4, Lu-7, Lu-1 and Lu-5, by using mRNA differential display analysis.[57] An analysis of this gene and its possible role in metastasis is in progress.

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