Acta Medica Okayama

Volume 46, *Issue* 4 1992 *Article* 6

AUGUST 1992

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Abstract

A cell line of human lung large cell carcinoma (LCC) was established directly from the metastatic skin tumor tissue. The clinical course of the patient who carried this carcinoma was peculiar; generalized lymphadenopathy, histologically resembling Hodgkin's disease, was found as the first clinical symptom. The lung tumor was not discovered until the time of autopsy. This cell line (KaMi) grew adherent to culture vessels with the population doubling time of 20.6h, formed colonies in soft agars with efficiency of 22.6%, and formed tumors in athymic nude mice. The authenticity of KaMi was confirmed by chromosomal analysis and isoenzyme patterns. KaMi cells bore a strong resemblance to the original tumor cells which were composed of small spindle cells, large polygonal cells, and multinucleated giant cells. Immunohistochemically, KaMi cells showed a weak tendency to differentiate to squamous cells, and these immunohistochemical reactivities were almost compatible to those of the original tumor cells, but ultrastructurally, KaMi cells were more immature than the original ones. Treatment with several reagents could not augment a differentiation of KaMi cells. Cytokeratin profiles showed a tendency of squamous cell differentiation. KaMi cells may aid in elucidating the pathogenesis and biology of LCC and its relationship to other lung tumors.

KEYWORDS: Large cell lung carcinoma, cell line, cytokeratin

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*PMID: 1279943 [PubMed - indexed for MEDLINE]

Acta Med Okayama 46 (4) 257-264 (1992)

Establishment and Characterization of a Cell Line, KaMi, from Human Lung Large Cell Carcinoma

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A cell line of human lung large cell carcinoma (LCC) was established directly from the metastatic skin tumor tissue. The clinical course of the patient who carried this carcinoma was peculiar; generalized lymphadenopathy, histologically resembling Hodgkin's disease, was found as the first clinical symptom. The lung tumor was not discovered until the time of autopsy. This cell line (KaMi) grew adherent to culture vessels with the population doubling time of 20.6 h, formed colonies in soft agars with efficiency of 22.6%, and formed tumors in athymic nude mice. The authenticity of KaMi was confirmed by chromosomal analysis and isoenzyme patterns. KaMi cells bore a strong resemblance to the original tumor cells which were composed of small spindle cells, large polygonal cells, and multinucleated giant cells. Immunohistochemically, KaMi cells showed a weak tendency to differentiate to squamous cells, and these immunohistochemical reactivities were almost compatible to those of the original tumor cells, but ultrastructurally, KaMi cells were more immature than the original ones. Treatment with several reagents could not augment a differentiation of KaMi cells. Cytokeratin profiles showed a tendency of squamous cell differentiation. KaMi cells may aid in elucidating the pathogenesis and biology of LCC and its relationship to other lung tumors.

Key words : **large cell lung carcinoma, cell line, cytokeratin**

Human lung cancers are histologically classified into 4 major categories, that is, squamous cell carcinoma (SQC), adenocacinoma (ADC), small cell carcinoma (SCC), and large cell carcinoma (LCC) (1). LCC is defined as a cancer composed of large cells with abundant cytoplasm, variable shapes and stainability of nuclei, prominent nucleoli, and usually well-defined cell borders, but without glandular lumina and architectural characteristics of stratified squamous epithelium. However, the ultrastructural analysis of LCC often reveals some differentiation to SQC as shown by the presence of tonofilaments, or to ADC as shown by the presence of intracytoplasmic canaliculi. Some tumor cells produce even mucin. Therefore, at least some LCC could be merely an undifferentiated variant of the other types rather than an independent entity. The establishment of human lung cancer cell lines will greatly contribute to the elucidation of pathogenesis and biological characterization of human lung cancer which is

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one of the most common human malignant neoplasms. Many cell lines have already been established for each category, but most of them have been derived from SCC and ADC (2-5). Cell lines from LCC are still few (2, 6-8) and their value has yet to be established.

We observed a case of large cell carcinoma of the lung which showed very peculiar histological features mimicking Hodgkin's disease in the metastatic foci of lymph nodes, We were able to establish a continuous cell line, KaMi, from the metastatic skin lesion.

The present report deals with hitological characterization of the primary tumor and biological and immunohistochemical characterization of the KaMi cells.

Materials and Methods

Case. The patient, a 53-year-old man, was entered hospital in September, 1984, with a dry cough, general fatigue, fever and generalized lymphadenopathy. Biopsy of the cervical lymph nodes was done in October and disclosed the peculiar histological findings suggestive of Hodgkin's disease, mixed cellularity (Fig. 1). The patient was regarded as having advanced Hodgkin's disease and received C-MOPP consisting of cyclophosphamide, vincristine, procarbazine and predonisolone.

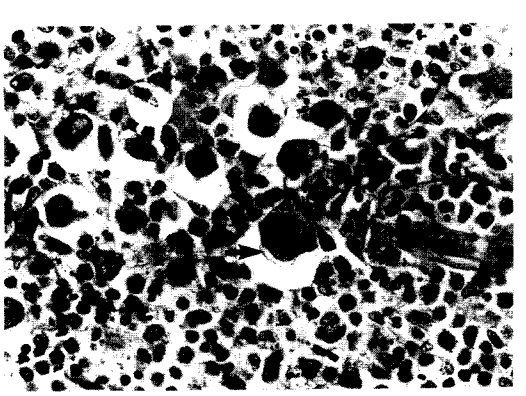

Fig. 1 Histological characteristics of the original tumor excised from the cervical lymph node. Several scattered atypical tumor cells are infiltrating, some of them look like Reed-Sternberg cell (arrow) (H & E: × 400).

He achieved a complete remission after 7 cycles of the therapy. In September, 1985, however, numerous subcutaneous nodules appeared throughout the body. Poorly differentiated squamous cell carcinoma was implied by the results of the biopsy of the subcutaneous tumors. Despite repeated chemotherapy, he died of intracranial hemorrhage. Autopsy revealed a bulky mass in the central portion near the left pulmonary hilus with wide spread metastases.

Culture. A metastatic nodule in the subcutaneous tissue was aseptically resected at the autopsy, minced with scissors, and treated with 0.25 % trypsin. The dispersed cells were cultured in RPMI1640 medium supplemented with 10 % fetal calf serum at 37°C in 5 % CO_2 plus air.

Immunoperoxidase staining. Tumor tissues and cell pellets of cultured cells were fixed in 10 % formalin, embedded in paraffin, and sectioned at 4 μm thickness. Cultured cells on cover slips were fixed in acetone for 5 min. After blocking endogenous peroxidase activity by treating with methanol containing 0.3 % H_2O_2 for 30 min, dewaxed sections and cultured cells on cover slips were stained by the peroxidase anti peroxidase (PAP) method (9) using rabbit polyclonal antibodies as the first antibody, or by the avidin-biotin complex (ABC) method (10) using monoclonal antibodies as the first antibody. Antibodies included monoclonal antibodies to squamous epithelium-specific and non-squamous epithelium-specific keratin (SE-and NSE-keratin) (Immunobiochemicals), vimentin and epithelial membrane antigen (EMA) (DAKO) and rabbit antibodies to cytokeratin, carcinoembrynoic antigen (CEA), secretory component and lysozyme (DAKO).

Electron microscopy. Cultured cells were scraped off with a rubber policeman and gently centrifuged. Cell pellets or tissues were fixed in 2 % glutaraldehyde, postifixed in 1 % OsO_4, and embedded in epoxy resin after dehydration in graded ethanol. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H-500 electron microscope.

Induction of differentiation. Sodium butyrate (2 mM), dexamethasone (50 μg/ml), dimethylsulfoxide (2 %), dibutyryl cAMP (1 mM), 5-azacitidine (5 μM), cytosine arabinoside (4×10^{-7}M), retinoic acid (10^{-6}M), TPA(10^{-9}M), or Ca^{2+} ionophore (A23187) (1 μg/ml) was added to the culture medium. Morphological changes and immunohistochemical stainability for keration, involcurin, secretory component, CEA, and vimentin were examined on the 1st, 2nd, 3rd and 4th days after the addition of these reagents.

Transplantaion. Ten million cells were transplanted subcutaneously into the back of three BALB/c nude mice.

Chromosome analysis. The cells which had been treated with colcemid (0.1 μg/ml) for 2 h were dispersed by trypsin digestion, treated with 0.9 % sodium citrate solution, and fixed in 1 : 3 mixture of absoulte methanol and glacial acetic acid. A small droplet of the fixed cell suspension was air-dried and analyzed for the banding pattern according to the standard Giemsa banding technique (11).

Colony formation in soft agar. The cells were dispersed using 0.05 % trypsin digestion. A single cell suspension containing 10^3 cells in culture medium was mixed with agar to give a final agar concentration of 0.3 % and plated on the bottom layer of 0.5 % agar in plastic Petri dishes (Corning 25010). The number of colonies was counted after 10 days.

Cytokeratin extraction. Cytokeratins were extracted from the cultured cells and human cutaneous and esophageal squamous epithelium as described by Nelson and Sun (12). Human skin and esophageal tissues were obtained at autopsy. Epidermis and esophageal mucosa were isolated mechanically by gentle scraping after incubation with 25 mM Tris-HCl solution (pH 7.4) containing a mixture of antipain (5 μg/ml), pepstain A (5 μg/ml), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Epidermis, esophageal squamous epithelium, and cultured cells scraped from culture dishes were homogenized by ultrasonication in the above solution. The water-insoluble fraction was dissolved by heating at 95 % for 10 min in 1 % SDS with 25 mM Tris-HCl (pH 7.4) and 10 mM DTT.

Western blot analysis. Solubilized proteins were electrophoresed on a 12.5 % polyacrylamide slab gel containing 0.1 % SDS as described by Laemmli (13). The separated proteins were electrophoretically transferred to nitrocellulose sheets. These blots were then stained with the mouse monoclonal anti-cytokeratin antibodies by ABC method. Anti-cytokeratin monoclonal antibodies included KL4 (Immunotech) which detects 50 -67 kd molecules and anti-type II cytokeratin (Amersham, RPN1164) which reacts with the majority of basic type II cytokeratins including cytokeratin 8 (14).

Isoenzyme determinations. The cells were typed for 5 polymorphic isoenzymes; glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LD), nucleoside phosphorylase (NP), malate dehydrogenase (MD), and phosphoglucomutase (PGM) by using the Corning Authenti Kit System (Corning).

Table 1 Summary of immunohistochemistry

Antigens	1st biopsy[a]	2nd biopsy[b]	KaMi	Transplanted tumor
Keratin	+*	+*	++	+
Keratin-SE	−	−	+	+*
Keratin-NSE	−	+	++	++
Involcin	−	−	−	−
CEA	−	−	+*	+*
Secretory component	−	−	−	−
EMA	++	+	+*	+*
Vimentin	+*	+*	++	+*
Lysozyme	−	−	−	−

*: only a part of cells were positive. *a*: Lymph node *b*: Subcutaneous tumor. Keratin: detected by polyclonal anti-keratin antibody. CEA: Carcinoembryonic antigen. EMA: Epithelial membrane antigen. Keratin-SE: Squamous epithelium-specific keratin. Keratin-NSE: Nonsquamous epithelium-specific keratin.

Results

Histopathology of the tumors. In the first biopsy material from the cervical lymph nodes, the normal architecture of lymph nodes was effaced by the diffuse, but sparsely scattered infiltration of large round or polygonal tumor cells with prominent nucleoli. Nuclei were vesicular and sometimes binuclear showing the occasional mirror image (Fig. 1). These histological features apparently mimicked a mixed cellularity type of Hodgkin's disease. The second biopsy specimen taken from the metastatic focus of the skin showed the diffuse proliferation of mainly spindle-shaped tumor cells. Some tumor cells were polygonal in shape and had clear cytoplasm. Many tumor cells were immunohistochemically positive for EMA, and a few were positive for vimentin and cytokeratin, but lysozyme and secretory component were never detected (Table 1). From these findings, poorly differentiated squamous cell carcinoma was suspected. The primary tumor of the left lung, taken in the autopsy, showed the histology of large cell carcinoma without mucin (Fig. 2A). Ultrastructural

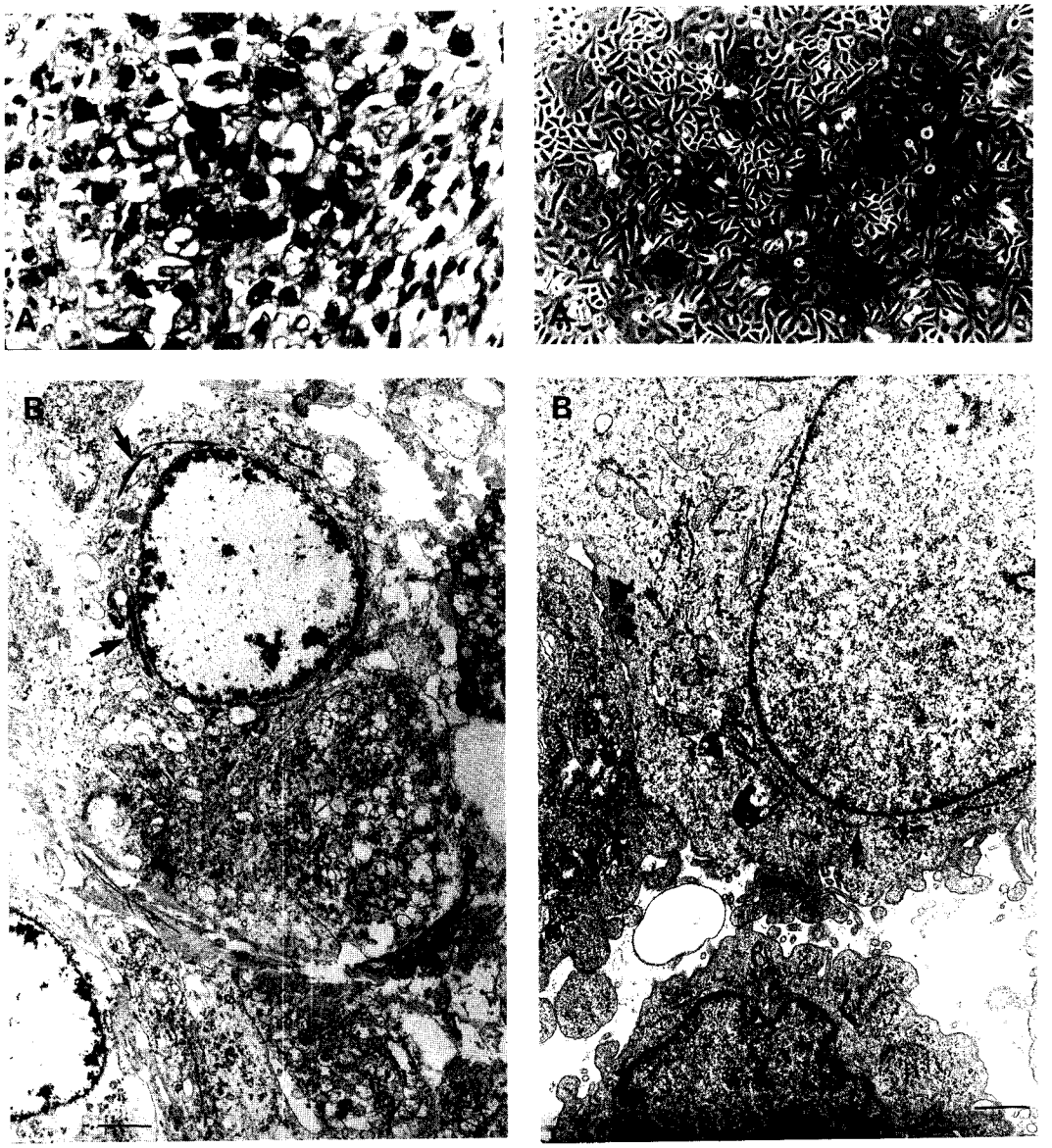

Fig. 2 (Left) Morphological characteristics of the original tumor at autopsy. A: Light microscopic level. The specimen was taken from the main tumor of the lung. Tumor cells were polygonal in shape and variable in cell size with no glandular structure and intercellular bridge (H & E: × 270). B: Ultrastructural level. The specimen was taken from the metastatic subcutaneous nodule. Arrows indicate tonofilaments in cytoplasma. Bar = 2 μm.

Fig. 3 (Right) Morphological characteristics of KaMi cells. A: Light microscopic level. Large polygonal cells and small spindle-shaped cells are seen (Phase contrast: × 70). B: Ultrastructural level. Arrow indicates a trace of tonofilament. Bar = 1 μm.

features in some tumor cells included a small number of tonofilaments in the cytoplasm (Fig. 3B). In summary, the tumor was diagnosed as large cell carcinoma at the level of conventional light microscopy, but showed a tendency to differentiate to squamous cell carcinoma at the electron microscopic and immunohistochemical levels.

Establishment and characteristics of KaMi. Tumor cells from metastatic skin lesions easily proliferated *in vitro* immediately after the cultivation and were serially subcultured, resulting in the establishment of a cell line, KaMi. KaMi cells were composed of large polygonal cells with abundant cytoplasm and small spindle-shaped cells, intermingled with a small number of multinucleated giant cells as seen in the primary tumor. They adhered to the plastic surface of culture dishes and formed a monolayer (Fig. 3A). KaMi cells had a doubling time of 20.6 h and cloning efficiency of 22.6 % in soft agar. Chromosome analysis revealed the pseudohypodiploid chromosome number with a modal number of 44 and marked aneuploidy. The isoenzyme phenotypes of KaMi cells were human for LD, G6PD, NP, and MD and distinct from those of HeLa cells as shown by the difference of migration pattern of PGM and the proportions of 5 isoenzymes of LD (data not shown). Ultrastructures of KaMi cells were more immature than the primary tumor cells. Cell union was weak, and desmosomes were hardly seen. Only a trace of tonofilaments were seen in a few cells (Fig. 3B). Immunohistochemically, strong positivity for NSE-keratin and vimentin and moderate to weak positivity for SE-keratin were demonstrated, but involcurin was negative (Table 1). CEA, secretory component, and EMA were very weakly positive for adherent cells, but negative for pelleted cells. Treatment with various reagents for induction of differentiation did not affect the immunohistochemical and morphological features of the cells.

Transplantation. All nude mice inoculated with 10^7 KaMi cells developed tumors reaching the size of pigeon egg about 3 months after the inoculation. The tumor was histologically and ultrastructurally similar to the primary tumor (Fig. 4A and 4B) and showed the intermediate immunohistochemical characters between the KaMi cells and the primary tumor cells (Table 1).

Western blotting analysis of cytokeratin. To

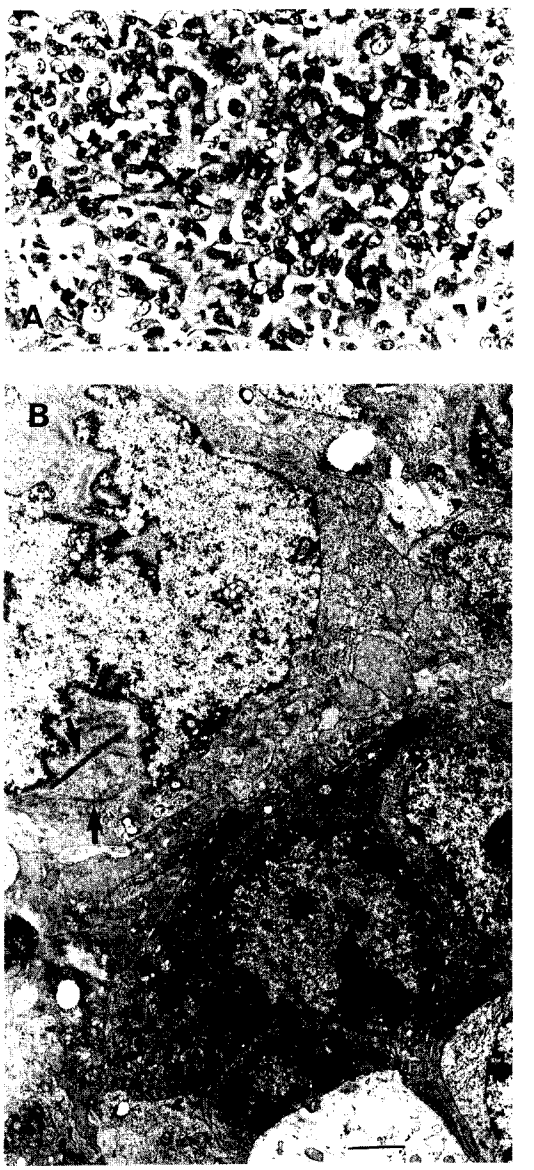

Fig. 4 Morphological characteristics of xenografts in nude mice. A: Light microscopic level. This histology resembles original lung large cell carcinoma (H & E: × 270). B: Ultrastructural level. Arrows indicate tonofilaments in cytoplasm. Bar = 1 μm.

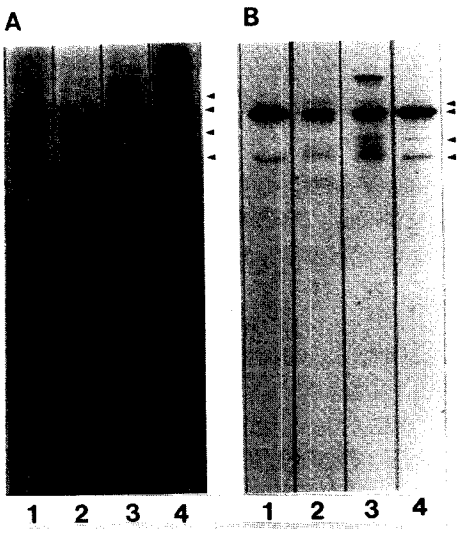

Fig. 5 Western blotting analysis of cytokeratins extracted from HeLa cells (lane 1), esophageal mucosa (lane 2), epidermis (lane 3) and KaMi cells (lane 4). A: Filter hybridized with KL4 monoclonal antibody. Triangles indicate 66kd, 62kd, 50kd and 48kd. B: Filter hybridized with RPN1164 monoclonal antibody. Triangles indicate 57kd, 53kd, 45kd, 43kd and 39kd.

determine more exactly characteristics of cytokeratins extracted from KaMi cells, Western blotting analysis was performed. Cytokeratins of KaMi cells that reacted with RPN1164 monoclonal antibody were identified in 5 bands corresponding to 57kd, 53kd, 45kd, 43kd and 39kd, among which the bands of 57kd, 45kd and 39kd cytokeratin polypeptides were fainter than the others. KL4 monoclonal antibody detected 4 bands corresponding to 66kd, 62kd, 50kd and 45kd (Fig. 5). Cytokeratin polypeptide pattern of KaMi cells was different from those of the epidermis, esophageal mucosa and HeLa cells. Pretreatment of KaMi cells with dexamethasone did not affect the pattern of cytokeratin polypeptides (data not shown).

Discussion

The histogeneses of 4 major hitological types of human lung cancers are still only partly clarified. It has been hypothesized that both SCC and non-SCC could be of common histogentic origin and may be derived from a common bronchial stem cell (15, 16). The histogenetic origin of LCC is particularly controversial. When ultrastructural and immunohistological studies are available, the majority of LCC show evidence of glandular or squamous differentiation (17-19). The possible relation to SCC has also been suggested by the observation *in vivo* (20) and *in vitro* (16) that disclosed the progressive transition of SCC cells to LCC morphology. The availability of continuous LCC lines may contribute to the histogenetic study of this tumor.

Histological features of biopsy specimens of metastatic lymph node apparently resembled those of Hodgkin's disease. However, immunohistological study revealed the presence of cytokeratin and EMA, and ultrastructural study of primary tumors of the lung demonstrated the presence of tonofilaments, disclosing the squamous cell character of the tumor. Involcurin, a soluble cytoplasmic precursor of the envelope protein present in human stratum corneum (21) was not expressed.

The authenticity of KaMi was established by the following criteria: KaMi cells were hypodiploid with only human chromosomes and showed human isoenzyme pattern different from that of HaLa cells. The immunohistochemical nature of KaMi cells was similar to that of the primary tumor.

KaMi cells were reactive with polyclonal anti-cytokeratin and monoclonal squamous or nonsquamous epithelium-specific antibodies. However, the reactivity of anti-cytokeratin antibodies to KaMi cells was stronger than to the tumor cells. The cultured cells were also strongly positive for vimentin. Vimentin, hewever, is often coexpressed in epithelia-derived cultured cells and has no significant histological implication

(22). The histological appearance of tumors which arose in nude mice after transplantation was also similar to that of the primary tumor.

According to Western blotting analysis, cytokeratin polypeptides extracted from KaMi cells mainly consisted of small-sized cytokeratins, but small amounts of intermediate- and large-sized cytokeratins, which had a relation to squamous cell differentiation of both normal and cancer cells (14, 23, 24), also existed. This result parallels the ultrastructural and immunohistological findings of KaMi cells. Large-sized cytokeratins, which were characteristic of the epidermis, were detected by KL4. However, the meaning of the presence of these high molecular cytokeratins remains unclear.

In summary, we have established the cell line from human LCC of the lung, which grew in a substrate adherent manner, formed tumors in nude mice, and showed a tendency to differentiate to squamous cells in ultrastructural level and cytokeratin profiles. This cell line may be useful in elucidating of pathogenesis and biology of LCC.

Acknowledgement. We thank Mr. Tadao Zohta, Miss Miyuki Shiotani, Miss Yuka Fujiwara and Miss Katsuko Urakami for technical assistance, and Mrs. Motoko Mitani for secretarial assistance.

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Received March 11, 1992; accepted March 25, 1992.