Establishment and Characterization of BALB/c Retroperitoneal Sarcoma with Spontaneous Liver Metastases

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The objective of this study was to examine the identity and characteristics of a spontaneously occurring murine retroperitoneal tumor of BALB/c mouse origin that selectively metastasized to the liver. From the primary tumor, a permanent cell line, termed LMFS (liver metastasis from sarcoma) was established *in vivo* and *in vitro*. After a subcutaneous injection of more than 1×10^5 cells in the side back of mice, the LMFS cells proliferated at the inoculation site (100% take) and induced metastatic nodules spontaneously in the liver, but not in the lung. By the limiting dilution technique, a cloned cell line, LMFS-1, was established *in vitro*. The LMFS-1 cell line had similar morphological characteristics to the LMFS cells both *in vitro* and *in vivo*. The doubling time of the LMFS-1 cell line was 10 h in passage 60. The number of chromosomes ranged from 71 to 108 and 93% of metaphases showed near-tetraploidy. In microscopic examination, no specific arrangement of the LMFS tumor cells was seen; the LMFS cell had medium- to large-sized atypical nuclei and clear and large cytoplasm. Electronmicroscopy showed that the cytoplasm of the LMFS cell had a moderate amount of rough-surfaced endoplasmic reticulum but no desmosomes or microvilli. Immunohistochemically, the LMFS cells were positive for vimentin, but showed no reaction for keratin or cytokeratin. Therefore, the LMFS tumor was considered to be an undifferentiated sarcoma. The LMFS cell line should be a useful tool not only for studies of metastasis, but also for experiments on the therapy of hepatic tumors.

Key words: Liver metastasis — Retroperitoneal sarcoma — LMFS cell line

One of the most urgent tasks in the management of malignant tumors is the prevention of metastasis. Metastasis occurs via a complex cascade of events, including detachment from the primary tumor site, penetration into the lymphatic and/or circulatory systems and invasion to form metastatic foci.[1–4] The phenomenon of organ-specific metastasis, namely, preferential metastasis to a specific secondary site, has been recognized for many years in human and experimental tumors.[5–7] The mechanisms of target organ selection by the tumor cells are, however, poorly understood. The liver is one of the organs most frequently involved in metastatic sites from primary tumors.[8, 9] Direct injection of tumor cells into the bloodstream has been used in animal model experiments on liver metastasis. In these studies, intraportal,[10] intrasplenic[11] and ileocolic[12] routes have been used for the injection. However, in an ideal model of site-specific metastasis, the primary tumor would spontaneously colonize to a specific secondary organ.

The present study describes the metastatic pattern of a spontaneously occurring murine retroperitoneal tumor of BALB/c origin. This tumor cell line selectively and reproducibly colonized the liver, irrespective of the route of administration.

MATERIALS AND METHODS

Animals Six- to eight-week-old female BALB/c mice were used throughout the experiments. They were purchased from Charles River Japan Co.

Establishment of tumor line A retroperitoneal tumor with liver metastases was found incidentally in a 2.3-year-old female BALB/c mouse. The tumor was minced with scissors and 2 or 3 cubes (1 mm³) were subcutaneously implanted into the side back of recipient mice. Subcutaneous implantation of the tumors was carried out 5 times in succession by using tumor cells that had metastasized to the liver. The tumor thus selected was named "liver metastasis from sarcoma (LMFS)."

The tumor cells from the liver were cultured in RPMI-1640 with 20% fetal calf serum after physical dispersion. After 37 *in vitro* passages, the cells were cloned by a limiting dilution technique. Colonies appeared within 7–10 days. Several cloned cell lines were obtained, but they

all showed similar characteristics. Therefore, a representative cloned cell line, named LMFS-1, was used in this study. The cells were expanded in a tissue culture flask and maintained *in vitro*.

Histology The tissues to be analyzed were fixed in 10% formalin phosphate buffer (pH 7.0). Five μm paraffin sections were prepared and stained with hematoxylin and eosin. For immunohistochemical observation, the LMFS cells were fixed in 4% formaldehyde and stained by the avidin-biotin-peroxidase method using Histofine SAB-PO Kits (Nichirei Corp.). The antibodies used were as follows: anti-vimentin monoclonal antibody (DAKO), anti-keratin polyclonal antibidy (DAKO) and anti-cyto-keratin monoclonal antibody (Boehringer).

Evaluation of tumor growth The subcutaneously implanted tumors were measured twice a week with calipers. The mean tumor diameter of individual tumors was calculated from measurements made at right angles. The mean tumor diameter in a group of mice was expressed as the mean of the individual tumor diameters.

Evaluation of metastasis The animals were inspected macroscopically and microscopically for metastasis after being killed under anesthesia. All the organs were routinely screened, although metastatic foci were preferentially detected in the liver.

In vivo **assay** Mice injected subcutaneously with LMFS cells (1×10^6 cells/mouse) were killed under anesthesia at day 7, 14 or 21. Immediately, the lungs and liver were removed and minced with scissors. About 50 mg of lung or liver tissues was subcutaneously implanted in the side back of recipient mice. Tumor growth at the injection site was observed for 10 weeks.

Karyotype analysis The LMFS-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. Colcemid was added for 2 h at the concentration of 0.01 μg/ml. Then, the cells were collected after simple trypsinization, treated with 0.5% KCl for 40 min at room temperature and fixed with a methanol:acetic acid mixture (3:1).

Chromosome preparations were made by the conventional air-drying technique. The slides were sequentially stained, and the chromosomes were analyzed first with G-banding and then with C-banding techniques. The G-banded metaphases were obtained by urea treatment as reported[13] with slight modifications. After destaining with methanol:acetic acid (3:1) and dehydration with methanol, the chromosomes were subjected to C-banding.

RESULTS

General characteristics of the tumor A retroperitoneal tumor mass located between the pancreas and the left kidney was found in a 2.3-year-old female BALB/c

mouse. The pancreas was displaced to the abdominal side but the size and shape was normal. Histology of the original tumor revealed that the solid tumor mass was located adjacent to the pancreas and there was no specific array of neoplastic cells (Fig. 1a). The mouse had multiple macroscopic tumor foci in the liver, which were considered to have metastasized from the tumor described above. In microscopic examination, the cells had medium- to large-sized atypical nuclei and clear and abundant cytoplasm (Fig. 1b, 1c).

The retroperitoneal tumor was transplanted to another BALB/c mouse. The tumor grew rapidly at the inoculation site. After the mouse had been killed, metastases to the liver were observed. Subcutaneous implantation of tumor cells from the metastatic site in the liver was repeated 5 times.

An electron micrograph of a tumor cell is shown in Fig. 2. Prominent nuclear indentation and heterochromatin were observed. The cytoplasm had a moderate amount of rough endoplasmic reticulum but no desmosomes or microvilli. Therefore, this tumor was considered to be undifferentiated sarcoma. No evidence of epithelial origin was found. Thus, the tumor was named LMFS.

At passage 15, the cells were subcutaneously injected into normal syngeneic mice at 3 different doses ranging from 1×10^4 to 1×10^6 cells. A tumor grew at the injection site in 4 of 6 mice receiving 1×10^4 cells. The mean survival time of these 4 mice was 48.8 ± 11.3 days and multiple hepatic metastases were observed macroscopically in all the mice. After an injection of more than 1×10^5 tumor cells, the LMFS cells formed tumor nodules at the inoculation site in 100% of the mice. The tumor became detectable (2 mm in diameter) 8 days after the inoculation and all mice had multiple metastatic foci in the liver, but not in the lung. A representative growth curve and survival rate data are presented in Fig. 3 and Table I, respectively. Mean survival time of mice injected with 1×10^5 tumor cells was 38.0 ± 4.7 days and that of mice injected with 1×10^6 cells was 31.8 ± 3.5 days.

Mice injected subcutaneously with LMFS cells (1×10^6 cells/mouse) were killed at weekly intervals and the presence of metastases was ascertained by gross and microscopic examinations. The results (Table II) indicate that hepatic metastasis could first be detected microscopically at day 8 in 2/2 mice (Fig. 4) and that macroscopical metastasis was detected at day 22 in 2/4 mice (Fig. 5). After a latent period of 5 weeks, micrometastasis were noted in spleen and ovaries in some mice, but not in the lungs, brain, lymphnodes and kidneys as evaluated by microscopic examination.

Five mice injected intravenously with LMFS cells (1×10^5 cells/mouse) were killed at day 30. All of the mice had multiple metastatic foci in the liver, but not in the
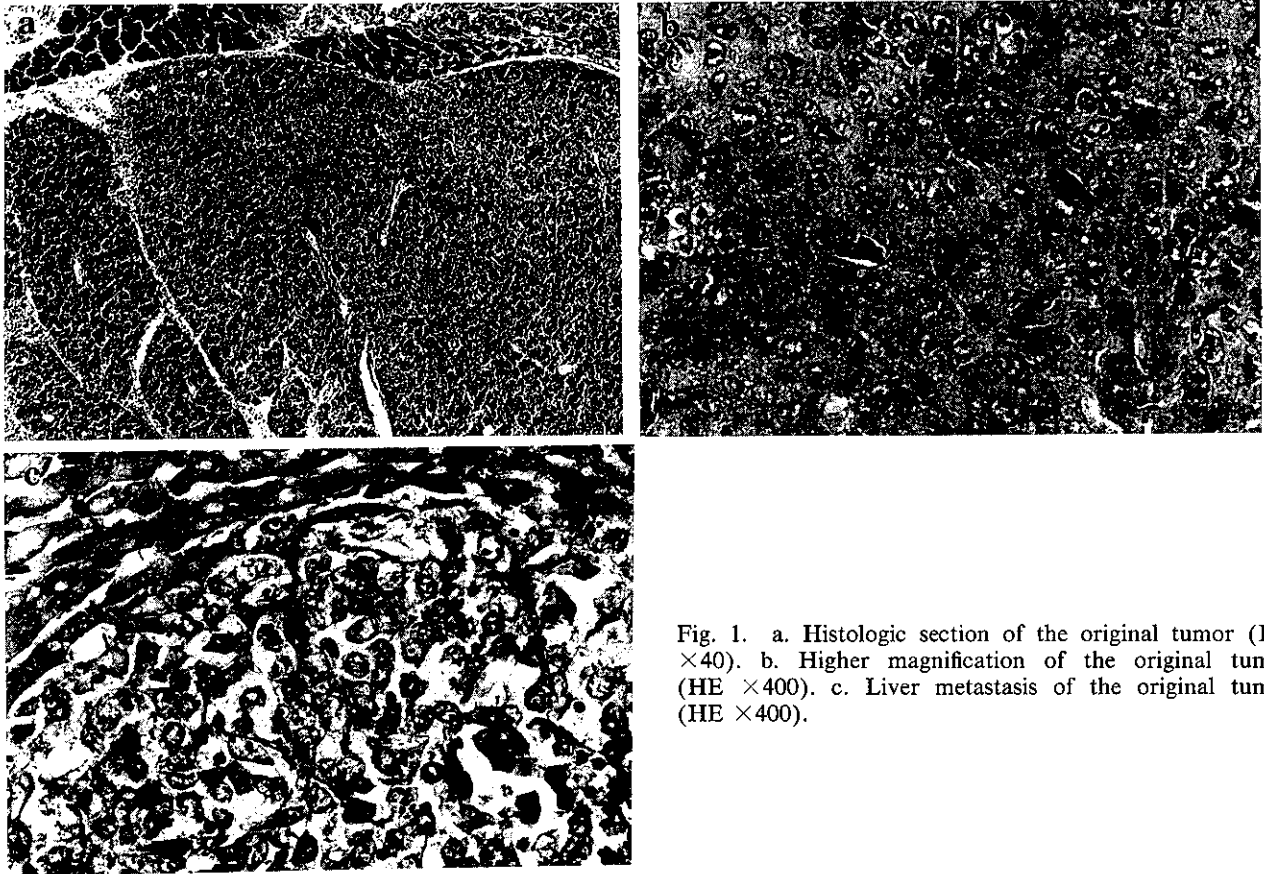
Fig. 1. a. Histologic section of the original tumor (HE ×40). b. Higher magnification of the original tumor (HE ×400). c. Liver metastasis of the original tumor (HE ×400).
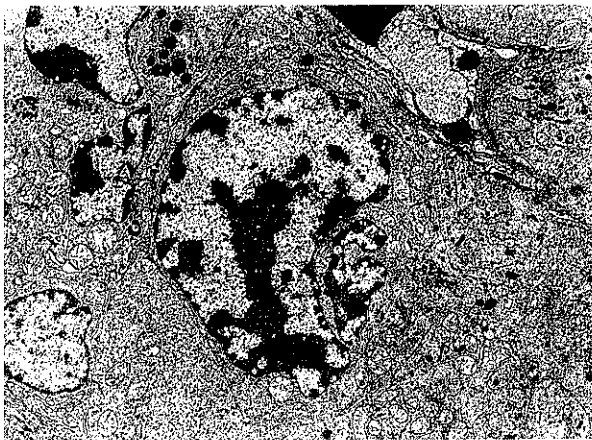

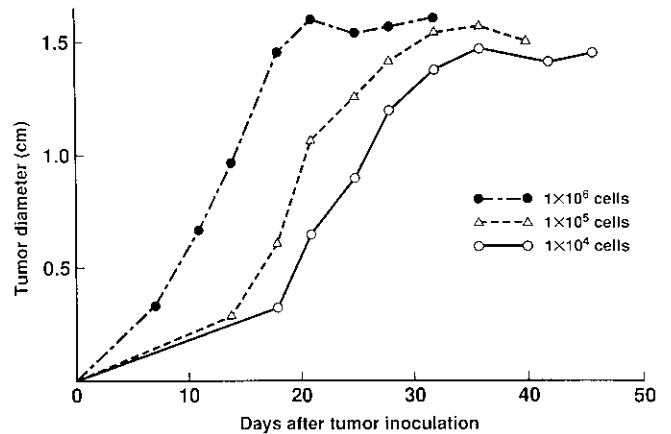Fig. 2. Electron micrograph of LMFS tumor cells (×8000).

Fig. 3. Tumor growth after subcutaneous inoculation of the LMFS cells.

lungs, brain or kidneys. In order to detect LMFS tumor cells in the lungs or the liver, *in vivo* assay was performed. Mice were injected subcutaneously into liver or lung tissues removed from LMFS tumor-bearing mice at day

7, 14 or 21 after transplantation. Neither the liver nor the lung tissues removed at day 7 formed a tumor at the injection site of the recipients. Four of 5 mice injected with lung tissues removed at day 14 and 2 of 5 injected with

Table I. Tumorigenicity and Mean Survival Time of Mice Inoculated Subcutaneously with LMFS Cells

Number of cells injected	Tumor take rate (%)	Mean survival time (days)	Hepatic metastases (%)
1×10^4	4/6 (67)	48.8±11.3	4/4 (100)
1×10^5	6/6 (100)	38.0±4.7	6/6 (100)
1×10^6	6/6 (100)	31.8±3.5	6/6 (100)

Table II. Relationship of Tumor Size to Metastasis

Days after tumor inoculation	Primary tumor site (mm)	Metastasis		
		Liver		Extrahepatic
		Macro	Micro	Micro
8	2.0	0/2	2/2	None
15	5.5	0/3	3/3	None
22	9.5	2/4	4/4	None
29	10.8	3/4	4/4	Spleen 1/4
36	13.0	4/4	4/4	Spleen 3/4 Ovary 1/4

Fig. 4. Liver metastasis of LMFS tumor cells 8 days after inoculation (HE ×250).

Fig. 5. Liver metastases after subcutaneous inoculation of LMFS tumor cells.

liver tissues formed tumor nodules at the injection sites. Therefore, the tumor uptake ratio in the lung tissues was 80% and that in the liver was 40%. Five of 5 mice injected with liver tissues and 5 of 5 injected with lung tissues removed at day 21 formed tumors (Table III).

Establishment of cloned cell lines Several cloned cell lines were obtained by a limiting dilution technique. All the clones were highly metastatic in nature and had similar morphological characteristics both *in vitro* and *in vivo*. A representative cell line was termed LMFS-1. The cells in monolayer culture showed a pavement arrangement and had a spindle-like shape. They had large nuclei and clear nucleoli (Fig. 6). The doubling time of the LMFS-1 cell line was about 10 h in passage 60 (data not shown). In immunohistochemical staining, the LMFS-1 cells were positive for vimentin, but showed no immunoreactivity for keratin or cytokeratin.

The LMFS-1 cells (1×10^6 cells/mouse) were subcutaneously injected into the side back of 10 mice. Tumors had grown at the inoculation site in all the mice at day 20, and at autopsy, multiple liver metastases of LMFS-1 were macroscopically observed. The LMFS-1 tumor at the inoculated site showed microscopically a much prominently cohesive arrangement (Fig. 7) than the LMFS tumors. The LMFS-1 cells had almost the same character as LMFS cells, but LMFS-1 grew more slowly than LMFS.

Karyotype analysis of a cloned cell line Sixty-eight metaphase spreads were analyzed for karyotype and chromosomal rearrangements. Three of 68 metaphases (4.4%) showed near-diploidy, with chromosome num-
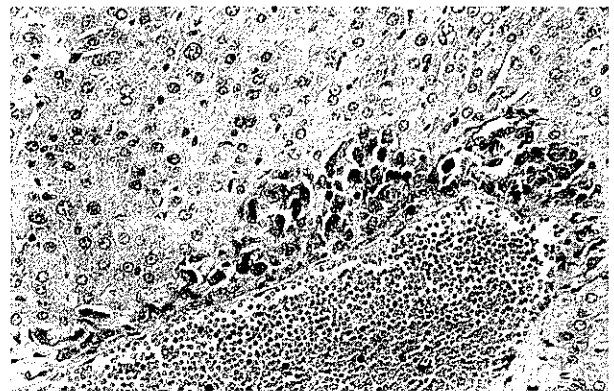
Table III. *In vivo* Bioassay

Days after tumor inoculation	Tumor take rate (%)	
	Lung	Liver
7	0/5 (0)	0/5 (0)
14	4/5 (80)	2/5 (40)
21	5/5 (100)	5/5 (100)

bers of 38, 41, and 44. Sixty-three metaphases (92.6%) showed near-tetraploidy, and the number of chromosomes ranged from 71 to 108. Two metaphases (2.9%) showed near-octaploidy, with 158 and 164 chromosomes.
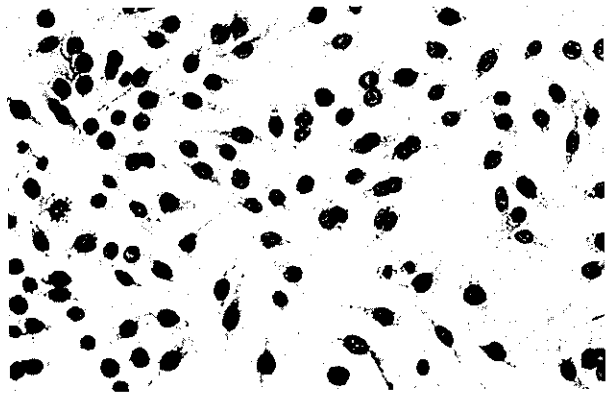
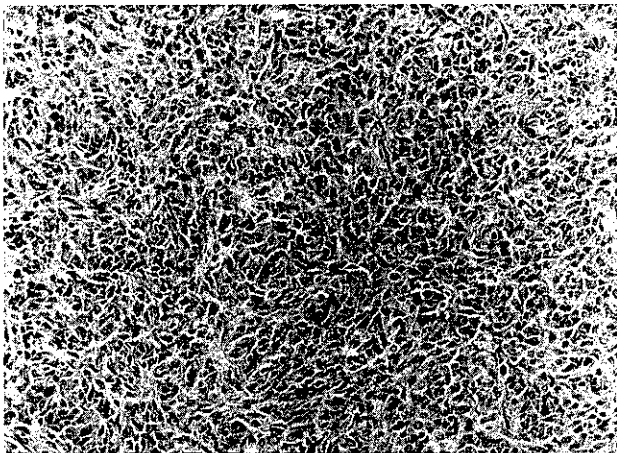Fig. 6. Phase-contrast photomicrograph of LMFS-1 (HE ×250).

Fig. 7. Histologic section of LMFS-1 tumor (HE ×100).

Fig. 8. G- and C-band karyotype of LMFS-1 cells. G-banded (a) and C-banded (b) chromosomes of the same metaphase spread from a near-tetraploid cell. Arrows indicate marker chromosome (m1 and m2) and satellite association between chromosome 11 and 17 (sa). (c) G-banded karyotype from another near-tetraploid cell with an excess chromosome 15. Three chromosome m1 detected from different cells are shown in the inset.

Twenty-one G- and C-banded metaphases were used for karyotype analysis according to Nesbitt and Francke.[14] One of the 21 metaphases (4.8%) was near-diploid, 19 (90.5%) were near-tetraploid (Fig. 8), and one (4.8%) was near-octaploid. Satellite association between chromosomes 11 and 17 was found at a significantly high rate (16/21 cells: 76.2%) (Fig. 8a and b), an excess number of chromosomes 11, 15, and 17 was observed frequently (23.8%, 42.9%, and 23.8%, respectively) (Fig. 8c), whereas near-tetraploidy was observed in another chromosome.

Two different types of marker chromosomes were found. Twenty of 21 cells (95.2%) possessed marker chromosome 1 (m1) with two C-positive bands at the proximal end and interstitial region. This chromosome had a weak secondary constriction at the proximal end (Fig. 8). Thirteen of 21 cells (61.9%) contained marker
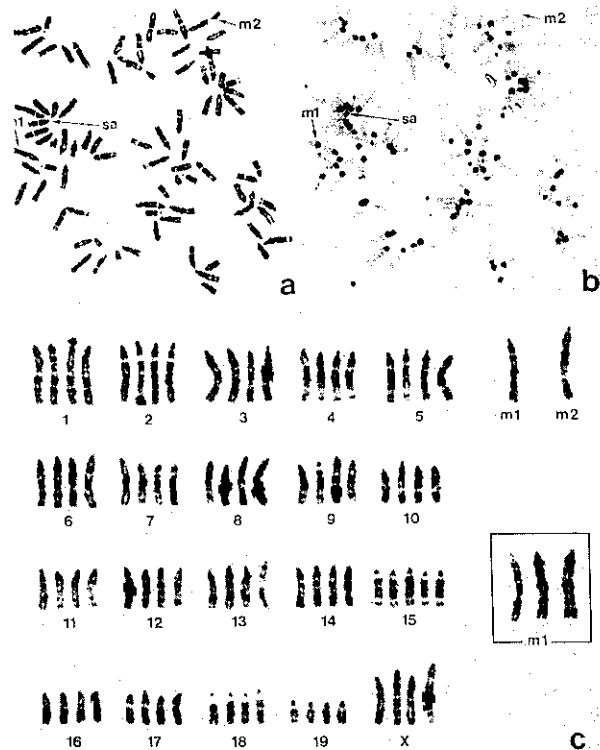
chromosome 2 (m2) (the largest chromosome in Fig. 8). The frequency of the cells bearing both m1 and m2 was 57.1% (12/21). No other type of chromosome rearrangements was found.

DISCUSSION

Liver metastasis is observed with high frequency in a variety of human cancers. As a first step in searching for a technique to treat liver metastasis, investigators have tried to find a method for the selective induction of experimental liver metastasis. Previous models of liver metastasis have involved intraportal,[10] intrasplenic[11] or intrahepatic implantation of cancer cells. In those cases, the mice required laparotomy, and investigators are required to have considerable technical expertise for the procedure to be successful; even then, not all animals develop tumors. Complications such as hemorrhage and tumor cell leakage are common.

The data presented in this paper characterize a spontaneously occurring murine retroperitoneal tumor. Immunohistochemically, the LMFS cells were positive for vimentin, but showed no reaction for keratin or cytokeratin. Both histological and electronmicroscopic studies suggested that the LMFS tumor is an undifferentiated sarcoma. The growth and progression of the LMFS tumor were observed to elucidate the natural history. Hepatic metastases were found when the original LMFS tumor was over 2.0 mm in size, 8 days after subcutaneous tumor inoculation. The pattern of hepatic micrometastases suggests that the LMFS tumor spreads via a hematological route rather than via a lymphatic route. The findings of tumor cells in the liver and the lung by *in vivo* assay and the growth of metastatic tumor mainly in the liver, in contrast to poor growth in the lung, support the interpretation that the environment at the site of tumor cell arrest, together with intrinsic characteristics of the tumor cells, influence metastatic development. Both cell adhesion molecules,[5, 15, 16] such as the cadherin, Ig, integrin, selectin and CD44 families, by which the tumor cells and organ interact with each other, and growth factors or cytokines[17-19] derived from tumor cells and/or host tissues may play an important role in organ-specific metastases. *In vivo* bioassay revealed that the LMFS cells had to traverse the lung capillaries before being trapped in the liver. These observations may support Paget's 'seed and soil' hypothesis.[20] The liver may serve a favorable microenvironment for LMFS tumor cell seeding.

In order to select a cell line with low metastatic ability the cells were cloned by the limiting dilution technique. Several cloned cell lines were obtained, but all had high metastatic abilities and displayed similar characteristics. We could not obtain a low-metastatic line.

Chromosome analysis showed that this cell line is characterized by the dominance of near-tetraploid cells with two types of marker chromosomes (m1 and m2). It is difficult to ascertain the origin of these marker chromosomes from the G- and C-banding patterns. However, chromosome m1 could be derived from an abnormal type of reciprocal translocation in which the region A–E of chromosome 5 attached to an acentric fragment of an unidentified chromosome and other acentric segments were eliminated in the subsequent cell division. Chromosome m2 could be derived from a reciprocal translocation between chromosome 3 with a breakpoint at region F and another unidentified choromosomal fragment. To identify the breakpoints and unknown chromosomal segments, high-resolution banding and fluorescence *in situ* hybridization are available. Because it is known that two genes related to oncogenesis, c-kit[21, 22] and N-myc,[23] and the liver-specific transcription factor gene (Hnf-1[24, 25]) are located around the terminal of region E and the proximal portion of region of F of choromosome 5,[26] which contains a breakpoint to generate m1, there may be a significant relationship between the presence of m1 at a very high rate (95.2%) and the nature of the original tumor. This could be clarified by *in situ* hybridization to metaphase choromosomes using appropriate probes.

In conclusion, the cell line we have established metastasizes spontaneously to the liver without metastasizing simultaneously to other organs. It should be a useful tool for studying many aspects of the pathogenesis and therapy of spontaneous liver metastases.

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