Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice

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Few experimental studies have been conducted to clarify the mechanism of development of metastasis in scirrhous carcinoma of the stomach. In the present study, we attempted to establish gastric carcinoma cell lines by incubation of cancer cells collected from the body fluids of patients with gastric cancer. At the same time, xenografting of these cells to nude mice was performed. It was found that, of the gastric carcinoma cell lines thus established, two cell lines, designated as HSC-44PE and HSC-58, formed s.c. tumors with a high infiltrative potential (often invading the lymphatics around the cancer tissue) when implanted. Metastasis to the lymph nodes and lungs was observed in 20–40% of all the animals, indicating that the two cell lines are also capable of metastasizing spontaneously. Through repeated selection, i.e., repeated cycles of removal, culture, and implantation of the HSC cancer cells from metastatic lesions, we obtained 5 subclones of HSC-44PE and HSC-58 (designated as m2509, m2615, m2792, m2917, and m2691), which, when implanted orthotopically, exhibited the following characteristics as compared to the parent cells: (1) a higher percentage take (survival), similar frequency of metastasis, shorter time to metastasis (less than 100 days), and consistent metastasizing potential; (2) a relatively high frequency of metastasis to lymph nodes, including distant metastasis to axillary lymph nodes; (3) the potential to cause occasional bloody ascites; (4) enhanced expression of dysadherin, CD44, and other molecules. This is the first report of cultured scirrhous gastric carcinoma cells showing the potential for spontaneous metastasis. (Cancer Sci 2004; 95: 575–582)

Although gastric carcinoma has recently shown a gradual decrease in prevalence, it still accounts for a significant proportion of cancer-related deaths in Japan. To improve the cure rate, more attention should be directed to early detection and prevention of metastasis of this cancer. Scirrhous carcinoma of the stomach, known as diffusely infiltrative carcinoma or Borrmann's type-IV carcinoma, or linitis plastica-type carcinoma, is characterized clinically as having the worst prognosis among the various types of gastric cancer, because it is frequently associated with metastases to lymph nodes and peritoneal dissemination. However, the mechanisms underlying this propensity for metastasis are not yet clearly understood. Therefore, establishment of relevant animal models of metastasis is considered to be extremely important for the elucidation of these mechanisms and establishment of appropriate therapeutic approaches. Transplants of human tumors into nude mice have been used increasingly as experimental systems for this purpose.[1)] Many human tumors can proliferate when injected s.c. into nude mice, but metastasis from the site of injection is rare.[2)] It has been found that in most models of human cancers, including gastric carcinoma, i.v. or intrasplenic injection, or orthotopic implantation of the tumor cells is necessary to generate metastasis.[3–6)] There are few reports of spontaneous metastasis from human gastric tumor xenografts in nude mice.[7, 8)] To date, only one experimental model of signet-ring cell or scirrhous carcinoma of the stomach has been reported.[9)] In order to address this problem, we previously established and characterized four cell lines from a primary gastric carcinoma and disseminated metastatic lesions of gastric scirrhous carcinoma.[10–12)] These cell lines did not exhibit the potential to form experimental or spontaneous metastases when injected s.c. or i.v., or implanted orthotopically into nude mice.

We now report the establishment and the biological characterization of new human signet-ring cell gastric carcinoma cell lines that exhibit the ability to metastasize spontaneously in nude mice.

Materials and Methods

Origin and establishment of the cell lines. The HSC-44PE cell line was established from the pleural fluid, obtained by thoracocentesis, of a 28-year-old female Japanese patient with scirrhous gastric carcinoma (linitis plastica-type). The previously reported HSC-45 cells,[12)] and the HSC-44PE cells reported in this paper, were derived from the same patient. The former was derived from the ascitic fluid in the early stage of scirrhous gastric carcinoma, while the latter was established by culture of tumor cells collected from the pleural fluid in the terminal stage of the cancer. The HSC-58 cell line was established from the ascitic fluid, obtained by peritoneocentesis, of a 57-year-old male Japanese patient with scirrhous gastric carcinoma. The HSC-60 cell line was established from the ascitic fluid, obtained by peritoneocentesis, of a 40-year-old male patient with scirrhous gastric carcinoma. Histopathologically, all of these primary tumors were diagnosed as signet-ring cell carcinoma with a scirrhous stromal reaction. The HSC-57 cell line was established from a 76-year-old male patient with well-differentiated tubular adenocarcinoma. The HSC-59 cell line was established from a 52-year-old female patient with poorly differentiated signet-ring cell adenocarcinoma. The HSC-64 cell line was established from a 41-year-old female Japanese patient with poorly differentiated adenocarcinoma. The sources of the other stomach cancer cell lines established before have been described previously.[10–12)] This study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all of the patients.

All the cell lines were established from malignant ascitic or

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Abbreviations: BSA, bovine serum albumin; CEA, carcinoembryonic antigen; CDM, chemically defined medium; FBS, fetal bovine serum; i.p., intraperitoneal(ly); s.c., subcutaneous(ly); i.v., intravenous(ly); TPA, tissue polypeptide antigen.

pleural fluid collected under aseptic conditions in heparinized syringes from patients with stomach cancer. The cells were harvested by centrifugation (700 rpm for 5 min) and seeded onto 10-cm dishes containing RPMI-1640 medium [Immuno-Biological Laboratories (IBL), Fujioka, Japan)] supplemented with 10% FBS (BioWhittaker, Walkersville, MD), 100 IU/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (IBL). The dishes were initially trypsinized (0.05% trypsin and 0.02% EDTA, IBL) to selectively remove overgrowing fibroblasts. In addition, we also attempted to remove the fibroblasts mechanically and transfer only the tumor cells. The cells were cultured on dishes at 37°C in a 5% CO_2/95% air atmosphere. Half of the medium was changed every 4–6 days. Dishes containing tumor cells were observed weekly under an inverted phase-contrast microscope. The cultures were first split after 3 to 7 months of cultivation, and the cells were passaged thereafter at a 1:10 or 1:20 ratio. They were judged, established, and designated (Table 1). All of the cell lines were routinely tested for *Mycoplasma* using a DNA fluorochrome staining kit (Flow Laboratories, McLean, VA), and no contamination was detected.

Animal experiments. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation, and the experiments were conducted in accordance with the Guideline for Animal Experiments of the National Cancer Center. Mice were purchased from CLEA Japan, Inc. (Tokyo), and maintained under specific-pathogen-free conditions. The mice were housed in filter-protected cages and reared on sterile water. The ambient light was controlled to provide regular 12-h light:12-h darkness cycles.

Tumorigenicity and metastasis assay. The tumorigenicity and spontaneous metastatic potential of the cell lines were tested by s.c. injection of $0.5–1\times10^7$ cells suspended in 0.2 ml of RPMI-1640 medium into 6- or 8-week-old BALB/c nude mice. Tumor growth was measured weekly, in terms of the tumor diameter, with calipers. The mice were sacrificed at appropriate intervals, or when moribund, and the tissues were examined macroscopically for metastasis in various organs and then processed for histological examination, as described.[10] Histological typing of the gastric carcinoma was conducted in accordance with the Japanese Classification of Gastric Carcinoma (1999), as sig (signet-ring cell carcinoma), sci (tumor with scirrhous stromal reaction), por (poorly differentiated adenocarcinoma), por 2 (non-solid type carcinoma), tub (tubular adenocarcinoma), tub 1 (well differentiated adenocarcinoma), or tub 2 (moderately differentiated adenocarcinoma).

The experimental metastatic phenotype was determined as described previously.[13] To determine the potential for peritoneal dissemination, viable cells were injected i.p. into nude mice. Four weeks later, the mice were sacrificed and peritoneal dissemination was evaluated from the number of tumor nodules in the mesentery.

Orthotopic implantation. Six-week-old female BALB/c nude mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol (Aldrich Chemical Co., Inc., Milwaukee, WI) at the dose of 0.28 mg/g body weight. Then, a small median abdominal incision was made in the anesthetized mice, and 2×10^6 cells in a 0.05 ml volume of RPMI medium were inoculated into the middle wall of the greater curvature of the glandular portion of the stomach using a 30-gauge needle (Nipro Co., Tokyo). The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed with an Autoclip Applier (Becton Dickinson, Sparks, MD). The mice were sacrificed at 150 days after the tumor cell inoculation or when moribund, and peritoneal dissemination was evaluated by counting the number of tumor nodules in the mesentery. The body organs were examined for metastasis and various tissues were processed for histological examination.

Immunohistochemical analysis. Mouse antibodies against human E-cadherin and β-catenin (Transduction Laboratories, Lexington, KY), anti-c-erbB-2 (550842), anti-CD44 (550392: both from BD Biosciences, San Diego, CA), anti-EGF receptor (E3138: Sigma-Aldrich, St. Louis, MO), anti-nm23 protein (NLC-nm23: Novocastra Laboratories, Newcastle, UK), anti-FGF-2/basic FGF (05-118: Upstate Cell Signaling Solutions, Lake Placid, NY), anti-human CD104 (integrin β4: CBL545: Chemicon International, Temecula, CA), anti-human IL-6 (MAB206: R&D Systems, Minneapolis, MN), anti-human c-met (18321) and anti-VEGF (18413: IBL) rabbit polyclonal antibodies, anti-human CD49f (integrin α6: MAB1378: Chemicon International) rat monoclonal antibody, CRIPT (sc-8573: Santa Cruz Biotechnology, Santa Cruz, CA) goat polyclonal antibody, anti-human IL-8 (ab7747: Abcam, Cambrige, UK) rabbit polyclonal antibody, and anti-human HGF (AF-294-NA: R&D Systems) goat monoclonal antibody were used in this study. Immunohistochemical staining was carried out as described previously.[14] Staining was repeated to check for possible technical errors, but the results were consistent. Scores for the expression of various genes were assigned semiquantitatively according to the percentage of the cells stained and the staining intensity.

Results

Establishment and characterization of gastric cancer cell lines. Six cell lines (HSC-44PE, HSC-57, HSC-58, HSC-59, HSC-60, and HSC-64) were newly established for the present study, from human gastric carcinomas. The HSC-44PE, HSC-58, and

Table 1. Biological characteristics of newly established gastric cancer cell lines

Cell line	Primary culture period (d)	Origin		Tumor[1] source	Histological typing[2]		Growth[3]			DT[4] (h)	Tumor markers[5]		
		Sex	Age		Original	Xenograft	Pattern	in CDM	in Agar		CA19-9 (units/ml)	CEA (ng/ml)	TPA (units/liter)
HSC-44PE	97	F	28	P	sig (sci)	sig	A/F	+	+	24	2570	43	>2000
HSC-58	121	M	57	As	por 2/sig (sci)	por/sig	A/F	+	+	23	40	51	>2000
HSC-60	197	M	40	As	sig (sci)	sig	A/F	–	+	26	2030	453	>2000
HSC-57	153	M	76	As	tub 1/sig	tub 1	A	–	+	28	8320	4000	>2000
HSC-59	89	F	52	As	por 2/sig	por/sig	A/F	+	+	22	1050	109	>2000
HSC-64	73	F	41	As	por	—	F	–	–	30	5106	1867	>2000

1) As, ascitic tumor; P, pleural effusion.
2) Histological typing of the gastric carcinoma as described in "Materials and Methods."
3) A, adherent type; F, floating type; A/F, mixed type; CDM, composed of DMEM/Ham's F-12 (1:1) medium supplemented with 0.05% BSA. +, positive; –, negative. Colony formation in semisolid agar was assayed as described previously.[15]
4) The doubling time of each line was determined as described previously.[13]
5) Secretion of CA19-9, CEA, and TPA was tested by radioimmunoassay and immunoradioassay at Otsuka Assay Laboratories (Tokushima, Japan).

HSC-60 cell lines were derived from gastric scirrhous carcinoma (Table 1). All the cell lines were strictly anchorage-independent (70 to 90% efficiency), and formed multi-layered sheets with clusters upon confluence (Fig. 1, A–C and D). The cytoplasm of many cells showed positive PAS staining, but the Alcian-blue staining reaction was negative. The morphology of the cells was similar to that of the signet-ring cell carcinoma cell lines described previously.[10–12] The HSC-57 line exhibited typical morphologic features of epithelial cells, characterized by sheets of polygonal cells in a pavement-like arrangement (Fig. 1E). The HSC-64 cells were floating and tended to aggregate (Fig. 1F). The HSC-44PE, HSC-58, and HSC-59 cell lines were able to grow in CDM in the absence of any polypeptide growth factor.[11] Almost all of the cell lines were found to secrete CA19-9, the concentration of which in the culture supernatants varied from 40 to 5106 units/ml. HSC-57 and HSC-64 cells secreted large amounts of CEA, which was found to be present at abnormally high concentrations in the patients' sera. Production of TPA was also detected from all of the cell lines. The doubling times of the cell lines varied approximately from 22–30 h in RPMI-1640 medium supplemented with 10% FBS.

Establishment of the cell lines and detection of their potential for spontaneous metastasis. Simultaneously with plating of cancer cells, a proportion of the cells was xenografted s.c. into nude mice. Implantation of primary culture cells, within one month of the start of culture, was also performed. As a result, tumor

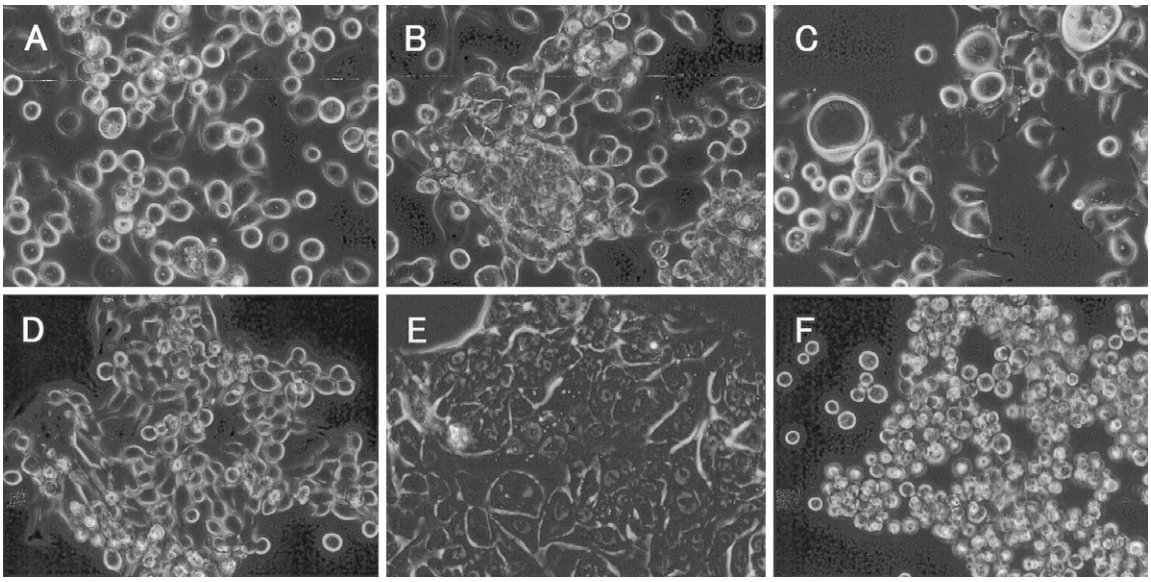

Fig. 1. Phase-contrast micrographs of 6 established HSC cell lines at the 25–30th passage. (A) HSC-44PE, (B) HSC-58, (C) HSC-60, (D) HSC-59, (E) HSC-57, (F) HSC-64. Original magnification, ×200.

Table 2. Tumorigenicity and spontaneous metastasis of original ascitic tumor cells, primary cultured cells and established cell lines in nude mice

Cell line	Source of cells	Number of cells injected	Route of injection	Incidence[1] of tumor formation	Incidences[2] of metastasis	
					Lymph node	Lung
HSC-44PE	Primary[3]	1×10^7	flank	4/5	0/4	1/4 (1)[4]
	HSC-44PE (10)[5]	5×10^6	flank	5/5	0/5	1/5 (2)
	HSC-44PE (46)	5×10^6	back	5/5	1/5	2/5 (1–3)
HSC-58	Ascitic tumor[6]	1×10^7	flank	3/5	1/3	1/3 (1)
	Primary	1×10^7	back	3/5	1/3	0/3
	HSC-58 (16)	5×10^6	flank	5/5	1/5	0/5
	HSC-58 (48)	5×10^6	back	6/6	2/6	1/6 (2)
HSC-60	Ascitic tumor	1×10^7	back	2/5	0/2	0/2
	Primary	1×10^7	flank	4/6	0/4	0/4
	HSC-60 (16)	5×10^6	back	4/4	0/4	0/4
HSC-57	Ascitic tumor	1×10^7	back	1/4	0/1	0/1
	HSC-57 (18)	5×10^6	flank	5/5	0/5	0/5
HSC-59	Ascitic tumor	1×10^7	back	2/4	0/2	0/2
	HSC-60 (23)	5×10^6	flank	5/5	0/5	0/5

1) Number of mice with tumors/number of mice given injection. Autopsy of mice bearing tumors was performed 180 days after injection.
2) Number of mice with metastasis/number of mice with tumors.
3) Primary cultured cells within 30 days.
4) Number of metastatic nodules.
5) Established cell lines derived from different cell culture passages were injected s.c.
6) Ascitic tumor cells were collected and cells were then inoculated s.c. into recipients previously injected with asialoGM1 antibody.

metastasis to the lymph nodes or lungs was observed in the mice implanted with cancer cells from the ascites of patient (HSC-58) or with primary culture cells of HSC-44PE (Table 2). The percent take was high for both the cell lines (3/5 and 4/5, respectively). However, it took a long period of time (over 3 months) for a 10-mm-diameter tumor to be formed in both cases. When the animals were autopsied 180 days after implantation, the number of macroscopically detectable lung metastases was small (1 to 3), but micrometastases were detected histopathologically at high frequency. In these metastatic lesions, not only actively dividing cancer cells, but also a small number of apoptotic cells were detected. Thus, we confirmed that even cells passaged for many generations *in vitro* possessed the potential for spontaneous metastasis (Table 2); indeed, no significant change in the frequency or extent of metastasis was observed as compared to the parent cells. In the mice s.c. implanted with one of the other established cell lines (HSC-60, HSC-57, or HSC-59 ascitic tumor cells), while tumor formation was seen, no metastasis was observed. On the other hand, HSC-64 did not even show tumorigenicity, either following implantation of the ascitic tumor cells into nude and SCID mice, or following repeated implantation of the established culture cells (Table 1).

Evaluation of the metastasizing potential of cultured cells established from human gastric carcinomas. The two cell lines found to possess spontaneous metastasizing potential in this study, and previously established human gastric carcinoma cell lines,[10–12] were implanted into nude mice and these mice were examined for tumorigenicity, spontaneous metastasis, experimental metastasis, and peritoneal dissemination (Table 3).

When injected s.c., all of the cultured cell lines established from human gastric carcinomas, except for HSC-64, survived and showed tumorigenicity. On the other hand, metastasis was observed only following implantation of HSC-44PE and HSC-58 cells. These two cell lines were found to be capable of spontaneously metastasizing to lymph nodes and lungs, even though the incidence of such metastasis was low (20–40%) (Fig. 2, A, B, and C). Histopathologically, the tumor tissue formed by cells such as HSC-39 was covered with a thick fibrous capsule, whereas the capsules of the tumors formed by HSC-44PE and HSC-58 cells were very thin and poorly demarcated, suggesting that the latter possessed a high infiltrative potential. Tumor invasion of the surrounding lymphatics was also seen in the case of the tumors formed by the latter two cell lines (Fig. 2D), confirming their high infiltrative potential. These features were not observed in the case of the s.c. tumors formed by the other cell lines tested.

When the potential for experimental metastasis was examined following injection of tumor cells via the caudal vein, it was found that HSC-44PE cells tended to metastasize to the lungs, while HSC-58 cells tended to metastasize to lymph nodes. HSC-44PE and HSC-45 cells showed a tendency to metastasize to the axillary lymph nodes.

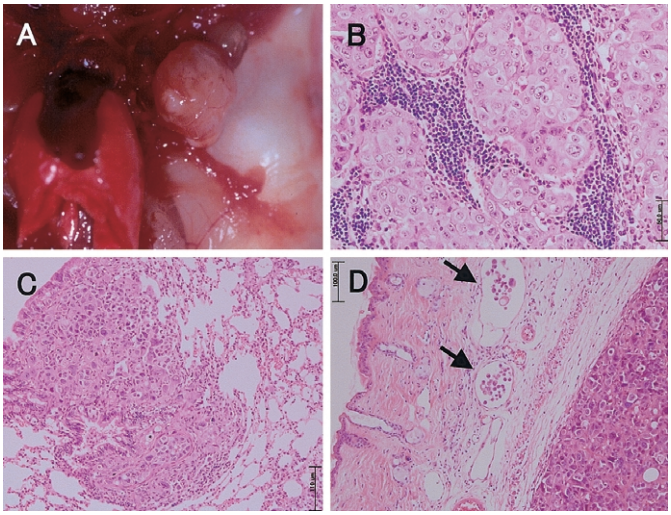

Fig. 2. (A) Macroscopic view of lymphatic metastasis of HSC-44PE cells after s.c. injection. (B) Histology of lymph node metastasis developing after s.c. injection of HSC-58 cells into the back of nude mice. H&E stain. (C) Histology of lung metastasis developing after s.c. injection of HSC-44PE cells into the back of nude mice. H&E stain. (D) Histology of tumor developing after s.c. injection of HSC-44PE cells into a nude mouse. Proliferation of signet-ring cells can be seen among actively growing tumor cells. Arrows show infiltration of lymphatics by the tumor cells. H&E stain.

Table 3. Activity for metastasis and peritoneal dissemination of various gastric cancer cell lines in nude mice

Cell line	Tumor[1] formation	Metastases				Peritoneal[3] dissemination
		Spontaneous		Experimental[2]		
		Lung	Lymph node	Lung	Lymph node	
HSC-44PE	5/5	2/5 (+)	1/5 (+)	3/5 (+)	0/5	3/5 (+)
HSC-58	5/5	0/5	2/5 (+)	1/5 (+)	2/5 (+)	5/5 (+)
HSC-60	5/5	0/5	0/5	0/5	0/5	0/5
HSC-57	5/5	0/5	0/5	0/5	0/5	0/5
HSC-59	5/5	0/5	0/5	0/5	0/5	0/5
HSC-64	0/5 (–)	0/5	0/5	0/5	0/5	0/5
HSC-39	5/5	0/5	0/5	0/5	0/5	4/5 (+)
HSC-40A	5/5	0/5	0/5	0/5	0/5	3/5 (+)
HSC-41	5/5	0/5	0/5	0/5	0/5	0/5
HSC-42	5/5	0/5	0/5	0/5	0/5	0/5
HSC-43	5/5	0/5	0/5	0/5	0/5	1/5 (+)
HSC-45	5/5	0/5	0/5	0/5	1/5 (+)	2/5 (+)
SH101-P4	5/5	0/5	0/5	0/5	0/5	2/5 (+)

1) Viable cultured cells (5×10^6/0.2 ml) were injected s.c. into 6-week-old BALB/c nude mice. Number of mice with tumors or metastatic nodules/number of mice given injection. Autopsy of mice bearing tumors was performed 180 days after injection.
2) Viable cells (1×10^6/1.0 ml) were injected into the lateral tail vein of nude mice. Six weeks later, mice were killed, the lungs were removed, and the number of metastases was counted under a dissecting microscope.[16]
3) Viable cells (1×10^7/1.0 ml) were injected i.p. into nude mice. Four weeks later, the mice were sacrificed and peritoneal dissemination was evaluated from the number of tumor nodules in the mesentery.

When the onset of peritoneal dissemination following direct inoculation of cancer cells into the peritoneal cavity was examined, dissemination of cancer cells to the mesentery, peritoneum, etc., was observed in all the animals inoculated with HSC-58 cells, and in a large proportion of the animals inoculated with HSC-44PE cells (Table 3). Peritoneal dissemination was also seen following inoculation of other cell lines derived from scirrhous gastric carcinomas (HSC-39, -40A, -43, and -45), but the incidence of such metastasis differed among the different cell lines.

Attempts to isolate highly metastatic cell lines and the results of s.c. or orthotopic implantation of these cells. Following the above-

Table 4. Metastasis and peritoneal dissemination after s.c. or orthotopic implantation of two human gastric cancer cell lines and their sublines

Cell line	Route of injection	Tumor formation	Ascites	Lymph node	Lung	Liver	Disseminated metastasis			
							Omentum	Mesenterium	Parietal peritoneum	Diaphragm
HSC-44PE	flank	10/10	0/10	3/10	4/10	0/10	0/10	0/10	0/10	0/10
m2509	flank	10/10	0/10	4/10	3/10	0/10	0/10	1/10	0/10	0/10
m2615	back	9/9	0/9	3/9	3/9	0/9	0/9	0/9	0/9	0/9
HSC-58	flank	10/10	0/10	4/10	2/10	0/10	0/10	0/10	0/10	0/10
m2792	flank	9/9	0/9	3/9	3/9	0/9	0/9	1/9	0/9	0/9
m2917	back	10/10	0/10	4/10	2/10	0/10	0/10	0/10	0/10	0/10
m2691	back	10/10	0/10	5/10	4/10	0/10	0/10	1/10	0/10	0/10
HSC-44PE	stomach	8/12 (60%)	0/8	2/8	0/8	1/8	2/8	2/8	0/8	0/8
m2509	stomach	9/10	1/9	2/9	0/9	1/9	3/9	2/9	2/9	0/9
m2615	stomach	10/10	1/10	4/10	0/10	3/10	4/10	3/10	3/10	1/10
HSC-58	stomach	10/12 (83%)	0/10	3/10	0/10	1/10	2/10	2/10	2/10	0/10
m2792	stomach	10/10	2/10	4/10	1/10	1/10	4/10	3/10	3/10	2/10
m2917	stomach	9/10	1/9	3/9	0/9	2/9	6/9	3/9	2/9	2/9
m2691	stomach	10/10	3/10	6/10	1/10	3/10	6/10	5/10	4/10	3/10

Mice were sacrificed at 150 days after inoculation. Data are shown as the number of mice bearing metastasis at the site/total number of mice bearing tumor.

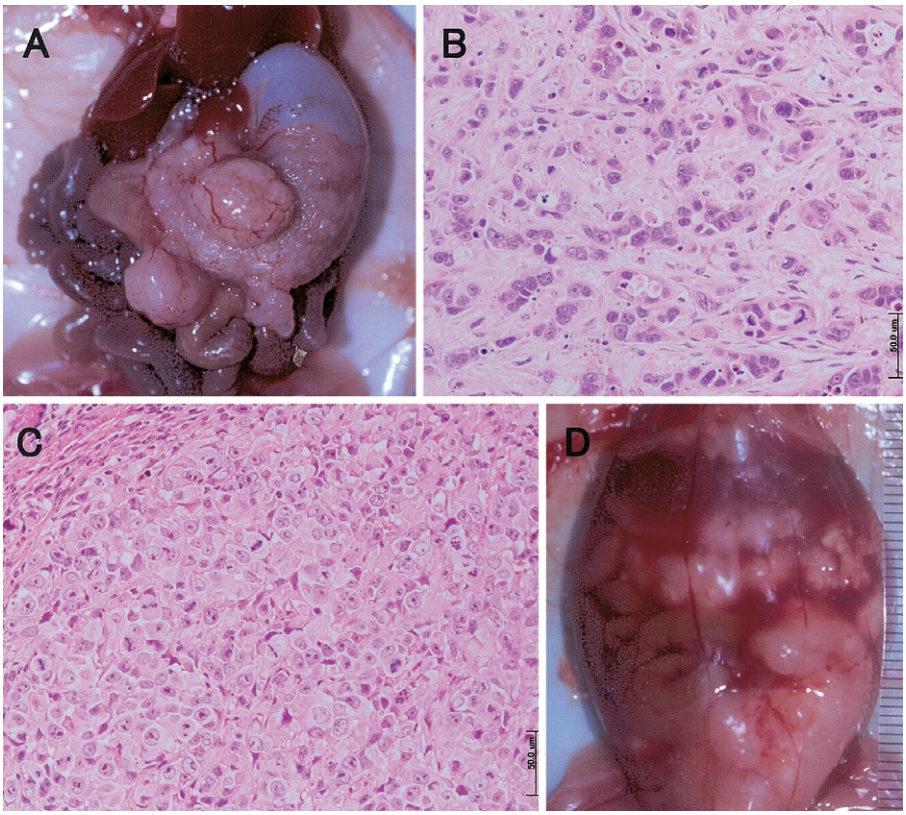

Fig. 3. (A) Macroscopic appearance of stomach tumor with peritoneal dissemination developing after orthotopic implantation of HSC-58 cells. Extensive dissemination of tumor is observed. (B) Histology of stomach tumor developing after orthotopic implantation of HSC-44PE cells into a nude mouse. Extensive fibrosis with only sparse tumor cell infiltration is seen. H&E stain. (C) Histology of stomach tumor developing after orthotopic implantation of HSC-58 cells into a nude mouse. Proliferation of signet-ring cells can be seen among actively growing tumor cells. H&E stain. (D) Macroscopic appearance of peritoneal dissemination after orthotopic implantation of m2691 subclone. Extensive dissemination of tumor and bloody ascites are observed.

mentioned findings, we attempted to isolate cell lines possessing high and consistent metastatic potential. Cancer cells were removed from metastatic lesions (lymph nodes and lungs) derived from the s.c. tumors and cultured; these cells were then implanted s.c. into mice. This sequence of manipulations was repeated over 10 cycles. In this way, we isolated 5 subclones from the HSC-58 and HSC-44PE cell lines (m2509 and m2615 from HSC-44PE, and m2792, m2917, and m2691 from HSC-58).

These subclones and their parent cell lines were implanted s.c. into mice (Table 4, upper column). The potential for metastasis following s.c. injection did not differ markedly between any parent cell line and its subclones. Thereupon, these cell lines were implanted orthotopically into the gastric wall of mice (Table 4, lower column). The percent take on the gastric wall was 60% for HSC-44PE cells and 83% for HSC-58 cells, but it was higher (90–100%) and more consistent for all of the subclones. Implantation of these subclones resulted in tumor formation at the sites of injection as well as thickening of the gastric wall, associated with an increase in the stomach weight (1.5- to 3-fold increase in the stomach weight), as shown in Fig. 3A. When the tumors were examined histopathologically, active proliferation of the cells in the submucosal tissue, with eventual invasion of the musculature was found. Following implantation of HSC-44PE cells, interstitial hyperplasia resembling that in scirrhous gastric carcinoma was marked in some parts of the gastric wall (Fig. 3B), but on the whole, the interstitial elements were few, and the signet-ring cells assumed a restiform appearance and showed medullary proliferation (Fig. 3C). Similar findings were observed following orthotopic im-

Table 5. Expression of metastasis-related genes in various gastric cancer cell lines

Cell line	Perito. dissemi.	Cell adhesion					Oncogenes					Angiogenesis				*nm23*
		CD44	*E-cadherin*	*Dysadherin*	*β-catenin*	*integrin α6β4*	*EGFR*	*c-erbB-2*	*cript*	*c-met*	*HGF*	*bFGF*	*VEGF*	*IL-6*	*IL-8*	
HSC-44PE	+	++ A	++	++	++	– ++	++	–	+	+	+	–	+	–	–	+
m2615	++	++ A	++	++	++	– –	++	–	+	+	–	–	+	–	–	–
HSC-58	+	+	–	–	++	– –	–	–	+	++ A	–	+	–	–	–	–
m2691	++	++	–	++	++	– ++	++	–	+	++ A	++	+	+	–	–	–
HSC-60	–	–	++	++	+	– –	++	–	+	–	–	–	–	+	–	–
HSC-57	–	++	++	+	++	– ++	+	–	++	+	+	+	+	+	–	–
HSC-59	–	++	–	–	+	– –	+	–	+	–	–	–	–	+	–	–
HSC-64	–	ND	–	–	ND	– –	ND	–	–	–	–	ND	ND	ND	–	–
HSC-39	–	++ A	++	–	+	– –	++	–	++	+	+	–	–	–	–	–
HSC-40A	–	++ A	++	–	+	– –	++	–	++	+	+	–	–	–	–	–
HSC-41	–	+	–	+	++	– +	–	–	–	–	–	–	–	–	–	–
HSC-42	–	++	–	+	++	– –	+	–	–	+	ND	+	–	–	–	–
HSC-43	–	++	++	++	++	– ++	–	–	+	+	+	+	–	–	–	–
HSC-45	+	++ A	++	+	++	– ++	+	–	+	+	+	–	+	–	–	+
SH101-P4	–	+	–	–	–	– –	–	–	–	–	–	–	–	–	–	–

Immunohistochemical staining was carried out as described previously.[14] ++, moderate or strong staining intensity, or staining of >75% of the cells; +, weak staining intensity, or staining of <25% of the cells; –, negative staining, or staining of <1% of the cells. A, gene amplification; ND, not done.

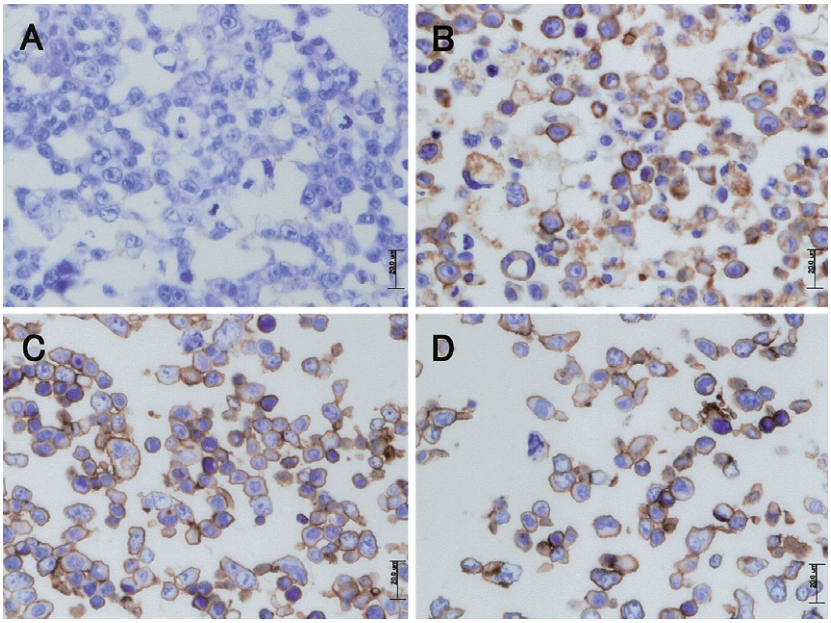

Fig. 4. (A) Immunoreactivity for dysadherin was completely absent from HSC-58 cells. (B) Expression of dysadherin is observed in the membranes of the m2691 subclone. (C & D) Immunoreactivity for dysadherin observed at the cell-cell boundaries in both HSC-44PE and its subclone, m2615.

plantation of the HSC-58 cell line and its subclones.

Following orthotopic implantation of the HSC-44PE and HSC-58 cells, tumor dissemination to the greater omentum, mesenery, parietal peritoneum, liver, etc., was observed, although the incidence of such dissemination was low (Table 4). Implantation of the subclones of these cell lines was associated with a higher incidence of metastasis to the lymph nodes, not only to those near the stomach, but also to distant neck and axillary lymph nodes, compared to those of the parent cell lines. Following implantation of the subclones, the incidence of peritoneal dissemination was similar to that following implantation of the parent cell lines, but the time from implantation to dissemination was shorter (within 100 days). The metastasizing potential of these subclones was consistent, and the subclones sometimes caused bloody ascites (Fig. 3D) (this was particularly marked for subclone m2691). No such findings were observed following orthotopic implantation of any parent cell line.

Expression of metastasis-associated genes. Expression of metastasis-associated genes was compared immunohistochemically between the two aforementioned cell lines and their subclones that exhibited metastatic potential (HSC-44PE, HSC-58, m2615, and m2691), and cell lines not exhibiting any metastatic potential (HSC-39, etc.) (Table 5). HSC-44PE cells showed intense expression of E-cadherin and CD44. On the other hand, HSC-58 cells showed no E-cadherin expression, but intense expression of c-met. None of the genes examined showed specifically intense expression in the two cell lines that exhibited metastatic potential. However, the levels of expression of dysadherin, CD44, and integrin β4 differed markedly between the parent cells and their subclones. Dysadherin was not expressed on HSC-58 cells, but intense expression of this protein was observed in the highly metastatic subclone m2691 (Fig. 4, A and B). On the other hand, dysadherin was expressed on both the HSC-44PE cell line and its subclone m2615 (Fig. 4, C and D). β-Catenin, c-met, VEGF, etc., were expressed on both HSC-44PE and HSC-58 cells. The expression of integrin β4 was weak on HSC-58.

Discussion

It is now established that animal models resembling clinical cases are indispensable for studying the mechanisms underlying cancer metastasis. However, very few reports have been published concerning spontaneous metastasis following s.c. implantation of cultured cancer cells derived from human cancers. Most of the reports published previously pertained to clones of cell lines exhibiting strong metastatic potential.[17, 18] No previous reports have dealt with cells showing strong metastatic potential even before (i.e., during the stage of primary culture itself) they were established as clones of cell lines. In the present study, we attempted to establish carcinoma cells lines by culturing tumor cells collected from the body fluids of patients with gastric cancers. Of the 6 cell lines thus established, two cell lines derived from scirrhous gastric carcinoma (HSC-44PE and HSC-58) were found to exhibit a strong potential for spontaneous metastasis.

To ensure the supply of culture materials, we implanted a part of the cultured material or primary culture cells (in the early stage of cell line development) into nude mice, while simultaneously inoculating the tumor cells onto culture dishes. To our surprise, we observed tumor take and metastasis even in mice implanted with cancer cells collected from ascitic fluid (HSC-58 cells) and primary culture cells (HSC-44PE cells). It is well known that cells with a strong metastatic potential can appear as a result of selection and mutation during multiple passages of cultured cells.[19] In the present study, metastatic lesions were observed even in animals implanted with ascitic tumor cells and primary culture cells. This finding strongly suggests that cells with considerably high malignant potential and strong metastatic potential *in vivo* had already been present in the clinical isolates.

Because the incidence of metastasis of HSC-44PE and HSC-58 cells was low (20–40%), and their incidence of forming metastatic lesions was also low, we attempted to isolate cell lines with a strong potential for lymphatic metastasis. Repeated selection (over 10 cycles) yielded 5 subclones. Initially, we checked for metastasis following s.c. injection of these subclones. S.c. injection refers to heterotopic implantation of gastric cancer cells. It is known that cells implanted orthotopically proliferate within the submucosal tissue.[20] Therefore, we performed orthotopic implantation of tumor cells into the gastric wall and examined the time-course of the tumor/metastasis development. When implanted orthotopically, the subclones exhibited the following characteristics as compared to their parent cells: (1) a similar frequency of metastasis and peritoneal dissemination, more consistent metastatic potential, and shorter time to metastasis; (2) a relatively high frequency of metastasis to lymph nodes, including metastasis to distal neck and axillary lymph nodes; (3) the capability to cause occasional bloody ascites. Formation of ascites, which was not observed with any of the parent cell lines, was seen following implantation of the subclones, especially subclone m2691. The pool of bloody ascites resembled that seen in clinical cases of cancerous peritonitis.

Why do only HSC-44PE and HSC-58 cells possess the potential for spontaneous metastasis among all the cultured cells derived from lesions of cancerous peritonitis or pleuritis? It is known that the onset and progression of gastric cancer are an outcome of accumulation of mutations in several genes.[21] Factors known to be involved in tumor metastasis and infiltration include expression of cell adhesion-associated molecules, such as E-cadherin,[22, 23] β-catenin,[22] integrin α6β4,[14] dysadherin,[24] CD44,[25] factors involved in cellular proliferation, loss of intercellular adhesion and degradation of matrix, such as EGF, c-erbB-2,[26] and cript,[27] factors associated with cell motility, such as HGF and c-met,[28] factors associated with vascularization such as VEGF,[29] IL-6,[30] IL-8,[31] and bFGF,[32] and genes suppressing metastasis, such as *nm23*.[33] In the present study, dysadherin, recently identified as a molecule causing inactivation of the cadherin-cell adhesion system, was specifically expressed in both HSC-44PE and HSC-58 cells. The fact that dysadherin was not detected in HSC-58 cells which exhibited relatively weak metastatic potential, but was detected in subclones that exhibited strong metastatic potential suggests that the gene encoding dysadherin may be closely involved in the progression of tumor metastasis, including peritoneal dissemination. Dysadherin was also detected in parent HSC-44PE cells, as in the cell lines exhibiting strong metastatic potential, suggesting that while the parent HSC-44PE cells also have metastatic potential, some other molecules may also be involved in the development of metastasis. Further study is needed on this subject. The two cell lines shown to exhibit strong metastatic potential in this study had the following characteristics. In the case of HSC-44PE cells, the macromolecule E-cadherin was expressed in them (attributable to modification of the sugar chain), and the gene encoding E-cadherin showed a mutation (exon 9 deletion).[34] Furthermore, amplification and overexpression of the CD44 molecule, which assumed a v8-10 variant isoform, was also observed.[35] In the case of the HSC-58 cells, no expression of E-cadherin was observed, which may suggest gene mutation or methylation of the promoter. HSC-58 cells also showed intense expression and amplification of the *c-met* gene (unpublished data). It is an important question, that remains under debate, as to how these features of the cells examined in this study may be correlated with their metastatic potential. There were few molecules whose expression levels

differed between the parent cell lines and their subclones. This suggests the necessity of isolating cell lines with consistent and strong metastatic potential.

In conclusion, we have described the establishment, for the first time, of two very aggressive human cancer cell lines exhibiting spontaneous metastatic potential in nude mice, which is expected to provide the opportunity for studying the biological aspects of human scirrhous gastric carcinoma cells, especially the process of metastasis of these cells. Isolation of highly malignant peritoneal metastatic sublines from the lymph node metastases is in progress.

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