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A Novel Experimental Mouse Model of Peritoneal Dissemination of Human Gastric Cancer Cells: Analysis of the Mechanism of Peritoneal Dissemination Using cDNA Macroarrays

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We established a new cell line, NUGC-3P4T, with high peritoneal metastatic disseminating potential in nude mice. NUGC-3P4T cells were derived from the human gastric carcinoma line NUGC-3, which has low capacity for peritoneal dissemination. NUGC-3P4T cells developed peritoneal dissemination in 10/10 (100%) mice, whereas the parental NUGC-3 cells developed dissemination in 1/5 (20.0%) mice. The metastatic foci in the peritoneum showed essentially the same histological appearance as those induced by parental cells. The tumorigenicity, the motile activity and the adhesive activity to the laminin of NUGC-3P4T cells were stronger than those of NUGC-3 cells. Production of IL-8 was significantly higher in NUGC-3P4T than in NUGC-3. cDNA macroarrays analysis showed that a variety of cytokines, interleukins, and other immunomodulators and their receptors were up- or down-regulated at the mRNA level in NUGC-3P4T cells, compared with NUGC-3 cells. Thus, this unique cell line and *in vivo* model might be useful to study the biology of peritoneal dissemination of human gastric cancer.

Key words: Gastric cancer lines — Peritoneal dissemination model — Nude mice — cDNA macroarray

The prognosis of gastric cancer has been improving owing to the improvement of diagnostic techniques and treatment methods for gastric cancer, but peritoneal dissemination is the main cause of recurrence after curative resection of advanced cancer.[1)] Although some trials to prevent peritoneal dissemination of gastric cancer have been performed,[2)] including chemotherapy, hyperthermia and peritonectomy, no real prolongation of survival has been attained in most patients.[3)] The establishment of relevant animal models of metastasis is extremely important to understand and design new therapeutic modalities for gastric cancer. To elucidate the biology of metastasis, several excellent metastatic models have been established by intrasplenic injection[4–6)] and the orthotopic implantation technique.[7–9)] We have already established the following three cell lines of human gastric cancer derived from the same parental AZ521 cells: AZ-H5c[10)]; highly liver metastatic line, AZ-L5G[11)]; highly lymph node metastatic line and AZ-P7a[12)]; highly peritoneal disseminating line.[10–13)] However, only a few peritoneal dissemination models have been reported for human gastric cancer.[14, 15)]

In this study, we established NUGC-3P4T cells, with a high potential for peritoneal dissemination in nude mice, derived from the parental NUGC-3 cells. Subsequently, we characterized these NUGC-3P4T cells and NUGC-3 cells comparatively in term of their tumorigenicity, motility, adhesive activity, and production of cytokines. Furthermore, to investigate the molecular mechanism of peritoneal dissemination, a comparative analysis of cDNA macroarrays between NUGC-3 and NUGC-3P4T cells was also performed.

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MATERIALS AND METHODS

Cell line and animals Athymic female BALB/c *nu*/*nu* mice, 6–7 weeks old and weighing 20–22 g, which originated from the Central Institute for Experimental Animals (Kawasaki), were purchased from CLEA Japan (Tokyo). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and provided with sterile food, water and cages. NUGC-3 cells were obtained from a metastatic tumor in the brachial muscle of a 72-year-old male patient in February, 1982 and pathohistological findings of these cells were poorly differentiated adenocarcinoma. The maintenance of these cells was previously reported.[10–13)]

Establishment of highly peritoneal disseminating cell lines NUGC-3 (1×10^7) was inoculated intraabdominally into nude mice. After 5 weeks, the mice were killed and a

few peritoneal dissemination nodules were harvested aseptically. Cells were treated with trypsin and were established in culture. Primary cultures were passaged *in vitro* 3–7 times and then cells were harvested by tripsinization and injected into the peritoneal cavity. The selection cycle was repeated 4 times to yield an original line designated as NUGC-3P4T.

Evaluation of the growth rate and disseminating potential of cell lines To evaluate the *in vivo* tumorigenicity of the parental NUGC-3 and metastatic NUGC-3P4T cells, cultured cells (5×10^6 cells) were inoculated subcutaneously into three regions each of three nude mice. The mice were surveyed daily and the tumors were measured with calipers and their volumes were estimated by using the following formula: $V=L\times W\times H/2$ (*V*, volume; *L*, length; *W*, width; *H*, height). To evaluate the peritoneal disseminating potential, cultured cells (1×10^7) from each cell line were inoculated into the peritoneal cavity of nude mice. Between 1 and 5 weeks after the inoculation, the mice were killed and examined macroscopically for the presence of peritoneal dissemination.

Motility assay A 24-well transwell cell culture chamber (Coster, Cambridge, MA) was used for the motility assay, as previously described.[10, 12, 13] NUGC-3 and NUGC-3P4T (4×10^4 cells/well) were suspended in serum-free RPMI 1640 medium and plated on the upper chamber. The lower chamber contained serum-free RPMI 1640 medium and human cellular fibronectin (50 μg/ml) as a chemoattractant. After incubation for 72 h at 37°C in a CO_2 incubator, the cells on the surface of the filter of the upper chamber were cleaned by wiping. Thereafter, cells were fixed with methanol and stained with Giemsa solution. Motile activity was quantified by counting cells that had migrated to the back surface of the filter. Five fields were counted in each filter under a light microscope at a magnification of ×125. The experiment was performed in duplicate.

Adhesion assay To examine the effect of extracellular matrix (ECM) components and serum on cell proliferation, a 96-well transwell cell culture chamber coated with fibronectin, type IV collagen and laminin (Becton Dickinson Labware, Bedford, MA) was used for the adhesion assay, as previously described.[4] NUGC-3 and NUGC-3P4T cells (4×10^4 cells/well) were suspended in RPMI 1640 medium supplement with 10% FCS (fetal calf serum; JRH Biosciences, Lenexa, KS) and incubated for 1 h at 37°C. After unattached cells had been removed gently by washing with PBS, the number of cells attaching to each ECM was estimated using the MTT (3{4,5}-dimethylthiazol-2,5-diphenyl tetrazolium bromide; Sigma Chemical, St. Louis, MO) assay, as previously described.[10, 12, 13] The absorbance of each well was quantified with a Nova-Path microplate reader (Corona Electric, Hitachinaka) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The experiment was performed in triplicate.

Production of cytokines NUGC-3 and NUGC-3P4T cells were cultured in 96-well plates at a cell density of 1×10^4 cells/well in RPMI 1640 medium supplemented with 10% FCS for 24 h and the next day the culture medium was changed to serum-free RPMI 1640. After 2 days, supernatants were collected and concentrations of vascular endothelial growth factor (VEGF) and IL-8 were measured using enzyme-linked immunosorbent assay kits (VEGF: IBL, Fujioka; IL-8: TFB, Tokyo). The experiment was performed in duplicate.

Analysis of gene expressions Differential gene expression between the two cell lines was measured. First, Poly(A)$^+$ mRNA was extracted from cultured cell pellets using a Fast Track mRNA Isolation Kit (Invitrogen, Leek, the Netherlands), according to the manufacturer's instructions, and quantified spectrophotometrically. The Human Cytokine Labeling Primers (Sigma-genosys, The Woodlands, TX) were annealed to 1 μg of the mRNA template and then ^{32}P-dCTP and Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) were added to initiate cDNA synthesis reaction using a Panorama cDNA Labeling and Hybridization Kit (Sigma-genosys). A Sephadex G-25 spin column (Sigma-genosys, component of the kit) was used to remove the excess, unincorporated radioactive nucleotides from the labeling reaction mixture. Then, the radioactively labeled cDNA was hybridized to the Panorama Human Cytokine Gene Array (Sigma-genosys). After washing the arrays, they were exposed to X-ray films overnight at −80°C. To quantitate the gene expression signals, the X-ray films were analyzed using the software ImageQuaNT (Molecular Dynamics, Sunnyvale, CA). The process of analysis involved the following steps: 1) subtracting the background signal (1× TE Buffer negative control spot) from each spot, 2) determining the average signal (pixel intensity) of the pair of duplicate spots representing each gene, 3) normalizing relative signals from each array to the averaged signal of a housekeeping gene (positive control) that did not change between the samples, 4) comparing normalized signals and dividing the normalized values from the "experimental" array by the normalized values from the "control" array. The resulting values were taken as "gene expression level," and higher or lower values corresponding to a 2-fold difference or more were considered significant.

Statistical analysis All data were analyzed using the unpaired *t* test and expressed as the mean±SE. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Macroscopic and histopathologic findings As shown in Fig. 1, the mice injected with NUGC-3P4T developed peritoneal dissemination that appeared as numerous white nodules on the peritoneum and the abdominal organs with

marked peritoneal hyperplasia. Ascites was not recognized. Liver and/or pulmonary metastasis was not observed within the experimental period. The histopathological findings of these foci corresponded to poorly differentiated adenocarcinoma, and had essentially the same appearance as the foci of the parental NUGC-3 tumors (Fig. 2).

Peritoneal metastasis and tumorigenicity The characteristics of each cell line established in a stepwise fashion are summarized in Table I. Three weeks after intraabdominal injection NUGC-3P4T cells had disseminated around the peritoneal cavity in 10/10 (100%) mice, whereas parental NUGC-3 had developed in 1/5 (20.0%) mice. Therefore, NUGC-3P4T was the most highly peritoneal disseminating cell line.

First we determined the *in vivo* tumorigenicity of the NUGC-3 and NUGC-3P4T cell lines. NUGC-3 and NUGC-3P4T cells were injected s.c into nude mice. As shown in Table II, NUGC-3P4T tumors grew faster and became larger than NUGC-3 tumors.

Motile activity *in vitro* Since motile activity is reported to be an important factor in several steps of tumor metastasis,[16] we compared the motile activity between NUGC-3 and NUGC-3P4T cells. The number of cells that migrated to the back surface of the filter was clearly higher for NUGC-3P4T compared with NUGC-3 cells, with or without 50 μg/ml human cellular fibronectin. The numbers of migrating NUGC-3 and NUGC-3P4T cells were 2.2±0.4/field and 7.4±1.0/field in random assay, and 3.6±0.4/field and 13.2±1.0/field in chemotaxis, respectively ($*P<0.01$) (Table II).

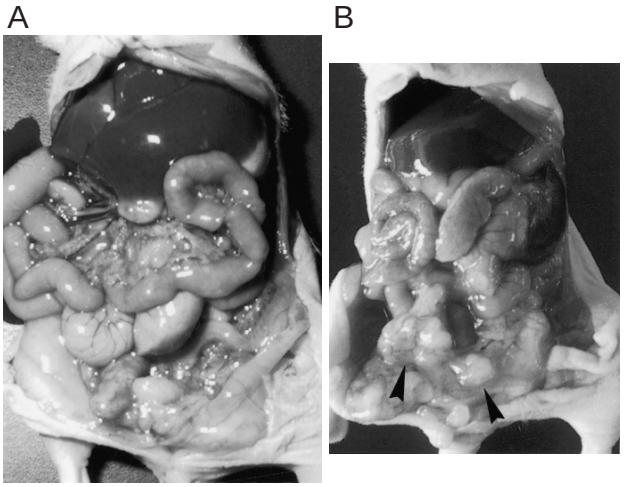

Fig. 1. Macroscopic views of peritoneal disseminations at three weeks after intraabdominal injection. A, NUGC-3; B, NUGC-3P4T. In NUGC-3P4T, great numbers of disseminated tumors were observed in the mesentery, pelvic space and omentum, with marked peritoneal hyperplasia (black arrow).

Adhesion activity *in vitro* Since among the sequential steps of tumor metastasis, the adhesion of tumor cells to ECM is known to be critical,[3, 17] we determined whether NUGC-3 and NUGC-3P4T could adhere to such ECMs as fibronectin, type IV collagen and laminin. As shown in Table II, the *in vitro* adhesive activity of NUGC-3P4T cells to laminin was higher than that of NUGC-3 cells ($*P<0.05$), whereas, adhesive activity to fibronectin and type IV collagen showed little difference.

Production of cytokines To investigate the implication of metastasis-related cytokines, such as VEGF and IL-8, in peritoneal dissemination, we determined the concentrations of VEGF and IL-8 produced by NUGC-3 and NUGC-3P4T cells. As shown in Table II, IL-8 production by NUGC-3P4T was higher than that by NUGC-3, but VEGF production by NUGC-3P4T was not significantly higher than that by NUGC-3.

Differential gene expression in NUGC-3 versus NUGC-3P4T cells Recent developments in gene array technology have facilitated investigation of the relative levels of mRNA of hundreds of genes simultaneously in a single

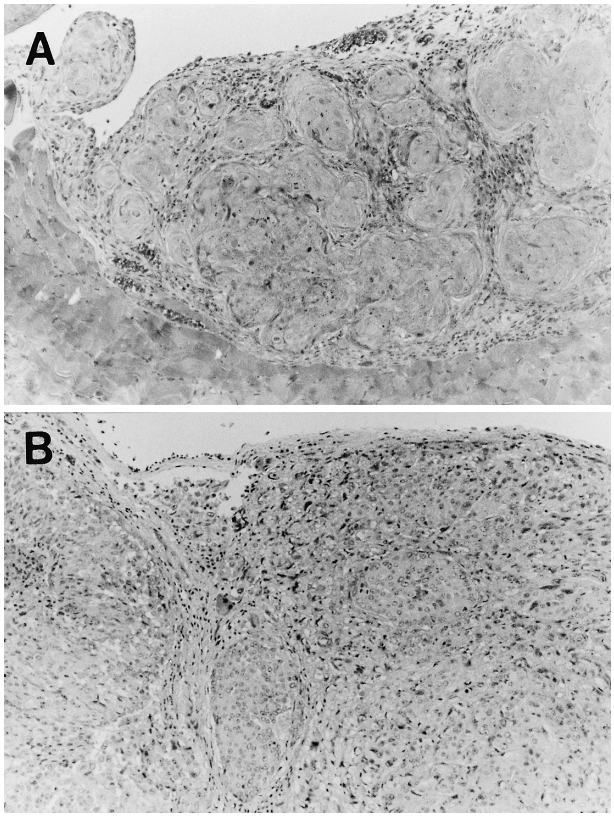

Fig. 2. Microscopic views of developed tumors. A, NUGC-3; B, NUGC-3P4T. Histological findings showed a poorly differentiated adenocarcinoma and essentially the same appearance for parental NUGC-3 tumors and NUGC-3P4T tumors (×100).

experiment. In this study, the experimental differential gene expression in NUGC-3 versus NUGC-3P4T cells, obtained by using Panorama Human Cytokine Gene Arrays, is listed in Table III. Integrin β1, 6, NT-4, fibroblast growth factor (FGF)-R and FGF-R3 were significantly up-regulated, while integrin α2 and transforming growth factor (TGF) β-R were down-regulated in NUGC-3P4T cells, compared with NUGC-3 cells. However the extent of down-regulation of genes in NUGC-3P4T was not significant.

DISCUSSION

We established a new cell line, NUGC-3P4T, of human gastric carcinoma with high peritoneal disseminating potential. By repeated intraabdominal injection of parental NUGC-3 cells, NUGC-3P4T cells were successfully obtained. To investigate the mechanism of peritoneal dissemination in digestive cancer, several animal models have been established and characterized.[18, 19] Additionally we established and characterized the highly peritoneal disseminating line AZ-P7a[12] from a gastric cancer line, and HPC-3P4a[20] and HPC-4P4a, from a pancreatic cancer line. Nevertheless, the detailed mechanisms of peritoneal dissemination have not been extensively studied as yet.[21, 22] At present, it is thought to involve several sequential steps[22]; namely, local growth, invasion to the serosa, exfoliation, "free" cancer cell, adhesion to the peritoneum, invasion, proliferation around the vessels and angiogenesis. At each step, the tumor cells interact with a variety of MMPs (matrix metalloproteinases), adhesion molecules and cytokines, and cell motility plays a central role throughout the process of tumor metastasis.[23–28] Since our model was established by intraabdominal injection, it may reflect the sequential steps of cancer cell dissemination in the abdominal cavity. However, patients with free cancer cells in their abdominal cavity do not always develop peritoneal dissemination clinically, and thus, it is critical to investigate the behavior of cancer cells injected into the abdominal cavity.

Table I. Metastatic Rate after Intraabdominal Injection

Gastric carcinoma lines	Cell dose	Autopsy (w)	No. of mice with peritoneal dissemination/ total no. of mice (%)
NUGC-3	1×10^7	3	1/5 (20)
NUGC-3	1×10^7	5	2/5 (40)
NUGC-3P1T	1×10^7	4	2/2 (100)
NUGC-3P2T	1×10^7	3	2/4 (50)
NUGC-3P3T	1×10^7	6	2/3 (66.7)
NUGC-3P4T	1×10^7	1	2/5 (40)
NUGC-3P4T	1×10^7	2	4/5 (80)
NUGC-3P4T	1×10^7	3	10/10 (100)

Table II. Differences in Biological Malignancy of Established Cell Lines

	NUGC-3 (mean±SE)	NUGC-3P4T (mean±SE)
Tumorigenicity (mg) (*in vivo*)[a]	182.2±52.1	479.7±93.4*
Motility assay (cells/field)		
random assay[b]	2.2±0.4	7.4±1.0**
chemotaxis[c]	3.6±0.4	13.2±1.3**
Adhesive assay (absorption)		
fibronectin	0.015±0.003	0.012±0.001
collagen IV	0.035±0.002	0.045±0.005
laminin	0.033±0.006	0.075±0.007*
Production of cytokines (pg/ml)		
VEGF	1173.2±91.2	1414.5±45.6
IL-8	1164.0±11.3	1418.1±22.0**

SE: standard error.
a) Tumor weight on day 24 after innoculation.
b) In the absence of human fibronectin.
c) In the presence of 50 μg/ml human fibronectin.
* $P<0.05$ NUGC-3 versus NUGC-3P4T.
** $P<0.01$ NUGC-3 versus NUGC-3P4T.

Table III. Differential Gene Expression in NUGC-3 versus NUGC-3P4T Cell

Gene name	Gene group	Accession number	Gene expression
Upregulated genes in NUGC-3P4T			
Integrin-β6	Integrin	9122250	34.8
NT-4	Neutrophic factor	M59371	27.03
FGF R3	Fibroblast growth factor	AB009391	20.78
Integrin-β1	Integrin	Z38102	9.85
Cysteine-rich FGF R	Fibroblast growth factor	J05081	5.96
Downregulated genes in NUGC-3P4T			
Integrin α2	Integrin	M28249	0.53
TGF β R1	TGF β superfamily	M38449	0.87

First, in a variety of biologic comparisons between parental NUGC-3 cells and highly metastatic NUGC-3P4T cells, the *in vivo* tumorigenicity, the metastatic ability, the motile ability and the ability to adhere to laminin were more strongly enhanced in the NUGC-3P4T cells. Similar results have been reported in other gastroenterological cancer models as well as for gastric cancer, and our data showed that highly metastatic variants selected from heterogenous human tumors produced malignant populations of higher metastatic potential.[11, 12, 20)]

Though cell motility is critical under normal physiological conditions, increased motile activity is also an important central process in cancer metastasis.[3, 16, 29)] We revealed that the motile activity of NUGC-3P4T cells *in vitro* was significantly stronger than that of NUGC-3 cells, and the same result was obtained for our original AZ-P7a, HPC-3P4a and HPC-4P4a cells.[12, 20)] Our data suggested that enhanced motile activity might play a very important role in dissemination of tumor cells in the abdominal cavity.[30, 31)]

It is well known that various cytokines are produced by tumor cells and they mediate the host-tumor cell interaction in their microenvironment. Some of them promote fibrosis and angiogenesis, while others inhibit tumor growth.[24)] VEGF is a cytokine essential for angiogenesis in tumor proliferation and is associated with hematogenous metastasis.[4, 32)] In our study, the production of VEGF by NUGC-3P4T cells was not significantly higher than that by NUGC-3. But the production of IL-8 by NUGC-3P4T cells was significantly higher than that by NUGC-3. Although IL-8 has been shown to be an angiogenic factor,[33)] it was reported that the biological consequences of increased IL-8 production by melanoma cells and ovarian carcinoma cells and the role of IL-8 in the metastatic process remain unclear.[34, 35)] These findings suggested that increased angiogenesis might be less implicated in the development of peritoneal dissemination than it is in the development of hematogenous metastasis. We previously reported similar results in assays using AZ-P7a[12)] and HPC-3P4a cells.[20)]

The improvement of novel array technologies has led to new possibilities for studying the molecular mechanisms of cancer metastasis. In this study, the relative mRNA levels of genes expressed in the two cell lines were identified using cDNA macroarrays. The experimental results showed that the differential expression profile of cytokines, chemokines and other immunomodulatory factors, and their receptor genes varied in the development of cancer dissemination. The array analysis suggested that genes for β1-, β6-integrins, FGF-R, etc., were up-regulated, while no genes were significantly down-regulated in the NUGC-3P4T cells. Recent reports showed that many kinds of integrins, for example β1-integrin family,[36, 37)] might be closely associated with peritoneal dissemination of gastric cancer, but the role of integrins in peritoneal dissemination is not fully clarified. In this array analysis, the expression level of β1-integrin was up-regulated in the NUGC-3P4T cells. Expression of large numbers of genes can be investigated easily by using cDNA arrays, though it is difficult to clarify which genes or which steps are implicated in the development of peritoneal dissemination. However, a comparison between gene expression profiles of parental and highly metastatic cell lines might identify coordinate genes which function as biological markers of peritoneal dissemination, possibly leading to new therapeutic modalities.

In conclusion, we established a new human gastric cancer cell line with high peritoneal disseminating potential by repeated intraabdominal injection. It is interesting that increased motility and adhesive activity to laminin were observed in this model. This unique, *in vivo* model cell line should be useful to study the biology of peritoneal dissemination of human gastric cancer.

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REFERENCES

1) Tateishi, M., Ichiyoshi, Y., Kawano, T., Toda, T., Minamisono, Y. and Nagasaki, S. Recurrent pattern of digestive tract carcinoma in the Japanese: comparison of gastric cancer to colon cancer. *Int. Surg.*, **80**, 41–44 (1995).
2) Okamoto, K., Yamaguchi, T., Otsuji, E., Yamaoka, N., Yata, Y., Tsuruta, H., Kitamura, K. and Takahashi, T. Targeted chemotherapy in mice with peritoneally disseminated gastric cancer using monoclonal antibody-drug conjugate. *Cancer Lett.*, **122**, 231–236 (1998).
3) Igarashi, N., Kubota, T., Otani, Y., Matsuzaki, S. W., Watanabe, M., Teramoto, T., Kumai, K., Tamaki, K., Tanzawa, K., Kobayashi, T. and Kitajima, M. Preventive effect of matrix metalloproteinase inhibitor, R-94138, in combination with mitomycin C or cisplatin on peritoneal dissemination of human gastric cancer cell line TMK-1 in nude mice. *Jpn. J. Cancer Res.*, **90**, 116–121 (1999).
4) Shishido, T., Yasoshima, T., Hirata, K., Denno, R., Mukaiya, M., Ura, H., Yamaguchi, K., Kawaguchi, S. and Sato, N. Establishment and characterization of human pancreatic carcinoma lines with a high metastatic potential in the liver of nude mice. *Jpn. J. Surg.*, **29**, 519–525 (1999).
5) Vezeridis, M. P., Tzanakakis, G. N., Meitner, P. A., Doremus, C. M., Tibbets, L. M. and Calabresi, P. *In vivo* selection of a highly metastatic cell line from a human pancreatic carcinoma in the nude mouse. *Cancer*, **69**, 2060–

2063 (1991).
6) Fidler, I. J. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metastasis Rev.*, **5**, 29–49 (1986).
7) Yamashita, T. Manifestation of metastatic potential in human gastric cancer implanted into the stomach wall of nude mice. *Jpn. J. Cancer Res.*, **79**, 945–951 (1988).
8) Fu, X., Besterman, J. M., Monosov, A. and Hoffman, R. M. Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimen. *Proc. Natl. Acad. Sci. USA*, **88**, 9345–9349 (1991).
9) Furukawa, T., Kubota, T., Watanabe, M., Kitajima, M. and Hoffman, M. A novel "patient-like" treatment model of human pancreatic cancer constructed using orthotopic transplantation of histologically intact human tumor tissue in nude mice. *Cancer Res.*, **53**, 3070–3072 (1993).
10) Denno, R., Yasoshima, T., Hirata, K. and Hirata, K. Tumorigenicity, motility and liver metastasis of human gastric carcinoma lines with high metastatic potential in the liver of nude mice. *Tumor Res.*, **30**, 57–65 (1996).
11) Yamaguchi, K., Ura, H., Yasoshima, T. and Hirata, K. Establishment and characterization of a human gastric carcinoma cell line that is highly metastatic to lymph nodes. *J. Exp. Clin. Cancer Res.*, **19**, 113–120 (2000).
12) Nishimori, H., Yasoshima, T., Denno, R., Shishido, T., Hata, F., Okada, Y., Ura, H., Yamaguchi, K., Isomura, H., Sato, N. and Hirata, K. A novel experimental mouse model of peritoneal dissemination of human gastric cancer cells: different mechanisms in peritoneal dissemination and hematogenous metastasis. *Jpn. J. Cancer Res.*, **91**, 715–722 (2000).
13) Yasoshima, T., Denno, R., Kawaguchi, S., Sato, N., Okada, Y., Ura, H., Kikuchi, K. and Hirata, K. Establishment and characterization of human gastric carcinoma lines with high metastatic potential in the liver: changes in integrin expression associated with the ability to metastasize in the liver of nude mice. *Jpn. J. Cancer Res.*, **87**, 153–160 (1996).
14) Yashiro, M., Chung, Y. S., Nishimura, S., Inoue, T. and Sowa, M. Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. *Clin. Exp. Metastasis*, **14**, 43–54 (1996).
15) Sato, T., Sato, N., Takahashi, S., Okubo, M., Yagihashi, A., Koshiba, H., Usui, T., Katsuramaki, T., Yamada, T., Hirata, K., Shiramatsu, K., Hayasaka, H. and Kikuchi, K. Establishment and characterization of new cell lines derived from human pancreatic cancers. *Tumor Res.*, **21**, 9–19 (1986).
16) Sakurai, Y., Sawada, T., Chung, Y. S., Funae, Y. and Sowa, M. Identification and characterization of motility stimulating factor secreted from pancreatic cancer cells: role in tumor invasion and metastasis. *Clin. Exp. Metastasis*, **15**, 307–317 (1997).
17) Karasawa, K., Sugiura, N., Hori, Y., Suzuki, S., Onaya, J., Sakurai, K. and Kimata, K. Inhibition of experimental metastasis and cell adhesion of murine melanoma cells by chondroitin sulfate-derivatized lipid, a neoproteoglycan with anti-cell adhesion activity. *Clin. Exp. Metastasis*, **15**, 83–93 (1997).
18) Yoshimura, T., Manabe, T., Imamura, T., Imanishi, K., Ohshio, G., Yamabe, H., Kitamura, O., Matsumoto, M., Ogasahara, K., Takasan, H. and Tobe, T. Flow cytometric analysis of nuclear DNA content of duct cell carcinoma of the pancreas. *Cancer*, **70**, 1069–1074 (1992).
19) Matsuoka, H., Seo, Y., Fujikawa, K., Yano, K. and Tomoda, H. A newly established mouse model of peritoneal dissemination in human pancreatic cancer. *Anticancer Res.*, **17**, 951–954 (1997).
20) Nishimori, H., Yasoshima, T., Denno, R., Shishido, T., Hata, F., Honma, T., Ura, H., Yamaguchi, K., Yagihashi, A., Kawaguchi, S., Isomura, H., Sato, N. and Hirata, K. A new peritoneal dissemination model established from the human pancreatic cancer cell line. *Pancreas*, **22,** 348-356 (2001).
21) Nakashio, T., Narita, T., Sato, M., Akiyama, S., Kasai, Y., Fujiwara, M., Ito, K., Takagi, H. and Kannagi, R. The association of metastasis with the expression of adhesion molecules in cell lines derived from human gastric cancer. *Anticancer Res.*, **17**, 293–300 (1997).
22) Nishimura, S., Chung, Y. S., Yashiro, M., Inoue, T. and Sowa, M. Role of α2β1- and α3β1-integrin in the peritoneal implantation of scirrhous gastric cancer. *Br. J. Cancer*, **74**, 1406–1412 (1996).
23) Nakajima, M., Morikawa, K., Fabra, A., Bucana, C. D. and Fidler, I. J. Influence of organ environment on extracellular matrix degradative activity and metastasis of human colon carcinoma cells. *J. Natl. Cancer Inst.*, **82**, 1890–1898 (1990).
24) Ito, R., Yasui, W., Kuniyasu, H., Yokozaki, H. and Tahara, E. Expression of interleukin-6 and its effect on the cell growth of gastric carcinoma cell lines. *Jpn. J. Cancer Res.*, **88**, 953–958 (1997).
25) Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M. and Buck, C. A. Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. *Cancer Res.*, **50**, 6757–6764 (1990).
26) Nakashio, T., Narita, T., Akiyama, S., Kasai, Y., Kondo, K., Takagi, H. and Kannagi, R. Adhesion molecules and TGF-β1 are involved in the peritoneal dissemination of NUGC-4 human gastric cancer cells. *Int. J. Cancer*, **70**, 612–618 (1997).
27) Xianming, C., Hokita, S., Nuruki, K., Toyoyama, H., Tanabe, G., Baba, M., Takao, S., Aikou, T. and Kuroshima, K. Abnormal expression of β- and γ-catenins in early gastric cancer. *Int. J. Clin. Cancer*, **3**, 233–239 (1998).
28) Tomokazu, K., Konno, H., Tanaka, T., Baba, M., Matsumoto, K., Nakamura, S., Yukita, A., Asano, M., Suzuki, H. and Baba, S. Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. *Int. J. Cancer*, **77**, 933–936 (1998).
29) Vogelmann, R., Kreuser, E. D., Adler, G. and Lutz, M. P.

Integrin α6β1 role in metastatic behavior of human pancreatic carcinoma cells. *Int. J. Cancer*, **80**, 791–795 (1999).
30) Hasegawa, A., Takahashi, T., Sawai, K., Taniguchi, H., Shimotsuma, M., Okano, S., Sakakura, C., Tsujimoto, H., Osaki, K., Sasaki, S. and Shirasu, M. Milky spots as the implantation site for malignant cells in peritoneal dissemination in mice. *Cancer Res.*, **53**, 687–692 (1993).
31) Tsujimoto, H., Takahashi, T., Hasegawa, A., Shimotsuma, M., Sakakura, C., Osaki, K., Sasaki, S., Shirasu, M., Sakakibara, T., Ohyama, T., Sakuyama, A., Ohgaki, M., Imanishi, T. and Yamasaki. J. Site-specific implantation in the milky spots of malignant cells in peritoneal dissemination: immunohistochemical observation in mice inoculated intraperitoneally with bromodeoxyuridine-labelled cells. *Br. J. Cancer*, **71**, 468–472 (1995).
32) Saito, H., Tsujitani, S., Kondo, A., Ikeguchi, M., Maeta, M. and Kaibara, N. Expression of vascular endothelial growth factor correlates with hematogenous recurrence in gastric cancer. *Surgery*, **125**, 195–201 (1999).
33) Fujimoto, J., Sakaguchi, H., Aoki, I. and Tamiya, T. Clinical implication of expression of interleukin-8 related to angiogenesis in uterine cervical cancer. *Cancer Res.*, **60**, 2632–2635 (2000).
34) Luca, M., Huang, S., Gershenwald, J., Singh, R., Reich, R. and Bar-Emi, M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am. J. Pathol.*, **151**, 1105–1113 (1997).
35) Xu, L., Xie, K., Mukaida, N., Matsushima, K. and Fidler, I. Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res.*, **59**, 5822–5829 (1999).
36) Kawamura, T., Endo, Y., Yonemura, Y., Nojima, N., Fujita, H., Fujimura, T., Obata, T., Yamaguchi, T. and Sasaki, T. Significance of integrin alpha2/beta1 in peritoneal dissemination of a human gastric cancer xenograft model. *Int. J. Oncol.*, **18**, 809–815 (2000).
37) Matsuoka, T., Yashiro, M., Nishimura, S., Inoue, T., Fujihara, T., Sawada, T., Kato, Y., Seki, S. and Hirakawa-Ys. Chung, K. Increased expression of alpha2beta1-integrin in the peritoneal dissemination of human gastric carcinoma. *Int. J. Mol. Med.*, **5**, 21–25 (2000).