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 injection4–6) 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-H5c10); highly liver metastatic line, AZ-L5G11); highly lymph node metastatic line and AZ-P7a12); 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.

MATERIALS AND METHODS

Cell line and animals Atlhmic 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 (1×10⁷) 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 tripсинization 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 the following formula: 

\[ V = L \times W \times H / 2 \]

(where; \( V \) = volume; \( L \) = length; \( W \) = width; \( H \) = height). To evaluate the peritoneal disseminating potential, cultured cells (1 × 10^3) 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. 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 chemoattrac tant. 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. NUGC-3 and NUGC-3P4T cells (4 × 10^5 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. The absorbance of each well was quantified with a NovaPath 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 32P-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 \( \mu g/ml \) 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).

Adhesion activity *in vitro* Since among the sequential steps of tumor metastasis, the adhesion of tumor cells to ECM is known to be critical,15) 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. 1. Macroscopic views of peritoneal disseminations at three weeks after intraabdominal injection. A, NUGC-3; B, NUGC-3P4T.](image1)

![Fig. 2. Microscopic views of developed tumors. A, NUGC-3; B, NUGC-3P4T.](image2)
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-P7a2) from a gastric cancer line, and HPC-3P4a20) 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 steps22); 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 metastasis23–28) 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                  | 1×10⁷     | 3           | 1/5 (20)                                                      |
| NUGC-3                  | 1×10⁷     | 5           | 2/5 (40)                                                      |
| NUGC-3P1T               | 1×10⁷     | 4           | 2/2 (100)                                                    |
| NUGC-3P2T               | 1×10⁷     | 3           | 2/4 (50)                                                      |
| NUGC-3P3T               | 1×10⁷     | 6           | 2/3 (66.7)                                                   |
| NUGC-3P4T               | 1×10⁷     | 1           | 2/5 (40)                                                      |
| NUGC-3P4T               | 1×10⁷     | 2           | 4/5 (80)                                                      |
| NUGC-3P4T               | 1×10⁷     | 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) | 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 |
|-----------|------------|------------------|-----------------|
| Intelectin-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.\(^{1, 12, 20}\)

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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