Novel models for human scirrhous gastric carcinoma *in vivo*

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Human scirrhous gastric carcinoma, a diffusely infiltrating type of poorly differentiated gastric carcinoma also known as linitis plastica type carcinoma, is characterized by cancer cell infiltration and proliferation accompanied with extensive stromal fibrosis. We established two new gastric cancer cell lines, designated OUCM-8 and OCUM-11, which developed the characteristic biology of scirrhous gastric carcinoma upon orthotopic implantation in mice. Involvement of lymph nodes and liver metastasis was also found in both orthotopic models. Histologically, these orthotopic models showed proliferation with extensive fibrosis, resembling human scirrhous gastric cancer. Both cell lines were derived from ascites of patients with scirrhous gastric cancer. The growth of OCUM-8 and OCUM-11 cells following the addition of KGF, FGF, and EGF was increased significantly relative to untreated cells. An increase in the number of attached and spreading cells occurred following the addition of TGF-β1 in both cell lines. OCUM-11 cells showed microsatellite instability. Although subcutaneous scirrhous gastric cancer cells show medullary growth, most *in vivo* studies of scirrhous gastric cancer have used xenografted tumors implanted subcutaneously. Only in a few cases was it confirmed that these scirrhous gastric cancer cell lines retained the original histologic characteristics. Our orthotopic models should contribute to the elucidation of disease progression *in situ* and to the development of therapy for scirrhous gastric cancer. (Cancer Sci 2004; 95: 893–900)

Human scirrhous gastric carcinoma, a diffusely infiltrating type of poorly differentiated gastric carcinoma also known as linitis plastica type carcinoma, is characterized by cancer cell infiltration and proliferation accompanied with extensive stromal fibrosis.[1)] Gastric cancer has been a major cause of cancer death and scirrhous gastric cancer has the highest mortality of all gastric cancers. Common features of scirrhous gastric cancer include remarkable fibrosis, rapid invasive progress and a high frequency of metastasis to peritoneum and lymph nodes.[2)] Development of a new therapeutic approach based on this characteristic biologic behavior is an important issue. However, the mechanisms responsible for proliferation and metastasis remain unclear.[3)] For elucidation of these mechanisms, the establishment of a suitable scirrhous gastric carcinoma mouse model is needed.[4)]

Since the report of Sekiguchi *et al*., there have been several reports of the establishment of scirrhous gastric carcinoma cell lines.[5–11)] Most of these cell lines have been used as models of scirrhous gastric carcinoma only because they were established from human scirrhous gastric carcinoma. In only a few cases was it confirmed that these scirrhous gastric cancer cell lines retained the original characteristics of cancer cell proliferation accompanied with extensive stromal fibrosis.[12, 13)] For studies of the character of scirrhous gastric carcinoma using cancer cell lines, it is important to use gastric cancer cell lines with similar behavior to human scirrhous gastric carcinoma, including histologic characteristics and metastatic ability to lymph nodes *in vivo*. We have established two new scirrhous gastric cancer cell lines, designated OUCM-8 and OCUM-11, which lead to scirrhous gastric carcinoma with extensive fibrosis and lymph node spread following orthotopic inoculation.

Materials and Methods

Patients. OCUM-8 was derived from ascites of a 67-year-old woman in 1999. The patient had peritoneal dissemination on admission. An upper GI series and gastro-fiberscopy showed scirrhous gastric carcinoma. Histology showed a poorly differentiated adenocarcinoma (Fig. 1A). OCUM-11 was derived from ascites of an 82-year-old woman in 2000. The gastroscopic diagnosis was scirrhous gastric carcinoma. Histological findings showed a poorly differentiated adenocarcinoma (Fig. 1B). Her father and brother had died of gastric cancer.

Cell culture. Effusion from each patient was collected aseptically into a bottle with heparin then centrifuged at 1000 rpm for 5 min. Each cell pellet was suspended in 10 ml of culture medium (see below) and seeded into 100 mm culture dishes (Falcon, Lincoln Park, NJ). Initial culture was performed in a humidified incubator at 37°C in an atmosphere of 5% carbon dioxide and 95% air. The culture medium was Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Kyoto, Japan) with 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), 100 IU/ml penicillin (ICN Biomedical, Costa Mesa, CA), 100 μg/ml streptomycin (ICN Biomedical), and 0.5 m*M* sodium pyruvate (Cambrex, Walkersville, MD). Floating cells and adherent mesothelial cells were found in each dish. Floating cells were collected and re-suspended in medium. Serial passages were carried out every 4 to 7 days. The cells were passaged routinely at a ratio of 1:5 or 1:10. The floating cell lines were designated OCUM-8 and OCUM-11. Adherent mesothelial cells were designated MS-8 and MS-11. OCUM-8 and OCUM-11 cells were cultured for more than 24 months and passaged for more than 160 generations. The cells were tested for *Mycoplasma* contamination with a Hoechst staining kit (Flow, Tokyo). The doubling time of each cell line at the 25th passage was determined. Briefly, suspensions of 10^4 cells were incubated in 48-well dishes with 0.5 ml of DMEM containing 10% FCS. Cells were counted every 6 to 24 h using a Coulter Z2 counter (Beckman Coulter, Fullerton, CA). The doubling times were determined from the growth curve. MKN74 cells derived from a differentiated adenocarcinoma of human stomach were also cultured in DMEM with 10% FCS.

Xenografted tumor. OCUM-8 and OCUM-11 cells (5.0×10^6, 10^6, 10^5) suspended in a volume of 0.2 ml were inoculated subcutaneously into female athymic 4-week-old BALB/c nude mice (Oriental Kobo, Osaka, Japan). The mice were observed for 8 weeks. Tumor incidence was then determined. Mice were sacrificed and tumors were removed and fixed in 10% formalin for paraffin sections. The sections were stained with hematoxylin and eosin (H&E).

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Orthotopic implantation in stomach. Under ether anesthesia, a median abdominal incision was made in 4-week-old female athymic nude mice (Oriental Kobo). The stomach was exposed carefully and various numbers (5.0×10^6, 10^6, 10^5) of OCUM-8 and OCUM-11 cells in a volume of 20 µl of DMEM were inoculated orthotopically at the greater curvature of the antrum using 30-gauge needles (Handaya, Saitama, Japan).[3] MKN74 cells (5.0×10^6) were inoculated orthotopically. Four weeks after inoculation, mice were sacrificed; the gastric tumor, lymph nodes, and liver were fixed in formalin for H&E staining.

Intraperitoneal inoculation. Cells (2.0×10^7, 10^7, 5.0×10^6) suspended in a volume of 0.5 ml DMEM were injected into the peritoneal cavity of female athymic 4-week-old nude mice (Oriental Kobo). The mice were observed for 8 weeks. Bloody ascitic effusion was observed in the abdominal cavity. After 8 weeks, the mice were sacrificed. All experiments with nude mice were performed in accordance with the guidelines approved by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR).

Production of tumor-associated antigen. A suspension of 10^6 cells in 100 mm plastic culture dishes was incubated for 5 days in culture medium, then tumor-associated antigen was measured. As a control, DMEM containing 10% FCS was used.

Effect of growth factors on the growth of cell lines and their morphology. The cell suspension (10^4 cells/0.5 ml) was incubated in 48-well plates with 0.1, 1, 10, and 100 ng/ml of each growth factor, including epidermal growth factor (EGF; Becton Dickinson, Bedford, MA), basic fibroblast growth factor (b-FGF; Genzyme, Cambridge, MA), hepatocyte growth factor (HGF; Becton Dickinson), insulin like growth factor-I (IGF-I; Austral Biologicals, San Ramon, CA), keratinocyte growth factor (KGF; Pepro Tec, Rocky Hill, NJ), platelet derived growth factor-AA (PDGF-AA; Sigma, St. Louis, MO), transforming growth factor-β (TGF-β1; Austral Biologicals), and vascular endothelial growth factor (VEGF; Genzyme). After 72 h, the cells were counted. The cell suspension was finally incubated with 10 ng/ml of growth factor, and the cells were counted at various time points to 120 h. Morphology was investigated with a phase-contrast microscope.

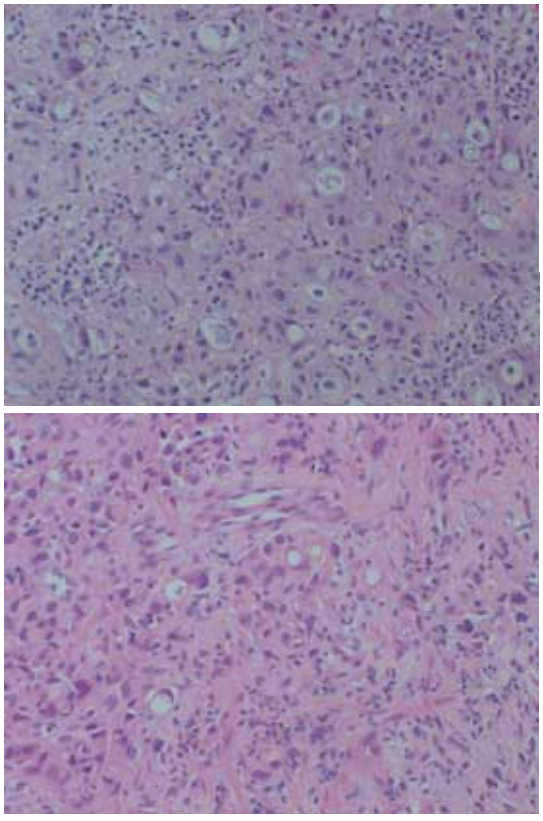

Fig. 1. Histology of original tumors. The original tumors of OCUM-8 (A) and OCUM-11(B) showed the characteristics of poorly differentiated adenocarcinoma.

Chromosome analysis. Cells were karyotyped using a standard air-drying method[14] following treatment with a final concentration of 0.05 µg/ml colcemid for 2 h when the cells were in an exponential growth phase. They were analyzed using trypsin G banding. A total of 50 metaphase spreads was counted to determine the modal number. Karyotyping was performed according to the International System for Human Cytogenetic Nomenclature.[14] DNA histogram analysis and chromosome analysis were carried out on the two cell lines at the 40th passage.

Microsatellite instability and loss of heterozygosity analysis. Genomic DNA was extracted with the Blood & Cell Culture Mini kit (Qiagen, Hilden, Germany). Microsatellite instability (MSI) and loss of heterozygosity (LOH) were analyzed as follows. A 6 µl aliquot of reaction mixture was formulated using 0.4 µl of DNA extracted from OCUM-8 cells and OCUM-11 cells, MS-8 cells, and MS-11 cells, 4.5 µl of each primer marker set, 0.06 µl Ampli*Taq* Gold (Applied Biosystems, Foster City, CA), and 1.04 µl of distilled water. Eight-primer marker sets, *D18S35*, *D1S2883*, *D2S123*, *D3S1611*, *D5S346*, *D7S501*, *TP53Dint*, and *TP53Penta*, were linked to the *DCC* locus on 18q21, the *HPC1* locus on 1p24, the *hMSH2* locus on 2p16, the *hMLH1* locus on 3p23-21.3, the *APC* locus on 5q21, the *c-met* locus on 7q31, and the two-*p53* loci on 17p13. *BAT25* and *BAT26* were also examined. The polymerase chain reaction (PCR) conditions were as follows: 95°C for 10 min followed by 45 cycles (96°C for 10 s, 55°C for 30 s, 70°C for 3 min), with a final elongation at 70°C for 30 min. Each sample was analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). A novel band shift was considered to be present when more than 20% of the electropherograms with informative microsatellite markers exhibited numerous extra alleles in the tumor sample. MS-8 and MS-11 were used as the controls for OCUM-8 and OCUM-11. We utilized the stringent criteria for determination of MSI from a National Cancer Institute workshop.[15] A tumor was identified as exhibiting LOH if there was absence or more than a 50% reduction in the peak height of one allele of the tumor sample compared to the normal mesothelial cell allele.

Frameshift mutations of *TGFβRII*, *BAX*, *IGFIIR*, *hMSH3*, and *hMSH6*. *TGFβRII* (transforming growth factor β receptor type II), *BAX*, *IGFIIR* (insulin-like growth factor II receptor), *hMSH3*, and *hMSH6* encompass the $(A)_{10}$, $(G)_8$, $(G)_8$, $(A)_8$, and $(C)_8$ mononucleotide tracts, respectively. Frameshift mutations at *TGFβRII*, *BAX*, *IGFIIR*, *hMSH3*, or *hMSH6* were examined as previously reported.[16]

Statistical analysis. Data were analyzed using Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

Scirrhous gastric carcinoma models *in vivo*. Gastric tumors after orthotopic inoculation of 5×10^6 OCUM-8 cells into the middle wall of the stomach were found in 4 of 6 mice. Following the inoculation of 5×10^6 OCUM-11 cells into the stomach wall the formation of cancer was seen in 3 of 6 mice (Table 1). Macroscopic findings of orthotopic tumors, OCUM-8 (Fig. 2A) and OCUM-11 (Fig. 2B), showed linitis plastica type carcinoma accompanied by pyloric stenosis. Involvement of lymph nodes and liver was found in both orthotopic tumor models. The normal stomach consists of mucosa and tunica muscularis propria with a thin submucosal layer in nude mice (Fig. 3A). Orthotopic tumors generated by OCUM-8 cells (Fig. 3, B and C) and

Table 1. Tumorigenicity after subcutaneous inoculation, orthotopic inoculation, and intraperitoneal inoculation in nude mice

Cell line	Inoculation site	Cell number			
		2×10^7	5×10^6	1×10^6	1×10^5
OCUM-8	Subcutaneous tumor	U.D.[1)]	12/12[2)] (100%)	12/12 (100%)	4/12 (33%)
	Orthotopic tumor	U.D.	4/6 (67%)	3/6 (50%)	0/6 (0%)
	liver metastasis	U.D.	1/6 (17%)	0/6 (0%)	0/6 (0%)
	lymph node metastasis	U.D.	4/6 (66%)	3/6 (50%)	0/6 (0%)
	Intraperitoneal metastasis	6/6 (100%)	3/6 (50%)	3/6 (50%)	U.D.
OCUM-11	Subcutaneous tumor	U.D.	12/12 (100%)	12/12 (100%)	2/12 (17%)
	Orthotopic tumor	U.D.	3/6 (50%)	0/6 (0%)	0/6 (0%)
	liver metastasis	U.D.	1/6 (17%)	0/6 (0%)	0/6 (0%)
	lymph node metastasis	U.D.	3/6 (50%)	0/6 (0%)	0/6 (0%)
	Intraperitoneal metastasis	6/6 (100%)	4/6 (67%)	4/6 (67%)	U.D.

1) U.D., undone.
2) Number of mice bearing a tumor/total number of mice.

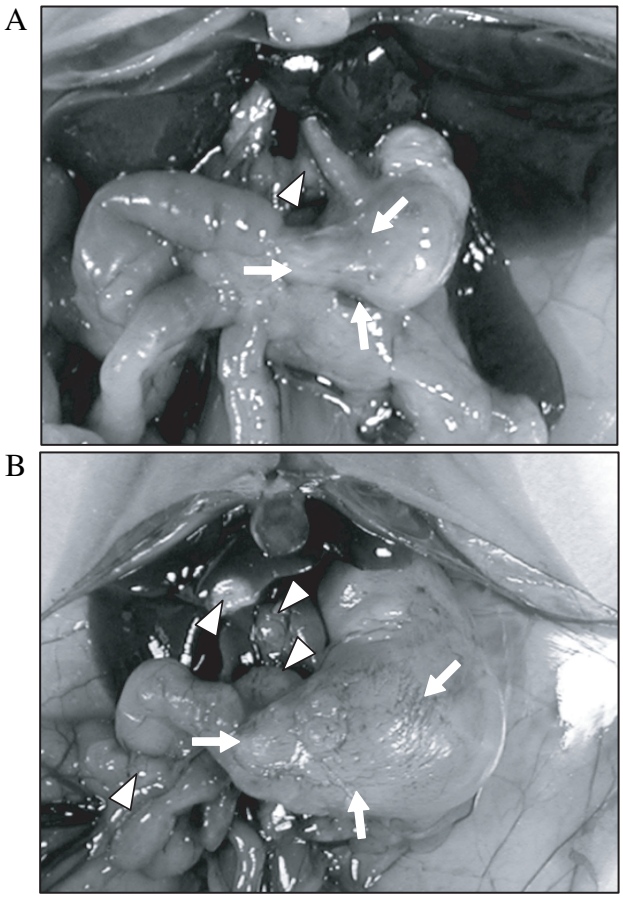

Fig. 2. Orthotopic implantation model. Orthotopically implanted OCUM-8 cells (A) in the stomach of nude mice (arrows) yielded multiple involved lymph nodes (arrowhead) 4 weeks after inoculation. Orthotopically implanted OCUM-11 cells (B) in the stomach of nude mice (arrows) yielded multiple involved lymph nodes (arrowheads) and liver metastases (arrowhead) 4 weeks after inoculation.

by OCUM-11 cells (Fig. 3, E and F) showed extensive fibrosis with the occasional presence of poorly differentiated adenocarcinoma cells which resembled scirrhous gastric carcinoma. The orthotopic tumors of OCUM-8 (Fig. 3D) and OCUM-11 (Fig. 3G) cells presented lymphatic invasion, while sparse tumor cells infiltrating desmoplastic stroma were seen infrequently. Extensive stromal fibrosis was found in all cases of orthotopic tumors derived from OCUM-8 cells and OCUM-11 cells. Reproducibility of the extensive stromal fibrosis was 100%. In contrast, the histologic findings of orthotopic tumors formed by 5×10^6 MKN74 cells, a line derived from a differentiated adenocarcinoma of the human stomach, showed medullary growth with fewer fibroblasts in all cases (Fig. 3, H and I). Stromal fibrosis was scanty in the orthotopic tumors derived from MKN74 cells. The orthotopic tumors showed different histologic features from the original tumor, from which the cancer cells were derived. Subcutaneously xenografted OCUM-8 (Fig. 4A) and OCUM-11 (Fig. 4B) showed medullary growth, while xenograft formation was seen in all mice receiving 5×10^6 OCUM-8 or OCUM-11 cells (Table 1). Liver (Fig. 4C (OCUM-8), 4D (OCUM-11)) and lymph node tumors (Fig. 4E (OCUM-8), 4F (OCUM-11)) showed medullary growth with less fibrosis in both orthotopic tumor models.

Peritoneal dissemination models *in vivo*. Intraperitoneal injection of 2×10^7 cells caused bloody ascites due to dissemination 8 weeks after inoculation in all mice. Injection of 10^5 cells caused bloody ascites in 50% of mice injected with OCUM-8 cells and in 67% of mice injected with OCUM-11 cells (Table 1). The normal peritoneum of nude mice showed a monolayer mesothelium with a thin layer of connective tissue (Fig. 4G). In contrast, histologic findings of peritoneal metastatic tumors formed following injection of cancer cells into the intraperitoneal cavity showed infiltration of cancer cells with extensive fibrosis (Fig. 4H(OCUM-8), 4I(OCUM-11)).

Characterization of OCUM-8 cells and OCUM-11 cells. Doubling times for OCUM-8 and OCUM-11 were 13.4 h and 11.3 h. In OCUM-8, tumor-associated antigens CEA, CA19-9, and SPan-1 were detected at 20.6 ng/ml, 6000 U/ml, and 1000 U/ml, respectively, while αFP, SLX, and STN were within the normal ranges. In OCUM-11, the concentrations of CEA, CA19-9, SPan-1, and SLX were 10.3 ng/ml, 2100 U/ml, 520 U/ml and 51 U/ml, respectively, while αFP and STN were within the normal ranges.

Effect of growth factors on proliferation. The growth of OCUM-8 and OCUM-11 cells following the addition of KGF, FGF, or EGF was increased significantly relative to untreated cells at 72 h. VEGF increased the growth of OCUM-8 cells. The growth of OCUM-11 cells was decreased by TGF-β1 (Fig. 5). Both cell lines were round, floating, and partly adherent (Fig. 6, A and B). An increase in the number of attached and spreading cells occurred following the addition of TGF-β1 in both OCUM-8 (Fig. 6C) and OCUM-11 (Fig. 6D) cells, while most cells were round without TGF-β1 at 72 h (Fig. 6, A and B).

Chromosome analysis. Fig. 7 shows the distribution of chromosomes. The chromosome number in OCUM-8 cells ranged from

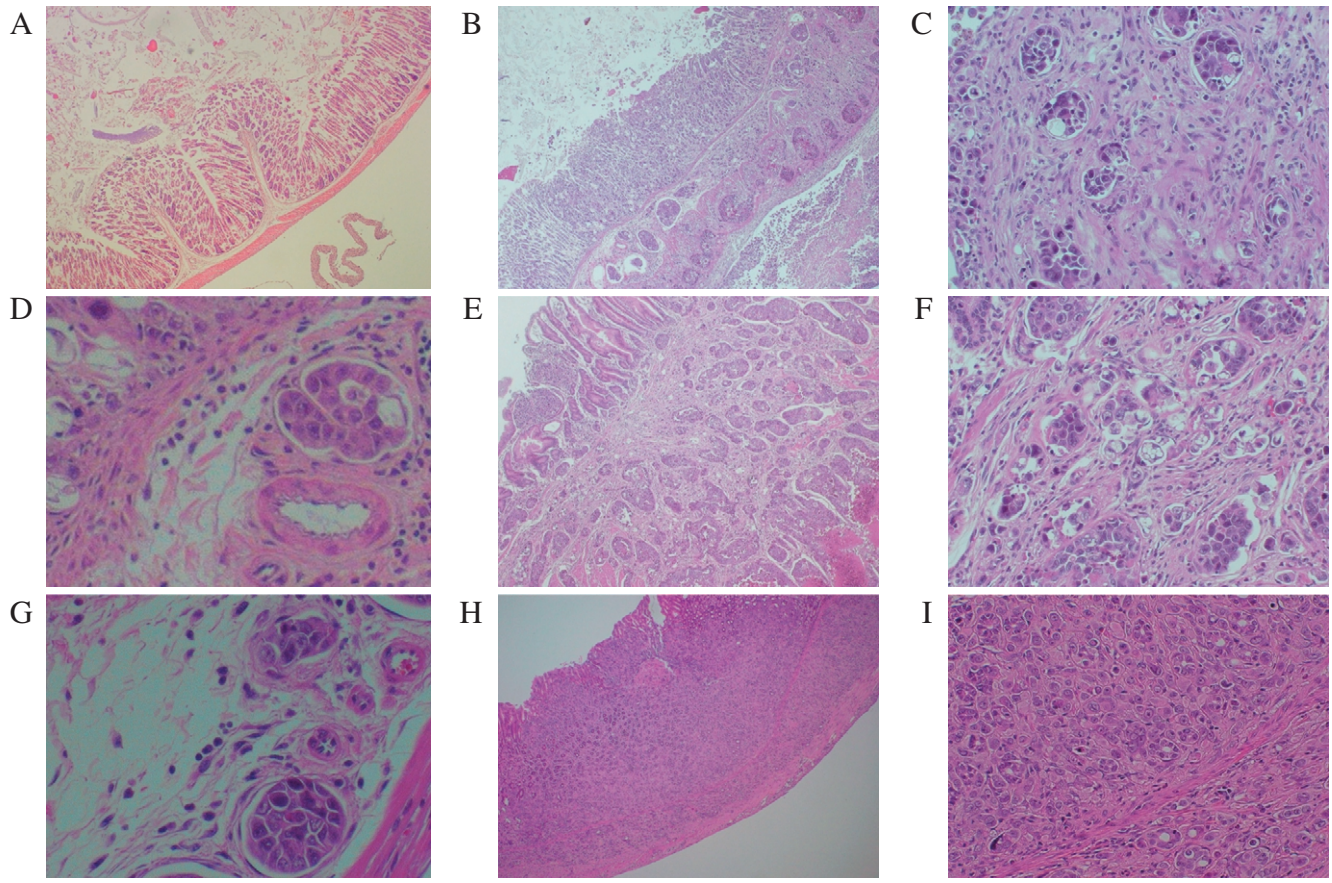

Fig. 3. Histologic findings of inoculated OCUM-8 and OCUM-11 tumors. The normal stomach of nude mouse consists of mucosa and tunica muscularis propria with a thin submucosal layer (A). Orthotopic tumors with OCUM-8 (B, C) and OCUM-11 (E, F) showed extensive fibrosis with the occasional presence of poorly differentiated adenocarcinoma cells. Orthotopic tumors presented lymphatic invasion: OCUM-8 (D) and OCUM-11 (G). The growth of stromal fibrosis was scanty in the orthotopic tumors formed by MKN74 cells (derived from differentiated adenocarcinoma of human stomach). (H, I) H&E staining.

75 to 83 (Fig. 7A), while that in OCUM-11 cells ranged from 59 to 65 (Fig. 7B). Fifteen of 50 metaphase spreads examined were karyotyped. Fig. 7, C and D show the major karyotypic features. The arrowheads indicate rearranged chromosomes. OCUM-8 (Fig. 7C): 75 82<3*n*, XXX, +X [6], +X [3], +1[10], +add (1)(p32)[2], −3[4], +4[2], add (4)(p11)[10], add (4)[9], +7[7], +8[6], +9[10], +10[4], +14[2],−15[3], +16[7], −17[8], +18[2], +20[7], +21[9], +2~4mar, and 14~74dmin. OCUM-11 (Fig. 7D): 62 65<3*n*, X, −X [4], add (X)(p11)[10], del (X) del (X)(p?) del (X)(q)[6], add (1)(p11)[10], del (2)(q21q31)[10], −3[10], add (3)(q11)[10], −4[10], add (4)(q35)[10], add (6)(p21)[10], add (6)(q21)[10], −7[10], −9[10], add (9)(p22)[10], −10[10], del (10)(p11)[10], −11[10], −13[10], add (14)(q24)[10], add (16)(p11)[10], −17[10], −18[10], −19[9], +20[10], −21[10], del (22)(q13)[8], +der (?) t (?; 3)(?;q11)[10], +mar1[10], +mar2[10], +mar3[9], +mar4[7], and +0~1mar.

Genetic alterations. OCUM-8 cells had LOH at the *APC (D5S346)* and *c-met (D7S501)* loci, and a band shift *(D3S1611)* was found. OCUM-11 cells showed 3 of 11 (27%) microsatellite loci, *D2S123, P53-Dinucl, and P53Penta*, with a novel band shift, and OCUM-11 cells had LOH at the *APC (D5S346)* locus (Fig. 8). OCUM-11 cells were defined as MSI-positive (Table 2), but no mutations were found in *Fas antigen, BAX, TGFβRII, IGFR II, hMSH3* and *hMSH6*.

Discussion

We have developed two new scirrhous gastric cancer models *in vivo*. Histologically, these orthotopic models showed proliferation with extensive fibrosis, resembling human scirrhous gastric cancer. No objective criteria for deciding the extent of stromal fibrosis have been set for gastric carcinoma. The Japanese Classification of Gastric Carcinoma defines the scirrhous type subjectively; stroma is abundant.[17] Our orthotopic tumors resembled the scirrhous type presented in the Japanese Classification of Gastric Carcinoma,[18] and were therefore concluded to be of scirrhous type. Most *in vivo* studies of scirrhous gastric cancer have used xenografted tumors implanted subcutaneously,[12, 19–22], and subcutaneous scirrhous gastric cancer cells show medullary growth. Our orthotopic models may be more useful to study scirrhous gastric cancer than subcutaneous xenograffed tumor. Organ specificity may contribute to the histology of gastric cancer,[1] and a microenvironment inducing fibroblasts may be important for cancer development.[20, 23] We previously reported that stromal cells are important for differentiation of scirrhous gastric carcinoma.[23] KGF produced by orthotopic fibroblasts affected the development of scirrhous gastric carcinoma in a paracrine manner,[1] and KGF production was different among organ-specific stromal cells. Differences in KGF production in organ-specific microenvironments may produce differences in histologic type between gastric tumors and subcutaneous tumors. In addition, it has been reported that the proliferation of gastric fibroblasts was increased by conditioned medium from scirrhous gastric cancer cells *in vitro*.[24] These findings suggested that growth-promoting factors from gastric cancer cells and organ-specific fibroblasts might mutu-

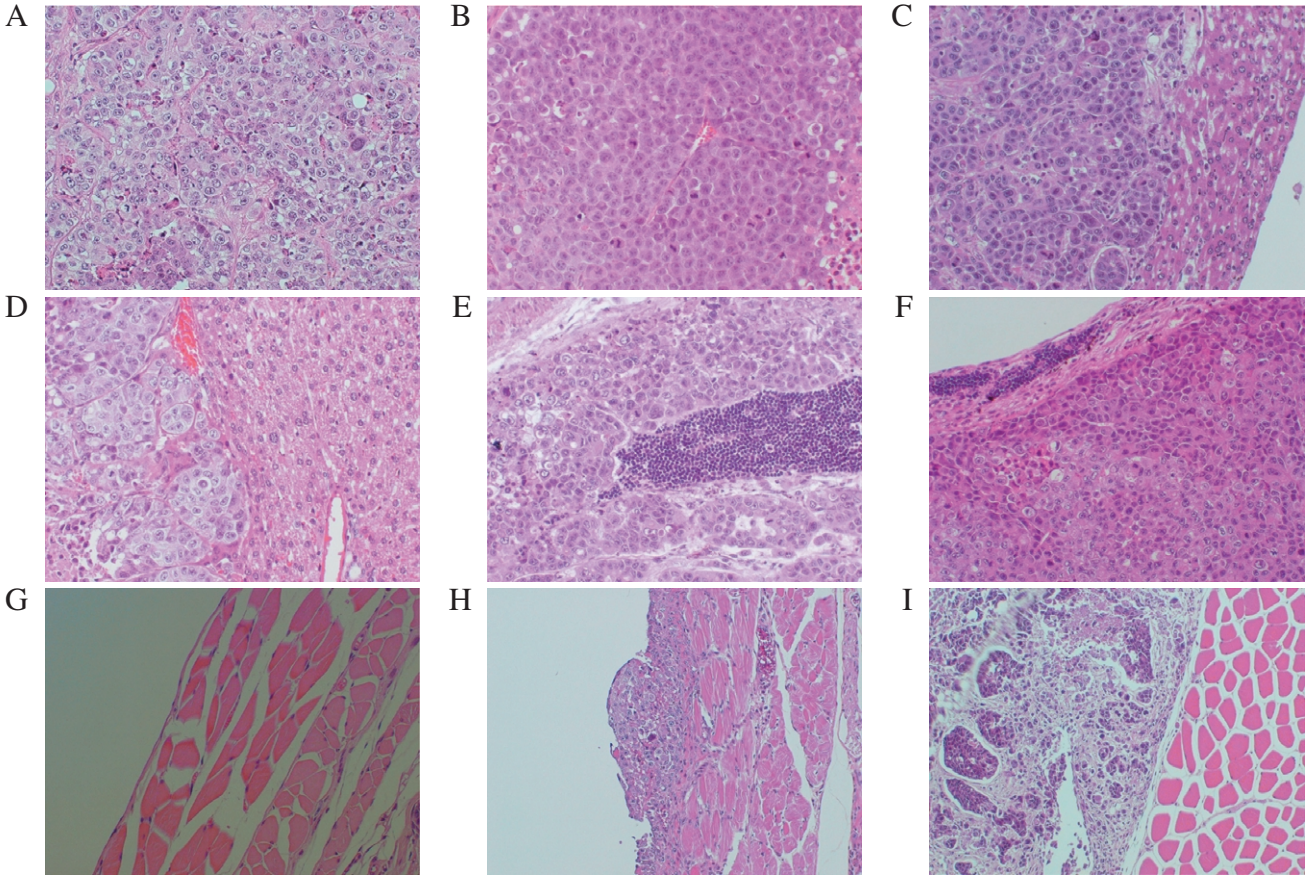

Fig. 4. Histologic findings of metastatic tumors. Xenografted tumors, OCUM-8 (A) and OCUM-11 (B), showed medullary growth. Liver tumor (C, OCUM-8; D, OCUM-11) and lymph node tumor (E, OCUM-8; F, OCUM-11) showed medullary growth with less fibrosis. The normal peritoneum of nude mice consists only of a monolayer mesothelium with a thin layer of connective tissue (G). Peritoneal metastatic tumor after injection of cells i.p. (H, OCUM-8; I, OCUM-11) showed metastatic cancer cells in the thickened fibrous peritoneum. H&E staining.

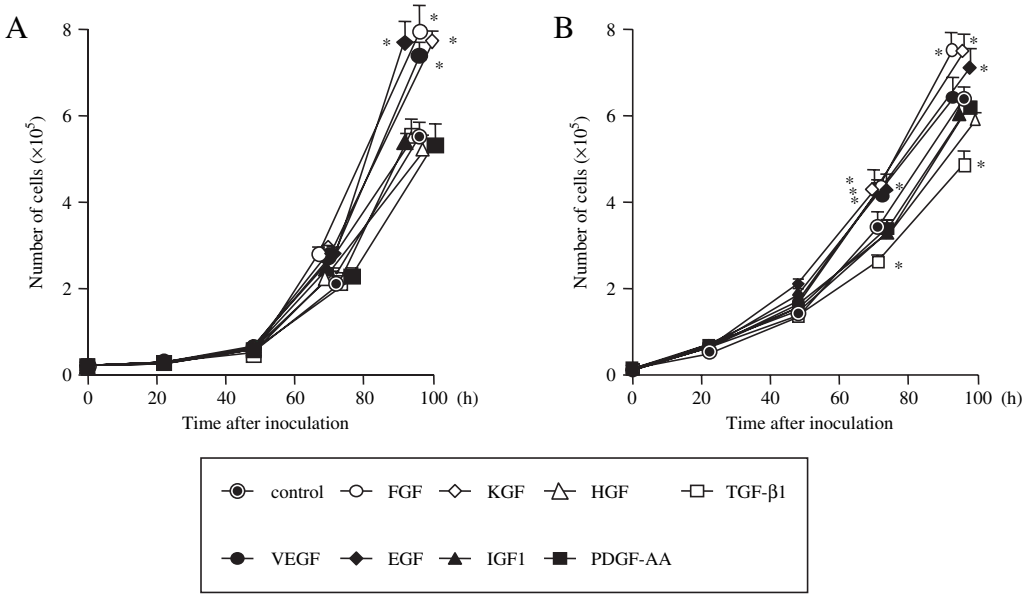

Fig. 5. Effect of growth factors on proliferation of OCUM-8 and OCUM-11 cells. OCUM-8 (A) and OCUM-11 cells (B) were cultured for 100 h at factor concentrations of 10 ng/ml. Growth of OCUM-8 and OCUM-11 cells was increased significantly by adding KGF, FGF, or EGF. VEGF showed a meaningful increase only for OCUM-8 cells. TGF-β1 showed a greater inhibitory effect on OCUM-11 cells. Other growth factors had no significant effect. Results are presented as the mean for three independent experiments; bars indicate SD. * $P<0.01$ *versus* control. EGF, epidermal growth factor; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; IGF-I, insulin-like growth factor-I; b-FGF, basic fibroblast growth factor; TGF-β1, transforming growth factor β1.

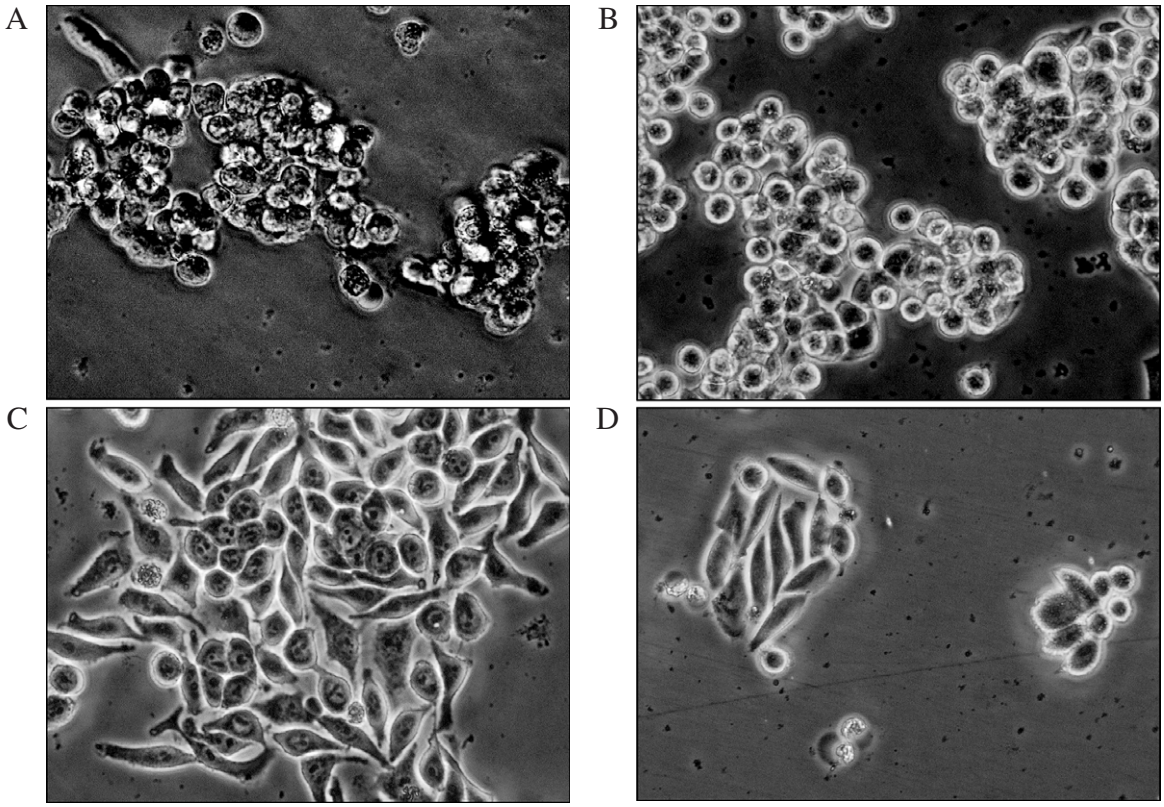

Fig. 6. Morphology of OCUM-8 and OCUM-11 cells. Both OCUM-8 (A) and OCUM-11 cells (B) were round and formed loose cell aggregates. An increase in the number of attached and spreading cells (C, OCUM-8; D, OCUM-11) was seen following the addition of 10 ng/ml TGF-β1 after 72 h of culture.

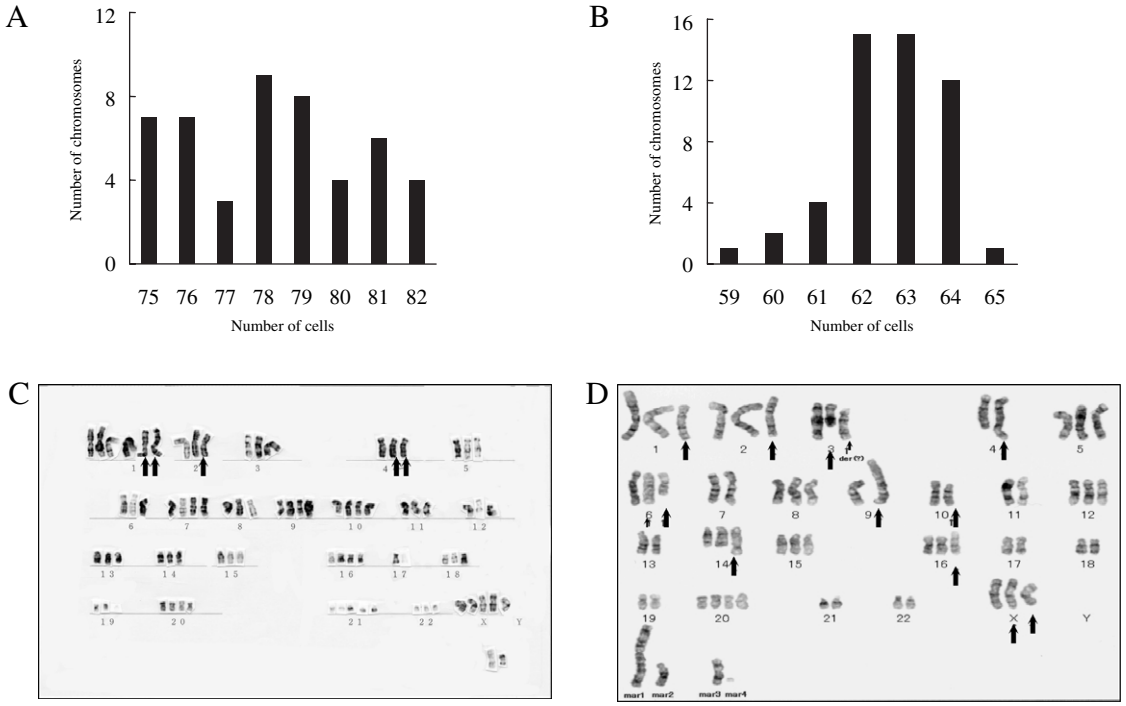

Fig. 7. Distribution of chromosome numbers. OCUM-8 (A) and OCUM-11 (B) cells were analyzed for chromosome number. G-Banded karyotypes of OCUM-8 (C) and OCUM-11 (D). Arrows, breakpoints present.

ally increase proliferation of the other cell type, which might result in the characteristic histology of scirrhous gastric carcinoma. These orthotopic implantation models developed pyloric stenosis and expansion of the stomach in mice, similar to the

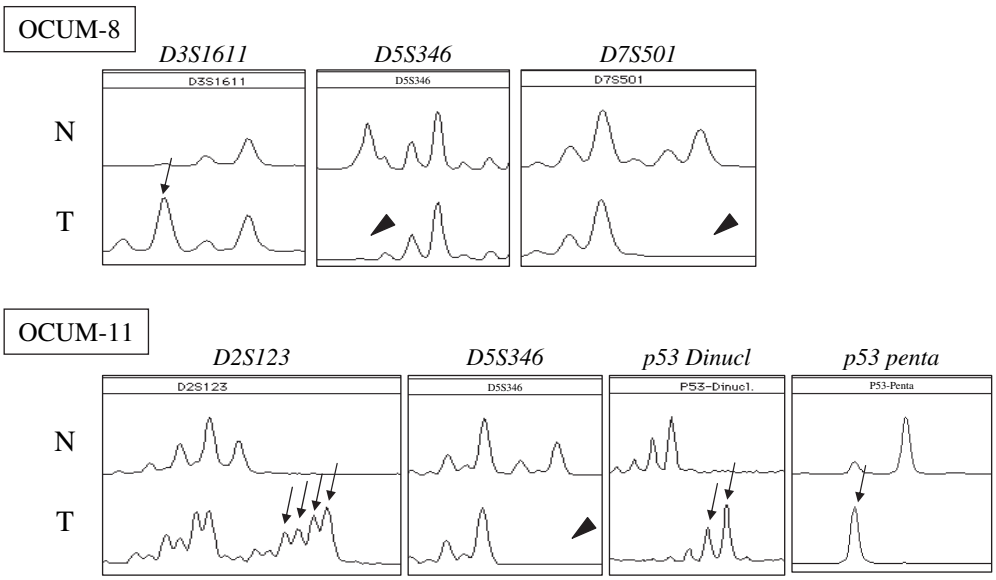

Fig. 8. LOH and MSI at microsatellite markers. DNA from OCUM-8 or OCUM-11 cells (T) and MS-8 or MS-11 (N) was analyzed. Arrowheads show LOH, and arrows show band-shifts in the cancer cell lanes.

Table 2. Genetic alterations in OCUM-8 cells and OCUM-11 cells

Cell line	Microsatellite marker											MSI
	D1S2883 (1q, *HPC1*)	*D2S123* (2p, *hMSH2*)	*D3S1611* (3p, *hMLH1*)	*D5S346* (5q, *APC*)	*D7S501* (7q, *c-met*)	*NM23* (17q, *nm23*)	*P53-Dinucl* (17p, p53)	*P53-Penta* (17p, p53)	*D18S35* (18q, *DCC*)	*BAT25*	*BAT26*	
OCUM-8	–	–	Shift	LOH	LOH	–	–	Ho	–	–	–	–
OCUM-11	–	Shift	Ho	LOH	Ho	–	Shift	Shift		–	–	+

LOH, loss of heterozygosity; Ho, homozygous; Shift, markers with a novel allele when compared to normal tissue; –, no LOH or microsatellite stable; MSI, microsatellite instability.

findings in human scirrhous gastric carcinoma. These models, formed by the orthotopic implantation of OCUM-8 or OCUM-11 cells, could therefore contribute to the elucidation of proliferative mechanisms *in situ* and to the development of therapy.

Liver metastases and lymph node disease developed in our orthotopic implantation models. The latter is the most frequent metastatic pattern in human scirrhous gastric carcinoma.[2, 25] The orthotopic tumors of OCUM-8 and OCUM-11 cells showed infiltrating growth and indistinct borders from the surrounding tissue in part. In addition, these orthotopic tumors presented lymphatic invasion. These findings suggested that orthotopic tumors of OCUM-8 and OCUM-11 can grow invasively. Liver metastasis was found in only one mouse in each of the groups inoculated with the two cell lines, and no peritoneal metastasis was found. Orthotopic tumors presented lymphatic invasion. Although it is difficult to clarify the mechanism of liver metastasis on the basis of one mouse, lymphogenous metastasis might be associated with this model. Thus, our models might not be useful for the study of liver metastasis and peritoneal metastasis. In contrast, these orthotopic models showed the scirrhous type, and developed frequent lymph node metastasis, so they should be useful for the study of therapeutic approaches for primary tumor and lymph node metastasis. Peritoneal dissemination was found after i.p. inoculation of the two cell lines. Such metastatic patterns are the most frequent cause of death and recurrence in scirrhous gastric cancer.[2] These metastatic models may be useful for analysis of the mechanisms of the metastatic process and for the development of therapy. Histologically, the peritoneal metastatic lesion was scirrhous, while liver metastases, and lymph node involvement showed medullary growth. These histologic differences in metastatic loci might be useful for analysis of the biology of metastasis.

Scirrhous gastric carcinoma cells have been reported to grow singly or in clusters in culture medium.[26] Our two cell lines exhibited these features. The doubling times were 13.4 h and 11.3 h, which are high compared with other reports.[5, 8–10, 13] Most gastric cancer cells produce some tumor-associated antigens[26]; our cells produced the tumor-associated antigens CEA, CA19-9, SPan-1, and SLX at high concentrations.

KGF was a strong stimulator of proliferation for both cell lines *in vitro*. KGF, a member of the fibroblast growth factor (FGF) family also known as FGF-7,[27] is produced by mesenchymal cells in various tissues.[28, 29] KGF exerts its effect in a paracrine manner limited to epithelial cells, while other FGF family members stimulate growth of cultured endothelial cells and fibroblasts.[27, 29] Several types of FGF receptor (FGFR) have been reported, and FGFR-2 or KGF receptor (KGFR) is identical with the K-*sam-II* gene product. The K-*sam-II* gene was first amplified and identified in an extract from the human scirrhous gastric cancer cell line KATO-III.[5] K-*sam-II* has been reported to be expressed preferentially in scirrhous gastric cancer.[30] OCUM-8 and OCUM-11 strongly expressed *KGFR* mRNA.[1] These findings suggest that KGF is important in progression of scirrhous gastric cancer.

EGF and bFGF also significantly stimulated growth in both cell lines. There have been reports that the level of EGF/EGFR is correlated with tumor invasion, frequency of metastasis, and prognosis for human gastric carcinoma.[31] On the other hand, following addition of TGF-β1, growth of OCUM-11 cells, but not OCUM-8 cells, decreased significantly. TGF-β1 has been reported to decrease the growth of epithelial cells; however, the growth of some gastric carcinoma cell lines was not decreased by TGF-β1 because of escape from negative regulation by TGF-β1 at the receptor level.[32] Morphologic changes, includ-

ing attached and spreading cells, were recognized in both cell lines following the addition of TGF-β1. It was reported that TGF-β1 differentially regulates cell activation and proliferation through Smad2-dependent and Smad3-dependent pathways, respectively.[33, 34] OCUM-8 cells may have a different TGF-β1 signal for proliferation as opposed to morphology because of a dysfunction of Smad3. TGF-β1 has been reported to induce invasion by cells. These morphologic changes induced by TGF-β1 may be associated with invasiveness.

Recent studies have revealed that genetic instability is an important predisposition for human multistep carcinogenesis.[35] MSI was observed in 10 to 20% of gastric carcinoma cases,[15] while the reported prevalence of MSI in human scirrhous gastric carcinoma has varied between studies, ranging from 5% to 75%.[35, 36] Although some colorectal cancer cell lines have MSI status,[15] few researchers have pursued the establishment of MSI-positive gastric carcinoma cell lines. OCUM-11 cells had MSI status, and therefore might be useful for the study of the characteristic features of MSI in scirrhous gastric carcinoma. no frameshift mutation of the *TGFβ-RII*, *BAX*, or *hMSH3* genes was detected in OCUM-11 cells, whereas *TGFβ-RII*, *BAX*, and *hMSH3* frameshift mutations have been reported to be frequent in MSI colon cancers.

In conclusion, we have established two new scirrhous gastric cancer cell lines, designated OUCM-8 and OCUM-11, which have the characteristic biology of scirrhous gastric carcinoma *in situ*. The two cell lines may be useful for analyzing disease progression and for developing novel therapeutic options.

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