

Establishment and characterization of novel gastric signet-ring cell and non signet-ring cell poorly differentiated adenocarcinoma cell lines with low and high malignant potential

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Abstract

Background Poorly differentiated signet-ring cell carcinoma (SRCC) and non signet-ring cell carcinoma (NSRCC) are prevalent histological subtypes of gastric cancers with distinct morphological features. To date, however, the molecular basis of their growth, differentiation, and metastasis still remains unclear, because of the limitation of available cell lines.

Methods In the present study, we established novel SRCC and NSRCC cell lines (designated GPM-2 and GPM-1) derived from the ascites of two individual gastric cancer patients with peritoneal metastasis.

Results Immunohistochemical and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis revealed that GPM-2 cells showed both gastric and intestinal differentiation phenotypes (E-cadherin+

MUC5AC+/MUC6+/Villin+), and formed xenografted tumors with typical SRCC histology in nude mice. In contrast, GPM-1 cells only weakly expressed differentiation markers, showing a phenotype of E-cadherin^{low}+ / MUC2-/MUC5AC-/Villin^{low}+. Characteristically, GPM-2 cells were found to highly express both membrane-bound mucin (MUC1/MUC4) and secreted mucin glycoproteins (MUC5AC/MUC6), whose expression is regulated by an epigenetic mechanism such as histone acetylation. GPM-2 cells also secreted a large amount of sTn antigen into the culture medium. These mucin profiles of GPM-2 cells are distinct from those of conventional SRCC cell lines (KATO III and HSC-39), which preferentially express intestinal MUC2/MUC4 as well as sLe^x and sLe^A antigens. In addition, GPM-2 cells showed a slow growth rate, and a lower metastatic potential than GPM-1 cells.

Conclusions These results indicate that the cells of the new SRCC line, GPM-2 cells, are more differentiated and less aggressive than NSRCC-type GPM-1 cells, and would thus offer an excellent model for understanding the molecular mechanism underlying the growth, differentiation, and mucin production of an SRCC gastric cancer cell line.

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Introduction

Although the survival of patients with gastric cancer has improved due to the development of new diagnostic and therapeutic modalities, the disease remains one of the leading causes of cancer death in east-Asian countries, as

well as in some western countries [1]. Gastric cancers are histologically heterogeneous and have been subclassified as intestinal and diffuse types of adenocarcinoma [2]. Diffuse-type gastric cancers consisting of single or small clusters of tumor cells correspond to poorly differentiated gastric cancers in the WHO classification and include heterogeneous subtypes such as signet-ring cell (SRCC) type and non signet-ring cell (NSRCC) type [3]. SRCCs have been considered to be closely associated with scirrhous-type gastric cancers upon progression and therefore are generally characterized by their poor prognosis [4]. In fact, some investigators have reported that SRCC is a major and independent factor for poor prognosis due to its specific characteristics, including a high rate of lymphatic spread and peritoneal dissemination [4]. However, others have reported that the survival of patients with early SRCC was not significantly worse or was even better than that of patients with other types of early gastric carcinoma [5–7]. Thus, the results of the survival studies and studies comparing the malignant potential of SRCC and NSRCC are conflicting. The reasons for the differences reported in the pathobiological behavior of SRCC in different studies are because few reproducible *in vitro* and *in vivo* analyses of the biological behavior of SRCC have been conducted using established cell lines. Therefore, the development of an *in vitro* model system is an important approach to clarify the pathobiological characteristics of SRCC. At present, there are more than 20 available gastric cancer cell lines; however, few SRCC gastric cancer cell lines have been reported (KATO III, HSC-39, and Mz-Sto-1), and these cell lines have not been fully delineated so far [8–10].

The unique biological feature of SRCC is the production and accumulation of abundant mucin in the cytoplasm and plasma membrane. Altered glycosylation and splicing forms of mucin core proteins have been widely used as tumor biomarkers to evaluate the risk of various malignancies [11]. In addition, recent studies have provided evidence on the important biological roles of secreted and membrane-bound mucins in the pathogenesis of gastrointestinal malignancies, such as their roles in growth and metastasis [12, 13]. In gastric cancers, tumor cells produce MUC1, MUC5AC, and MUC6, as does normal stomach mucosa, and the *de novo* expression of MUC2 and MUC4 is also shown in intestinal metaplasia and related malignancies [14]. MUC1 and MUC4, which are membrane-bound mucins, could be involved in dysregulated cell proliferation through increased receptor-mediated signal transduction [15, 16]. On the other hand, MUC2, MUC5AC, and MUC6, which are known to be secreted-type mucins, form gels that serve as physiological barriers [17]. However, the regulatory role played by these MUC glycoproteins in the growth, differentiation, and metastasis of gastric cancers still remains largely unknown.

In the present study, to increase knowledge about the pathobiology of poorly differentiated gastric cancers, we established new SRCC and NSRCC gastric cancer cell line. To develop new therapeutic approaches for these cancers, we also established xenografted tumors from these cells in nude mice. In these clinically relevant cell lines, we found that the new SRCC line had characteristics that were distinct from those of both the NSRCC cell line and conventional SRCC lines, in terms of growth, invasion/metastasis, and mucin production.

Materials and methods

Source of cell lines

The NSRCC cell line GPM-1 and the SRCC cell line GPM-2 were established from malignant ascites obtained from a 57-year-old Japanese man and a 37-year-old Japanese man, respectively, both suffering from stage IV gastric cancer with peritoneal dissemination. The ascites used in this study were obtained from the patients, with written informed consent, at the Department of Gastroenterological Surgery, Aichi Cancer Center Hospital.

Establishment of cell lines

Ascites (100 ml) from each patient was collected at the beginning of each of their operations and aspirated into tubes. Intact cells that were collected from the ascites by centrifugation at 1,200 rpm for 10 min at 4°C were rinsed with phosphate-buffered saline (PBS). Cell pellets were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin in plastic dishes (Falcon, Franklin Lakes, NJ, USA) and incubated for 1–2 h on plastic dishes at 37°C to allow for fibroblasts and mesothelial cells to attach to the plastic dishes. Medium containing floating tumor cell clusters was collected and centrifuged. The cell pellets were resuspended in fresh DMEM with 10% FBS and cultured on plastic dishes (Falcon, BD Labware, Franklin Lakes, NJ, USA) in a humidified 5% CO₂ incubator at 37°C. After several weeks, growing colonies were harvested with trypsin–ethylenediamine tetraacetic acid (EDTA) (0.125%/2 mM) and passaged several times on plastic dishes. During this time, any remaining fibroblasts were removed by mechanical scraping and a differential attachment selection method with trypsin–EDTA, after which pure epithelial cell cultures were obtained as described previously [18]. Other cell lines (GCIY and KATO III) were obtained from the RIKEN Cell Bank

(Tokyo, Japan). The HSC-39 cell line was kindly provided by Professor K. Yanagihara (Yasuda Women's University, Hiroshima, Japan). These cell lines have now been cultured for 1 year without apparent phenotypic change. There was no contamination of these cell lines with *Mycoplasma pulmonis* or mouse hepatitis virus, as confirmed by a polymerase chain reaction (PCR) method.

In vitro cell growth assay

GPM-1 and GPM-2 cells were plated at 1×10^4 cells/96-well plastic plate in DMEM supplemented with 10% FBS. The number of viable cells was counted with a hemocytometer, in triplicate, every 24 h for 3 days after seeding.

Quantitative reverse transcription (qRT)-PCR analysis

Total RNA was extracted from cells dissolved in ISOGEN (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using SuperScript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resultant first-strand cDNA was stored at -80°C until analysis. Single-step real-time RT-PCR using the Universal ProbeLibrary system (Roche Diagnostic, Mannheim, Germany) was performed using specific oligonucleotide primer pairs and Taqman probes (Roche Diagnostic, Mannheim, Germany) on a LightCycler (Roche Diagnostic, Mannheim, Germany) instrument as described previously [19]. The sequences (5'-3') of the primer pairs and the Taqman probe number (#) used in this study are shown in Table 1S (electronic supplementary material).

Effects of sodium butyrate (NaBT), trichostatin (TSA), and 5-Aza-2'-deoxycytidine (5-Aza) on MUC mRNA expression

GPM-2 and KATO III cells were trypsinized and seeded at 1×10^6 cells/60-mm dish and allowed to grow overnight at 37°C . Cells were treated with the histone deacetylase (HDAC) inhibitors NaBT (1 mM) (Sigma Chemical, St. Louis, MO, USA) and TSA (50 ng/ml) (Biomol, Plymouth Meeting, PA, USA), and an inhibitor of DNA methylation, 5-Aza (5 μM) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cells were rinsed with PBS, dissolved in ISOGEN, and stored -80°C until processing for total RNA extraction.

Assay for tumor-associated carbohydrate antigens

Carbohydrate antigen (CA) 19-9 and sialyl Lewis X antigen (sLe^x) (CSLEX, Nittobo Medical Co., Tokyo, Japan) levels in the conditioned medium of 4 cell lines (GPM-1, GPM-2, KATO III and HSC-39) were examined using an enzyme immunoassay (EIA), and CA72-4 levels in the

medium were measured using an electronic chemiluminescent enzyme immunoassay (ECLIA) in a commercial laboratory (SRL, Tokyo, Japan).

Immunohistochemistry

Xenografted tumors in nude mice were removed and fixed in 10% buffered formalin for 24 h and embedded in paraffin. Tissue sections at 5- μm thickness were stained with hematoxylin and eosin (H&E) for histopathological examination. Immunohistochemical staining was carried out as described previously [18] with the following mouse monoclonal antibodies against the human antigens; MUC2 (CLH2, Novocastra Laboratories, Newcastle, UK), MUC5AC (clone CLH2, Novocastra Laboratories), MUC6 (CLH5, Novocastra Laboratories), villin (clone 12, Transduction Laboratories, Lexington, KY, USA), epidermal growth factor receptor (EGFR, PharmDx Kit; DakoCytomation, Glostrup, Denmark), carcinoembryonic antigen (CEA, clone II-7; DaKoCytomation), P53 (DO-7, DaKoCytomation), and E-cadherin (clone NCH-38, DakoCytomation). Rabbit polyclonal antibodies to human human epidermal growth factor receptor 2 (HER2) (DakoCytomation) were also used. For antigen retrieval, the sections were microwave-treated at 98°C for 10 min. After the blocking of nonspecific reactions by normal serum for 30 min, these sections were incubated at 4°C overnight with the primary antibodies, thoroughly washed in PBS, then incubated with biotinylated secondary antibody. The sections were washed again with PBS and incubated with streptavidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) for 60 min. The sites of peroxidase binding were visualized using 0.01% diaminobenzidine (DAB) as a chromogen.

Assay for tumorigenesis and metastasis in nude mice

For the examination of tumorigenicity and the spontaneous metastatic potentials of the GPM-1, GPM-2, and KATO III cell lines in nude mice, growing cells were harvested with trypsin/EDTA and washed with PBS, and each cell line [5×10^6 to 1×10^7 cells in 0.2 ml Hank's balanced salt solution (HBSS)] was injected subcutaneously into the abdominal flanks of 7-week-old male nude mice of the KSN strain (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) maintained under specific-pathogen-free (SPF) conditions. Tumor growth was measured weekly with a sliding caliper. Tumor volume (V) was calculated using the formula: $V = a^2b/2$, where a is the minimum width and b is the maximum length. For orthotopic transplantation, cells (2×10^6 cells in 0.05 ml HBSS) were injected into the stomach serosa of nude mice, under 2, 2, 2-tribromoethanol anesthesia, with an abdominal incision.

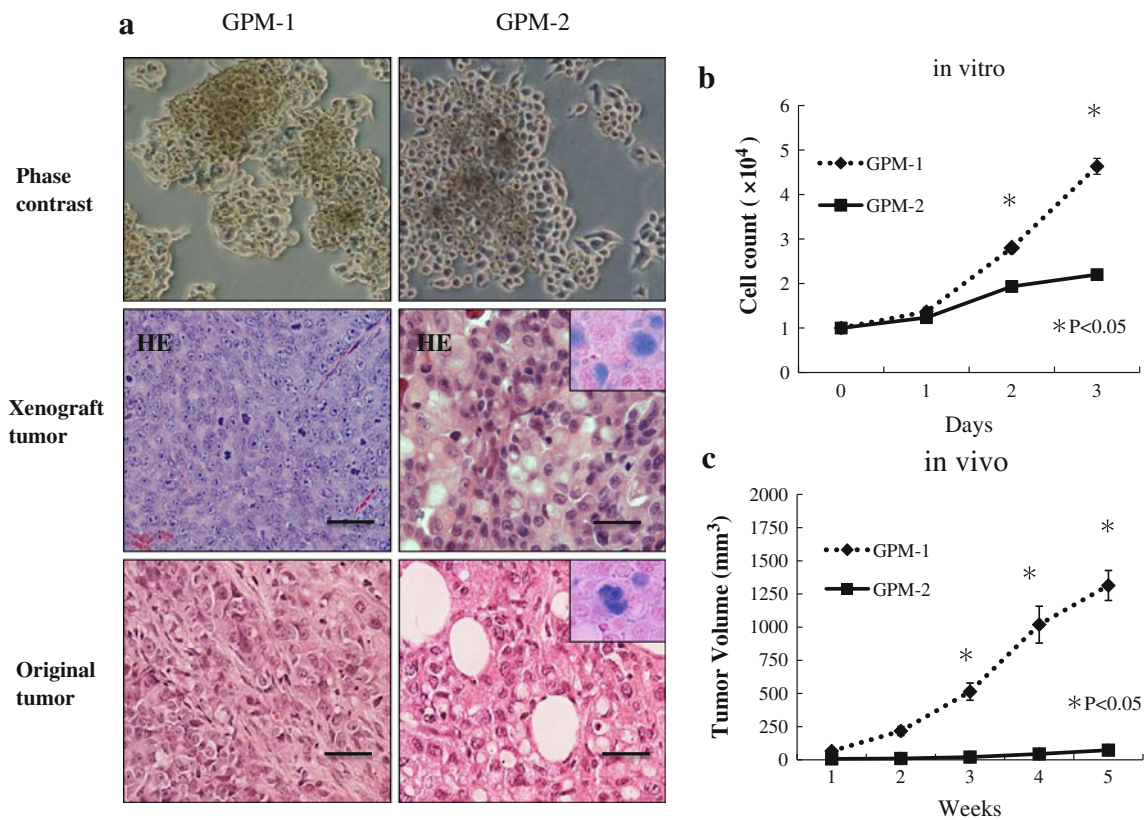


Fig. 1 Morphology and growth characteristics of GPM-1 and GPM-2 cells. **a** Upper images, phase-contrast photomicrographs of GPM-1 and GPM-2 cells in culture. GPM-1 cells showed a multilayered growth pattern, whereas GPM-2 cells grew partly in suspension. Bars 50 μm . **a** Middle and lower images, histology of xenografted tumor in

nude mouse and original stomach tumor, respectively (H&E staining). Insets Alcian blue staining. Bars 50 μm . **b** In vitro growth of GPM-1 and GPM-2 cells. Bars SE. **c** Subcutaneous tumor growth of GPM-1 and GPM-2 cells xenografted in nude mice. Bars SE. * $P < 0.05$

Mice bearing subcutaneous tumors or stomach subserosal tumors were autopsied 1–3 months after injection. Subcutaneous tumors, para-aortic lymph nodes, and stomach subserosal tumors in the abdominal cavity were removed and fixed in 10% buffered formalin. For examining peritoneal metastasis, 5×10^6 cells of the GPM-1, GPM-2, and KATO III cell lines resuspended in 0.4 ml HBSS were injected intraperitoneally into nude mice. Mice were sacrificed and peritoneal metastasis was examined 1–2 months after injection, both macroscopically and histologically. All experiments were carried out with the approval of the Institutional Ethics Committee for Animal Experiments of Aichi Cancer Center Research Institute and met the standard defined by the United Kingdom Co-ordinating Committee on Cancer Research guidelines.

Statistics

Student's *t*-test was used to evaluate statistically significant differences between groups. Significant differences were considered as $P < 0.05$.

Results

Morphological and growth characteristics of GPM-1 and GPM-2 cells

Phase-contrast microscopy showed that GPM-1 cells grew in a multilayered epithelial pattern, whereas GPM-2 cells grew in a pile-up pattern composed of mixed round and spindle-shaped cells with a partial floating growth pattern. Some GPM-2 cells contained intracytoplasmic vacuoles (Fig. 1a, upper column). Histological analysis of subcutaneous tumors in nude mice revealed that GPM-2 tumors were SRCC with Alcian blue-positive intracytoplasmic mucin, whereas GPM-1 tumors were poorly differentiated NSRCC without any glandular structures (Fig. 1a, middle column). The original stomach tumor of the patient from whose GPM-1 cells were established showed essentially the same histology as the xenografted tumors in nude mice (Fig. 1a, lower column). The growth rate of GPM-2 cells in vitro was significantly lower than that of the GPM-1 cell line (Fig. 1b). The in vivo growth of GPM-2 cells after

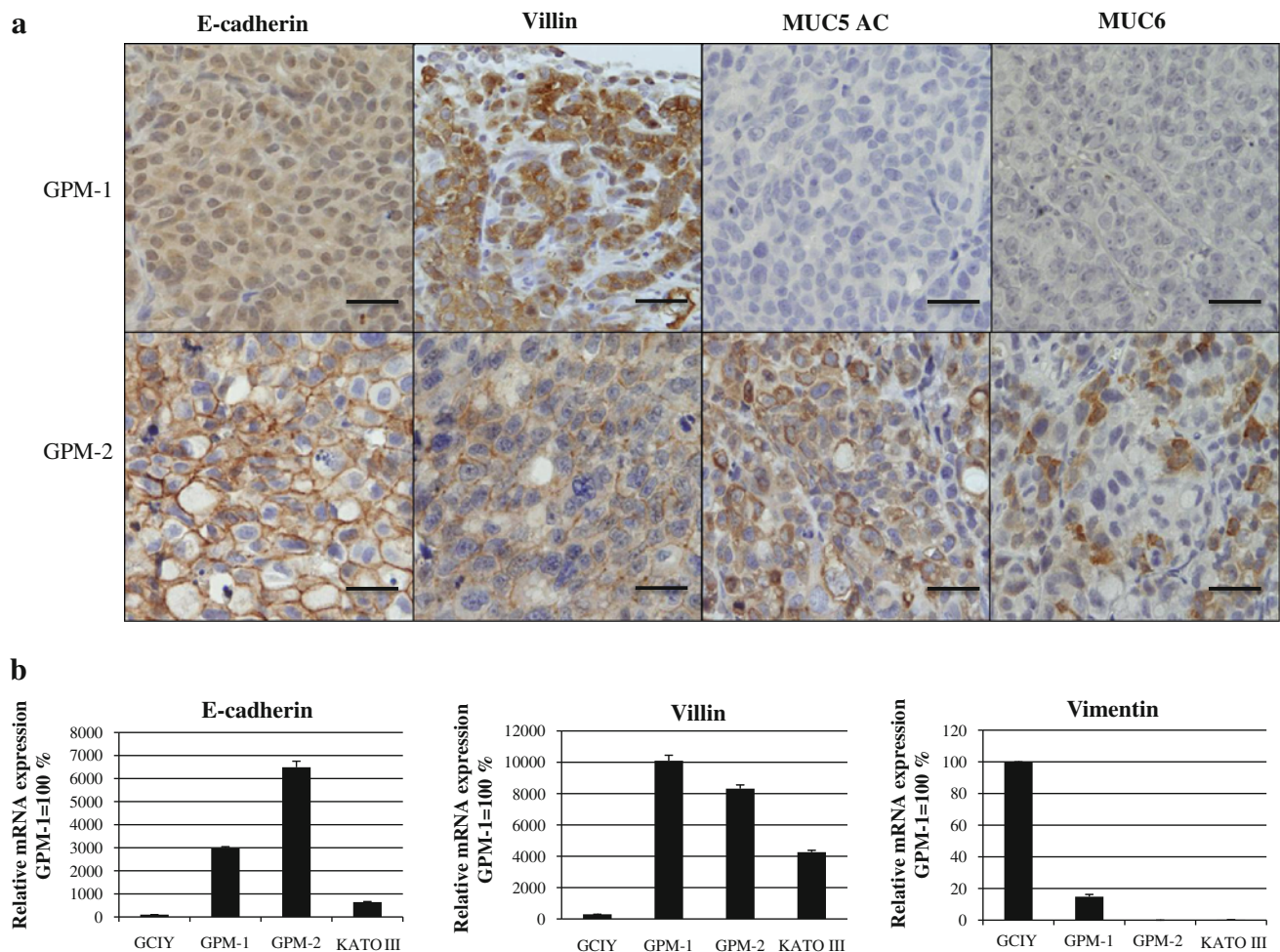


Fig. 2 Immunohistochemistry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of differentiation phenotypes of GPM-1 and GPM-2 cells. **a** Immunohistochemical staining of subcutaneous tumors in nude mice for E-cadherin,

subcutaneous injection into nude mice was also much slower than that of GPM-1 cells (Fig. 1c). At the periphery of the subcutaneous tumors, a strong inflammatory response was observed in GPM-2 tumors, but not in GPM-1 tumors, suggesting an anti-tumor immune response, probably against mucins secreted by GPM-2 cells.

Immunohistochemical and RT-PCR analysis of differentiation status

Immunohistochemical analysis of the subcutaneous tumors in nude mice revealed that GPM-1 cells were weakly positive for E-cadherin and for vimentin. As for gastrointestinal differentiation markers, MUC2, MUC5AC, and MUC6 were all negative, but villin was weakly positive. In contrast, GPM-2 cells were E-cadherin-positive and they were also positive for the intestinal markers villin/MUC4 as well as the gastric markers MUC5AC/MUC6 (Fig. 2a), indicating the mixed gastric and intestinal differentiation

MUC5AC, MUC6, and villin. Bars 50 μ m. **b** RT-PCR analysis of E-cadherin, villin, and vimentin mRNA expression of GPM-1 and GPM-2 cells in culture. Bars SE

phenotype of GPM-2 cells. Membranous CEA/EGFR and nuclear p53-positive staining patterns were also observed in both cell lines (data not shown).

qRT-PCR analysis of cell lines confirmed that GPM-2 cells highly expressed E-cadherin compared with GPM-1 and KATO III cells, another SRCC cell line. GPM-1 cells but not GPM-2 cells expressed a significant level of vimentin, but the expression level was much lower than that in GCIY cells showing typical epithelial mesenchymal transition (EMT) features. Both GPM-1 and GPM-2 cells expressed villin to a similar extent (Fig. 2b).

mRNA expression of the MUC glycoprotein family

RT-PCR analysis showed that GPM-2 cells highly expressed members of the MUC gene family, including MUC1, MUC4, MUC5AC and MUC6, but not MUC2. This expression pattern of MUCs was different from that of the other SRCC cell lines (HSC-39 and KATO III cells),

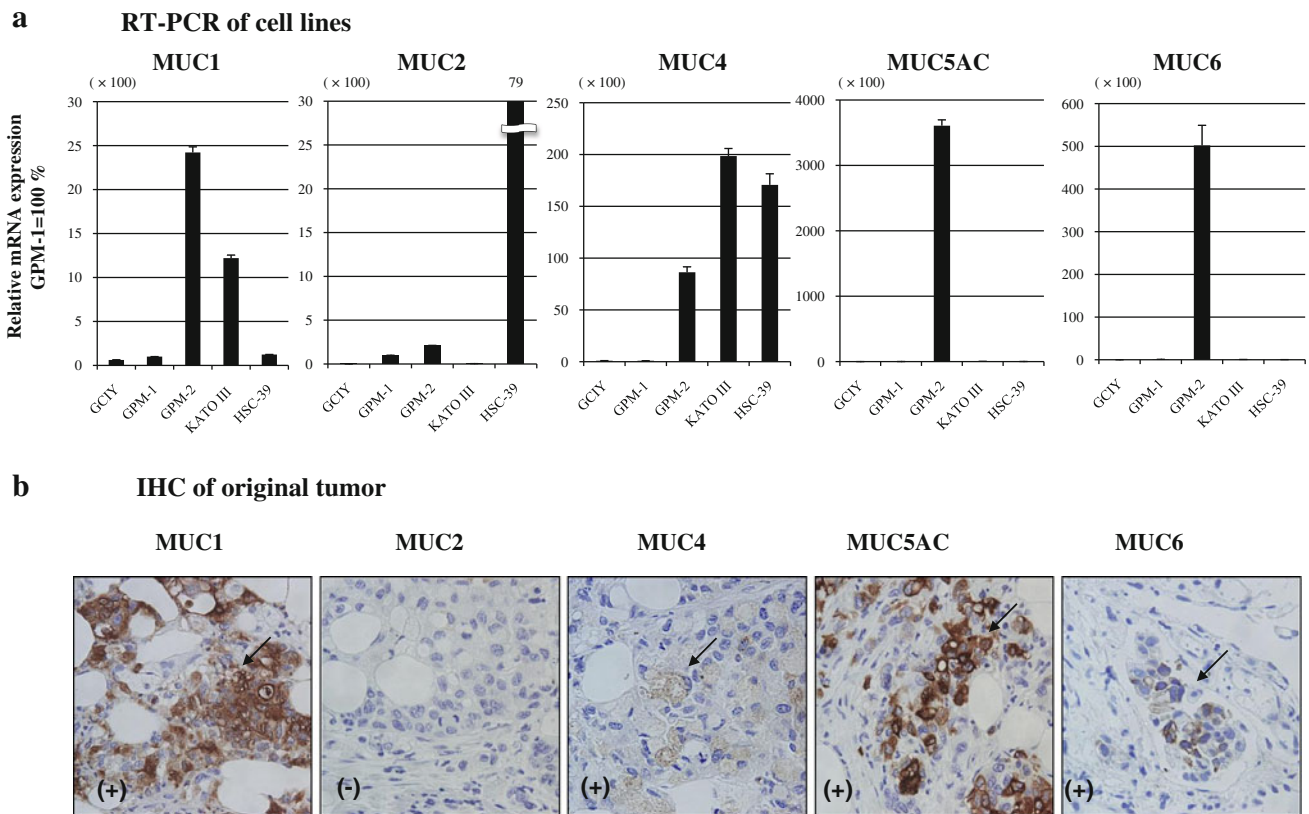


Fig. 3 Expression of a series of MUC glycoproteins in GPM-1 and GPM-2 cells and in the original stomach tumors of patients. **a** mRNA expression of MUC1, MUC2, MUC4, MUC5AC, and MUC6 in GPM-1 and GPM-2 cells. mRNA expression in other signet-ring cell carcinoma (SRCC) cell lines (KATO-III and HSC-39) and a non signet-ring cell carcinoma (NSRCC) (GCIY) cell line was also

measured as positive and negative controls. *Bars* SE. **b** Immunohistochemical staining of original stomach tumors for MUC1, MUC2, MUC4, MUC5AC, and MUC6. (+) and (-) indicate positive and negative staining, respectively. Positively stained tumor cells (*arrows*) are shown (objective, $\times 20$)

which express primarily MUC2/MUC4 and MUC1/MUC4, respectively. Gastric-type MUC such as MUC5AC and MUC6 was not expressed in the HSC-39 or KATO III cell lines. In contrast, GPM-1 cells did not substantially express any of these MUCs (Fig. 3a).

Immunohistochemical analysis of the original stomach tumor from which the GPM-2 cells were derived revealed that the original tumor cells significantly expressed MUC1, MUC4, MUC5AC, and MUC6, the pattern of which was quite similar to that of established cell lines (Fig. 3b).

Epigenetic regulation of mucin gene expression

up-regulated by the treatment with NaBT and TSA in the KATO III cell line (Fig. 4).

Secretion of tumor-associated carbohydrate antigens into culture medium

GPM-2 cells secreted abundant CA72-4 (sTn) into the culture medium, whereas GPM-1 cells did not secrete any carbohydrate antigens. In contrast to GPM-2 cells, KATO III and HSC-39 cells secreted CA19-9 (sLe^a) and CA19-9 plus CSLEX (sLe^x), respectively, (Table 1), indicating that GPM-2 cells had a secretion pattern of carbohydrate antigens different from that of KATO III and HSC-39 cells.

Metastatic potentials of GPM-1 and GPM-2 cells in nude mice

The tumorigenicity and metastatic potentials of GPM-1 and GPM-2 cells in nude mice and non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice are summarized and compared with those of conventional

In the GPM-2 cell line, the mRNA expressions of MUC1, MUC2, MUC4, MUC5AC, and MUC6 were strongly up-regulated by the treatment with the HDAC inhibitor NaBT. MUC2 and MUC4 expressions were also enhanced by TSA. On the other hand, 5-Aza treatment did not significantly affect MUC expression at the mRNA level, indicating the involvement of an epigenetic mechanism such as histone acetylation in the regulation of these MUC glycoproteins. MUC2, MUC4, and MUC6 were also

Fig. 4 Induction of MUC mRNA expression in GPM-2 and KATO III cells after treatment with histone deacetylase (HDAC) inhibitors (sodium butyrate [NaBt] and trichostatin [TSA]), and an inhibitor of DNA methylation (5-Aza-2'-deoxycytidine [5-Aza]), as measured by qRT-PCR. Bars SE. * $P < 0.01$

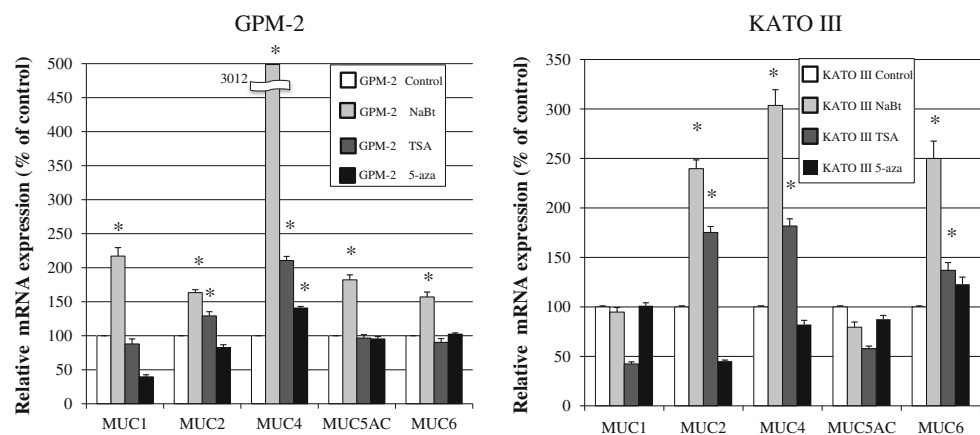


Table 1 Quantification of tumor-associated carbohydrate antigens (Units/ml) secreted into the culture medium

	GPM-1	GPM-2	KATO III	HSC-39
CA19-9 (sLea)	12.4	1.0	66.1	684
CSLEX (sLex)	2.5	2.5	2.5	24
CA72.4 (sTn)	1.7	133	1.5	2.3

Table 2 Tumorigenicity and metastatic potentials of GPM cell lines in nude mice compared with conventional gastric cancer cell lines

Cell lines	NSRCC	SRCC		
	GPM-1	GPM-2	KATO III	HSC-39 ^a
Tumorigenicity (sc)				
Nude mouse	6/6	4/6	1/8	5/5
NOD/SCID mouse	4/4	2/4	NT	ND
Orthotopic transplantation	4/4	1/4	NT	ND
Metastatic potential				
Peritoneum	3/3	0/3	0/4	4/4
Lymph node	4/7	0/6	NT	ND

SRCC Signet-ring cell carcinoma, NSRCC non signet-ring cell carcinoma, NOD/SCID non-obese diabetic/severe combined immune deficiency, sc subcutaneous, ND not described, NT not tested

^a Data cited from reference [9]

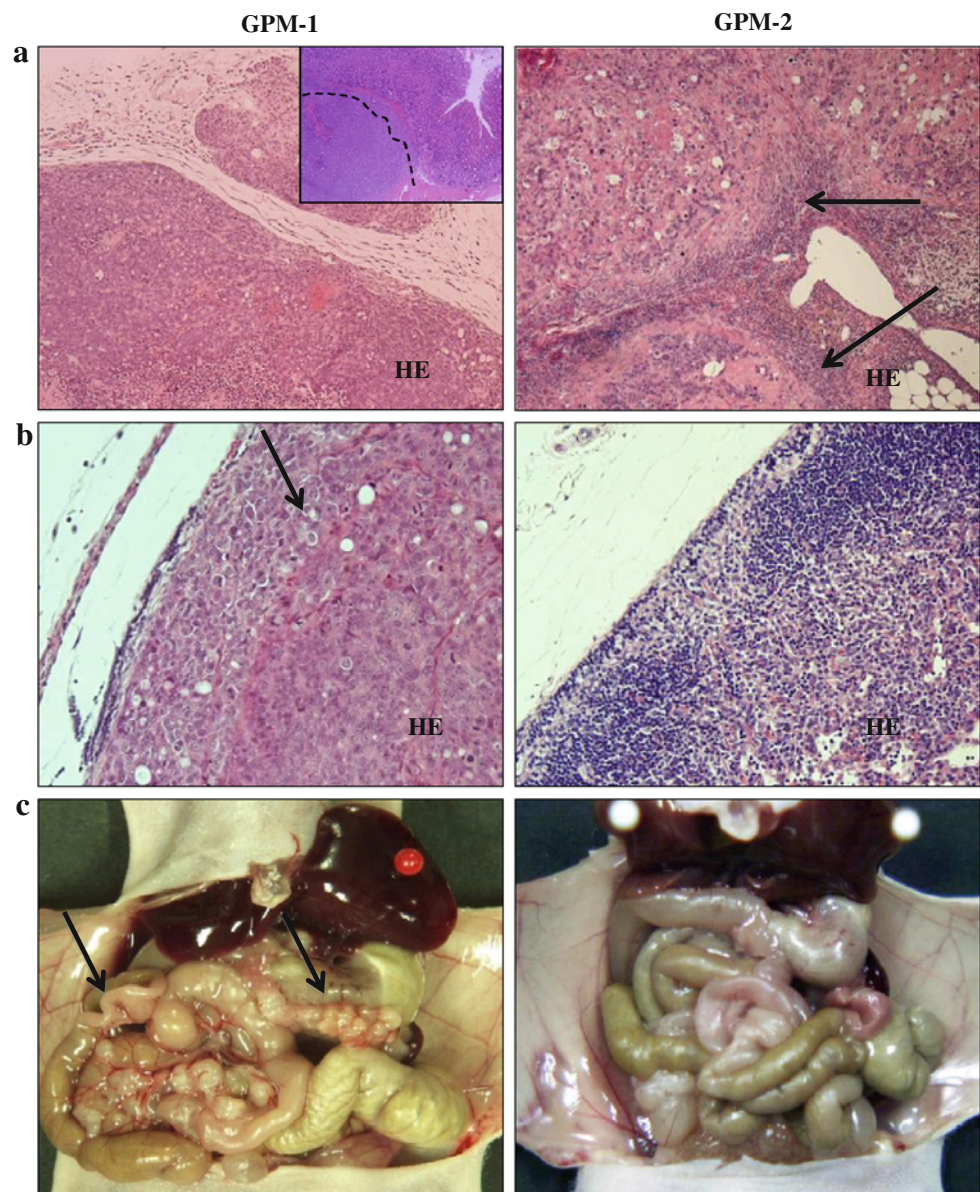
SRCC cell lines in Table 2. GPM-1 cells showed high tumorigenicity (100% = 6/6 or 4/4) in nude mice and NOD/SCID mice, whereas GPM-2 cells exhibited relatively low tumorigenicity (66% in nude mice and 50% in NOD/SCID mice). Furthermore, successful orthotopic transplantation into the stomach serosa (Fig. 5a, inset) was observed at an incidence of 100% (4/4) and 25% (1/4), respectively, for GPM-1 cells and GPM-2 cells. Apoptosis of tumor cells and inflammatory cell infiltration around the tumor nest were observed only in GPM-2 tumors in nude mice (Fig. 5a). As for metastasis to other organs, GPM-1 cells showed para-aortic lymph node metastasis and peritoneal metastasis at an incidence of 57% (4/7) and 100% (3/3), respectively. In contrast, GPM-2 cells showed no lymph node or peritoneal metastasis (Fig. 5b, c).

Discussion

In the present study, we established novel SRCC (GPM-2) and NSRCC (GPM-1) cell lines from the ascites fluids of two gastric cancer patients with peritoneal metastasis. The GPM-2 cell line formed tumors with typical SRCC histology in nude mice, similar to the histology of the conventional SRCC lines KATO III and HSC-39 cells, but the GPM-2 histology showed unique characteristics distinct from those of the conventional SRCC lines, as follows: (1) GPM-2 cell line tumors consisted predominantly of adherent cells and partly of floating cells in culture. In contrast, KATO III and HSC-39 cells grow totally in suspension on plastic [8, 9]. (2) GPM-2 cells exhibit more differentiated phenotypes, such as E-cadherin, MUC, and

Fig. 5 Primary tumor and lymph node and peritoneal metastasis of GPM-1 and GPM-2 cells in nude mice.

a Subcutaneous tumor in nude mouse. Inflammatory cell infiltrations around the tumor periphery are seen only in the GPM-2 tumor (*arrows*). Orthotopically transplanted GPM-1 tumor in the stomach serosa of nude mouse, showing invasion into mucosa, is seen in the *inset*. **b** Para-aortic lymph node metastasis formed 12 weeks after subcutaneous injection of tumor cells. Lymph node is replaced by GPM-1 tumor cells (*arrow*). **c** Macroscopic findings of intraperitoneal metastasis in nude mice after intraperitoneal injection of GPM-1 and GPM-2 cells. Peritoneal metastases (*arrows*) are seen only in the mouse bearing GPM-1 tumors



villin expression, than KATO III and HSC-39 cells. (3) GPM-2 cells exhibit an expression pattern of MUC core proteins and cancer-associated carbohydrate antigens that is distinct from the pattern in KATO III and HSC-39 cells. GPM-2 cells express both gastric-type mucins (MUC1/MUC5AC/MUC6) and intestinal-type mucins (MUC2/MUC4) and secrete abundant sTn antigen extracellularly. In contrast, KATO III and HSC-39 cells predominantly express intestinal-type mucin (MUC2/MUC4) and secrete sLe^x and sLe^a antigens. (4) GPM-2 cells have low tumorigenic potential, are slow-growing both in vitro and in vivo, and have no metastatic potential, suggesting low malignant potential. GPM-2 cells would thus offer an excellent gastric SRCC model for understanding the carcinogenesis, progression, and heterogeneity of gastric SRCC.

To date, the prognostic significance of SRCC is still unclear. In the present study, we found that a new SRCC line, GPM-2 cells, exhibited a differentiated phenotype and low aggressive behavior. In addition, we found an inflammatory response around GPM-2 subcutaneous tumors in nude mice, probably in response to extracellularly secreted mucins. These results suggest that the growth retardation of GPM-2 tumors in nude mice may be due not only to the low growth rate of the tumor cells themselves, but may also be due to the host inflammatory or immune response exerted by macrophages and natural killer (NK) cells [20]. These experimental findings are consistent with the clinical observation that early SRCC appears to have a behavior distinct from that of NSRCC, and a better prognosis [5]. Therefore, the GPM-2 cell line could be useful

for further study to experimentally clarify whether or not the transition or progression from SRCC to scirrhous carcinoma can occur after an additional gain of genetic or epigenetic changes.

GPM-2 cells overexpressed wide varieties of high-molecular-weight mucin glycoproteins. Among these glycoproteins, secreted mucins, including MUC2/MUC5AC and MUC6, form mucus and are known to serve as a barrier against physiological stress as well as bacterial infection. These glycoproteins also act as differentiation markers for specialized gastrointestinal epithelial cells, where MUC2 serves as a marker for intestinal goblet cells, MUC5AC for gastric foveolar cells, and MUC6 for pyloric glandular cells in normal gastric mucosa [17]. We found that GPM-2 cells highly expressed MUC5AC and MUC6, whereas KATO III and HSC-39 cells do not express MUC5AC and MUC6 at all. Nguyen et al. [21] investigated the mucin profiling of clinical gastric SRCC and demonstrated that the majority of 21 gastric SRCC cases expressed an intestinal mucin profile, such as MUC2/MUC4, but did not express gastric-type mucins such as MUC5AC/MUC6. These results suggest that GPM-2 cells, unlike conventional SRCC cell lines and clinical gastric cancer cases, have a unique MUC5AC and MUC6 double-positive mucin profile. On the other hand, membrane-bound mucins such as MUC1 and MUC4 are known to participate in the activation of EGFR/HER2 and downstream signal transduction through the EGF-like domain in the juxtamembrane region [22]. In fact, we found that GPM-2 cells expressed considerable amounts of EGFR and HER2 on their surfaces. Therefore, it is likely that MUC1 and MUC4 participate in the regulation of EGFR/HER2 signaling pathways, although the precise role of MUC1 and MUC4 in the signal transduction of GPM-2 cells remains to be elucidated. As for the regulation of MUC gene expression, Cdx2 and Sox2 are known to regulate MUC2 gene expression at the transcriptional level [23], but the mechanism of the gene regulation of other mucins remains largely unknown. We found that the HDAC inhibitor, NaBT, markedly enhanced the mRNA expression of all the MUC family members in GPM-2 cells. TSA, but not 5-Aza, also significantly increased the expression of MUC2 and MUC4 in GPM-2 cells, suggesting that an epigenetic mechanism, mainly via histone acetylation, is involved in the overexpression of the MUC gene family in GPM-2 cells, as reported previously [24, 25].

The expression of Lewis antigens (sLe^a and sLe^x) was high in 3 of the 4 gastric cancer cell lines we tested (GPM-1, KATO III, and HSC-39), whereas the expression of simple mucin-type antigen, sTn, was high in only 1 (GPM-2) of the 4 gastric cancer cell lines tested. These findings are consistent with previous reports, in that type 1 and type 2 Lewis antigens are widely expressed in most gastric

cancer cell lines, while simple mucin-type antigen expression is low or absent in gastric cancer cell lines [26, 27]. These findings suggest that GPM-2 cells overexpress sTn antigen, which is probably carried by core proteins of various MUCs, and therefore, is also useful for the functional analysis of the sTn antigen in gastric cancer.

In contrast to GPM-2 cells, our new NSRCC line, GPM-1 cells, showed the following unique characteristics: (1) they expressed a modest EMT phenotype, such as low vimentin and snail expression without fibroblastic morphology. (2) They showed rapid growth and high metastatic potential to lymph nodes and the peritoneal cavity, especially the latter, in a pattern of diffuse peritoneal carcinomatosis, indicating their high malignant potential.

In conclusion, we have established new SRCC and NSRCC cell lines and have demonstrated that these two lines are biologically quite distinct entities, although SRCC and NSRCC have both been categorized as poorly differentiated adenocarcinomas. These models promise to provide new insights into the biology and oncogenic mechanisms of gastric SRCC and NSRCC, and to lead to new therapeutic strategies for these tumors.

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Conflict of interest The authors declare no conflict of interest.

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