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Establishment and characterization of three novel human gastric cancer cell lines with differentiated intestinal phenotype derived from liver metastasis

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Abstract

Gastric cancers with liver metastasis are fatal diseases with rapid progression and poor patient outcome. To date, however, the molecular basis of their growth and metastasis remains essentially unknown, largely because of the presence of few available gastric cancer cell lines established from liver metastasis. In the present study, we developed two novel cultured cell lines (designated GLM-1 and GLM-2) and one transplantable line in nude mice (designated GLM-3) derived from liver metastasis of gastric cancer patients. These GLM cell lines share unique biological features such as differentiation, growth and metastasis. They form moderately differentiated tumors with CD10 positive and MUC2 negative intestinal absorptive phenotype when injected into nude mice. Their growth is stimulated by EGF and TGF-α *in vitro* like other gastric cancer cell lines. However, GLM cells differ from conventional gastric cancer cell lines in their high apoptotic rate, even in the absence of apoptosis inducing stimuli as revealed by Caspase3/7 assay and the TUNEL method. This apoptosis is further enhanced by phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), but not by MEK1/2 inhibitor (U0126), indicating the strong dependency of their survival on PI3K/Akt pathway rather than MAPK pathway, the major downstream signaling pathways of EGFR. GLM-1 cells can metastasize to the liver after intrasplenic injection, and GLM-3 cells have spontaneous lung metastatic potential after subcutaneous transplantation, respectively. These results indicate that the GLM series are the first cell lines reflecting the intestinal-type differentiated adenocarcinoma, a major subtype of gastric cancer with liver metastasis. Therefore, they would be excellent models for understanding the mechanism of metastatic growth and the development of a new molecular targeting therapy for gastric cancer with liver metastasis.

Abbreviations: EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; MMP – matrix metalloproteinase; PI3K – phosphatidylinositol 3-kinase; PTEN – phosphatase and tensin homologue deleted on chromosome ten; RT-PCR – reverse transcriptase polymerase chain reaction; TGF-α – transforming growth factor alpha; VEGF – vascular endothelial growth factor

Introduction

Liver metastasis is one of the major routes for metastasis of gastric cancer. It occurs 10–15% of recurrence of gastric cancer patients after curative resection in Japan and Western countries [1, 2]. Gastric cancer with liver metastasis is a non-curable, fatal disease with a 5-year survival of less than 10%. This is therefore one of the important prognostic factors in gastric cancer patients [3]. This lesion is known to consist of three histological types, differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and a special type including endocrine carcinoma and hepatoid carcinoma [4–8]. The differentiated type grows in a papillary or tubular pattern. The poorly differentiated type exhibits a medullary growth pattern, in sharp contrast to poorly differentiated gastric cancer of scirrhous type which shows a desmoplastic stromal reaction and has high peritoneal metastatic potential [9]. These subtypes have unique characteristics, but share common pathological features such as scant fibrous stroma and abundant tumor blood vessels.

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Several investigators have reported that overexpression of growth factors, adhesion molecules and ECM degrading enzymes such as c-Met, VEGF, LFA-3, E-selectin and heparanase and transcription factor including β-catenin are correlated or inversely correlated with liver metastasis in gastric cancer, and suggested their clinical significance as prognostic markers for liver metastasis [10–17]. However, molecular mechanism of liver metastasis and its subsequent growth remain essentially unknown. Experimental analysis of liver metastasis using relevant animal models is therefore extremely important to understand the molecular mechanism and to develop new therapeutic approaches for gastric cancer patients with liver metastasis.

To date, more than 20 gastric cancer cell lines are available, but most of them were derived from ascites, pleural effusion and lymph node metastasis [18–21]. Gastric cancer cell lines derived from liver metastasis are still few. In addition, models with hematogenous metastatic potential, not only spontaneous metastatic potential but also experimental metastatic potential in nude mice, are few and have not been fully delineated thus far [22–24]. In the present study, we established three novel gastric cancer cell lines derived from liver metastasis and characterized their growth, differentiation and metastatic potential.

Materials and methods

Source of cell lines

Tumor tissues used for establishing GLM-1, GLM-2 and GLM-3 cell lines were obtained from liver metastatic foci, which were surgically resected (GLM-1, GLM-2) or biopsied (OLM-3) with informed consent from three Japanese gastric cancer patients at the Department of Gastroenterological Surgery, Aichi Cancer Center Hospital. Characteristics of patients and primary tumors are shown in Table 1. In brief, three male patients aged 67, 61 and 61 were suffering from two stage IV and one stage I gastric cancer with synchronous or metachronous liver metastasis, respectively. The depth of cancer invasion (pT categories), the number of metastatic lymph nodes (pN categories) and liver metastases (pM categories) were evaluated according to the Tumor-Node-Metastasis Classification by UICC [25]. The histology of the original primary tumors of GLM-1 and GLM-2 were respectively diagnosed as papillotubular adenocarcinomas with moderately differentiated type, and the remaining GLM-3 was poorly differentiated adenocarcinoma with medullary type containing partially differentiated component, based on the Japanese Classification of Gastric Carcinoma [26]. The GLM-1 cell line is a cultured cell line established from transplanted tumor in nude mice and the GLM-2 cell line is cultured cell line directly established from the metastatic liver tumor in gastric cancer patients. The GLM-3 line, on the other hand, is a transplantable line in nude mice, which was established by serial subcutaneous transplantation of original metastatic liver tumor into female nude mouse of KSN strain (Shizuoka Laboratory Animal Center, Inc., Hamamatsu, Japan).

Establishment of cultured cell lines

GLM-1 and GLM-2 cell lines were established according to the method as reported previously [27]. Fresh metastatic tumor tissues from the liver were washed with Hank's balanced salt solution (HBSS), cut into small pieces with sterile scissors, treated with 50 U/m1 Dispase (Godo Shusei, Tokyo, Japan) for 30–60 min at 37 °C in a water bath, and after vigorous pipetting were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 100*g*. Cell pellets were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd. Tokyo, Japan) containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin, and then incubated for 1 h on plastic dishes at 37 °C. Medium containing floating tumor cell clusters was collected and centrifuged. The cell pellets were resuspended in fresh DMEM with 10% FBS plus MITO (serum extender admixed with growth factors and hormones) (BD Biosciences, Bedford, MA) and cultured on plastic dishes (Falcon, BD Labware, Franklin Lakes, NJ) or type I collagen-coated dishes (Iwaki, Asahi Techno Glass, Japan) in a humidified 5% CO_2 incubator at 37 °C with weekly change of medium. After several weeks, growing colonies were harvested with trypsin-EDTA (0.125%/2 mM) and passaged several times on plastic dishes for GLM-1 cells or type I collagen-coated dishes for GLM-2 cells. 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. GLM-1 and GLM-2 cell lines have now been

Table 1. Characteristics of original gastric cancers from which GLM series of cell lines were obtained.

Cells	Age/sex	Liver metastasis	Stage (TNM)	Histology	Established lines
GLM-1	67/M	Synchronous	IV (T3N2M1)	Mode	Cultured
GLM-2	61/M	Metachronous	I (T1N0M0)	Mode	Cultured
GLM-3	61/M	Synchronous	IV (T2N0M1)	Por	Transplantable

M – male; mode – papillotubular adenocarcinoma of moderately differentiated type; Por – poorly differentiated adenocarcinoma with solid type (Japanese Classification of Gastric Carcinoma). Stages were evaluated according to the Tumor-Node-Metastasis Classification by UICC.

cultured for more than 2 years without apparent phenotypic change. No contamination of these cell lines with Mycoplasma pulmonis and Mouse hepatitis virus was confirmed by culture and PCR method, respectively (Central Institute of Laboratory Animals, Tokyo, Japan). Other cell lines i.e., MKN-28, MKN-45, MKN-74 and GCIY, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HSC-43 cell line was kindly provided by Dr. K. Yanagihara [19].

In vitro *cell growth assay*

To examine the growth rate of GLM-1 and GLM-2 cell lines *in vitro*, the cells were plated at 5×10^4 cells/24 well plastic plate in DMEM supplemented with 10% FBS. The number of viable cells was counted with a hemocytometer in triplicate after 4–5 days culture. For studying growth factor responsiveness of GLM-1 and GLM-2 cells, the cells were plated into 24-well plastic plates in basal medium containing 0.1% FBS with or without various growth factors as supplements and the number of cells was counted 4–5 days after seeding. Concentrations of growth factors used were as follows: insulin/transferrin (5 μg/ml, each) (Sigma, St. Louis, MO), EGF (1 or 5 ng/ml) (R&D systems, Minneapolis, MN), and TGF- α (5 ng/ml) (Sigma). To examine the substrate dependency of the growth of GLM cells, the cells were plated at 5×10^4 cells/24 well plastic plate or type I collagen-coated dishes in DMEM supplemented with 10% FBS. The number of viable cells was counted 4 days after seeding.

Apoptosis assay

Apoptosis was detected both morphologically (TUNEL method) and biochemically (Caspase-3/7 activity). TUNEL assay was performed using an *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany). GLM-1 and GLM-2 cells were cultured on a Labtek chamber slide in growth medium for 1–2 days and fixed in 4% paraformaldehyde for 30 min, followed by wash with PBS. After permeation with citrate buffer containing 0.1% Triton X-100 for 2 min, the specimens were incubated in TUNEL solution containing enzyme and fluorescein-labeled dUTP at 37 °C for 1 h, followed by wash with PBS and then observed by fluorescence microscopy or immunohistochemistry.

For caspase assay, the cells were harvested with trypsin/EDTA and were plated at 1×10^4 cells/96 well plastic plate (Falcon) or type I collagen-coated plate (IWAKI) in DMEM supplemented with 10% FBS for 1–2 days. In some experiment, cells were cultured in the presence of PI3 kinase inhibitor (LY294002, 50 μM) (Cayman Chemical, Ann Arbor, Mich) or MEK1/2 inhibitor (U0126, 10 μM) (Cell Signaling Tech, Beverly, MA) for 2–3 days. Caspase-3/7 activity of the cells was then measured using Apo-One Homogenous caspase-3/7 Assay Kit (Promega Corp., Madison, WI). An aliquot of caspase reagent was added to each well, mixed on a plate shaker for 1 h at room temperature with light protection, and was measured for fluorescence.

Flow cytometry

GLM-1 and GLM-2 cells were harvested with trypsin/EDTA and resuspended in PBS. They were then incubated with mouse monoclonal antibodies, anti-human EGFR mAb (NeoMarkers, Fremont, CA) and anti-Fas mAb (Sigma, St. Louis, MO). After washing with PBS, they were exposed to FITC-conjugated anti-mouse IgG (MBL, Nagoya, Japan) for 30 min. The labeled cells were washed, and the intensity of fluorescence was measured in a flow cytometer, FACS sort (BD Biosciences, San Diego, CA).

Galatin zymography

MMP-2 and MMP-9 activities of the tumor cells was determined by sodium dodecyl sulfate polyacryl amide gel (SDS-PAGE) gelatin zymography. Aliquot of conditioned medium (CM) prepared after 48 h culture of the cells in serum-free DMEM medium were mixed with the sample buffer (63 mM Tris–Hcl, pH 6.8, 10% glycerol, 2% SDS and 0.0025% bromophenol blue) and incubated at room temperature for 10 min. The lysates were then applied to SDS-PAGE using a 8.5% gel containing 0.1% gelatin. After electrophoresis, the gel was rinsed with renaturing buffer for 30 min, and the gel was incubated in developing buffer for 48 h at 37 °C. After incubation, the gel was stained with coomassie brilliant blue R250 (Wako, Ltd., Osaka, Japan). The MMPs were detected as transparent bands on the blue–black ground of the coomassie blue-stained slab gel.

RT-PCR

To examine angiogenic potentials of GLM-1 and GLM-2 cells, VEGF, VEGF-B, VEGF-C and VEGF-D mRNA expression was measured by the RT-PCR method. Total RNA was extracted from cultured tumor cells dissolved in ISOGEN (Nippon Gene, Tokyo, Japan) with a guanidinium–isothiocyanate–phenol–chloroform based method. Total RNA preparation was digested with RNase-free DNase (Nippon Gene, Tokyo, Japan) at 37 °C for 30 min to remove contaminated DNA. Purified total RNA was then reverse transcribed at 37 °C for 1 h with SuperScript II reverse transcriptase (Invitrogen, Gaithersburg, MD) and resultant cDNA was used for PCR amplification using AmpliTaq Gold (Perkin-Elmer, NJ). Primer pairs used for VEGF, VEGF-B, VEGF-C and VEGF-D are as follows: VEGF sense primer [5′-CGA AAC CAT GAA CTT TCT GCT GTC-3′] and VEGF antisense primer [5′-TCA CCG CCT CGC CTT GTC ACA T-3′]; VEGF-B sense primer [5′-AGC ACC AAG TCC GGA TG-3′] and VEGF-B antisense primer [5′-GTC TGG CTT CAC AGC ACT G-3′]; VEGF-C sense primer [5′-CAG TTA CGG TCT GTG TCC AGT GTA G-3′] and VEGF-C antisense

primer [5′-GGA CAC ACA TGG AGG TTT AAA GAA G-3′]; VEGF-D sense primer [5′-GTA TGG ACT CTC GCT CAG CAT-3′]; and VEGF-D antisense primer [5′-AGG CTC TCT TCA TTG CAA CAG-3′].

Assay for tumorigenesis and metastasis in nude mice

To examine tumorigenicity and spontaneous metastatic potentials of GLM-1 and GLM-2 cell lines in nude mice, growing cells were harvested with trypsin-EDTA, washed with PBS, and 5×10^6 cells in 0.2 ml HBSS were injected subcutaneously into the left abdominal flanks of 8-week-old male nude mice of KSN strain (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) maintained under specific-pathogen-free (SPF) conditions. In GLM-3 line, subcutaneous tumor tissue serially transplanted in nude mice was obtained, cut into small pieces and were re-transplanted subcutaneously into the left abdominal flanks of nude mice with a trocar. Tumor size was measured in 2 dimensions with a slide caliper every week. KSN mice bearing GLM-1, GLM-2 and GLM-3 subcutaneous tumor were autopsied 8 weeks after injection; then inguinal lymph nodes, para-aortic lymph nodes and lungs were removed and fixed in 10% buffered formalin. The numbers of macroscopic lung metastases were determined by counting visible parietal nodules and were confirmed by histological examination with H-E stained specimens. For experimental liver metastasis assay, 1×10^6 tumor cells in 0.05 ml HBSS were injected intrasplenically. For peritoneal metastasis, 5×10^6 tumor cells in 0.3 ml HBSS were injected intraperitoneally. After 4 weeks post injection, mice were sacrificed, and liver and peritoneal metastasis were examined both macroscopically and histologically.

Immunohistochemical analysis

Subcutaneous GLM-1, GLM-2 and GLM-3 tumors in nude mice were removed and fixed in 10% buffered formalin and embedded in paraffin. Four micrometer thick-sections were deparaffinized with xylene and dehydrated in alcohol. For antigen retrieval, the sections were then treated with microwave at 98 °C for 10 min. These sections were immersed in methanol with 0.3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase activity, followed by normal serum for 30 min to block nonspecific reactions. The sections were incubated at 4 °C overnight with mouse monoclonal antibody against CD10, MUC-2 and MUC-5AC (all antibodies were obtained from Novocastra Labs, UK) with optimal dilution in phosphate-buffered saline (PBS, pH 7.2) containing 1% bovine serum albumin. After washing with PBS, the sections were incubated with biotinylated second antibodies for 30 min. The sections were washed again with PBS, then incubated with streptavidin–peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 60 min. The chromogen was developed with 0.01% diaminobenzidine (DAB) and the sections were counterstained with Meyer's hematoxylin.

Results

Characteristics of original liver tumors in gastric cancer patients

Table 1 shows the characteristics of original metastatic liver tumors in gastric cancer patients. GLM-1 was derived from synchronous liver metastasis which was surgically resected from a 67-year-old stage IV (T3N2M1) patient, GLM-3, on the other hand, was derived from synchronous multiple liver metastases biopsied from 61-year-old stage IV (T2N0M1) gastric cancer patients. GLM-2 was obtained from metachronous liver metastasis that recurred 2 years after the first resection in stage I (T1N02M0P0) 61-year-old gastric cancer patients. Histologically, both GLM-1 and GLM-2 original liver metastatic tumors were papillotubular adenocarcinoma of moderately differentiated type, whereas GLM-3 tumor was poorly differentiated adenocarcinoma with a medullary growth pattern containing a partial tubular adenocarcinoma component (Figure 1a). All three tumors showed an intestinal absorptive phenotype as verified by CD10-positive, MUC2-negative and MUC5AC-negative immunohistochemical staining patterns. These tumors were also negative for chromogranin A and AFP, indicating a lack of endocrine and hepatoid differentiation (data not shown).

Differentiation phenotype

Established GLM-1 and GLM-2 cell lines showed multilayered and monolayered growth in culture, respectively (Figure 1b). We examined whether these cell lines preserve their original differentiation phenotype after establishment *in vitro*. GLM-1 and GLM-2 cell lines formed tumors in nude mice with the histology of tubular adenocarcinomas of moderately differentiated type (Figures 2a and b), whereas the transplantable GLM-3 line formed poorly differentiated adenocarcinoma of medullary type with partial microglandular structures in nude mice (Figure 2c). The histology of these transplanted tumors in nude mice proved to be essentially the same as those observed in the original metastatic liver tumors (Figure 1a). Immunohistochemical analysis revealed that all subcutaneous tumors in nude mice were CD10-positive (Figures 2d, e and f), MUC2-negative (data not shown) and MUC5AC-negative (Figures 2g, h and i). Similar negative result was obtained from staining for MUC6. These results indicate that GLM cells preserve intestinal absorptive phenotype of the original liver metastases, but not gastric phenotype.

Growth characteristics

The growth rate of GLM-2 cell line *in vitro* is significantly lower than that of the GLM-1 cell line and is dependent on Type 1 collagen as a substrate (Figure 3a).

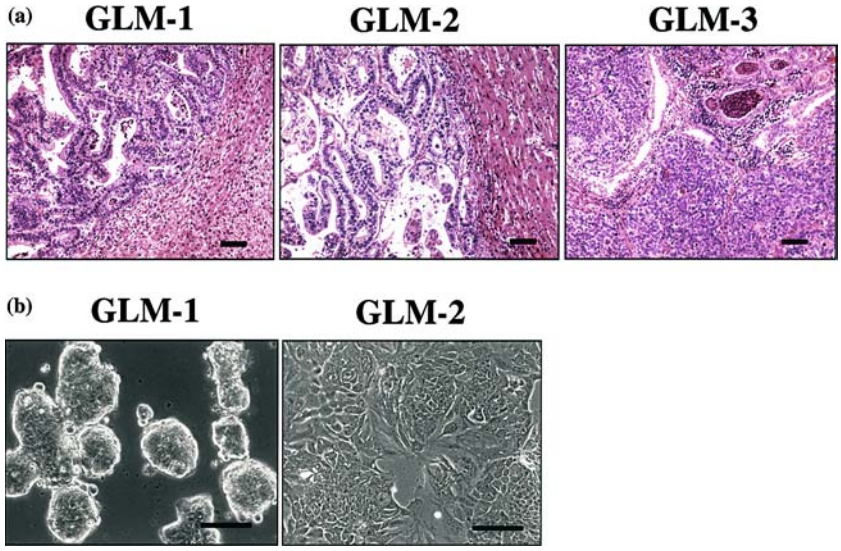

Figure 1. Morphology of original tumors in gastric cancer patients from which GLM-1, GLM-2 and GLM-3 cell lines derived. (a) Histology of original liver metastasis (GLM-1 and GLM-2) and original primary gastric tumor (GLM-3). H&E staining. Bars indicate 100 μm. (b) Phase contrast microscopy of GLM-l and GLM-2 cell lines in culture. Bars indicate 100 μm.

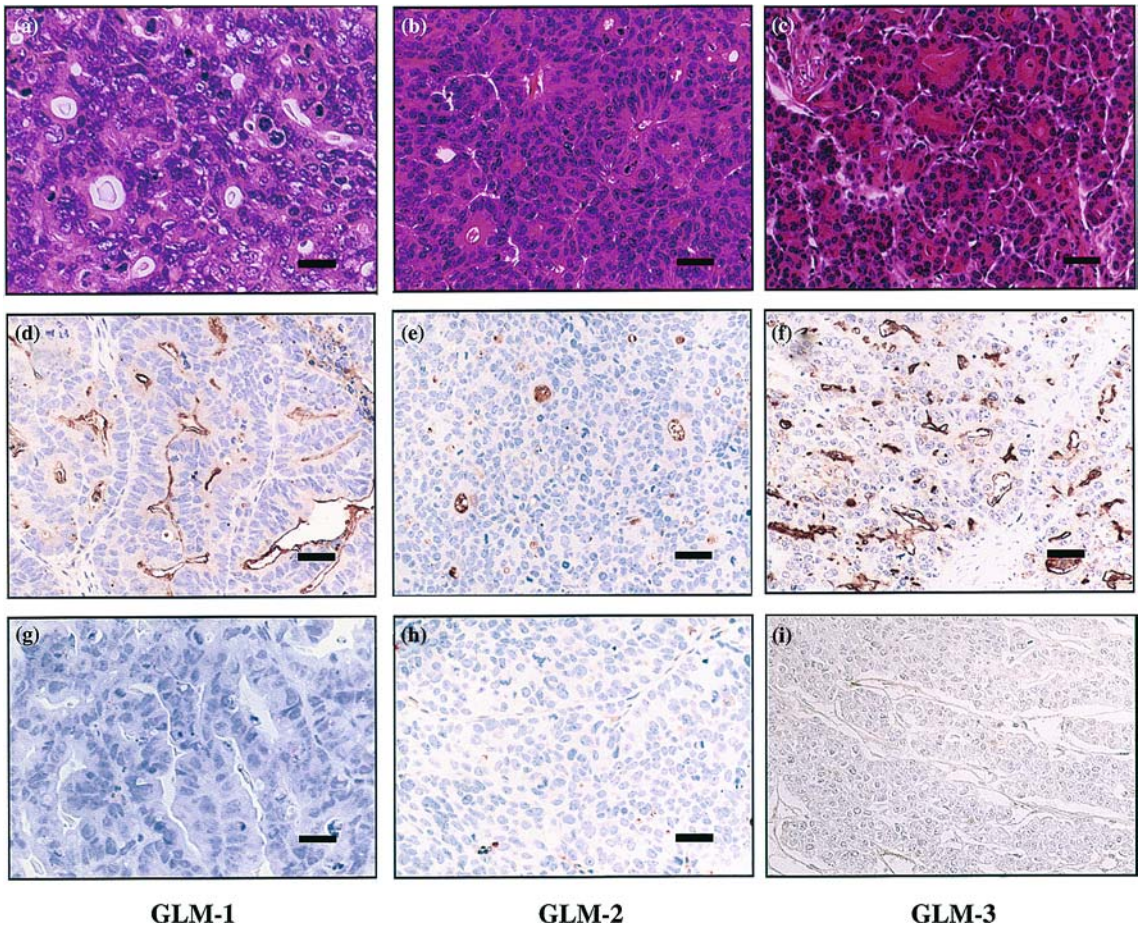

Figure 2. Differentiation phenotype of subcutaneous tumors in nude mice formed after s. c. injection of GLM-1 (a, d, g), GLM-2 (b, e, h) and GLM-3 (c, f, i) cells. (a–c) Histology of subcutaneous tumor in nude mice. Papillary or tubular structures were observed in all the tumors. Poorly differentiated component of GLM-3 tumor was similar to the original gastric tumor as shown in Figure 1a. H&E staining. (d–f) CD10 immunohistochemistry. (g–i) MUC5AC immunohistochemistry. All the GLM tumors were positive for CD10, but were negative for MUC5AC. Bars indicate 50 μm.

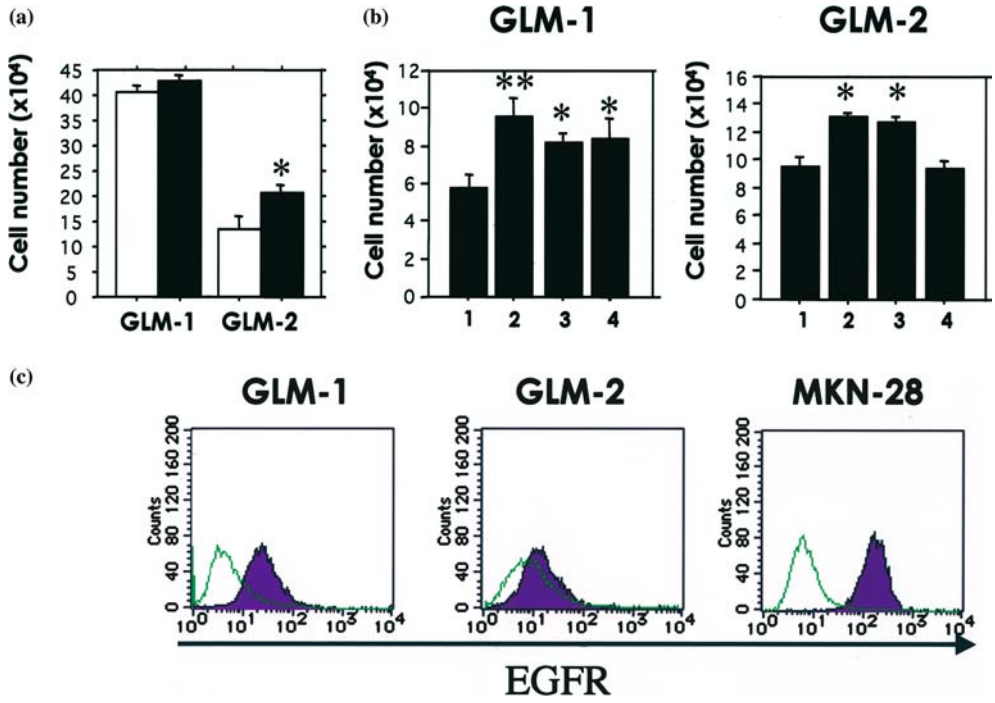

Figure 3. Growth characteristics of GLM-1 and GLM-2 cells *in vitro*. (a) Substrate dependence. GLM-1 and GLM-2 cells were plated at 1×10^5 cells/35 mm plastic dishes (□) or type I collagen-coated dishes (■) in DMEM supplemented with 10% FBS. The number of viable cells was counted 4 days after seeding, (b) Growth factor responsiveness. GLM-1 cells and GLM-2 cells were plated at 5×10^4 cells/24-well plastic plate or type I collagen-coated plate in DMEM containing 0.1% FBS (basal medium) supplemented with growth factors, respectively. The number of viable cells was counted 4 days after seeding. Lane 1, Control; lane 2, EGF; lane 3, TGF- α; lane 4, Insulin/transferrin. The data are means ± s.d. in triplicate. $^*P < 0.01$, $^{**}P < 0.001$ (vs control). (c) Flow cytometric analysis of EGFR expression on the cell surface of GLM-1 and GLM-2 cells as compared with MKN-28 cells.

Addition of EGF and TGF-α to cell culture in the absence of FBS significantly stimulated the growth of both cell lines ($P < 0.01$). Insulin/transferrin significantly stimulated the growth of only GLM-1 cells (Figure 3b). Flow cytometric analysis proved EGF receptor expression on the cell surface of both GLM-1 and GLM-2 cells, but the expression was relatively weak in GLM-1 and even more so in GLM-2 cells as compared with conventional gastric cancer cell lines such as MKN-28 cells (Figure 3c).

Apoptotic characteristics

In the culture of GLM-1, GLM-2 cells, many floating dead cells are apparent in the medium without apoptosis-inducing stimuli. To clarify the reason for this, we examined whether apoptosis increased in these cells or not by caspase3/7 assay and TUNEL method. Caspase3/7 activities of GLM-1 and especially GLM-2 cells were significantly higher than those of the conventional gastric cancer cell lines ($P < 0.001$) (Figure 4a). We confirmed many apoptotic bodies in the GLM-1 and GLM-2 cells by TUNEL method (Figure 4b). Therefore, these GLM cells are supposed to up-regulate the anti-apoptotic signaling pathway to achieve survival. To clarify this point, we further examined the effect of MAPK pathway and phosphatidylinositol 3-kinase (PI3K)/Akt pathway on apoptosis of these cells using specific MEK (U0126) and PI3K inhibitors (LY294002), respectively. The results showed that LY294002 induced apoptosis intensively in GLM-1 and GLM-2 cells ($P < 0.001$) (Figure 4c), whereas MEK inhibitor induced significant apoptosis only in GLM-2 cells ($P < 0.001$) (Figure 4d). Furthermore, neither expression of death receptors such as Fas nor induction of apoptosis by Fas ligand was observed in either types of GLM cells (data not shown).

MMP activities

Gelatin zymography of conditioned medium prepared from culture of GLM-1, GLM-2 cells and other gastric cancer cells are shown in Figure 5a. Only GLM-2 cells showed both pro form (92 kDa) and active form of MMP-9 (82 kDa), and the pro form (72 kDa) and active form of MMP-2 (62 kDa) activities. Other cell lines secrete only pro MMP-9 and pro MMP-2 in varying degrees.

VEGF mRNA expression

RT-PCR results are shown in Figure 5b. Four RT-PCR products for VEGF corresponding to $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ isoforms were observed. Expression of VEGF was higher in GLM-2 cells than other gastric cancer cell lines, and VEGF-C

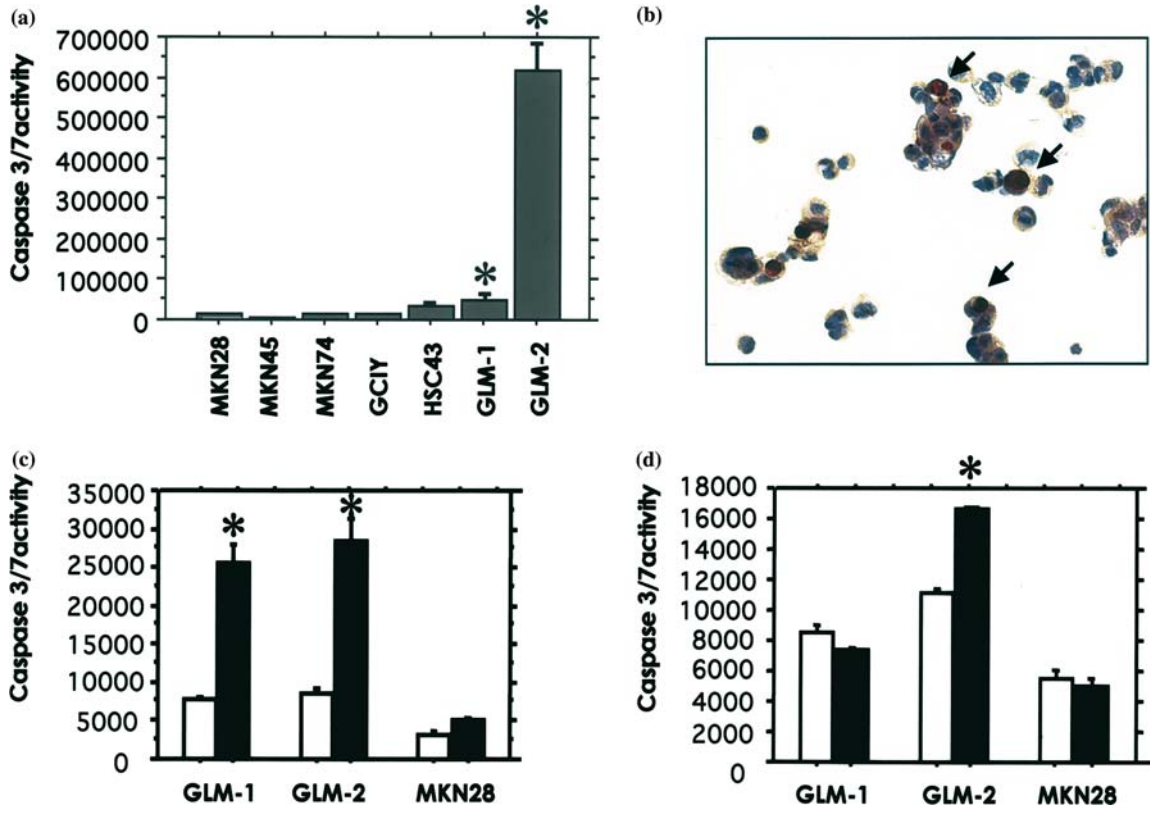

Figure 4. Apoptotic characteristics of GLM-1 and GLM-2 cells *in vitro*. (a) Spontaneous apoptosis of various gastric cancer cell lines observed in the absence of apoptosis inducing stimuli as evaluated by caspase 3/7 assay. (b) Apoptosis of GLM-2 cells grown in the medium without any inducers as demonstrated by the TUNEL method. Arrows indicate apoptotic bodies. (c and d) Induction of apoptosis by P13K inhibitors and MEK inhibitors, respectively. Cells were plated at 1×10^4 cells/96 well plate and 50 μM of PI3K (c) and 10 μM of MEK inhibitors (d) were added 1 day after seeding and cultured for 2–3 days in growth medium supplemented with above inhibitors.

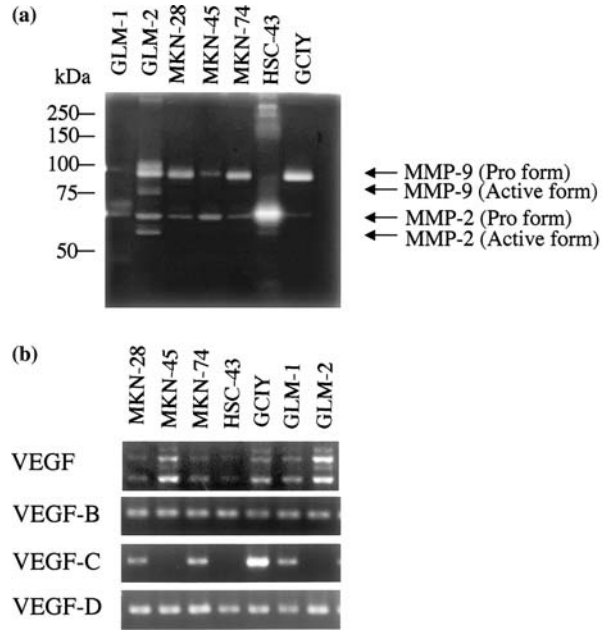

Figure 5. MMP activities and VEGF expression of GLM-1 and GLM-2 cells. (a) Conditioned medium was prepared from culture of GLM-1 and GLM-2 cells for 48 h in serum-free medium, and their MMP activities were measured by gelatin zymography. (b) VEGF family mRNA expression of GLM-1 and GLM-2 cells was measured by RT-PCR analysis.

expression, a major growth factor specific for lymphatic endothelial cells, was evident in GLM-1 cells as well as in GCIY cells, other lymph node metastatic cell line. No significant differences in VEGF-B and VEGF-D mRNA expression were observed between GLM-1, GLM-2 cells and the conventional gastric cancer cell lines.

Metastatic potentials in nude mice

The tumorigenicity and metastatic potentials of GLM-1, GLM-2 and GLM-3 cells in nude mice are summarized in Table 2. GLM-1 cells metastasized to the liver when 1×10^6 cells were injected into the spleen at an incidence of 40% (2/5), with the numbers of metastatic nodules per liver ranging from 1 to 10 (Figures 6a and b). No spontaneous liver metastasis was observed even after orthotopic transplantation into the stomach serosa. GLM-2 cells showed low tumorigenicity (40% = 2/5) in nude mice, and therefore, it is impossible to evaluate their metastatic potential. The GLM-3 line spontaneously metastasized to the lung in nude mice at an incidence of 75% (3/4) after subcutaneous transplantation. They form tumor thrombus with fibrin clot and grow exclusively intravascularly. The lung metastasis was, therefore, microscopic and difficult to identify macroscopically (Figure 6d). In the primary tumor, GLM-3 cells form hypervascular tumor with dilated blood vessels and showed intravasation into blood vessels not infrequently (Figure 6c). As for metastasis to other organs, GLM-1 showed lymph node metastasis and peritoneal metastasis at an incidence of 40% and 80%, respectively (Table 2). Histological features of these metastatic foci in the liver, lung, lymph node and peritoneal cavity were essentially the same as those of the respective primary tumors in nude mice (Figure 2).

Discussion

In the present study, we developed a new series of gastric cancer cell lines derived from liver metastasis. These cell lines are unique for the following reasons. First, GLM-1 and GLM-2 are rare cultured gastric cancer cell lines of papillotubular adenocarcinomas originated from liver metastasis, a major subtype of gastric cancer with liver metastasis. At least more than 20 gastric cancer cell lines are now available, but most of them were derived from

Table 2. Tumorigenicity and metastatic potentials of GLM cell lines in nude mice.

Cell lines	Tumorigenicity	Metastatic potential			
		Liver (isp)	Lung (sc)	Lymph node (sc)	Peritoneum (ip)
GLM-1	5/5	2/5	0/5	2/5	4/5
GLM-2	2/5	NT	1/2	1/2	NT
GLM-3	4/5	NT	3/4	0/4	NT

Tumorigenicity – subcutaneous tumor formation in nude mice; isp – intrasplenic injection; sc – subcutaneous injection; ip – intraperitoneal injection; NT – not tested.

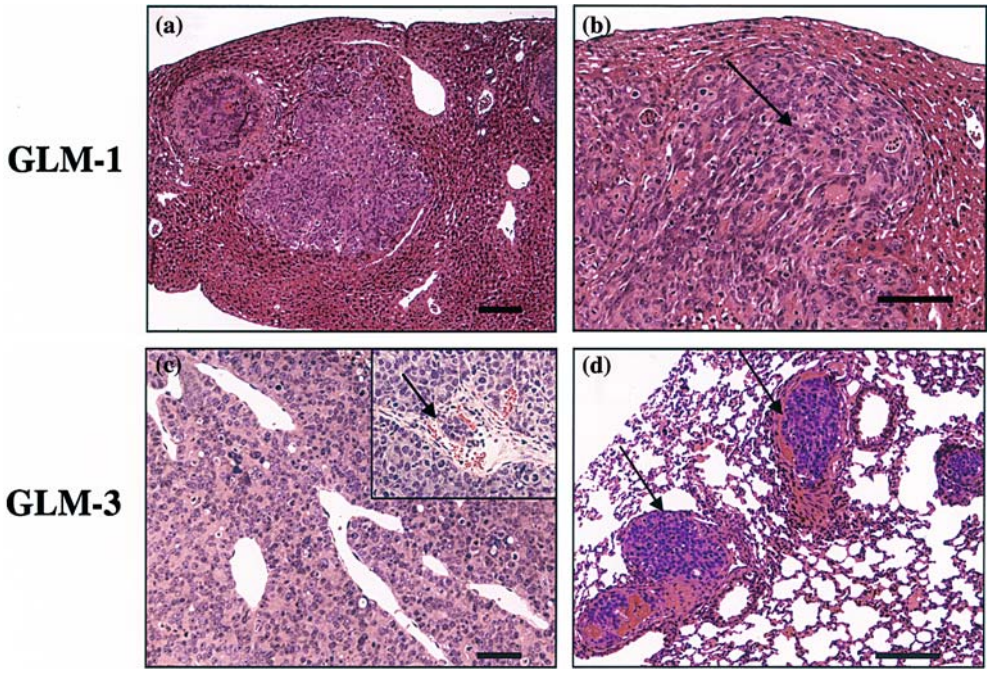

Figure 6. Metastatic potentials of GLM-1 and GLM-3 lines in nude mice. (a) and (b) Liver metastasis formed 2 weeks after intrasplenic injection of 1×10^6 GLM-1 cells. Microglandular structures (arrow) were seen in high power view (b). (c) and (d) Primary tumor (c) and lung metastasis (d) formed 6–8 weeks after subcutaneous transplantation of GLM-3 line. Intravasation (arrow in inset of c) and intravascular growth with fibrin clots were seen (arrows in d). Bars indicate 100 μm.

ascites, pleural effusion and lymph node metastasis. To date, MKN-45, MKN-74 and N87 cells are reportedly gastric cancer cell lines derived from liver metastasis [18, 20], However, MKN-45 cell line is a poorly differentiated adenocarcinoma of medullary type, another histological subtype of gastric cancer with liver metastasis, which is known to be highly metastatic and aggressive. To our knowledge, the MKN-74 and N87 cell lines are the only differentiated adenocarcinoma cell lines from liver metastasis, but MKN-74 cell line has lost its original differentiated phenotype based on their histology of transplanted tumors in nude mice in which no clear glandular structures are seen. N87 cells reportedly preserve the histology of tubular adenocarcinoma, but they exhibit a gastric phenotype such as MUC 6 positivity [20]. In contrast to these cell lines, our GLM-1 and GLM-2 cells exhibited apparent glandular differentiation with an additional intestinal absorptive phenotype, judging from the positive staining for CD10 and villin, but not MUC2. We recently found that the intestinal phenotype is a major phenotype of gastric cancer with liver metastasis (unpublished result), suggesting that GLM series are the first cell lines well reflecting this type of gastric cancer with liver metastasis.

Second, the transplantable GLM-3 line is a poorly differentiated adenocarcinoma of medullary type partially admixed with a tubular adenocarcinoma component. The tubular component harbored CD10-positive intestinal absorptive phenotype, similar to the GLM-1 and GLM-2 cells as described above. The poorly differentiated component shows some morphological similarity to neuroendocrine carcinoma or hepatoid carcinoma. However, the GLM-3 line expressed chromogranin A only weakly and no alpha-fetoprotein, indicating that it is not a special type of gastric cancer with liver metastasis, but is relevant to the poorly differentiated adenocarcinoma of medullary type, the second major subtype of gastric cancer with liver metastasis [7–9]. Third, GLM-1, GLM-2 and GLM-3 lines share common pathobiological features such as an intestinal absorptive phenotype, high apoptotic rate (except for GLM-3) and metastatic potential. Thus, our newly established cell lines would have great advantages over conventional gastric cancer cell lines of heterogenous origin for analyzing the mechanism underlying gastric cancer liver metastasis and the subsequent growth.

GLM-1 and GLM-2 cells exhibited a remarkably higher apoptotic rate than the conventional gastric cancer cell lines. Interestingly, PI3K inhibitor (LY294002) induced further apoptosis in GLM-1 cells and GLM-2 cells, whereas MEK1/2 inhibitor (U0126) had only a limited apoptotic effect on GLM-2 cells. A preliminary experiment showed that of the two major downstream signaling pathways of EGFR, PI3K/Akt pathway, but not MAPK pathway, is constitutively activated (phosphorylated) in GLM-1 cells, irrespective of ligand (EGF) stimulation (data not shown). These findings suggest that the growth of GLM-1 and GLM-2 cells is dependent on the strong survival signal via PI3K/Akt pathway rather than MAPK pathway. Osaki et al. reported that the PI3K/Akt pathway is activated in MKN-45 and other gastric cancer cell lines, but the inhibition of this pathway alone is not sufficient for significant induction of apoptosis; additional stimulation such as death signal via Fas/anti-Fas antibody is required for induction of apoptosis [28], suggesting that the survival in other gastric cancer cell lines are more complexly regulated than GLM-1 and GLM-2 cells by a multiple signaling pathway or crosstalk between them [29]. Although the mechanism by which the PI3K/Akt pathway is constitutively activated in GLM cell lines remains unknown, it is possible that amplification of the EGFR family such as HER2 and loss of PTEN, a negative regulator for PI3K/Akt pathway, by mutation, LOH or hypermethylation may contribute to the activation of this pathway [30–32]. The reason for the increased apoptosis of GLM-1 and GLM-2 cells also remains unclear. We found no overexpression of death receptor such as Fas and no induction of apoptosis by stimulation with Fas ligand in GLM cells, indicating that at least an extrinsic pathway of apoptosis such as activation of death signal by Fas/FasL, is not responsible for this increased apoptosis of GLM-1 and GLM-2 cells. It seems more likely that activation of an intrinsic pathway occurs via cytchrome-*c* release from mitochondria caused by an unknown stress or imbalance between negative (Bcl-2 family) and positive (Bax family) apoptosis regulatory molecules [33]. Taken together, these findings suggest that GLM cell lines have unique signaling pathways for growth, apoptosis and survival, and therefore, would be excellent tools for developing a new molecular targeting therapy for gastric cancer with liver metastasis. Further studies are now ongoing in our laboratory to clarify these points.

We found that GLM-1 cells can indeed metastasize to the liver at an incidence of 40% when injected intrasplenically and the transplantable GLM-3 line metastasizes to the lung spontaneously at a high incidence (75%) when injected subcutaneously. In contrast, the metastatic potential of GLM-2 cells cannot be assessed because of their low tumorigenicity (40%) in nude mice, although GLM-2 cells express some metastasis-associated phenotypes such as high expression of VEGF mRNA and production of the active form of MMP-2 and MMP-9. GLM-1 cells form micrometastases in portal areas of the liver at an incidence of almost 100% at the initial stage (1–2 days post injection), when a considerable number of tumor cells show apoptotic or degenerative change. This is consistent with the high apoptotic rate of GLM-1 cells *in vitro*. Therefore, the relatively low liver metastatic potential of GLM-1 cells may be due to the slow or impaired initial growth after arrest of tumor cells in the terminal portal venules of the liver [34]. GLM-1 cells can also metastasize to the lymph node at a relatively low incidence (40%). Moderate expression of VEGF-C, a crucial factor for tumor lymphangiogenesis, in the GLM-1 cell line may be involved in this lymph node metastasis [35].

Transplantable GLM-3 line forms micrometastases in the lung where they form tumor thrombus with fibrin clot and grow expansively within blood vessels, but they never extravasate individually into the lung parenchyma. Another transplantable gastric cancer line we previously established from liver metastasis (HY-1) also has spontaneous metastatic potential in the lung and the same histology with GLM-3 (poorly differentiated adenocarcinoma of medullary type) [23]. HY-1 cells showed intravascular growth without penetration of the vascular wall until the late stage in the lung like GLM-3, suggesting that such poorly differentiated gastric cancer of medullary type are metastatic but weakly invasive in the metastatic site. To date, attachment to the vascular endothelium → extravasation of vascular wall → extravascular growth in the lung parenchyma is believed to be the main route for lung metastasis [36]. However, our present and past findings suggest the presence of an alternative lung metastasis sequence, consisting of formation of tumor embolus in the venules → attachment to the vascular BM → intravascular growth without extravasation [23, 34]. Evidences in line with such a metastasis pathway are now accumulating [37, 38]. In addition, Sugino et al. demonstrated intravasation of tumor cell nests surrounded by sinusoidal blood vessels and subsequent intravascular tumor growth in the lung, and proposed the concept of invasion-independent hematogenous metastasis [39, 40]. In agreement with this hypothesis, GLM-3 cells form hypervascular tumors with dilated sinusoidal blood vessels and showed not infrequent intravasation with or without endothelial covering.

In conclusion, we established and characterized three novel gastric cancer cell lines, two differentiated types and one poorly differentiated type, which include major types of gastric cancers with liver metastasis. These cell lines would be useful tools for understanding the molecular bases of liver metastasis and subsequent growth and the development of a new molecular targeting therapy against gastric cancer with liver metastasis.

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References

1. Ohno S, Fujii T, Ueda S. Predictive factors and timing for liver recurrence after curative resection of gastric carcinoma. Am J Surg 2003; 185(3): 258–63.
2. Marrelli D, Roviello F, De Stefano A. Risk factors for liver metastases after curative surgical procedures for gastric cancer: A prospective study of 208 patients treated with surgical resection. J Am Coll Surg 2004; 198(1): 51–8.
3. Shirabe K, Shimada M, Matsumata T. Analysis of the prognostic factors for liver metastasis of gastric cancer after hepatic resection: a multi-institutional study of the indications for resection. Hepatogastroenterology 2003; 50(53): 1560–3.
4. Zacherl J, Zacherl M, Scheuba C. Analysis of hepatic resection of metastasis originating from gastric adenocarcinoma. J Gastrointest Surg 2002; 6(5): 682–9.
5. Sakamoto Y, Ohyama S, Yamamoto J. Surgical resection of liver metastases of gastric cancer: An analysis of a 17-year experience with 22 patients. Surgery 2003; 133(5): 507–11.
6. Saiura A, Umekita. N, Inoue S. Clinicopathological features and outcome of hepatic resection for liver metastasis from gastric cancer. Hepatogastroenterology 2002; 49(46): 1062–5.
7. Terracciano LM, Glatz K, Mhawech P. Hepatoid adenocarcinoma with liver metastasis mimicking hepatocellular carcinoma: An immunohistochemical and molecular study of eight cases. Am J Surg Pathol 2003; 27(10): 1302–12.
8. Adachi Y, Tsuchihashi J, Shiraishi N. AFP-producing gastric carcinoma: Multivariate analysis of prognostic factors in 270 patients. Oncology 2003; 65(2): 95–101.
9. Kaibara N, Kimura O, Nishidoi H. High incidence of liver metastasis in gastric cancer with medullary growth pattern. J Surg Oncol 1985; 28:195–8.
10. Amemiya H, Kono K, Itakura J. c-Met expression in gastric cancer with liver metastasis. Oncology 2002; 63(3): 286–96.
11. Kamei S, Kono K, Amemiya H. Evaluation of VEGF and VEGF-C expression in gastric cancer cells producing alpha-fetoprotein. J Gastroenterol 2003; 38(6): 540–7.
12. Maruo Y, Gochi A, Kaihara A. ICAM-1 expression and the soluble ICAM-1 level for evaluating the metastatic potential of gastric cancer. Int J Cancer 2002; 100(4): 486–90.
13. Alexiou D, Karayiannakis AJ, Syrigos KN. Clinical significance of serum levels of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in gastric cancer patients. Am J Gastroenterol 2003; 98(2): 478–85.
14. Mayer B, Lorenz C, Babic R. Expression of leukocyte cell adhesion molecules on gastric carcinomas: Possible involvement of LFA-3 expression in the development of distant metastases. Int J Cancer 1995; 64(6): 415–23.
15. Maehara Y, Kakeji Y, Kabashima A. Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. J Clin Oncol 1999; 17(2): 607–14.
16. Takaoka M, Naomoto Y, Ohkawa T. Heparanase expression correlates with invasion and poor prognosis in gastric cancers. Lab Invest 2003; 83(5): 613–22.
17. Ebert MP, Yu J, Hoffmann J. Loss of beta-catenin expression in metastatic gastric cancer. J Clin Oncol 2003; 21(9): 1708–14.
18. Motoyama T, Hojo H, Watanabe H. Comparison of seven lines derived from human gastric carcinomas. Acta Pathol Jpn 1986; 36(1): 65–83.
19. Yanagihara K, Kamada N, Tsumuraya M. Establishment and characterization of a human gastric scirrhous carcinoma cell line in serum-free chemically defined medium. Int J Cancer 1993; 54: 200–207.
20. Park JG, Frucht H, LaRocca RV. Characteristics of cell lines established from human gastric carcinoma. Cancer Res 1990; 50(9): 2773–80.
21. Nozue M, Nishida M, Todoroki T. Establishment and characterization of a human scirrhus type gastric cancer cell line, GCIY, producing CA19–9 (in Japanese). Human Cell 1991; 4: 71–5.
22. Li H, Zhang YYC, Tsuchihashi Y. Invasion and metastasis of SY86B human gastric carcinoma cells in nude mice. Jpn J Cancer Res 1988; 79: 750–6.
23. Nakanishi H, Yasui K, Yamagata S. Establishment and characterization of a new spontaneous metastasis model of human gastric carcinoma in nude mice. Jpn J Cancer Res 1991; 82(8): 927–33.
24. Yamaguchi K, Ura H, Yasoshima T. Liver metastatic model for human gastric cancer established by orthotopic tumor cell implantation. World J Surg 2001; 25(2): 131–7.
25. Sobin LH, Wittekind C. TNM Classification of Malignant Tumors 5. New York: John Wiley and Sons 1997.

26. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma, 2nd English edition. Gastric Cancer 1998; 1: 10–24.
27. Nakanishi H, Taylor RM, Hawkins AL. Hormone dependent and hormone–independent mammary cancer cell line derived from spontaneous mammary tumors in F344 rats. Int J Cancer 1994; 58(4): 592–601.
28. Osaki M, Kase S, Adachi K. Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN-45. J Cancer Res Clin Oncol 2004; 130(1): 8–14.
29. Kanda N, Seno H, Konda Y. STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. Oncogene 2004; 23(28): 4921–9.
30. Moasser MM, Basso A, Averbuch SD. et al., The tyrosine kinase inhibitor ZD1839 (''Iressa'') inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells Cancer Res 2001; 61(19): 7184–8.
31. Byun DS, Cho K, Ryu BK. Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma. Int J Cancer 2003; 104(3): 318–27.
32. Kang YH, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. Lab Invest 2002; 82(3): 285–91.
33. Luu Y, Bush J, Cheung KJ Jr The p53 stabilizing compound CP-31398 induces apoptosis by activating the intrinsic Bax/mitochondrial/caspase-9 pathway. Exp Cell Res 2002; 276(2): 214–22.
34. Ito S, Nakanishi H, Ikehara Y. Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. Int J Cancer 2001; 93(2): 212–17.
35. Duff SE, Li C, Jeziorska M. Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy. Br J Cancer 2003; 89(3): 426–30.
36. Morris VL, Koop S, MacDonald IC. Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metast 1994; 12(6): 357–67.
37. Al-Mehdi AB, Tozawa K, Fisher AB Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: A new model for metastasis. Nat Med 2000; 6(1): 100–2.
38. Wong CW, Song C, Grimes MM. Intravascular location of breast cancer cells after spontaneous metastasis to the lung. Am J Pathol 2002; 161(3): 749–53.
39. Sugino T, Kusakabe T, Hoshi N. An invasion-independent pathway of blood-borne metastasis: A new murine mammary tumor model. Am J Pathol 2002; 160(6): 1937–9.
40. Sugino T, Yamaguchi T, Ogura G. Morphological evidence for an invasion-independent metastasis pathway exists in multiple human cancers. BMC Med 2004; 2(1): 9.