Establishment of Highly Tumorigenic Human Gastric Carcinoma Cell Lines from Xenograft Tumors in Mice

Kyung-A Song¹, Jihyun Park², Ha-Jung Kim³, Myung Soo Kang^{4,†} and Sun Young Kim^{3,†}

¹Philips Institute for Oral Health Research, Virginia Commonwealth University School of Dentistry, Richmond, VA23298,

²Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul 06351, Korea

³Department of Medicine, Division of Hematology-Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, Korea

⁴Institute of Life Technology, Intron Biotechnology Inc., Seongnam 13202, Korea

Patient's primary tumor-derived tumor cell lines likely represent ideal tools for human tumor biology *in vitro* and *in vivo*. Here, we describe eight human gastric carcinoma cell lines derived from established tumors *in vivo* upon subcutaneous transplantation of primary gastric carcinoma specimens in BALB/c nude mice. These xenografted gastric tumor cell lines (GTX) displayed close similarity with primary gastric tumor tissues in their *in vivo* growth pattern and genomic alterations. GTX-085 cells were resistant to cisplatin, while GTX-087 was the most sensitive cell line. GTX-085 was the only cell line showing a metastatic potential. Epithelial cell adhesion molecule (EPCAM) expression was especially strong in all tissue samples, as well as in cell cultures. GTX-139, the largest tumor graft obtained after injection, displayed distinct expression of CD44v6, fibroblast growth factor receptor 2 (FGFR2), and prominin 1 (PROM1, also known as CD133). In summary, we established eight xenograft gastric cancer cell lines from gastric cancer patient tissues, with their histological and molecular features consistent with those of the primary tumors. The established GTX cell lines will enable future studies of their responses to various treatments for gastric cancer.

Key Words: Gastric cancer, Xenograft, Cell line, *FGFR2* amplification, Targeted therapy

INTRODUCTION

Although the gastric carcinoma (GC) mortality rate has decreased worldwide, it is still high in Korea (43/100,000), especially among men (Shin et al., 2004; Ushijima and Sasako, 2004). Metastasis is the major cause of gastric cancer death (Shridhar et al., 2013). Therefore, thorough understanding

of the biological nature of gastric cancer is vital to develop proper therapeutic methods and to improve the current prognosis of the disease. To achieve these goals, permanently growing cell lines derived from cancer patients may play an invaluable role in research studies.

Cancer cell lines are established from pathologically proven gastric tumors, either directly or after xenotransplantation to athymic nude mice (Park et al., 1990). Although GC has

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†Corresponding author: Sun Young Kim. Department of Medicine, Division of Hematology-Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, Korea.

Tel: +82-2-2148-7293, Fax: +82-2-3410-7385, e-mail: enthalpy98@gmail.com

†Corresponding author: Myung Soo Kang. Institute of Life Technology, Intron Biotechnology Inc., Sagimakol-ro 137, Jungwon-gu, Seongnam, Gyeonggi-do 13202, Korea.

Tel: +82-31-739-5315, Fax: +82-31-739-5353, e-mail: msk915@intron.co.kr

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been considered to be very difficult to culture (Park et al., 1990), more than 36 gastric cancer cell lines have been established to date, mostly from Japan and Korea, where gastric cancer mortality rates are still very high (Ushijima and Sasako, 2004; Ku et al., 2012). Established cell lines were derived either from ascites of patients with signet-ring cell GC (Yanagihara et al., 1991) or from human primary and metastatic GCs (Park et al., 1990; Park et al., 1997), and there have been few reports of spontaneous metastasis from human gastric tumor xenografts in nude mice (Yanagihara et al., 2004). Thus, it has been reported that two human signet-ring cell GC cell lines exhibited the ability to spontaneously metastasize to lymph nodes and lungs in nude mice (Yanagihara et al., 2004) and three gastric cancer cell lines derived from the liver showed a metastatic potential for the liver, lung, lymph nodes, and peritoneum (Nakanishi et al., 2005). However, the availability of cell lines to investigate the heterogeneity of GCs is still limited.

In 2007, advantages of the establishment of a cancer cell line from a tumor fragment through a xenograft stage, rather than directly from a primary tumor, were first demonstrated for colon carcinoma (Dangles-Marie et al., 2007). The authors claimed that it was technically difficult to obtain representative human colon cancer cell lines from fresh tumors. In particular, it was shown that a prior xenograft increased the efficiency of the cell line establishment compared with that of the direct establishment from fresh tumors (Dangles-Marie et al., 2007). Moreover, human colon cancer cell lines derived from the same tumor fragment using the two protocols displayed similarity in major phenotypic and genotypic characteristics such as the ability to form compact spheroids, karyotype alterations, and distinctive additional chromosomal aberrations, as well as in the expression levels of genes selected for their role in oncogenesis (Dangles-Marie et al., 2007). Although Daniel and coauthors (Daniel et al., 2009) argued that a number of tumor-specific genes expressed in primary small-cell lung cancer and xenografts were lost during the transition to tissue culture and not regained when the tumors were reestablished as secondary xenografts, such changes in gene expression may be a common feature of many cancer cell culture systems. Therefore, xenotransplantation of tumor fragments in mice before the

establishment of cell lines can be considered a practical method enabling the generation of novel human cancer cell lines for the investigation of cancer cell biology and providing the opportunity to reproduce the diversity of the diseases.

Epstein-Barr virus (EBV) has a pathogenic role in several lymphomas and solid cancers and is considered to cause GC (Yoo et al., 2015). EBV-associated GC comprises nearly 10% of all GC cases worldwide (Fukayama, 2010). EBV-positive gastric cancers have been demonstrated to be more prevalent in males than in females and less likely to be found in the gastric antrum than in the cardia or body of the stomach (Young and Murray, 2003). Despite extensive studies, the mechanism underlying the gastric carcinogenesis caused by EBV remains unknown, which might be due to the lack of naturally infected EBV-positive cell lines available for studies. So far, only a few EBV-positive gastric cancer cell lines such as SNU-719, NCC-24, and YCCE1 have been established and characterized (Oh et al., 2007; Ku et al., 2012).

In the present study, we report the establishment and tumorigenic characterization of eight human GC cell lines (GTX-006, GTX-007, GTX-085, GTX-086, GTX-087, GTX-103, GTX-116, and GTX-139), established from seven Korean male and one female gastric cancer patients whose ages ranged from 59 to 76 years old. All of these eight cell lines were indirectly established from xenografts after serial heterotransplantation of gastric tumors in nude mice. Seven cell lines (GTX-006, GTX-007, GTX-085, GTX-086, GTX-087, GTX-116, and GTX-139) showed high *in vivo* tumorigenicity when injected subcutaneously, and five of them (GTX-006, GTX-007, GTX-085, GTX-116, GTX-139) also formed tumors upon injection in an orthotopic site (gastric wall) in nude mice. Interestingly, the GTX-085 cell line showed a metastatic ability to the ovary, which is rarely observed in the case of gastric cancer cell lines.

MATERIALS AND METHODS

Primary tissue xenograft screening

Twenty-two surgical specimens of primary GC were used for a xenograft model test. All patients provided written informed consent, and the collection and use of materials

for research purposes were approved by the Institutional Review Board of the Samsung Medical Center. The tissues (0.2 cm³) were subcutaneously implanted into three BALB/c nude mice (Orient Bio, Inc., Gyeonggi, Korea). Xenograft tumors were established for 12~16 weeks, and their sizes were monitored. The 22 primary tissues showed tumorigenicity 3 weeks after the mouse subcutaneous implantation. A serial passage was performed *in vivo* as described above, using a tumor fragment when the xenograft tumor size reached 1,000 mm³. A clinicopathological summary of the 22 tumors used for xenografts is described in Supplementary Table 1.

Xenograft cell line establishment and culture

The xenograft tumor tissues derived from primary tissues subcutaneously implanted into the mice were mechanically and enzymatically dissociated. After washing with phosphate-buffered saline (PBS), minced tissue was mixed with an enzyme cocktail containing 0.4 mg/mL collagenase (Gibco, Waltham, MA, USA), 0.5 mg/mL DNase I (Roche, Basel, Switzerland), and 0.2 mg/mL dispase (Gibco, Waltham, MA, USA) in RPMI 1640 medium (Gibco, Waltham, MA, USA) and incubated at 37°C for 2 h with shaking. Dissociated tissue was filtered through a 40-µm cell strainer (BD Biosciences, San Jose, CA, USA) and washed with PBS. Isolated cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Waltham, MA, USA). *Mycoplasma* contamination was examined using a MycoAlert kit (Lonza, Basel, Switzerland).

Array comparative genomic hybridization analysis

Genomic DNA (gDNA) was extracted from tissue for pathology quality control using a QIAmp DNA mini kit (Cat. No. 51306, Qiagen, Hilden, Germany). The amount and quality of extracted gDNA were assessed using a Nanodrop spectrophotometer (ND-1000). Array comparative genomic hybridization (aCGH) analysis was performed using a SurePrint G3 human CGH microarray kit 8×60K (Agilent, G4450A) following the manufacturer's instructions. Briefly, gDNA (500 ng) was digested with the restriction endonucleases *AluI* and *RsaI* at 37°C for 20 min. Then, random

primers were added to the sample, and it was incubated for fluorescent labeling at 95°C for 3 min and on ice for 5 min. Experimental gDNA and reference gDNA were incubated with labeling master mixtures containing cyanine 3-dUTP and cyanine 5-dUTP at 37°C for 2 h and at 65°C for 10 min, respectively (Agilent genomic DNA enzymatic labeling kit, p/n 5190-0449). After the incubation, samples were cleaned using an Amicon Ultra-0.5 Ultracel-30 membrane, 30 kDa (Millipore, Billerica, MA, USA).

DNA fingerprinting

gDNA from each cell line was amplified using an Amp-FiSTR identifier polymerase chain reaction (PCR) amplification kit (Applied Biosystems, Foster City, CA, USA). A single PCR amplified 15 tetranucleotide repeat loci (D8-S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13-S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) and the amelogenin sex determination marker at loci containing highly polymorphic microsatellite markers. Amplified products were analyzed using the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Cell viability assay with antitumor drugs

5-Fluorouracil (5-FU) was obtained from Choongwae Pharma Corp. (Gyeonggi, Korea), and cisplatin was obtained from Dong-A Pharmaceutical (Seoul, Korea). Cell viability was evaluated using a CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) per manufacturer's instructions. Briefly, 5,000 cells were treated with five different concentrations of 5-FU (0.9375 to 240 µg/mL) and cisplatin (0.5 to 8 µg/mL) in a 96-well plate for 72 h. Cell growth inhibition was detected as a luminescent signal of survived cells, and the concentration required to inhibit cell growth by 50% (IC₅₀) was determined. Each experiment was conducted three times.

Cell line xenograft assay

Gastric tumor xenograft (GTX) cell lines were resuspended in a 1:1 mixture of Hank's balanced salt solution (HBSS; Gibco, Waltham, MA, USA) and Matrigel (BD Biosciences, San Jose, CA, USA) to give a final volume of 100

μL. Cells ($1\sim 2.5 \times 10^6$) were injected subcutaneously to 7-week-old female BALB/c nude mice (for EBV-negative cell lines) or NSG mice (for EBV-positive cell lines). Tumors were measured with calipers every 2 days (the largest length and width), and the mice were weighed. The mice were housed in filter-top cages containing a maximum of five mice per cage at a 12-h light/dark cycle and were provided free access to food and water. Tumor volumes (mm^3) were calculated using the following formula: $\text{width}^2 (\text{mm}^2) \times \text{length} (\text{mm}) \times 0.5$. For orthotopic injection, $1\sim 2.5 \times 10^6$ cells resuspended in 100 μL of HBSS were injected into the middle wall of the greater curvature of an anesthetized mouse. Magnetic resonance imaging was performed every week to detect tumor growth. At 6~12 weeks after the injection, the grafts were dissected and processed for further studies.

Immunocytochemistry

To determine whether novel established cell lines were of epithelial origin, we measured expression of cytokeratin by immunofluorescence. Cells were grown in RPMI 1640 medium on cover slips placed in 6-well plates. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and incubated with 5% (w/v) bovine serum albumin in PBS for 30 min at room temperature. The cells were immunostained using a fluorescein isothiocyanate (FITC)-conjugated anti-cytokeratin antibody (Milenyi Biotech, Inc., Auburn, CA, USA) to visualize their immunoreactivity. The stained cells were mounted with the Prolong gold antifade reagent containing 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) and examined using a spectral confocal system (LSM 710, Zeiss, Oberkochen, Germany).

Hematoxylin and eosin staining and immunohistochemistry

A tumor mass was fixed in 4% formalin for 24~48 h, embedded in paraffin, and sectioned at 4 μm. Tissues on slides were stained with hematoxylin and eosin (H&E) for 30 s, respectively. Serial sectioned tissue slides were deparaffinized in xylene, rehydrated in 70, 80, 90, 95, and 100% alcohol for 5 min, and washed with distilled water. The

hydrated slides were treated with an antigen retrieval solution (Dako REALTM target retrieval solution, 10×, pH 6.0) for 40 min in a water bath at 98°C and then blocked in a Dako REALTM peroxidase blocking solution (0.3% H₂O₂ in methanol). Sections were incubated with primary antibodies for 1 h at room temperature. After washing with PBS-Tween 20 (0.1 M PBS, 0.5% Tween 20, pH 7.4), an EnVisionTM detection system was used according to the manufacturer's instruction (Dako, Santa Clara, CA, USA). To obtain clear results, the following optimal antibodies were used: anti-CD44v6 (Abcam, 1:200), anti-CD133/1 (Milenyi Biotech, 1:200), anti-EPCAM (Santa Cruz Biotechnology, 1:200), anti-FGFR2 (Abcam, 1:150), and anti-THY1 (Cell Signaling Technology, 1:100).

Immunoblotting

Total proteins were isolated using a Pro-Prep protein extraction solution (Intron Biotechnology, Gyeonggi, Korea). Then, 25~30 μg of protein was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 and then probed overnight at 4°C with CD44v6 (1:500; R&D Systems, Minneapolis, MN, USA), FGFR2 (1:500; Abcam, Cambridge, UK), EPCAM (1:250; Santa Cruz Biotechnology, Dallas, TX, USA), THY1 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) antibodies. A horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as a secondary antibody, and signals were detected by chemiluminescence.

Statistical analysis

To compare results, the Student's *t*-test or Mann-Whitney *U*-test was performed for independent variables, and the paired *t*-test or Wilcoxon signed-rank test was performed for related variables as appropriate, using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Establishment of novel gastric tumor xenograft cell lines and culture

Eight cell lines (GTX-006, GTX-007, GTX-085, GTX-086, GTX-087, GTX-103, GTX-116, and GTX-139) were established from 22 xenograft tissues. Cells were dissociated from xenograft tissues of primary gastric cancer tissues and successfully cultured in 10% FBS-supplemented RPMI 1640 media (Fig. 1A). Supplementary Table 1 shows a summary of clinicopathologic data. Interestingly, four cell lines (GTX-006, GTX-007, GTX-085, and GTX-139) were poorly differentiated tubular adenocarcinomas, whereas moderately differentiated tumors constituted more than 50% of the 22 xenografts of primary gastric cancer tissues. The *in vivo* passage numbers of the xenograft tissues used for cell culture were P2-P6 (Table 1). When analyzing their growth properties (Fig. 1A), GTX-006 and GTX-007 cells showed a round shape, and some floating cell aggregates were observed. GTX-085 cells grew as a round-cell monolayer, with most cells attached and some cells floating. GTX-086 cells formed an attached dense monolayer; and some round-shaped suspended cells were observed. GTX-087 cells were adherent and grew as round cells in aggregates. GTX-103 cells grew as a monolayer, and individual cells displayed a

polygonal shape. GTX-116 and GTX-139 cells were round; most cells were attached, and some cells were floating as single cells. All results are described in Table 1.

Identification and characterization of GTX cell lines

To confirm that the xenograft tumors corresponded to the primary patient tumors, we conducted histological analysis and DNA fingerprinting. Based on the Lauren classification, the primary tumors of the GTX-006 and GTX-007 cell lines were of diffuse type, and those of the GTX-085, GTX-087, GTX-116, and GTX-139 cell lines were of intestinal type. The xenograft tissues used for cell line culture displayed similarity with the primary gastric tumor tissues as shown in Fig. 1B and 1C. Using DNA fingerprinting, we detected heterogeneity among 15 tetranucleotide repeat loci and the amelogenin sex determination marker between the primary gastric tumor tissues and xenograft tissues used for cell culture (Table 2). STR profile of xenograft tissues were concordant to those of the primary tissues. Based on the aCGH analysis, abnormal chromosome ploidy levels in the primary gastric tumor tissues corresponded to those in the xenograft tissues used for cell culture (Supplementary Fig. 1). In some cases (e.g., GTX-086 and GTX-103), the xenograft tissues showed more severe genomic abnormalities, indicating that xenograft tissues were more similar to tumor cells than to primary tissues. To examine the sensitivity of the novel cell

Table 1. Characteristics of GTX cell lines *in vitro* culture

Cell lines	Xenograft cell culture					
	Lauren	Passage*	Growth characteristics	Cell morphology	IC ₅₀ (μg/mL)	
					5FU	Cisplatin
GTX-006	Diffuse	P6	Adherent/Suspended	Round	6.70	5.29
GTX-007	Diffuse	P5	Adherent/suspended	Round	7.27	4.70
GTX-085	Intestinal	P4	Adherent/suspended	Round	4.39	R
GTX-086	ID	P6	Adherent	Spindle	ND	ND
GTX-087	Intestinal	P5	Adherent	Round	2.97	3.10
GTX-103	ID	P5	Adherent	Polygonal	4.89	3.29
GTX-116	Intestinal	P2	Adherent/suspended	Round	ND	ND
GTX-139	Intestinal	P2	Adherent/suspended	Round	ND	ND

ID, Indeterminate; ND, Not Done; R, Resistant

**In vivo* passage number of xenograft tissue for cell culture

Fig. 1. Morphology of established cell lines. (A) Phase-contrast microscopy of eight established GTX cell lines (magnification 20×). (B) Histology of primary tumors. (C) H&E histology staining of xenograft tumors from which GTX cell lines originated. Scale bar = 100 μm. (D) Immunofluorescence of cytokeratin in cell culture. Confocal microscopy images show immunofluorescence of cytokeratin, marked with a FITC-conjugated antibody (green), in eight GTX-cell lines.

lines to antitumor drugs, cells were treated for 72 h with 5-FU and cisplatin, and the IC_{50} values were determined (Table 1). GTX-085 cells were resistant to cisplatin, while

GTX-087 was the most sensitive cell line. GTX-085 was the only cell line showing a metastatic potential as described in the next section. Immunocytochemistry of cytokeratin per-

Table 2. DNA fingerprinting analysis of 16 STR loci of primary gastric tumor tissues (GT) and xenograft tissues (GTX) for newly established cell lines

Locus	Chromosome location	GT-006	GTX-006	GT-007	GTX-007	GT-085	GTX-085	GT-086	GTX-086	GT-087	GTX-087	GT-103	GTX-103	GT-116	GTX-116	GT-139	GTX-139
D8S1179	8	11, 14	11, 14	12, 14	12, 14	15, 17	15, 17	13, 14	13, 14	10, 12	10, 12	15	15	11, 12	11, 12	10, 11	10, 11
D21S11	21q11.2-q21	29, 31	29, 31	29, 30	29, 30	29, 34	29, 34	29, 31	29, 31	29, 30	29, 30	30, 32.2	30, 32.2	28, 33.2	28, 33.2	31.2	31.2
D7S820	7q11.21-22	8, 11	8, 11	10, 11	10, 11	10, 12	10, 12	10, 11	10, 11	10, 12	10, 12	8, 13	8, 13	8, 12	8, 12	10, 12	10, 12
CSF1PO	5q33.3-34	12	Fail	12, 13	12, 13	8, 11	8, 11	12	12	11	11	11	11	8, 9	8, 9	9, 12	9, 12
D3S1358	3p	16, 17	16, 17	16, 17	16, 17	16, 17	16, 17	15, 16	15, 16	16	16	15, 18	18	17	17	13, 14	13, 14
TH01	11p15.5	7	7	6, 7	6, 7	9, 10	9, 10	7, 9	7, 9	9	9	9, 9.3	9, 9.3	7, 9	7, 9	7, 9	7, 9
D13S317	13q22-31	10, 12	10, 12	10, 11	10	9, 14	9, 14	10, 12	10, 12	8, 9	8, 9	8	8	11, 12	11, 12	8, 9	8, 9
D16S539	16q24-qter	10, 11	Fail	8, 11	8, 11	10, 13	10, 13	9, 11	11	9, 10	9, 10	11, 12	12	10, 11	10, 11	9	9
D2S1338	2q35-37.1	23	23	19, 23	19, 23	23, 24	23, 24	20, 24	20, 24	23	23	23, 24	23, 24	20	20	17, 25	17, 25
D19S433	19q12-13.1	13, 14	13, 14	13, 14.2	13, 14.2	11, 14.2	11, 14.2	14, 14.2	14, 14.2	13, 15	13, 15	14, 15	14, 15	11, 12	11, 12	12, 14	12, 14
vWA	12p12-pter	14, 18	14, 18	18, 19	18, 19	18, 20	18, 20	14, 18	14, 18	16, 18	16, 18	17, 18	17, 18	18, 19	18, 19	19, 22	19, 22
TPOX	2p23-2per	8	8	9, 11	9, 11	8, 9	8, 9	11	11	8	8	8	8	9, 11	9, 11	8, 9	8, 9
D18S51	18q21.3	18, 19	Fail	13, 14	13, 14	12, 13	12, 13	14	14	14	14	17	17	16, 17	16, 17	15, 17	15, 17
X	X:p22.1-22.3 Y:p11.2	XY	X	X, Y	X	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X	X	X	X	X, Y	X, Y
D5S818	5q21-31	10, 13	10, 13	9, 10	9, 10	8, 11	8, 11	7, 10	7, 10	9, 12	12	10, 11	10, 11	10, 11	10, 11	10, 12	10, 12
FGA	4q28	23, 25	23, 25	22, 23	22, 23	20, 21	20, 21	23, 26	23, 26	22	22	20, 22	20	19, 21	19, 21	21, 22	21, 22

Table 3. Summary of *in vivo* tumorigenicity screening analysis of GTX cell lines

Cell lines	Subcutaneous		Orthotopic		
	Number of cells injected	Tumor*	Number of cells injected	Tumor*	Metastasis
GTX-006	2.46×10^6	2/3	2.46×10^6	2/2	x
GTX-007	1.13×10^6	2/2	1.13×10^6	1/2	x
GTX-085	1.00×10^6	1/2	1.00×10^6	2/3	Ovary
GTX-086	2.00×10^6	2/2	ND	ND	ND
GTX-087	2.50×10^6	1/2	2.50×10^6	0/2	x
GTX-103	2.50×10^6	0/2	2.00×10^6	0/2	x
GTX-116	1.00×10^6	1/1	1.00×10^6	1/3	x
GTX-139	2.50×10^6	2/2	2.50×10^6	2/3	x

*Number of mice with tumor growths / total number of mice with injection

formed on the GTX cell lines showed that cytokeratin was expressed in all the GTX cell lines (Fig. 1D).

High tumorigenicity of gastric tumor xenograft cell lines

The tumorigenicity and metastatic potential of the eight GTX cell lines in mice are summarized in Table 3. Seven cell lines, except GTX-103, showed high tumorigenicity in

4~6 weeks after subcutaneous injection to nude mice (Fig. 2A). In the case of GTX-086, which was derived from an EBV-positive gastric cancer, cells were injected to NSG mice (Fig. 2B). After cells were orthotopically injected into the stomach serosa, five cell lines (GTX-006, GTX-007, GTX-085, GTX-116, and GTX-139) formed tumor grafts (Fig. 2C). Among these, the GTX-085 line spontaneously metas-

Fig. 2. Screening of eight newly established GTX cell lines for tumorigenicity. (A) Tumor growth after subcutaneous injection of six GTX cell lines (GTX-006, GTX-007, GTX-085, GTX-087, GTX-116, and GTX-139) to nude mice. Mice were sacrificed 4~5 weeks after the injection. (B) Tumor growth after subcutaneous injection of GTX-086 to NSG mice. Mice were sacrificed 6 weeks after the injection. (C) Orthotopic tumorigenicity screening of newly established GTX cell lines. Magnetic resonance (top), macroscopic imaging (middle), and H&E staining (bottom) of the tumors. Autopsy of mice was carried out 3~4 months after the injection.

tasized to the ovary in nude mice (GTX-085-meta; Fig. 2C). Histological features of these xenograft tumors were similar to those of their primary tumors.

Distribution of cancer stem cell markers in GTX cell lines

Cancer stem cells (CSCs) are regarded as the driving force of tumor formation *in vivo*, responsible for antitumor

Fig. 3. Expression of cancer stem cell markers in GTX cell lines and xenograft tumors derived from GTX cell lines in nude mice. (A) Western blot of CSC markers in seven GTX cell lines. (B) Immunohistochemistry of CSC markers in xenograft tumor tissues generated by subcutaneous injection from GTX cell lines.

resistance. To identify the presence of gastric CSCs, expression of several markers was evaluated by Western blotting in seven GTX cell lines (except GTX-086) (Fig. 3A). Epithelial cell adhesion molecule (EPCAM) was expressed in all the GTX cell lines, and CD44v6 was expressed in GTX-007, GTX-85, GTX-087, GTX-116, and GTX-139. Fibroblast growth factor receptor 2 (FGFR2) was strongly expressed in GTX-087, and THY1 membrane glycoprotein was expressed in five GTX cell lines, except GTX-087 and GTX-103.

Immunohistochemistry was performed to detect the expression of CSC markers on tumor grafts obtained after subcutaneous injection of GTX cell lines to nude mice (Fig. 3B). EPCAM expression was especially strong in all samples, similar to the cell cultures, and GTX-139, the largest tumor graft formed after the injection (Fig. 2A), displayed distinct expression of CD44v6, FGFR2, and prominin 1 (PROM1, also known as CD133).

DISCUSSION

Most of the current research on the biology of gastric cancer is based on *in vivo* and *in vitro* experiments performed with gastric cancer cell lines. Because authentic cultured cells can be applied to many studies, when tissue specimens are

not available, permanent cell lines established from human cancers have played an important role in the understanding of the biology of cancers (Ku et al., 2012). The present study describes eight gastric cancer cell lines established from xenograft tumor tissues, which originated from transplantation of primary tissue. Each cell line was shown to be unique and related to the primary tissue at the DNA level, as shown by fingerprinting analysis. None of the cell lines was contaminated by mycoplasmas or bacteria.

Animal models play a crucial role in drug development and research on cancer biology. Various animal models have been established for the investigation of carcinogenic mechanisms and prediction of targeted therapies (Richmond and Su, 2008). However, generation of animal models using primary tumor tissue is time-consuming and expensive, and thus its application is limited in the research of anticancer drug responses. The eight cell lines from our study showed high tumorigenicity in a few weeks, and their relevant tumor biology mirrored the histochemical and biochemical features of the primary gastric tumors. However, further investigation on the differences between direct culture of primary tissue and xenograft tissue culture cell lines should be performed for elucidating the efficiency of GTX cell lines.

The CD44 isoform overexpression is significantly associ-

ated with tumor progression, tumorigenicity, growth, metastasis, and prognosis of many carcinomas in humans. Binding of CD44 with its ligands may induce the production of autocrine growth factors, which play an important role in tumor growth (Kodama et al., 2017). Our data from the *in vivo* tumorigenesis screening of the GTX cell lines revealed that CD44v6 expression was correlated with the tumor growth rate and tumor size. Thus, GTX-006 showed a low level of expression of CD44v6 and low tumorigenicity, while GTX-116 exhibited high tumorigenicity and overexpression of CD44v6.

Metastasis of gastric cancer occurs via hematogenous and lymphogenous routes, peritoneal seeding, and direct invasion. Metastasis is a very complex process, involving multiple consecutive steps (Fidler, 2002). A mouse model of metastatic gastric cancer is an extremely valuable tool to study the metastatic process. In the present study, the mice injected orthotopically with GTX-085 developed tumor growth at the injected position and ovary metastasis. This cell line facilitated research of Krukenberg tumor, poor prognostic features associated with GC metastasis to the ovary (Lee et al., 2015; Jung et al., 2017).

Recent studies have reported an association of EBV with 6~16% of gastric cancer cases worldwide (Shibata et al., 1993; van Beek et al., 2002). EBV-positive gastric cancer cell lines such as SNU-719, NCC-24, and YCCE1 have been established and characterized (Oh et al., 2007; Ku et al., 2012). However, attempts to obtain stable EBV-positive cell lines from carcinoma tissues have been largely unsuccessful since the EBV genome tends to be lost during *in vitro* passages (Lin et al., 1994). Our data on the naturally EBV-infected GTX-086 line, obtained in the present study, also showed that the EBV RNA disappeared as the passage number increased (Supplementary Fig. 2).

In summary, we established eight xenograft cell lines from gastric cancer patient tissues, with their histological and molecular features consistent with those of the primary tumors. Patient-derived xenograft cell lines represent ideal tools for studying tumorigenicity of human stomach cancers *in vitro* and *in vivo*. The established GTX cell lines will enable future studies on their responses to different treatments of gastric cancer.

ABBREVIATIONS

aCGH, array comparative genomic hybridization
CSC, cancer stem cell
EBV, Epstein-Barr virus
EPCAM, epithelial cell adhesion molecule
FBS, fetal bovine serum
FGFR2, fibroblast growth factor receptor 2
FITC, fluorescein isothiocyanate
5-FU, 5-fluorouracil
GC, gastric carcinoma
gDNA, genomic DNA
GTX, gastric tumor xenograft
HBSS, Hank's balanced salt solution
H&E, hematoxylin and eosin
PBS, phosphate-buffered saline
PCR, polymerase chain reaction

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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