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ORIGINAL ARTICLE

Intravenous paclitaxel against metastasis of human gastric tumors of diffuse type

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Abstract
Purpose Gastric cancer is one of the leading cancerous diseases worldwide. It is diagnosed often at the advanced stage for which chemotherapy is the main treatment option. The prognosis remains poor for metastatic, especially the diffuse type, gastric cancers. We investigated the efficacy of intravenously administered paclitaxel treating metastases of locally disseminated gastric tumors of diffuse type.
Methods Transfection of green fluorescent proteins (GFP)-expressing plasmid into human gastric cancer MKN45 cells of diffuse type was performed, and MKN45-GFP cells constitutively expressing GFP were isolated. The MKN45-GFP cells were orthotopically inoculated into the mouse peritoneal cavity, and tumor growth and organ metastases were monitored. Liver metastases were harvested, re-inoculated, monitored for liver metastases again, and harvested for further inoculation. This in vivo selection procedure was repeated to isolate a subline with high metastatic abilities demonstrated by in vitro invasion abilities using Transwell® system. By visualizing the GFP-expressing tumors, the effects of intravenously administered paclitaxel against the growing peritoneally disseminated and metastasized tumors in nude mice without laparotomy were measured.
Results An in vivo selected gastric cancer cell line MKN45-GFP-ip4 with high metastatic ability was established. Its invasion ability was inhibited by paclitaxel treatments in vitro. The growths of metastatic and intraperitoneally disseminated MKN45-GFP-ip4 tumors were significantly suppressed by intravenous paclitaxel treatments in nude mice.
Conclusions We found that intravenous paclitaxel is active against the metastases of human gastric cancer of peritoneal diffuse type, which warrants further investigations on optimizing the perioperative regimens with intravenous paclitaxel therapy for gastric cancer in patients.

Keywords Stomach · Taxol · Paclitaxel · Metastasis · Chemotherapy

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Introduction

Gastric cancer is one of the most common human cancers [1] and the second most common cause of cancer death in the world [2]. Gastric cancer has a wide variation in the geographical distribution. An estimated 21,500 new cases were diagnosed with an estimate of 10,880 deaths in the United States in 2008 [3]. The gastric cancer incidence is particularly high in Asia, South America, and Eastern Europe [4, 5], ranked as the second and the fourth overall

leading cause of cancer death in Japan and Taiwan, respectively [6, 7]. The prognosis for patients with advanced gastric cancer remains dismal, and little improvement in survival has been achieved in recent years [8, 9]. Gastric cancer is often diagnosed at advanced stages, with approximately half of all patients presenting with unresectable, locally advanced or metastatic diseases [9, 10], recurrence after curative resection for gastric cancer remains high, and the prognosis is generally poor with a 5-year relative survival at 3.4% with cancer in the distant stage and 26.6% in the regional cancer stage [9, 11]. Gastric carcinomas of diffuse, intestinal, and mixed types accounted for 47, 34, and 19% of 217 patients examined, respectively, and diffuse type showed a survival rate (42%) poorer than that (64%) of intestinal-type gastric cancer [10]. Therefore, gastric cancer of diffuse type remains the major problem in treating gastric cancer, and the development of effective drugs and regimens against this cancer type is warranted.

The subcutaneously xenografted growing human tumor model in immunodeficient mice has been a conventional tool used in drug discovery and development for several decades including gastric tumors. It has been challenged as not sufficiently representing the clinical condition of tumor growth in patients, especially regarding to tumor metastasis and drug sensitivity [12]. A concept of orthotopic growing human tumors in animals in the same type of organs from which the human tumors are originated has been practiced in tumors of different organ types [12–14]. A number of orthotopic human tumor models have been established for delineating molecular and biological events and for evaluations of anticancer agents in animals [12–14]. It is of significance to establish a clinically relevant preclinical animal model of human diffuse-type gastric cancer. The model can thus be utilized as an investigational tool for further discovery of drugs treating human diffuse-type gastric cancer.

Paclitaxel is a natural compound purified from the bark of the Pacific yew [15]. It binds to microtubules, promotes microtubule polymerization, and stabilizes microtubules [16] resulting in intervention of the cell cycle and induction of apoptosis in cancer cells, including human gastric cells [17]. Paclitaxel has been used in treating gastric cancer in patients of advanced diseases [18, 19]. However, the clinical outcomes have not been consistent in different studies [20]. Intuitively and as demonstrated previously, locally administered intraperitoneal paclitaxel reached a high concentration in the nude mouse peritoneum and was, therefore, effective against the locally disseminated human gastric tumor cells [21]. In the same study, intravenously administered paclitaxel, however, did not show activities against the locally growing human gastric tumors cells in the peritoneum [21]. Gastric tumors are frequently observed as metastases in the patient liver [22]. It was reported that the paclitaxel tissue concentration was higher in the liver when systemically given via intravenous administration in animals [23]. It is interesting to further explore if systemically administered paclitaxel is efficacious in treating the human gastric carcinomas of diffuse type in a clinically relevant mouse model resembling the tumor metastasis processes.

By mimicking the clinical condition in which the diffuse-type gastric cancer cells are disseminated and growing locally in the patient peritoneum and/or in the secondary metastasized organs, we have established a highly invasive diffuse-type human gastric cancer cell subline by in vivo intraperitoneal selection in nude mice. An orthotopic tumor model with intraperitoneal and metastasized growing tumors given rise from the human gastric cancer cell subline was established in nude mice. The orthotopic gastric cancer model was used to investigate the antitumoral activity of systemically administered paclitaxel by intravenous injection.

Materials and methods

Cell line and transfection

Human gastric cancer cell line MKN45 was purchased from the Japanese Collection of Research Bioresources, Human Science Research Resources Bank (Osaka, Japan). The cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C and 5% CO_2. To establish a stable green fluorescence protein (GFP)-expressed MKN45 cell line, MKN45 cells were seeded onto 24-well culture plate and grown to 70–90% confluence, and incubated with a mixture of 1 μg GFP-expression plasmid pEGFP-C1 (Cat# 6084), 98 μl OPTI-MEM (Cat# 31985), and 2 μl Lipofectamine™ 2000 (Cat# 11668) from Invitrogen Corp. (Carlsbad, CA, USA). After 48 h of the incubation, the treated cells were incubated with 1 mg/ml G418 (Cat# 11811) from GIBCO, and the stable GFP-expressing MKN45 cell line, MKN45-GFP, was selected. All culture materials were purchased from GIBCO (Grand Island, NY, USA). Expressed GFP and cell morphology were viewed using an inverted fluorescent microscope DM IRB Leica (Wetzlar, Germany).

In vivo selection of invasive MKN45-GFP sublines

The established MKN45-GFP cells were peritoneally inoculated into BALB/c nude mice at 5×10^6 cells/0.25 ml per mouse mixed with Matrigel™ from BD Biosciences (Bedford, MA, USA) in a 1:1 ratio. Twelve weeks later, tumors invaded the mouse liver were harvested and minced using sterile blades into small pieces in RPMI 1640 containing

1 mg/ml collagenase IV from Sigma (St. Louis, MO, USA). The tumor tissues were then incubated for enzymatic digestion at 37°C for 3–5 h. After the incubation, the digested suspensions were centrifuged for 1 min at 100–200 rpm (10*g*) to remove tissue debris. Dissociated cells were washed three times in phosphate-buffered saline (PBS) to remove collagenase IV and injected into nude mice intraperitoneally (5×10^6 cells/0.25 ml per mouse mixed with Matrigel™ in a 1:1 ratio). Twelve weeks later, all mice were killed, and the peritoneal tumor nodules that invaded liver were dissected. The tumor nodules were dissociated again for intraperitoneal inoculation to nude mice. The intraperitoneal inoculation and tumor cell dissociation procedures were repeated for a total of four times and a cell line, MKN45-GFP-ip4, with a high potential of peritoneal dissemination and tissue invasion was collected. The care and use of the animals were approved by the Institutional Animal Care and Use Committee of The National Health Research Institutes.

Measurement of cell doubling time and growth inhibition by paclitaxel

Modified from the previously reported [24], the gastric cancer cell proliferation and growth inhibitory activities by paclitaxel were measured using a colorimetric MTS/PMS assay. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, was purchased from Promega (Madison, WI, USA), and PMS, phenazine methosulfate an electron-coupling reagent was from Sigma (St. Louis, MO, USA). Briefly, cells were seeded onto the 96-well microtiter plates at a density of 4,500 cells/well in 100 µl and incubated at 37°C overnight in an atmosphere of 5% CO_2 before drug exposure. Cancer cells in culture were collected every 24 h for 5 days, cell numbers were measured, and the cell doubling time was estimated using SigmaPlot from SPSS Inc. (Chicago, IL, USA). For measurement of growth inhibition by paclitaxel, cancer cells in culture were replenished with the medium containing paclitaxel from Sigma (St. Louis, MO, USA) in a range of concentrations from 0.01 nM to 1 µM dissolved in 0.01% dimethyl sulfoxide (DMSO) for 72 h. After washing the cells with PBS, MTS/PMS solution of 100 µl was added to each well followed by a 2-h incubation at 37°C. The number of cells correlated by the optical density of the formazan reaction solution was measured at the wavelength of 490 nm using a microplate reader Zenyth 340st from Anthos Labtec (Cambridge, UK). The concentrations that inhibited the growth activity by 50%, IC_{50} of paclitaxel in these gastric cancer cells was then determined. Data values of each drug concentration were resulted from five replicated wells and from at least three experiments carried out independently.

Western blot assay

MKN45-GFP and MKN45-GFP-ip4 cells seeded at 4×10^5 cells per well of 6-well culture plates overnight were treated with culture media containing paclitaxel of 0.1, 1, and 10 µM for 16 h. Cell lysates were prepared using RIPA buffer from Sigma Chemicals (St. Louis, MO, USA), and proteins of 20 µg per loading lane were separated by 10% SDS–PAGE and transferred to PVDF membrane from Millipore (Billerica, MA, USA). Primary antibodies against matrix metalloproteinase 9 (MMP9) (1:200 dilution) and internal control GAPDH (1:1,000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were incubated with the membrane at 4°C overnight. The HRP-conjugated secondary antibodies against goat and rabbit IgGs for MMP9 and GAPDH from LabFrontier Co. (Seoul, Korea) were, respectively, incubated at 1:5,000 dilution with the membrane for 1 h at room temperature. The antibodies amplified signals were detected using ECL Western blotting detection system from Millipore with Kodak BioMax light films. The MMP9 protein levels were quantified as integrated optical densities using Image-Pro Plus from Media Cybernetics, Inc. (Bethesda, MD, USA) and normalized, correspondingly, to those of GAPDH. To compare the MMP9 expression levels among the cells, a relative protein expression ratio was calculated by dividing the normalized MMP9 expression level of the paclitaxel-treated cells by that of nontreated for individual cells.

In vitro invasion assay

Measurement of cell invasion activity using the matrix-coated Transwell™ system was performed according to the previously reported [25] with modifications. The upper-chambers of the Transwell™ plates of 24-well from Corning (Acton, MA, USA) were coated each with 50 µl per well of a mixture of Matrigel™ from BD Biosciences (Bedford, MA, USA) and phenol-red free RPMI1640 in 1:20 (v:v) ratio. The coated plates were dried in room temperature over night, and the cancer cells were seeded onto the coated semi-permeable membrane in the upper-chamber at 1×10^5 cells in 200 µl of RPMI 1640 with 1% FBS. The lower-chambers were filled with 600 µl of RPMI 1640 supplemented with 10% FBS. The cancer cells were pretreated with paclitaxel at a range of concentrations from 0.01 to 100 nM for 12 h before seeded on the upper-chamber for measurement of invasion activity. After incubation at 37°C for 72 h, the culture wells were fixed with 3.7% formaldehyde in PBS for 15 min. The cells and Matrigel™ on the upper surface of the membrane were scraped by cotton swab. The cells that invaded the coated Matrigel™ and penetrated through the Transwell™ membrane to the lower surface of the membrane were stained for nuclei with

hematoxylin. The cells were then visualized and cell number counted from at least 3 different fields of 100-fold magnification for each concentration group under a microscope. The IC_{50}, a concentration needed to inhibit 50% of the invasion ability of MKN45-GFP-ip4 cells, was estimated using a previously reported sigmoidal pharmacodynamic model [26].

Metastatic and intraperitoneal tumor growth and paclitaxel treatments

Male athymic BALB/c nude mice of 6- to 8-weeks old purchased from the National Laboratory Animal Center (Taipei, Taiwan) were housed in sterilized cages with water and feed ad libitum under 12-h light/dark cycles. MKN45-GFP-ip4 cells were suspended in the RPMI1640 phenol-red free medium, mixed with Matrigel™ in a ratio of 1:1 (v:v), and inoculated at 5×10^6 cells/mouse intraperitoneally to the nude mice. Intraperitoneal growing tumors in mice were visualized using a stereo-fluorescence microscope MZ FLIII from Leica (Wetzlar, Germany). Excitation and emission for GFP visualization were carried out with a D470/40× bandpass filter and a 495DCLR dichroic mirror. Paclitaxel was dissolved in a mixture of DMSO/Cremophor EL/saline (5/20/75) at 2 mg/ml and intravenously (tail veins) administered at 10 mg/kg once per week for two consecutive weeks. Paclitaxel treatments were initiated 2 weeks after the intraperitoneal inoculation of MKN45-GFP-ip4 cells. Control animals received 100 µl of the vehicle each accordingly. The organ-invaded metastatic and intraperitoneal disseminated tumors were counted twice per week. At the end of the experiment, each mouse was laparotomized, and the number of total tumors per mouse was examined. Observation of fluorescent tumors in the peritoneal cavity was accomplished by LT-9500 Illumatool TLS a fluorescent imaging system. The harvested tumors were fixed in 3.7% formalin, alcohol-dehydrated, and paraffin embedded using a Hypercenter XP Tissue Processor and Histocentre Embedding Center from Thermo Shandon (Waltham, MA, USA). Tumor tissue sections of 5 µm thick were collected onto microscopic slides. The images of tissue sections on slides were taken in fluorescent mode first, and then the slides were processed for hematoxylin and eosin staining before taking bright field images using an inverted fluorescent microscope DM IRB Leica. The care and use of the animals were approved by the Institutional Animal Care and Use Committee of The National Health Research Institutes.

Statistic analysis

Data were analyzed for a significant difference using *ANOVA* or *ANOVA* followed by multiple comparisons using *Student–Newman–Keuls Test*. A significant difference between groups was considered with a p value <0.05.

Results

Establishment of MKN45-GFP-ip4 cells

Stable GFP-expressing MKN45-GFP cells shown in Fig. 1a, b were established by using transfection of the GFP-expressing plasmids followed by in vitro selection with G418. The MKN45-GFP cells were further selected in vivo after 4 cycles of intraperitoneal growth selection in nude mice, and a metastatic subline MKN45-GFP-ip4 with high invasion ability was collected from a liver-metastasized tumor. The morphologies of MKN45-GFP-ip4 cells were shown in Fig. 1c, d. There is no obvious morphologic change after the in vivo selection showing only a tendency of more MKN45-GFP-ip4 cells in spindle shape as shown in Fig. 1a, c. The fluorescence of GFP allows observing and monitoring the tumor growth in vivo in nude mice.

Invasion ability of MKN45-GFP-ip4 cells

After a 72-h incubation, the representative images of the invaded cells on the bottom surface of the Transwell™ membrane stained for nuclei with hematoxylin were shown in Fig. 2a (MKN45), b (MKN45-GFP), and c (MKN45-GFP-ip4). As shown in Fig. 2d, MKN45-GFP-ip4 cells exhibited a significantly (approximately four–five fold) higher invasion ability than those of MKN45 and MKN45-GFP. While MKN45-GFP-ip4 was more invasive, MKN45-GFP shared a similar invasion ability to that of the parental MKN45 cells, indicating no phenotypic changes in the invasion ability caused by the GFP transfection and expression.

Inhibition of the invasive ability of MKN45-GFP-ip4 by paclitaxel treatments

Paclitaxel pretreatments for 12 h inhibited the invasion of MKN45-GFP-ip4 cells. Two representative photographs of treated MKN45-GFP-ip4 cells with paclitaxel of 1 and 100 nM were shown in Fig. 3a, b. As shown in Fig. 3c, paclitaxel pretreatments significantly inhibited the invasion ability of MKN45-GFP-ip4 cells in a concentration-dependent relationship with an IC_{50} of 0.64 nM. Data were collected from at least three repeated experiments. Figure 3d showed that MMP9 protein levels were not different between MKN45-GFP and MKN45-GFP-ip4 cells. Paclitaxel treatments at 0.1, 1, and 10 µM for 16 h did not cause significant changes of the MMP9 protein expression in both cell lines. The results suggested that paclitaxel inhibited the

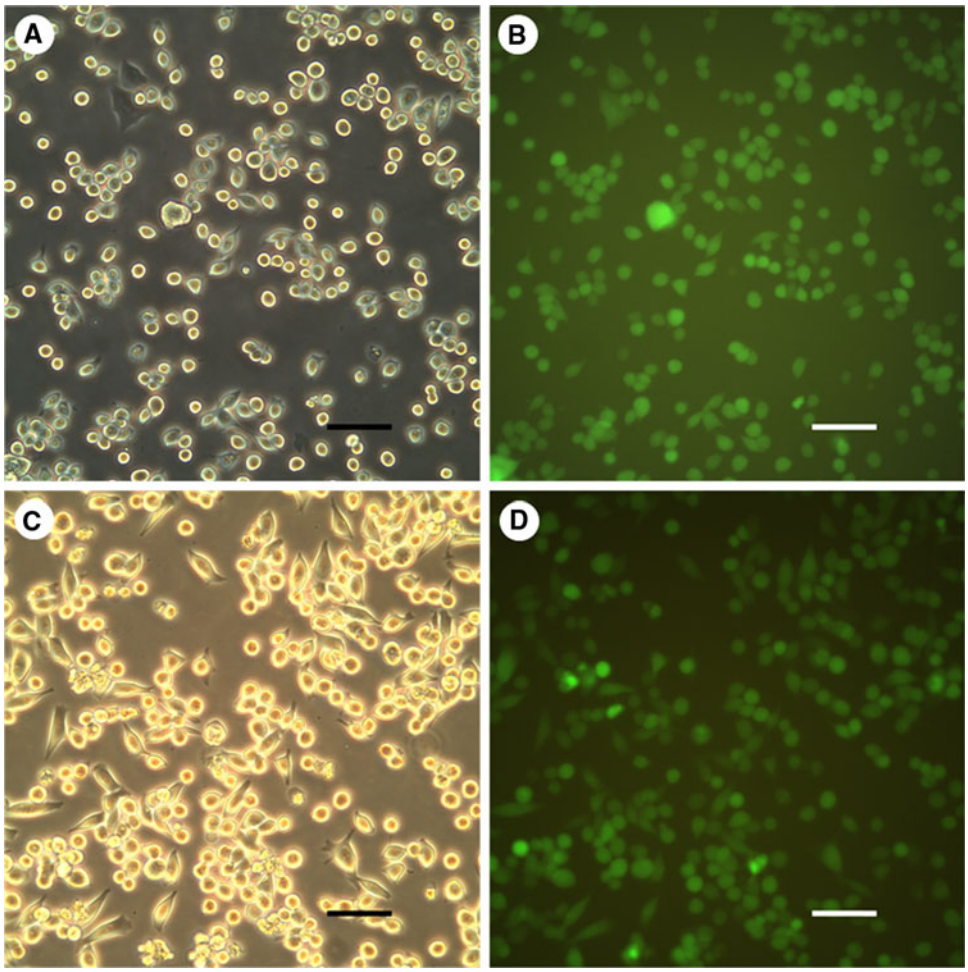

Fig. 1 Morphology of MKN45-GFP and MKN45-GFP-ip4 cells. The established stable constitutively GFP-expressing cells, MKN45-GFP (**a** and **b**) transfected with GFP-expression plasmids and MKN45-GFP-ip4 (**c** and **d**) in vivo selected with invasion and liver metastasis abilities, were observed under bright and fluorescent fields, respectively. *Scale bar* 50 μm

invasion of MKN45-GFP-ip4 cells through a MMP9-independent signaling pathway.

Growth inhibition in MKN45 sublines by paclitaxel treatments

Paclitaxel treatments inhibited the growth of MKN45 cancer sublines. The IC_{50} values of paclitaxel treatments for 72 h against MKN45, MKN45-GFP, and MKN45-GFP-ip4 cells were listed in Table 1. Paclitaxel showed no significantly different activities in the growth inhibition between MKN45 and MKN45-GFP cells and exerted a significant and more potent inhibitory activity on MKN45-GFP-ip4 cells. The results suggested that the MKN45-GFP-ip4 cells were more sensitive to paclitaxel than its parental MKN45 and MKN45-GFP cells.

Orthotopic growth of MKN45-GFP-ip4 cells intraperitoneally inoculated in nude mice

Orthotopic tumor growth of the intraperitoneally inoculated MKN45-GFP-ip4 cells in nude mice was monitored and found that the MKN45-GFP-ip4 tumor cells grew as disseminated and metastasized tumors on and/or into the mesentery, pancreas (data not shown), intestines (data not shown), stomach, and liver. Representative photographs were shown in Fig. 4a, b in which the disseminated tumors in the mesenteries and the metastasized tumors in the stomach and liver were shown. As shown in Fig. 4c, d, photographs of the whole-body fluorescence imaging of a nude mouse with MKN45-GFP-ip4 tumors were taken before and after laparotomy. Hematoxylin- and eosin-stained tissue sections of the liver-metastasized MKN45-GFP-ip4 tumors were examined under microscope and found that MKN45-GFP-ip4 tumors exhibited both poorly differentiated and well-differentiated glandular histological structures as shown in Fig. 4e, f, respectively. Liver invasive MKN45-GFP-ip4 cells were shown in Fig. 4g, h. After intraperitoneally inoculated at 5×10^6 cells per mouse, MKN45-GFP cells also gave rise to tumors growing intraperitoneally in nude mouse with a less frequency of organ metastasis (not shown). A mouse with an intraperitoneally disseminated growing MKN45-GFP tumor was shown in Fig. 4i. Intraperitoneally growing MKN45-GFP-ip4 cells

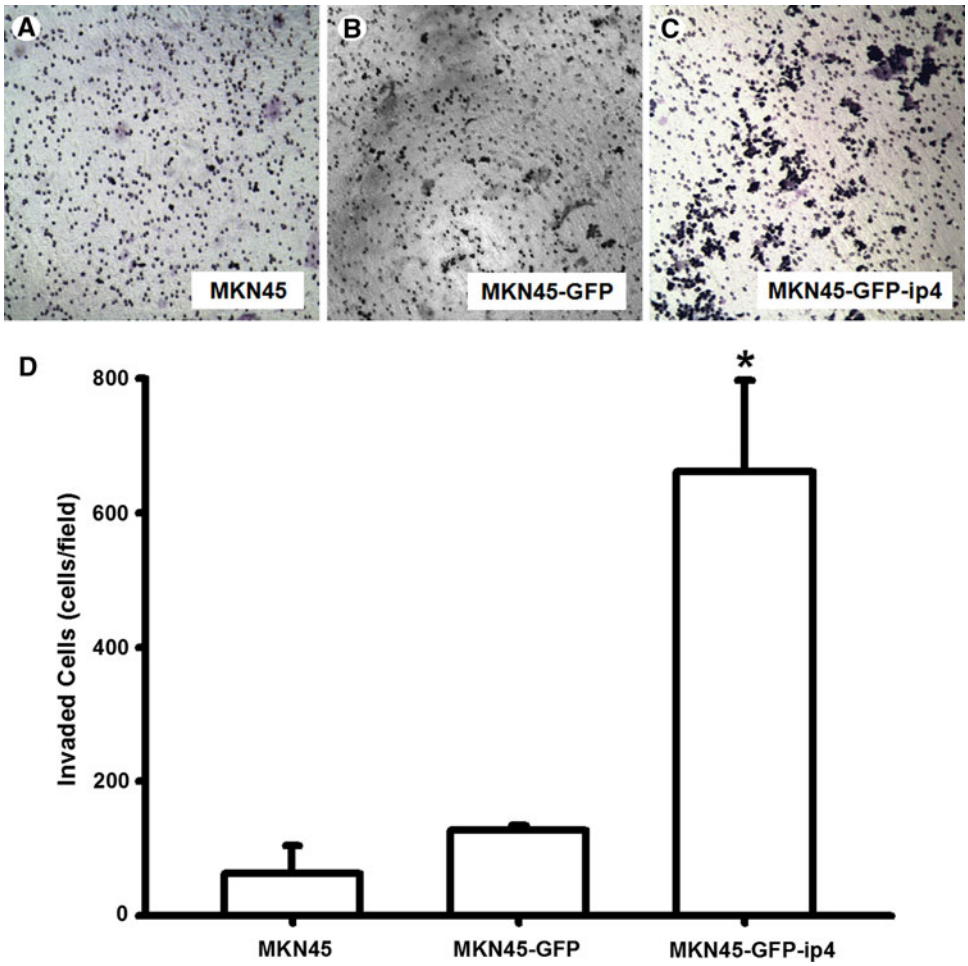

Fig. 2 Increased invasion ability of MKN45-GFP-ip4 cells. MKN45, MKN45-GFP, and MKN45-GFP-ip4 cells invaded through the Matrigel™-coated Transwell® membrane in culture for 72 h were stained with hematoxylin and representative photographs were shown in (**a**), (**b**), and (**c**), respectively. The relative invasion abilities of the three cell lines were measured. MKN45-GFP-ip4 cells showed a significantly higher invasive ability than those of MKN45 and MKN45-GFP cells as shown in (**d**). (*$p < 0.05$, *Student–Newman–Keuls Test*)

metastasized and grew invasively into the liver in the nude mouse.

Paclitaxel inhibited the orthotopic growth of MKN45-GFP-ip4 cells in nude mice

Monitoring the total number of metastasized and intraperitoneally disseminated MKN45-GFP-ip4 tumors per mouse revealed that only one or two residual intraperitoneally disseminated MKN45-GFP-ip4 tumors were found in the paclitaxel-treated nude mouse. No metastatic tumors in stomach or liver can be found in the paclitaxel-treated mice. The total number of growing tumors in the nude mice was significantly less in the paclitaxel-treated group than that of the vehicle control group, respectively, at 1.6 ± 0.3 vs. 4.2 ± 0.2 (mean $\pm$ SEM, $n = 9$, $p < 0.01$, *ANOVA*) tumors per mouse on the 24th day after the initiation of the intravenous paclitaxel treatments. The observed mean numbers of the orthotopic growing metastasized and intraperitoneally disseminated tumors in the paclitaxel-treated and vehicle control nude mice over the observation time were shown in Fig. 5. Paclitaxel, intravenously given at 10 mg/kg once per week for 2 weeks, significantly inhibited the MKN45-GFP-ip4 tumor growth in the nude mice. The body weights of the two treatment mouse groups were not different (Fig. 5b).

Discussion

Diffuse-type gastric cancer is frequently diagnosed with more peritoneal dissemination, lymph node or organ metastases, and nerve permeation than intestinal-type gastric cancer leading to a poor prognosis of clinical outcome [27]. Diffuse-type gastric cancer is characterized as lack of cell cohesion and malignant growth infiltrating the surrounding tissues as single or small clusters of cells and/or poorly differentiated tumors without formation of glandular lumina [28, 29]. Differential spectra of genetic changes were observed between poorly differentiated and well-differentiated tumors collected from patients [30]. The intraperitoneally disseminated growing tumors in animals mimicked the metastatic processes of patient gastric cancer cells in vivo [31]. By having taken the advantages of the in vivo environment for selection of human cancer cell sublines with invasive and metastatic properties in mice, several putative

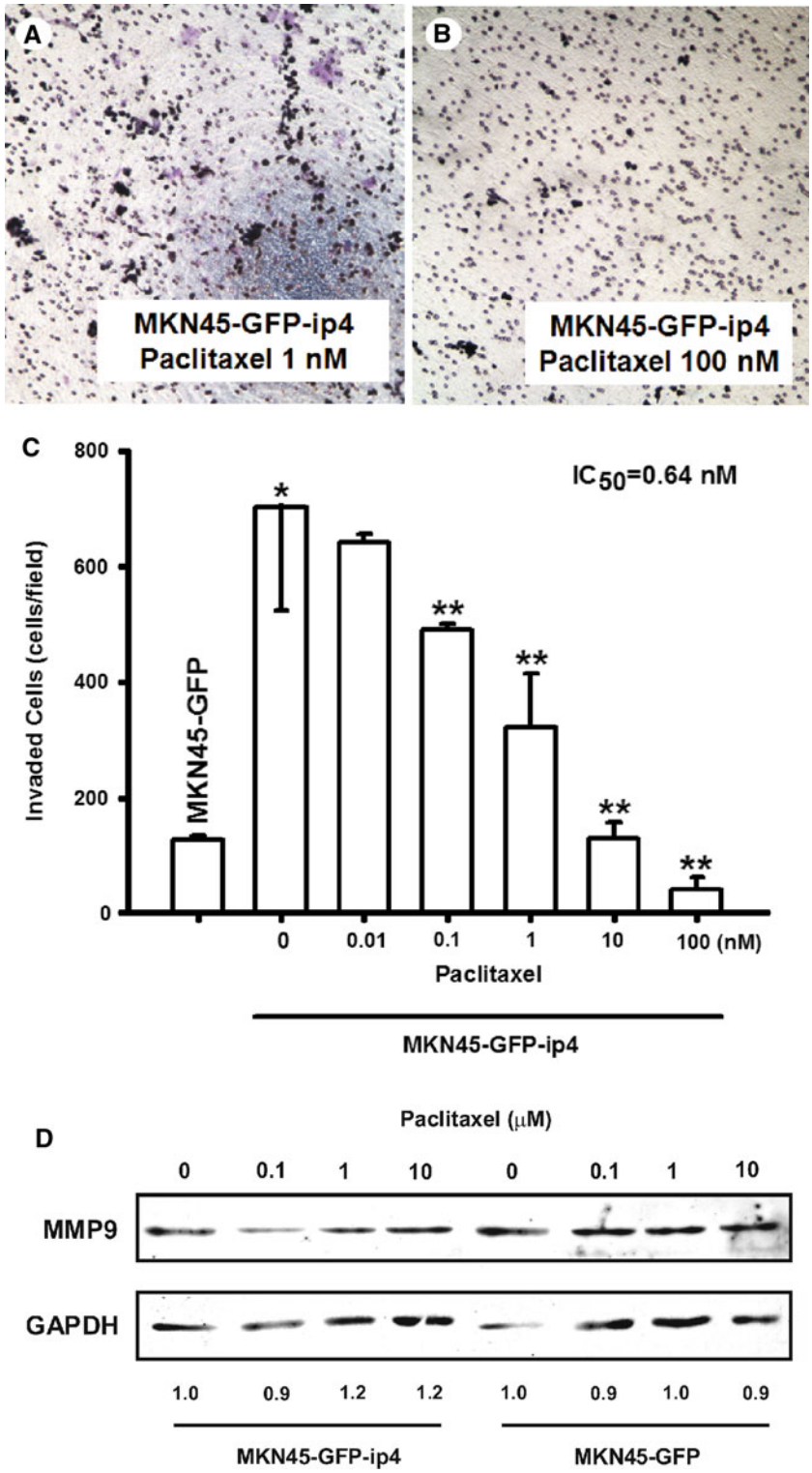

Fig. 3 Concentration-dependent inhibition on the invasion ability of MKN45-GFP-ip4 by paclitaxel treatments. The invasion ability of MKN45-GFP-ip4 cells was inhibited by a pretreatment of paclitaxel at 0.01–100 nM for 12 h. Two representative images with the hematoxylin-stained cells on the Transwell® membrane were shown in (**a**), 1 nM and (**b**), 100 nM. Quantitative measurements of the invaded cells were shown in (**c**), $*p < 0.05$ vs. MKN45-GFP; $**p < 0.05$ vs. nontreated MKN45-GFP-ip4, *Student–Newman–Keuls Test*. (**d**), Protein levels of MMP9 were not different in MKN45-GFP and MKN45-GFP-ip4 cells and showed no significant changes after paclitaxel treatments for 16 h

tumor metastasis-associated genes have been discovered [32–34]. We adopted the orthotopic implantation concepts [12, 35] and, by serial in vivo intraperitoneal selection processes, set up a noninvasive real-time whole-body fluorescence imaging system to visualize metastatic and intraperitoneally disseminated growing human gastric tumors in nude mice. MKN45 is a human gastric cancer cell of diffuse type and has been widely used in studies of human gastric cancer [36]. We have established a highly metastatic MKN45 gastric cancer subline, MKN45-GFP-ip4, with constitutively expressing reporter GFP of high fluorescence intensity. Matrix metalloproteinases such as MMP2 and

Table 1 Activities of paclitaxel against the MKN45 cell sublines

Cell lines	Doubling time (day)	IC_{50} (nM)[a]
MKN45	1.5 ± 0.3	20.8 ± 3.8
MKN45-GFP	1.3 ± 0.2	18.8 ± 2.2
MKN45-GFP-ip4	1.5 ± 0.1	8.4 ± 0.8*

[a] IC_{50}, the concentration required to reduce the number of the cancer cells to 50% of the control

* $p < 0.05$ vs. MKN45 and MKN45-GFP, *Student–Newman–Keuls Test*

MMP9 have played an important role in tumor angiogenesis and tumor metastasis [37]. It was, however, reported that MKN45 cells did not express MMP2 [38], as we also observed no MMP2 expression in both MKN45-GFP and MKN45-GFP-ip4 cells (data not shown). Together with our observations on MMP9 (Fig. 3d), it is likely that paclitaxel inhibits the invasion of MKN45-GFP-ip4 cells through interacting with MMP2- and MMP9-independent regulatory machineries, and the underlying mechanism is an interesting subject to be further studied.

MKN45-GFP-ip4 is capable of metastatic growth from mouse omentum to the liver, in which two distinct, poorly differentiated and well differentiated, histology formations were observed. Notably, the formation of well-differentiated glandular lumina in the liver-metastasized MKN45-GFP-ip4 tumors was observed. The finding warrants further investigations for MKN45 cells were originally isolated from a poorly differentiated gastric adenocarcinoma [36]. It would be interesting to demonstrate whether a differential expression of protein(s) may be responsible for change of the differentiation status in vivo as reported in MKN45 cells in vitro [39]. The well-differentiated glandular tumors were also observed after the in vivo intraperitoneal selection and during the metastatic growth conditions that mimic the pathological growing condition of cancer cells in intraperitoneal

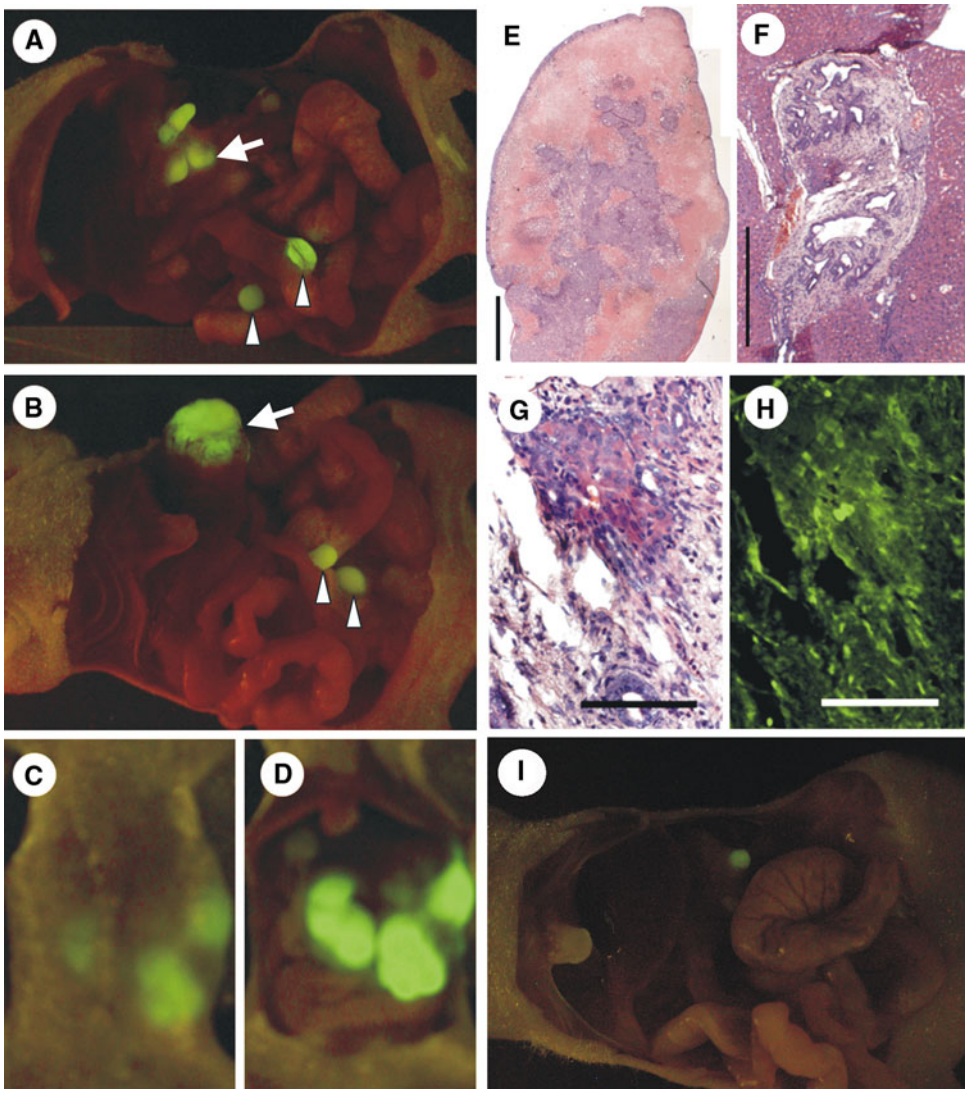

Fig. 4 Metastatic growth of orthotopically inoculated intraperitoneally MKN45-GFP-ip4 cells. MKN45-GFP-ip4 cells, intraperitoneally injected at 5×10^6 cells per mouse, grew as peritoneally disseminated and metastasized tumors in nude mice. The tumor cells grew into tumors to the mesenteries (*arrowheads*) and metastasized into the mouse stomach (**a**) and liver (**b**) as *arrows* indicated, respectively. MKN45-GFP-ip4 tumors were visualized using the whole-body fluorescence imaging for GFP-expressing tumors in nude mice (**c**) and after laparotomy (**d**). Invasion of MKN45-GFP-ip4 tumors into the mouse livers was demonstrated by the hematoxylin- and eosin-stained tissue sections, in which both the poorly differentiated (**e**) and well differentiated (**f**) glandular-like formation of the MKN45-GFP-ip4 tumors were observed. A tissue section of a mouse liver with invaded MKN45-GFP-ip4 tumor in bright and fluorescent fields was shown in (**g**) and (**h**), respectively. A nude mouse with an intraperitoneally disseminated growing tumor after intraperitoneally injected with 5×10^6 MKN45-GFP cells (**i**). *Scale bars* 1 mm (**e**), 500 μm (**f**), 100 μm (**g**) and (**h**)

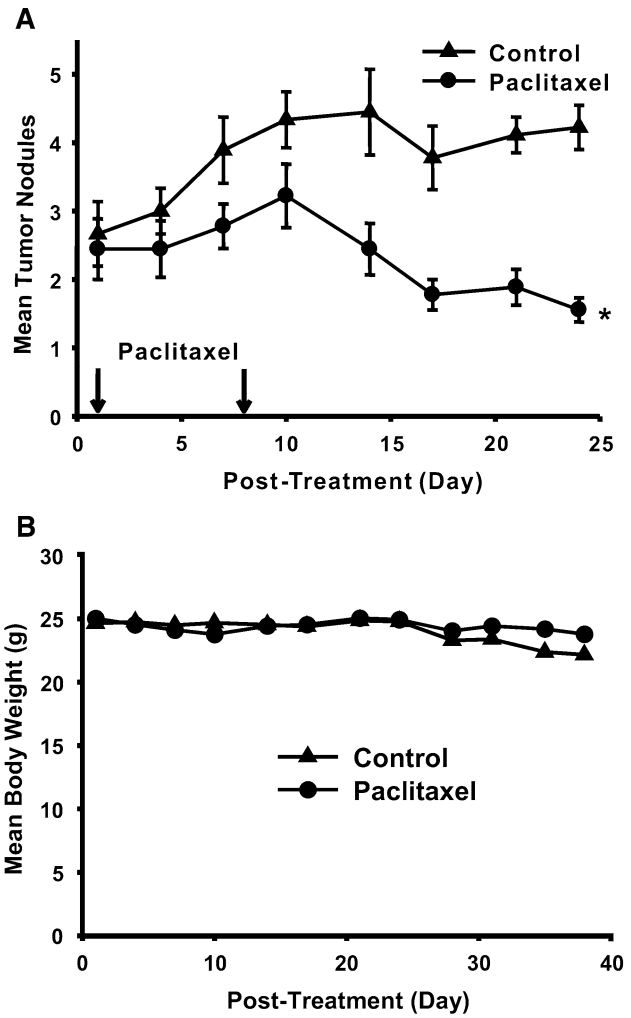

Fig. 5 The growth of MKN45-GFP-ip4 tumors was inhibited by paclitaxel treatments. The metastatic organ invaded and intraperitoneally disseminated MKN45-GFP-ip4 tumors growing in the nude mice were visualized by GFP fluorescence, and the tumor numbers were measured without laparotomy (**a**). Body weights of the intravenous paclitaxel and vehicle control groups were not different (**b**). The averaged data of each group were collected from 9 mice/group intravenously treated with 2 weekly doses of 10 mg/kg paclitaxel as the *arrows* indicated and the vehicle, respectively. (*$p < 0.05$, *ANOVA*)

ascites of patients with gastric cancer. The findings implied that ascites growth of poorly differentiated gastric cancer cells lead to not only peritoneal metastases but also liver metastasis with well-differentiated glandular tumors in patients. It has been reported that well-differentiated gastric tumors were more sensitive to chemotherapy [40]. Our findings, therefore, suggest a combination use of intravenous paclitaxel treatments with surgery for treating advanced gastric cancer with both localized and metastasized tumors.

It was reported that intraperitoneally, but not intravenously, administered paclitaxel showed good activities against peritoneal micrometastases of gastric cancer cells inoculated intraperitoneally in mice [21]. The MKN45 cells used in the previous report were not in vivo selected for invasive abilities and, therefore, only peritoneal micrometastases were observed. The locally distributed disseminating tumors inside the peritoneum were more accessible to the intraperitoneally administered paclitaxel. As supported, intraperitoneally administration delivers paclitaxel locally to the abdominal cavity, produces a relatively high concentration to which the intraperitoneal organs are exposed, and thus provides a better therapeutic efficacy [21, 23]. Soma et al. compared tissue distribution of paclitaxel after intraperitoneal and intravenous administrations in rabbits [23] and showed that intraperitoneal paclitaxel administration caused a higher exposure (i.e., area under the tissue concentration curve) to the omentum and mesenteric lymph nodes. On the other hand, intravenous paclitaxel administration produced a higher tissue exposure to the rabbit liver. In agreement with these findings, we observed that intravenous paclitaxel treatments inhibited liver metastases of the invasive human gastric tumors in mice.

Surgery has been the first line treatment for primary gastric adenocarcinoma. However, relapses after curative surgery were found in 40% patients showing both local and/or distant metastases [41]. The 5-year survival rate after gastrectomy for large (≥10 cm) gastric tumors and for those with liver metastasis or with peritoneal dissemination was 0–3.4% [9, 42]. Chemotherapy remains one of important and effective therapeutic options treating the gastric cancer since the uses of epirubicin, cisplatin, and fluorouracil as combination chemotherapy and has shown promising response rates and survival benefit in patients with locally advanced gastric cancer [43–46]. Perioperative chemotherapy has been suggested for treating gastric cancer [47]. Nonetheless, controversial findings were observed in studies using the conventional drugs (cisplatin, epirubicin, and 5-fluoruracil) as adjuvant chemotherapy [47, 48]. New combination therapies have, therefore, been suggested using taxanes (paclitaxel and docetaxel), irinotecan, and oral fluoropyrimidines [48]. Our results suggested intravenous paclitaxel is active against gastric cancer metastases, while, supported by previously reported, intraperitoneal paclitaxel is active preferentially against intraperitoneally disseminated gastric cancer growths. It would be interesting to further investigate whether intravenous and intraperitoneal paclitaxel treatments can both be included together in the perioperative regimens and, therefore, provide a lower incidence of relapse in patients with advanced gastric cancer.

In conclusion, we have established an in vitro and in vivo metastasis-prone human gastric cancer MKN45 cell subline, MKN45-GFP-ip4. We further demonstrated the antimetastasis efficacy of intravenously administered paclitaxel against the orthotopically growing MKN45-GFP-ip4 cells in the nude mouse peritoneum. Our findings suggest

that systemic exposure of paclitaxel by intravenous administration is effective against gastric tumor metastasis. Clinical regimens of intravenous paclitaxel therapies combined with intraperitoneal paclitaxel therapies and surgery for treating advanced gastric cancer are to be further investigated.

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Conflict of interest statement None.

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