Propagermanium Induces NK Cell Maturation and Tends to Prolong Overall Survival of Patients With Refractory Cancer

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Abstract. *Background/Aim: Propagermanium (PG) inhibits the CCL2/CCR2 axis, and has been shown to function as an immune modulator. This study investigated its anti-tumor mechanism in patients with refractory cancers. Materials and Methods: Five healthy volunteers and 23 patients with refractory oral (n=8) or gastric (n=15) cancer received PG (30 mg/day). We performed flow cytometry (FCM) of peripheral blood mononuclear cells and in vitro killing assays. Results: FCM revealed that CD16^{+}/CD56[Dim] NK cells (i.e., mature, cytolytic subset) increased, and the apoptosis induction rate of cancer cells increased after PG administration. Among gastric cancer patients, median OS was 172.0 days. Two patients showed complete remission of lung or liver metastasis. Survival of patients with oral cancer also tended to be prolonged. Conclusion: PG induces NK cell maturation, and may potentiate anti-tumor activity.*

Propagermanium (PG, 3-oxygermylpropionic acid polymer, Serocion®, Sanwa Kagaku Kenkyusho, Nagoya, Aichi, Japan) is directly obtained through hydrolysis of 3-(trichlorogermyl) propanoic acid. It has been shown to function as a multifunctional immunomodulator (1). Although its mechanism is still unclear, PG activates cytotoxic T cells and natural killer (NK) cells to destroy cells infected with the hepatitis B virus, and was therefore approved in 1994 as a treatment for hepatitis B. PG is also known as an antagonist

of C-C motif chemokine receptor-2 (CCR2); it inhibits mobilization of monocytes and multiple leukocyte subsets from the bone marrow to target tissues (2). PG also attenuates diet-induced insulin resistance, adipose tissue inflammation, and non-alcoholic steatohepatitis due to its inhibition of monocyte/macrophage trafficking (3, 4). Moreover, PG reduces chronic colitis-associated carcinogenesis in mice by decreasing the number of infiltrating macrophages in tumors and nuclear accumulation of β-catenin in tumor cells (5).

Cancer cells secrete CC chemokine ligand-2 (CCL2), which is a ligand of CCR2. Metastatic cancer cells form a suitable environment for proliferation by attracting monocytes and bone marrow-derived stromal cells from bone marrow using the CCL2/CCR2 axis. CCL2 derived from breast cancer cells, recruits inflammatory monocytes that express CCR2 at primary tumor and metastasis sites; CCL2 neutralization in a mouse model inhibited metastasis by retaining monocytes in the bone marrow (6). In a recent study, PG showed anti-tumor effects *via* inhibition of the C-C motif CCL2/CCR2 axis, even in non-inflammatory models (7). Nakayama *et al.* clearly showed that PG attenuated cancer metastasis as a non-specific anti-tumor effect in mouse models, because it inhibited recruitment of bone marrow-derived stromal cells (8). We hypothesized that PG administration would be a new treatment to attenuate metastasis by two different mechanisms: modification of both immune cells and the cancer microenvironment.

In past clinical studies, Tsutsumi *et al.* reported that PG was effective for multiple myeloma (9); and Mimori *et al.* confirmed its safety in Phase I trials for breast cancer (UMIN-CTR041-001). However, its immunomodulatory effects, efficacy, and safety in patients with refractory cancer have not been yet examined. The present study was a proof-of-concept investigation that analyzed changes in NK cells from healthy donors (HD) treated with PG. We also

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examined the effectiveness of PG as innate immunotherapy for patients with refractory gastric or oral cancers.

Materials and Methods

Healthy donors and patients. Written informed consent was obtained from five healthy donors (HD) with no history of cancer or disease being treated. Peripheral blood was collected before, and after 4 weeks and 8 weeks (drug withdrawal) of PG administration (10 mg, three times a day [TID]) for PBMC isolation and blood testing. For the clinical study, we recruited patients with pathologically confirmed, relapsed or refractory gastric cancer (adenocarcinoma, n=15) or oral cancer (squamous cell carcinoma, n=8) who failed previous chemotherapies and/or could not tolerate other treatments. These cancer patients received PG (10 mg TID), for as long as possible.

All clinical samples were approved for analysis by the ethical committees of the Hyogo College of Medicine (No. 1911, March 2015–March 2019). Written informed consent was obtained from all patients with cancers analyzed in this study.

This investigation was performed as an open-labeled, single-institutional, single-armed, exploratory clinical study. The study was conducted in accordance with Good Clinical Practice guidelines, the ethical principles of the Declaration of Helsinki, and local regulations. This clinical study was registered at UMIN-CTR (http://.umin.ac.jp/ctr/index-j.htm), identification number UMIN000017123. Peripheral blood analysis of healthy volunteers was registered at UMIN000030952.

As historical controls, we screened survival outcomes for 47 gastric cancer patients and 15 oral cancer patients, treated with best supportive care (BSC) in Hyogo College of Medicine between Jan 04, 2008 and Dec 31, 2015. Overall survival (OS) was measured from date of final antitumor treatment to date of death in historical controls. In the patients with PG administration, OS was measured from the date of enrolment in the study until death or last follow-up, because four gastric cancer patients (27%) and four oral cancer patients (50%) did not receive any prior chemotherapy.

Sample collection. Blood samples from HD, and patients with gastric or oral cancers were collected from the HD and patients with informed consent, in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in a heparinized tube (Vacutainer CPT®, BD, Franklin Lake, NJ, USA) under sterile conditions. PBMC were either used immediately or frozen (1×10^6 cells/ml) in liquid nitrogen using CellBanker®1 Freezing Medium (Zenoaq, Tokyo, Japan). Mononuclear cells were collected and washed in phosphate-buffered saline twice. The serum was isolated from the peripheral blood of cancer patients (n=16) and HD (n=5) for 3-4 weeks with PG administration, and all samples were stored at –80°C.

Cancer cell lines. HeLa (squamous cancer, RCB-0007) was supplied from the Riken Cell Bank (Tsukuba, Ibaraki, Japan). Cancer cell lines were maintained in RPMI1640 medium (Nacalai Tesque, Inc., Tokyo, Japan) supplemented with 10% FBS (BioWest, Riverside, MO, USA), and 100 units/ml each of penicillin and streptomycin (Gibco BRL, Paisley, UK).

Serum concentration of PG. Measurement of PG concentration by Liquid Chromatography-Mass spectrometry (LC/MS) was performed using high performance liquid chromatography (ACQUITY UPLC I-Class, Waters, Milford, MA, USA) and mass spectrometry (LTQ-Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA). Xcalibur (Thermo Fisher Scientific) was used for measurement and data analysis.

Multicolor flow cytometry. Immunofluoroscence labeled antibodies were purchased form Bio Legend (San Diego, CA, USA), BD Bioscience (Franklin Lakes, NJ, USA), and TONBO (Kobe, Hyogo, Japan). Details are shown in Table I. Fresh isolated or thawed PBMCs were stained with conjugated antibodies (NK cell panel #1~3 protocol) for 30 min at 4°C in the dark. Stained cells were incubated with BD FACS Lysing Solution (BD Biosciences,) for 10 min to lyse red blood cells completely. After two washes, PBMCs (200,000 cells per assay) were read on an SP-6800 Spectral Cell Analyzer (SONY Biotechnology, Tokyo, Japan). Dead cells, monocytes, and T and B lymphocytes were gated out, and NK cell subsets were analyzed, based on the differential expressions of CD56 and CD16.

In vitro killing assay. HeLa cells were pre-cultured overnight with Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Kyoto, Japan) in 35-mm imaging dishes with four compartments (ibidi μ-Dish 35 mm Quad; Martinsried, Munich, Bavaria, Germany) with a volume of 600 μl (5×10^3 cells) in each well. The culture medium was then replaced with F-12K nutrient mixture (Gibco Thermo Fisher, MA, USA) supplemented with 10% (v/v) FBS. PBMCs were thawed and incubated in DMEM overnight, collected, counted, re-suspended with F-12K medium (Gibco BRL), and added to each well (1×10^5 cells). PBMCs from HD (effector cells) and HeLa cells (target cells) were co-cultured at 20:1. PBMCs collected before starting medication, at Week 4, and at Week 8 (4 weeks after drug withdrawal) were assayed at the same time and compared to control wells containing only target HeLa cells. Caspase-3/7 green reagent (5 μM, IncuCyte, Essen Bioscience/Sartorius, Ann Arbor, MI, USA) was added to each well. The co-culture dishes were set into a time-lapse imaging system BioStation IM-Q (Nikon Instruments Inc., Tokyo, Japan). Time-lapse images in phase contrast and green fluorescent channel were acquired every 30 min for 72 h. The number of apoptotic cancer cells was counted by a green fluorescent signal and the number of live cells was counted using the images in the phase contrast. Green fluorescent signals derived from apoptotic PBMCs were distinguished by size and excluded, using Nikon NIS elements software (Nikon Instruments Inc.).

Treatment and assessments. The patients received 30 mg/day of PG as an oral drug, 10 mg after each meal, for up to 1 year, or until confirmed progression, intolerable toxicity, or patient's or investigator's decision to discontinue. Patients could continue this treatment after progressive disease appeared if the following criteria were met: evidence of investigator-assessed clinical benefit; tolerance of study drug; stable performance status; treatment would not delay intervention to prevent serious complications of disease progression; and the patient provided written informed consent before continuing treatment. Treatment was repeated until the appearance of unacceptable adverse effects (AEs), or patient refusal. The PG was supplied by Sanwa Kagaku Kenkyusho Co., Ltd. (Nagoya, Aichi, Japan).

Within two weeks before commencing the study, all patients underwent computed tomography (CT) of the chest and abdomen, a complete blood cell count, and measurement of blood chemistry values.

Table I. *Immunofluorescence-labeled antibodies for multicolor flow cytometry. Upper panel: Panel #1~3 indicates fluorescence for spectral cell analyzer and specific antigen (clone name). Lower panel: specific antigen, clone name, and manufacturer.*

Panel # Fluorescence/Name	#1 NK cell surface	#2 NK cell intracellular	#3 NK cell isotype control
FITC	CD69 (FN50)	GranzymeB (GB11)	Mouse IgG1k (MOPC-21)
PE	CD3 (Hit3a)	CD3 (Hit3a)	CD3 (Hit3a)
Ghost Dye Violet 450	Dead cell stain		
PE-cy7	CD8 (SK1)	CD8 (SK1)	CD8 (SK1)
PerCp-cy5.5	CD19 (HIB19)	CD19 (HIB19)	CD19 (HIB19)
APC/ Alexa 647	CD56 (MY31)	CD56 (MY31)	CD56 (MY31)
APC-cy7	CD16 (3G8)	CD16 (3G8)	CD16 (3G8)
BV421	CD14 (MφP9)	CD14 (MφP9)	CD14 (MφP9)

Fluorescence	Molecule	Clone	Manufacturer
FITC	Human CD69	FN50	BioLegend
FITC	Human Granzyme B	GB11	BD Biosciences
FITC	Mouse IgG1 k	MOPC-21	BioLegend
PE	Human CD3	Hit3a	TONBO
PE/Cy7	Human CD8	SK1	BioLegend
PerCP/Cy5.5	Human CD19	HIB19	TONBO
APC	Human CD56	MY31	TONBO
APC/Cy7	Human CD16	3G8	BioLegend
BV421	Human CD14	MφP9	BD Biosciences
Ghost Dye Violet 450	N/A	N/A	TONBO

On registration day, all patients had their histories recorded, and also underwent physical examinations; measurements of body weight, blood pressure, and body temperature; and assessment of performance status. Treatment-related AEs were assessed using the Common Terminology Criteria for Adverse Events, version 4.0 (NCI-CTCAE ver.4.0). During the period of assessing AEs, physical examinations, complete blood cell counts with differential leukocyte counts, and serum chemistry assessments were performed at least every two weeks; thereafter, these assessments were carried out monthly.

Statistical analysis. Kaplan–Meier plots were used to evaluate OS. The incidence of AEs was also calculated. Data were summarized as proportions and percentages for categorical variables, and as median and ranges for continuous variables. For FCM and biomarker evaluation (*i.e.*, cell-surface and intracellular markers), categorical groups were examined using FlowJo® software version 10.6 (Ashland, OR, USA). Statistical analyses were performed using GraphPad Prism v6.01 (GraphPad Software, Inc., San Diego, CA, USA). Percentage of NK cell subsets were calculated in total NK cell at Pre, 4wks, and 8wks. Each sample was analyzed using the two-tailed, parametric paired t-test.

Results

Subset analysis of NK cells by oral PG administration. We used multicolor staining of PBMCs to classify NK cells (CD3$^-$/CD19$^-$ lymphocytes) into four subsets according to the staining levels of CD16 and CD56. They are CD16$^-$/CD56^{Bright} (proliferative subset), CD16$^+$/CD56^{Bright} (intermediate subset), CD16$^+$/CD56^{Dim} (dominant, cytolytic subset), CD16$^+$/CD56$^-$ (CD56$^-$ subset). The CD16$^+$/CD56^{Dim} subset is the predominant subset in peripheral blood; it has abundant granzyme B (Figure 1), which induces apoptosis against microorganisms and abnormal cells. Five HD took PG for 4 weeks; medication was withdrawn at that point and PBMCs were collected at 8 weeks. As a characteristic example, FCM of HD #3 is shown (Figure 2). Among the 5 HDs, at Week 4, CD16$^+$/CD56^{Dim} subsets tended to increase (Figure 3A) and the CD16$^+$/CD56^{Bright} subsets tended to decrease (Figure 3B), although not significantly so; at Week 8, their CD16$^+$/CD56^{Bright} subsets were significantly decreased to levels before PG administration (Figure 3B, p=0.474, 95% CI=−8.587- −0.08077).

Serum concentration of PG. After PG administration to cancer patients and HD for 3 to 4 weeks, serum PG concentration was measured by LC/MS. Serum PG concentrations did not show abnormally high values in refractory cancer patients (n=16) and healthy donors (n=5) in Table II. Serum PG concentration was within the range 8.2-218.0 ng/ml, which is consistent with the pharmacokinetic level reported for healthy donors in the 2010 Japanese interview form of the approved medicine.

In vitro killing assay. PBMCs taken before and after 4 weeks of PG administration were co-cultured with HeLa cells, and apoptotic HeLa cells were counted every 30 min. In 48 h, apoptotic HeLa cells were indicated (Figure 4A, green
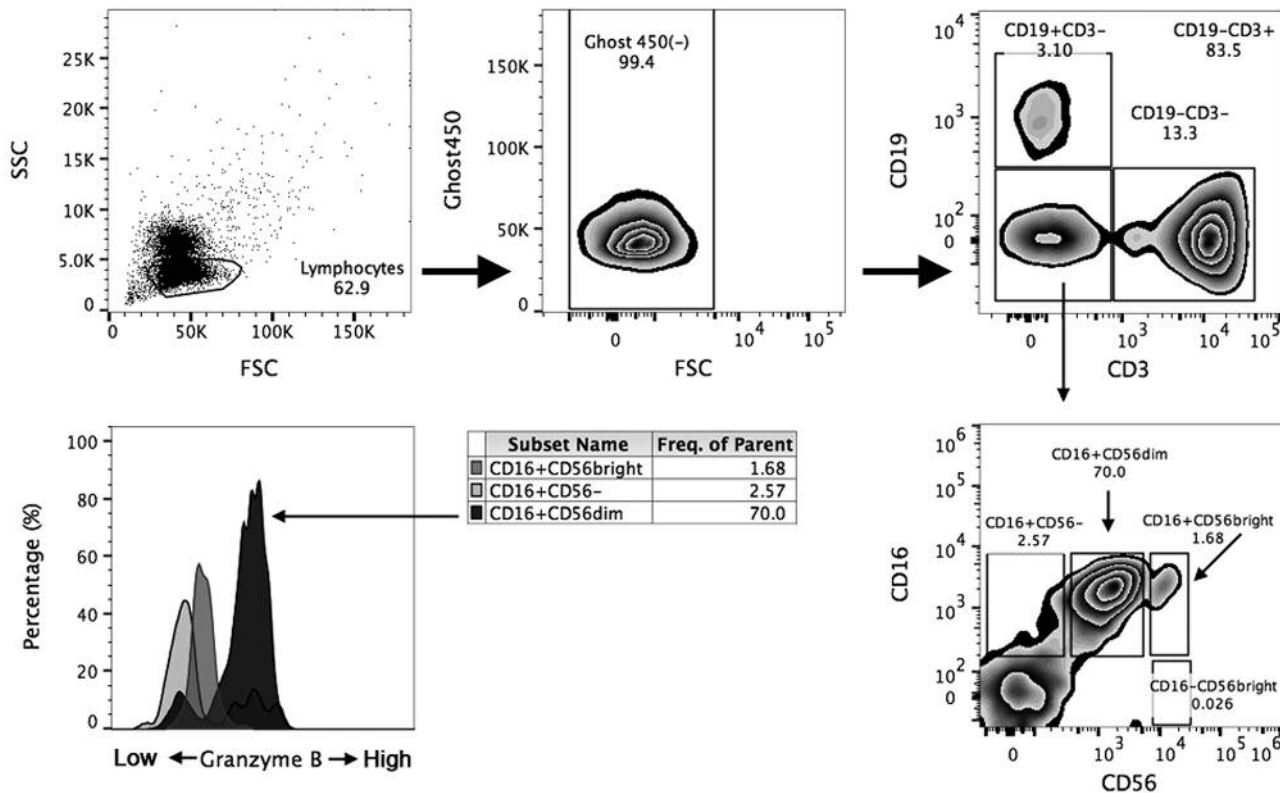
Figure 1. *Subset analysis of NK cells by oral administration of propagermanium. Using multicolor staining of PBMCs, CD3⁻/CD19⁻ lymphocytes were classified as NK cells into four subsets from levels of CD16 and CD56 staining. They were CD16−/CD56Bright (proliferative subset), CD16⁺/CD56Bright (intermediate subset), CD16⁺CD56Dim (dominant; cytolytic subset), CD16⁺/CD56⁻ (CD56-negative subset). CD16⁺/CD56Dim is the dominant subset in peripheral blood. It has abundant Granzyme B that induces apoptosis against micro-organisms and abnormal cells (left lower panel, black color).*

color). Figure 4B shows the number of apoptotic HeLa cells per 1,000 cells counted every 12 h. After PG administration, PBMCs had enhanced ability to induce apoptosis compared to Pre (no PG medication).

Characteristics of patients and AEs of PG monotherapy. Fifteen patients with advanced or relapsed refractory gastric cancer were enrolled between May 2015 and January 2017. Their median age was 78 years (range=40-87 years). Gender (Male: Female=8:7) and Eastern Cooperative Oncology Group (ECOG) PS (PS 0-1, n=8 *vs.* PS 2, n=7) were almost equal. Nine patients (60%) had received two or more regimens as prior treatment. All patients had multiple metastatic lesions. Safety analyses were performed in all treated patients. Anemia (grade 1-2) was reported in 10 patients (66.7%); aspartate transaminase elevation (40.7%), alkaline phosphatase elevation (53.0%), constipation (26.7%), γ-glutamyltransferase (40.0%), and creatinine elevation (26.7%) were also reported. Neutropenia and

autoimmune reactions were not reported among treated patients. Anemia was frequent but it did not exacerbate during PG treatment in 10 patients. One patient suspended PG treatment due to bleeding from the primary gastric cancer lesion and received irradiation to manage the bleeding.

Efficacy. The final analysis was carried out when 15 (gastric cancer) and 8 (oral cancer) events had occurred. Figure 5A, B show Kaplan–Meier curves for gastric cancer patients who received PG medication and for historical controls who received BSC. Hazard ratios (HRs) were calculated for PS 0-1 *vs.* PS 2 in each panel. Median OS was 172 days in the PS 0-1 group and 44 days in the PS 2 group (Figure 5A). The HR for death was 0.236 (95%CI=0.023-0.327, *p*=0.011) in the PS 0-1 group. In the historical control group, median OS was 66 days in the PS 0-1 group and 57.5 days in the PS 2 group (Figure 5B). The HR for death was 0.590 (95%CI=0.197-1.245, *p*=0.1519) in the PS 0-1 group. Figure 5C and D shows Kaplan–Meier
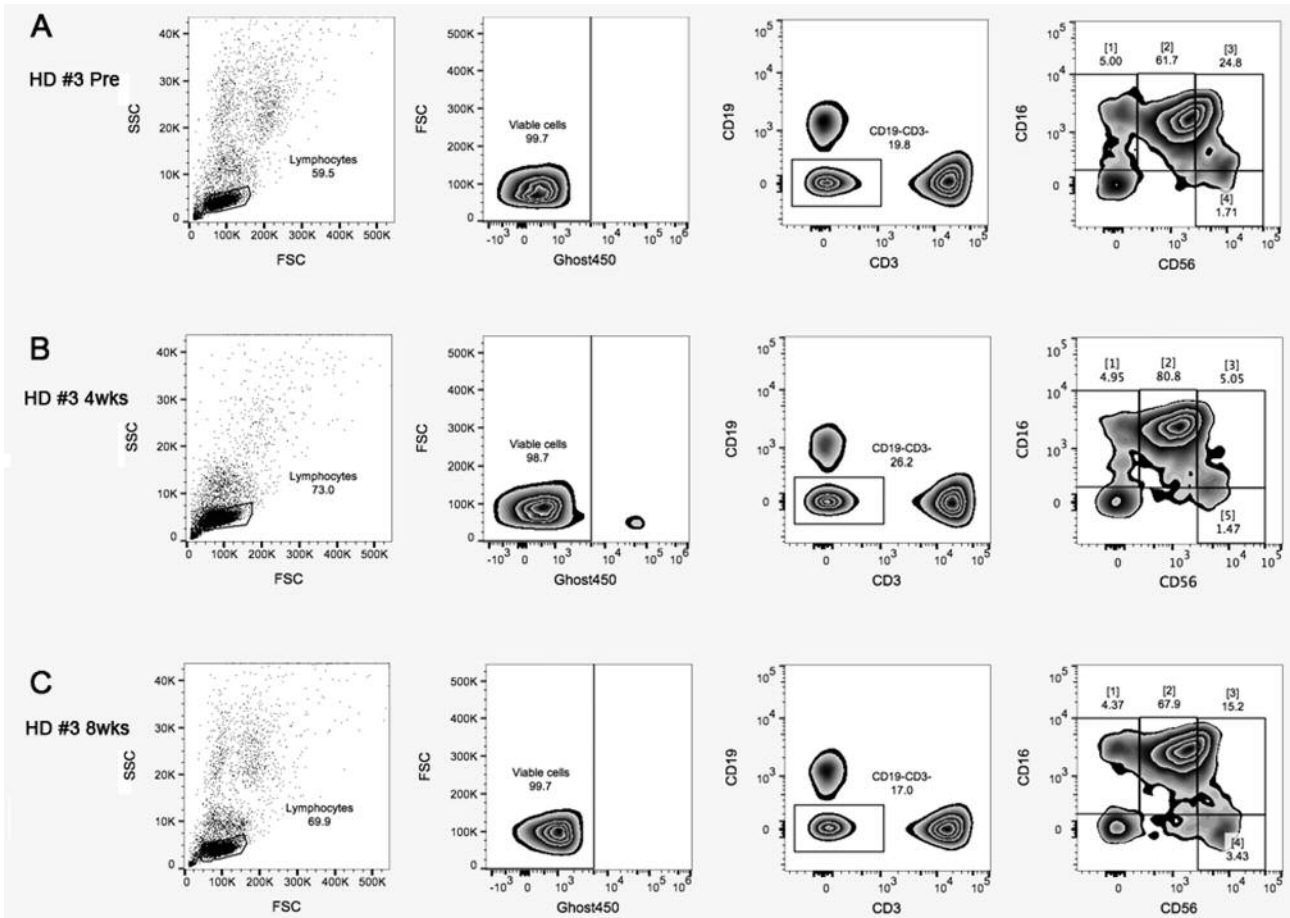
Figure 2. *Transition of NK cell subset by multicolor flow cytometry (FCM) analysis. As a characteristic example, FCM of healthy donor (HD) #3 is shown. Right column: The CD19⁻/CD3⁻ subset was divided into four subsets by expression of CD16 and CD56. The CD16⁺/CD56^{Bright} subset (shown as [3]) decreased from 24.8% (A, right panel) to 5.05% (B, right panel) after 4 weeks' medication. The CD16⁺/CD56^{Dim} (cytolytic) subset (shown as [2]), increased from 61.7% (B, right panel) to 80.8% (B, right panel) after 4 weeks' medication. After 4 weeks of drug withdrawal (8wks), an intermediate subset (shown as [3]) increased to 15.2% (C, right panel).*

OS curves for patients with oral cancer who received PG medication, and for historical controls who were treated with BSC. Median OS was 87.5 days in PG medication and 36.0 days in historical controls.

Figure 6 shows a complete remission of metastatic lung tumors in a patient after a total gastrectomy for advanced gastric cancer. This patient had multiple lung tumors and a small amount of pleural effusion (adenocarcinoma). In this patient, PG treatment was stopped after 12 months. Drug cessation did not induce secondary relapse after more than two years. Another objective response was observed in another patient, who had advanced gastric cancer with pyloric stenosis and multiple liver metastases (Figure 7). The stomach had expanded due to pyloric stenosis, but liver metastases were in remission after 6 weeks of PG treatment (Figure 7C, 7D).

Table II. *Serum concentration of propagermanium. Patient #1~8 (gastric cancer patients), patient #9~16 (oral cancer patients), HD # 1~5 are healthy donors. PG concentration in serum was measured by LC-MS after 3~4 weeks PG administration. In cancer patients, the concentration was ranged from 8.2 to 208.0 ng eq. /ml, which were similar to HD #1~5 (8.3 to 218.0 ng eq./ml).*

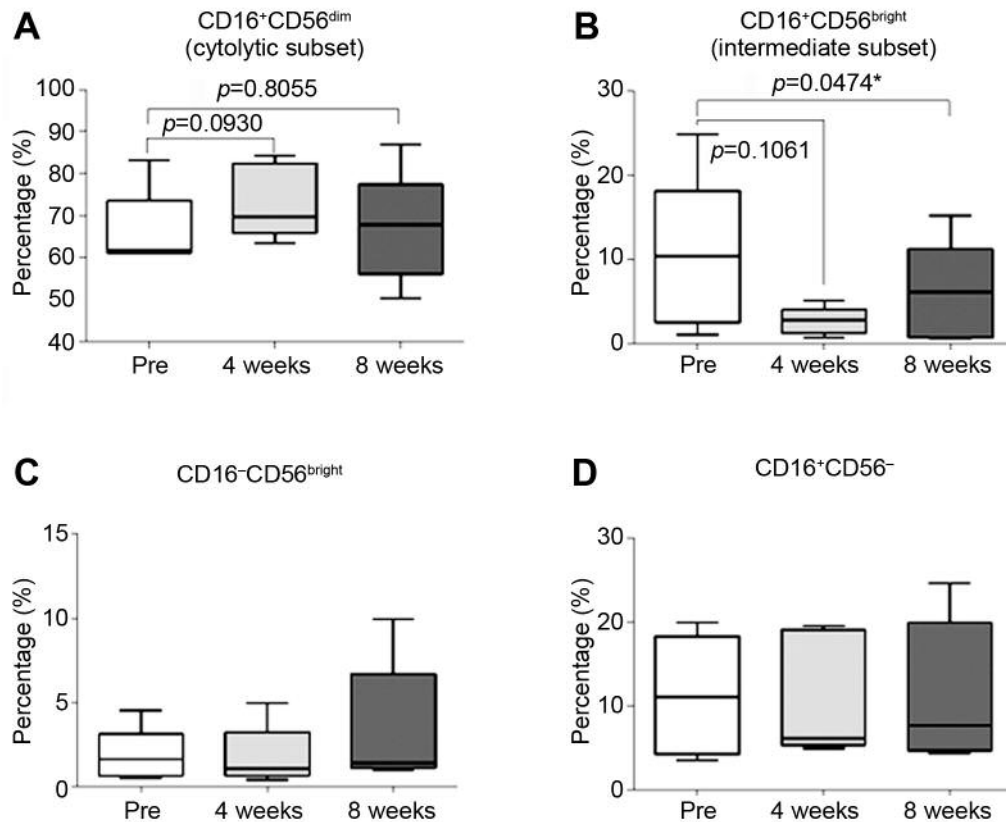| Serum concentration of propagermanium (ng eq./ml)					
Patient #1	32.7	Patient #9	126.0	HD #1	218.0
#2	12.4	#10	9.7	HD #2	8.3
#3	121.0	#11	34.2	HD #3	19.0
#4	34.9	#12	97.7	HD #4	10.3
#5	135.0	#13	8.2	HD #5	18.6
#6	61.8	#14	81.3		
#7	78.2	#15	24.1		
#8	208.0	#16	29.3		
Median	70.0		31.8		18.6
Standard deviation	65.5		44.3		91.3

Figure 3. *Transition of NK cell subsets in 5 healthy donors (HD) with no history of cancer. Subset analysis of NK cells before (Pre: white box) and after starting propagermanium (10 mg/day; TID) at 4 weeks (grey box) and 8 weeks (drug withdrawal, dark gray box). A: Cytolytic cell subsets tended to increase at 4 weeks. B: The intermediate subset decreased at 4 and 8 weeks. C and D: No significant changes were observed in CD16-CD56^{Bright} and CD16^{+} CD56^{-} subsets. Each datum is indicated by a bar (standard deviation) and was tested by the parametric paired t-test.*

Discussion

Gastric cancer is the fifth most common cancer and the third leading cause of cancer death in the world (10). Outcomes for patients have improved because of the global application of lymph node dissection and adjuvant chemotherapy (CHT) with several drugs (11). Platinum compounds plus fluoropyrimidines are the most common first-line treatment options for patients with advanced or metastatic gastric cancer. For patients who are refractory to first-line CHT, treatment options include taxanes or irinotecan monotherapy, or the anti-vascular endothelial growth factor receptor 2 (VEGFR-2) antibody, ramucirumab, alone, or combined with taxanes (12-14). However, complete tumor remission is rare due to biological heterogeneity and malignant potential of gastric cancer, and most relapsed or metastatic cancers continue to progress even after treatment (15, 16). In patients treated with the BSC, OS is reportedly only 2.4-3.8 months (17, 18). Several candidate drugs have been tested as 3rd-line treatment, but a standard 3rd-line therapy has not been established. Recently, nivolumab, an immune checkpoint inhibitor, showed efficacy in heavily pretreated patients with advanced gastric cancer in a large phase III study (19). Nivolumab resulted in a mean OS benefit of 1.1 months over placebo in patients who had been heavily pretreated. In our study, the median OS of BSC patients with refractory gastric cancer was 172.0 days (5.7 months) and two patients showed complete remission of lung or liver metastasis. In addition, PG treatment did not present any drug-induced AEs. Most patients have uncontrolled multiple metastases, which are common causes of death.

The prognosis for intractable oral cancer is even worse. In the EXTREME trial (NCT00122460), median OS for recurrent metastatic oral cancer for which first-line CHT was ineffective was 4.4 months (20). However, few scientifically reliable data are available regarding survival of oral cancer in patients who undergo secondary CHT or palliative care. At our facility, median OS for oral cancer with BSC was
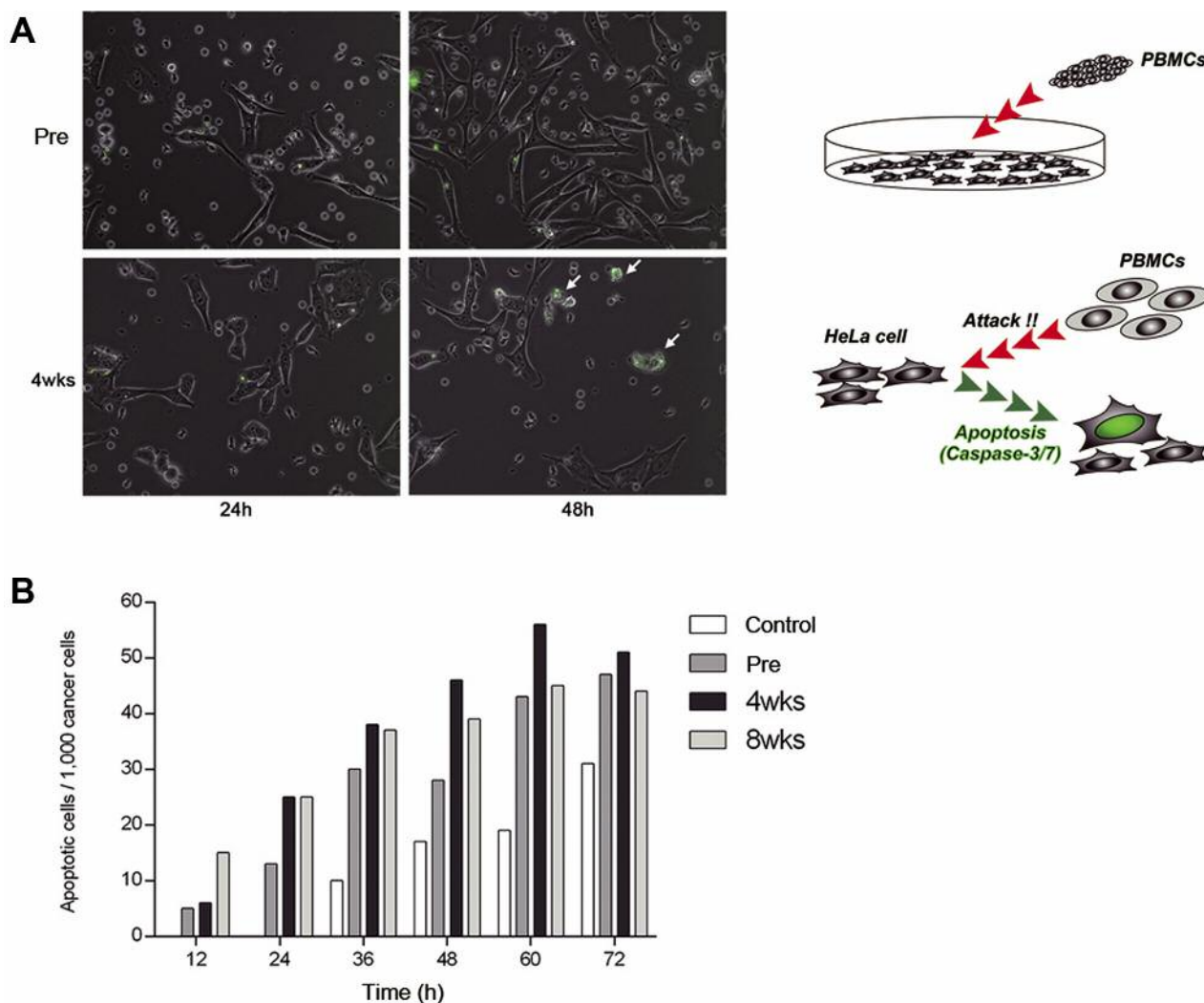
Figure 4. *In vitro killing assay. PBMCs (small round cells) isolated before (Pre), after 4 weeks of propagermanium (PG) administration (4 wks), and drug withdrawal (8 wks) were co-cultured with HeLa cells (large, spread cells). Apoptotic cancer cells were counted every 30 min. A: After 48 h, apoptotic cancer cells were indicated (A, lower right panel, green cells indicated with white arrows). B: The number of apoptotic HeLa cells per 1,000 cancer cells every 12 h. Each column shows control (HeLa cell alone, white), Pre (dark gray), after 4 weeks PG (black), and after 8 weeks (drug withdrawal, light gray). After PG administration, PBMCs showed enhanced ability to induce apoptosis.*

36.0 days (Figure 5D). Generally, heavy, multi-drug regimens deteriorate patients' quality of life and the function of critical organs. Thus, new treatment options with fewer AEs are needed to control metastasis in these patients.

Over the 40+ years since the discovery of NK cells, their best-known function has been their ability to kill tumor cells and virus-infected cells. Their mechanism of action involves apoptosis of target cells and interaction with other immune cells and non-immune cells by cytokine and chemokine secretion (21). NK cells are a key facilitator of innate immunity and are strictly controlled by numerous activating and inhibitory receptors for maintaining immune homeostasis and pregnancy, and the ability to attack abnormal cells. For example, a mechanism for attacking cells with deficient or decreased expression of HLA-class 1 molecules of their own by "missing self" recognition is known (22). Apart from that, NK cell is classified into several subsets by the expression of CD56 (neural cell adhesion molecule, NCAM) and CD16 (also known as FcrR3); each characteristic is reportedly diverse. Differentiation of the NK cell is coupled with functional changes. The CD16^{+}/CD56^{Dim} subset is the most dominant and matured type in peripheral blood; it retains a large amount of the serine protease granzyme B, and was classically described as a large granular lymphocyte. The
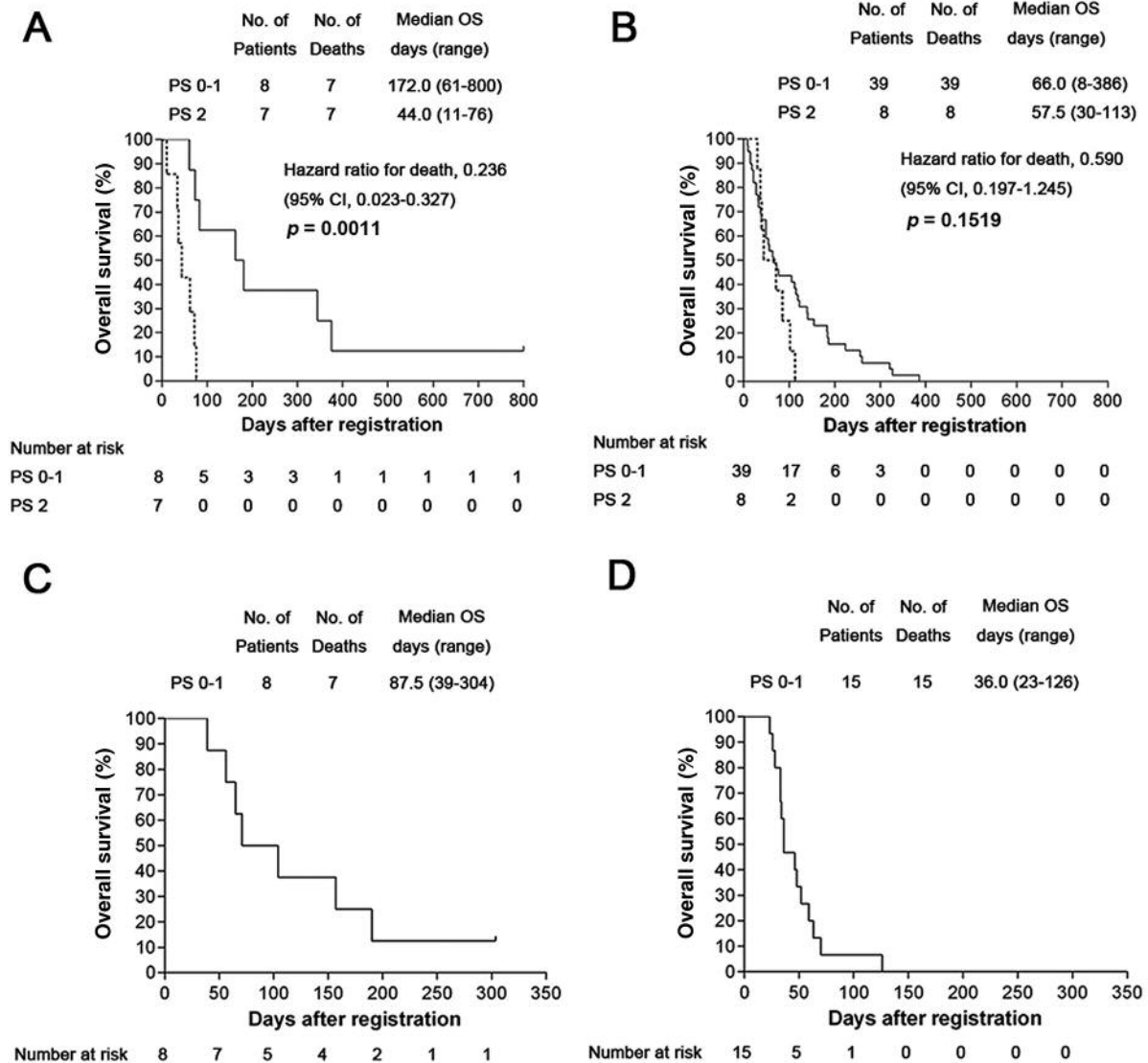
Figure 5. *Clinical efficacy of propagermanium in patients with refractory gastric or oral cancers. Kaplan−Meier curve indicates overall survival of gastric cancer patients (A and B), and oral cancer patients (C and D). A: Among 15 enrolled patients with refractory advanced or relapsed gastric cancer, median OS was 172 days in the PS 0-1 group and 44 days in the PS 2 group. B: In the historical control group, median OS was 66 days in the PS 0-1 group and 57.5 days in the PS 2 group. C: Among eight enrolled patients with refractory advanced or relapsed oral cancer, median OS was 87.5 days. D: In the historical control group, median OS was 36 days.*

CD16$^-$/CD56^{Bright} (immature) subset has the longest telomere and is considered to be a proliferative subset (23). Since CD16$^+$/CD56^{Bright} changes to CD16$^+$/CD56^{Dim} *in vitro*, the CD16$^+$/CD56^{Bright} subset is considered to be a functional intermediate subset (24). Although the CD16$^+$/CD56$^-$ subset was previously considered to be dysfunctional, Human Immunodeficiency Virus and hepatitis c virus patients have shown that the CD16$^+$/CD56$^-$ subset produces macrophage inflammatory proteins such as CCL3, CCL4, CCL5, *etc*. (25). NK cells constitute a large number of diverse populations,

some of which are speculated to be induced to differentiate by various developmental or environmental factors. Although NK cell biology has progressed, details regarding their differentiation and maturation *in vivo* are still unclear. In this study, we used flow cytometry (FCM) to analyze the *in vivo* effects of PG on NK cells of healthy donors.

The mature/cytolytic subset of NK cell is the dominant subset in the peripheral blood of healthy individuals. In addition, a cytolytic subset of NK cells in peripheral blood is important also in preventing blood-borne metastasis. The
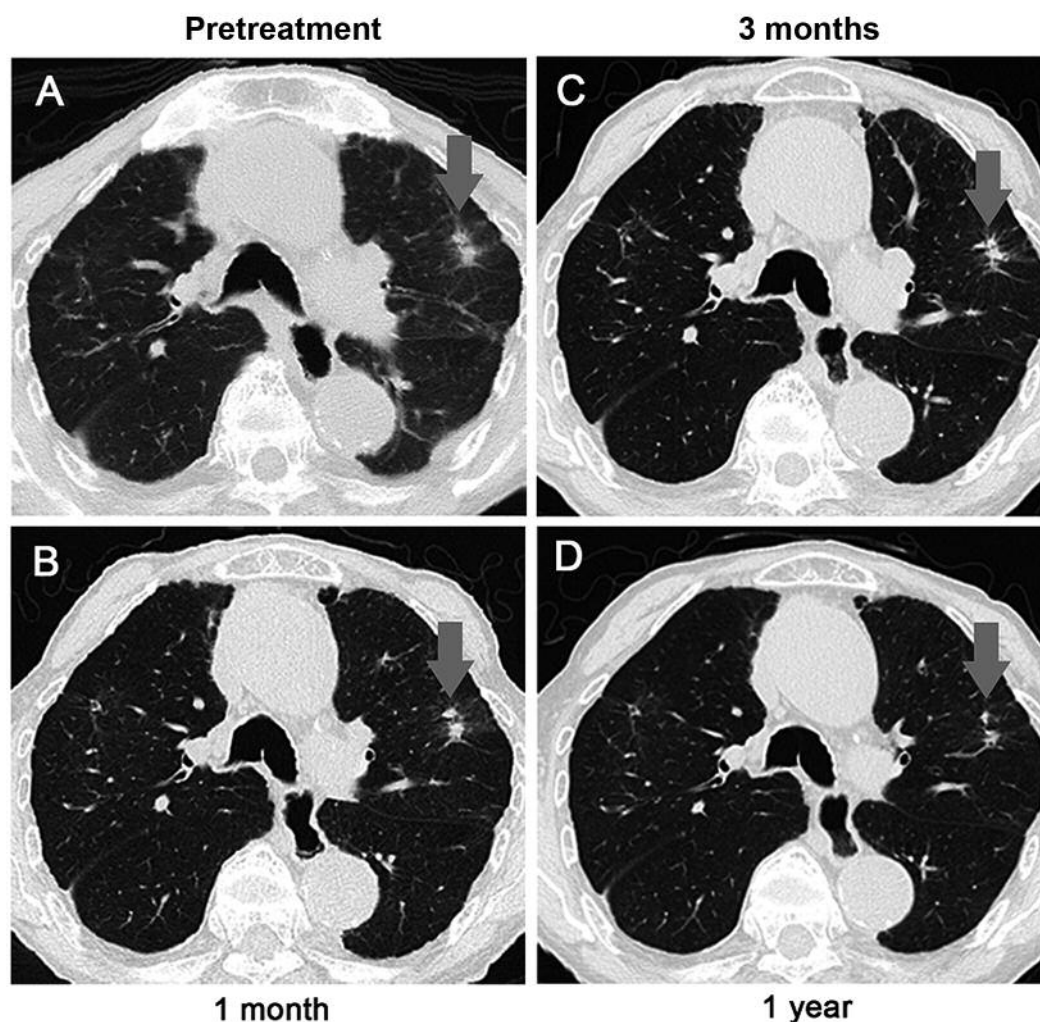
Figure 6. *A: CT scan shows complete remission of metastatic lung tumors in a patient after total gastrectomy (gastric adenocarcinoma). This patient had multiple lung tumors and a small amount of pleural effusion (adenocarcinoma). Gray arrow: tumor. B, C: After receiving propagermanium, the tumor was smaller. D: Complete remission was observed at 1 year.*

mechanism that links PG and NK cell maturation has not been fully elucidated. NK cells are effective in combating infections and tumors and are considered attractive for adoptive transfer therapy. Therefore, *in vitro* activation and expansion have been attempted, including various combinations of cytokines (26). We isolated and prepared NK cells of a HD using a MACS magnetic bead column (Miltenyi Biotec, Bergisch Gladbach, NRW, Germany) and we performed a differentiation assay with different concentrations (250 ng–10 μg/ml) of PG for 14 days. However, FCM did not show the activation or maturation of NK cells, PG did not apparently induce maturation of NK cells directly (data not shown).

We performed serial blood sampling three times prior to PG administration, for 4 weeks, and after withdrawal in 5 HD. We analyzed changes in NK cell subsets, T cells, B cells surface antigens, and activation markers by PG administration. The $CD16^+CD56^{Bright}$ NK subset tended to decrease at week 4 after PG administration (Figure 3B, gray box), and was significantly decreased after drug withdrawal compared with before receiving PG (Figure 3B, dark gray box). The life span of NK cells is relatively long, several weeks, and the effects of PG may remain after drug withdrawal. $CD16^+/CD56^{Dim}$ (cytolytic subset) tended to increase (although not significantly so) under PG treatment (Figure 3A). In HD # 3, the intermediate NK subset decreased and the cytolytic NK subset increased under PG administration (Figure 2, right panels). The frozen PBMCs of HD #3 was used to perform a killing assay on HeLa cells. As a result, the ability to induce apoptosis in cancer cells was clearly enhanced by PG. Although further detailed analysis is required, changes in the NK subset suggest PG's clinical efficacy.
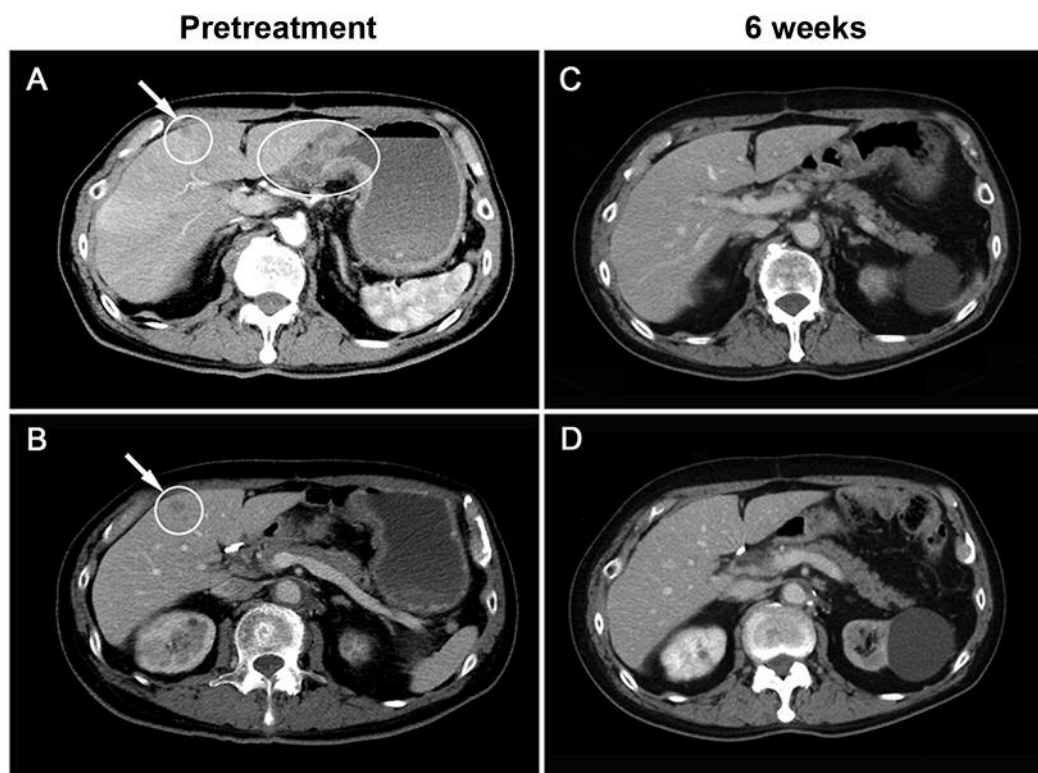
Figure 7. *CT scan shows untreated advanced gastric cancer with multiple liver metastases. A, B: Before propagermanium (PG) treatment. C, D: After 6 weeks' PG administration. White ovals: advanced gastric cancer with pyloric stenosis; white circle: liver metastasis. Gastric cancer lesions were decreased and pyloric stenosis was reduced. Multiple liver metastases were in complete remission.*

For patients with refractory cancers, after first-line CHT, combined CHT and/or molecular-targeting drugs, including immunotherapy are applied as second-line treatment. During heavy CHT, most patients suffer severely reduced quality of life from drug-induced AEs. However, even among cancer patients treated under these conditions, PG did not cause obvious AEs, and compliance was good.

In terms of cost, multi-drug regimens and molecular targeting therapies, including immune checkpoint inhibitors, are very expensive, and this is another problem for patients with refractory cancers. However, PG is a low-cost, already-approved oral medicine. The cost of a regular dose (10 mg TID) to treat hepatitis is less than about ten dollars per day. Our clinical data showed some clinical and economic benefits of PG to treat refractory gastric and oral cancers.

As shown by Yumimoto *et al.*, PG administration strongly suppressed lung metastasis by malignant melanoma and breast cancer through inhibition of the CCL2/CCR2 axis (7). Our data suggest that PG has an anti-tumor activity through NK cell maturation. In conclusion, PG may be effective on several cancer types, and may be a promising option for patients with refractory cancers.

Conflicts of Interest

Authors' Contributions

Acknowledgements

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