

# Nano-particulate Toll-like Receptor 9 Agonist Potentiates the Antitumor Activity of Anti-Glypican-1 Antibody

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**Abstract.** *Background/Aim:* Monoclonal antibodies (mAbs) that target tumor antigens have recently been developed. Their antitumor activity is mainly achieved through antibody-dependent cellular cytotoxicity (ADCC) via effector cells such as tumor-infiltrated macrophages and natural killer (NK) cells. CpG oligodeoxynucleotides (ODNs) have potent antitumor activity and are considered to increase the tumor infiltration of macrophages and NK cells; however, a completely solubilized novel CpG-schizophyllan (SPG) complex, K3-SPG, displays more potent antitumor activity. We recently reported the significant antitumor activity of anti-glypican-1 (GPC1) mAb against GPC1-positive esophageal squamous cell carcinoma

*(ESCC) via ADCC. The aim of this study was to evaluate the potential synergistic antitumor activity of anti-GPC1 mAb and K3-SPG and elucidate the underlying mechanisms using a xenograft model of GPC1-positive human ESCC cells. Materials and Methods:* The established human esophageal cancer cell line TE14 was subcutaneously injected into SCID mice. Xenograft mice were treated with anti-GPC1 mAb, K3-SPG, or their combination. Antitumor activity was evaluated by measuring the tumor volume. For FACS analysis, agents were administered, and tumors were resected 1 day after the final treatment. *Results:* Anti-GPC1 mAb or K3-SPG monotherapy showed dose-dependent antitumor activity, and combination therapy with anti-GPC1 mAb and K3-SPG showed antitumor activity ( $p=0.0859$ ). Flow cytometry revealed significantly increased numbers of macrophages ( $p=0.0133$ ) and of the ratio of activated NK cells/total NK cells ( $p=0.0058$ ) following K3-SPG or combination therapy. *Conclusion:* Combination therapy with K3-SPG and anti-GPC1 mAb or another antitumor mAb may represent a new cancer treatment option acting via ADCC.

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**Key Words:** Antitumor antibody, glypican 1, CpG oligodeoxynucleotides, macrophage, natural killer cell.



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Esophageal cancer is the sixth leading cause of cancer-related deaths worldwide (1). Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal cancer in eastern Asia and Africa (2). Despite improvements of multimodal therapy, including surgery, chemotherapy, and radiotherapy, the prognosis of patients with ESCC remains poor (3). Therefore, there is an urgent need to identify novel treatment options for ESCC.

Monoclonal antibodies (mAbs) are increasingly being used for cancer therapy given their main advantage of high specificity with their targeted antigens, which results in less severe side effects than conventional cytotoxic agents (4). Cancer therapy with an anti-tumor mAb depends on the antibody-dependent cellular cytotoxicity (ADCC) that is achieved *via* interactions with immune effector cells such as tumor-infiltrating natural killer (NK) cells and macrophages (5). However, cancer-induced immunosuppression *via* NK cells has been reported in various types of cancers, which can reduce the ADCC effect. Therefore, immunostimulants are promising agents to enhance the numbers of tumor-infiltrating NK cells and macrophages to induce efficient ADCC in patients with cancer. For example, Kohrt *et al.* reported that stimulation of NK cells with a CD137 mAb enhanced the efficacy of trastuzumab in xenograft models of breast cancer (6).

CpG oligodeoxynucleotides (ODNs) are potent immunostimulants that are recognized by Toll-like receptor 9 on dendritic and B cells (7). We previously reported that a CpG ODN exerted synergistic antitumor activity with anti-bone marrow stromal antigen 2 mAb using xenograft mouse models (8). Recently, a nanoparticle K CpG ODN (K3) wrapped by the nonagonistic Dectin-1 ligand schizophyllan (SPG), K3-SPG, was developed, demonstrating the most potent anti-tumor activity of all CpG ODN and CpG-SPG complexes reported to date (9).

Glypican-1 (GPC1) is a cancer antigen for ESCC that was identified by a quantitative proteomics approach focused on cell surface membrane proteins (10). Moreover, GPC1 overexpression was significantly associated with a poor prognosis and chemoresistance of patients with ESCC (10). GPC1 is a cell-surface heparan sulphate proteoglycan, which acts as a co-receptor for the heparin binding of mitogenic growth factors (11). We previously developed a chicken/mouse chimeric mAb against human GPC1, which induced tumor growth inhibition in ESCC xenograft models *via* ADCC and showed complement-dependent and -independent cytotoxicity (12).

The aim of the present study was to evaluate the potential synergistic antitumor activity of anti-GPC1 mAb and K3-SPG and to elucidate the underlying mechanisms using a xenograft model of GPC1-positive human ESCC cells.

## Materials and Methods

**Cell lines and culture.** The human ESCC cell line TE14 (RCB2101, RRID: CVCL\_3336) was obtained from the RIKEN BRC cell bank (Tsukuba, Japan). The identity of the cell line was confirmed using DNA fingerprinting *via* short tandem repeat profiling. TE14 cells were mycoplasma free. TE14 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>.

**ESCC cell xenograft mouse models.** To establish the xenograft model, TE14 cells were implanted in female CB17/Icr-Prkdc<sup>scid</sup>/CrjCrj (SCID) mice (6-8 weeks old; Charles River Japan, Yokohama, Japan). For cell inoculation, 2.0×10<sup>6</sup> cells in 100 µl of 1:1 (v/v) phosphate-buffered saline (PBS)/Matrigel (Becton Dickinson, Bedford, MA, USA) were injected subcutaneously into the flanks of the mice. When the tumor volume reached approximately 100 mm<sup>3</sup>, the animals received anti-GPC1 mAb clone1-12, K3-SPG, combination treatment, or PBS for 3 weeks twice a week. For anti-GPC1 mAb treatment, the mice received intraperitoneal (*i.p.*) injection of 400 µl of mouse IgG2a or anti-GPC1 mAb (25, 100, and 400 µg in 400 µl of PBS/mouse) (six to eight per group). For K3-SPG treatment, the mice received intratumoral (*i.t.*) injection of PBS or K3-SPG (2.5, 10, and 20 µg in 25 µl of PBS/mouse) (seven per group). For evaluation of the effects of combination therapy, the mice were divided into four groups (six per group), treated by *i.p./i.t.* injection of (a) PBS/PBS, (b) PBS/K3-SPG (2.5 µg/mouse), (c) anti-GPC1 mAb (25 µg/mouse)/PBS, and (d) anti-GPC1 mAb (25 µg/mouse)/K3-SPG (2.5 µg/mouse), respectively. Tumor sizes were measured twice a week throughout the study. Tumor volumes were determined by measuring the length (L) and width (W), calculated as (W<sup>2</sup> × L)/2.

All animal experiments were conducted according to the ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation (Osaka, Japan). The CpG ODNs and the preparation and characterization of K3-SPG were previously described (9). Construction of chicken-mouse IgG2a chimeric antibody and production of anti-human GPC1 mAb clone1-12 were previously described (12).

**Flow cytometric analysis.** Xenograft tumor cells were collected on day 2 and on day 10 during treatment from TE14-bearing female SCID mice in the four groups, in which treatment was administered on days 1, 4, and 7. Resected xenograft tumors were washed in PBS (Nacalai Tesque). Tumor tissues were cut into small pieces by scissors in RPMI1640 medium (Wako, Osaka, Japan) with 10% FBS, collagenase D, and DNase I and incubated for 30 min at 37°C. Tumor cells were isolated as reported previously (13).

After preparation of the tumors, tumor-infiltrated cells were stained with anti-CD45 (30-F11), anti-F4/80 (BM8), anti-CD49b (DX5), and anti-CD69 (H1.2F3) antibodies (Biolegend, San Diego, CA, USA). Canto II Flow Cytometer (Becton Dickinson, Mountain View, CA, USA) was used to assess the CD45<sup>+</sup> CD49b<sup>+</sup> cell numbers and the CD69<sup>+</sup>/CD49b<sup>+</sup> ratio on day 2, and to determine the CD45<sup>+</sup> F4/80<sup>+</sup> cell numbers on day 10. The data were analyzed using the Flow JO (Tree Star, Stanford, CA, USA).

**Statistical analysis.** Data are shown as mean±SE values for *in vivo* experiments. One-way ANOVA followed by Turkey's *post hoc* test was used to test for statistically significant differences between groups. Differences were considered significant at *p*<0.05. All analyses were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd. Tokyo, Japan).

## Results

**Anti-GPC1 mAb and K3-SPG exhibit dose-dependent antitumor activity.** To determine the optimum concentration of anti-GPC1 mAb and K3-SPG for combination therapy, we first evaluated their individual dose-dependent antitumor

activities. Treatment with 400 µg anti-GPC1 mAb resulted in reduction in tumor volume and weight compared with mouse IgG2a treatment (Figure 1A and B), and the effect seemed to be greater than those observed with lower doses. Similarly, the K3-SPG at 10 µg and 20 µg groups exhibited a significant reduction in tumor volume and weight (all  $p < 0.0001$ ) compared with the PBS group (Figure 1C and D), and the effects were stronger than that observed with 2.5 µg K3-SPG. These results demonstrated that both anti-GPC1 mAb and K3-SPG have dose-dependent antitumor activity.

*Anti-GPC1 mAb and K3-SPG exhibit synergistic therapeutic effects.* Treatment with combination regimen (d) resulted in antitumor activity tendency compared with the control regimen (a) in terms of reductions in tumor volume and weight ( $p = 0.0859$  and  $p = 0.0928$ , respectively) (Figure 2A and B). The reductions were also greater than those of the individual treatments with anti-GPC1 mAb (c) and K3-SPG (b). These results demonstrate that the combination therapy exhibits synergistic antitumor activity.

*K3-SPG induces tumor macrophage and NK cell infiltration.* The dot plots in Figure 3A show representative results of the flow cytometric analysis of F4/80<sup>+</sup> macrophages. In tumors treated with regimen (b) and (d), the number of macrophage cells was significantly higher ( $p = 0.0408$  and  $p = 0.0133$ , respectively) than that of the control regimen (a) (Figure 3B). The dot plots and histograms in Figure 3C show representative results of flow cytometry analysis of CD49b<sup>+</sup> NK cells and CD69 expression on CD49b<sup>+</sup> NK cells. In tumors treated with regimen (b) and (d), the CD49b<sup>+</sup> CD69<sup>+</sup> activated NK cells/CD49b<sup>+</sup> total NK cells ratio was significantly higher than that of the control regimen (a) ( $p = 0.0029$  and  $p = 0.0058$ , respectively) (Figure 3D).

## Discussion

This study represents the first confirmation of the individual dose-dependent antitumor activity of anti-GPC1 mAb and K3-SPG. Thus, a high concentration of the mAb is required to achieve greater efficacy, which may be a concern for increasing the risk of adverse effects given that slight expression of GPC1 has been observed in the testis, ovary, and heart (12). In addition, a high therapeutic dose of an antibody requires the expensive large-scale capacity for antibody production. K3-SPG also exhibited a stronger antitumor effect at high doses, which could also pose a risk since a high dose of a K CpG ODN induced splenomegaly in a mouse model (14). In a human clinical trial, a CpG ODN showed dose-dependent local injection reactions or systemic flu-like reactions (15). Thus, the benefits of a CpG ODN must be balanced against the risk of adverse reactions. In addition, CpG ODN monotherapy can be sufficient for relatively small tumors, but often needs to be

combined with other agents such as mAbs to induce antitumor activity in larger tumors (16). Here, we demonstrated the significant therapeutic activity of combination therapy with anti-GPC1 mAb and K3-SPG at low respective doses in a GPC1-positive ESCC xenograft model. K3-SPG increased the activity of anti-GPC1 mAb at low doses, suggesting that the combination therapy may allow the use of a decreased therapeutic dose of anti-GPC1 mAb to potentially reduce the possibility of adverse effects.

We used SCID mice to establish the ESCC xenograft model, which are competent in NK cells and macrophages but lack B-cells and T-cells. We found that K3-SPG monotherapy increased the tumor infiltration of macrophages and activated NK cells. These results suggest that K3-SPG might potentially augment the ADCC of anti-GPC1 mAb by inducing an immune response with NK cells and macrophages without an immune response *via* B-cells (17).

In addition to ESCC, increased expression of GPC1 has been reported in pancreatic cancer, breast cancer, cervical cancer, and glioma, with common effects of promoting the mitogenic metastatic and angiogenic properties of these cancers (11, 18-23). Therefore, anti-GPC1 mAb may have antitumor efficacy for these GPC1-positive cancers. We previously reported several clones of anti-GPC1 mAb and found that clone 01a033 had particularly high internalizing activity (21). Furthermore, we conjugated the anti-GPC1 mAb clone 01a033 with the cytotoxic agent monomethyl auristatin F (MMAF) and found that this GPC1-targeted antibody-drug conjugate had antitumor activity against GPC1-positive uterine cervical cancer and pancreatic cancer (21, 23). Anti-GPC1 mAb named clone 1-12 was found to be the suitable clone in terms of ADCC, with specific binding to GPC1 positive cell lines and high binding affinity to GPC1 protein (12). We hypothesize that K3-SPG augmented the ADCC activity of anti-GPC1 mAb and the combination therapy had the strongest anti-tumor effect in this study (Figure 2). Therefore, anti-GPC1 mAb has potential as a novel therapeutic modality for patients with GPC1-positive cancers.

There are several limitations to this study that should be mentioned. First, we were able to demonstrate an increase in tumor-infiltrating macrophages and activated NK cells in xenografted immunodeficient SCID mice; however, the effects of B-cell or T-cell responses are still unclear. Second, *i.t.* injection is required for the effective treatment with K3-SPG. In ESCC, *i.t.* injection is possible for localized cancer. However, a recent study demonstrated the antitumor activity of the systemic administration of K3-SPG, which suggests that K3-SPG can also be used for the treatment of advanced ESCC with metastasis (24).

In summary, combination therapy with anti-GPC1 mAb and K3-SPG exerts antitumor activity and induces significant infiltration of macrophages and activated NK cells in K3-SPG-treated tumors in a xenograft model of ESCC. Thus,

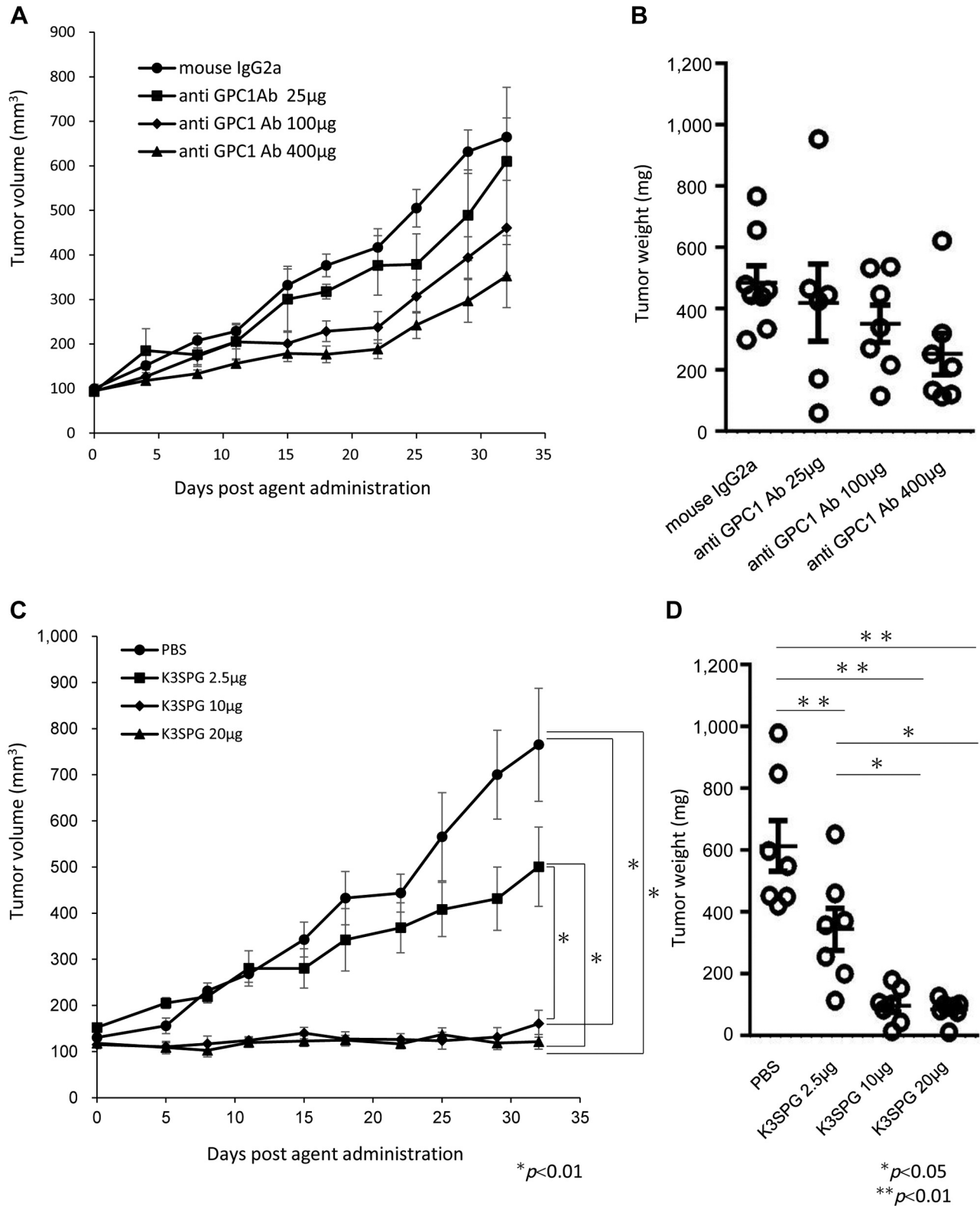


Figure 1. Anti-GPC1 mAb or K3-SPG monotherapy shows dose-dependent antitumor activity in SCID mice. (A, B) Xenografted SCID mice treated with 400 µl of mouse IgG2a or anti-GPC1 mAb (25, 100, and 400 µg in 400 µl of PBS/mouse) by intraperitoneal (i.p.) injection. There were 8 mice in the mouse IgG2a group, 6 mice in the anti-GPC1 mAb 25 µg group, and 7 mice in the rest two groups. (C, D) Xenografted SCID mice treated with PBS or K3-SPG (2.5, 10, and 20 µg in 25 µl of PBS/mouse) by intratumoral (i.t.) injection. Seven mice were used in each group.

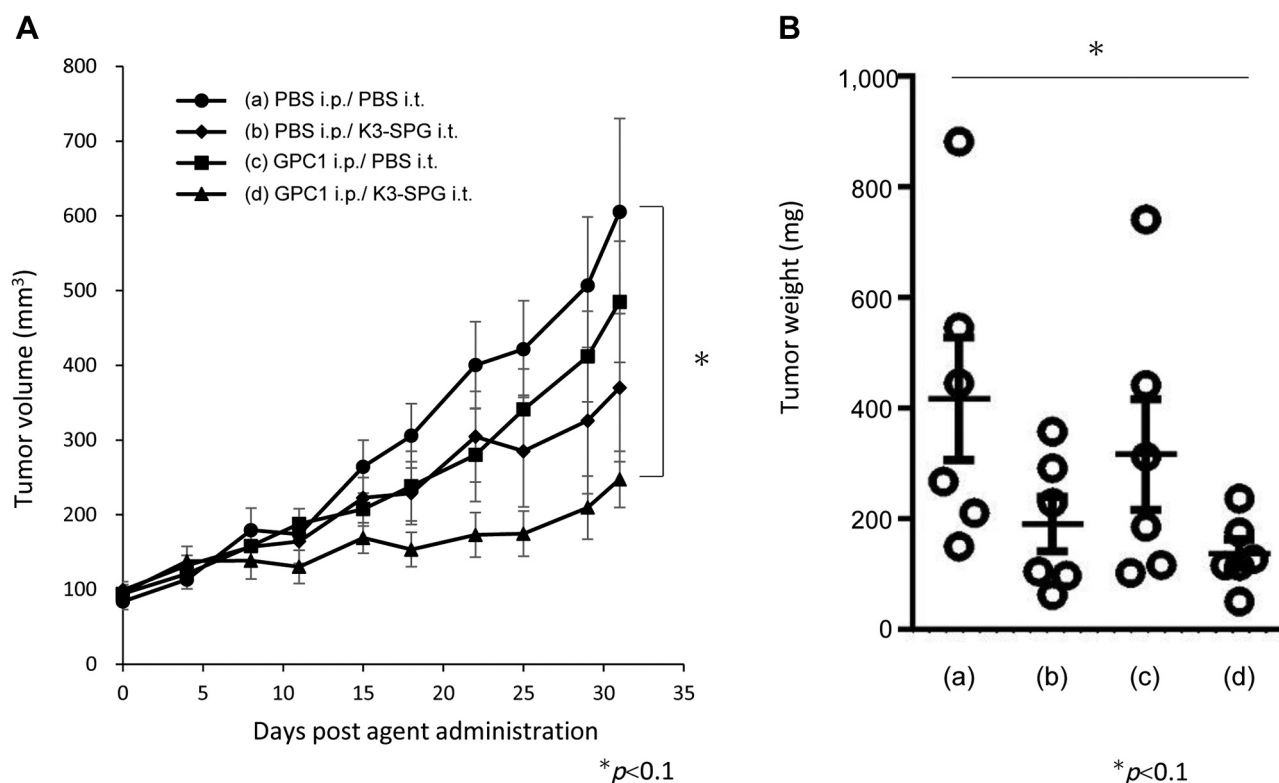


Figure 2. Combination therapy with anti-GPC1 mAb and K3-SPG exerted synergistic antitumor activity. (A, B) Xenografted SCID mice were treated by intraperitoneal/intratumoral injection of (a) PBS/PBS, (b) PBS/K3-SPG (2.5  $\mu$ g/mouse), (c) anti-GPC1 mAb (25  $\mu$ g/mouse)/PBS, and (d) anti-GPC1 mAb (25  $\mu$ g/mouse)/K3-SPG (2.5  $\mu$ g/mouse). Six mice were used in each treatment group.

combination therapy with K3-SPG and anti-GPC1 mAb or other antitumor agents depending on ADCC may represent a novel treatment option for cancer.

## Conflicts of Interest

All Authors have no conflicts of interest to declare in relation to this study.

## Authors' Contributions

Conception and design: Yurina Saito, Tsuyoshi Takahashi, Ken J Ishii and Tetsuji Naka; Development of methodology: Yurina Saito, Tsuyoshi Takahashi, Satoshi Serada, Minoru Fujimoto, Tomoharu Ohkawara, Ken J Ishii and Tetsuji Naka; Acquisition of data: Yurina Saito, Tsuyoshi Takahashi, Kosuke Hiramatsu, Satoshi Serada, Minoru Fujimoto, Tomoharu Ohkawara, Takahito Sugase and Takahiko Nishigaki; Analysis and interpretation of data: Yurina Saito, Tsuyoshi Takahashi, Satoshi Serada, Minoru Fujimoto and Tetsuji Naka; Writing, review and/or revision of manuscript: Yurina Saito, Tsuyoshi Takahashi, Kosuke Hiramatsu, Satoshi Serada, Minoru Fujimoto, Tomoharu Ohkawara, Takahito Sugase, Takahiko Nishigaki, Koji Tanaka, Yasuhiro Miyazaki, Tomoki Makino, Yukinori Kurokawa, Kiyokazu Nakajima, Makoto Yamasaki, Ken J Ishii, Hidetoshi Eguchi, Yuichiro Doki, and Tetsuji Naka; Study supervision: Tetsuji Naka.

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## References

- Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, Abdelalim A, Abdoli A, Abdollahpour I, Abdulle ASM, Abebe ND, Abraha HN, Abu-Raddad LJ, Abualhasan A, Adedeji IA, Advani SM, Afarideh M, Afshari M, Aghaali M, Agius D, Agrawal S, Ahmadi A, Ahmadian E, Ahmadpour E, Ahmed MB, Akbari ME, Akinyemiju T, Al-Aly Z, AlAbdulKader AM, Alahdab F, Alam T, Alamene GM, Alemnew BTT, Alene KA, Alinia C, Alipour V, Aljunid SM, Bakeshei FA, Almadi MAH, Almasi-Hashiani A, Alsharif U, Alsowaidi S, Alvis-Guzman N, Amini E, Amini S, Amoako YA, Anbari Z, Anber NH, Andrei CL, Anjomshoa M, Ansari F, Ansariadi A, Appiah SCY, Arab-Zozani M, Arabloo J, Arefi Z, Aremu O, Areri HA, Artaman A, Asayesh H, Asfaw ET, Ashagre AF, Assadi R, Ataeinia B, Atalay HT, Ataro Z, Atique S, Ausloos M, Avila-Burgos L, Avokpaho EFGA, Awasthi A, Awoke N, Ayala Quintanilla BP, Ayanore MA, Ayele HT, Babae E, Bacha U, Badawi A, Bagherzadeh M, Bagli E, Balakrishnan S, Balouchi

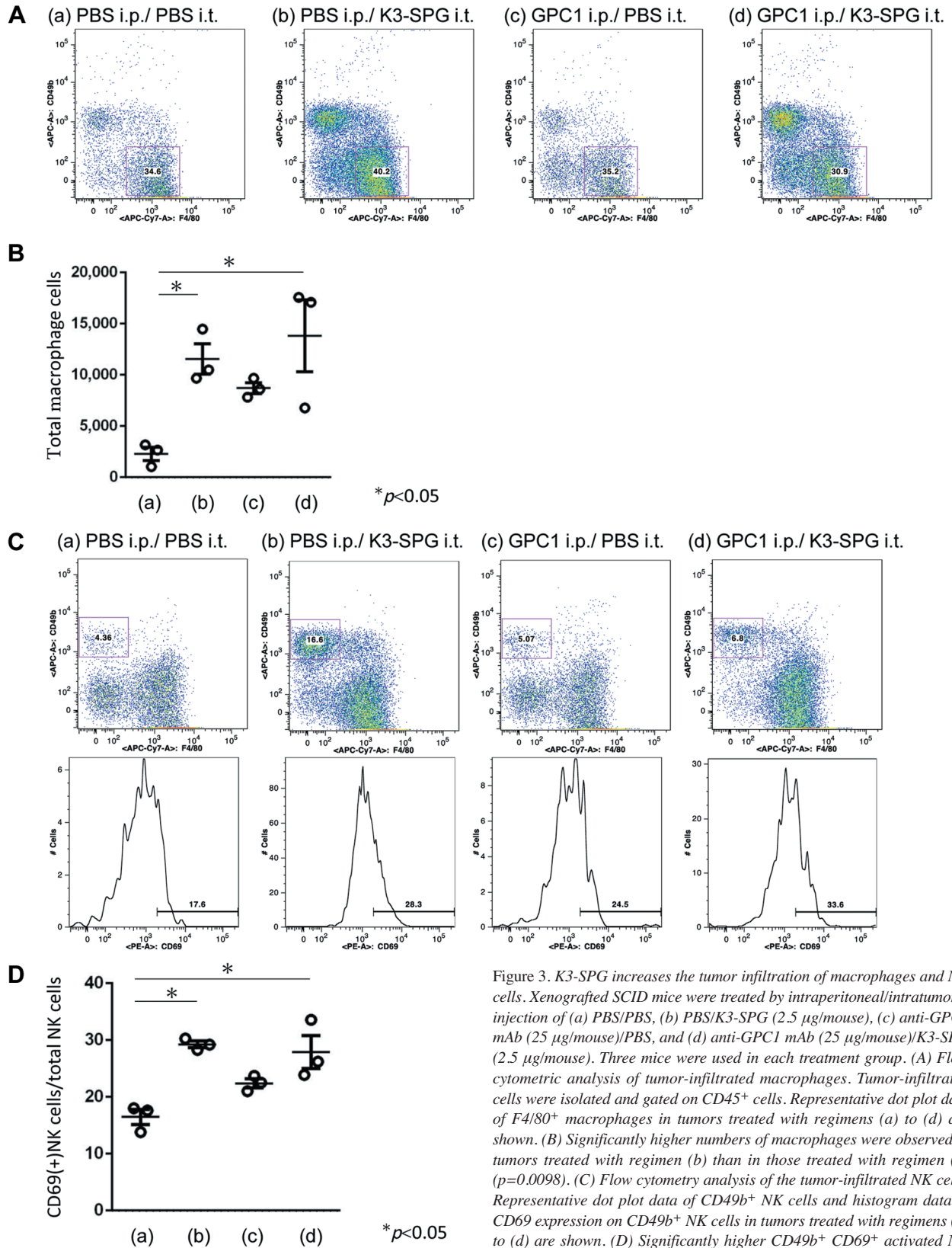


Figure 3. K3-SPG increases the tumor infiltration of macrophages and NK cells. Xenografted SCID mice were treated by intraperitoneal/intratumoral injection of (a) PBS/PBS, (b) PBS/K3-SPG (2.5  $\mu\text{g}/\text{mouse}$ ), (c) anti-GPC1 mAb (25  $\mu\text{g}/\text{mouse}$ )/PBS, and (d) anti-GPC1 mAb (25  $\mu\text{g}/\text{mouse}$ )/K3-SPG (2.5  $\mu\text{g}/\text{mouse}$ ). Three mice were used in each treatment group. (A) Flow cytometric analysis of tumor-infiltrated macrophages. Tumor-infiltrated cells were isolated and gated on CD45<sup>+</sup> cells. Representative dot plot data of F4/80<sup>+</sup> macrophages in tumors treated with regimens (a) to (d) are shown. (B) Significantly higher numbers of macrophages were observed in tumors treated with regimen (b) than in those treated with regimen (a) ( $p=0.0098$ ). (C) Flow cytometry analysis of the tumor-infiltrated NK cells. Representative dot plot data of CD49b<sup>+</sup> NK cells and histogram data of CD69 expression on CD49b<sup>+</sup> NK cells in tumors treated with regimens (a) to (d) are shown. (D) Significantly higher CD49b<sup>+</sup> CD69<sup>+</sup> activated NK cells/CD49b<sup>+</sup> total NK cells ratio in tumors treated with regimen (b) than in those treated regimen (a) ( $p=0.0354$ ).

- A, Bärnighausen TW, Battista RJ, Behzadifar M, Behzadifar M, Bekele BB, Belay YB, Belayneh YM, Berfield KKS, Berhane A, Bernabe E, Beuran M, Bhakta N, Bhattacharyya K, Biadgo B, Bijani A, Bin Sayeed MS, Birungi C, Bisignano C, Bitew H, Björge T, Bleyer A, Bogale KA, Bojia HA, Borzi AM, Bosetti C, Bou-Orm IR, Brenner H, Brewer JD, Briko AN, Briko NI, Bustamante-Teixeira MT, Butt ZA, Carreras G, Carrero JJ, Carvalho F, Castro C, Castro F, Catalá-López F, Cerin E, Chaiah Y, Chaniew WF, Chattu VK, Chaturvedi P, Chauhan NS, Chehrizi M, Chiang PP, Chichiabellu TY, Chido-Amajuoyi OG, Chimed-Ochir O, Choi JJ, Christopher DJ, Chu DT, Constantin MM, Costa VM, Crocetti E, Crowe CS, Curado MP, Dahlawi SMA, Damiani G, Darwish AH, Daryani A, das Neves J, Demekke FM, Demis AB, Demissie BW, Demoz GT, Denova-Gutiérrez E, Derakhshani A, Deribe KS, Desai R, Desalegn BB, Desta M, Dey S, Dharmaratne SD, Dhimal M, Diaz D, Dinberu MTT, Djalalinia S, Doku DT, Drake TM, Dubey M, Dublinan E, Duken EE, Ebrahimi H, Effiong A, Eftekhari A, El Sayed I, Zaki MES, El-Jaafary SI, El-Khatib Z, Elemineh DA, Elkout H, Ellenbogen RG, Elsharkawy A, Emamian MH, Endalew DA, Endries AY, Eshtrati B, Fadhil I, Fallah Omrani V, Faramarzi M, Farhangi MA, Farioli A, Farzadfar F, Fentahun N, Fernandes E, Feyissa GT, Filip I, Fischer F, Fisher JL, Force LM, Foroutan M, Freitas M, Fukumoto T, Futran ND, Gallus S, Gankpe FG, Gayesa RT, Gebrehiwot TT, Gebremeskel GG, Gedefaw GA, Gelaw BK, Geta B, Getachew S, Gezae KE, Ghafourifard M, Ghajar A, Ghashghaee A, Gholamian A, Gill PS, Ginindza TTG, Girmay A, Gizaw M, Gomez RS, Gopalani SV, Gorini G, Goulart BNG, Grada A, Ribeiro Guerra M, Guimaraes ALS, Gupta PC, Gupta R, Hadkhale K, Haj-Mirzaian A, Haj-Mirzaian A, Hamadeh RR, Hamidi S, Hanfore LK, Haro JM, Hasankhani M, Hasanzadeh A, Hassen HY, Hay RJ, Hay SI, Henok A, Henry NJ, Herteliu C, Hidru HD, Hoang CL, Hole MK, Hoogar P, Horita N, Hosgood HD, Hosseini M, Hosseinzadeh M, Hostiuc M, Hostiuc S, Househ M, Hussien MM, Ileanu B, Ilic MD, Innos K, Irvani SSN, Iseh KR, Islam SMS, Islami F, Jafari Balalami N, Jafarinia M, Jahangiry L, Jahani MA, Jahanmehr N, Jakovljevic M, James SL, Javanbakht M, Jayaraman S, Jee SH, Jenabi E, Jha RP, Jonas JB, Jonnagaddala J, Joo T, Jungari SB, Jürisson M, Kabir A, Kamangar F, Karch A, Karimi N, Karimian A, Kasaeeian A, Kasahun GG, Kassa B, Kassa TD, Kassaw MW, Kaul A, Keiyoro PN, Kelbore AG, Kerbo AA, Khader YS, Khalilarjmandi M, Khan EA, Khan G, Khang YH, Khatab K, Khater A, Khayamzadeh M, Khazae-Pool M, Khazaei S, Khoja AT, Khosravi MH, Khubchandani J, Kianipour N, Kim D, Kim YJ, Kisa A, Kisa S, Kissimova-Skarbek K, Komaki H, Koyanagi A, Krohn KJ, Bicer BK, Kugbey N, Kumar V, Kuupiel D, La Vecchia C, Lad DP, Lake EA, Lakew AM, Lal DK, Lami FH, Lan Q, Lasrado S, Lauriola P, Lazarus JV, Leigh J, Leshargie CT, Liao Y, Limenih MA, Listl S, Lopez AD, Lopukhov PD, Lunevicius R, Madadin M, Magdeldin S, El Razek HMA, Majeed A, Maleki A, Malekzadeh R, Manafi A, Manafi N, Manamo WA, Mansourian M, Mansournia MA, Mantovani LG, Maroufizadeh S, Martini SMS, Mashamba-Thompson TP, Massenbourg BB, Maswabi MT, Mathur MR, McAlinden C, McKee M, Meheretu HAA, Mehrotra R, Mehta V, Meier T, Melaku YA, Meles GG, Meles HG, Melese A, Melku M, Memiah PTN, Mendoza W, Menezes RG, Merat S, Meretoja TJ, Mestrovic T, Miazgowski B, Miazgowski T, Mihretie KMM, Miller TR, Mills EJ, Mir SM, Mirzaei H, Mirzaei HR, Mishra R, Moazen B, Mohammad DK, Mohammad KA, Mohammad Y, Darwesh AM, Mohammadbeigi A, Mohammadi H, Mohammadi M, Mohammadian M, Mohammadian-Hafshejani A, Mohammadoo-Khorasani M, Mohammadpourhodki R, Mohammed AS, Mohammed JA, Mohammed S, Mohebi F, Mokdad AH, Monasta L, Moodley Y, Moosazadeh M, Moossavi M, Moradi G, Moradi-Joo M, Moradi-Lakeh M, Moradpour F, Morawska L, Morgado-da-Costa J, Morisaki N, Morrison SD, Mosapour A, Mousavi SM, Muche AA, Muhammed OSS, Musa J, Nabhan AF, Naderi M, Nagarajan AJ, Nagel G, Nahvijou A, Naik G, Najafi F, Naldi L, Nam HS, Nasiri N, Nazari J, Negoi I, Neupane S, Newcomb PA, Nggada HA, Ngunjiri JW, Nguyen CT, Nikniaz L, Ningrum DNA, Nirayo YL, Nixon MR, Nnaji CA, Nojomi M, Nosratnejad S, Shiadeh MN, Obsa MS, Ofori-Asenso R, Ogbo FA, Oh IH, Olagunju AT, Olagunju TO, Oluwasanu MM, Omonisi AE, Onwujekwe OE, Oommen AM, Oren E, Ortega-Altamirano DDV, Ota E, Ostavnov SS, Owolabi MO, P A M, Padubidri JR, Pakhale S, Pakpour AH, Pana A, Park EK, Parsian H, Pashaei T, Patel S, Patil ST, Pennini A, Pereira DM, Piccinelli C, Pillay JD, Pirestani M, Pishgar F, Postma MJ, Pourjafar H, Pourmalek F, Pourshams A, Prakash S, Prasad N, Qorbani M, Rabiee M, Rabiee N, Radfar A, Rafiei A, Rahim F, Rahimi M, Rahman MA, Rajati F, Rana SM, Raoofi S, Rath GK, Rawaf DL, Rawaf S, Reiner RC, Renzaho AMN, Rezaei N, Rezapour A, Ribeiro AI, Ribeiro D, Ronfani L, Roro EM, Roshandel G, Rostami A, Saad RS, Sabbagh P, Sabour S, Saddik B, Safiri S, Sahebkar A, Salahshoor MR, Salehi F, Salem H, Salem MR, Salimzadeh H, Salomon JA, Samy AM, Sanabria J, Santric Milicevic MM, Sartorius B, Sarveazad A, Sathian B, Satpathy M, Savic M, Sawhney M, Sayyah M, Schneider IJC, Schöttker B, Sekerija M, Sepanlou SG, Sepehrimanesh M, Seyedmousavi S, Shaahmadi F, Shabaninejad H, Shahbaz M, Shaikh MA, Shamshirian A, Shamsizadeh M, Sharafi H, Sharafi Z, Sharif M, Sharifi A, Sharifi H, Sharma R, Sheikh A, Shirkoobi R, Shukla SR, Si S, Siabani S, Silva DAS, Silveira DGA, Singh A, Singh JA, Sisay S, Sitas F, Sobngwi E, Soofi M, Soriano JB, Stathopoulou V, Sufiyan MB, Tabarés-Seisdedos R, Tabuchi T, Takahashi K, Tamtaji OR, Tarawneh MR, Tassew SG, Taymoori P, Tehrani-Banihashemi A, Temsah MH, Temsah O, Tesfay BE, Tesfay FH, Teshale MY, Tessema GA, Thapa S, Tlaye KG, Topor-Madry R, Tovani-Palone MR, Traini E, Tran BX, Tran KB, Tsadik AG, Ullah I, Uthman OA, Vacante M, Vaezi M, Varona Pérez P, Veisani Y, Vidale S, Violante FS, Vlassov V, Vollset SE, Vos T, Vosoughi K, Vu GT, Vujcic IS, Wabinga H, Wachamo TM, Wagnew FS, Waheed Y, Weldegebreel F, Weldesamuel GT, Wijeratne T, Wondafrash DZ, Wonde TE, Wondmieni AB, Workie HM, Yadav R, Yadegar A, Yadollahpour A, Yaseri M, Yazdi-Feyzabadi V, Yeshaneh A, Yimam MA, Yimer EM, Yisma E, Yonemoto N, Younis MZ, Yousefi B, Yousefifard M, Yu C, Zabeh E, Zadnik V, Moghadam TZ, Zaidi Z, Zamani M, Zandian H, Zangeneh A, Zaki L, Zendehele K, Zenebe ZM, Zewale TA, Ziapour A, Zodepy S and Murray CJL: Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the Global Burden of Disease study. *JAMA Oncol* 5(12): 1749-1768, 2019. PMID: 31560378. DOI: 10.1001/jamaoncol.2019.2996
- 2 Malhotra GK, Yanala U, Ravipati A, Follet M, Vijayakumar M and Are C: Global trends in esophageal cancer. *J Surg Oncol* 115(5): 564-579, 2017. PMID: 28320055. DOI: 10.1002/jso.24592
  - 3 Colvin H, Mizushima T, Eguchi H, Takiguchi S, Doki Y and Mori M: Gastroenterological surgery in Japan: The past, the present and the future. *Ann Gastroenterol Surg* 1(1): 5-10, 2017. PMID: 29863129. DOI: 10.1002/ags3.12008

- 4 Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G and Slamon DJ: Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 17(9): 2639-2648, 1999. PMID: 10561337. DOI: 10.1200/JCO.1999.17.9.2639
- 5 Villegas FR, Coca S, Villarrubia VG, Jiménez R, Chillón MJ, Jareño J, Zuñil M and Callol L: Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer* 35(1): 23-28, 2002. PMID: 11750709. DOI: 10.1016/s0169-5002(01)00292-6
- 6 Kohrt HE, Houot R, Weiskopf K, Goldstein MJ, Scheeren F, Czerwinski D, Colevas AD, Weng WK, Clarke MF, Carlson RW, Stockdale FE, Mollick JA, Chen L and Levy R: Stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer. *J Clin Invest* 122(3): 1066-1075, 2012. PMID: 22326955. DOI: 10.1172/JCI61226
- 7 Akira S, Takeda K and Kaisho T: Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2(8): 675-680, 2001. PMID: 11477402. DOI: 10.1038/90609
- 8 Hiramatsu K, Serada S, Kobiyama K, Nakagawa S, Morimoto A, Matsuzaki S, Ueda Y, Fujimoto M, Yoshino K, Ishii KJ, Enomoto T, Kimura T and Naka T: CpG oligodeoxynucleotides potentiate the antitumor activity of anti-BST2 antibody. *Cancer Sci* 106(10): 1474-1478, 2015. PMID: 26498112. DOI: 10.1111/cas.12738
- 9 Kobiyama K, Aoshi T, Narita H, Kuroda E, Hayashi M, Tetsutani K, Koyama S, Mochizuki S, Sakurai K, Katakai Y, Yasutomi Y, Saijo S, Iwakura Y, Akira S, Coban C and Ishii KJ: Nonagonistic Dectin-1 ligand transforms CpG into a multitask nanoparticulate TLR9 agonist. *Proc Natl Acad Sci USA* 111(8): 3086-3091, 2014. PMID: 24516163. DOI: 10.1073/pnas.1319268111
- 10 Hara H, Takahashi T, Serada S, Fujimoto M, Ohkawara T, Nakatsuka R, Harada E, Nishigaki T, Takahashi Y, Nojima S, Miyazaki Y, Makino T, Kurokawa Y, Yamasaki M, Miyata H, Nakajima K, Takiguchi S, Morii E, Mori M, Doki Y and Naka T: Overexpression of glypican-1 implicates poor prognosis and their chemoresistance in oesophageal squamous cell carcinoma. *Br J Cancer* 115(1): 66-75, 2016. PMID: 27310703. DOI: 10.1038/bjc.2016.183
- 11 Matsuda K, Maruyama H, Guo F, Kleeff J, Itakura J, Matsumoto Y, Lander AD and Korc M: Glypican-1 is overexpressed in human breast cancer and modulates the mitogenic effects of multiple heparin-binding growth factors in breast cancer cells. *Cancer Res* 61(14): 5562-5569, 2001. PMID: 11454708.
- 12 Harada E, Serada S, Fujimoto M, Takahashi Y, Takahashi T, Hara H, Nakatsuka R, Sugase T, Nishigaki T, Saito Y, Hiramatsu K, Nojima S, Mitsuo R, Ohkawara T, Morii E, Mori M, Doki Y, Kaneda Y and Naka T: Glypican-1 targeted antibody-based therapy induces preclinical antitumor activity against esophageal squamous cell carcinoma. *Oncotarget* 8(15): 24741-24752, 2017. PMID: 28445969. DOI: 10.18632/oncotarget.15799
- 13 Naito H, Kidoya H, Sakimoto S, Wakabayashi T and Takakura N: Identification and characterization of a resident vascular stem/progenitor cell population in preexisting blood vessels. *EMBO J* 31(4): 842-855, 2012. PMID: 22179698. DOI: 10.1038/emboj.2011.465
- 14 Takahashi H, Misato K, Aoshi T, Yamamoto Y, Kubota Y, Wu X, Kuroda E, Ishii KJ, Yamamoto H and Yoshioka Y: Carbonate apatite nanoparticles act as potent vaccine adjuvant delivery vehicles by enhancing cytokine production induced by encapsulated cytosine-phosphate-guanine oligodeoxynucleotides. *Front Immunol* 9: 783, 2018. PMID: 29720976. DOI: 10.3389/fimmu.2018.00783
- 15 Manegold C, Gravenor D, Woytowicz D, Mezger J, Hirsh V, Albert G, Al-Adhami M, Readett D, Krieg AM and Leichman CG: Randomized phase II trial of a toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer. *J Clin Oncol* 26(24): 3979-3986, 2008. PMID: 18711188. DOI: 10.1200/JCO.2007.12.5807
- 16 Krieg AM: Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov* 5(6): 471-484, 2006. PMID: 16763660. DOI: 10.1038/nrd2059
- 17 Klinman DM: Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 4(4): 249-258, 2004. PMID: 15057783. DOI: 10.1038/nri1329
- 18 Aikawa T, Whipple CA, Lopez ME, Gunn J, Young A, Lander AD and Korc M: Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells. *J Clin Invest* 118(1): 89-99, 2008. PMID: 18064304. DOI: 10.1172/JCI32412
- 19 Kleeff J, Ishiwata T, Kumbasar A, Friess H, Büchler MW, Lander AD and Korc M: The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer. *J Clin Invest* 102(9): 1662-1673, 1998. PMID: 9802880. DOI: 10.1172/JCI4105
- 20 Duan L, Hu XQ, Feng DY, Lei SY and Hu GH: GPC-1 may serve as a predictor of perineural invasion and a prognosticator of survival in pancreatic cancer. *Asian J Surg* 36(1): 7-12, 2013. PMID: 23270819. DOI: 10.1016/j.asjsur.2012.08.001
- 21 Matsuzaki S, Serada S, Hiramatsu K, Nojima S, Matsuzaki S, Ueda Y, Ohkawara T, Mabuchi S, Fujimoto M, Morii E, Yoshino K, Kimura T and Naka T: Anti-glypican-1 antibody-drug conjugate exhibits potent preclinical antitumor activity against glypican-1 positive uterine cervical cancer. *Int J Cancer* 142(5): 1056-1066, 2018. PMID: 29055044. DOI: 10.1002/ijc.31124
- 22 Su G, Meyer K, Nandini CD, Qiao D, Salamat S and Friedl A: Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells. *Am J Pathol* 168(6): 2014-2026, 2006. PMID: 16723715. DOI: 10.2353/ajpath.2006.050800
- 23 Nishigaki T, Takahashi T, Serada S, Fujimoto M, Ohkawara T, Hara H, Sugase T, Otsuru T, Saito Y, Tsujii S, Nomura T, Tanaka K, Miyazaki Y, Makino T, Kurokawa Y, Nakajima K, Eguchi H, Yamasaki M, Mori M, Doki Y and Naka T: Anti-glypican-1 antibody-drug conjugate is a potential therapy against pancreatic cancer. *Br J Cancer* 122(9): 1333-1341, 2020. PMID: 32152502. DOI: 10.1038/s41416-020-0781-2
- 24 Kitahata Y, Kanuma T, Hayashi M, Kobayashi N, Ozasa K, Kusakabe T, Temizoz B, Kuroda E, Yamaue H, Coban C, Yamamoto T, Kobiyama K, Aoshi T and Ishii KJ: Circulating nanoparticulate TLR9 agonist scouts out tumor microenvironment to release immunogenic dead tumor cells. *Oncotarget* 7(31): 48860-48869, 2016. PMID: 27384490. DOI: 10.18632/oncotarget.10379

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