Neoadjuvant Use of Oncolytic Herpes Virus G47Δ Enhances the Antitumor Efficacy of Radiofrequency Ablation

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G47Δ is a triple-mutated oncolytic herpes simplex virus type 1 designed to induce antitumor immune responses efficiently. We examine the usefulness of G47Δ as a neoadjuvant therapy for radiofrequency ablation (RFA), a standard local treatment for certain cancers such as liver cancer, but remote recurrences within the same organ often occur. In A/J mice harboring bilateral subcutaneous Neuro2a tumors, the left tumors were treated with G47Δ intratumoral injections followed by RFA. Whereas the RFA-treated tumors were all eradicated, the growth of the right tumors was evaluated and tumor-infiltrating lymphocytes were analyzed. The G47Δ + RFA treatment caused smaller volumes of right tumors, accompanied by increased CD8+CD45− T cells, compared with G47Δ monotherapy. After depletion of CD8+ T cells, the enhanced efficacy on the contralateral tumors was completely abolished. Neoadjuvant G47Δ led to rejection of rechallenged tumors, which was caused by efficient induction of specific antitumor immune responses shown by enzyme-linked immunospot (ELISPOT) assays. Treatment of tumor-harboring animals with an anti-programmed cell death 1 (PD-L1) antibody led to even greater efficacy on contralateral tumors. Our study indicates that the neoadjuvant use of G47Δ effectively enhances the efficacy of RFA via CD8+ T cell-dependent immunity that is further augmented by an immune checkpoint inhibitor.

INTRODUCTION

Oncolytic viruses can selectively replicate in and kill cancer cells. Oncolytic herpes simplex virus 1 (HSV-1) is attractive for cancer therapy, because it infects a wide variety of cell types, exhibits strong cytotoxicity, and induces antitumor immune responses. Genetically engineered HSV-1 with multiple mutations exhibits minimal toxicity to normal tissues, which is important for clinical application. G47Δ is a triple-mutated, third-generation oncolytic HSV-1 constructed from G207, a double-mutated HSV-1, by adding another deletion mutation within the nonessential α47 gene. The third mutation conferred improved replication capability in cancer cells and augmentation of antitumor immunity, resulting in an enhanced antitumor effect of G47Δ while maintaining the safety profile.1 G47Δ has been shown to have efficacy in a variety of solid cancers in vivo and to kill cancer stem cells.2−4 G47Δ has been tested in clinical trials in Japan, including those for glioblastoma, prostate cancer, olfactory neuroblastoma, and malignant mesothelioma,5 and a phase II trial has recently shown a high efficacy in residual or recurrent glioblastoma patients. Among other oncolytic viruses, talimogene laherparepvec (T-VEC), a double-mutated oncolytic HSV-1 expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), showed a significantly higher durable response rate in patients with advanced melanoma when injected repeatedly into the tumors compared with control patients who received subcutaneous injections of GM-CSF, and was approved by the US Food and Drug Administration (FDA) as a new drug in 2015.6 An oncolytic poliovirus reportedly generated long-term survival in a portion of patients with glioblastoma in a phase I trial,7 and an oncolytic vaccinia virus seemed effective in patients with hepatocellular carcinoma (HCC) in a phase II trial but failed to show the efficacy in a phase III trial that followed.8 Oncolytic HSV-1 has shown efficacy for HCC in preclinical studies.9,10 Thus, oncolytic viruses, including G47Δ, seem promising for a variety of cancers, although they are yet to be developed for HCC.

Radiofrequency ablation (RFA) is widely used for cancer treatment.11 Especially for HCC, RFA is considered the standard local treatment.12 Although a recurrence of HCC at an RFA-treated site is rare, remote new lesions often appear within the liver.13 Many agents such as c-MET inhibitors, immune checkpoint inhibitors (ICIs), and Toll-like receptor 9 agonists reportedly enhance the antitumor effect of RFA, but none of them is considered efficacious enough to be a standard combination partner for RFA, and new agents that can reduce remote recurrence after RFA treatment are yet to be developed.14–16 The FDA approved nivolumab, an ICI, for HCC in 2017. In a phase I/II trial, the disease control rate was greater than 50%, but the objective response rate was less than 20%.17 Whereas RFA is reported to release tumor-associated antigens,18,19 clearly, an additional treatment that can turn those non-responsive “cold” tumors “hot” is required.

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Oncolytic HSV-1 can elicit specific antitumor immunity in the course of cancer cell-selective viral replication, and therefore a combination with various ICIs is being investigated. In a mouse brain tumor model, the efficacy of G47Δ-expressing murine interleukin (IL)-12 was enhanced by a combination with ICI. In a phase Ib clinical trial, the efficacy of T-VEC in advanced melanoma patients was improved by a combination with an anti-programmed cell death (PD-1) antibody. In the successful cases, those tumors that received T-VEC injections showed infiltration of interferon (IFN)-γ-producing CD8⁺ T cells. Another report showed that the antitumor immunity elicited by oncolytic Maraba virus was maintained after the resection of virus-injected tumors in a mouse subcutaneous tumor model, suggesting that oncolytic virus therapy prior to surgery can reduce tumor recurrence. In this study, we examined the usefulness of G47Δ as a neoadjuvant therapy for RFA and, furthermore, ICIs as a booster.

RESULTS

G47Δ Exerts Broad Oncolytic Activity against Human HCC Cell Lines

Because RFA is the current standard local treatment for HCC, we first sought to perform this study in models using HCC cells. To evaluate the cytopathic effect of G47Δ on HCC in vitro, five human HCC cell lines (Hep3B, SNU-398, HuH-7, HepG2, and PLC/PRF/5), a murine HCC cell line (Hepa1-6), and a murine neuroblastoma cell line (Neuro2a) were used. The cells were infected with G47Δ at a multiplicity of infection (MOI) of 0.1 or 0.01. G47Δ showed cytopathic activity in all cell lines tested, killing more than 80% of cells by day 4 after infection at a MOI of 0.1 in all cell lines tested, and more than 35% at a MOI of 0.01, except for SNU-398 cells (Figure 1A). To evaluate the infectivity with G47Δ, X-gal (5-bromo-4-chloro-3-indolyl β-d-galactoside) staining was performed in the six HCC cell lines, plus Vero cells as control, after infection with G47Δ at a MOI of 1 or 3. G47Δ efficiently infected all cell lines evaluated (Figure S1A). We further examined the replication capabilities of G47Δ, and all HCC cell lines tested except for SNU-398 (p = 0.061) showed significantly better virus yields at 48 h compared with 24 h (Figure S1B).

G47Δ Is Efficacious for Human HCC In Vivo

Established subcutaneous tumors of HepG2 or Hep3B in athymic mice were injected intratumorally with G47Δ (2 × 10⁶ plaque-forming units [PFU]) on days 0 and 3. In both the HepG2 and Hep3B subcutaneous tumor models, G47Δ exhibited significant antitumor effects, indicating that these HCCs are highly susceptible to G47Δ treatment in vivo (Figure 1B).

To mimic the clinical settings, orthotopic tumors were generated in athymic mice using Hep3B cells and were treated with a single intratumoral injection with G47Δ (2 × 10⁶ PFU) on day 35. For each animal, the abdominal cavity was opened at the time of injection, the established tumor in the liver was confirmed, and G47Δ was injected under direct view into the tumor. G47Δ treatment significantly improved the overall survival of the tumor-bearing mice compared with mock treatment (Figure 1C). Necropsy at the time of death confirmed that all animals died from tumor burden. These results suggest that G47Δ is useful for the treatment of human HCC.

Neoadjuvant Use of G47Δ in Combination with RFA Improves the Antitumor Efficacy in Immunocompetent Mice

Next, we hypothesized that the antitumor immunity induced by intratumoral injection with G47Δ prior to RFA would enhance the antitumor efficacy of RFA. Because an immunocompetent tumor model is necessary to test the hypothesis, we initially tried to use a murine HCC cell line, MH134-TC. Bilateral subcutaneous tumors were generated in C3H mice and the left tumors were treated with RFA or sham-RFA on day 4. However, regardless of whether the RFA treatment was performed, all contralateral right tumors shrank after day 15, indicating that MH134-TC cells were too immunogenic and the model was inappropriate for this study (Figure S2). Two other murine HCC cell lines, Hepa1-6 and BNL1, did not form subcutaneous tumors consistently in respective syngeneic mice (data not shown). Because none of the murine HCC cell lines available, including MH134-TC that formed subcutaneous tumors, was suitable for generating a tumor model for RFA, we decided to use poorly immunogenic, HSV-1-susceptible Neuro2a cells for this proof-of-concept study. Using A/J mice with established bilateral subcutaneous tumors of syngeneic Neuro2a, the left tumors were treated with intratumoral injections with G47Δ (2 × 10⁶ PFU) or mock on days 6, 8 and 10, followed by RFA on day 12. In all RFA-treated animals, with or without neoadjuvant G47Δ, the treated tumors were eradicated, and no local recurrence is observed by day 26, so the contralateral tumor growth is followed in this study (Figure 2A). In all sham-RFA-treated animals, the sham-treated tumors grew, and the tumor growth could be inhibited with G47Δ monotherapy (data not shown). G47Δ monotherapy inhibited the growth of the contralateral, non-treated tumors significantly compared with control or RFA monotherapy on day 18 (G47Δ versus control, p < 0.01; G47Δ versus RFA, p < 0.05). Mice with tumors that reached 20 mm in diameter in mock-treated groups were sacrificed on day 18, whereas G47Δ-treated groups could be followed until day 24. RFA monotherapy did not demonstrate a significant antitumor effect on the contralateral tumors by day 18 (RFA versus control, p = 0.12 on day 18). The G47Δ + RFA
Neoadjuvant G47Δ in Combination with RFA Results in Recruitment of CD8+ T Cells and an Increase in Serum IFN-γ Levels

To explore the immunological impact of neoadjuvant G47Δ in combination with RFA on the contralateral tumors, we evaluated tumor-infiltrating lymphocytes (TILs) in these tumors by flow cytometric analysis and determined the numbers of CD8+ T cells, CD4+ T cells, natural killer cells, regulatory T cells, and helper T cells among CD45+ lymphocytes (Figure S3). The proportion of CD8+ T cells among CD45+ cells was significantly increased by G47Δ monotherapy compared with control or RFA monotherapy (G47Δ versus control, p < 0.01; G47Δ versus RFA, p < 0.05). The combination therapy further increased the proportion of CD8+ T cells among CD45+ cells compared with each monotherapy (G47Δ + RFA versus RFA, p < 0.01; G47Δ + RFA versus G47Δ, p < 0.05; Figure 3A). The proportion of regulatory T cells of the combination therapy was smaller than that of the control (p < 0.01), although it was not significantly different from either monotherapies (Figure 3B). Moreover, histological analysis of the CD8+ T cell in the contralateral tumors revealed greater infiltration of CD8+ T cells after G47Δ + RFA combination therapy compared with each monotherapy (G47Δ + RFA versus G47Δ, p < 0.05; G47Δ + RFA versus RFA, p < 0.001; Figure 3D).

CD8+ T Cell Depletion Abolishes the Augmented Antitumor Effect by the Combination Therapy

To examine the contribution of CD8+ T cells to the augmented antitumor effect by the combination therapy, A/J mice harboring bilateral subcutaneous Neuro2a tumors were depleted of CD8+ T cells by intraperitoneal injections with an anti-CD8 monoclonal antibody initiated 1 day before G47Δ injection (Figure 4A). The augmented antitumor effect of G47Δ + RFA in the right, non-treated tumors was completely abolished after depletion of CD8+ T cells (G47Δ + RFA + anti-CD8 versus G47Δ + RFA, p < 0.01; G47Δ + RFA + anti-CD8 versus G47Δ, p < 0.001; Figure 4B), suggesting that CD8+ T cells are required for the augmented antitumor effect of the combination therapy (Figure 4B).

Neoadjuvant G47Δ in Combination with RFA Leads to Rejection of Tumor Rechallenge

Next, we examined whether neoadjuvant use of G47Δ in combination with RFA can prevent tumor recurrence. To mimic remote
Figure 3. Flow Cytometric Analyses of TILs and Serum Cytokine Analyses in the G47Δ and RFA Combination Therapy
A/J mice with bilateral subcutaneous Neuro2a tumors were treated as scheduled in Figure 2A. (A) The right, non-treated tumors were collected on day 14, and immune cells infiltrating the subcutaneous tumors were evaluated by flow cytometry. The G47Δ + RFA therapy increased the proportion of CD8+ T cells in the right tumors compared with each monotherapy. The data are means (n = 4-5); bars represent SEM. (B) The G47Δ + RFA therapy decreased the proportion of regulatory T cells in the right tumors compared with control but not with each monotherapy. (C) Immunohistological analysis of CD8+ T cells in right tumors revealed a greater quantity of CD8+ T cells infiltrating the tumor after the combination therapy. Scale bars, 50 μm. (D) CD8+ cells were counted in five fields/section. (E) The G47Δ + RFA therapy increased serum IFN-γ levels. Serum was collected from the mice on days 13 (POD 1), 15 (POD 3), and 19 (POD 7). IFN-γ was measured using the Bio-Plex system. IFN-γ was significantly increased in the G47Δ + RFA group compared with control but not with each monotherapy. *p < 0.05, **p < 0.01, ***p < 0.001. POD, postoperative day.
cell lines available, including MH134-TC, the only murine HCC cell line that consistently formed subcutaneous tumors, were found to be too immunogenic in syngeneic mice for immunological studies. As a result of seeking a model, we show that G47Δ infects efficiently, exhibits cytopathic activities against, and replicates well in human HCC cells. Also, using athymic mice bearing HCC subcutaneously or orthotopically, we show that intratumoral injections with G47Δ cause a significant efficacy. G47Δ has been tested in multiple clinical trials and shown to be safe even when injected repeatedly into the human brain at a high dose of $10^9$ PFU. In clinical settings, most HCCs are easily accessed by ultrasonography-guided needle interventions, and therefore G47Δ should be useful for the treatment of human HCC.

Nivolumab for advanced HCC treatment showed durable objective responses in the CheckMate 040 study and is available for HCC treatment in many countries. Several factors, such as the extent of cytotoxic T cell infiltration and the PD-L1 expression level on the surface of cancer cells, are reportedly useful for estimating the efficacy of ICIs. Because the key feature of G47Δ is its high capability as an in situ vaccination where the cancer cells are efficiently recognized by the host immune system via cancer cell-specific virus replication, simultaneous administration of an ICI should augment the efficacy of G47Δ. In fact, we show that an anti-PD-L1 antibody, when used as a booster, significantly enhances the efficacy of the G47Δ and RFA combination therapy. The anti-PD-L1 antibody was used intentionally at a low dose of 50 μg, so that the antibody alone showed no effect on the growth of subcutaneous Neuro2a tumors. Others have used an anti-CTLA-4 antibody or an anti-PD-1 antibody in combination with G47Δ armed with IL-12 and shown improvement of survival in a mouse glioma model. Our results have an important clinical implication that the sequential use of G47Δ, RFA, and an ICI may be useful as a treatment strategy that can newly replace RFA monotherapy.

In conclusion, the neoadjuvant use of G47Δ can effectively enhance the efficacy of RFA via CD8+ T cell-dependent antitumor immunity, which may be a practical therapeutic approach for patients treated with RFA, including those with liver cancer. The sequential use of G47Δ, RFA, and an ICI should further improve the antitumor efficacy.

**MATERIALS AND METHODS**

**Cell Lines and Virus**

*Vero* (African green monkey kidney), *Hep3B* (human HCC), *SNU-398* (human HCC), and *Sai/N* (*A/J* mouse-derived fibrosarcoma) cell lines were purchased from the American Type Culture Collection. *HuH-7* (human HCC), *HepG2* (human HCC), and *PLC/PRF/5* (human HCC) cell lines were purchased from the Japanese Collection of Research Bioresources. The *Hepa1-6* (murine HCC) cell line was purchased from Riken Cell Bank. The MH134-TC (*C3H* mouse-derived HCC) cell line was purchased from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. *Neuro2a* (*A/J* mouse-derived neuroblastoma) cells were purchased from the Health Science Research Resources Bank. Cells were maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum or 10% heat-inactivated fetal bovine serum at 37°C under 5% CO2. G47Δ is a conditionally replicating oncolytic HSV-1 virus that was constructed as described previously.

**In Vitro Cytotoxicity Studies**

*In vitro* cytotoxicity studies were performed as described previously. The number of surviving cells was counted daily using a Coulter counter (Beckman Coulter, Pasadena, CA, USA) and expressed as the percentage of the number of mock-infected control cells.
X-gal Staining
Vero cells, five human HCC cell lines (Hep3B, SNU-398, HuH-7, HepG2, and PLC/PRF/5), and a murine HCC cell line (Hepa1-6) were seeded in six-well plates (3 × 10^5/well) and infected with G47Δ at a MOI of 1 or 3 at 37°C for 1 h and incubated for another 4 h. Cells were fixed with 0.2% glutaraldehyde/2% paraformaldehyde and incubated with X-gal substrate solution (PBS, X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride) at 37°C for 2 h.

Virus Replication Assay
The five human HCC cell lines, Hepa1-6 cells, and Vero cells were seeded in six-well plates (3 × 10^5/well) and infected with G47Δ at a MOI of 0.01. After incubation at 37°C for 24 or 48 h, cells were collected and virus yields were assessed using Vero cells by a plaque assay.

Animal Studies
BALB/c nu/nu female mice (6–7 weeks old), A/J mice (5–7 weeks old), and C3H female mice (6 weeks old) were purchased from Japan SLC. All animal studies were approved by the Ethics Committee for Animal Experimentation of the University of Tokyo.

Subcutaneous Tumor Models
Subcutaneous tumors were generated by injecting HepG2 or Hep3B cells (5 × 10^6) into BALB/c nu/nu mice, MH134-TC cells (5 × 10^6) into syngeneic C3H mice, and Neuro2a cells (5 × 10^6) into syngeneic A/J mice; the cells were added to 70 μL of serum-free medium before subcutaneous injection into the left or bilateral flanks. When the subcutaneous tumors in the athymic mice reached 5 mm in diameter, G47Δ (2 × 10^6 PFU) or mock (defined as 20 μL of PBS supplemented with 10% glycerol) was administered intratumorally on days 0 and 3. Mice were sacrificed when tumors reached 20 mm in diameter in bilateral models and 24 mm in diameter in unilateral models as approved by the Ethics Committee for Animal Experimentation of the University of Tokyo.

Orthotopic Tumor Model
As an orthotopic mouse tumor model of liver cancer, several reports describe generating tumors in the liver by injecting HCC cells into the subcapsular space of the spleen. Orthotopic HCC was generated in athymic mice by splenic subcapsular implantation of Hep3B cells (3 × 10^6) in 30 μL of serum-free medium as described previously, with modifications. Tumors were treated with an intratumoral injection of G47Δ or the mock treatment on day 35.

Neoadjuvant G47Δ Therapy and ICI Booster
A/J mice with bilateral Neuro2a tumors were treated with intratumoral injections with G47Δ (2 × 10^6 PFU) in the left-side tumors on days 6, 8, and 10, followed by RFA on day 12. For the ICI experiment, 50 μg of anti-PD-L1 antibody (clone 10F.9G2, Bio X Cell, West Lebanon, NH, USA) or isotype control antibody (rat IgG2b clone LTF-2, Bio X Cell, West Lebanon, NH, USA) was administered...
intraperitoneally on days 6, 9, 12, and 15. For CD8+ T cell depletion, 250 μg of anti-CD8 antibody (clone 53-6.82, BioXCell, West Lebanon, NH, USA) or the isotype control antibody (rat IgG2a clone 2A3, BioXCell, West Lebanon, NH, USA) was administered intraperitoneally on days 5, 8, 12, 17, and 22. Tumor growth was determined by measuring the tumor volume (length × width × height) twice weekly. Animals were sacrificed when the maximum diameter of the tumor on either side reached 20 mm.

Combination drug interactions were determined based on a previously reported method. The FTV was calculated as the experimental tumor volume/mean control tumor volume. The expected FTV was calculated as the FTV after RFA/FTV after G47Δ. If the ratio of the expected FTV to observed FTV is greater than 1, the effect is expected to be synergistic. A ratio less than 1 suggests that the effect is additive.

RFA Application
Subcutaneous tumors were treated with RFA using the VIVA RF generator and electrode (STARmed, Goyang, Republic of Korea). An active electrode (5 mm long) was inserted directly into the tumor for ablation at 5 W for 60–80 s.

Flow Cytometric Analysis
For extraction of TILs, mice bearing bilateral subcutaneous Neuro2a tumors were treated with G47Δ (2 × 10^6 PFU) on days 6, 8, and 10, followed by RFA on day 12, and contralateral tumors were removed on day 14. A mouse tumor dissociation kit, gentleMACS dissociator, and gentleMACS C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to prepare single tumor cells according to the manufacturer’s protocol. Red blood cells (RBCs) were removed using RBC lysis buffer (eBioscience, Santa Clara, CA, USA). TILs were stained with several antibodies (see Table S1) and analyzed following the gating strategy (see Figure S2A). For intranuclear staining, the True-Nuclear transcription factor buffer set (BioLegend, San Diego, CA, USA) was used according to the manufacturer’s protocol. Flow cytometric analysis was performed on the CytoFLEX flow cytometer (Beckman Coulter, Pasadena, CA, USA).

Histological Analyses
Mice were sacrificed on day 14, and tumors were fixed in 10% formaldehyde neutral buffer solution (Sigma-Aldrich, St. Louis, MO, USA) for 72 h and embedded in paraffin. Sections were rehydrated through an alcohol gradient and subjected to heat-mediated antigen retrieval using target retrieval solution S1700 (Dako, Santa Clara, CA, USA). The sections were stained with an anti-mouse CD8 antibody (Abcam, Cambridge, UK) followed by 3,3′-diaminobenzidine as the chromogenic substrate. For semiquantitative analysis, positive cells were counted in five fields/section.

Cytokine analysis
Serum collected from mice on days 13, 15, and 19 was used for cytokine analysis. The levels of IFN-γ, IL-12, and IL-6 were evaluated using the Bio-Plex multiplex assay and Bio-Plex 200 system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol.

Rechallenge Studies
Neuro2a cells (5 × 10^6) were implanted subcutaneously into the left flanks of A/J mice, and the resulting tumors were injected intratumorally with G47Δ (2 × 10^6 PFU) or mock treatment on days 6, 8, and 10, followed by RFA on day 12. Whereas 5 × 10^6 cells, larger than the minimal number, are used for rapid establishment of...
subcutaneous Neuro2a tumors, the minimum number of cells that gives 100% engraftment is found to be $1 \times 10^6$ according to our preliminary experiments (data not shown). Therefore, mice were rechallenged with subcutaneous implantation of $1 \times 10^6$ Neuro2a cells into the contralateral right flanks on the same day of RFA administration, and tumor growth was observed twice a week. Because we observe that subcutaneous Neuro2a tumors do not regress spontaneously once they exceed 25 mm$^3$, tumors larger than 25 mm$^3$ were defined as engrafted, and the rate of tumor engraftment was determined.

**ELISPOT Assay**

Neuro2a cells ($5 \times 10^6$) were implanted subcutaneously into the left flanks of A/J mice, and the resulting tumors were injected intratumorally with G47Δ ($2 \times 10^6$ PFU) or mock on days 6, 8, and 10, followed by RFA on day 12. Mice were sacrificed on day 26, and a single-cell suspension of splenocytes was prepared. After the splenocytes were stimulated by Neuro2a or Sal/N cells, IFN-γ secretion from tumor-reactive splenocytes was evaluated using an IFN-γ ELISPOT$^\text{PLUS}$ kit (Mabtech, Nacka Strand, Sweden). Assays were conducted following the manufacturer’s protocol. Specific spots were counted and analyzed using the ImmunoSpot analyzer and ImmunoSpot software (CTL, Cleveland, OH, USA).

**Statistical Analysis**

Two-tailed Student’s t tests were used for all comparisons, except those of overall survival and engraftment rate, for which the log-rank test was used. In all cases, $p < 0.05$ was considered to indicate significance. All statistical analyses were performed using JMP Pro 14.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.08.010.

**AUTHOR CONTRIBUTIONS**

T.Y. and T.T. were involved with the conception, design, and performance of experiments, statistical analyses, interpretation of results, and writing the manuscript. M.I. assisted with designing and performing the experiments. R.T. and K.K. were involved with the conception and design of experiments. All authors reviewed and edited the manuscript.

**CONFLICTS OF INTEREST**

T.T. owns the patent right for G47Δ in Japan. The remaining authors declare no competing interests.

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