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Photosensitizer With Illumination Enhances *In Vivo* Antitumor Effect of Anti-ROBO1 Immunotoxin on Maxillary Sinus Squamous Cell Carcinoma

NORIKO KOMATSU[1,2], MIKU KOMATSU[3], RIUKO OHASHI[4,5], AKIRA HORII[3], KAZUTO HOSHI[1], TSUYOSHI TAKATO[1,6], TAKAHIRO ABE[1] and TAKAO HAMAKUBO[2]

[1]*Department of Oral and Maxillofacial Surgery, The University of Tokyo Hospital, Tokyo, Japan;*
[2]*Department of Protein-protein Interaction Research, Institute for Advanced Medical Sciences, Nippon Medical School, Kawasaki, Japan;*
[3]*Department of Molecular Pathology, Tohoku University of School of Medicine, Miyagi, Japan;*
[4]*Histopathology Core Facility, Niigata University Faculty of Medicine, Niigata, Japan;*
[5]*Division of Molecular and Diagnostic Pathology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan;*
[6]*JR Tokyo General Hospital, Tokyo, Japan*

Abstract. *Background/Aim: Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of cancer worldwide. Our study focused on the axon guidance receptor roundabout guidance receptor 1 (ROBO1) as a target for monoclonal antibody therapy of HNSCC. We previously showed that saporin-conjugated anti-ROBO1 (B5209B) immunotoxin (IT-ROBO1) enhanced cytotoxic effects on HNSCC cells in combination with the photosensitizer aluminum phthalocyanine disulphonate (AlPcS2a) and illumination. We examined the effects of this combination therapy in a mouse xenograft model. Materials and Methods: IT-ROBO1 was intraperitoneally administered to HSQ-89 (derived from Japanese maxillary sinus squamous carcinoma, RCB0789; RIKEN, Tsukuba, Japan) xenografted mice. After 3 days, AlPcS2a was injected subcutaneously around the tumor and the area was illuminated at 650 nm for 30 min. The growth of the tumor was evaluated and the effects on the tumor were examined. Results: Pronounced anti-tumor effects were elicited by the administration of IT-ROBO1 and AlPcS2a with light illumination on tumor size and pathological characteristics. Conclusion: The results showed that photosensitizer treatment with illumination robustly enhanced the antitumor effect of the IT-ROBO1 immunotoxin.*

Correspondence to: Takao Hamakubo, Department of Protein–protein Interaction Research, Institute for Advanced Medical Sciences, Nippon Medical School, 1-396, Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan. E-mail: hamakubo@qbm.rcast.u-tokyo.ac.jp; Takahiro Abe, Department of Oral and Maxillofacial Surgery, The University of Tokyo Hospital, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. E-mail: abet-ora@h.u-tokyo.ac.jp

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The annual number of deaths from head and neck cancer worldwide was recently reported to be 300,000 (1, 2). Triple therapy has been the standard treatment for Head and neck squamous cell carcinoma (HNSCC), *i.e.* the combination of surgery, radiation therapy and chemotherapy. Various complications, however, such as postoperative aesthetic issues, masticatory disorders, dysphagia, articulatory disorders, respiratory disorders, mucositis, and long-term osteomyelitis have been matters of concern with regard to quality of life. As the number of patients suffering from HNSCC is increasing with the aging of society, the development of new treatments with less functional impairment and higher therapeutic effect is an urgent issue.

Monoclonal antibody treatment is one of the approaches expected to afford improved care. Cetuximab and nivolumab have been approved for HNSCC treatment by the Food and Drug Administration (FDA) (3). Several methods to enhance antitumor effects have been tried, such as antibody drug conjugates, immunotoxin (IT), and radioimmunotherapy (RIT) (4). Trastuzumab-emtansine as an antibody drug conjugate was approved for inoperable or recurrent breast cancer that is human epidermal growth factor receptor 2 (HER2)-positive by the FDA in 1988 (5). IT was also approved for CD25-positive T-cell lymphoma (6) by the FDA in 2019. We demonstrated that a radioactive antibody

to roundabout guidance receptor 1 (ROBO1) labeled with ^{90}Y displays antitumor activities against small-cell lung carcinoma and Hep G2 xenograft in mice (7, 8).

Photodynamic therapy (PDT) has attracted attention as a non-invasive antitumor treatment. For example, talaporfin sodium (Laserphyrin^R) was approved for the PDT of lung cancer, primary brain malignancy and local remnant recurrence esophageal cancer after chemotherapy or irradiation (9). Many other photosensitizers have also been developed, such as porfimer sodium (Photofrin^R; 630 nm) for a variety of cancer types, and verteporfin (Visudine^R; 689 nm) for age-related macular degeneration.

Photochemical internalization (PCI) is a technique used to localize a photosensitizer to endosomal membranes for drugs which distribute to endosomes and induce endocytosis in cancer cells. When the cell is excited by light of a specific wavelength, the photosensitizer generates singlet oxygen, which destroys the endosomal membrane and releases the drug into the cytoplasm (10). Photosensitizers utilizing PCI are not in themselves designed to induce a cytotoxic effect (11). PCI-utilizing photosensitizers such as di-sulfonated tetraphenyl chlorin (TPCS2a), di-sulfonated aluminum phthalocyanine having sulfonate groups on the adjacent phthalate rings (AlpCS2a), and *meso*-tetraphenyl porphine di-sulfonate (TPPS2a), have been reported (12, 13).

ROBO1, an axon guidance receptor, has received considerable attention as a possible drug target in various cancer types, including liver, colon, breast, pancreatic, and SCC of the head and neck (14-16). It has been reported that the slit guidance ligand 2/ROBO1 signal in cancer plays important roles in invasion and migration of career cells, in the epithelial–mesenchymal transition (14), and in tumor-induced angiogenesis (14). We previously reported the cytotoxic effects of IT-ROBO1 on the HNSCC cell line, HSQ-89, as being enhanced in combination with PCI.

The purpose of this study is to examine the antitumor effect of IT-ROBO1 with PCI in a mouse model with maxillary sinus SCC (HSQ-89) xenograft.

Materials and Methods

Cells. The HNSCC cell line HSQ-89 (derived from the maxillary sinus, RCB0789) was purchased from RIKEN (Tsukuba, Japan). HSQ-89 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) with antibiotics (90 Units/ml penicillin, 90 μg/ml streptomycin (Thermo Scientific, Waltham, MA, USA) and incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Dulbecco's phosphate-buffered saline (D-PBS) was purchased from Wako (Osaka, Japan).

Chemicals and photochemical equipment. Antibody against ROBO1 (B5209B) was generated as previously described (7, 17). The antibody was biotinylated and conjugated with saporin as described elsewhere (18). The saporin-conjugated antibody against ROBO1 is called IT-ROBO1.

AlPcS2a was used as the PCI photosensitizer in this study. AlPcS2a was purchased from Frontier Scientific (Logan, UT, USA). A light-emitting diode lamp (54 W) with a peak wavelength of 650 nm was purchased from King Do Way (Amazon.co.jp, Seattle, WA, USA). AlPcS2a stock solution diluted with D-PBS (5 mg/ml) was kept at 4˚C in aliquots and protected from light.

HSQ-89 xenograft in mice. All procedures involving mice were carried out in accordance with the protocols approved by the University of Tokyo (RAC130109-2). Semiconfluent HSQ-89 cells cultured in 10 cm dishes were dissociated by trypsin, washed twice and centrifuged. The cells were supplemented with D-PBS and adjusted to a density of 2×10^7 cells/ml. Equal amounts of cell suspension and basement membrane matrix gel (Matrigel; Corning, Corning, NY, USA) (19) were mixed together. Male BALB/cSlc-nu/nu mice of 5-6 weeks old (20-23 g) were used. HSQ-89 tumor cells, 2×10^6/200 μl, were inoculated subcutaneously into the right shoulder of each mouse. Water and food were provided *ad libitum*.

IT-ROBO1 with PCI. The growth of the tumor was monitored by measuring the tumor size every day. The tumor size was calculated using the following formula:

$V=\pi/6\times length_{tumor}\times width_{tumor}r\times depth_{tumor}$ (20).

The experiments were initiated when the tumor size reached 40 mm^3. At that time, the mice weighed on average 20-25 g (6-8 weeks of age). Mice were randomly divided into four groups (n=5) as follows: i) IT-ROBO1 (*i.p.*) plus PCI:16 μg IT-ROBO1 in 200 μl D-PBS *i.p.* plus 100 μg AlPcS2a in 100 μl D-PBS subcutaneously (*s.c.*); ii) IT-ROBO1 only: 16 μg IT-ROBO1 in 200 μl D-PBS *i.p.* plus 100 μl D-PBS *s.c.*; iii) AlPcS2a only: 100 μl D-PBS *i.p.* plus 100 μg AlPcS2a in 100 μl D-PBS *s.c.*; iv) PBS control: 200 μl D-PBS *i.p.* plus 100 μl D-PBS *s.c.* IT-ROBO1 was administered on day 0 and AlPcS2a was injected subcutaneously around the tumor on day 3. Thirty 30 minutes after AlPcS2a injection, the tumors were illuminated with red light at 650 nm with a dose of 62.7 mW/cm^2, 113 J/cm^2. The mouse leg was covered with thick paper that had an opening for the tumor with a 2-3 mm free margin, to which the light was exposed. The mice were kept under inhalation anesthesia (Isoflurane; Wako) during light exposure. All of the animals received light illumination at the tumor as described above. There was only one application of AlPcS2a/light illumination for an animal. When the size of tumor reached 1,000 mm^3 or the weight of the mice decreased drastically (loss of more than 25% of body weight in a week), the mice were sacrificed.

Histopathological analysis. Two weeks after the injection of IT-ROBO1, two mice from each group were sacrificed for histopathological analysis. Tissues were separated from mice after sacrifice and put in 10% neutral buffered formalin solution (Muto Pure Chemicals, Tokyo, Japan) for several days. After routine processing and paraffin embedding, tissues were serially sectioned. Sectioned tissues were mounted on slide glasses and stained with hematoxylin and eosin for histopathological diagnosis.

Data analysis. Data are shown as the means±SD. Statistical evaluation was performed using analysis of variance (ANOVA) followed by Tukey Honest Significant Differences test. Differences with a value $p<0.01$ were taken to be statistically significant.

Results

Antitumor effects of IT-ROBO1 with PCI in vivo. In order to examine effect of immunotoxin with PCI, we prepared tumor-bearing mice by inoculating HSQ-89 cells mixed with matrix gel subcutaneously into the shoulder of 5- to 6-week-old mice as described in the Materials and Methods section. Since we had observed that *i.p*. administration of 100 μg AlPcS2a in 100 μl D-PBS under anesthesia resulted in leg paralysis in some of the mice, we opted for local *s.c*. administration around the tumor prior to application of red light. No apparent acute adverse effect was seen with the *s.c*. administration of AlPcS2a, there was only slight adhesive scar formation surrounding tumor observed in those treated with AlPcS2a..

As shown in Figure 1A, tumor growth was conspicuously reduced only in the group treated with IT-ROBO1 and PCI (ANOVA, $p<0.01$). There was no significant difference found between the other groups. The weight loss was significantly inhibited in the group treated with IT-ROBO1 combined with PCI (ANOVA, $p<0.01$) (Figure 1B). The appearance of a representative mouse from each of the groups 14 days after the treatment is shown in Figure 1C. The remaining tumors in mice of the IT-ROBO1 with PCI group were slightly detectable by palpation (Figure 1C) but the margin was unclear upon macroscopic inspection.

Edema was observed in the area surrounding the tumor in the groups treated with AlPcS2a, alone and in combination with AlPcS2a on the day after the localized exposure to 650 nm light. The edema lasted a few days and gradually diminished. An ulcer appeared in the same area about 2 days after light exposure in animals of these groups. Thereafter, formation of a crust was observed (Figure 1C), which fell off after about 10 days.

Histopathological examination. The tumors exhibited only small areas of intratumoral hemorrhage accompanied by degenerated cancer cells with pyknosis (shrinkage or condensation) and karyorrhexis (fragmentation of the nuclei) that may correspond to spontaneous necrosis (Figure 2A). The IT-ROBO1 only-treated tumors displayed large areas of intratumoral hemorrhagic necrosis with focal fibroblast proliferation adjacent to the tumor cells and small areas of granulation tissue comprised of a proliferation of fibroblasts, collagen fibers, endothelial cells, capillary-like microvessels and an infiltration of mononuclear inflammatory cells such as lymphocytes and macrophages around the tumor (Figure 2B). The PCI only-treated tumors displayed large areas of intratumoral hemorrhagic necrosis without any intratumor fibroblast proliferation small foci of granulation tissue formation around the tumor (Figure 2C). The tumors treated with the combination of IT-ROBO1 and AlPcS2a displayed small areas of intratumoral hemorrhagic necrosis accompanied by coagulatory tumor necrosis and granulation tissue formation. Roughly one-fourth the volume of the mass was replaced by granulation tissue surrounding irregular tumor cell nodules (Figure 2D).

Discussion

The first evidence that ROBO1 is a tumor-specific surface antigen of hepatocellular carcinoma was reported by Ito *et al*. (16). It was subsequently reported that ROBO1 is specifically expressed in a wide range of malignant cells or neoangiogenic endothelial cells (21, 22). Thus, ROBO1 has been widely studied as a good molecular target for cancer therapy (7, 8, 14, 16, 18, 21-24). Maiti *et al*. reported the expression of ROBO1 in HNSCC (23). We previously confirmed the expression levels of *ROBO1* mRNA and protein in several HNSCC cell lines (18). We demonstrated the expression of ROBO1 in HSQ-89 cells to be at the same level as in the hepatocellular carcinoma cell line HEP G2.

Monoclonal antibodies have been anticipated as useful therapies for the conservation of quality of life in patients with HNSCC. In order to enhance the antitumor effect of the antibodies, several methods have been investigated, such as antibody drug conjugates, IT, and RIT (4). RIT with the ^{90}Y-labelled B5209B antibody to ROBO1 has been shown to be effective for reducing xenografted HEP G2 tumors in mice (7).

Previously, we observed that B5209B ROBO1 IT conjugated with saporin exerted a small cytotoxic effect on HSQ-89 cells (18). The poor activity was considered to be due to low internalization of IT. Since photosensitizer administration and light exposure enhanced the cytotoxic effect of IT-ROBO1 enormously, we concluded that the cytotoxicity results from the problem of endosomal escape (10, 11, 18). This was also supported by our observation that treatment with saponin, which facilitates the internalization of IT, and the endosomal release of saporin, augmented cytotoxicity and enhanced the tumor-reducing effect both *in vitro* and *in vivo* (24).

Bostad *et al*. reported the utility of PCI-based endosomal escape technology. They showed that the CD133 targeting IT, AC133-saporin, along with the photosensitizer TPCS2a and light irradiation of 652 nm (15J/cm^2, 90 mW/cm^2) augmented the effects of IT on colorectal adenocarcinoma WiDr cells, resulting in inhibition and antitumor effects in a xenograft mouse model (25).

In this study, we sought to examine the *in vivo* antitumor effect of IT-ROBO1 with AlpCS2a for PCI treatment using HSQ-89 HNSCC cell xenografts in a mouse model. In order to avoid the systemic side-effects of PCI such as neuronal toxicity (26), we utilized subcutaneous administration of the photosensitizer near the tumor. As a result of *s.c*. administration, no symptoms suggestive of acute adverse effects occurred. Various degrees of ulcer formation were observed in the AlPcS2a and light-treated mice. They

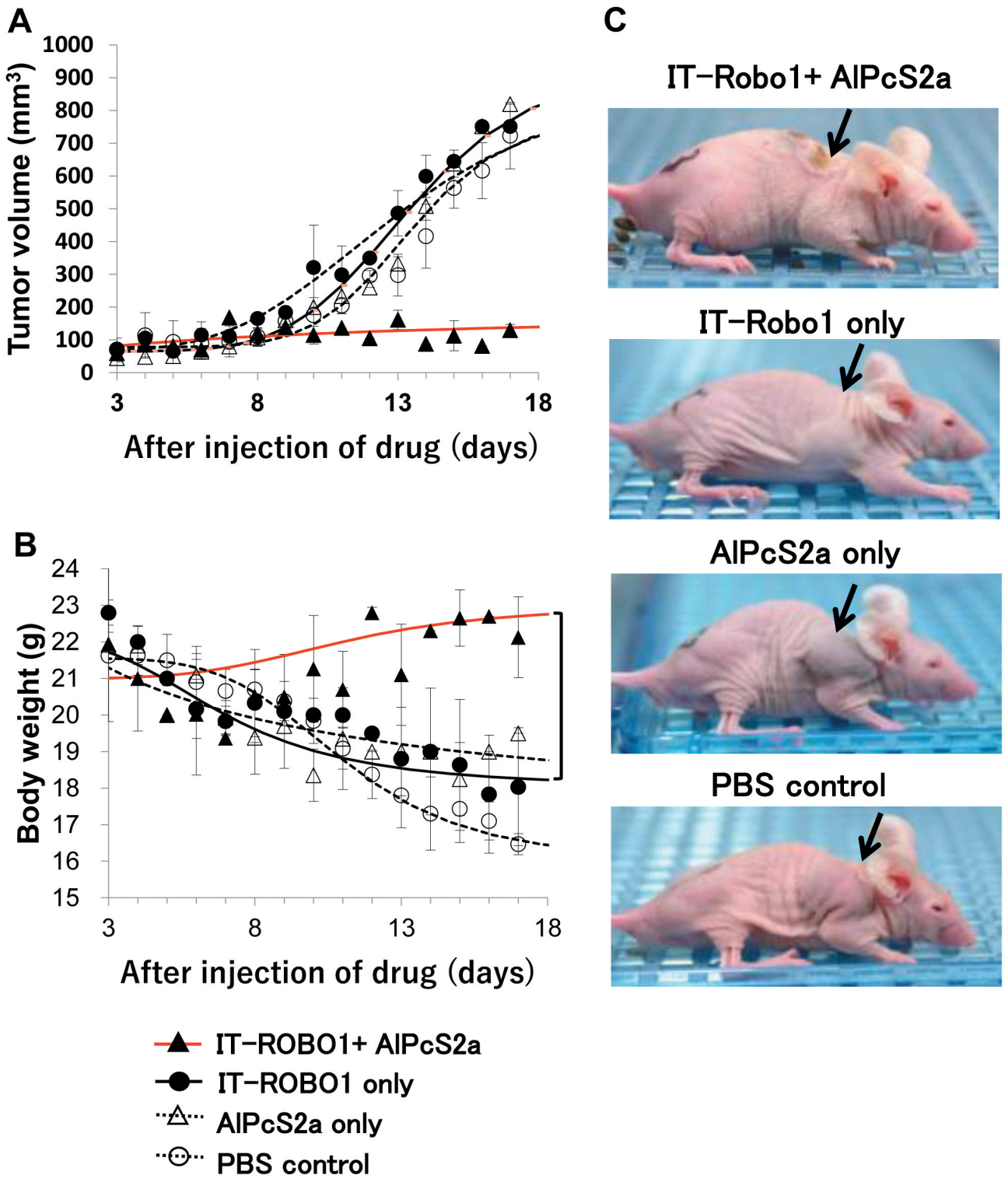

Figure 1. *A: The growth of tumors in animals treated with Dulbecco's phosphate-buffered saline (PBS control), Saporin-conjugated anti-roundabout guidance receptor 1 (ROBO1) (B5209B) immunotoxin (IT-ROBO1) only, aluminum phthalocyanine having sulfonate groups on the adjacent phthalate rings (ALPcS2a) only, and IT-ROBO1 combined with AlPcS2a. The tumor growth in the mice treated with IT-ROBO1 combined with AlPcS2a was inhibited compared to the mice treated with IT-ROBO1 alone (ANOVA, p<0.01). B: A reduction in body weight was significantly prevented in tumors treated with IT-ROBO1 combined with AlPcS2a compared to other groups, IT-ROBO1 only, ALPcS2a only, or PBS control (ANOVA, p<0.01). C: Representative image of a mouse from each treatment group at 14 days after light exposure. The tumor is designated by an arrow.*

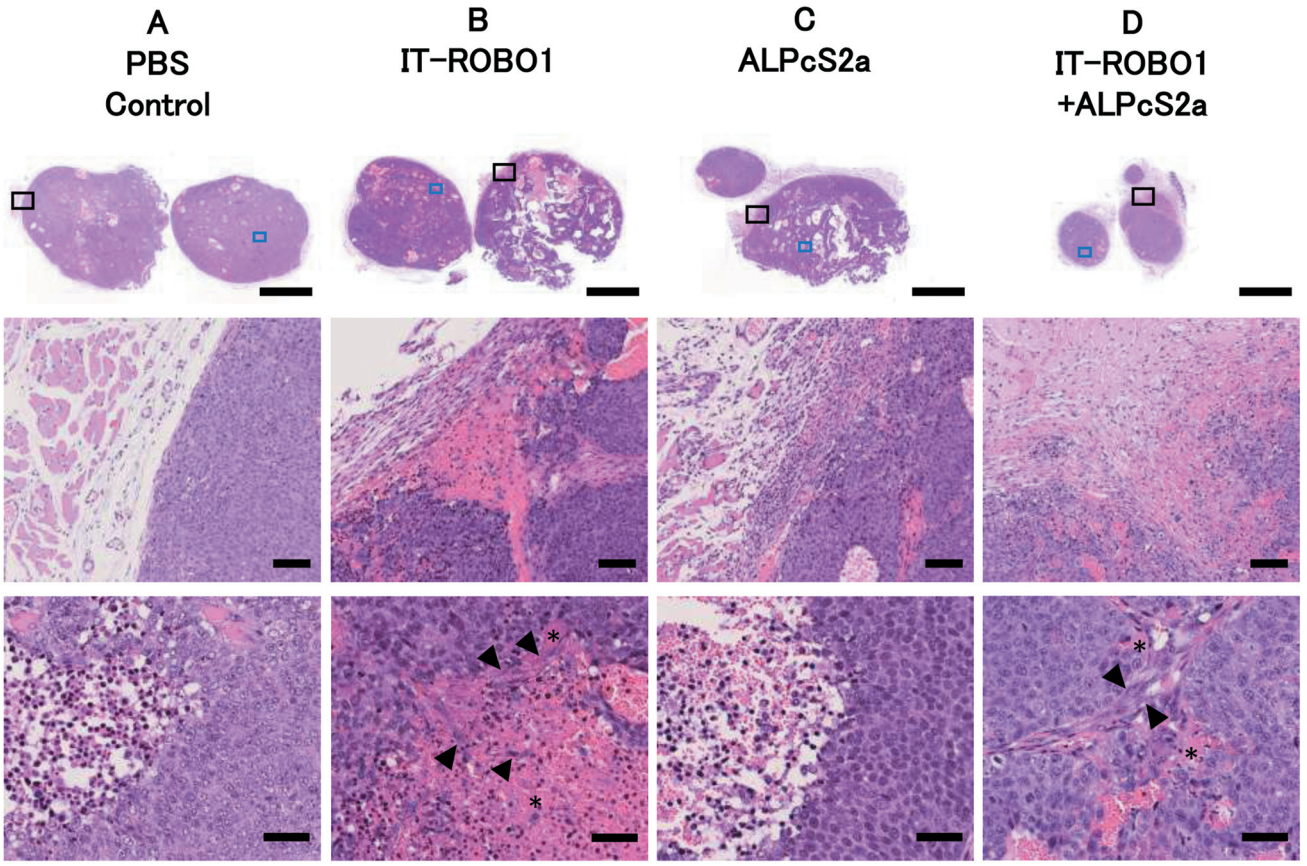

Figure 2. *Hematoxylin and eosin staining showing histopathological effects on tumors 2 weeks after treatment with Dulbecco's phosphate-buffered saline (PBS control), Saporin-conjugated anti-roundabout guidance receptor 1 (ROBO1) (B5209B) immunotoxin (IT-ROBO1) (B), aluminum phthalocyanine having sulfonate groups on the adjacent phthalate rings (AlPcS2a) only (C), and IT-ROBO1 with AlPcS2a (D). Upper row: Low-magnification views (scale bar=2 mm). Middle row: Higher-power view of the peritumoral area shown in the black box in the upper row (scale bar=100 μm). Bottom row: Higher-power view of the intratumoral area shown in the blue box in the upper row (scale bar=50 μm). Intratumoral fibroblast proliferation (arrowheads; spindle cells) with small foci of coagulation necrosis (asterisks; amorphous, pink, necrotic material) were observed in those treated with ROBO1 alone and in combination with AlPcS2a. Formation of peritumoral granulation tissue with microvessel proliferation and an infiltration of inflammatory cells such as lymphocytes and macrophages were observed in all groups except the control.*

resolved spontaneously without any treatment, accompanied by the formation of a crust that subsequently fell off. Thus, these adverse effects are considered to be tolerable.

As shown in Figure 1, treatment using IT-ROBO1 and AlPcS2a with light illumination resulted in a significant reduction of the tumor volume and a retention of body weight. This significant effect was not observed in the mice treated solely with IT-ROBO1 nor those administered AlPcS2a alone (group 3).

The pathological examination revealed conspicuous focal hemorrhagic necrosis with granulation tissue formation inside or outside the tumor in the mice receiving IT-ROBO1 with AlPcS2a for PCI, suggesting tumoricidal effects and initiation of the healing process (Figure 2D). In the group treated with IT-ROBO1 only (Figure 2B), there was a large hemorrhagic necrotic area observed in the center of the tumor, suggesting some cytotoxic effect on cancer cells. These findings concur with the previous observation of a synergistic antitumor effect of IT-ROBO1 with PCI *in vitro* (18).

In this study, we showed that the local administration of AlPcS2a along with application of light enhanced the antitumor effect of saporin-based IT and thus widens the therapeutic window for the molecular targeted treatment of cancer. In particular because HNSCC is a comparatively superficial tumor, this technique will be applicable to the clinical setting.

Conclusion

We have presented evidence that ROBO1 comprises a novel immunotherapeutic target in HNSCC that may be utilized by

the application of toxin conjugate and photosensitive drug with light application, and the drug delivery system developed here should prove to be applicable to other targets of low abundance in cancer cells, thus widening the therapeutic window for rare cancer types.

Conflicts of Interest

There is no conflict of interest.

Authors' Contributions

Concept design: Noriko Komatsu, Takahiro Abe, Takao Hamakubo. Literature search: Noriko Komatsu, Takao Hamakubo. Experiments: Noriko Komatsu and Miku Komatsu contributed Figure 1. Noriko Komatsu and Riuko Ohashi contributed Figure 2. Article preparation: Noriko Komatsu, Miku Komatsu, Riuko Ohashi, Akira Horii, Takahiro Abe, Takao Hamakubo. Article review: Kazuto Hoshi, Tsuyoshi Takato.

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