

An Antihuman Epidermal Growth Factor Receptor 2 Monoclonal Antibody (H₂Mab-19) Exerts Antitumor Activity in Glioblastoma Xenograft Models

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Overexpression of human epidermal growth factor receptor 2 (HER2) has been reported in glioblastoma as well as breast, gastric, lung, colorectal, and pancreatic cancers. Its expression is associated with poor clinical outcomes. Anti-HER2 antibodies have provided significant survival benefits to patients with HER2-overexpressing breast and gastric cancers. We recently developed an anti-HER2 monoclonal antibody (mAb), H₂Mab-19 (IgG_{2b}, kappa), by immunizing mice with the extracellular domain of HER2, which is expressed in LN229 glioblastoma cells. In this study, we investigated the antitumor activity of H₂Mab-19 in an LN229 glioblastoma xenograft model. H₂Mab-19 showed high binding affinity (K_D : 1.1×10^{-8} M) against LN229 cells. Furthermore, H₂Mab-19 significantly reduced tumor development in an LN229 xenograft. These results suggest that treatment with H₂Mab-19 may be a useful therapy for patients with HER2-expressing glioblastomas.

Keywords: HER2, monoclonal antibody, antitumor activity, glioblastoma

Introduction

GLIOMAS REPRESENT THE MOST COMMON primary brain tumor, and glioblastoma multiforme is the most frequent and malignant type of glioma.⁽¹⁾ Despite advances in surgical techniques, radiation therapy, and adjuvant chemotherapy, their prognoses remain poor. Many antigens, including epidermal growth factor receptor (EGFRwt), its glioma-associated deletion variant EGFRvIII, tenascin, chondroitin sulfate proteoglycans, and lacto-series gangliosides, have been found in gliomas, and upregulation of those molecules has been observed in brain tumor cells.⁽²⁾ Although these molecules are under investigation as therapeutic targets, multiple entities may ultimately have to be targeted to compensate for tumor heterogeneity. Human epidermal growth factor receptor 2 (HER2) has also been reported to be expressed in glioblastomas, and represents one of the molecular targets for immunotherapy.^(3–8)

A novel anti-HER2 monoclonal antibody (mAb) (H₂Mab-19; IgG_{2b}, kappa) was recently developed by immunizing mice with the purified recombinant ectodomain of HER2.⁽⁹⁾ H₂Mab-19 exerts antitumor activity in BT-474, HSC-2, and SAS xenografts by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), suggesting that treatment with H₂Mab-19 may be a useful therapy for patients with HER2-expressing breast and oral cancers. In this

study, we investigated whether H₂Mab-19 exhibits antitumor activity in a mouse xenograft model of glioblastoma.

Materials and Methods

Cell lines

LN229 (glioblastoma cell line) was obtained from the American Type Culture Collection (Manassas, VA). LN229/HER2 was established in a previous study.⁽¹⁰⁾ LN229 and LN229/HER2 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Flow cytometry

LN229 or LN229/HER2 cells were harvested by brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline (PBS), LN229 or LN229/HER2 cells were treated with 1 µg/mL anti-HER2 (H₂Mab-19) for 30 minutes at 4°C and subsequently with

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Alexa Fluor 488-conjugated antimouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using an EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of the binding affinity

LN229 cells were suspended in 100 μ L serially diluted H₂Mab-19 (6 ng/mL–100 μ g/mL), followed by the addition of Alexa Fluor 488-conjugated antimouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using an EC800 Cell Analyzer (Sony Corp.). The dissociation constant (K_D) was obtained by fitting binding isotherms to built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Antitumor activity of H₂Mab-19 in the xenografts of glioblastoma

Sixteen 6-week-old female BALB/c nude mice were purchased from Charles River Laboratories and used at 7 weeks

of age. All animal experiments were performed in accordance with relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal studies for antitumor activity were approved by the institutional committee for experiments of the Institute of Microbial Chemistry (Permit number: 2019-021). Mice were monitored for health and weight every 2 to 4 days. The duration of the experiments was 3 weeks. A bodyweight loss exceeding 25% and a maximum tumor size exceeding 3000 mm³ were designated as humane endpoints. Mice were euthanized by cervical dislocation and death was verified by respiratory arrest and cardiac arrest.

LN229 cells (0.3 mL of 1.33×10^8 cells/mL in DMEM) were mixed with 0.5 mL BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA). One hundred microliters of this suspension (5×10^6 cells) was injected subcutaneously into the left flank. After day 1, 100 μ g of H₂Mab-19 and control mouse IgG (Sigma-Aldrich Corp.) in 100 μ L of PBS were injected intraperitoneal (i.p.) into treated and control mice, respectively. Additional antibodies were

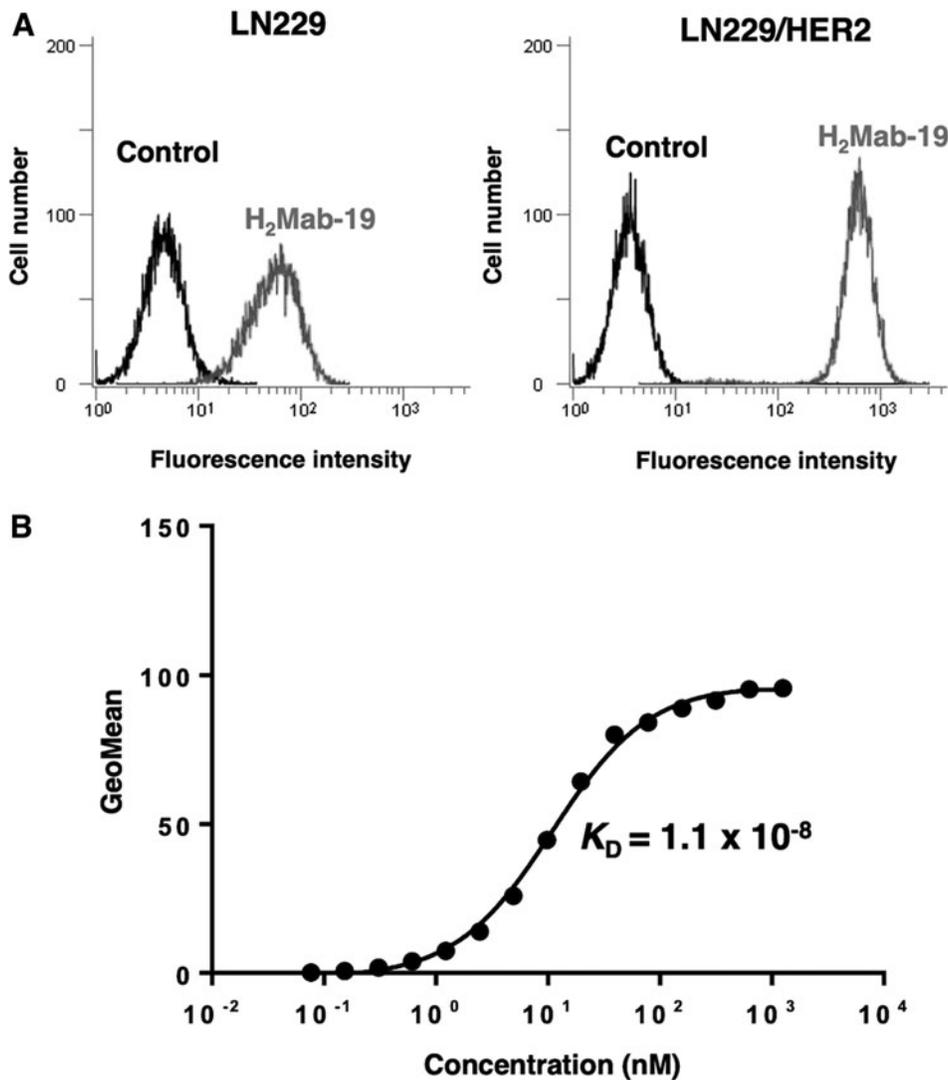


FIG. 1. Characterization of H₂Mab-19 using flow cytometry. (A) LN229 cells were treated with H₂Mab-19. The black line represents the negative control (blocking buffer). (B) Determination of the binding affinity of H₂Mab-19 for LN229 cells using flow cytometry. H₂Mab-19, anti-human epidermal growth factor receptor 2 monoclonal antibody.

then injected on days 6 and 14. Twenty days after cell implantation, all mice were euthanized by cervical dislocation and tumor diameters and volumes were determined as previously described.⁽¹¹⁾

Statistical analyses

All data are expressed as mean \pm standard error of the mean. Statistical analysis used analysis of variance and Tukey–Kramer’s test with R statistical. p values < 0.05 were considered statistically significant.

Results

Characterization of H₂Mab-19 against a glioblastoma cell line

We first measured the surface expression of HER2 in the LN229 human glioblastoma and HER2-overexpressing LN229/HER2 cell lines. As expected, H₂Mab-19 recognized endogenous HER2 in LN229 cells and overexpressed HER2 of LN229/HER2 cells by flow cytometry (Fig. 1A).

The binding affinity (K_D) of H₂Mab-19 to LN229 cells was 1.1×10^{-8} M (Fig. 1B), indicating that H₂Mab-19 exhibits a

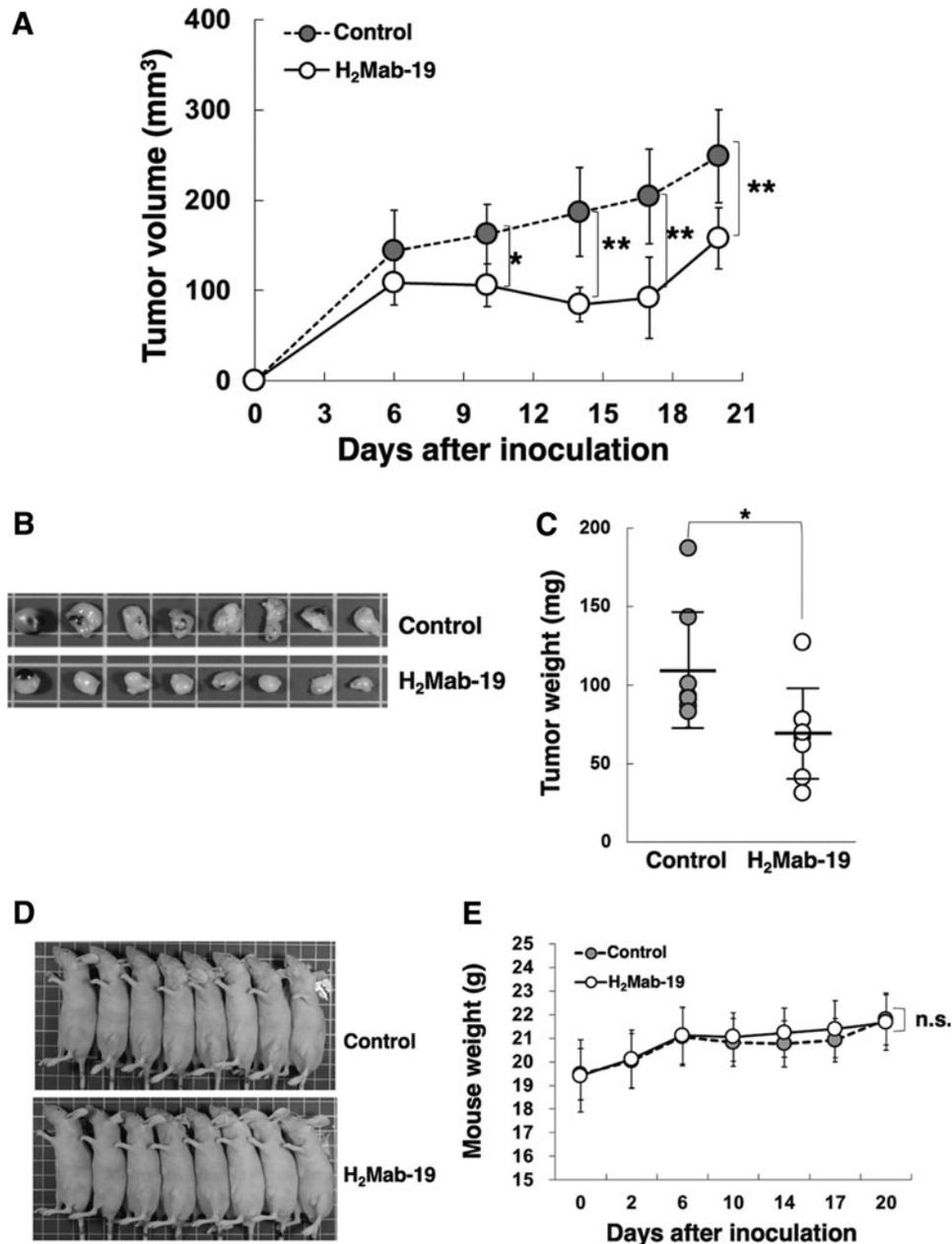


FIG. 2. Evaluation of the antitumor activity of H₂Mab-19. (A) Tumor volume was measured in LN229 xenografts. (B) Resected tumors of LN229 xenografts (day 20). (C) Tumor weight was measured in LN229 xenografts (day 20). (D) Appearance of treated mice on day 20. (E) Body weights of the mice with the LN229 xenografts were measured for 20 days. Values represent the mean \pm SEM. ** $p < 0.01$, * $p < 0.05$, the Tukey–Kramer’s test. n.s.: not significant. SEM, standard error of the mean.

high affinity for HER2-expressing glioblastoma cell lines. These results suggest the possibility of targeting HER2 as an antigen for immunotherapy.

Antitumor activity of H₂Mab-19 in mouse xenografts of glioblastoma

Next, we investigated whether H₂Mab-19 induces cytotoxic activity against glioblastoma *in vivo*. To study the antitumor activity of H₂Mab-19 on cell growth *in vivo*, LN229 cells were implanted subcutaneously in the flanks of nude mice. H₂Mab-19 and control mouse IgG were injected i.p. three times (days 1, 6, and 14 after cell injection) into treated and control mice, respectively. Tumor formation was observed in both the H₂Mab-19-treated and control groups. H₂Mab-19 treatment significantly reduced tumor development compared with development in control mice on day 10 ($p < 0.05$), day 14 ($p < 0.01$), day 17 ($p < 0.01$), and day 20 ($p < 0.01$; Fig. 2A). Resected tumors are depicted in Figure 2B. Tumor weight from H₂Mab-19-treated mice was significantly less than that of the IgG-treated control mice ($p < 0.05$; Fig. 2C). Mice on day 20 are depicted in Figure 2D. Total body weight was not significantly different between the two groups (Fig. 2E). These results indicate that H₂Mab-19 exerts a significant antitumor effect against HER2-expressing glioblastomas.

Discussion

HER2 overexpression has been reported in breast and gastric cancers, and is associated with poor clinical outcomes.^(12–14) The humanized anti-HER2 mAbs, trastuzumab and pertuzumab, have been used in the treatment of HER2-positive breast cancer.^(15–17) Treatment with trastuzumab resulted in significant survival benefits in these patients.⁽¹⁸⁾ The combination of trastuzumab and pertuzumab with chemotherapy has led to significant improvements in overall survival.⁽¹⁹⁾ Recently, trastuzumab deruxtecan (DS-8201), which consists of three components, a novel enzyme-cleavable linker, and a topoisomerase I inhibitor, exerts antitumor activity even in low-HER2-expressing tumors.^(20,21) Therefore, we still require additional sensitive and specific anti-HER2 mAbs, the epitope of which is different than trastuzumab and pertuzumab. We have tried to develop many anti-HER2 mAbs that react with not only breast cancers,⁽¹⁰⁾ but also colon cancers,⁽²²⁾ pancreatic cancers,⁽²³⁾ and glioblastoma using CasMab technology.⁽²⁴⁾

We previously developed anti-HER2 mAbs, H₂Mab-77,⁽¹⁰⁾ H₂Mab-119,⁽²³⁾ and H₂Mab-139.⁽²²⁾ These antibodies are useful for flow cytometry, Western blot, and immunohistochemical analyses. The subclass of these mAbs was determined to be mouse IgG₁. Therefore, they do not possess ADCC or CDC activities. We further tried to develop an anti-HER2 mAb of the IgG_{2b} subclass using CasMab technology, because mouse IgG_{2b} antibodies exhibit ADCC and CDC activity.⁽²⁵⁾ A novel anti-HER2 mAb (H₂Mab-19) of IgG_{2b} was recently established,⁽⁹⁾ which exhibited antitumor activity in breast or oral cancer xenografts by ADCC and CDC. In this study, we investigated whether H₂Mab-19 exhibits antitumor activities in LN229 mouse xenograft models, since HER2 has been reported to be expressed in glioblastomas.^(3–8)

Antigen–antibody affinity is thought to be an important factor influencing the outcome of antibody-based therapy.⁽²⁶⁾ The binding affinity (K_D) of H₂Mab-19 to LN229 was determined to be 1.1×10^{-8} M using flow cytometry, indicating that H₂Mab-19 shows high affinity to HER2-expressing glioblastoma cell lines. This is similar to the K_D of H₂Mab-19 to BT-474 (2.3×10^{-8} M), HSC-2 (9.5×10^{-9} M), and SAS (5.5×10^{-9} M).⁽⁹⁾

We selected an LN229 cell line for the *in vivo* study, since LN229 is useful for glioblastoma xenograft models.⁽²⁷⁾ In our previous study, H₂Mab-19 treatment significantly reduced tumor development in breast and oral cancer xenograft models.⁽⁹⁾ H₂Mab-19 also exerted antitumor activity against LN229 xenografts in this study (Fig. 2). Further studies using other glioblastoma xenografts should be performed to confirm that H₂Mab-19 could represent a viable therapy for patients with HER2-expressing glioblastomas.

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Author Disclosure Statement

No competing financial interests exist.

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