Abstract. HER3 belongs to the epidermal growth factor receptor (EGFR) family and is known to form an active heterodimer with other three family members EGFR, HER2, and HER4. HER3 is overexpressed in lung, breast, colon, prostate, and gastric cancers. In the present study, we developed and validated an anti-HER3 monoclonal antibody (mAb), H3Mab-17 (IgG2a, kappa), by immunizing mice with HER3-overexpressed CHO-K1 cells (CHO/HER3). H3Mab-17 was found to react specifically with endogenous HER3 in colorectal carcinoma cell lines, using flow cytometry. The $K_D$ for H3Mab-17 in CHO/HER3 and Caco-2 (a colon cancer cell line) were determined to be 3.0x10$^{-9}$ M and 1.5x10$^{-9}$ M via flow cytometry, respectively, suggesting high binding affinity of H3Mab-17 to HER3. Then, we assessed the H3Mab-17 antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against Caco-2, and evaluated its antitumor capacity in a Caco-2 xenograft model. In vitro experiments revealed H3Mab-17 had strongly induced both ADCC and CDC against Caco-2 cells. In vivo experiments on Caco-2 xenografts revealed that H3Mab-17 treatment significantly reduced tumor growth compared with the control mouse IgG. These data indicated that H3Mab-17 could be a promising treatment option for HER3-expressing colon cancers.

Introduction

The epidermal growth factor receptor (EGFR) family, also known as HER or ErbB, has a tyrosine kinase domain in its intracellular region (1). The EGFR family transduces extracellular to intracellular signals through the activation of tyrosine kinase domain (1). By binding to the ligand, the extracellular domain promotes the formation of homodimers or heterodimers between the EGFR family receptors (2,3). This dimerization is essential for the activation of tyrosine kinase and intracellular signaling pathways such as Ras/MAPK, PI3K/Akt, and JAK/STAT (4,5).

The EGFR family consists of four members [EGFR (HER1, ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4)] and each member has different ligands: EGFR binds to seven ligands such as EGF, TGF-$\alpha$, and epigen; HER3 binds to neuregulin1 and neuregulin2; HER4 binds to seven ligands such as heparin binding-EGF, betacellulin, and epiregulin. In contrast, there is no ligand for HER2 (6,7). Although HER3 has a tyrosine kinase domain, its kinase activity is impaired (8,9). Therefore, transphosphorylation by other members of the EGFR family is required to activate HER3. HER3 can form an active heterodimer with the other three members of the EGFR family (2,10-13).

The EGFR family plays an essential role in regulating cell growth and in the differentiation, proliferation, and survival of normal cells. Insufficient EGFR signaling is associated with Alzheimer's disease and multiple sclerosis (14), while the overexpression of EGFR family is associated with the development of tumors (15-17). The EGFR family has been found to be overexpressed in many cancers as below: EGFR in breast, non-small...
cell lung, and prostate cancers (18); HER2 in breast, colon, lung, and pancreatic cancers (18); HER3 in lung, breast, colon, prostate, and stomach cancers (2,19); HER4 in non-small cell lung, and ovarian cancers (20,21). Therefore, the EGFR family is thought to be a valid target for candidates in cancer therapy.

High expression of HER3 is thought to be an established negative prognostic factor in several solid tumors including colorectal cancer (22,23). Metastatic colorectal cancer is one of the most aggressive tumors, associated with high mortality rates worldwide (24). In a previous study, 79% of primary tumors were found to present a high HER3 expression and there was a correlation between HER3 expression in primary tumors and corresponding lymph node metastases in 236 colorectal cancer patients (25). In addition, elevated HER3 expression was associated with shorter overall survival and disease-free survival in patients with colorectal cancer (25). Furthermore, HER3 downregulation in colorectal cancer cell lines caused G2-M cell-cycle arrest, leading to apoptosis and abrogated cell proliferation, migration, and invasion (22). Altogether, these results suggest that HER3 can be a potential therapeutic target for colorectal cancer.

Several monoclonal antibodies (mAbs) have been established as an innovative immunotherapy against tumors. Programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are inhibitory receptors for immune checkpoints, which are expressed on the surface of T cells (26-29). Anti-PD-1 and anti-CTLA-4 mAbs have been reported as potential anticancer drugs (30,31). Nivolumab and pembrolizumab are anti-PD-1 mAbs, which were approved by the US Food and Drug Administration (32-34).

Nivolumab and pembrolizumab are anti-PD-1 mAbs, which block the interaction between PD-1 and its ligand, PD-L1, respectively, into CHO-K1 cells using the Neon Transfection System (42). Moreover, pembrolizumab and nivolumab have highly upregulated expression of multiple immune checkpoint proteins, including PD-1 compared with microsatellite stable tumors; therefore, nivolumab and pembrolizumab are available for the treatment of DNA mismatch repair deficiency and microsatellite instable subset of colorectal cancer (35-37).

Several antibody drugs have been developed against ligands, such as transforming growth factor (TGF)-α and EGF, or receptors, such as EGFR (38). These mAbs neutralize the interaction between ligands and receptors. Antibody-drug conjugate (ADC) is a complex molecule, which is composed of an antibody, linker, and an anticancer drug, and delivers the anticancer drug to target cells (39). Moreover, some mAbs possess antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Cetuximab, a mouse/human chimeric IgG1 against EGFR, binds to the ligand-binding site of EGFR, and inhibits the activation and dimerization of EGFR (38). Cetuximab has been used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer, and head and neck squamous cell carcinomas (HNSCC) (38). Trastuzumab, a humanized mAb against HER2, has been used to treat HER2-positive cancers, such as breast cancers and gastric cancers (40,41). Trastuzumab binds to the extracellular domain of HER2 and downregulates activation of AKT (42). Moreover, trastuzumab exhibited ADCC in a mouse model (43). However, it has been shown that some types of cancers are resistant to cetuximab and trastuzumab (44,45).

Most HNSCCs are resistant to cetuximab, because cetuximab treatment induces HER2/HER3 dimerization and HER3 activation in HNSCC cell lines (44). It has been reported that anti-HER3-ADC exerts antitumor effect on breast cancer cells, which have resistance to trastuzumab (45). For this reason, the development of anti-HER3 mAbs has been required for cancer therapy. Seribantumab and lumretuzumab are anti-HER3 mAbs, which block HER3-neuregulin interaction and inhibit HER3 heterodimerization and phosphorylation (46,47). Lumretuzumab is also known to have ADCC activity (46). Phase II and phase Ib/II trial are now ongoing concerning seribantumab and lumretuzumab, respectively (47,48). An anti-HER3-ADC (U3-1402), composed of an anti-HER3 mAb (patritumab) and a novel topoisomerase I inhibitor (DX-8951 derivative; DXd) has entered phase I and II trials for the treatment of HER3-positive non-small cell lung cancers (NCT04676477), metastatic breast cancers (NCT02980341), and colorectal cancers (NCT04479436) (49-51). Preliminary results demonstrate that U3-1402 treatment appears to be safe and exhibits antitumor activity, suggesting that HER3-targeting therapy may be effective for HER3-overexpressing metastatic breast cancers (50).

It has been reported that one amino acid substitution in EGFR in tumors causes acquisition of resistance to gefitinib after gefitinib treatment (52,53); therefore, HER3 may also acquire resistance to seribantumab and lumretuzumab in the future. To characterize the HER3 and HER3-targeting cancer therapy, the development of further anti-HER3 specific mAbs is required. In this study, we developed a novel anti-HER3 mAb against colon cancers using a Cell-Based Immunization and Screening (CBIS) method (54). Furthermore, we investigated whether a novel anti-HER3 mAb shows ADCC/CDC activities or antitumor activities for colon cancers.

Materials and methods

Construction of plasmids. The Genome Network Project clone IRAK17418 (HER3) was provided by the RIKEN BioResource Research Center through the National BioResource Project of the MEXT and AMED agencies of Japan. HER3 DNA plus N-terminal PA16 tag, recognized by NZ-1, was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corp.) and named pCAG/PA16-HER3. HER3 DNA plus C-terminal PA tag, recognized by NZ-1, was subcloned into a pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corp.) and named pCAG/HER3-PA.

Cell lines. A mouse myeloma cell line (P3X63Ag8U.1; P3U1), Chinese hamster ovary (CHO)-K1 cells, a glioblastoma cell line (LN229), colorectal adenocarcinoma cell lines (Caco-2, LS 174T, COLO 201, HCT-8, SW1116, and HT-29), and a colorectal carcinoma cell line (HCT 116) were obtained from the American Type Culture Collection, Colon adenocarcinoma cell lines (HCT-15, COLO 205, and DLD-1) and a breast adenocarcinoma cell line (MCFT7) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University. CHO/PA16-HER3 and CHO/HER3-PA were established by transfecting pCAG/PA16-HER3 and pCAG/HER3-PA, respectively, into CHO-K1 cells using the Neon Transfection System.
A few days after transfection, cells positive for anti-HER3 mAb (clone D22C5; cat. no. 12708; Cell Signaling Technology, Inc.) were sorted using a cell sorter (SH800; Sony Biotechnology Corp.). CHO/mock (Ble) and CHO/mock (Neo) were established by transfection of the pCAG-Ble vector and pCAG-Neo vector, respectively. Stable transfectants of CHO/mock (Ble) and CHO/PA16-HER3 cells were cultured at 37°C for 14 days on media containing 0.5 mg/mL of Zeocin (InvivoGen), and stable transfectants of CHO/mock (Neo) and CHO/HER3-PA were cultured at 37°C for 14 days on media containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc.).

Preparation of the purified antibodies. Purified mouse IgG (cat. no. 18765) and mouse IgG2a (cat. no. M7769) were purchased from Sigma-Aldrich; Merck KGaA. An anti-HER3 mAb was purified using Protein G-Sepharose (Thermo Fisher Scientific, Inc.).

Hybridoma production. Female BALB/c mice (6 weeks old) were purchased from CLEA Japan and kept under specific pathogen-free conditions. All animal experiments were conducted in accordance with the relevant guidelines and regulations in order to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University approved all the animal experiments (permit no. 2019NiA-001). Mice were euthanized by cervical dislocation under inhalation anesthesia using 2% of isoflurane (Thermo Fisher Scientific, Inc.) (Fig. 1). Two eight-week-old BALB/c mice were intraperitoneally (i.p.) immunized with 1 µg/ml of anti-HER3 mAbs, for 30 min at 4°C, and with Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min, followed by incubation with peroxidase-conjugated anti-rabbit immunoglobulins (diluted 1:1,000; cat. no. P0448; Agilent Technologies Inc.) and peroxidase-conjugated anti-mouse immunoglobulins (diluted 1:1,000; cat. no. P0260; Agilent Technologies Inc.), respectively, at room temperature for 30 min. Blots were developed using ImmunoStar LD (cat. no. 290-69904; FUJIFILM Wako Pure Chemical Corp.) or Pierce™ ECL Plus Western Blotting Substrate (cat. no. 32132; Thermo Fisher Scientific, Inc.) and imaged with a Sayaca-Imager (DRC Co., Ltd.). Qcapture Pro software (DRC Co., Ltd.) was used for the densitometry.

Flow cytometry analyses. Cells (2x10^5 cells/ml) were harvested after brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After being washed with 0.1% bovine serum albumin (BSA; Nacalai Tesque, Inc.) in PBS, cells were treated with 1 µg/ml of anti-HER3 mAbs, for 30 min at 4°C, and with Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000; cat. no. 4408; Cell Signaling Technology, Inc.). Fluorescence data were collected using a flow cytometer: the EC800 Cell Analyzer (Sony Biotechnology Corp.).
from Charles River Laboratories, Inc. Mice were kept under specific pathogen-free condition on an 11-h light/13-h dark cycle at a temperature of 23±2°C and 55±5% humidity with food and water supplied ad libitum during the experimental periods. After euthanasia by cervical dislocation, spleens were removed aseptically, and single-cell suspensions were obtained by forcing spleen tissues through a sterile cell strainer (product no. 352360; Corning, Inc.) with a syringe. Erythrocytes were lysed with a 10-sec exposure to ice-cold distilled water. The splenocytes were washed with DMEM and resuspended in DMEM with 10% FBS; this preparation was designated as effector cells. The target tumor cells were labeled with 10 µg/ml Calcein-AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. The target cells were then transferred to 96-well plates, at 2x10⁴ cells/well, and mixed with effector cells at an effector-to-target ratio of 100:1, along with 100 µg/ml of anti-HER3 antibodies or control mouse IgG2a. After a 4.5-h incubation at 37°C, Calcein release into the supernatant was measured for each well. Fluorescence intensity was assessed using a microplate reader (PowerScan HT; BioTek Instruments, Inc.) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Cytolytic activity was measured as a percentage of lysis and calculated using the equation: Percentage of lysis (%) = (E−S)/(M−S) x100, where E is the fluorescence measured in combined cultures of target and effector cells, S is the spontaneous fluorescence of the target cells, and M is the maximum fluorescence measured after lysis of all cells with buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM EDTA. Animal studies for ADCC were approved by the Institutional Committee for experiments of the Institute of Microbial Chemistry (permit no. 2020-024).

**Figure 1. Production of anti-HER3 mAbs. Procedure of the Cell-Based Immunization and Screening (CBIS) method.** (A) CHO/PA16-HER3 cells were immunized into BALB/c mice using an intraperitoneal injection. (B) Spleen cells were fused with P3U1 cells. (C) Culture supernatants were screened using flow cytometry. (D) After limiting dilution and several additional screenings, an anti-HER3 mAb was finally established. mAb, monoclonal antibody.  

**Antitumor activity of an anti-HER3 mAb in xenografts of colon cancers.** Sixteen five-week-old female BALB/c nude mice (mean weight, 15±3 g) were purchased from Charles River Laboratories, Inc. All animal experiments were performed in accordance with institutional guidelines and regulations to minimize animal suffering and distress in the laboratory. The Institutional Committee for experiments of the Institute of Microbial Chemistry (permit no. 2020-024) approved the animal studies for antitumor activity here described. Mice were maintained in a pathogen-free environment on an 11-h light/13-h dark cycle at a temperature of 23±2°C and 55±5% humidity with food and water supplied ad libitum throughout the experiments. Mice were monitored for health and weight every three or five days. Experiments on mice were conducted in four weeks. Weight loss exceeding 25% or tumor volume exceeding 3,000 mm³ were identified as humane endpoints for euthanasia. At humane and experimental endpoints, mice were euthanized by cervical dislocation, and death was verified by validating respiratory and cardiac arrest.

After a one-week acclimation period, these mice were used in experiments at six weeks of age (mean weight, 16±2 g). Caco-2 cells (0.3 ml of 1.3x10⁶ cells/ml in DMEM) were mixed with 0.5 ml BD Matrigel Matrix Growth Factor Reduced (BD Biosciences), and 100 µl of this suspension (5x10⁶ cells) was injected subcutaneously into the left flank of each animal. On the eighth day post-inoculation, 16 mice were divided into two groups (n=8 in each group) with equal mean tumor volume: An anti-HER3 mAb group or a control mouse IgG group. Then, 100 µg of an anti-HER3 mAb or control mouse IgG in 100 µl PBS was injected i.p. Additional antibody inoculations were performed on days 15 and 23. Twenty-six days after cell implantation, all mice were euthanized by cervical dislocation, and tumor diameters and volumes were measured and recorded.

**CDC activity of an anti-HER3 mAb.** CDC inducement by HER3 was assayed as follows. Target cells were labeled with 10 µg/ml Calcein-AM (Thermo Fisher Scientific, Inc.) and resuspended in medium and plated in 96-well plates, at 2x10⁴ cells/well, with 15% rabbit complement (Low-Tox-M rabbit complement; Cedarlane Laboratories), 100 µg/ml of anti-HER3 antibodies, or control IgG (mouse IgG₂) added to each well. After 4.5 h of incubation at 37°C, Calcein release into the supernatant was measured for each well. Fluorescence intensity was calculated as described in the ADCC section above.
Statistical analyses. All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted with ANOVA and Tukey's multiple comparisons tests for ADCC and CDC, ANOVA and Sidak's multiple comparisons tests for tumor volume and mouse weight, and Welch's t-test for tumor weight. All calculations were performed with GraphPad Prism 8 (GraphPad Software, Inc.). A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Development of anti-HER3 mAbs. We employed the CBIS method to develop anti-HER3 mAbs using CHO/PA16-HER3 cells both for the immunization and flow cytometry screening (Fig. 1). The developed hybridomas were seeded into 96-well plates and cultivated for 10 days. Supernatants positive for CHO/HER3-PA and negative for CHO-K1 were selected by flow cytometry analysis. Colon cancer cell lines, such as Caco-2, HCT116, HCT-15, HT-29, LS174T, COLO201, COLO205, HCT-8, SW1116, and DLD-1 cells were treated with 1 µg/ml of H3Mab-17, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Black line, negative control.

Confirmation of HER3 expression by western blot analysis. We established CHO/mock (Ble), CHO/PA16-HER3, CHO/mock (Neo), and CHO/HER3-PA, and investigated whether HER3 was overexpressed in those cell lines. As shown in Fig. 2A, overexpression of HER3 in CHO/PA16-HER3 and
CHO/HER3-PA was confirmed by western blot analysis using an anti-HER3 mAb (clone D22C5). Endogenous HER3 expression in MCF7 cells was also detected by an anti-HER3 mAb. In contrast, knockout of endogenous HER3 in BINDS-30 (MCF7/HER3-KO) was confirmed by western blot analysis using an anti-HER3 mAb.

**Flow cytometry analyses of H₃Mab-17.** We performed flow cytometry using H₃Mab-17 against CHO-K1, CHO/HER3-PA, MCF7, and BINDS-30 (MCF7/HER3-KO). H₃Mab-17 recognized the CHO/HER3-PA cells, but not the parental CHO-K1 cells (Fig. 2B). H₃Mab-17 also recognized the endogenous HER3 in MCF7 breast cancer cells (Fig. 2B). The reaction of H₃Mab-17 to BINDS-30 was lost after the knockout of HER3 in MCF7 cells (Fig. 2B), indicating the specificity of H₃Mab-17 for HER3.

Next, we investigated whether H₃Mab-17 reacts with colon cancer cell lines. As shown in Fig. 2C, H₃Mab-17 reacted with 10 colon cancer cell lines, Caco-2, HCT 116, HCT-15, HT-29, LS 174T, COLO 201, COLO 205, HCT-8, SW1116, and DLD-1. Among them, Caco-2 was known to be useful for the mouse xenograft model (55). Therefore, we used Caco-2 cells for the ADCC/CDC assay or in vivo xenograft models.

**Determination of the binding affinity of H₃Mab-17.** A kinetic analysis of the interactions of H₃Mab-17 with CHO/HER3-PA and Caco-2 cells was then conducted using flow cytometry. The $K_D$ for H₃Mab-17 in CHO/HER3-PA and Caco-2 cells were $3.0 \times 10^{-9}$ and $1.5 \times 10^{-9}$ M, respectively (Fig. 3), indicating high binding affinity of H₃Mab-17 against HER3-expressing cells.

**ADCC and CDC activities of H₃Mab-17 in colon cancer cell lines.** We then examined whether H₃Mab-17 (mouse IgG₂a) induced ADCC and CDC activity in HER3-expressing Caco-2 colon cancer cell lines. H₃Mab-17 exhibited higher ADCC (14.8% cytotoxicity) in Caco-2 cells than that of control mouse IgG₂a (5.2% cytotoxicity; $P<0.05$) or control PBS (3.2% cytotoxicity; $P<0.05$) treatment (Fig. 4A). H₃Mab-17 was also associated with a more robust CDC activity (30.4% cytotoxicity) in Caco-2 cells than the control mouse IgG₂a (7.7% cytotoxicity; $P<0.05$) or the control with PBS treatment (8.8% cytotoxicity; $P<0.05$) (Fig. 4B). These favorable ADCC/CDC activities indicated that H₃Mab-17 may induce strong antitumor activity against colon cancer cells in vivo as well as in vitro.

**Antitumor effect of H₃Mab-17 in mouse xenografts of colon cancer cells.** Tumor formation of 16 Caco-2-bearing mice was observed on day eight. Then, these 16 Caco-2-bearing mice...
were divided into an H3Mab-17-treated group and a control group. On days 8, 15 and 23 after Caco-2 cell injections into the mice, H3Mab-17 (100 µg) or control mouse IgG (100 µg) were injected i.p. into the treated and control mice, respectively. Additional antibodies were then injected on days 15 and 23. Tumor volume was measured on days 8, 11, 15, 18, 23 and 26. Values are mean ± SEM. Asterisk indicates statistical significance ("P<0.01; n.s., not significant, ANOVA and Sidak’s multiple comparisons test). O, control; △, H3Mab-17. (B) Tumors of Caco-2 xenografts were resected from H3Mab-17 and control mouse IgG groups. Tumor weight on day 26 was measured from excised xenografts. Values are mean ± SEM. Asterisk indicates statistical significance ("P<0.01, Welch’s t-test). O, control; △, H3Mab-17. (C) Resected tumors of Caco-2 xenografts from H3Mab-17 and control mouse IgG groups on day 26. The tumor in the square dotted region was the largest tumor in this experiment. The vertical and horizontal lengths for Caco-2 cells were 1.6 and 1.3 cm, respectively (estimated tumor volume, 1,352 mm3, tumor weight, 661 mg). Scale bar, 1 cm.

Discussion

Many commercially available anti-HER3 mAbs have been developed using recombinant HER3 protein, peptide or cDNA as an immunogen. Seribantumab was developed by phage display (56,57) and lumretuzumab was developed using recombinant HER3 extracellular domain as an immunogen (46). In this study, we succeeded in the development of an anti-HER3 mAb using the CBIS method, which used HER3-expressed cells for both immunization and screening. The CBIS method can help us effectively develop mAbs that are useful in flow cytometry. We recently succeeded in developing numerous useful mAbs that target membrane
proteins, including podoplanin (58-61), CD20 (62), CD44 (63), CD133 (54), and TROP2 (64,65). Importantly, these mAbs are very useful for various experiments, not including not only flow cytometry, but also western blot analysis and immunohistochemistry. Furthermore, those mAbs possess ADCC/CDC activities and antitumor activities (61). Using the CBIS method, proteins for immunogen expressed on cells maintain its native confirmation and glycosylation pattern. Previously, we successfully established a cancer-specific mAb (CasMab) against podoplanin, which recognizes the cancer cell-specific glycosylation of podoplanin (66). Therefore, we may develop CasMab against HER3 using the CBIS method in the future. The CBIS method is advantageous for the development for specific and sensitive mAbs for antibody therapy.

Newly highly accurate therapeutic options are possible to treat most solid tumors. In the case of colorectal cancer, HER3 overexpression is found in ~17-75%, although the definition of its cutoff signals for HER3 expression are different in each immunohistochemical study (67). It has been reported that the incidence of HER3 overexpression in metastatic colorectal cancer is much higher than that of HER2 (68). In this study, we developed an anti-HER3 mAb, H3Mab-17, which specifically reacted with endogenous HER3 in colorectal carcinoma cell lines in flow cytometry. The $K_c$ for H3Mab-17 in CHO/HER3-PA and Caco-2 cells were determined to be 3.0x10⁻⁹ and 1.5x10⁻⁹ M, respectively, suggesting high binding affinity of H3Mab-17 for HER3. In vitro experiments revealed strong ADCC and CDC inducement against Caco-2 cells by H3Mab-17. In vivo experiments on Caco-2 xenografts revealed that the treatment with H3Mab-17 significantly reduced the tumor growth, compared with the control mouse IgG. Based on these findings, H3Mab-17 may be useful in therapeutic approach for patients with colorectal cancer.

Although H3Mab-17 recognizes both overexpressed and endogenous HER3 by flow cytometric analyses, it is not applicable to western blot and immunohistochemical analyses (data not shown). H3Mab-17 did not recognize denatured HER3, such as SDS-treated and formalin-fixed HER3 probably because it might recognize the three-dimensional structure of HER3. Since the antitumor activity mechanism of H3Mab-17 has not been clarified, we need to identify the epitope of H3Mab-17 and investigate the inhibitory activity of HER3-neureglin interaction of H3Mab-17. Furthermore, HER3-ADC and HER3-chimeric antigen receptor (CAR)-T should be developed in future research.

Acknowledgements

We would like to thank Ms. Miyuki Yanaka, Ms. Saori Handa, and Mr. Yu Komatsu (Department of Antibody Drug Development, Tohoku University Graduate School of Medicine) for technical assistance in the in vitro experiments, and Ms. Akiko Harakawa [Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation] for technical assistance in the animal experiments.

Funding

This research was supported in part by the Japan Agency for Medical Research and Development (AMED) under grant nos. JP21am040103 (to YK) and JP21am0101078 (to YK), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant nos. 21K15523 (to TA), 21K07168 (to MKK), 19K07705 (to YK) and 20K16322 (to MS).

Availability of data and materials

The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request.

Authors' contributions

TA, TO, TN, RN, HHo, TT, and MS performed the experiments. JT and MKK analyzed the experimental data. HHa, MK, and YK designed the present study. TA, TO, and YK wrote the manuscript. All the authors read and approved the final manuscript for publishing.

Ethics approval and consent to participate

The Animal Care and Use Committee of Tohoku University approved all the animal experiments (permit no. 2019NiA-001). Animal studies for ADCC and the antitumor activity were approved by the Institutional Committee for experiments of the Institute of Microbial Chemistry (permit no. 2020-024).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Gschwind A, Fischer OM and Ullrich A: The discovery of receptor tyrosine kinases: Targets for cancer therapy. Nat Rev Cancer 4: 361-370, 2004.
2. Yarden Y and Siwickowski MJ: Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2: 127-137, 2001.
3. Schlessinger J: Ligand-induced, receptor-mediated dimerization and activation of EGFR receptor. Cell 110: 669-672, 2002.
4. Scialtri M and Basela J: The epidermal growth factor receptor pathway: A model for targeted therapy. Clin Cancer Res 12: 5268-5272, 2006.
5. Schreiber AB, Libermann TA, Lax I, Yarden Y and Schlessinger J: Biological role of epidermal growth factor-receptor clustering. Investigation with monoclonal anti-receptor antibodies. J Biol Chem 258: 846-853, 1983.
6. Linggi B and Carpenter G: ErbB receptors: New insights on mechanisms and biology. Trends Cell Biol 16: 649-656, 2006.
7. Harris RC, Chung E and Coffey RJ: EGFR receptor ligands. Exp Cell Res 284: 2-13, 2003.
8. Citri A, Skaria KB and Yarden Y: The deaf and the dumb: The biology of ErbB-2 and ErbB-3. Exp Cell Res 284: 54-65, 2003.
9. Guy PM, Platko JV, Cantley LC, Cerione RA and Carraway KL III: Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci USA 91: 8132-8136, 1994.
10. Holbro T, Berriti KR, Maurer F, Koziczak M, Barbas CF 3rd and Hynes NE: The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci USA 100: 8933-8938, 2003.
11. Akhtar S, Chandrasekhar B, Attur S, Drauhsim GS, Yousif MH and Benter IF: Transactivation of ErbB family of receptor tyrosine kinases is inhibited by angiotsin-(1-7) via its mas receptor. PLoS One 10: e0141657, 2015.
12. Ceresa BP and Vanlindingham PA: Molecular mechanisms that regulate epidermal growth factor receptor inactivation. Clin Med Oncol 2: 47-61, 2008.

13. Heselmans L, Grandal MY, Knudsen SL, van Deurs B and Grøvdal LM: Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. PLoS One 8: e58148, 2013.

14. Bublil EM and Yarden Y: The EGF receptor family: Spearheading a merger of signaling and therapeutics. Curr Opin Cell Biol 19: 124-134, 2007.

15. Nishimura H, Minato N, Nakano T and Honjo T: Immunological mechanism of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. J Immunol 177: 1052-1061, 2006.

16. Hendler FJ and Ozanne BW: Human squamous cell lung cancers with mutations in the EGFR gene. Cancer Invest 74: 647-651, 2004.

17. Kraus MH, Popsucu NC, Amsbaugh SC and King CR: Overexpression of the EGFR receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6: 605-610, 1987.

18. Appert-Collin A, Hubert P, Crémel G and Benaasroune A: Role of erbB receptors in cancer cell migration and invasion. Front Pharmacol 6: 283, 2015.

19. Lai WW, Chen FF, Wu MH, Chow NH, Su WC, Ma MC, Su PF, Chen H, Lin MY and Tseng YL: Immunohistochromatological analysis of epidermal growth factor receptor family members in stage I non-small-cell lung cancer. Ann Thorac Surg 72: 1868-1876, 2001.

20. Koutsopoulos AV, Mavroudis D, Dambaki KI, Souglakos J, Tzortzaki EG, Drosis J, Delides GS, Georgoulas V and Stathopoulos EN: Simultaneous expression of c-erbB-1, c-erbB-2, c-erbB-3 and c-erbB-4 receptors in non-small-cell lung carcinomas: Correlation with clinical outcome. Lung Cancer 57: 193-200, 2007.

21. Davies S, Holmes A, Lomo L, Steinkamp MP, Kang H, Muller CY and Wilson BS: High incidence of ErbB3, ErbB4, and MET expression in ovarian cancer. Int J Gynecol Pathol 33: 402-410, 2014.

22. Beji A, Horst D, Engel J, Kirchner T and Ullrich A: Toward the prognostic significance and therapeutic potential of HER3 receptor tyrosine kinase in human colon cancer. Clin Cancer Res 18: 956-968, 2012.

23. Ocana A, Vera-Badillo F, Seruga B, Templeton A, Pandiella A and Amir E: HER3 overexpression and survival in solid tumors: A meta-analysis. J Natl Cancer Inst 105: 266-273, 2013.

24. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.

25. Ledel F, Hallstrom M, Ragnhammar P, Ohrling K and Edler D: HER3 activity and HER2/HER3 dimerization: Evidence from cell line and Patient-Derived xenograft models. Clin Cancer Res 23: 677-686, 2017.

26. Nishimura H, Nose M, Hiai H, Minato N and Honjo T: Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 11: 141-151, 1999.

27. Nishimura H, Minato N, Nakano T and Honjo T: Immunological studies on PD-1 deficient mice: Implication of PD-1 as a negative regulator for B cell responses. Int Immunol 10: 1563-1572, 1998.

28. Engelhardt JJ, Sullivan TJ and Allison JP: CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. J Immunol 177: 1052-1061, 2006.

29. Sharma P and Allison JP: The future of immune checkpoint blockade. Nat Rev Cancer 12: 553-563, 2012.

30. Roveri M, Cimino L, Cefalà C, Rigante D, Verrotti A, D'Amico G and Manni G: The role of HER2 expression in colorectal cancer. Oncologist 24: 1095-1102, 2019.

31. Slamon DJ, Leyland-Jones B, Paton V, Bajramovic-Livoni A, Fleming N, Eiermann W,oller J, Pegram M et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344: 783-792, 2001.

32. Gandullo-Sánchez L, Capone E, Ocaña A, Iacobelli S, Sala G and Distante A: HER3 overexpression and survival in solid tumors: A meta-analysis. J Natl Cancer Inst 105: 266-273, 2013.

33. Li S, Schmitz KR, Jeffrey PD, Kussie P and Metcalf D: Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 7: 301-311, 2005.

34. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, Desai J, Hill A, Axelsson M, Moss RA, et al: Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): An open-label, multicentre, phase 2 study. Lancet Oncol 18: 1182-1191, 2017.

35. Xiao Y and Freeman GF: The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy. Cancer Discov 5: 16-18, 2015.

36. blondeau JL, Cruise M, Wicks EC, Hechenleitner EM, Taube JM, Brossler RL, Fan H, Wang H, Luber BS, et al: The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov 5: 43-51, 2015.

37. Doi T, Uram JN, Yang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, et al: PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 372: 2590-2590, 2012.

38. Kute T, Lack CM, Willingham M, Bishowkama B, Williams H, Barrett K, Mitchell T and Vaughn J: Development of Herceptin resistance in breast cancer cells. Cytometry A 57: 86-93, 2004.

39. Clynes RA, Towers TL, Presta LG and Ravetch JV: Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med 6: 443-446, 2000.

40. Wang D, Qian G, Zhang H, Magliocco KR, Nannapaneni S, Amin AR, Rossi MS, Patel M, El-Deiry W, Wadsworth JT, et al: HER3 targeting sensitizes HNSCC to Cetuximab by reducing HER2-positive advanced gastric or gastro-esophageal junction cancer (ToGA): A phase 3, open-label, randomised controlled trial. Lancet 376: 687-697, 2010.

41. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sang BM, Chavan A, Lomoto S, Fu S, Ocampo Y, Sarab T, et al: Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): A phase 3, open-label, randomised controlled trial. Lancet 376: 687-697, 2010.

42. Aldridge AA, Huang MH, O'Connell M, Walker E, Hyland B, Haddad R, Johnson LM, Muller MC, Fong EB, Topalian SL et al: PD-1 blockade plus ipilimumab for advanced melanoma. N Engl J Med 368: 2449-2459, 2013.

43. Aldrich SR, LoRusso PM, Oscier DG and Pardoll D: The role of checkpoint inhibitors in the immunotherapy of advanced cancers. Nat Rev Clin Oncol 14: 55-67, 2017.

44. Adler JR, Sosman JD, Atkins MB, Herbst RS, Puzanov I, Salgia R, Long GV, Brahmer J, Gordon MS, Sondak VK et al: PD-1 blockade plus ipilimumab for advanced melanoma. N Engl J Med 370: 1572-1582, 2014.

45. Gandhi-Sanchez L, Capone E, Ocaña A, Iacobelli S, Sala G and Pandiella A: HER3 targeting with an antibody-drug conjugate bypasses resistance to anti-HER2 therapies. EMBO Mol Med 12: e11498, 2020.

46. Mirschberger C, Schiller CB, Schrämli M, Dimouids N, Friess T, Grøvdal LM: Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. J Mol Biol 347: 409-413, 2017.
51. Hashimoto Y, Koyama K, Kamai Y, Hirotani K, Ogitani Y, Zembutsu A, Abe M, Kaneda Y, Maeda N, Shiose Y, et al: A Novel HER3-Targeting Antibody-Drug Conjugate, U3-1402, exhibits potent therapeutic efficacy through the delivery of cytotoxic payload by efficient internalization. Clin Cancer Res 25: 7151-7161, 2019.

52. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2: e73, 2005.

53. Itai S, Fujii Y, Nakamura T, ChangYW, Yanaka M, Saidoh N, Handa S, Suzuki H, Harada H, Yamada S, et al: Establishment of CMab-43, a sensitive and specific Anti-CD133 monoclonal antibody, for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 36: 231-235, 2017.

54. Kato Y, Ohishi T, Yamada S, Itai S, Furusawa Y, Sano M, Nakamura T, Kawada M and Kaneko MK: Anti-CD133 Monoclonal Antibody CMab-43 exerts antitumor activity in a mouse xenograft model of colon cancer. Monoclon Antib Immunodiagn Immunother 38: 75-78, 2019.

55. Schoeberl B, Pace EA, FitzGerald JB, Harms BD, Xu L, Nie L, Lingg B, Kalra A, Paragas V, Bukhalid R, et al: Therapeutically targeting ErbB3: A key node in ligand-induced activation of the ErbB receptor-P13K axis. Sci Signal 2: ra31, 2009.

56. Furusawa Y, Yamada S, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, Kaneko MK and Kato Y: Establishment of a monoclonal antibody PMab-231 for immunohistochemical analysis of horse podoplanin. Biochem Biophys Rep 18: 100616, 2019.

57. Furusawa Y, Kaneko MK and Kato Y: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 4: 5924, 2014.

58. Wang Y, Yang H and Duan G: HER3 over-expression and overall survival in gastrointestinal cancers. Oncotarget 6: 42868-42878, 2015.

59. Stahler A, Heinemann V, Neumann J, Crispin A, Schalhorn A, Stintzing S, Giessen-Jung C, Fischer von Weikersthal L, Vehling-Kaiser U, Staub M, et al: Prevalence and influence on outcome of HER2/neu, HER3 and NRG1 expression in patients with metastatic colorectal cancer. Anticancer Drugs 28: 717-722, 2017.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.