Structural insights into cancer cell-specific recognition by H_2Mab-214, a novel anti-HER2 antibody

Monoclonal antibodies bind to target molecules with high specificity and affinity and are used as therapeutic agents for various diseases. In cancer therapy, antibody drugs typically target proteins that are overexpressed in cancer cells and are significantly more abundant on the surface of cancer cells than on normal cells (Fig. 1). However, such proteins have been extensively studied, making the discovery of novel target molecules increasingly challenging. Moreover, if the target molecule is expressed in normal cells even at relatively low levels, there is a risk of adverse effects.

Human epidermal growth factor receptor 2 (HER2) is a well-known therapeutic target that is overexpressed in several cancers, including breast, gastric, bladder, ovarian, and non-small cell lung cancers [1-3]. Multiple anti-HER2 antibodies, such as trastuzumab (Herceptin®, approved by Food and Drug Administration in 1998), have been developed and are clinically used. However, these antibodies also bind to HER2 on normal cells, causing concerns regarding their cardiotoxic side effects. Recently, we succeeded in obtaining a novel antibody, H_2Mab-214, which specifically binds to HER2 on cancer cells, through the differential screening of numerous hybridomas. In flow cytometry analysis, trastuzumab reacted with breast cancer cells (SK-BR-3), as well as with human mammary epithelial cells (MCF 10A) and human embryonic kidney epithelial cells (HEK293T), whereas H_2Mab-214 showed reactivity only with SK-BR-3 cells [4]. ELISA-based assessments showed that the affinity of H_2Mab-214 for the HER2 ectodomain (HER2ec) is equivalent to that of trastuzumab (K_D values of H_2Mab-214 and trastuzumab are 0.53 and 0.31 nM, respectively), indicating that the cancer selectivity of H_2Mab-214 is not dependent on its affinity for HER2 (or the HER2 expression level).

To elucidate the mechanism underlying the cancer cell-selectivity of H_2Mab-214, we identified the binding site (that is, epitope) of H_2Mab-214 by evaluating its binding to various HER2 fragments. As a result, it was found that the antibody recognizes a sequence of only seven residues, spanning Pro612 to Asp618, in HER2 domain IV. It is well-known that proteins produced by cancer cells often contain sugar chains with abnormal chemical compositions or amino acid mutations that can serve as cancer-specific markers. However, the identified epitope does not exhibit these chemical characteristics, leaving the mechanism by which H_2Mab-214 distinguishes between cancer and normal cells unclear. To investigate further, we conducted a crystallographic analysis. For this, H_2Mab-214 was converted into a small and hyper-crystallizable antibody fragment, Fv-clasp [5]. Crystals of the H_2Mab-214 Fv-clasp were successfully obtained in the presence of an epitope peptide (a synthetic peptide corresponding to HER2 residues 611–618 was used for crystallization). X-ray diffraction data were collected at SPring-8 **BL44XU**, and the complex structure was obtained at a resolution of 1.75 Å (Fig. 2(a)).

Numerous interactions were observed between H_2Mab-214 and the peptide in the crystal structure, reflecting high affinity between the two (Fig. 2(b)). Interestingly, when we compared the conformation of the peptide bound to H_2Mab-214 with that of the corresponding region in the known HER2ec structures, we found that they are entirely different. The epitope peptide bound to H_2Mab-214 adopts a U-shaped conformation, whereas the corresponding region in HER2ec adopts an extended conformation, part of which forms a β-strand (Figs. 2(c) and 2(d)). This finding suggests that H_2Mab-214 recognizes the structurally compromised HER2. However, because our crystal structure represents H_2Mab-214 complexed with the epitope peptide rather than with HER2ec itself, it remains unclear whether H_2Mab-214 actually recognizes misfolded HER2 on the cell surface. To address this, we artificially disrupted the tertiary structure of HER2 on HEK293T cells and investigated antibody binding using flow cytometry. The extracellular region of HER2 contains many disulfide bonds, which contribute to the maintenance

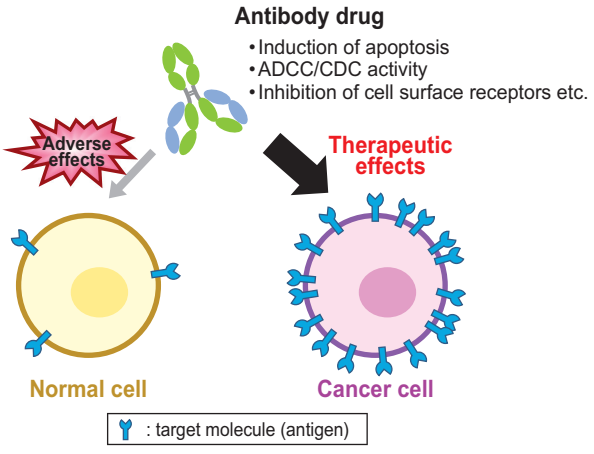

Fig. 1. Conventional strategies for cancer treatment using monoclonal antibodies. ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

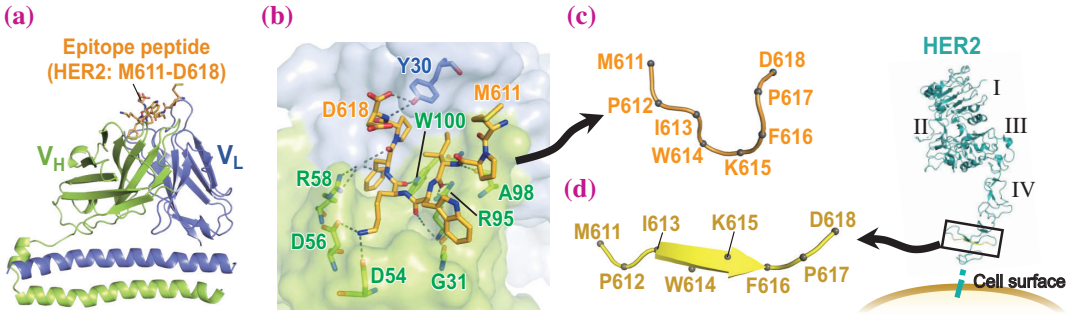

Fig. 2. Crystal structure of H_2Mab-214 in complex with its epitope peptide. **(a)** Overall structure of the H_2Mab-214 Fv-clasp/peptide complex. **(b)** Close-up view of the antigen-binding site of H_2Mab-214. Hydrogen bonding interactions are indicated with dashed lines. **(c,d)** Comparison of the conformation of the epitope peptide bound to H_2Mab-214 with the corresponding region in the HER2ec crystal structure (PDB: 3n85).

of the correct tertiary structure. Therefore, we cultured HEK293T cells in medium containing the reducing agent dithiothreitol (DTT, 1 mM) for 1 h to cleave the disulfide bonds of HER2 on the cell surface. After DTT treatment, trastuzumab binding to cells was nearly abolished, confirming the structural disruption of HER2. In contrast, H_2Mab-214 binding increased significantly after DTT treatment (Fig. 3(a)), indicating that the epitope of H_2Mab-214 was exposed due to structural disruption. Furthermore, when a disulfide bond near the epitope of H_2Mab-214 was specifically broken through mutations, H_2Mab-214 binding increased. This suggests that the H_2Mab-214 epitope region in HER2 is particularly susceptible to structural defects, even with minor destabilization of the surrounding conformation [4].

Our results suggest that locally misfolded HER2 molecules are produced in cancer cells, and that H_2Mab-214 recognizes these molecules to achieve cancer specificity (Fig. 3(b)). Considering the abnormal state of cancer cells, similar local misfolding may occur in other cell surface molecules, which can be a promising target for the development of new cancer-specific antibodies.

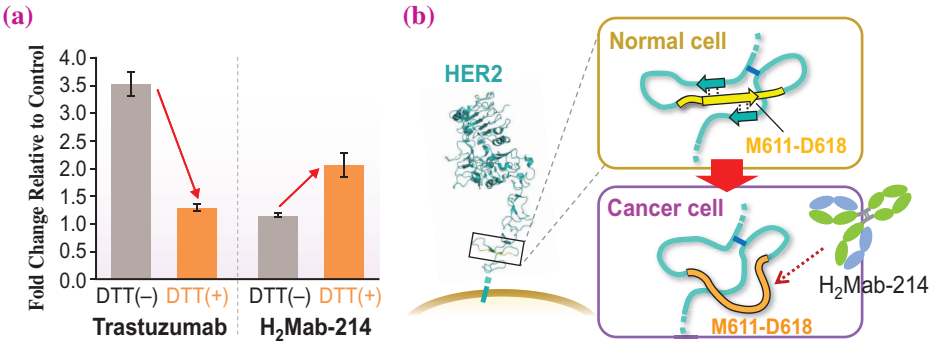

Fig. 3. H_2Mab-214 recognizes misfolded HER2. **(a)** Effects of DTT treatment of HEK293T cells on antibody binding. HEK293T cells were treated with 1 mM DTT for 1 h and stained with trastuzumab or H_2Mab-214, followed by flow cytometric analysis. Results are reported as the fold-change in the median fluorescence intensity compared with the buffer control. Data are presented as the mean ± standard error (SEM) of four independent experiments. **(b)** Proposed model of local structural differences in HER2 between normal and cancer cells.

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