Title page

1 **Study of discordance of positive test results among different primary antibodies used**
2 **for immunohistochemistry HER2 testing in breast cancer: From a comparison**
3 **between Histofine® HER2 Kit (MONO) SV2-61γ antibody and Ventana I-VIEW**
4 **PATHWAY™ HER2 4B5 antibody**
5

6 Kei Fujita [a,*], Takashi Yao [a]
7 [a] Juntendo University Graduate School of Medicine Department of Human Pathology,
8 Tokyo, Japan
9 ***Corresponding Author:** Kei Fujita, Ph.D.
10 Juntendo University Graduate School of Medicine Department of Human Pathology, 2-
11 1-1 Hongo Bunkyoku-ku,Tokyo, Japan
12 Tel.: +81 3 3813 3111 E-mail: kfujita@juntendo.ac.jp

Study of discordance of positive test results among different primary antibodies used for immunohistochemistry HER2 testing in breast cancer: From a comparison between Histofine® HER2 Kit (MONO) SV2-61γ antibody and Ventana I-VIEW PATHWAY™ HER2 4B5 antibody

Abstract

Introduction

For immunohistochemistry (IHC) assay to assess human epidermal growth factor receptor 2 (HER2) expression status, different primary antibodies cause different IHC scores.

Materials and methods

We assessed HER2 expression status by IHC score using two types of kits, i.e., Roche Ventana I-VIEW PATHWAY™ HER2 using the 4B5 antibody that recognizes the intracellular domain (ICD) and Nichirei Biosciences Histofine® HER2 Kit (MONO) using the SV2-61γ antibody that recognizes the extracellular domain (ECD). In addition, we determined the presence or absence of the ECD in samples by Western blotting (WB) and examined its relationship with the IHC score.

Results

Of all 101 samples, 44 and nine samples received a score of 2+/3+ by IHC with 4B5 and SV2-61γ, respectively. A verification by WB in samples with a discrepancy of IHC scores found that samples with the score of 2+/3+ with SV2-61γ showed a significantly higher protein intensity of p185HER2 with the ECD, whereas samples with a score of

2+/3+ with 4B5 but as 0/1+ with SV2-61γ showed a significantly higher protein intensity of p95HER2 without the ECD.

Conclusion

The nature of primary antibodies used for IHC and the presence or absence of the ECD in samples may contribute to different positive rates by IHC between 4B5 and SV2-61γ.

1. Introduction

Human epidermal growth factor receptor 2 (HER2) gene amplification and its associated HER2 protein overexpression are seen in 15% to 25% of invasive breast cancer cases and have been associated with a higher tumor grade and a poorer prognosis.[1-3] At the same time, the advent of molecular targeted agents against the HER2 protein has led to dramatic improvement in clinical outcomes and the status of HER2 protein expression is now an important predictive factor for the therapeutic effect of breast cancer therapy.

The HER2 protein is a transmembrane receptor protein. It comprises three domains, an extracellular domain (ECD), a transmembrane domain, and an intracellular domain (ICD) (Sup. 1) and occurs as full-length p185HER2 (which has a molecular weight of 185 kDa and all the three domains) or p95HER2 (which has a molecular weight of 95 kDa and lacks the ECD). p95HER2 is a poor prognostic factor.[4-6]

Breast cancer with HER2 gene amplification and HER2 protein overexpression has responded to molecular targeted therapy with the humanized anti-HER2 antibody trastuzumab.[7] Trastuzumab binds Fcγ receptor III (RIII) on the surface of immune effector cells to induce tumor cell death through antibody-dependent cellular cytotoxicity.[8,9] In addition, it also has several mechanisms of action such as inhibition of the production of truncated p95HER2.[10,11]

However, these effects are targeted at the ECD (Sup. 2) and expression of p95HER2 lacking the ECD in cancers can be a potential cause of drug resistance because trastuzumab could not elicit any anti-tumor effects due to its inability to bind to the ECD.[12]

Although trastuzumab is indicated for patients with invasive breast cancer, it is effective in cases with HER2 gene amplification or HER2 protein overexpression.

Therefore, before treatment with trastuzumab, it is necessary to determine the status of HER2 expression based on surgical or biopsy samples. Methods for determining the status of HER2 expression include Southern blotting and in situ hybridization (ISH) for detection of DNA amplification levels, reverse transcription polymerase chain reaction (RT-PCR) for detection of RNA levels, and Western blotting (WB), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA) for detection of protein levels.[13]

The guidelines of the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) currently recommend the use of HER2 tests by IHC, ISH, and fluorescence in situ hybridization (FISH). [13,14] IHC assay scores tumors on a 4-point scale (0, 1+, 2+, and 3+) based on the intensity of membrane staining and the percentage of positive cells, with IHC 0/1+, 2+, and 3+ categorized as HER2-negative, HER2-equivocal, and HER2-positive, respectively. IHC 2+ tumors are then tested for gene amplification by FISH to assess the status of HER2 overexpression.[13,14]

In Japan, a total of six IHC reagents from four companies are approved as ex-vivo diagnostics. Among these reagents, SV2-61γ, a reagent in the Histofine® HER2 Kit (MONO), is the only antibody that recognizes the ECD as trastuzumab does, and the five other primary antibodies are all those that recognize the ICD.[12,13,15] In addition, Japan is not the only case where these ICDs and ECDs are mixed and used for diagnosis or research. It is reported in the quality control assessment of NordiQC that such cases also exist in other countries and regions.[16] There have been reports that positive HER2 test results vary among different reagents or assays used by different facilities and that IHC scores vary among different antigen recognition sites of primary antibodies.[17-19] However, all of the previous reports only describe comparisons of IHC results, and no verification

has been conducted to demonstrate differences in the recognition site of primary antibodies. Therefore, interpretations of test results are a matter of speculation. HER2 status can be directly assessed by FISH testing even without IHC testing, but FISH assays require more cost, time, and effort than IHC assays and IHC testing is therefore performed first. Elucidation of the factor for differences in test accuracy to approach treatment with the proper understanding of test results is an issue.

We hypothesized that the discordance of positive rates based on IHC scores might be attributable to different recognition sites of primary antibodies, i.e., the presence of two forms of the HER2 protein targeted by antibodies; full-length p185HER2 with a molecular weight of 185 kDa and ECD-deficient p95HER2 with a molecular weight of 95 kDa. To test this hypothesis, the 4B5 antibody that recognizes the ICD and the SV2-61γ antibody that recognizes the ECD, both of which are used in reagent kits approved in Japan, were used for comparison. Samples with a discrepancy of HER2 status (positive or negative) between the reagent kits were measured for HER2 molecular weight and protein intensity by IHC score by WB and data were analyzed. In addition, in light of these data, we reviewed the advantages and disadvantages of the 4B5 and SV2-61γ antibodies based on the concordance with the concordance of results of the IHC assay with the humanized murine 4D5 antibody.

2. Materials and Methods

2.1. Subjects

In this study, comparisons were made on paraffin-embedded blocks prepared from tissue samples from 101 patients with untreated primary invasive ductal carcinoma who underwent surgery at Juntendo University Hospital between January and December 2018.

2.2. Preparation of paraffin-embedded tissue blocks and tissue sections

10% neutral-buffered formalin-fixed, paraffin-embedded blocks were used as tissue samples to be included in the study. Tissue sections 4 μm thick for IHC and 10 μm thick for WB were cut.

2.3. HER2 testing

IHC staining was performed using the Ventana I-VIEW PATHWAY™HER2 (4B5) kit (Roche Diagnostics K.K., Tokyo, Japan), the Histofine® HER2 Kit (MONO) (Nichirei Biosciences Inc., Tokyo, Japan), and trastuzumab (4D5) (Absolute Antibody Ltd., Oxford, UK). IHC results were interpreted as 0 to 3+ by a pathologist at Juntendo University Hospital according to the guidelines for HER2 pathological diagnostics in breast cancer.[13]

2.4. WB analysis

For WB assay, samples obtained by solubilizing proteins of the formalin-fixed, paraffin-embedded tissue sections used for IHC assay with heat were used.[20] HER2 proteins were solubilized and extracted from paraffin-embedded sections 10 μm thick using the Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany),[20] and obtained samples were electrophoresed. Electrophoresis was performed using SDS sample buffer (0.125 M Tris-HCl, 4.3% SDS, 30% Glycerol, 0.01% BPB [pH6.8]) (Cosmo Bio, Tokyo, Japan) and polyacrylamide gel (MULTIGEL II mini 4/20 [13W]) (Cosmo Bio, Tokyo, Japan).

Subsequently, proteins isolated by electrophoresis were transferred on a PVDF membrane using Protein Transfer Kit for Semidry Electroblotting (Cosmo Bio, Tokyo, Japan), blocked with 5% bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan) for

30 minutes, and incubated with Polyclonal Rabbit Anti-Human c-erbB-2 Oncoprotein A0485 (1:500) (DAKO) as a primary antibody at 4° C overnight. The proteins were then incubated with Rabbit IgG (H&L) Antibody Biotin Conjugated Pre-Adsorbed (Rockland Immunochemicals, Inc., PA, USA) (1:500) as a secondary antibody at room temperature for two hours. In addition, the proteins were incubated with the VECTASTAIN® ABC kit (Vector Laboratories, Inc., CA, USA) for one hour. Ez West Blue (ATTO CORPORATION, Tokyo, Japan) was used as a chromogenic substrate. Band extraction and analysis were performed using LuminoGraph II, CS Analyzer (ATTO CORPORATION, Tokyo, Japan). The protein intensities of HER2 proteins at the molecular weights of 185 kDa and 95 kDa were calculated by WB assay.

Furthermore, for verification, proteins treated with 5% bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan) for 30 minutes were incubated with 4D5 (Absolute Antibody Ltd., Oxford, UK) as a primary antibody at 4° C overnight in the same manner. The proteins were then incubated with Clean Blot IP Detection Reagent (1:500) (Thermo Fisher Scientific MA USA) at room temperature for two hours, and protein intensities at the molecular weights of 185 kDa and 95 kDa were calculated by WB assay.

2.5. Methods for evaluation

All of the 101 samples were scored by IHC. In addition, all samples were measured for protein intensity by WB and assessed as described below:

1) IHC scores with the 4B5, SV2-61γ, and 4D5 antibodies were compared to calculate the degree of concordance among the antibodies. It should be noted that the 4D5 antibody was used only for this assessment as a control.

2) By IHC score category with the 4B5 and SV2-61γ antibodies, it was determined

whether there was a correlation between the IHC score and the protein intensity at a molecular weight of 185 kDa,95 kDa, and the sum of these two protein intensities (185 kDa plus 95 kDa) obtained by WB.

3) Among the cases with a score of 2+/3+ by IHC with 4B5, the protein intensities at the molecular weights of 185 kDa and 95 kDa were compared within each of the groups of cases with scores of 0/1+ and cases with scores of 2+/3+ by IHC with SV2-61γ. In addition, the protein intensities at the molecular weights of 185 kDa and 95 kDa were compared between the groups.

2.6. Statistical analysis

Continuous variables were expressed as mean ± standard error (SE). Inter-group comparisons were performed using a Student's t-test, and comparisons of paired data were performed using a paired t-test. The degree of concordance of the two types of score was assessed by calculating a Weighted Cohen's Kappa value. A correlation between HER2 protein intensity by WB and IHC score was assessed with a Kendall rank correlation coefficient.

All tests were two-sided with a level of significance of $P < 0.05$. Procedures such as the imputation of missing values or exclusion of outliers and extreme values were not performed, and all data were included for analysis. We use the BellCurve plugin for Excel (version 2.15; Japan) for analyses.

2.7. Ethical considerations

This study was conducted in accordance with "Declaration of Helsinki", "Ethical Guidelines for Medical and Health Research Involving Human Subjects", "Ethical

Guidelines for Human Genome/Gene Analysis Research" provided by "Ministry of Health, Labour and Welfare". This study was approved by the ethics committee of the Juntendo University School of Medicine. As this study involved no invasion or intervention to patients and used only information such as medical information and residual samples, an opt-out was provided to disclose information, and an opportunity for refusal was assured.

3. Results

3.1. Evaluation of IHC interpretation

Details of each IHC score category are shown in Table 1. An evaluation of the degree of concordance among the score categories is shown in Tables 2a, 2b, and 2c. The concordance between the results with SV2-61γ and 4D5 was almost perfect with $\kappa = 0.920$, but a κ value was only 0.267 between the results with 4B5 and SV2-61γ and only 0.211 between the results with 4B5 and 4D5. Pathological images are presented in Fig. 1.

3.2. Results of evaluation by WB (using A0485 as a primary antibody)

The relative intensity of HER2 protein with 4B5 and SV2-61γ is shown by the IHC score category in Table 3. When 4B5 was used, the correlation between HER2 protein intensity by WB and each IHC score category was higher for HER2 protein with a molecular weight of 95 kDa. The mean ± standard error of the relative intensity was 14.3 ± 0.6 for a score of 0, 21.8 ± 1.1 for a score of 1+, 30.2 ± 0.9 for a score of 2+, and 43.7 ± 3.6 for a score of 3+, with $\tau = 0.730$ (Kendall rank correlation coefficient) and $p < 0.001$, indicating evidence of a strong positive correlation. Also, HER2 protein with a molecular weight of 95 kDa plus 185 kDa indicated a strong positive correlation, too (τ=0.786、

$p<0.001$).

In contrast, when SV2-61γ was used, the correlation between HER2 protein intensity by WB and each IHC score category was higher for HER2 protein with a molecular weight of 185 kDa. The relative intensity was 15.6 ± 0.8 for a score of 0, 27.2 ± 0.3 for a score of 2, and 35.1 ± 2.1 for a score of 3, and a weak positive correlation was seen between HER2 protein intensity and IHC score with $\tau = 0.383$ and $p < 0.001$.

3.3. WB analysis in cases with a discrepancy in IHC positive results

Nine cases received scores of 2+/3+ by IHC with both 4B5 and SV2-61γ. These cases with scores of 2+/3+ by IHC with SV2-61γ all received scores of 2+/3+ by IHC with 4B5 (Table 2a). Therefore, the cases with scores of 2+/3+ with 4B5 were divided into groups of cases with scores of 0/1+ and 2+/3+ with SV2-61γ to compare the relative intensity of HER protein, as shown in Table 4. In the group of cases with scores of 2+/3+ with SV2-61γ, the relative intensity was 32.4 ± 1.9 for a molecular weight of 185 kDa alone and 26.6 ± 1.9 for a molecular weight of 95 kDa alone, indicating that the protein intensity for p185HER2 was significantly higher ($p = 0.002$). In the group of cases with scores of 0/1+ with SV2-61γ, the relative intensity was 22.0 ± 1.0 for a molecular weight of 185 kDa alone and 38.8 ± 2.3 for a molecular weight of 95 kDa alone, indicating that the protein intensity for p95HER2 was significantly higher ($p < 0.001$).

Comparisons between the groups showed that the protein intensity for p185HER2 was significantly higher in the group of cases with scores of 2+/3+ with SV2-61γ ($p < 0.001$), and the protein intensity for p95HER2 was significantly higher in the group of cases with scores of 0/1+ with SV2-61γ ($p = 0.011$).

3.4. Verification of images obtained by WB assay

Typical images obtained by WB are shown in Fig. 2. The results with A0485 (an antibody recognizing the ICD) used as a primary antibody showed that bands representing both p185Her2 and p95Her2 appeared under the conditions of 4B5 (+), SV2-61γ (-), 4B5 (+), and SV2-61γ (+) (Fig. 2a). In contrast, the results with 4D5 (an antibody recognizing the ECD) used as a primary antibody showed that only a band representing p185Her2 appeared under the conditions of 4B5 (+) and SV2-61γ (+), with no other bands appearing (Fig. 2b).

4. Discussion

This study found that the discordance of positive rates between 4B5 and SV2-61γ was attributed to differences in HER2 protein targeted by the antibodies.

Results of Table 2 and Table 4 mean that p185HER2 with the ECD was more likely to be detected and p95HER2 without the ECD was less likely to be detected when the SV2-61γ antibody was used, leading to a significantly higher p185HER2 protein intensity in IHC 2+/3+ cases and a significantly lower p95HER2 protein intensity in IHC 2+/3+ cases when the SV2-61γ antibody was used.

Results of Table 3 suggested that IHC tests with the 4B5 antibody reflected expression of both p95HER2 and p185HER2, whereas IHC tests with the SV2-61γ antibody reflected only expression of p185HER2 with the ECD, supporting the results shown in Table 4. As shown in Table 3, analysis of relative intensity by IHC score category showed that a τ value with p185HER2 was a little lower for SV2-61γ than 4B5. A factor for this may be that cases with a low protein amount are all scored as 0 because cases beyond a certain level of protein intensity are assigned a higher IHC score with

SV2-61γ. Therefore, a τ value provides a measure of which of either p95HER2 or p185HER2 is more likely to be reflected within the groups with SV2-61γ and 4B5, but is not regarded as a measure for comparison of differences in the strength of correlation between SV2-61γ and 4B5.

Although different positive results among different HER2 tests and different IHC scores among different antigen recognition sites of primary antibodies have been described,[17-19] this study has an enormous implication in that a factor for such differences was successfully demonstrated through examination of HER2 molecular weight and protein intensity by WB. Maeda et al. reported that there were many cases in which even samples assigned an HER2 score of 0 with SV2-61γ were assigned an HER2 score of 3+ with the A0485 polyclonal antibody, whereas samples assigned an HER2 score of 3+ with SV2-61γ are assigned an HER2 score of 3+ with the A0485 polyclonal antibody.[19] This suggests that the HER2 protein is overexpressed in a region recognized by the A0485 polyclonal antibody, a deficiency of the ECD recognized by SV2-61γ is observed in not a few cases, and an HER2 receptor lacking the ECD has overexpression of the ICD due to the lack of binding sites. As evidenced by this study, this may be underlain by the presence of two types of HER2: full-length p185HER2 and p95HER2 lacking the ECD due to effects of a disintegrin, a metalloproteinase10, or splicing.

In addition, we also evaluated the accuracy of tests with 4B5 and SV2-61γ. The results showed that the sensitivity of HER2 testing by IHC was higher with 4B5 than with SV2-61γ. There have been reports that the interpretation of HER2 testing by IHC varies depending on the test kit used.[13,17-19,21] Kitano et al. reported that HER2 IHC positive rates tended to be lower with the SV2-61γ antibody than with the CB11 antibody, which had a recognition site located in the ICD.[17] In addition, using two types of kits, i.e., SV2-61γ in

the Histofine® HER2 Kit (MONO), which this study used, and DAKO Hercep Test II with a primary antibody targeting the ICD and a polyclonal antibody, Maeda et al. reported that there were many cases with differences in IHC score of at least two levels and that IHC scores with the polyclonal antibody were higher in all cases with more individuals testing positive.[19] Similar results were also obtained in this study, in which only nine cases received scores of as 2+/3+ by IHC with SV2-61γ, whereas 44 cases received scores of 2+/3+ by IHC with 4B5, which recognizes the ICD. In contrast, the degree of concordance of IHC scores between SV2-61γ and 4D5 was high, indicating that IHC score results obtained using the SV2-61γ antibody might be useful in predicting the response to trastuzumab.

Although HER2 testing is performed to assess the eligibility for treatment with trastuzumab, trastuzumab is originally a drug that exhibits anti-tumor effects by specifically recognizing and binding to the ECD. This means that HER2-positive patients without the ECD may not adequately respond to trastuzumab. Carvajal-Hausdorf DE et al. reported that the presence of the ECD in the HER2 protein can be associated with a prolonged disease-free survival with trastuzumab, whereas overexpression of the HER2 protein lacking the ECD did not affect disease-free survival.[22] If HER2 protein lacking the ECD is recognized in IHC testing, leading to an HER2-positive test result, IHC score positive status and FISH-positive status are diagnosed; however, an adequate response to trastuzumab may not be achieved in spite of high cost and the risk of both cardiotoxicity and skin toxicity.

For IHC score and effectiveness of trastuzumab, Koh et al. described pathology and prognosis in patients with gastric cancer. According to the report, an evaluation of HER2-positive status and prognosis of trastuzumab therapy using trastuzumab as an

IHC primary antibody showed that progression-free survival and overall survival were higher in cases with an IHC score of 2+ or higher.[23] Given the observed discrepancy of results of IHC assay with 4B5 and SV2-61γ, it is difficult to predict the degree of effectiveness of trastuzumab only based on the results of testing with 4B5. In addition, Koh et al. evaluated HER2-positive status and prognosis in trastuzumab-naïve patients with gastric cancer using 4B5 and trastuzumab as IHC primary antibodies and reported that disease-free survival and overall survival were significantly different between patients with scores of ≥2+ and as <2+ by IHC with trastuzumab, but not between patients with scores of ≥2 and as <2+ by IHC with 4B5.[23] This also suggests that determination of the presence of the ECD by testing may be of significance.

This study has several limitations. This study demonstrated that a primary antibody used for IHC assay affected IHC scores and that differences in score were related to the form of the HER2 protein. However, the study does not indicate whether to administer trastuzumab in cases with a discrepancy of IHC scores with 4B5 and SV2-61γ and the response rate achieved by trastuzumab in the presence or absence of the ECD of HER2 protein. In addition, this study addressed 4B5 and SV2-61γ but cannot assure that the same is true for other antibodies. And because of limited number of cases and lack of treatment and follow-up data, we cannot provide predictive value to treatment with trastuzumab.

5. Conclusion

This study used WB analysis to examine the cause of discordance of positive rates by IHC with 4B5 and SV2-61γ in assessing HER2 expression status in breast cancer cells and suggested that IHC tests with the 4B5 antibody reflected expression of both

p185HER2 and p95HER2, and that IHC tests with SV2-61γ reflected only expression of p185HER2 with the ECD. Moreover, trastuzumab targets the ECD of HER2, and IHC scores with SV2-61γ may therefore be correlated with the drug's effect. Based on these considerations, selection of tests and assessment of test results are required. In addition, further study is needed to evaluate the results of pathology and the prognosis of trastuzumab therapy in order to establish a link to clinical practice and improve the accuracy of tests for the accurate selection of optimal therapies.

Funding

This research has not received specific aid from agencies from public or commercial sectors or non-profit entities.

Conflict of interests

The authors declare that no conflicts of interest exist regarding the publication of this paper.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 1987;235:177-82. https://doi.org/10.1126/science.3798106
2. Harold JB. The distinctive nature of HER2-positive breast cancers. *N Engl J Med.* 2005;353:1652-4. https://doi:10.1056/NEJMp058197
3. Sjögren S, Inganäs M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in

combination with other prognostic markers. *J Clin Oncol.* 1998;16:462-9. https://doi:10.1200/JCO.1998.16.2.462

4. Leahy DJ. A molecular view of anti-ErbB monoclonal antibody therapy. *Cancer Cell.* 2008;13:291-3. https://doi.org/10.1016/j.ccr.2008.03.010
5. Arribas J, Baselga J, Pedersen K, Parra-Palau JL. p95HER2 and breast cancer. *Cancer Res.* 2011;71:1515-9. https://doi.org/10.1158/0008-5472.CAN-10-3795
6. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* 2000;19:3159-67. https://doi.org/10.1093/emboj/19.13.3159
7. Baselga J, Albanell J. Mechanism of action of anti-HER2 monoclonal antibodies. *Ann Oncol.* 2001;12:S35-41. https://doi.org/10.1093/annonc/12.suppl_1.S35
8. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci. USA.* 1992;89:4285-9. https://doi.org/10.1073/pnas.89.10.4285
9. Pietras RJ, Pegram MD, Finn RS, Maneval DA, Slamon DJ. Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. *Oncogene.* 1998;17:2235-49. https://doi:10.1038/sj.onc.1202132
10. Baldassarre T, Truesdell P, Craig AW. Endophilin A2 promotes HER2 internalization and sensitivity to trastuzumab-based therapy in HER2-positive breast cancers. *Breast Cancer Res.* 2017;19:110. https://doi: 10.1186/s13058-017-0900-z.
11. Albanell J, Codony J, Rovira A, Mellado B, Gascón P. Mechanism of action of anti-HER2 monoclonal antibodies: scientific update on trastuzumab and 2C4. *Adv Exp Med Biol.* 2003;532:253-68. https://doi: 10.1007/978-1-4615-0081-0_21.

12. Scaltriti M, Rojo F, Ocaña A, Anido J, Guzman M, Cortes J, et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst.* 2007;99:628-38. https://doi.org/10.1093/jnci/djk134

13. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. Arch Pathol Lab Med. 2018;142:1364-1382. https://doi.org/10.5858/arpa.2018-0902-SA.

14. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. *J Clin Oncol.* 2018;36:2105-22. https://doi.org/10.5858/arpa.2018-0902-SA

15. Tsuda H, Tani Y, Hasegawa T, Fukutomi T. Concordance in judgments among c-erbB- 2 (HER2/neu) overexpression detected by two immunohistochemical tests and gene amplification detected by Southern blot hybridization in breast carcinoma. *Pathol Int.* 2001;51:26-32. https://doi.org/10.1046/j.1440-1827.2001.01163.x

16. NordiQC. HER2 IHC, https://www.nordiqc.org/epitope.php?id=11; [accessed 6 Feb 2022].

17. Kitano Y, Umemura S, Ohbayashi H, Takenaga M, Osamura RY. Assessment of a new anti-HER2 monoclonal antibody, SV2-61gamma: a best concordance with HER2 FISH. *Appl Immunohistochem Mol Morphol.* 2007;15:389-93.

https://doi: 10.1097/PAI.0b013e31802f411c

18. Nunes CB, Rocha RM, Buzelin MA, Balabram D, de Souza Foureaux F, Porto SS, et al. False positivity in HER2 testing of breast cancer: novel paths for approaching an old dilemma. *Comparative Study J Clin Pathol.* 2013;66:946-50. http://dx.doi.org/10.1136/jclinpath-2013-201647

19. Maeda Y, Oshiro S, Saga S, Yamashiro A, Goeku A, Kawakami J, et al. Study of HER2 positive rates among different primary antibodies used for IHC in breast cancer. *Medical journal of Naha City Hospital*. 2012;4(1):27-30. (in Japanese) https://mol.medicalonline.jp/en/archive/search?jo=eh2nahac&ye=2012&vo=4&issue=1

20. Becker KF, Schott C, Hipp S, Metzger V, Porschewski P, Beck R, et al. Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J Pathol.* 2007;211:370-8. https://doi.org/10.1002/path.2107

21. Tamura G, Osakabe M, Yanagawa N, Ogata S, Nomura T, Fukushima N, et al. Comparison of HER2 immunohistochemical results using a monoclonal antibody (SV2-61γ) and a polyclonal antibody (for Dako HercepTest) in advanced gastric cancer. *Pathol Int.* 2012;62:513-7 https://doi.org/10.1111/j.1440-1827.2012.02843.x

22. Carvajal-Hausdorf DE, Schalper KA, Pusztai L, Psyrri A, Kalogeras KT, Kotoula V, et al. Measurement of Domain-Specific HER2 (ERBB2) Expression May Classify Benefit From Trastuzumab in Breast Cancer. *J Natl Cancer Inst.* 2015; 107:djv136. https://doi.org/10.1093/jnci/djv136

23. Koh J, Nam SK, Lee YW, Kim JW, Lee KW, Ock CY, et.al. Trastuzumab Specific

Epitope Evaluation as a Predictive and Prognostic Biomarker in Gastric Cancer Patients. *Biomolecules*. 2019;9:782. https://doi.org/10.3390/biom9120782

Figure legend

Fig 1. Examples of pathological images by IHC

Fig 1A, 1B, 1C are examples of typical cases in HER2 IHC score 3+（Ａ: 4B5, Ｂ:SV2-61γ, C: 4D5)

Fig 1D, 1E, 1F are examples of discordant cases. (D: 3+ using 4B5, E: 0 using Sv2-61γ, F: 4D5)

Fig 2. Examples of results images by WB

Table 1 Disposition in each IHC score by antibody

primally Antibody	Her2 score			
	0	1 +	2+	3+
4B5	52	5	24	20
Sv2-61γ	92	0	3	6
4D5	93	0	6	2

Table 2

Table 2a Degree of concordance of IHC scores between 4B5 and SV2-61γ

		SV2-61γ			
		0	1+	2 +	3+
4B5	0	52	0	0	0
	1+	5	0	0	0
	2+	21	0	3	0
	3+	14	0	0	6

Squared weighted κ＝0.267

Table2b Degree of concordance of IHC scores between 4D5 and SV2-61γ

		SV2-61γ			
		0	1+	2 +	3+
4D5	0	92	0	1	0
	1+	0	0	0	0
	2+	0	0	2	4
	3+	0	0	0	2

Squared weighted κ＝0.920

Table 2c Degree of concordance of IHC scores between 4B5 and 4D5

		4D5			
		0	1+	2 +	3+
4B5	0	52	0	0	0
	1+	5	0	0	0
	2+	22	0	2	0
	3+	14	0	4	2

Squared weighted κ＝0.211

Table 3. Protein intensity by IHC score *

	4B5			SV2-61γ		
	185kDa+95kDa	185kDa	95kDa	185kDa+95kDa	185kDa	95kDa
Score 0	25.4±1.0	11.1±0.6	14.3±0.6	39.6±2.1	15.6±0.8	24.0±1.5
Score 1	39.1±0.7	17.3±0.5	21.8±1.1			
Score 2	51.2±0.8	21.0±1.1	30.2±0.9	51.4±2.4	27.2±0.3	24.3±2.1
Score 3	71.6±3.0	27.9±1.6	43.7±3.6	62.8±4.6	35.1±2.1	27.7±2.7
τ	0.786	0.660	0.730	0.257	0.383	0.131
p	<0.001	<0.001	<0.001	0.002	<0.001	0.108

τ: Kendall rank correlation coefficient for HER2 protein intensity by WB and IHC score

*HER2 protein intensity against the positive control human breast cancer cell line SKBR3

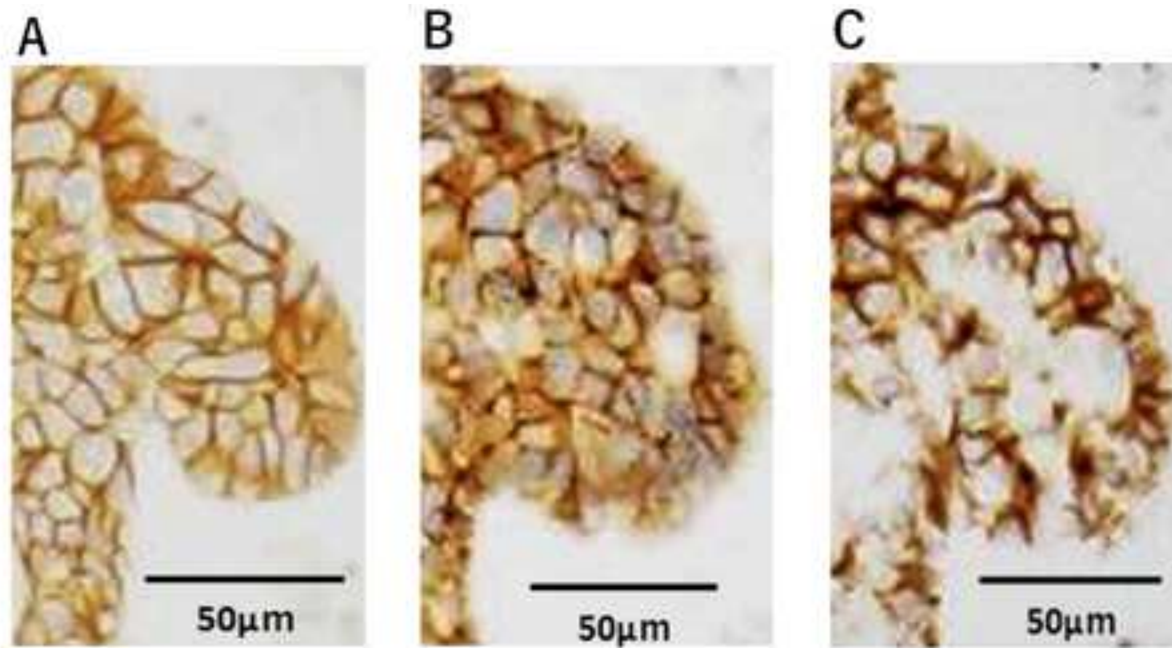
Table 4

Table 4 HER2 protein intensity in cases with a discrepancy in IHC positive results

4B5	SV2-61γ	n	185kDa	95kDa	p (paired t-test)
Score 2+,3+	Score 0,1+	35	22.0±1.0	38.8±2.3	<0.001
Score 2+,3+	Score 2+,3+	9	32.4±1.9	26.6±1.9	0.002
p (Stutent's t-test)			<0.001	0.011	

Fig. 1

Click here to access/download;Figura;fig1.tiff



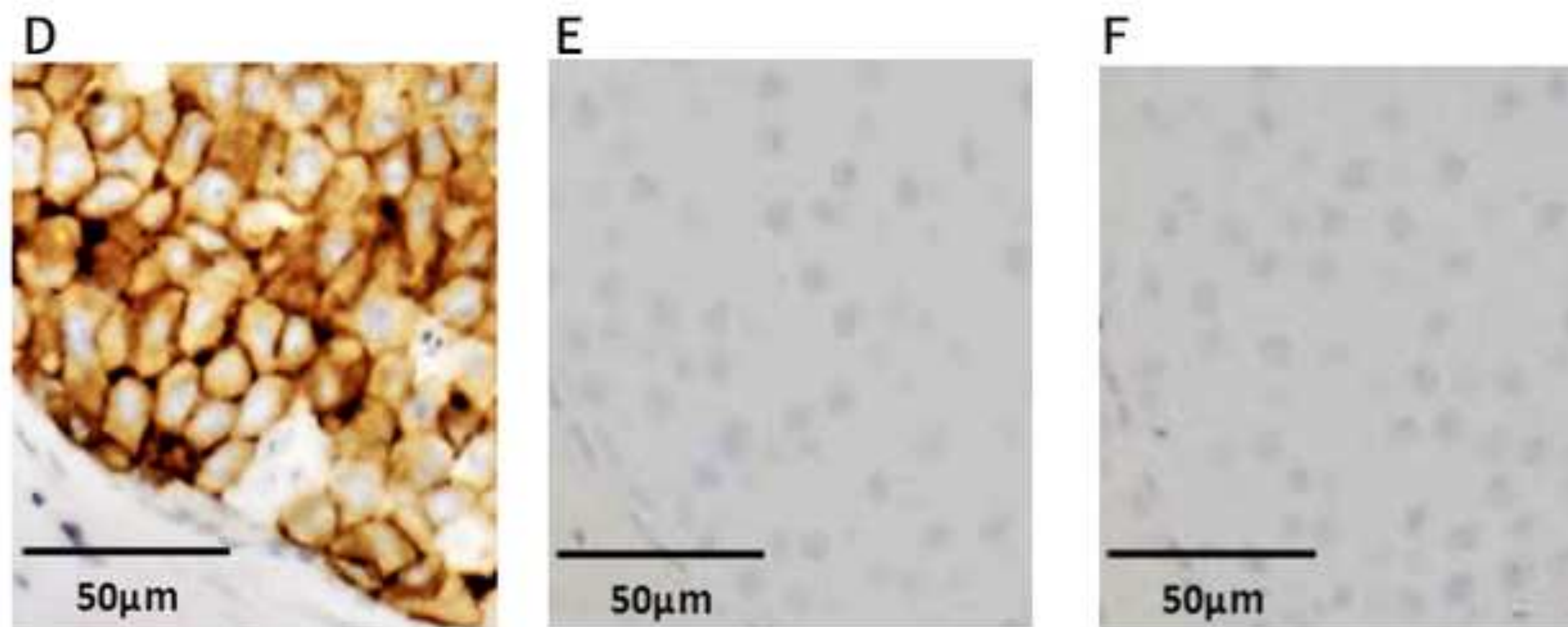
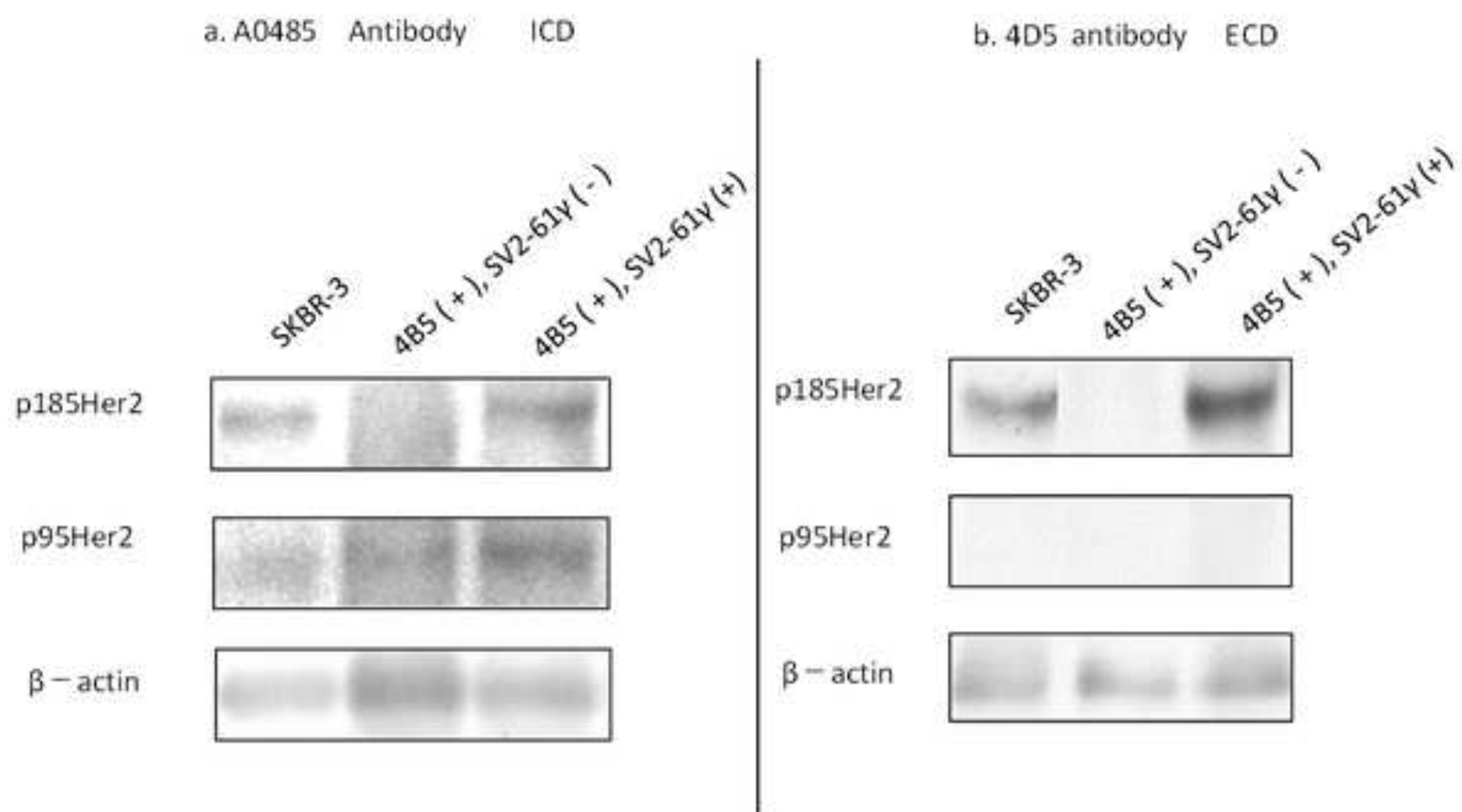
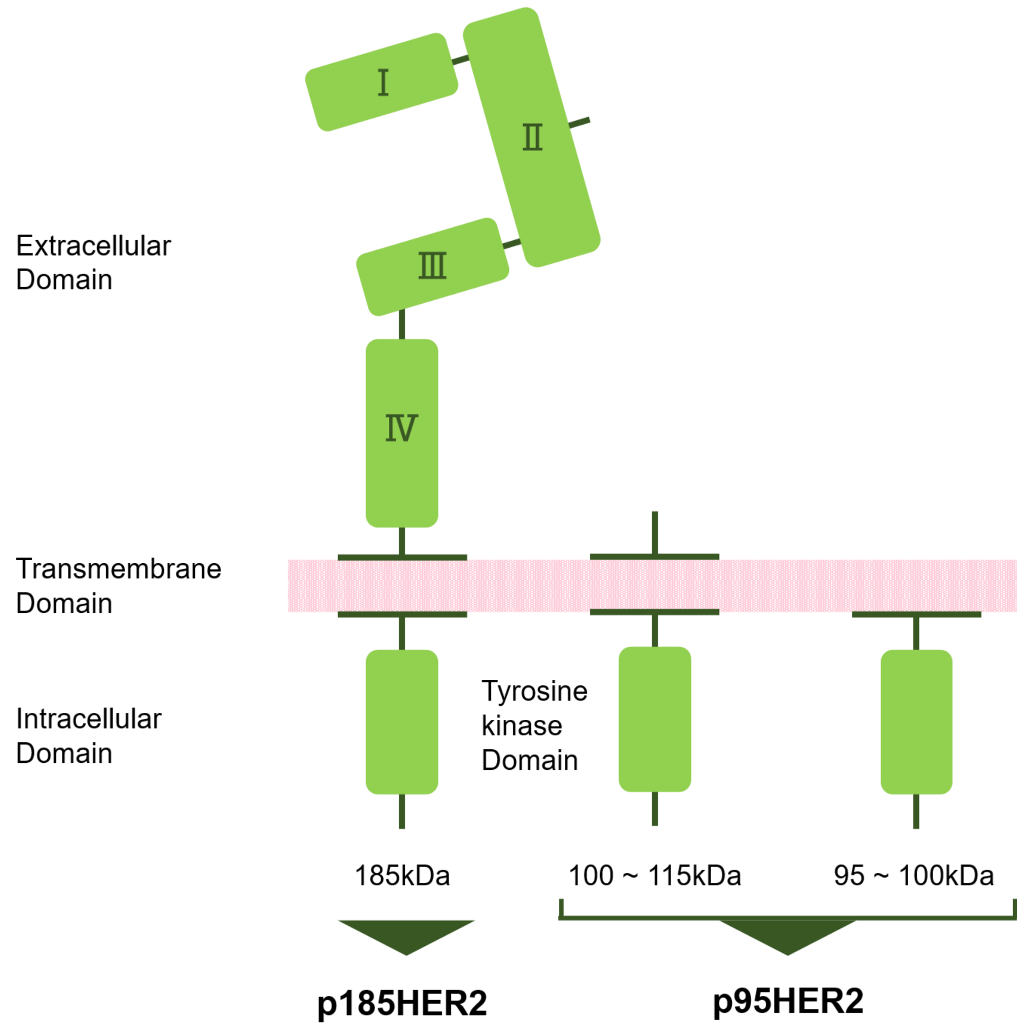


Fig. 2

Click here to access/download;Figura;fig2.tiff

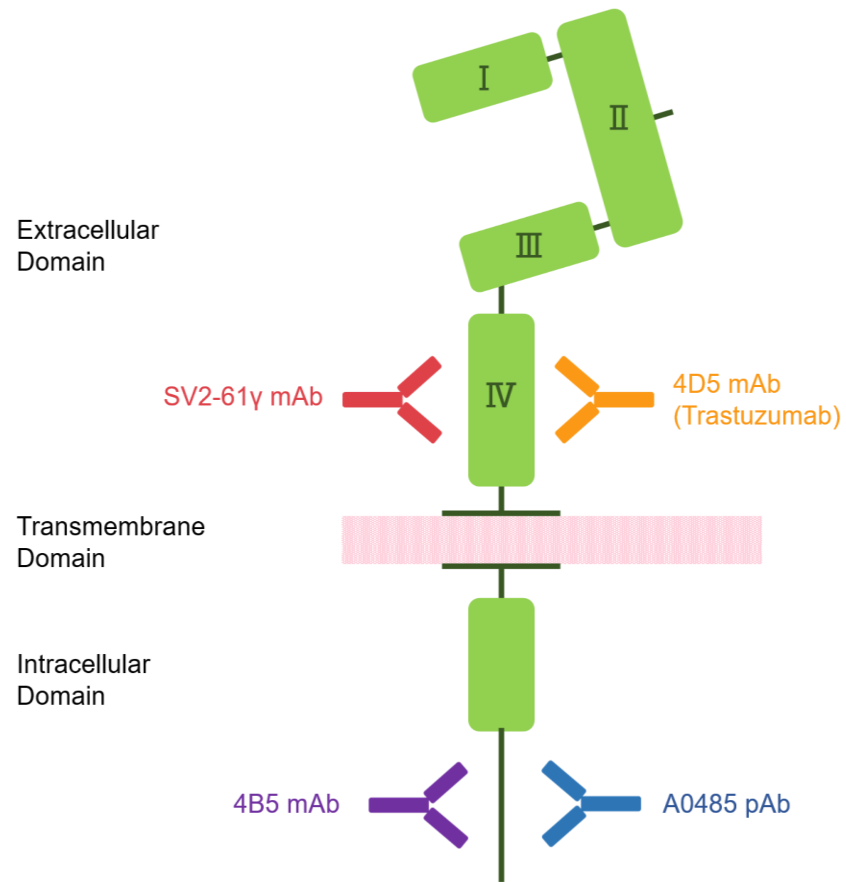


Sup 1. Structure of HER2



100 to 115 kDa receptors: Lost by alternative splicing at the stage of mRNA. 95 to 100 kDa receptors: Lost by protease of ADAM10, with 6 amino acid residues left.

Sup 2. Targets of the antibodies in HER2



mAb: monoclonal antibody; pAb: polyclonal antibody