A Human B cell Receptor Epitope-Based erbB-2 Peptide (N: 163-182) with Pan-Reactivity to the T cells of Japanese Breast Cancer Patients

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Abstract

Background: While monoclonal antibodies are widely accepted as being powerful in molecular targeting therapy, the activation of immunity by the B cell receptor epitope peptide has not been reported. Our objective was to evaluate a 20-mer multiple antigen peptide containing the anti-erbB-2 (HER2/neu) monoclonal antibody epitope (N: 163–182), designated as CH401MAP, as a general peptide vaccine for Japanese breast cancer patients.

Methods: The 20-mer CH401 epitope peptide binding to the human leukocyte antigen (HLA) of Japanese breast cancer patients was predicted by algorithms from the following databases: SYFPEITHI, Bioinformatics and Molecular Analysis Section (BIMAS), and Immune Epitope Database (IEDB). Peripheral blood mononuclear cells (PBMCs) were collected from 173 breast cancer patients and stimulated with the peptide in vitro. Cytokine secretion was examined by enzyme-linked immunosorbent assay (ELISA). Flow cytometric analysis was performed to detect CD4 and CD8 T cells and Treg cells.

Results: Using the algorithms used for the simulation, CH401MAP was predicted to bind to more than 90% of class I HLA and to 30–50% of class II HLA in breast cancer patients. In vitro stimulation of patients' PBMCs with CH401MAP induced a significant increase in interleukin-2 secretion, proliferation, and CD8 T cell ratios.

Conclusions: Our study demonstrates that CH401MAP induces T cell activation in PBMCs derived from breast cancer patients. This antibody epitope-based peptide with HLA motifs could be a candidate peptide vaccine for general breast cancer.

Keywords: CH401; Breast cancer; HER2; Peptide vaccine; B cell epitope

Abbreviations: HER2: Human Epidermal Growth Factor Receptor 2; BIMAS: BioInformatics and Molecular Analysis Section; IEDB: Immune Epitope Database; PBMC: Peripheral Blood Mononuclear Cells; ELISA: Enzyme-linked Immunosorbent Assay; HLA: Human Leukocyte Antigen; P13K: Phosphoinositide 3-kinase; CTLs: Cytotoxic Lymphocytes; MHC: Major Histocompatibility Complex; FISH: Fluorescence In Situ hybridization; HD: Healthy Donors; Mab: Monoclonal Antibody; MAP: Multiple Antigen Peptide; PBS: Phosphate Buffer saline; FCM: Flowcytometry; PE: Phycoerythrin; FITC: Fluorescin Isothiocyanate; PerCP-Cy5.5: Peridinin Chlorophyll Protein Complex-Cyanin 5.5; APC: Allophyocyanin; FOXP3: Forkhead box P3; SD: Standard Deviation

Introduction

Passive immune therapy with “trastuzumab” is a powerful tool for treating human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancers [1], which is observed in 20–30% of breast cancer patients [2]. HER2 positivity rate is similar between Japanese and Caucasians [3]. While amplification of the HER2 gene and overexpression of HER2 protein indicates rapid disease progression from early metastatic stages [4], trastuzumab treatment controls the progression of this type of breast cancer. However, several problems, such as occasional recurrence, occur under trastuzumab therapy, probably due to the acquisition of trastuzumab resistance. The mechanism of resistance acquisition has not been clarified, although Nahta et al. suggested that increased signaling via the phosphoinositide 3-kinase (PI3K)/Akt pathway might contribute to trastuzumab resistance [5]. Thus far, no means to overcome trastuzumab resistance have been reported. In the past decades, numerous attempts have been made to develop active immune therapies using appropriate peptide vaccines for breast cancer as an alternate or adjuvant of trastuzumab therapy. Some peptides, including GP2, E75, and AE37, were successively introduced into clinical trials [6-8]. However, to use these peptides for vaccination, it is crucial to determine the patients' human leukocyte antigen (HLA) types, because anchoring of the peptide to an individual's HLA is necessary to induce effective T cell activation with the peptide vaccines. However, limited types of HLA can present a specific peptide in general. Immunogenic peptides mentioned above (GP2, E75 and AE37) containing HER2 sequence can induce peptide-specific cytotoxic T lymphocytes (CTLs) that recognize tumor cells presenting themselves on major histocompatibility complex (MHC) class I molecules [9]. For example, the target of GP2 is HLA-2A, which is found in 45.8% of Caucasian, 39.0% of North American (NA) Black, 42.4% of Japanese, 45.9% of Chinese, and 43.0% of Hispanic source are credited.

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Received October 05, 2012; Accepted November 18, 2012; Published November 22, 2012

Citation: Tsuda B, Kametani Y, Goto Y, Saito Y, Suzuki Y, et al. (2012) A Human B cell Receptor Epitope-Based erbB-2 Peptide (N: 163-182) with Pan-Reactivity to the T cells of Japanese Breast Cancer Patients. J Vaccines Vaccin 3:159. doi:10.4172/2157-7560.1000159

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J Vaccines Vaccin
ISSN:2157-7560 JVV an open access journal

Volume 3 • Issue 7 • 1000159
populations [10]. HLA-A3 is found in 37.5% of Caucasian, 42.1% of NA Black, 45.8% of Japanese, 52.7% of Chinese, and 44.2% of Hispanic populations [10]. The targets of E75 are HLA-A2 and A3 [8]. None of these peptides can such a cover broad range of HLA types, including those of class I and class II.

Because the major HLA types are significantly different across races [10], it is necessary to select a peptide that binds to the HLA of patients of a particular race. Moreover, some results indicate that it is important to stimulate both CTLs and Th cells in order to acquire sufficient anticancer effect [11]. It is imperative to develop a ‘promiscuous’ peptide vaccine, which is broadly restricted.

We previously reported a 20-mer peptide containing a B cell receptor epitope of anti-HER2 monoclonal antibody “CH401,” which was reported to have apoptotic anticancer activity [12]. We named this peptide CH401MAP and proved that it can induce not only Th cell activation but also specific antibody production and Tc cell infiltration into HER2/neu-positive tumors in the BALB/c mouse system. CH401MAP showed an antitumor effect against HER2-overexpressing mouse B cell lymphomas transplanted into BALB/c mice [13].

In this study, we predicted the human class I and class II HLA anchor motifs of CH401MAP and examined if the peptide is a “promiscuous” one. We found that CH401MAP reacted with a vast majority of human lymphocytes of Japanese breast cancer patients, indicating the potential of a general breast cancer vaccine based on it in this population.

Material and Methods

Cells from patients and healthy donors

We enrolled 173 breast cancer patients, all of whom provided consent under a protocol approved by the Tokai University Hospital Institutional Review Board (Table 1). Enrollment criteria were women aged 20 years or more, no history of malignant disease in the past, and histologically confirmed breast cancer before their surgery at Tokai University Hospital, Kanagawa, Japan from Jan 2010 to July 2012. The HER2 expression level score was estimated in the main tumor by using immunohistochemistry.

We divided the patients into 3 groups: HER2 0, patients with HER2 expression immunohistochemistry (IHC) score 0 (n=66); HER2 1+2+, patients with HER2 expression IHC scores 1+ and 2+ (n=89); and HER2 3+, patients with HER2 expression IHC score 3+ (n=18). Fluorescence in situ hybridization (FISH) was conducted in case of HER2 2+. Healthy female donors (n=22) without any malignant disease in the past were selected to provide control blood. From patients, as well as healthy donors, 7.5 ml blood was obtained.

CH401MAP peptide

The peptide used for the stimulation was prepared as described previously [13]. Briefly, the 20-mer peptide containing the epitope of CH401, a monoclonal antibody (Mab) raised against HER2/neu developed by Ishida et al., was selected (N:163–182, YQDTILWKDIFHKNNQLALT) [12]. The multiple antigen peptide (MAP) was synthesized from Rink amide resin (0.4–0.7 mmol/g) using a peptide synthesizer ACT357 (Advanced ChemTech, Louisville, KY, USA), resulting in the formation of amide at the carboxyl termini. These MAPs were used for immunization and enzyme-linked immunosorbent assay (ELISA).

In vitro stimulation protocol

Blood from patients (7.5 ml) was drawn into Vacutainer ACD tubes (NIPRO Corporation, Japan, Osaka) in the morning of the day of surgery. Peripheral blood mononuclear cells (PBMCs) from 6 ml of the collected blood were isolated by density centrifugation (5,000g, 30 min, 20°C) using Ficoll-Hypaque (SIGMA-ALDRICH, UK, London). PBMCs were removed and washed with phosphate-buffered saline (PBS) for 5 min at 3,000g and 4°C. After the cell number was determined, cells were incubated with RPMI 1640 medium supplemented with 10% human serum (immortalized, 55°C, 30 min; Lonza, Walkersville, MD, USA), 1% l-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 0.5% penicillin-streptomycin in 96-well, round-bottom plates at 37°C in a 5% CO₂ atmosphere. All samples were treated within
12 h. The detailed protocol for the in vitro culture of the PBMCs is shown in figure 1. Briefly, cells were obtained from the 4 groups, HD, HER2 0, HER2 +1+2, and HER2 +3+, and 1×10^6 cells were used for flow cytometry (FCM) analysis. The remaining cells (5×10^6 cells/well) were plated and stimulated by CH401 or PBS. The surface markers of the cells were analyzed 21 days after the start of stimulation.

**HLA typing**

The remaining volume of patients’ blood (1.5 ml) was used for HLA typing analysis. Genotyping of the HLA-A, B, and DRB1 alleles were performed using the PCR-SSOP Luminex method using LABType SSO (One Lambda Inc., Canoga Park, CA, USA), a reverse SSO DNA typing system, according to the manufacturer’s instructions. A flow analyzer, LABScanTM 100, identified the fluorescent intensity of phycoerythrin on each microsphere. The determination of the HLA allele was based on the reaction pattern compared to patterns associated with published (http://www.hla.or.jp/) HLA allele sequences, which included all HLA allele types that are known until now.

### Peptide and MHC binding affinity simulation

Computer algorithms accessible from internet databases, namely, SYFPEITHI developed by Rammensee H. G. et al. (http://www.syfpeithi.de/) [14] epitope prediction based on a database for HLA class I and class II prediction, BioInformatics and Molecular Analysis Section (BIMAS) developed by Parker K. C. et al. (http://www-bimas.cit.nih.gov/molbio/hla_bind/) [15] for HLA class I prediction, and Immune Epitope Database (IEDB) and analysis resource developed by Peters B. et al. (http://www.immuneepitope.org/) [16] for HLA class II prediction, were used to predict CH401 MAP peptide binding affinity.

### Immunofluorescent staining and flow cytometry analysis

All antibodies, phycoerythrin (PE)-conjugated anti-human CD4 monoclonal antibodies (mAbs) (clone:L200), fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 mAbs (clone:G42-8), peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5)-conjugated anti-human CD3 mAbs (clone:HIT3a), allophycocyanin (APC)-conjugated anti-human CD25 mAbs (clone:M-A251), PE-conjugated

### Table 2a

| HLA type | Allele | Healthy control (n=20705) | Breast cancer patients (n=173) | SYFPEITHI score | Recognition site | BIMAS | Recognition site |
|----------|--------|--------------------------|-------------------------------|----------------|------------------|-------|------------------|
| A        | *24:02 | 35.9                     | 29.3                          | 12             | YQDTILWKDFHKNNQLALT | —     | —                |
|          | *02:01 | 11.5                     | 11.8                          | 20             | YQDTILWKDFHKNNQLALT | 2.8   | YQDTILWKDFHKNNQLALT |
|          | *02:06 | 9.2                      | 16.8                          | 0              | —                | —     | —                |
|          | *11:01 | 9.1                      | 8.7                           | 17             | YQDTILWKDFHKNNQLALT | 2.4   | YQDTILWKDFHKNNQLALT |
|          | *13:01 | 8.6                      | 20.5                          | 0              | —                | 3.6   | YQDTILWKDFHKNNQLALT |
|          | *26:01 | 7.5                      | 4                             | 0              | —                | —     | —                |
| B        | *52:01 | 11.1                     | 26                            | 13.2           | YQDTILWKDFHKNNQLALT | —     | —                |
|          | *51:01 | 9.0                      | 6.6                           | 14             | YQDTILWKDFHKNNQLALT | 4.4   | YQDTILWKDFHKNNQLALT |
|          | *35:01 | 8.2                      | 17.9                          | —              | YQDTILWKDFHKNNQLALT | 1     | YQDTILWKDFHKNNQLALT |
|          | *40:02 | 7.8                      | 7.1                           | 0              | —                | —     | —                |
|          | *15:01 | 7.8                      | 8.4                           | 11             | YQDTILWKDFHKNNQLALT | —     | —                |
|          | *54:01 | 7.6                      | 13.9                          | —              | —                | —     | —                |
|          | *44:03 | 6.5                      | 6                             | 0              | —                | 15    | YQDTILWKDFHKNNQLALT |
|          | *07:02 | 5.6                      | 3.3                           | 13             | YQDTILWKDFHKNNQLALT | —     | —                |
|          | *40:01 | 5.3                      | 5.3                           | 14             | YQDTILWKDFHKNNQLALT | —     | —                |
|          | *46:01 | 4.8                      | 7                             | —              | —                | —     | —                |
|          | *40:06 | 4.6                      | 4.7                           | —              | —                | —     | —                |
|          | *39:01 | 3.4                      | 3.3                           | 24             | YQDTILWKDFHKNNQLALT | 27    | YQDTILWKDFHKNNQLALT |
|          | *48:01 | 2.8                      | 4.4                           | —              | —                | —     | —                |
|          | *02:01 | 4.2                      | 3.7                           | 0              | —                | —     | —                |
|          | *09:02 | 4.2                      | 3.7                           | 0              | —                | —     | —                |
|          | *39:02 | 0.3                      | 0.6                           | 11             | YQDTILWKDFHKNNQLALT | —     | —                |

Table 2b

| AB       | Allele | Healthy control (n=20705) | Breast cancer patients (n=173) | Recognition site | IEDB Analysis | Recognition site |
|----------|--------|--------------------------|-------------------------------|------------------|---------------|------------------|
| DRB1     | *09:01 | 14.1                     | 35.9                          | 0                | 4.64          | YQDTILWKDFHKNNQLALT |
|          | *04:05 | 13.7                     | 8.4                           | 0                | —             | —                |
|          | *15:02 | 10.5                     | 10.8                          | 0                | 2.4           | YQDTILWKDFHKNNQLALT |
|          | *08:03 | 8.6                      | 7.9                           | 0                | 2.76          | YQDTILWKDFHKNNQLALT |
|          | *15:01 | 7.7                      | 8.7                           | 0                | 4.33          | YQDTILWKDFHKNNQLALT |
|          | *01:01 | 5.8                      | 3.7                           | 0                | —             | —                |
|          | *13:02 | 5.7                      | 5.5                           | 0                | —             | —                |
|          | *09:02 | 4.2                      | 3.7                           | 0                | —             | —                |
|          | *12:01 | 3.7                      | 7.5                           | 0                | —             | —                |
|          | *11:01 | 2.7                      | 3.7                           | 24               | YQDTILWKDFHKNNQLALT | —     | —                |

**Table 2:** HLA alleles of the patients and healthy donors with predicted affinity scores

a) The percentages of the HLA types of both healthy donors and breast cancer patients in Japan are shown in parallel with the SYFPEITHI, BIMAS, and IEDB analyses scores.

b) Recognized sequences are shown as underlined text. (—) means the allele and the peptide affinity could not be simulated by the algorithm.
450 nm was measured by SPECTRAmax250 (Molecular Devices, Japan, performed according to the manufacturer's instructions. Absorbance at vitro stimulated cell supernatants were collected, and ELISA was kits (BD OptEIA Set, BD Biosciences) and Immuno LockWell Module/Cytokine ELISA lymphocyte gate.

All the data used for the analyses were acquired from the 0.3.ff2b (BD Biosciences).

Fluorescence intensity of fluorochrome-labeled cells was measured samples were subjected to intracellular staining according to [12]. Fluorescence intensity of fluorochrome-labeled cells was measured by FCM (FACSCalibur, BD Biosciences) with Cell Quest Pro version Fluorescence intensity of fluorochrome-labeled cells was measured by FCM (FACSCalibur, BD Biosciences) with Cell Quest Pro version

CD25 mAbs (clone:BC96) were sourced from BioLegend Inc., USA, human CD3 mAbs (clone:M-A251), and FITC-conjugated anti-human anti-human CD4 mAbs (Clone:L200), PerCP-Cy5.5-conjugated anti-human CD25 mAbs (clone:BC96) were sourced from BioLegend Inc., USA, San Diego). The fixation/permeabilization concentrate, diluent, and permeabilization buffer were purchased from BD Biosciences (USA, California).

Immunoﬂuorescent staining was performed as reported previously [12]. Fluorescence intensity of ﬂuorochrome-labeled cells was measured by FCM (FACSCalibur, BD Biosciences) with Cell Quest Pro version 0.3.0f2b (BD Biosciences).

The CH401MAP peptide is predicted to possess highly reactive anchor motifs of both class I and class II HLAs in Japanese breast cancer patients examined were predicted to have afﬁnity with CH401MAP.

The CH401MAP peptide is predicted to possess highly reactive anchor motifs of both class I and class II HLAs in Japanese breast cancer patients. The pan-reactivity is superior to that of other breast cancer vaccines [17].

Lymphocyte response

The protocol for the in vitro culture of the PBMCs is summarized in figure 1. Cells were divided into 3 experimental groups. PBMCs were stimulated with the peptide and the peptide response analyzed. Mean scores of the groups' proliferation indexes were estimated as follows: (healthy control group, 1.008 ± 0.132; HER2 0 group, 1.165 ± 0.406; HER2 1+, 2+ group, 1.255 ± 0.331; HER2 3+ group, 1.655 ± 0.660). The scores of the patient groups were signiﬁcantly higher than those of the healthy control group (HER2 0 group, p=0.05; HER2 1+, 2+ group, p<0.01; HER2 3+ group, p<0.01).

Next, we compared cytokine secretion of stimulated PBMCs with unstimulated ones. The concentration of IL-2 in the stimulated supernatants was prone to increase in all the patient groups (Figure 3a). In particular, this concentration was signiﬁcantly increased in the HER2 1+2+ group at 48 h (p < 0.001) compared to control groups. We also measured IFN-γ (Figure 3b) and IL-4 (Figure 3c) concentrations. The secretion of these cytokines tended to increase, although the significant difference was not observed in total (IFN-γ: HER2 0 group, p=0.215; HER2 1+, 2+ group, p=0.206; HER2 3+ group, p=0.147). IL-4: HER2 0 group, p=0.335; HER2 1+, 2+ group, p=0.480; HER2 3+ group, p=0.955). Mean index (value of stimulated cells/value of unstimulated
Our results suggest that the CH401 epitope-containing 20-mer peptide is a promiscuous epitope peptide. Moreover, stimulation of patients’ PBMCs confirmed the broad applicability to adjunctive therapy. Trastuzumab is used for treatment in patients with high HER2 expression, because the recognition of the HER2 molecule on the surface of the cancer cells is directly correlated with the HER2 expression level. However, our results suggested that in the group with lower expression of HER2 (HER2 1-2+), PBMCs were stimulated to a greater extent with the peptide than were those of the HER2 3+ group (Figures 2-4). Accordingly, CH401MAP may react with HLAs of a significant number of Japanese breast cancer patients whose HER2 expression is intermediate. While this peptide possesses the B cell epitope and induces specific antibody production in a humanized mouse system [17], it activates T cells similarly to the observation in the mouse system [12]. CH401MAP is expected to induce antitumor effects in HER2-intermediate patients and prevent recurrence, suggesting its applicability to adjunctive therapy.

Acknowledgments
We are grateful to Rin Ohgiya MD, Risa Oshitanai MD, Mayako Terada MD, Mizuho Terada MD, Toru Morikawa MD, Takuo Okamura MD, and Naoko Nikura MD, PhD for their clinical support and to Syuya Mori, Shin Shimada, Erina Numao, Asuka Miyamoto, Shino Ohshima and Eri Kikkawa for their excellent technical support and to Asako Ando for her supportive advice.

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