Development of a Novel Inhibitory Chimeric Anti-HER2 Monoclonal Antibody

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ABSTRACT

Background: We have recently produced an inhibitory mouse anti-human HER2 mAb (2A8) which displayed potent anti-tumor activity in combination with trastuzumab. **Objective:** To describe chimerization and functional characterization of 2A8 mAb. **Methods:** The VH and VL genes of 2A8 mAb were amplified from cDNA of the mouse hybridoma, ligated to constant regions of human immunoglobulin, and expressed in CHO cell line. Reactivity with four members of human HER family, the inhibitory effects and antibody-dependent cell cytotoxicity (ADCC) of purified chimeric mAb (c2A8) were assessed by ELISA, XTT, H3-tymidine incorporation and lactate dehydrogenase assays. Inhibition of ERK and AKT downstream signaling pathways by the chimeric antibody were analyzed by Western blotting. **Results:** Chimeric 2A8 mAb bound to recombinant human HER2 and did not cross-react with the other members of HER family. Moreover, c2A8 was able to recognize HER2-overexpressing cancer cell line and inhibited growth and proliferation of these cells. The binding affinity of c2A8 was comparable to the mouse parental mAb. ADCC and Western blotting results showed that the mouse 2A8 mAb was successfully chimerized and could significantly inhibit phosphorylation of AKT in combination with trastuzumab. **Conclusion:** The c2A8 mAb is potentially a valuable tool for targeted immunotherapy of HER2 positive cancers.

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INTRODUCTION

HER2 is an 185kDa transmembrane glycoprotein that, along with other members (HER1, HER3 and HER4), belongs to epidermal growth factor (EGF) receptor family of receptor tyrosine kinases (1). HER2 gene is overexpressed in a number of human cancers including breast, colorectal, ovarian, and pancreatic cancers. Heterodimerization of HER2 with other members of ErbB family leads to cell growth and proliferation of tumor cells by activation of tyrosine kinases pathways (1,2). The oncogenic potential of HER2 has made it a valuable target for passive cancer immunotherapy by monoclonal antibodies (mAbs). Trastuzumab (Herceptin, Genentech Inc., CA, USA), which binds to domain IV of HER2, is the first mAb approved by FDA in 1998 for breast cancer treatment (3). Pertuzumab (Perjeta, Genentech Inc., CA, USA), which binds to a different epitope than trastuzumab on the domain II of HER2, has been recently approved by FDA in combination with trastuzumab and demonstrated improved survival in patients with breast cancer (4). The data from our studies and others suggest that targeting two distinct HER2 epitopes may result in increased growth inhibition paving the way to find more antibodies targeting new epitopes (5-8). Recently, several mouse mAbs against HER2 have been produced in our laboratory, which recognized different epitopes than trastuzumab (9). Two of these mAbs (1T0 and 2A8) in combination with trastuzumab showed potent anti-tumor activity compared to each mAb alone (8). These mAbs can be used for diagnostic and therapeutic purposes after reducing their immunogenicity by chimerization or humanization techniques. In our previous work, we have chimerized and humanized the 1T0 mAb successfully (10,11). In this study, we present data on chimerization of 2A8 mAb, which recognizes a different epitope other than those recognized by trastuzumab, pertuzumab and 1T0 mAb.

MATERIALS AND METHODS

Cell lines and antibodies. The hybridoma producing mAb against HER2, 2A8 (9), was grown in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA) and antibiotics (100 U/ml Penicillin, 100 µg/ml Streptomycin) at 37°C in a humidified atmosphere of 5% CO2. CHO-K1 and BT-474 cell line were purchased from National Cell Bank of Iran (NCBI, Tehran, Iran) and cultured under similar conditions. 10µg/ml bovine insulin (Sigma Chemical Co, St. Louis, MO, USA) was added to culture medium for growth of BT-474 cells.
Rabbit mAbs specific for phospho-P44/p42 MAPK (Erk1/2, #4370), phospho-AKT (ser473, #4060), AKT(#9272), P44/p42 MAPK (Erk1/2, #9102), β-actin (#8457) and HRP-conjugated anti-rabbit Ig antibody (#7074) were all purchased from Cell Signaling Technology (Boston, United States). HRP-rabbit anti-human Ig, FITC-rabbit anti-human Ig, FITC-rabbit-anti-mouse Ig, and HRP-rabbit anti-mouse Ig were purchased from Sina Biotech Company (Tehran, Iran), pertuzumab and trastuzumab were obtained from Roche (Basel, Switzerland). M-PER cell lysis buffer (#78501) and halt protease and phosphates inhibitor (#78443) were obtained from Thermo Fisher Scientific (Massachusetts, United States). Enhanced chemiluminescence (ECL) reagent (RPN2232) was purchased from Amersham (GE Healthcare, Buckinghamshire, UK).

Amplification, cloning and sequencing of mouse 2A8 mAb variable region and human IgG1 and IgCκ constant region genes. Total RNA was isolated with RNA Bee-RNA isolation reagent (AMS Biotechnology, Oxfordshire, UK) from $5×10^6$ murine hybridoma cells and the corresponding cDNA was synthesized with avian myeloblastosis virus RT using oligo dT as primer (Thermo Fisher Scientific Inc, MA, USA). The variable regions of heavy (VH) and light (VL) chains of the 2A8 mAb were respectively amplified using two pairs of forward degenerate primers, as previously described (10). Subsequently, the VH and VL genes of 2A8 mAb were cloned into the pGEM-T easy vector system (Promega, WI, USA) and sequenced. The human IgG1 and IgCκ region genes were amplified from chimeric 1T0 mAb construct, which have been already produced in our laboratory (10).

Construction of the mouse–human chimeric 2A8 mAb. The heavy and light chains of chimeric 2A8 mAb were produced by Splice Overlap Extension (SOE) PCR technique which has been described elsewhere (10). Based on the VH and VL genes sequences of 2A8 hybridoma, the forward primers containing SalI and KpnI restriction sites, Kozak and the leader sequences (VH-SalI-2A8-S and Vk-KpnI-2A8-S) and the reverse primers containing 15-bp complementary regions of the human CH and CL (Jk-2A8-AS and JH-2A8-AS) were designed and the complete VH and VL genes were amplified (Table 1).

Table 1. Sequences of PCR primers.

Primer	Amplified genes	Sequence 5'----3'	Amplicon size (bp)
Vκ-KpnI-2A8-S (EXT)	Vκ2A8	TT**GGTACC**GCCACCATGGTTT TCACACCTCAGATACTTG	381
Jκ-2A8-AS		TT**GTGCAG**CCACAGTTCGTTT TATTTCCAACTTTGTCCCCG	
VH-SalI-2A8-S (EXT)	VH2A8	**GTCGAC**GCCACCATGGGAGT GCTGATTCTTTTG	408
JH-2A8-AS		CTTTGGGGTGGAGGCTGAAG GAGACGGTCG	
Cκ-S	Cκ	ACTGTGGCTGCACCATCTGTC TTCATCTTCCC	318
Cκ-XhoI-AS (EXT)		TT**CTCGAG**CTAACACTCTCCC CTGTTGAAGCTCTTTGTGACG GGCGA	
CH-S	Cγ1	GCCTCCACCAAGGGCCCATC GGTC	990
CH-BamHI-AS (EXT)		TT**GGATCC**TCATTTACCCGGA GACAGGGAGAGGCTCTT	

Restriction sites are underlined. EXTs are external primers.

The VH (or VL) and CH (or CL) purified PCR products were employed as template for Soeing PCR. They were subsequently combined and extended to produce full-length chimeric VH–CH and VL–CL sequences using external primers (EXTs) which contain BamHI and XhoI restriction sites. For Soeing PCR, two sequential rounds of PCR were carried out: in the first round, a reaction was performed in 20 μl volume, containing 1 μl of PCR product extracted from VH and CH or VL and CL, 2 mM MgSO4, 1U/μl Pfu DNA polymerase (Thermo Fisher Scientific Inc, MA, USA) and 10X reaction buffer. After 3 min denaturation at 94°C, the PCR reaction was followed by five cycles of 1

min at 92°C, 1 minutes at 58°C and 1.5 min at 72°C. The temperature was retained at 92°C for 3 min and after the addition of external primers in 5 μl volume containing 10X reaction buffer, the second round of PCR was performed by 35 cycles of 1 min at 92°C, 1 min at 58°C, 1.5 min at 72°C and a final cycle at 72°C for 10 min.

The PCR product of VH-CH was digested with SalI and BamHI restriction enzymes and inserted into the pBudCE4.1 vector (Invitrogen, Grand Island, NY, USA). After sequence confirmation, the PCR product of VL-CL was digested with KpnI and XhoI restriction enzymes and inserted into the pBud-VH-CH and the final construct containing chimeric heavy and light chains of c2A8 mAb was established (pBud-c2A8).

Transfection of chimeric construct and establishment of stable transfected cell lines. Twenty-four hours before transfection, 5×10^5 CHO-K1 cells were seeded in a 12-well plate. The pBud-c2A8 construct was prepared using Plasmid Maxiprep (Qiagen, Stockholm, Sweden) and then CHO-K1 cells were transfected with Lipofectamine 3000 reagent (Thermo Fisher Scientific Inc, MA, USA) according to the manufacturer's instruction. After 48 hours, the supernatant of transfected cells was collected and analyzed for transient expression of c2A8 mAb by ELISA. To establish a stable cell line, the transfected cells were subsequently exposed to culture medium containing 1 mg/ml of Zeocin (Gibco, NY, USA) for a minimum of 2 weeks.

Productivity and activity assessment of chimeric antibody by ELISA. An antigen-based ELISA was designed for productivity and activity assessment of chimeric 2A8 antibody (10). Briefly, 2 μg/ml of the recombinant extracellular HER2 (eBioscience Inc., CA, USA) was coated into a 96-well ELISA plate (Maxisorp, Denmark) and incubated for 1.5 h at 37°C and blocked with PBS supplemented with 0.05% Tween20 (Sigma Chemical Co, St. Louis, MO, USA) and 3% non-fat skim milk as blocking buffer. Fifty microliter of supernatants of transfected CHO cells and trastuzumab, as a positive control, were added at 37°C for 1.5 h. After three times washing with PBS supplemented with 0.05% Tween20, appropriate dilution of HRP-conjugated rabbit anti-human immunoglobulin (Sina Biotech, Tehran, Iran) was added and the plate incubated for 1 h at 37°C. After washing, the reaction was revealed with TMB substrate (Pishytaz Teb Co., Tehran, Iran). HCl was added to stop the reaction and the optical density (OD) was measured by a multi-scan ELISA reader (Organon Teknika, Turnhout, Belgium) at 450 nm.

Structural characterization of chimeric antibody by ELISA and SDS PAGE. Stable transfected cells producing c2A8 were adapted to serum-free medium (EX-CELL™; Sigma Chemical Co, St. Louis, MO, USA). The supernatant was then purified using a 5 ml HiTrap Protein G HP column (Amersham Biosciences, NJ, USA). The presence of human IgG1 and IgCκ in purified c2A8 antibody was verified by ELISA. Briefly, a 96-well ELISA plate was coated with 5 μg/ml mouse monoclonal antibody against human IgG (prepared in our laboratory) in PBS. Purified c2A8 and trastuzumab, as a positive control, at different concentrations were added at 37°C for 1.5 h. For assessment of human IgG1 and IgCκ, appropriate dilution of HRP-conjugated rabbit anti-human IgG (Sina Biotech, Tehran, Iran) and sheep anti-human IgCκ were added separately and the plate was incubated for 1 h at 37°C. After three-times washing, the reaction was revealed with TMB substrate and stopped by HCl and then ODs were measured as mentioned above.

We used SDS-PAGE for analysis of the structure of c2A8 antibody. Briefly, 0.5 microgram of purified c2A8 antibody was separated on 10% polyacrylamide gel under

non-reducing and reducing conditions, and visualized with silver staining as describe elsewhere (10).

Analysis of specific binding of chimeric antibody to recombinant HER2 by Western blotting. Comparison of specific binding of mouse 2A8 and c2A8 to recombinant HER2 was assessed by Western blot technique. Fifty nanograms of recombinant HER2 was separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Roche Diagnostics, Mannheim, Germany). The membrane was blocked with blocking buffer (PBS-0.05%Tween20 containing 5% non-fat skim milk) overnight at 4°C. After three-time washing, mouse 2A8, c2A8 and trastuzumab antibodies were added at 10 µg/ml in blocking buffer at room temperature for 1.5 h while shaking. Washing steps were repeated and appropriate dilution of HRP-conjugated sheep anti-mouse Ig and sheep anti-human Ig (Sina Biotech, Tehran, Iran) were respectively added for mouse 2A8, c2A8, and trastuzumab. After washing, polyvinylidene difluoride membrane was treated with enhanced chemiluminescence (Amersham Biosciences, NJ, USA) and the bands were visualized on Kodak X-ray film (Eastman Kodak, NY, USA).

Binding assessment of mouse and chimeric 2A8 mAbs to other ErbB family members and Cynomolgus monkey HER2. Cross-reactivity of the mouse and chimeric 2A8 mAbs with other members of ErbB family (EGFR, HER3, and HER4) as well as Cynomolgus monkey HER2 was assessed by ELISA. Recombinant EGFR, HER3 and HER4 (Speed BioSystems, Rockville, MD, USA) and Cynomolgus HER2 (Sino Biological Inc, Beijing, China) proteins were coated at 1µg/ml in 96-well flat bottom microtiter plates (Maxisorp, Denmark) for 1.5 h at 37°C. After three-time washing, mAbs were added to wells by a final concentration of 5µg/ml dissolved in blocking buffer and incubated for 90 min at 37°C. Trastuzumab was used as a positive control. Finally, appropriate dilution of HRP-conjugated rabbit anti-mouse Ig or anti-human Ig (Sina Biotech Company, Tehran, Iran) were added for 1 h and OD values were measured as previously described. All recombinant proteins contain a C-terminal 6×histidine-tag, thus the HRP anti-His tag antibody was used to approve the presence of recombinant proteins in coating layers.

Affinity constant determination by ELISA. An ELISA-based method was used to define the binding affinity of mouse 2A8 and c2A8 as previously described (11, 12). Briefly, microtiter ELISA plates were coated with different concentrations (2–0.031 µg/ml) of the recombinant extracellular of HER2. After blocking, serial concentrations of mouse 2A8 and c2A8 in blocking buffer were added into coated wells and incubated for 1.5 h at 37°C. After washing, the wells were incubated with appropriate dilution of HRP-conjugated sheep anti-mouse Ig and sheep anti-human Ig (Sina Biotech, Tehran, Iran) at 37°C for 1.5 h. After the final wash step, TMB substrate solution was added followed by stopping solution and ODs were measured. Sigmoidal curves of ODs versus the logarithm of antibody concentrations were constructed and then the affinity constant value was calculated as described elsewhere (11).

Analysis of cell surface binding of chimeric antibody by flow cytometry. 106 BT-474 cells were trypsinized, washed twice with washing buffer (PBS; 0.1% NaN3) and incubated with 100 µl of 10 µg/ml of mouse 2A8 and c2A8 antibodies as primary antibodies for 1 hr at 4°C. Mouse IgG1 mAb, human IgG of irrelevant specificity (produced in our laboratory), and trastuzumab were included as negative and positive controls, respectively. The cells were subsequently incubated with FITC-conjugated sheep anti-mouse Ig or sheep anti-human Ig (Sina Biotech, Tehran, Iran) at 4°C for 1 h.

The cells were finally scanned by a flow cytometer (Partec, Nuremberg, Germany). Flomax flow cytometry analysis software (Partec) was used to analyze the data.

Epitope mapping of chimeric antibody. An indirect ELISA was conducted for epitope mapping of mouse and chimeric 2A8 mAbs. Briefly, recombinant full extracellular domain and subdomains including DI, DII, DIII, DIV and paired subdomains with 90bp overlap including DI+II, DII+III and DIII+IV of HER2 were amplified from HER2-pCMV-XL4 construct (OriGene Technologies, Rockville, MD, USA) and subcloned to mammalian expression pSecTag2A vector and then transiently expressed in CHO-K1 cell line and their presence in supernatants was confirmed by ELISA (13). Five micrograms of purified mouse and chimeric 2A8 were coated and the plate incubated at 37°C for 1.5 h and after blocking for 1 h at 37°C, the supernatants of recombinant proteins were added and the plate incubated again at 37°C for another hour. After washing step, since all recombinant proteins are terminated with a 6×histidine-tag in their C-terminal, HRP-anti His-tag antibody (Sina Biotech Company, Tehran, Iran) was used as a detector layer. TMB substrate was added into the wells and reaction was stopped by HCl and OD was measured as described previously.

Binding inhibition of trastuzumab and pertuzumab to HER2 by mouse and chimeric 2A8. HER2 binding inhibition of trastuzumab and pertuzumab by 2A8 and c2A8 was evaluated by an inhibition ELISA. Briefly, 0.5 µg/ml of recombinant HER2 was coated in ELISA plate wells for 1.5 hours at 37°C. After blocking with PBS-0.05% Tween 20 for 1h at 37°C, different concentrations of mAbs (50, 12.5, 3.1, 0.2, 0.05, 0.012 and 0.006 µg/ml) were added to the wells. Subsequently, appropriate dilutions of HRP-conjugated trastuzumab and pertuzumab were added separately. Finally, TMB substrate was added and the reaction was stopped and OD values measured as described above.

Assessment of tumor growth and proliferation inhibition by radioactive thymidine incorporation assay. 3×10^4 BT-474 cells/well were seeded in 96-well flat-bottom tissue culture plates. The antibodies were added to wells at different concentrations (12, 4, and 0.75 µg/ml) for 72h at 37°C in a humidified atmosphere of 5% CO_2. After incubation, 3H-thymidine (PerkinElmer, MA, USA) was added at 0.5 µCi per well for 18 h Cultures were then harvested and transferred to scintillation fluid for measurement of 3H-thymidine incorporation by a β-counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden). The following formula was used to estimate the proliferation inhibition rate:

Proliferation inhibition (%) = ([Counts per minute [CPM] without antibody-CPM with antibody]/CPM without antibody) × 100

For combination treatment, half concentrations of each mAb were employed. Cells with no treatment as well as trastuzumab and pertuzumab treated cells were used as background and positive controls, respectively, and all experiments were performed in triplicate.

Antibody-dependent cell cytotoxicity (ADCC) assay. ADCC was measured by lactate dehydrogenase-releasing assay kit (CytoTox96, Promega, Madison, WI) according to the manufacturer's instruction. The BT-474 cells were chosen as the target cells. Briefly, 2×10^4 BT-474 cells were incubated with 2A8, c2A8 and trastuzumab at various concentrations and culture medium alone for 20 min in phenol red-free RPMI-1640

culture medium on ice. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma Chemical Co, St. Louis, MO, USA) from healthy adult volunteers. Bt-474 cells and PBMCs were co-incubated at 50:1 effector: target ratio for 4 hr. in 200 μl phenol red-free RPMI containing 5% FBS in a 96-well U-bottomed plate. Percentage of cytotoxicity was calculated according to the amount of LDH released into the culture supernatant. Maximum release of target cells was determined by adding the lysis buffer provided in the kit.

Percentage of specific lysis was calculated according to the following formula:

% lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) $\times$ 100.

The spontaneous release represents the level of LDH detected in culture supernatant of untreated cells. All experiments were performed in triplicate at 37°C.

Effect of c2A8 on ERK and AKT downstream signaling pathways. 2×10^6 BT474 cells were seeded in T-25 culture flask and treated with 50μg/ml of 2A8, c2A8 and trastuzumab. After 24 h incubation at 37°C in a humidified atmosphere of 5% CO2, cells were washed with ice-cold PBS, trypsinized and lysed using the cell lysis buffer. Phosphatase-protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO, USA) was added right before preparation of cell lysates according to the manufacturer's instruction. Protein concentration of cell lysates was measured using the BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA).

The concentration of lysates was adjusted and mixed with 5x sample loading buffer and lysates were loaded on 10% SDS-PAGE. The protein bands were transferred to the nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, UK) and blocked with 5% non-fat dry milk in TBS without Tween 20 for 1 hour at room temperature. Blots were incubated with P-AKT, P-ERK and β-actin specific primary antibodies for overnight at 4°C and then with HRP-conjugated anti-rabbit Ig antibody as secondary antibody for 1 h at room temperature. The target proteins were detected with the ECL kit (Amersham Pharmacia, Buckinghamshire, UK). The whole procedure was repeated for the detection of total AKT and ERK proteins, after striping the blots as described elsewhere (14). Ultimately, protein bands were scanned and analyzed with ImageJ software. All experiments were repeated three times.

Statistical analysis. The results were calculated with One-Way ANOVA test using SPSS software (version 20, IBM SPSS statistics data editor). Differences between groups were considered significant at p values less than 0.05 (p<0.05).

RESULTS

Construction of heavy and light chains of chimeric 2A8. The VH, VL, Cκ and Cγ1 genes were successfully amplified using specific primers (Figures 1A and B). The open reading frames of the VH and VL region of the parental 2A8 mAb were 408 bp and 381 in length, which encoded 136 and 127-amino acid (aa) polypeptides, respectively, including the leader peptides. The signal leader nucleotide sequences in the N-terminal regions of VH and VL encode 18- and 20-aa polypeptides, respectively. The Cκ and Cγ1 genes encode 107- and 330- aa polypeptides, respectively. Finally, the VL2A8-Cκ and VH2A8-Cγ1 segments (Figures 1C and D) were fused to each other by the Soeing PCR technique.

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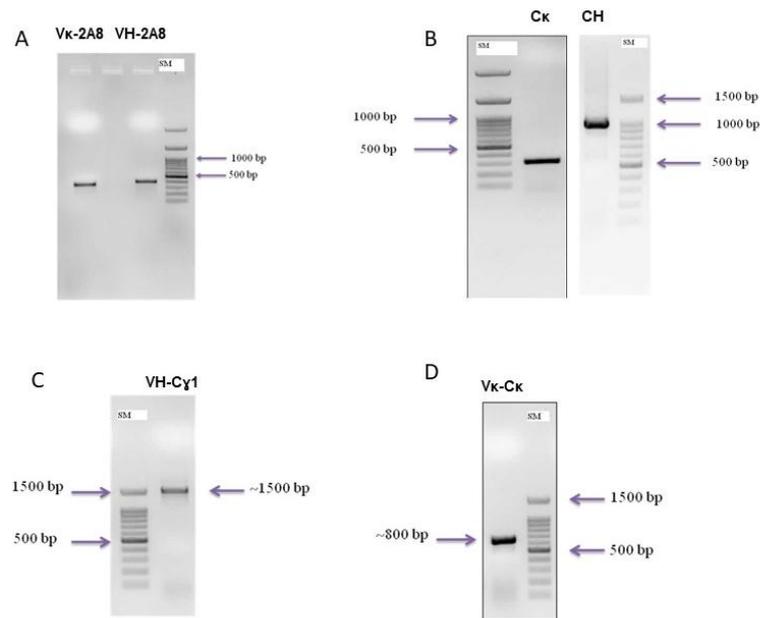
Figure 1. PCR amplification of VH-CH and VL–CL genes for construction of the chimeric 2A8 antibody. The mouse VH and VL chain genes from 2A8 hybridoma clone (A) and human constant region heavy chain of IgG1 (CH) and kappa light chain (Cκ) were amplified [10] (B). VH–Cγ1(C) and Vκ–Cκ (D) fragments were amplified by Soeing PCR. The PCR products were run on 1% agarose gel. SM: 100-bp size marker.

Expression and structural characterization of chimeric 2A8 mAb. The c2A8 construct was transfected in CHO-K1 cells by Lipofectamin transfection reagent and after selection in Zeocin and four rounds of single cell subcloning, a stable clone was selected. ELISA results showed that the expressed antibody bound to recombinant HER2 and contained human Cκ and Cγ1 (data not shown). The stable clone was maintained in a serum-free culture medium and c2A8 antibody was purified from the culture supernatant using SPG column. The purified chimeric antibody was analyzed by SDS-PAGE under non-reducing and reducing conditions. Silver staining of SDS-PAGE gel (Figure 2) showed the monomeric (~150 kDa) form of the chimeric antibody under non-reducing conditions (c2A8, lane 1) and monomeric light (~25 kDa) and heavy chains (~50 kDa) under reducing conditions (c2A8, lane 2). The parental mouse 2A8 mAb displayed a similar pattern under non-reducing and reducing conditions. Western blot analysis revealed that the chimeric and mouse 2A8 mAbs reacted only with the non-reduced recombinant extracellular HER2 protein (Figure 3), which indicates the recognition of a conformational epitope by 2A8. Trastuzumab was used as a positive control, which gave a similar pattern of reactivity with HER2.
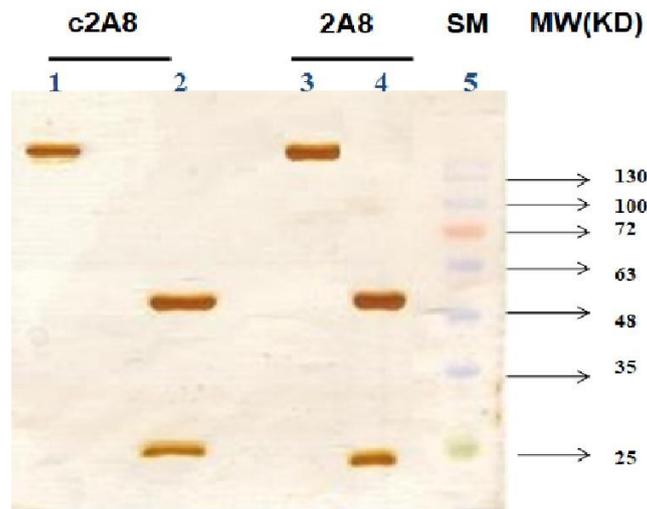
Figure 2. SDS-PAGE electrophoresis pattern of the purified chimeric antibody. SPG-purified c2A8 and mouse 2A8 IgG preparations were separated on 10% gel in non-reducing (Lanes 1 and 3) and reducing (Lanes 2 and 4) conditions. Lane 5 molecular weight size marker (SM).
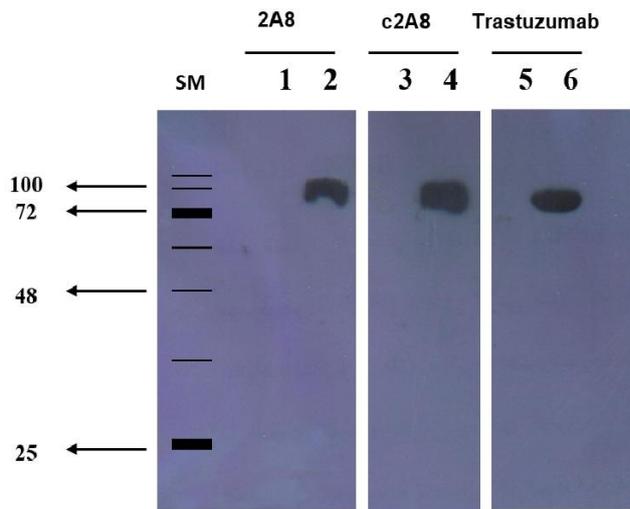
Figure 3. Western blot analysis of the purified chimeric antibody. Reduced (1, 3 and 5) and non-reduced (2, 4 and 6) forms of recombinant HER2 extracellular protein were separated on 10% SDS-PAGE gel and transferred onto PVDF membrane. The membrane was blotted with c2A8, mouse 2A8 and trastuzumab and then visualized by enhanced chemiluminescence, as described in the Materials and Methods. SM: protein size marker.

Epitope mapping of chimeric 2A8 antibody. An indirect ELISA was designed to determine reactivity of the mouse and chimeric mAbs with HER2 extracellular subdomains including I, II, III, IV, I+II, II+III and III+IV as well as full HER2-ECD. Trastuzumab and pertuzumab were used as positive control. ELISA results showed that the c2A8 similar to mouse 2A8 and trastuzumab detected subdomain DIII+IV of HER2, but not DIII or DIV alone. Pertuzumab did not recognize any single or paired recombinant subdomains of HER2 (Table 2).

Table 2. Binding profiles of mouse and chimeric 2A8 to recombinant subdomains of HER2 by ELISA.

mAbs	DI	DII	DIII	DIV	DI+DII	DIII+DIV	HER2-ECD
Chimeric 2A8	-	-	-	-	-	+	+
Mouse 2A8	-	-	-	-	-	+	+
Pertuzumab	-	-	-	-	-	-	+
Trastuzumab	-	-	-	-	-	+	+
Anti-His mAb	+	+	+	+	+	+	+

Pertuzumab and Trastuzumab were used as positive controls. Full HER2-ECD was also employed as positive control in the coating layer. Anti-His mAb was used to verify the coated recombinant proteins. The results represent OD values of <0.2 (-) and >2 (+).

Cross-reactivity of mouse and chimeric 2A8 mAbs with human HER family members and Cynomolgus monkey HER2. ELISA results showed that chimeric 2A8 like mouse 2A8 and trastuzumab reacts with recombinant human HER2, similar to Cynomolgus monkey HER2. However, none of them recognized other members of the human HER family, including HER1, HER3, and HER4 (Table 3).

Table 3. Cross-reactivity of mouse and chimeric 2A8 with human ErbB family members and Cynomolgus monkey HER2.

mAbs	HER1	HER2	HER3	HER4	HER2 (Cynomolgus)
Chimeric 2A8	-	+	-	-	+
Mouse 2A8	-	+	-	-	+
Trastuzumab	-	+	-	-	+
Pertuzumab	-	+	-	-	+
Anti-His Ab	+	+	+	+	+

Anti- His tag antibody was used to detect coated recombinant proteins.

Affinity constant determination. An ELISA method was used to determine affinity of mAbs to HER2. According to the binding curves obtained for the chimeric and mouse parental mAbs (Figure 4), the mean Kaff of mouse 2A8 and c2A8 were 1.7×10^8 M^{-1} and 2×10^8 M^{-1}, respectively.

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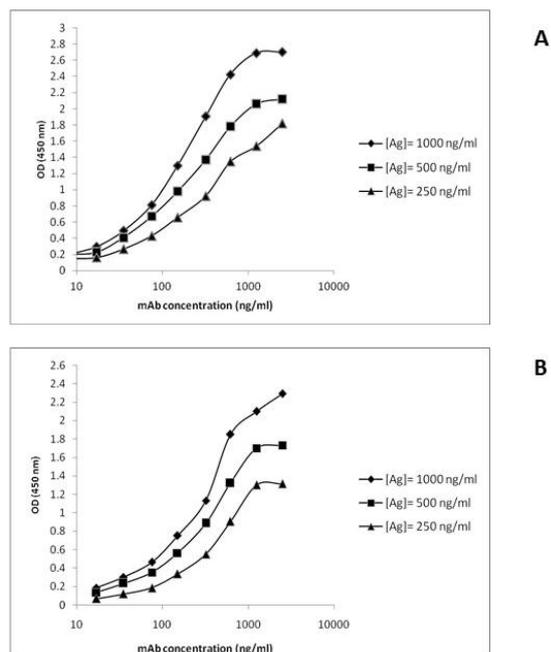
Figure 4. Affinity constant determination of the chimeric antibody. Experimental dose–response curves for (A) mouse 2A8 and (B) chimeric 2A8 mAbs at three different concentrations of recombinant extracellular HER2 protein (Ag). OD: optical density.

Cell binding activity of c2A8 by flow cytometry. Flow cytometry was conducted to determine the binding reactivity of c2A8 to the HER2-overexpressing cells. The 2A8, c2A8 and trastuzumab were used as first layer and sheep-anti human Ig-FITC and sheep anti mouse Ig-FITC as second layer, respectively. The results showed that c2A8 similar to the parental mouse 2A8 antibody detected natural HER2 expressed on the surface of BT-474 cells (Figure 5).
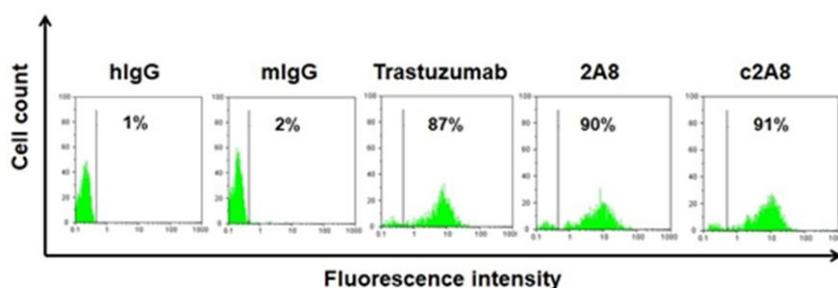
Figure 5. Binding assessment of chimeric 2A8 antibody to HER2-expressing BT-474 cells by flow cytometry. BT-474 cells were collected and stained with mouse and chimeric 2A8. Irrelevant mouse (mIgG) and human (hIgG) immunoglobulin and trastuzumab were used at the same concentration as negative and positive controls, respectively. Percentage of positive cells were represented in the figure.

Inhibition binding of trastuzumab and pertuzumab to HER2 by mouse and chimeric 2A8. The inhibition ELISA results showed that although unconjugated trastuzumab or pertuzumab binding to HER2 inhibited binding of the conjugated trastuzumab and pertuzumab in a dose-dependent manner, none of mouse or chimeric mAbs, even at the highest concentration, could inhibit binding of these mAbs to the recombinant HER2 (Figure 6).
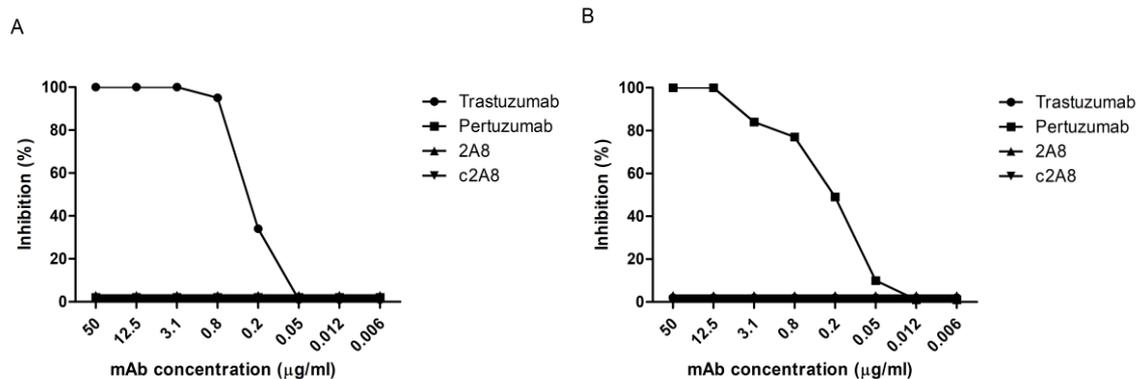
Figure 6. Cross-inhibition of trastuzumab and pertuzumab by mouse and chimeric 2A8. Binding inhibition of HRP-conjugated trastuzumab (A) and pertuzumab (B) to HER2 by different concentrations of conjugated mouse 2A8 or c2A8. Unconjugated trastuzumab and pertuzumab were used as control inhibitors.

Assessment of tumor growth and proliferation inhibition. In order to assess the effect of c2A8 on growth and proliferation of BT-474 cell line, 3H-thymidine incorporation assay was performed. The growth and proliferation inhibition rate of triplicate wells were determined and the percentage of inhibition was obtained by the formula described previously. Accordingly, c2A8 induced a dose-dependent growth and proliferation inhibition, similar to the parental mouse 2A8 (Figure 7A). Moreover, the combination of c2A8 with trastuzumab inhibited proliferation of tumor cells as efficiently as the combination of trastuzumab and pertuzumab therapeutic mAbs (Figure 7B).

Antibody-dependent cell cytotoxicity (ADCC). To evaluate the capacity of 2A8 and c2A8 to induce ADCC, BT-474 cells were incubated for 6 hr with decreasing concentrations of 2A8 and c2A8 (50, 5, 0.5, 0.05, and 0.005μg/ml). Trastuzumab was used as a control. The results demonstrated that in the existence of normal PBMC, c2A8 similar to trastuzumab triggers ADCC against HER2-positive BT-474 cells, dose dependently (Figure 8).

A

B
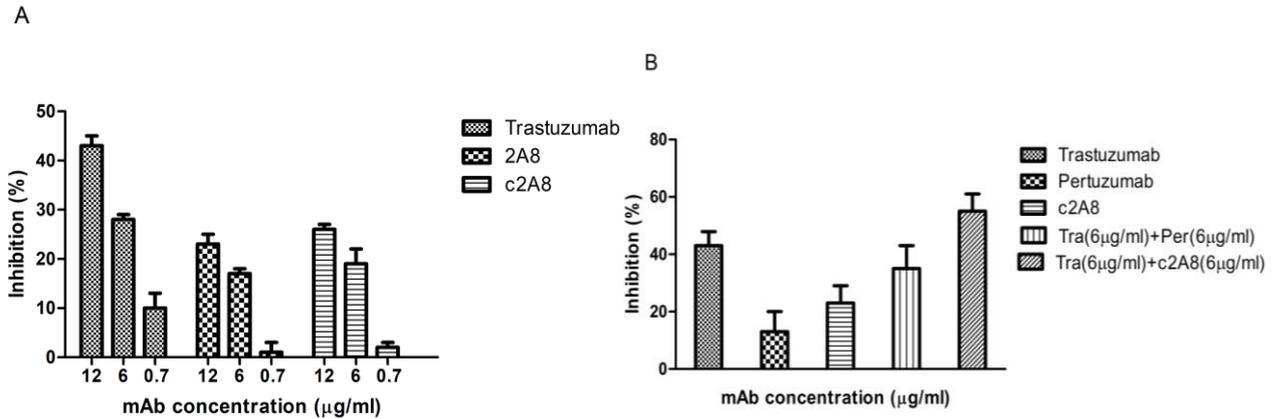
Figure 7. Assessment of tumor growth and proliferation inhibition activity of c2A8 by radioactive thymidine incorporation assay. Different concentrations of mouse 2A8, c2A8, pertuzumab, trastuzumab (A) and combination of them (B) were added to BT-474 cells. Percent of inhibition was measured as described in the Materials and Methods.

No ADCC activity, however, was seen in the existence of mouse 2A8 mAb (Figure 8) or MDA-MB-231 cell line as HER2-negative target cells (data not shown).
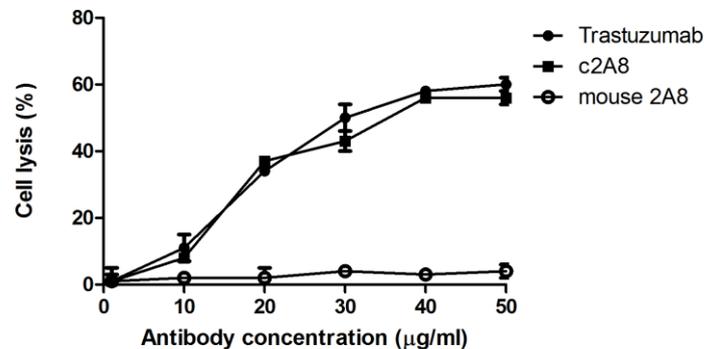
Figure 8. Antibody-dependent cell cytotoxicity (ADCC) induced by c2A8. ADCC was calculated by the level of released lactate dehydrogenase (LDH) from the BT-474 cells co-cultured with normal PBMC at 1:50 target to effector ratio after 6 h incubation. Trastuzumab and mouse 2A8 mAb were respectively used as positive and negative controls.

Effect of c2A8 on ERK and AKT downstream signaling pathways. Phosphorylation of ERK and AKT proteins was investigated following treatment of tumor cells with c2A8 by Western blot technique. The results showed that c2A8 similar to the mouse 2A8 and trastuzumab inhibited phosphorylation of ERK and AKT (Figure 9). The inhibitory effect was more evident in AKT signaling pathway. Combination of c2A8 with trastuzumab significantly reduced the level of phosphorylated AKT, while did not change the level of phosphorylated ERK, indicating its effect on AKT phosphorylation.

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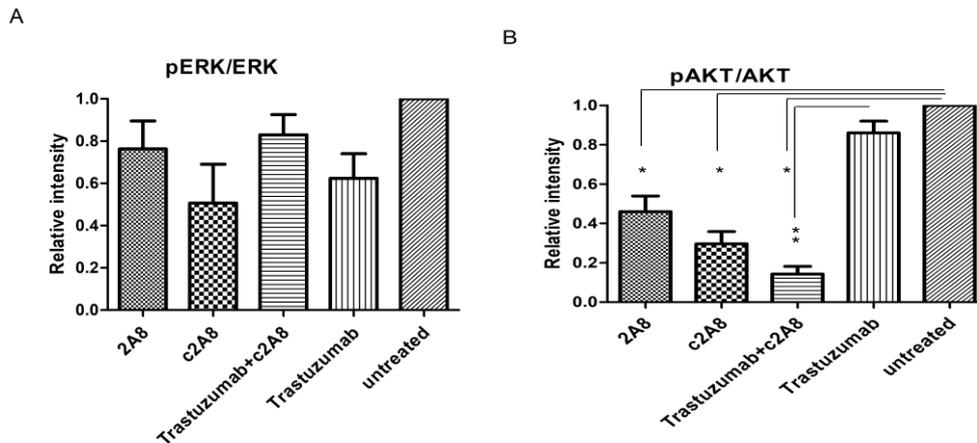
Figure 9. Influence of c2A8 mAb on ERK and AKT downstream signaling pathways. BT-474 cells were treated with 50 μg/ml of mouse 2A8, c2A8, trastuzumab and combination of trastuzumab and c2A8 for 24 hours. Cell lysates were collected and separated on 10% SDS-PAGE and transferred to PVDF membrane. The blots were stained with anti-p-AKT and p-ERK antibodies as primary and HRP-anti-rabbit as secondary antibody and then developed with ECL kit. The results are presented as band density ratio of p-ERK/total ERK (A) and p-AKT/total AKT (B). Statistically significant differences between treatment are presented; * $p \leq 0.001$ and ** $p \leq 0.0001$. Results represent the mean ± SD of three independent experiments.

DISCUSSION

HER2 is an oncoprotein which is overexpressed in breast cancer and has been used as a therapeutic target in several clinical studies using specific mAbs like trastuzumab, which binds to domain IV of HER2 and induces tumor growth inhibition (15). Primary and acquired resistance to trastuzumab is a major obstacle in clinical breast cancer therapy. One way to overcome this resistance is to use two mAbs, which recognize different non-overlaping epitopes on HER2 (16). Accordingly, pertuzumab, which binds to domain II of HER2, was approved by FDA for treatment of breast cancer in combination with trastuzumab (17). Recently, a panel of stimulatory and inhibitory anti-HER2 mouse mAbs has been produced in our laboratory, which binds to different non-overlapping epitopes of HER2 different from those recognized by trastuzumab and pertuzumab (9). Combination of trastuzumab with two of these inhibitory mAbs (1T0 and 2A8) induces a more vigorous antitumor activity than each mAb alone (8). We have already chimerized and humanized 1T0 (10,11) and in the present study, the data for chimerization of 2A8 as well as epitope mapping and its effects on signal transduction pathway have been presented.

The VH and VL genes were amplified from cDNA of 2A8 hybridoma clone by PCR. The results of sequencing show that they belong to the IGHV3-1*02 and IGκV5-43*01 immunoglobulin genes, respectively. The family specific leader sequences were designed and the full VH and VL genes were successfully amplified and joined to human CH and Cκ genes, respectively, and transferred to a dual expression vector. Finally, the construct was transfected to CHO-K1 cells. The results of ELISA on supernatant of transfected cells showed that the recombinant mAb binds specifically to recombinant HER2 and contains human CH and Cκ in its structure, which indicates successful chimerization. Generating human-mouse chimeric antibodies by

amplification of VH and VL genes from hybridoma has been reported in several studies (10,18,19). The results of immunoblotting showed that the c2A8 similar to its mouse counterpart binds to HER2 only under non-reducing condition, which suggests that it recognizes a conformational epitope. To further localize the epitope recognized by c2A8, different subdomains of HER2 including DI, DII, DIII, DIV and two adjacent subdomains DI+II and DIII+IV were designed, constructed and transiently expressed in CHO-K1 cells. ELISA results indicated that the c2A8 similar to the mouse 2A8 recognized only paired DIII+IV subdomains, which indicates that the epitope is placed within either DIII or DIV subdomain though it did not bind to DIII or DIV alone. According to trastuzumab results, the conformational epitope might be generated only by two adjacent subdomains. Similarly, Hu and coworkers (20) produced several eukaryotic recombinant single and paired subdomains of HER2 for epitope mapping of and anti-HER2 monoclonal antibody (A21). They suggested that the mAb A21 binds to DI+II, but does not bind to the single subdomain DI or DII of HER2 (20).

In another study, trastuzumab was found to recognize paired subdomains DIII+IV of HER2, but reactivity with single subdomain DIV was not tested. In contrast, Ko and coworkers (21) reported that trastuzumab could bind to DIV subdomain alone. It might be because of production of DIV as an Fc fusion protein in 293F system which maintains its three dimensional natural structure.

The results of cross-inhibition ELISA and cross-reactivity with other members of human HER family indicate that c2A8 similar to parental mouse mAb recognizes different epitope than trastuzumab and pertuzumab and does not cross-react with other members of ErbB family. The affinity constant of c2A8 antibody was highly similar to the parental mouse 2A8 mAb, which is in accordance to the other studies (10,22,23). Flow cytometry results indicates that c2A8 reacts to native HER2 on the surface of tumor cells as well as the mouse counterpart and trastuzumab, which suggests that this antibody could be employed to target HER2 positive tumor cells. Radioactive thymidine incorporation assay measures the DNA synthesis level of proliferating cells. The results of this technique demonstrated that c2A8 inhibits the proliferation of BT-474 cells dose-dependently, similar to trastuzumab and the mouse 2A8. In addition, the inhibitory effect induced by the combination of c2A8 and trastuzumab was comparable to the combination of trastuzumab and pertuzumab.

The ERK and AKT signaling pathways perform important roles in development and progression of human tumors (24). Analysis of these signaling pathways by Western blot showed that c2A8 could inhibit phosphorylation of ERK and AKT proteins similar to the mouse 2A8. Moreover, although the inhibitory effect of c2A8 and trastuzumab combination on ERK phosphorylation was similar to trastuzumab alone, this combination induced more significant inhibitory effect compared to trastuzumab alone on AKT phosphorylation. Similar results were reported by Li and coworkers (25). They showed that their humanized anti-HER2 mAb (HuA21) in combination with trastuzumab significantly inhibited ERK and AKT phosphorylation. In addition, Nahta and colleagues (26) showed that trastuzumab in combination with pertuzumab reduced the level of phosphorylated Akt more than each mAb alone. In contrast, the levels of phosphorylated p44/p42 MAPK were unaltered by the combination of these mAbs. In another study, Ko and coworkers (21) reported that their new mAb against HER2 (1E11) in combination with trastuzumab significantly downregulated both AKT and ERK phosphorylation while the total protein levels remained unchanged.

Interestingly, in our previous work we showed that the humanized 1T0 mAb similar to the parental mouse 1T0 could inhibit phosphorylation of both AKT and ERK signaling pathways. Unlike c2A8 in the present study, which can significantly inhibit only AKT pathway in combination with trastuzumab, combination of 1T0 and trastuzumab resulted in almost complete shutdown of phosphorylation of both AKT and ERK signaling pathways. This clearly indicates how combination of two or more mAbs, which recognize non-overlapping epitopes, can synergistically inhibit tumor cells growth by blocking different signaling pathways.

Since chimeric mAbs can mediate several immune effector functions through their Fc domain, ADCC was conducted to determine anti-tumor efficacy of c2A8 mAb in vitro. The results showed that unlike the mouse 2A8 mAb, the chimeric construct similar to trastuzumab induces tumor cells lysis in a dose-dependent manner. Similarly, Nishihara and coworkers (27) generated a chimeric antibody against pancreatic cancer (c-Nd2) and determined its ADCC activity. They reported that the ADCC activity of chimeric mAb was significantly higher than the parental mouse mAb.

Taken together, these data suggest that our chimeric anti-HER2 mAb (c2A8) is a novel inhibitory antibody with potential application for treatment of HER2-overexpressing malignancy.

ACKNOWLEDGEMENTS

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