In Vitro Selection of RNA Aptamer and Specific Targeting of ErbB2 in Breast Cancer Cells

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The ErbB family (also referred to as HER/neu or HER) of receptor tyrosine kinases plays major roles in the formation and progression of human tumors. Amplification and/or overexpression of ErbB2 have been reported in numerous cancers, including breast, ovarian, stomach, bladder, salivary, and lung cancers. As ErbB2 has been used as a target for the treatment of advanced cancer, RNA aptamers for the extracellular domain of the ErbB2 were selected from a RNA library consisting of 2'-fluorine-modified RNA transcripts. After 15 cycles of selection, high-affinity RNA aptamer was isolated. Binding patterns of the selected RNA aptamer clones were evaluated to choose RNA aptamers that were specific to the extracellular domain of ErbB2 protein. RNA aptamer 15-8 was the best candidate and its minimal version (mini-aptamer) was chemically synthesized. Surface plasmon resonance measurement showed that the mini-aptamer specifically bound to the ErbB2 protein with high affinity and specificity. To evaluate its potential as an ErbB2-targeting molecule in breast cancer cells, specific recognition of the mini-aptamer was confirmed with various breast cancer cell lines. We propose that the selected RNA aptamer is a potential cancer imaging agent by targeting malignant cells overexpressing the ErbB2 receptor.

Introduction

ERBB RECEPTORS ARE cell surface receptor tyrosine kinases. The human ErbB family consists of the epidermal growth factor receptor (EGFR or ErbB1) and its homologs ErbB2, ErbB3, and ErbB4 (also termed HER2/neu, HER3, and HER4, respectively) (Yarden and Sliwkowski, 2001). ErbB receptors are essential mediators of cell proliferation and differentiation in the developing embryo and in adult tissues (Olayioye et al., 2000). Overexpression of several members of this receptor family, especially EGFR and ErbB2, is associated with a variety of solid tumor malignancies. Overexpression of ErbB2 is found in 20%–30% of breast cancers (Slamon et al., 1987) as well as in ovarian, stomach, bladder, salivary, and lung cancers (Schneider et al., 1989; Berchuck et al., 1990; Coombs et al., 1991; Lemoine et al., 1991; Press et al., 1994). Crystal structure of ErbB2 reveals that extracellular domain of the receptor is always in an open conformation, meaning that ErbB2 can be activated in the absence of ligand engagement (Cho et al., 2003; Baselga and Swain, 2009). Moreover, heterodimerization of ErbB2 with the other ErbB receptors shows more aggressive behavior in growth (Baselga and Swain, 2009).

In patients with breast cancer, ErbB2 overexpression is associated with poor prognosis, aggressiveness of the disease, as well as resistance to chemotherapy and hormonal therapy. Thus, strategies to target ErbB2 are important in treating advanced breast cancer. ErbB2-targeted therapy typically utilizes Herceptin (trastuzumab), a humanized mouse monoclonal antibody 4D5. Herceptin binds to extracellular domain IV of ErbB2 (Cho et al., 2003) and is used as a treatment of ErbB2-overexpressing breast cancers (Pegram et al., 1999). As Herceptin was approved by the U.S. Food and Drug Administration for patients with invasive breast cancers, it is necessary to monitor in advance the expression of ErbB2 in a patient to guide Herceptin therapy.

Aptamers are molecules selected from a high-complexity library by a repeated *in vitro* selection procedure known as systematic evolution of ligands by exponential enrichment (Tuerk and Gold, 1990). RNA aptamers exhibit high affinity and exquisite specificity, comparable to those of antibodies, but the average sizes are one-tenth of antibodies (Soontornworajit and Wang, 2011). Also, RNA aptamers are easily generated with various forms of modifications by site-specific incorporation of radiolabels,

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fluorescent probes, or crosslinking reagents depending on their utilities (Lee et al., 2006). For example, the use of fluorine in the 2-position significantly enhances the half-life of RNA aptamers in serum (Ulrich et al., 2004). Such advantages of RNA aptamers make them as good targeting molecules for cancer imaging and therapy. Several reports demonstrated the validity of 2 RNA aptamers (tenascin-C aptamer and prostate-specific membrane antigen aptamer) as cancer cell imaging (Lupold et al., 2002; Hicke et al., 2006) and targeting tools by conjugation with radioisotope or fluorescent dye labeling (Bagalkot et al., 2007). RNA aptamer for ErbB3 has been developed, but it only binds to the oligomeric form of ErbB3, limiting its utility as a cancer-targeting tool (Chen et al., 2003; Park et al., 2008).

In the present study, we isolated the RNA aptamer for ErbB2 and showed it to have high affinity to extracellular domain of ErbB2 protein. We also generated a minimized version of RNA aptamer (mini-aptamer), which demonstrated high specificity to ErbB2-positive, but not EGFR-positive, cancer cell lines. The selected RNA aptamer is a potentially useful cancer imaging agent by targeting ErbB2-overexpressing malignant cells.

Materials and Methods

Preparation of the RNA library and target protein

A DNA library consisting of random 50 nucleotide (nt) sequences was synthesized by Integrated DNA Technologies (Coralville, IA). In the library, a random region is flanked by defined sequences including T7 promoter and several restriction enzyme sites for *in vitro* transcription and the cloning purposes. The 5' and 3' defined sequences were 5'-CGG AATTCGTAATACGACTCACTATAGGGACGCGTGGTACC-3' and 5'-TTGGATCCCCGCGGAAGCTT-3', respectively. The 2'-fluorine-modified RNA (2' FY-RNA) library consisted of T7 *in vitro* transcription with the random DNA libraries (1.0×10^{14} molecules) and modified NTPs (Epicentre, Madison, WI). After transcription, the template DNA was removed by DNase I digestion and the resulting 2' FY-RNA was purified by phenol:chloroform extraction and ethanol precipitation. The 2' FY-RNA band of expected size was cut out from a 6% polyacrylamide/7M urea gel and eluted with 0.5 M ammonium acetate, 0.2% sodium dodecyl sulfate, and 1 mM EDTA for 3 hours at 37°C. The purified 2' FY-RNA libraries were quantified with an ultraviolet spectrophotometer. Recombinant glutathione S-transferase (GST)-tagged ErbB2 protein (22–122 amino acids) was purchased from Abnova (Taipei, Taiwan).

In vitro selection of RNA aptamers

Selections were performed with 4 µg of the original 2' FY-RNA libraries in RNA binding buffer [20 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, 2 mM dithiothreitol, and 40 U RNase inhibitor] with an RNA:GST-ErbB2 protein molar ratio of 5:1. The basic selection procedure was similar to that previously described (Kim et al., 2002). After 11th round of selection, RNA:GST-ErbB2 protein molar ratio was reduced to 40:1 to strengthen the selection pressure. After 15 rounds of reiterated selection, the ErbB2-binding RNA was converted to DNA and amplified with primers containing restriction sites for *EcoRI* (in the 5'

primer) and *BamHI* (in the 3' primer). Using these restriction sites, the DNA sequences were inserted into pUC19 and the recombinant DNA was transformed into *Escherichia coli* DH5 α . Plasmid DNAs were prepared from 20 independent colonies and their sequences were determined with an ABI Prism automatic sequencer (Applied Biosystems, Carlsbad, CA).

RNA binding assay

The affinity of the RNA for ErbB2 was assessed by a GST pull-down assay with radiolabeled RNA and GST-ErbB2 protein. To measure binding affinity of the selected RNA, 1 nM of the labeled original RNA or the selected RNA was incubated with either GST or GST-ErbB2 fusion protein (50 nM) in the RNA binding buffer for 30 minutes at room temperature. RNA-protein complex was precipitated with glutathione-sepharose 4B bead. Unbound RNA was removed by bead washing and the bound RNA was eluted from the bead by phenol extraction and loaded onto a 6% polyacrylamide/7M urea gel. The gel was dried and the bound RNA was directly visualized by autoradiography.

Synthesis of minimal RNA

The mini-RNA (5'-AGCCGCGAGGGGAGGGGAUAGGG UAGGGCGGCGCU-3') and control RNA (5'-GGGGCAUA CUUGUGAGACUUUUAUGUCACCCCC-3') were synthesized with amino-terminal-conjugated biotin residues by Samchully Pharm. (Seoul, Republic of Korea).

Biosensor assay

A BIAcore 3000 (GE Healthcare, Buckinghamshire, United Kingdom) was used in surface plasmon resonance (SPR) experiments. To attach the biotin-conjugated minimal RNA to the CM5 sensor chip, the surface of the chip was preequilibrated with HEPES and activated with 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide by modifying the carboxymethyl groups of dextran. After preactivation, streptavidin was injected into 2 of the flow cells. After immobilizing the streptavidin, the surface of the chip was deactivated with 1 M ethanolamine hydrochloride (pH 8.5). The biotin-conjugated mini-RNA was injected into one of the flow cells and binding buffer was injected into the other. When the baselines had stabilized, various concentrations of GST-ErbB2 were injected. Following each experiment, the sensor chip was regenerated with 10 mM NaOH. Kinetic parameters were obtained with the BIA evaluation program. Three independent measurements were performed with 8 concentration points of the protein.

Cells and cell culture

Human MDA-MB-453, T47D, KPL4, and MCF-7 breast cancer cells (American Type Culture Collection, Rockville, Maryland) were cultured in Dulbecco's modified Eagle's medium (WelGENE, Seoul, Republic of Korea) with 10% fetal bovine serum (WelGENE). Human A431 epidermoid carcinoma cells were cultured in RPMI 1640 medium (WelGENE) supplemented with 10% fetal bovine serum.

Immunocytochemistry

Cells were grown on coverslips and fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. They were washed and blocked in 10% normal calf serum and 0.5% gelatin in phosphate-buffered saline for 1 hour at room temperature. Staining was carried out with biotin-labeled mini-RNA for 1 hour at room temperature. The quantum dot-conjugated streptavidin (Invitrogen, Carlsbad, CA) was applied at room temperature for 50 minutes. The cells were counterstained with Hoechst33258 and visualized by microscopy using an Axio-plan 2 microscope (Carl Zeiss, Jena, Germany).

Results

Selection of RNA aptamers to ErbB2

To select high-affinity aptamers for ErbB2 protein, 100 amino acids from the extracellular domain of the ErbB2 were used as a target protein. We used an RNA library consisting of 2' FY-RNA, because 2' FY-RNA is completely resistant to RNase A and has improved stability in storage (Ulrich et al., 2004). Individual RNA molecules were isolated from the RNA pools after 15 cycles; we identified 6 clones with similar sequences (Fig. 1A). To estimate the binding affinity of the RNA aptamers, we performed a GST pull-down assay with 6 RNA

A SE15-6 **AAATACCAATAAGGAAGCAA**. **GGCAGAACGGG**. **GCCTACTGTGTATATCATC** (1)
SE15-8 **AAAAGTTGTGAGGGGAGGGATAGGGTAGGGCA****CGACTAGTCAAGAAAATG** (1)
SE15-10 **CAATACTGAAAAGGAAGCGATAGGAA****CGAGTG**. **TGCAAAATTAATAGTGCA** (1)
SE15-15 **. . TCACAGAGACTATAGAGGGAGGGGAGTGGGAGGATAGTTACTGTGTTTT** (2)
SE15-19 **AAAGGTTGTGAAGGGAGGGATAGGGTAGGGCA****CGACTAGTCAAGAAAATG** (1)
Consensus **AAATACTGAGAAGGGAGGGATAGGGGAGGGCG**. **GACTAGTCAATAAAATG**

FIG. 1. Selection of ErbB2-binding RNA aptamers. **(A)** Sequences of the selected RNA aptamers. Numbers shown in parentheses are the numbers of independent clones with the same sequence. Identical nucleotides are shown in bold. **(B)** Glutathione S-transferase (GST) pull-down analysis. Pull-down was performed with labeled RNA aptamer (1 nM) and GST (50 nM) or GST-ErbB2 (50 nM) protein. Bound RNA was resolved in a 6% polyacrylamide/7M urea gel. B, bead; G, GST; E, GST-ErbB2. **(C)** GST pull-down analysis. Pull-down was performed with labeled RNA aptamer (1 nM) and GST or various concentrations of GST-ErbB2 (0–50 nM) protein. RNA was resolved in a 6% polyacrylamide/7M urea gel.

aptamer clones. Most of the clones displayed relatively high affinity in comparison to the original library, clone SE15-8 being the best binding RNA aptamer (Fig. 1B). To assess the binding affinity of RNA aptamer SE15-8 in an equilibrium condition, a trace amount of this RNA aptamer was incubated with various concentrations of the ErbB2 protein. Binding affinity was estimated as the half-binding concentration of the

ErbB2 protein; that of RNA aptamer SE15-8 was estimated to be about 10^{-9} M (Fig. 1C).

Characterization of mini-RNA aptamer

To be used as a cancer-targeting tool, the size of the targeting molecules should be carefully modulated to increase the accessibility to the cancer target site, but not to be rapidly cleared from the body. Therefore, we decided to minimize the selected high-affinity RNA aptamer because of potential difficulty as a full-length RNA molecule. First, we analyzed the structure of the RNA aptamer by use of the mfold (<http://mfold.rna.albany.edu/?q=mfold>) and pknotsRG RNA folding program (<http://bibiserv.techfak.uni-bielefeld.de/pknotsrg/submission.html>) to predict the secondary structure of the high-affinity aptamer SE15-8 (Fig. 2A). Defined sequences were found at the ends (positions 1–15 and 66–82) of RNA aptamer SE15-8, whereas the selected RNA sequences were mostly predicted to form a long stem-loop structure. We therefore generated mini-RNA aptamer (mini-aptamer) with 34 nt that contained the stem-loop structure (spanning nt 19–52) as that of the full-length RNA aptamer SE15-8. The mini-aptamer was synthesized with amino-terminal-conjugated biotin residues to be used as the streptavidin-binding ligand. Binding affinity of the mini-aptamer was investigated by SPR analysis. After immobilizing the biotinylated mini-aptamer to the streptavidin-coated sensor chip, various concentrations of the ErbB2 protein were injected. An example of SPR profile shown in Fig. 2B clearly shows that the binding was stable with low dissociation rate, and the binding affinity of the mini-aptamer was determined to be about $3.49 \pm 1.3 \times 10^{-9}$ M by BIA evaluation program (Fig. 2C).

FIG. 2. Characterization of mini-RNA aptamer binding to ErbB2 protein. (A) Predicted secondary structures of RNA aptamer SE15-8 (Full-length) and SE15-8 truncated mini RNA aptamer (Mini). (B) Surface plasmon resonance binding profile of mini-RNA aptamer. Arrow indicates increasing concentration of ErbB2 (150, 300, 600 nM). GST protein (1000 nM) was used as a control. (C) Dissociation constants (Kd) are presented as means of 3 independent surface plasmon resonance measurements.

Specific binding to ErbB2-expressing cancer cells

Previous biochemical analyses clearly demonstrated the high affinity of RNA aptamer as well as mini-aptamer on recombinant ErbB2 protein. However, it was very important to characterize the specific binding of the RNA aptamer to the expression of the ErbB2 protein on the cancer cell surface. To assess the utility of the ErbB2-binding aptamer, we utilized various cancer cell lines that did or did not express ErbB2. To detect ErbB2 on the cancer cell surface, we conjugated the mini-RNA aptamer to Qdots and tested its recognition on ErbB2-positive MDA-MB-453 breast cancer cells. As expected, the mini-aptamer stained the MDA-MB-453 cells as a membrane-bound form (Fig. 3A). The signal seemed to be specific, because we could not detect any when the RNA with similar size was used. Similar to MDA-MB-453 cells, mini-aptamer stained T47D breast cancer cells (data not shown). The other ErbB2-positive breast cancer cell, KPL-4, was also specifically stained with mini-aptamer (Fig. 3B). However, the mini-RNA aptamer did not show any staining of the ErbB2-negative MCF-7 cell line (Fig. 3C). Most significantly, we could not detect any signal from the A431 ErbB1-positive cell line (Fig. 3D), which strongly supports our notion that we selected an ErbB2-specific aptamer that can be used for specific targeting of the ErbB2 in cancer tissue *in vivo*.

Discussion

Here, we report the isolation of the RNA aptamer for extracellular domain of ErbB2 protein and showed high affinity and specificity to ErbB2-expressing breast cancer cell lines.

FIG. 3. Specific recognition of ErbB2-expressing cells by the mini-RNA aptamer. Cells were incubated with the biotin-labeled RNA aptamer (50 nM) and Qdot (20 nM). Scale bar: 20 μ m. (A) MDA-MB-453; (B) KPL-4; (C) MCF-7; (D) A431. Color images available online at www.liebertonline.com/nat

Therefore, the selected RNA aptamer might be potentially useful as a cancer imaging agent by targeting ErbB2-over-expressing malignant cells.

ErbB2 overexpression is correlated with aggressive tumor behavior and poor clinical outcome. Therefore, ErbB2 has become an important prognostic and predictive factor as well as a target for molecular therapies. On this account, monoclonal antibody or affibody are approached as ErbB2-targeting agents for imaging tool. Clinical study using the monoclonal antibody zirconium-89–trastuzumab showed that optimal dosage and time of administration of zirconium-89–trastuzumab determined for positron emission tomography imaging of ErbB2-positive lesions in patients with metastatic breast cancer (Dijkers et al., 2010). However, the clinical application of antibodies to imaging is limited because of their large size. Small scaffold protein affibody is stable and relatively small compared with antibody, so ErbB2-specific affibody Z_{HER2:342} was used as a specific near-infrared probe for *in vivo* optical imaging of ErbB2-expressing tumors (Lee et al., 2008).

In comparison to antibody or affibody, aptamers present several advantages for imaging: high affinity and specificity, smaller size for a better tissue penetration, and easy incorporation of chemical modification. In addition to aptamer as a therapeutic tool, it could be used as an imaging tool for *in vivo* diagnostic, such as tenascin-C imaging aptamer (Hicke et al., 2006). We hope to develop ErbB2-binding aptamer reporter here as an imaging tool for advanced breast cancer patients.

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Author Disclosure Statement

No competing financial interests exist.

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