Isolation of RNA Aptamers Targeting HER-2-overexpressing Breast Cancer Cells Using Cell-SELEX

Hye Suk Kang, Yong Min Huh,[†] Soyoun Kim,[‡,*] and Dong-ki Lee[*]

Global Research Laboratory for RNAi Medicine, Department of Chemistry and BK21 School of Chemical Materials Science, Sungkyunkwan University, Suwon 440-746, Korea. [*]E-mail: dklee@skku.edu
[†]Department of Radiology & Department of Biochemistry and Molecular Biology, Yonsei University, Seoul 120-752, Korea
[‡]Department of Biomedical Engineering, Dongguk University, Seoul 100-715, Korea. [*]E-mail: skim@dongguk.edu
Received May 14, 2009, Accepted June 16, 2009

Ligand molecules that can recognize and interact with cancer cell surface marker proteins with high affinity and specificity should greatly aid the development of novel cancer diagnostics and therapeutics. HER-2/ErbB2/Neu (HER-2), a member of the epidermal growth factor receptor family, is specifically overexpressed on the surface of breast cancer cells and serves as both a useful biomarker and a therapeutic target for breast cancer. In this study, we aimed to isolate RNA aptamers that specifically bind to a HER-2-overexpressing human breast cancer cell line, SK-BR-3, using Cell-SELEX strategy. The selected aptamers showed strong affinity to SK-BR-3, but not to MDA-MB-231, a HER-2-underexpressing breast cancer cell line. In addition, we confirmed the specific targeting of HER-2 receptor by aptamers using an unrelated mouse cell line overexpressing human HER-2 receptor. The HER-2-targeting RNA aptamers could become a useful reagent for the development of breast cancer diagnostics and therapeutics.

Key Words: HER-2, Cell-SELEX, Aptamer, Breast cancer, siRNA

Introduction

Ligand molecules which can bind to a specific cancer cell type, by interacting with cancer cell surface proteins with high affinity and specificity, is critical for developing cancer diagnostics and therapeutics. The ligand molecule in turn can be utilized to identify the cancer biomarker proteins.[1] Understanding the biology of these biomarkers can further facilitate the development of novel cancer therapeutics.

HER-2/ErbB2/Neu (HER-2) is a transmembrane receptor tyrosine kinase, which is a member of the epidermal growth factor receptor family.[2] Overexpression of HER-2 is observed in 20 to 30% of breast cancers, and predicts for a poor clinical outcome.[3] A HER-2 ectodomain-directed monoclonal antibody (trastuzumab or Herceptin) has been approved for the treatment of breast cancer.[2] HER-2 is also an attractive target for *in vivo* imaging[4] and targeted drug delivery for breast cancer.[5]

Aptamers are single-stranded DNA or RNA oligonucleotides which can fold into specific three-dimensional conformation to bind targets with high affinity and specificity.[6] The targets for aptamers include small molecules, peptides, proteins, and even whole cells.[7] High affinity aptamers for specific targets can be isolated from a randomized oligonucleotide library *in vitro* by using the Systematic Evolution of Ligands by EXponential enrichment (SELEX) process.[8-10]

Aptamers have several advantages over antibodies, such as smaller size, better tissue penetration, ease of chemical modification, and the lack of immune responses. For *in vivo* imaging applications, aptamers are especially superior over antibodies with faster kinetics and higher signal-to-noise ratio, owing to their smaller size.[11] Aptamers can decorate the surface of magnetic nanoparticles used for magnetic resonance imaging (MRI), to enhance the specificity of imaging.[12] As a cancer therapeutics, aptamers can either directly inhibit the cancer growth signaling pathway by blocking growth factor or growth factor receptors,[13] or be used as "escort aptamers"[11] to deliver therapeutic molecules such as cytotoxic agents, radionuclides, or even small interfering RNAs (siRNAs) to the target cells. Therefore, generation of specific aptamers targeting different cancer cell types and cancer cell surface proteins would greatly aid the development of novel cancer diagnostics and therapeutics.

However, isolation of aptamers targeting specific cancer cell types is challenging because for many cancer cell types, specific surface biomarker proteins are not known. Even if the biomarker protein specific to certain cancer type is known, membrane protein is typically difficult to express and purify, or the purified membrane protein might not have the native conformation as it is present on the cell membrane, so that aptamers selected against purified protein might not recognize the same protein on the cell surface.[14] Therefore, a method is required to isolate aptamers targeting membrane proteins in its native form as presented on the cell surface.

Recent development of Cell-SELEX strategy allows researchers to generate aptamers specifically targeting proteins on the cell surface,[14] or specific cancer cell types even when the biomarker protein is not known.[15] In this study, we report the selection of RNA aptamers targeting HER-2-overexpressing SK-BR-3 cells using Cell-SELEX strategy. The selected aptamers showed strong affinity to SK-BR-3, but not to MDA-MB-231, a HER-2-underexpressing breast cancer cell line. Specific targeting of HER-2 receptor by aptamers was further

confirmed by demonstrating that the aptamer could bind to an unrelated mouse cell line, NIH3T3, when it overexpresses the human HER-2 receptor.

Experimental Section

Cell and culture conditions. Human breast cancer cell lines SK-BR-3, MDA-MB-231, and a mouse fibroblast cell line, NIH-3T3, were obtained from the American Type Culture Collection and cultured at 37 °C in McCoy's 5A media (Welgene) for SK-BR-3, RPMI-1640 media (Gibco BRL) for MDA-MB-231 and DMEM media (Gibco BRL) for NIH-3T3 supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 ug/mL streptomycin, Gibco BRL), respectively. NIH3T6.7 cell, which is a HER-2 overexpressing NIH-3T3 cell line, was maintained in DMEM media. Cells were regularly subcultured to maintain exponential growth.

Transfection. For counter SELEX using siHER-2, SK-BR-3 cells were plated in 60 mm plates in complete medium without antibiotics and incubated for 24 h until they reached 50% confluency. 10 nM of siRNA was then transfected using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. After 24 h, the transfected cells were counted and 1×10^6 cells were incubated with the aptamer pool.

SELEX library and primers. The PAGE-purified random library 40 mer (5'-ATA CCA GCT TAT TCA ATT NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NAG ATA GTA AGT GCA ATC T-3') was used to generate the RNA library. N40 upstream primer (5'-GGTAATACGACTCACTATAGGGAGATACCAGCTTATTCAATT-3') and downstream primer (5'-AGATTGCACTTACTATCT-3') were used for library PCR. A TAMRA-labeled 3'-primer (5'-TAMRA-AGATTGCACTTACTATCT-3') was annealed with the aptamer for affinity test using fluorescence microscopy.

Cell SELEX. Cells were harvested by trypsinization and 1×10^6 cells were recovered in complete media at 37 °C for 30 min. 160 pmole of RNA library was dissolved in binding buffer (4.5 g/L glucose, 5 mM $MgCl_2$, 0.1 mg/mL yeast tRNA, 1 mg/mL BSA in Dulbecco's PBS) and denatured at 95 °C for 5 min followed by cooling on ice immediately. The recovered cells were washed twice with washing buffer (4.5 g/L glucose, 5 mM $MgCl_2$ in Dulbecco's PBS) and incubated with prepared RNA library in binding buffer at 4 °C for 45 min. Cells were washed twice with washing buffer, and then the bound RNA aptamers were eluted by heating at 95 °C for 5 min and separated by phenol:chloroform:isoamyl alcohol (PCI, Bioneer) and chloroform extraction. The obtained RNA was reverse-transcribed using ImProm-II™ Reverse Transcription System (Promega) and PCR-amplified. The purified PCR product was subjected to *in vitro* transcription using T7 polymerase (Ambion). In the case of negative selection the bound RNA pool was incubated with the HER-2 negative cell line (MDA-MB-231) or siHER-2-transfected SK-BR-3 cells, respectively.

Quantitative RT-PCR. Both the pre-bound (in-put) aptamer pool and post-bound (out-put) aptamer pool were used as a template for cDNA synthesis, which was performed with the ImProm-II™ Reverse Transcription System (Promega) according to the manufacture's protocol. Aliquots (1/250) of the cDNA reaction mixture were analyzed by quantitative real-time PCR on a Step-One real-time PCR machine (Applied Biosystems) according to the manufacturer's protocol. Ratio of input to the output signal was calculated.

Fluorescence microscopy. For binding with SK-BR-3 and MDA-MB-231 cells, 1 μM 3'-TAMRA-labeled aptamer was incubated with cells for 45 min at 4 °C. In the case of NIH-T6.7 cell line, cells were incubated with 500 nM aptamers for 20 min at 37 °C. Following a series of two washing steps to remove unbound aptamers, fluorescence signal was detected using microscopy (Olympus) with 400 X magnification.

Kd determination. Diluted S6 aptamer (10 nM to 1 μM) and pool in the binding buffer were incubated with 1×10^5 cells at 4 °C for 45 min in 60 mm culture dish. After two-times washing, cells collected into eppendorf tube, then bound RNAs were eluted with same procedure as mentioned in the Cell-SELEX process. The amount of aptamers that were bound to the cells was quantified by qPCR. Saturation curves were plotted based on qPCR data and the dissociation constants of aptamer calculated by non-linear regression analysis.

Western blot analysis. The expression of HER-2 protein in cultured cells was detected by Western blot analysis. Cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). After cell lysis at 4 °C for 30 min with vortexing, supernatant was collected after centrifugation of the samples at 12000 rpm for 10 min, and protein was quantified with Protein Assay Reagent (Pierce). 50 μg of protein was separated by SDS-PAGE and electrotransfered onto a polyninylidene difluoride (PVDF) membrane (Millipore). The blots were blocked with 5% skim milk in Tris-buffered saline (TBS) at room temperature for 1 h and incubated with 1 : 500 diluted primary antibody overnight at 4 °C. The blots washed three times in TBS-Tween 20 for 5 min and then incubated with 1 : 2000 diluted horseradish peroxidas-labeled goat anti-mouse IgG in TBS-Tween 20 for 1 h at room temperature. After washing 5 times in TBS-Tween 20 for 6 min, the proteins were visualized with an enhanced chemiluminescence (ECL plus) reagent (Amersham Bioscience). Mouse monoclonal anti-c-ErbB2/c-Neu antibody and goat anti-mouse HRP conjugate were purchased from Calbiochem and BIO-RAD, respectively.

Results and Discussion

To isolate aptamers with high affinity and specificity to the native, membrane-presented form of HER-2, we performed Cell-SELEX using a well-known HER-2-overexpressing breast cancer cell line, SK-BR-3.[16] We started the SELEX procedure by using a RNA library of 40 nt randomized region, with 3×10^{13} complexity (Fig. 1). First, we performed positive selection by retrieving aptamers that bind to SK-BR-3 cells. After 14 rounds of positive selection, the binding affinity of RNA library reached saturation (data not shown). For the next six rounds, we included negative selection using two separate methods in parallel (Fig. 1). For one selection, we used MDA-MB-231 cell line, another breast cancer cell line with little HER-2 expression, as a negative cell line, and removed any

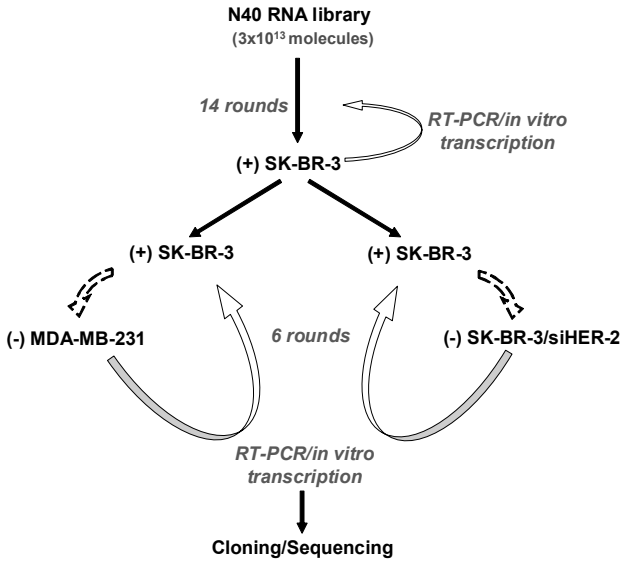

Figure 1. Cell-SELEX Scheme. See the text for details. (+); positive selection, (-); negative selection.

aptamers that bind to this cell line. For the other selection, we adopted a novel negative selection strategy, by knocking-down HER-2 expression in SK-BR-3 using HER-2 specific siRNA. After siRNA treatment, we incubated aptamers with the cell line and removed any binders.

After negative selection, we performed affinity test using first counter SELEX product. As shown in Fig. 2a, both negative selection using negative cell line (MDA-MB-231), and siRNA-based negative selection resulted in good discrimination between two cell lines. This suggests that the negative selection using siRNA was successful.

After the successful enrichment of aptamers with high affinity to SK-BR-3 cells, individual aptamers were cloned and sequenced. Aptamers from two different counter-selection approaches had similar sequences so that they were grouped together. We were able to group individual aptamer sequences into three groups, as shown in Fig 2b. Interestingly, Group II could be further divided into two subgroups, Group II-I and II-II (Fig. 2b). The two subgroups shared conserved 3'-half of the sequences, but there 5'-half sequences were divergent. In addition, we noticed that some aptamers had longer than 40 nt randomized region, suggesting that some sequences were inserted during the SELEX cycle.

To visualize the binding of aptamer to breast cancer cell lines, we indirectly labeled individual aptamers by annealing with a tetramethylrhodamine (TAMRA)-labeled oligonucleotide complementary to the 3'-constant region of aptamers. We then incubated the aptamers with breast cancer cell lines, and the bound aptamers were visualized by fluorescence microscopy (Fig. 2c). Whereas pool RNA did not show strong affinity to both cell lines tested, selected aptamers showed preferential binding to SK-BR-3 cell line over MDA-MB-231

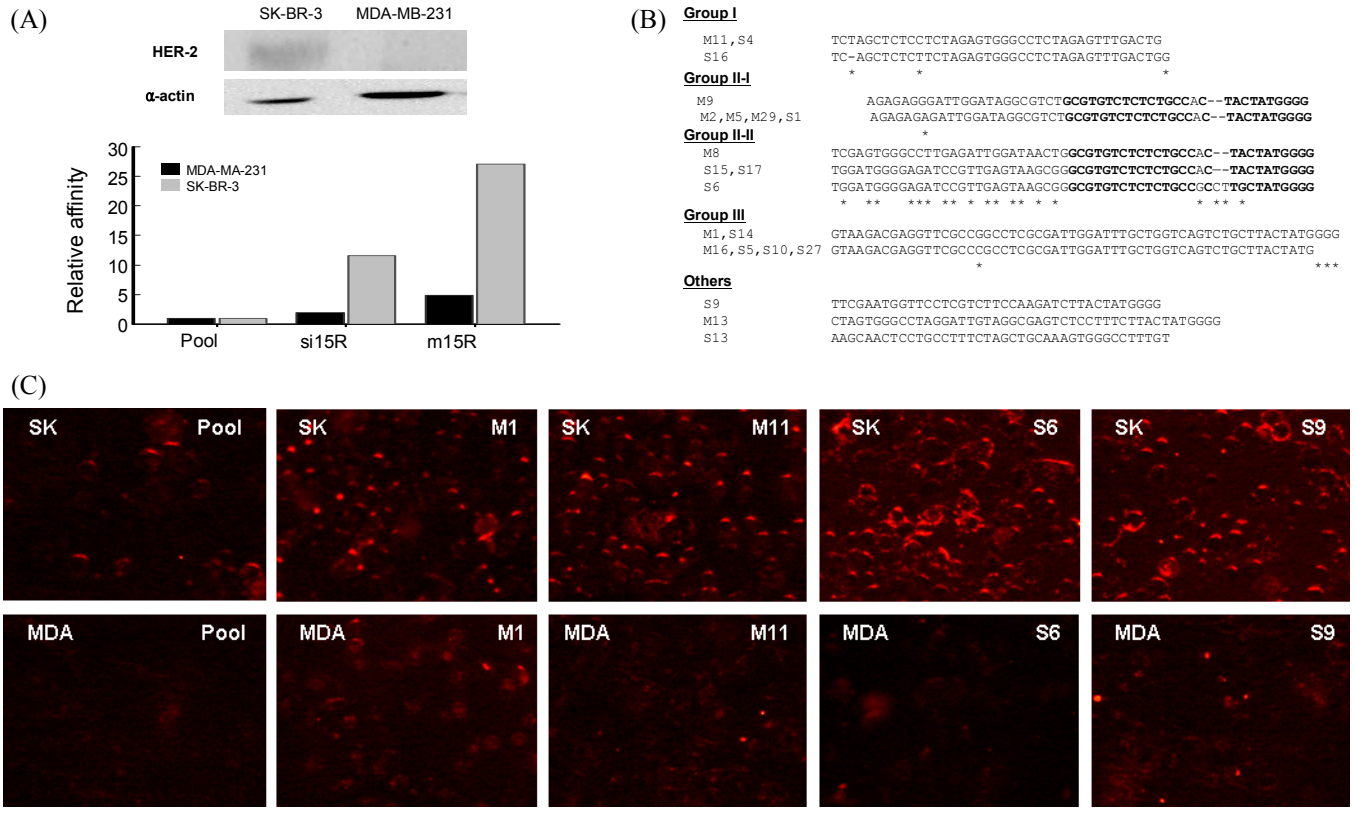

Figure 2. (A) (left panel) HER-2 expression level in SK-BR-3 and MDA-MB-231 cell lines was detected by western blot analysis. (Right panel) Affinity test using qPCR. Comparision of aptamer pool affinity before and after counter selection. si15R and m15R represent HER-2 siRNA treated SK-BR-3 and MDA-MB-231 counter SELEX product. (B) Sequence alignment of selected aptamers. (C) Affinity of 5'-TAMRA labeled individual aptamer clones visualized by fluorescence microscopy.

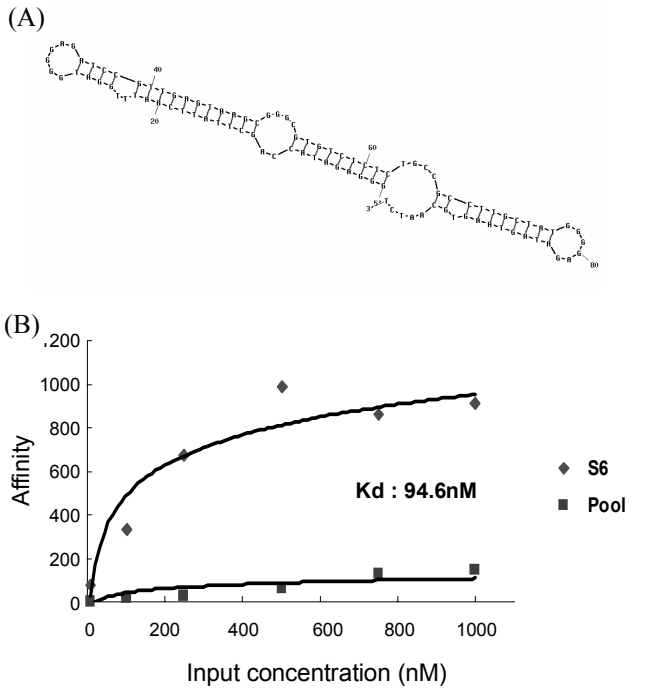

Figure 3. (A) secondary structure of S6 aptamer predicted by Mfold (B) Determination of the dissociation constant (Kd) for S6 aptamer.

cell line (Fig. 2c). Among these aptamers tested, the S6 aptamer showed strongest binding to the SK-BR-3 cell line but bound only weakly to the MDA-MB-231 cell line (Fig. 2c). Therefore, we further characterized S6 aptamer.

The secondary structure of S6 aptamer was predicted using Mfold program (Fig. 3a). We then measured the equilibrium dissociation constant (K_d) for the S6 aptamer by using a constant number of cells incubated with varying concentrations of RNAs. Using a non-linear regression analysis, the Kd value of S6 aptamer was determined as 94.6 nM (Fig. 3b).

While the S6 aptamer showed preferential binding to HER-2-overexpressing SK-BR-3 cells over HER-2-underexpressing MDA-MB-231 cells, there is a possibility that the target of S6 aptamer may not be the HER-2 receptor, but another cell surface protein which is enriched more in SK-BR-3 cells than in MDA-MB-231 cells. To confirm that the S6 aptamer targets the HER-2 receptor, we tested S6 aptamer's affinity to NIH-3T3/HER2, a NIH-3T3 cell line overexpressing human HER-2. As shown in Fig. 4, both fluorescence microscopy analysis and RT-qPCR-based affinity test demonstrated that S6 aptamer has higher affinity to the NIH-3T3/HER2 cell line than to the original NIH-3T3 cell line. These data confirms that the target of S6 aptamer is the human HER-2 receptor, not other proteins expressed on the surface of SK-BR-3 cells.

In this study, using Cell-SELEX, we isolated RNA aptamers which can bind with high affinity and specificity to HER-2. We also developed a siRNA-based counter-selection approach, which could be broadly applied to future Cell-SELEX experiments targeting specific cell surface receptors. The advantage of using siRNA-based counter-selection approach is that one

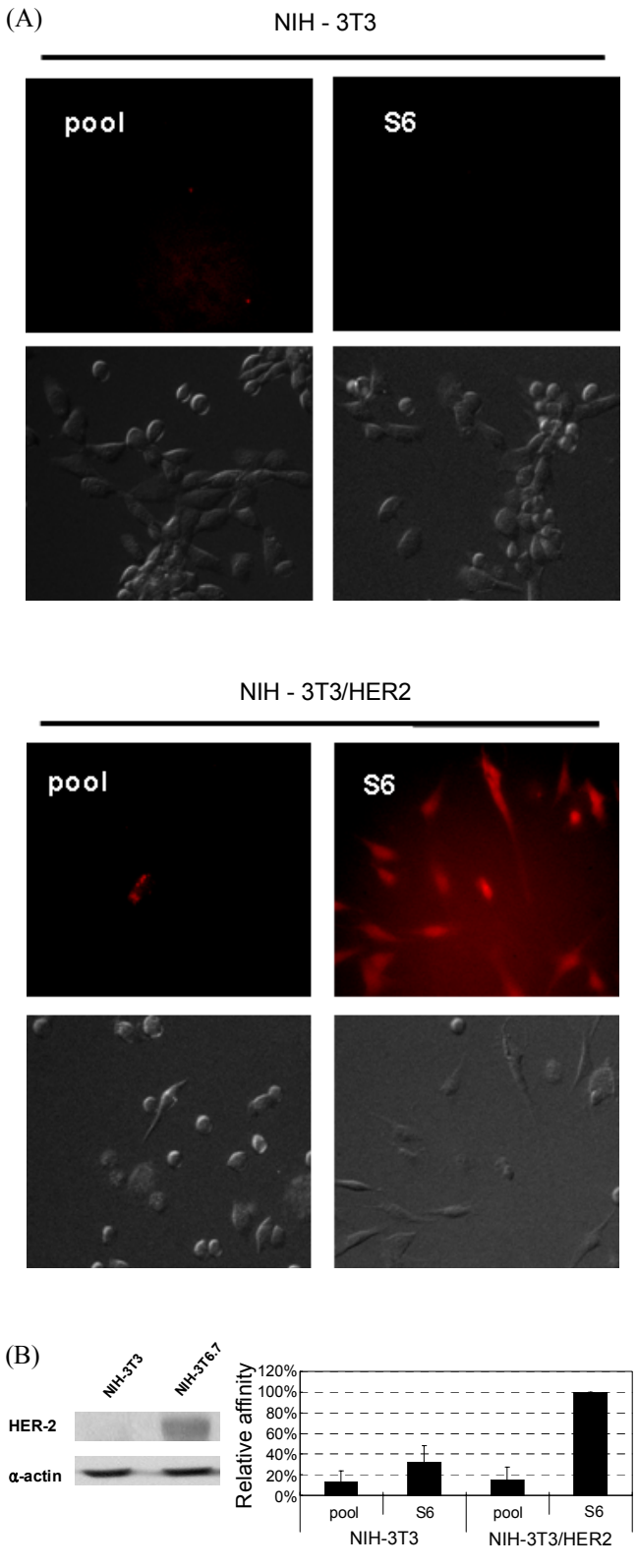

Figure 4. Specificity of S6 aptamer against HER-2 overexpressing cells. (A) Affinity of 5'-TAMRA labeled S6 aptatmer to NIH3T6.7 cells visualized by fluorescence microscopy. Aptamer was incubated with cells for 20 min at 37 °C. (B) (left panel) HER-2 expression level in NIH3T6.7 cell line was detected by western blot analysis. (Right panel) Affinity test of S6 aptamer to NIH3T6.7 cells by qPCR at the same condition with (A).

does not need to clone and express the receptor protein in unrelated cell lines to execute Cell-SELEX.

For *in vivo* diagnostic and therapeutic applications, the selected aptamers need to be optimized further. To confer resistance to serum nucleases, chemical modifications such as 2'-OMe or 2'-F in the ribose sugar should be introduced.[17] In addition, minimal domain required for HER-2 binding can be identified, and could be multimerized to maximize the avidity of aptamers against the target.[18] Upon these modifications, the HER-2-targeting RNA aptamers could provide a starting point to develop novel strategies for in vivo diagnostics, targeted drug delivery, and therapeutic applications against breast cancer.

Acknowledgments. We thank Pooja Dua for critical reading of the manuscript. D.-K. L. was supported by the Global Research Laboratory grant from KICOS (grant no. 2008-00582), and National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (grant 0520200-3). S. K. acknowledges the support from KOSEF (National Research Laboratory) and Ministry of Knowledge Economy (no. 10032 113). This work was also supported by the Small and Medium Business Administration (#08-1-059).

References

1. Shangguan, D.; Cao, Z.; Meng, L.; Mallikaratchy, P.; Sefah, K.; Wang, H.; Li, Y.; Tan, W. *J. Proteome Res.* **2008**, *7*, 2133.
2. Roskoski, R., Jr. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 1.
3. Xia, W.; Bisi, J.; Strum, J.; Liu, L.; Carrick, K.; Graham, K. M.; Treece, A. L.; Hardwicke, M. A.; Dush, M.; Liao, Q.; Westlund, R. E.; Zhao, S.; Bacus, S.; Spector, N. L. *Cancer Res.* **2006**, *66*, 1640.
4. Tolmachev, V. *Curr. Pharm. Des.* **2008**, *14*, 2999.
5. Chiu, S. J.; Ueno, N. T.; Lee, R. J. *J. Control. Release* **2004**, *97*, 357.
6. Nimjee, S. M.; Rusconi, C. P.; Sullenger, B. A. *Annu. Rev. Med.* **2005**, *56*, 555.
7. Dua, P.; Kim, S.; Lee, D. K. *Recent Pat. DNA Gene Seq.* **2008**, *2*, 172.
8. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.
9. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818.
10. Robertson, D. L.; Joyce, G. F. *Nature* **1990**, *344*, 467.
11. Hicke, B. J.; Stephens, A. W. *J. Clin. Invest.* **2000**, *106*, 923.
12. Yigit, M. V.; Mazumdar, D.; Kim, H. K.; Lee, J. H.; Odintsov, B.; Lu, Y. *Chembiochem* **2007**, *8*, 1675.
13. White, R. R.; Sullenger, B. A.; Rusconi, C. P. *J. Clin. Invest.* **2000**, *106*, 929.
14. Cerchia, L.; Duconge, F.; Pestourie, C.; Boulay, J.; Aissouni, Y.; Gombert, K.; Tavitian, B.; de Franciscis, V.; Libri, D. *PLoS Biol.* **2005**, *3*, e123.
15. Shangguan, D.; Meng, L.; Cao, Z. C.; Xiao, Z.; Fang, X.; Li, Y.; Cardona, D.; Witek, R. P.; Liu, C.; Tan, W. *Anal. Chem.* **2008**, *80*, 721.
16. Faltus, T.; Yuan, J.; Zimmer, B.; Kramer, A.; Loibl, S.; Kaufmann, M.; Strebhardt, K. *Neoplasia* **2004**, *6*, 786.
17. Bunka, D. H.; Stockley, P. G. *Nat. Rev. Microbiol.* **2006**, *4*, 588.
18. Shi, H.; Hoffman, B. E.; Lis, J. T. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10033.