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Massive Identification of Cancer-Specific Nucleic Acid Ligands

Young Ju Lee and Seong-Wook Lee*

Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, Seoul 140-714, Korea

Abstract

Targeting of complex system such as human cells rather than biochemically pure molecules will be a useful approach to massively identify ligands specific for the markers associated with human disease such as cancer and simultaneously discover the specific molecular markers. In this study, we developed *in vitro* selection method to identify nuclease-resistant nucleic acid ligands called RNA aptamers that are specific for human cancer cells. This method is based on the combination of the cell-based selection and subtractive systematic evolution of ligands by exponential enrichment (SELEX) method. These aptamers will be useful for cancer-specific ligands for proteomic research to identify cancer-specific molecular markers as well as tumor diagnosis and therapy.

Keywords: cancer, proteomics, RNA aptamers, SELEX

RNA can adopt stable tertiary structures to specifically bind target molecules with high affinities and specificities and encode easily amplificable genetic information. Therefore, RNAs could be a very useful agent for basic research and diagnostic and therapeutic aims (Gold *et al.*, 1993; Burgstaller *et al.*, 2002). Such RNA ligands with short size, termed RNA aptamers, have been identified for a host range of targets including proteins, carbohydrates, and small chemicals, etc., from a random RNA library using *in vitro* iterative selection techniques, called systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Recently, several RNA aptamers were reported to have potentials of diagnostic ligands and even therapeutic potentials from animal disease models (Sullenger and Gilboa, 2002; Hwang *et al.*, 2003; Rusconi *et al.*, 2004). Targeting a complex target mixture such as human red blood cell ghosts was reported to identify a pool of aptamers using selection procedure, called 'complex targets SELEX' (Morris *et al.*, 1998). Such targeting of complex systems rather than biochemically pure molecules will be useful to massively identify specific molecular markers associated with human disease and simultaneously identify ligands specific for the markers without prior knowledge of any molecular changes associated with the disease state (Daniels *et al.*, 2003).

In this study, we established *in vitro* SELEX method which is based on the combination of the cell-based selection with subtractive SELEX method for the selection of nuclease-resistant RNA aptamers specific for the cancer cell surface.

SELEX procedure for RNA aptamers to cancer cell

We selected RNase-resistant RNA aptamers to specifically recognize the target cancer cell lines, Jurkat T leukemia cells, but not normal peripheral blood mononuclear cells (PBMC). A random RNA library of ~10^{14} different molecules was created with every pyrimidine modified at its 2' position by a fluoro group. The sequence of the RNA library was 5'-GGGAUACCA GCUUAUUCAAUUN$_{60}$AGAUAGUAAGUGCAAUCU-3', where N_{60} denotes 60 nucleotides (nts) with the equimolar incorporation of A, G, C, and U at each position. Therefore, each molecule in the library harbored 60-nt long region derived from a randomized sequence flanked by defined sequences. The modification of the 2' position of RNA improved its stability in human serum more than 10,000 fold, when compared with unmodified 2' hydroxyl RNA (Seo and Lee, 2000; Lee and Sullenger, 1996, 1997). We employed subtractive SELEX procedure to preclude nonspecific RNAs that bind to normal cells as follows. Five μg of the RNA library was first incubated at 37℃ for 1 hr with 1.5x10^5 of normal PBMC in 200 μl of a binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA), and any RNAs that had bound to the molecules on the normal cell surface have been discarded by centrifugation. The

*Corresponding author: E-mail SWL0208@dankook.ac.kr, Tel +82-2-709-2905, Fax +82-2-798-4733
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•23: TCCCGGGTGAGTTGCCCAAGCATAGATAGTCCGTGGTATCCTGGCTATCTAGTGTCCTGT
•3:          TG-GCTGCACACG-GTAAGCA-TCCTTGGTATCC-GGTTTTTAAGT-TGGT-TTGATTGTTCGCG

•5:  CCGTTGGTTTCCTC-GAAAT--CCGTTCAAGC--GAG--GGGTATCC--TTTA-CGTTTGTGAGCTGTGG
•20:           CCACCGCACTTTCTGGACTAGCTAGAGCCGGATATCCCGTTTGTCATATCCCATCCGTG

•12: GAATACATCGTGAGC-TGGC---TGTAG--CTACCTGAGAATAGGACTGCG--TTTAAACGTG---CGGTG
•26:            GGGCATGGCGGATGCAGAGCTTCCCCACATTAGTCCTACAACTTTGTTTGTGTCACGGTG

•14: GTTACTATCGCC-GA-GTTGCGG--CTAGGTATCGCTGTCGTGATCTT-ACTCGA--CTAGT-TC--CCG
•25:         GTCCAGACGTA-CGGGCCTAGA-ATTGCTAGCAAGAGCTTGACACTATGCATGTATCACCCG

•5:            CCGTTGGTTTCCTCGAAATCCGTTCAAGCGAGGGGTATCCTTTACGTTTGTGAGCTGTGG
•21: GTCGATGCTTAAGT-GATTTA-TC---CTCAATACAA-CGAGTGG-ATCAAT---GTTTGTACGC-GTGG

•22: GAGCGTA-CG-A-GC-GAACCACACTACATT---CC--CGA--TTGTTT-TCTC-CGACTGTCCGTTGTGTTG
•26: GGGCATGGCGGATGCAGAGCTTCCCCACATTAGTCCTACAACTTTGTTTGTGTCACGG-TG

•29: TGACAGCTGTGAGAGTAATTGTC-CATCGGTA-TC-CAC----GTCAGAGCGAAT--TCCAC--CTCG
•30:        CGCTGAGTGTATTTGACTCAACGGTAATCGC-CTGTTGTCAG-GCCCATGATCCGCAGCTCG
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Fig. 1. Sequences of selected 2'-fluoro RNA aptamers. Selected RNAs against Jurkat cells were reverse transcribed, and the resulting cDNAs were PCR amplified and subcloned. Fourteen different clones were sequenced. Clones containing similar sequences were grouped together. C and U in this figure correspond to 2'-fluoro C and 2'-fluoro U, respectively.

precleared supernatant RNA pools were then reacted with 1.5×10^5 of the target Jurkat cells, and the leukemia cell-RNA complexes precipitated. Bound RNAs were amplified and transcribed to generate RNA for the next cycle of selection, as previously described (Bae *et al.*, 2002; Lee and Sullenger, 1996, 1997). The subtractive procedure was performed prior to every selection round. After total 13 rounds of selection, the amplified cDNA was cloned and sequenced. Different RNAs were selected probably due to diverse target molecules on the surface of the Jurkat cells (Fig. 1).

Cancer-specific binding of the selected RNA aptamers

To assess the enrichment of the selected RNAs, pool of amplified cDNAs of the selected RNAs was *in vitro* transcribed, and the purified RNA (200 ng) was incubated with 5×10^5 of PBMC or Jurkat cells. The bound RNAs were then reverse transcribed, and the resulting cDNA was amplified (forward primer, 5'-GGGTAATACGACTCACTATAGGGATACCAGCTT ATTCATT: reverse primer, 5-AGATTGCACTTACTATCT) with 10 thermal cycles. The semi-quantitative RT-PCR analysis of the selected RNA aptamers bound to cells showed that the RNAs were enriched specifically to the Jurkat cells (data not shown).

To confirm that the pool of the selected RNAs specifically bound to the target cells, flow cytometry

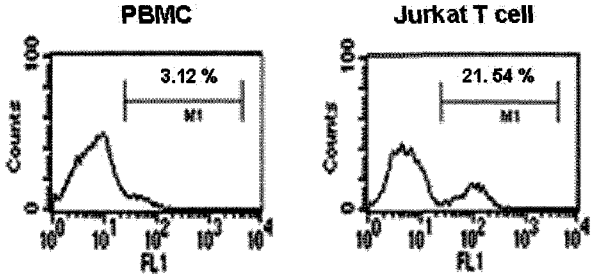

Fig. 2. Flow cytometry analysis of FITC labeled RNA aptamers. FITC-labeled 13th round pool RNA aptamers were incubated with PBMC or Jurkat T cells, and the RNA binding was monitored by FACS analysis.

analysis was performed with FITC-labeled aptamers (Fig. 2). FITC-labeled RNA aptamers were prepared as described (Rosemeyer *et al.*, 1995). Briefly, *in vitro* transcribed RNA aptamers (121 pmol) were labeled at their 3' ends by incubation with 5X reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml BSA, 0.5 mM dNTP), 0.5 mM FITC-dUTP (Roche), 5 mM $CoCl_2$, and 20 U terminal deoxynucleotide transferase (Roche) in a final volume of 30 $\mu\ell$ for 30 min at 37℃. FITC-labeled RNA aptamers (30 pmoles) were then incubated with 1×10^6 normal PBMC or Jurkat cells. FITC fluorescence was monitored using FACScan apparatus (Becton Dickinson). Results were represented as frequency distribution histograms of log fluorescence. FITC-labeled RNA pools after 13^{th} round bound more strongly to Jurkat T cells, by about 7-fold in fluorescence intensity, than to the normal PBMC. By contrast, FITC-labeled RNA library hardly bound to either PBMC or Jurkat cells (data not shown). These results indicate that the selected RNA aptamers specifically bound to the Jurkat T leukemia cells.

Recently, large efforts are focused on the identification of specific messages associated with a wide range of human diseases including cancers (Strausberg *et al.* 2004). Conventional SELEX procedure could be conducted to isolate cancer-specific RNA aptamers with purified proteins after expression of the specific messages. However, an obvious advantage of the cell-based subtractive SELEX developed here is that a specific disease such as cancer could be targeted without prior knowledge of any molecular alterations related to the disease state (Daniels *et al.*, 2003). Noticeably, any nonspecific RNAs that can bind to nontargeted cell surface could be easily eliminated by employing *in vitro* preclusion step which is simply incubating nondesirable cells and discarding RNAs binding to the cells. These aptamers can be applied to specific diagnosis, imaging, and therapy of tumor (Lupold *et al.*, 2002). Moreover, characterization of molecular targets against the RNA aptamers will be of important use to identify novel specific molecular markers of the cancer.

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