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In Vitro Selection of Cancer-Specific RNA Aptamers

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Abstract In this study, nuclease-resistant RNA aptamers that are specific for Jurkat T leukemia cells were selected by a subtractive systemic evolution of ligands by exponential enrichment (SELEX) method. A randomized nuclease-resistant RNA library was incubated with normal peripheral blood mononuclear cells (PBMC) in each round to preclude RNAs that recognize the common cellular components on the surface of normal and cancer cells. The precluded RNAs were used for the selection of Jurkat T cell-specific aptamers, and the specific RNAs were then gradually enriched from start to the following selections. After 16 rounds of the subtractive SELEX, the selected aptamers were found to preferentially bind to Jurkat T cells, but not to the normal PBMC, evidenced by fluorescence-activated cell sorting analysis. Thus, the subtractive SELEX can be used to identify ligands to cancer-specific biological markers without prior knowledge of the nature of markers. The aptamers could be applied to specific cell sorting, tumor therapy, and diagnosis, and moreover, to find cancer cell-specific markers.

Key words: Cancer, FACS, Jurkat T cell leukemia, RNA aptamers, SELEX

Characteristics of RNAs that can adopt complex structures to specifically bind target molecules with high affinities and encode easily amplifiable genetic information make the RNA a potentially very useful tool for basic research, as well as for diagnostic and therapeutic purposes [2, 5]. Such short RNA ligands, termed RNA aptamers, have been identified to be specific for a wide variety of targets from a random RNA library, using *in vitro* iterative selection techniques, called systemic evolution of ligands by exponential enrichment (SELEX) [4,24]. Selected RNA aptamers show high specificity for their targets and can distinguish even the very similar domains of individual proteins in the same classes [1] or nearly identical low molecular weight compounds [10, 11]. The therapeutic potential of several RNA aptamers has successfully been evaluated even in animal disease models [6, 20, 23].

Recently, a selection procedure, called complex targets SELEX, has been reported to identify a pool of aptamers, which could recognize different targets in a complex target mixture such as human red blood cell ghosts [16]. Such targeting of complex systems rather than biochemically pure molecules would be a very useful approach to discover specific molecular markers associated with human diseases such as cancer, and simultaneously identify ligands specific for the markers. A combination of the cell-based selection with the subtractive SELEX method, which involves the subtraction procedure before each round of selection to remove nonspecific aptamers, would be helpful to further identify specific aptamers for specific sub-cell types [25]. If aptamers, which specifically bind certain type of cancers but not normal tissues, could be isolated, these aptamers would be useful in the therapeutics, imaging, and diagnosis of cancers [15].

In the present study, we established a cell-based subtractive SELEX for the selection of nuclease-resistant RNA aptamers that bind with high specificity to molecules on the cell surface of Jurkat T cells (ATCC TIB-152), which was derived from a human T cell leukemia.

An RNA library of ~10^{14} different molecules was first generated with every pyrimidine nucleotide substituted at its 2' position with a fluoro group (2'-fluoro-pyrimidines) by the *in vitro* transcription of synthetic DNA templates with 2'-deoxy-2'-fluoro CTP and UTP (Epicentre Technologies) and normal GTP, ATP, and T7 RNA polymerase [21]. This modification of the 2' position of RNA enhanced its stability in human serum more than 10,000-fold, compared with unmodified RNA [12, 13, 21]. In addition, RNAs with 2'-fluoro group have high affinity, because the RNAs form substantially strong intramolecular helices, thereby leading to thermodynamically stable and rigid secondary structures [17]. The sequence of the resulting RNA library

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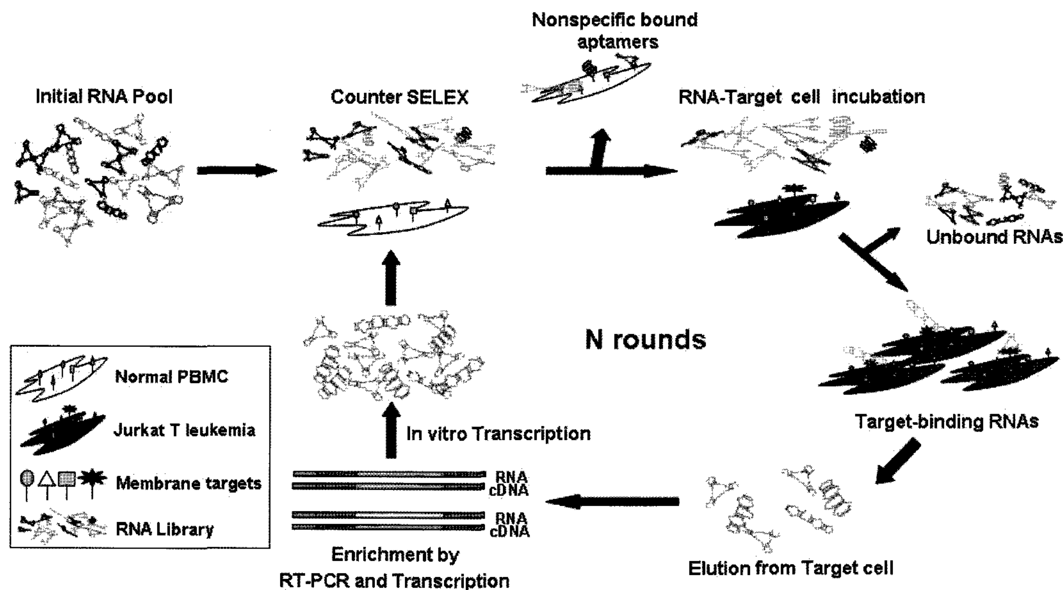

Fig. 1. Schematic diagram of the subtractive SELEX procedure.
A random sequence 2'-fluoro pyrimidine RNA library was first incubated with normal PBMC. Unbound RNA pools were then incubated with target Jurkat T leukemia cells, and the RNAs bound to the target cells were eluted and amplified using RT-PCR and *in vitro* transcription. The iterative procedure of the subtraction and SELEX was carried out to identify cancer cell specific RNA aptamers. The finally enriched RNAs were amplified, and the corresponding cDNAs were cloned and sequenced.

was 5'-GGGAUACCAGCUUAUUCAAUUN_{60}AGAUA-GUAAGUGCAAUCU-3', where N_{60} represents 60 nucleotides (nts) with the equimolar incorporation of A, G, C, and U at each position. Thus, each molecule in the library contained a 60-nt long region derived from a randomized sequence flanked by defined sequences.

Because a large number of available target molecules that are common to normal cells could also exist on the surface of cancer cells, a preclusion step to remove RNAs that bind to the common targets is needed for screening of cancer-specific aptamers. To eliminate any RNAs that bind to the normal cell surface, a subtractive procedure was developed with normal peripheral blood mononuclear cells (PBMC) from a normal donor (Fig. 1). PBMC were isolated from 100 ml of a normal donor peripheral blood, which was collected into a lithium heparin collection tube, diluted 1:1 with phosphate-buffered saline (PBS), and then centrifuged on a Lymphoprep™ (Axis-Shield) cushion for 20 min at 2,000 rpm at 20°C. The mononuclear band was collected, washed twice with PBS, and resuspended in RPMI with 10% FBS on a 35-mm culture dish to give 5×10^5 cells per ml. The cells were treated with 25 ng/ml phobol myristate acetate (Sigma-Aldrich) and 2 μM ionomycin (Sigma-Aldrich), incubated at 37°C for five to six days in a humidified incubator, and used for following SELEX experiments. Five μg of the RNA library was first incubated with 1.5×10^5 PBMC at 37°C for 1 h 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) in the presence of additional 750 pmol of tRNA prior to each round of selection. The cell-RNA complexes were separated by centrifugation, and any RNAs bound to the molecules on the normal cell surface were discarded. The precleared supernatant RNA pools unbound to the PBMC were then transferred to a new tube, and incubated with the 1.5×10^5 target Jurkat T cells at 37°C for 1 h using the binding buffer with tRNA. The leukemic cell-RNA complexes were separated by centrifugation, washed five to seven times with 0.5 ml of the binding buffer, and resuspended in 100 μl of TE buffer with 10 mM EDTA. The bound RNAs were recovered by heating at 75°C for 5 min and phenol extracted, amplified, transcribed, and used for next rounds of selection, as previously described [1, 12, 13]. Subtraction of RNAs with normal PBMC was employed twice (rounds 5–6) or three times (rounds 7–16) to increase stringency. In rounds 14–16, selection was performed with a 10-fold reduced amount of the selected RNA (0.5 μg). After total 16 rounds of selection, the amplified cDNA was cloned using pGEM-T vector (Promega) and sequenced. The finally selected RNAs were *in vitro* transcribed from the cloned cDNA [7, 8], and used for aptamer binding assay or flow cytometry analysis.

Enrichment of RNA aptamers specific to the Jurkat cells was monitored using semiquantitative RT-PCR of RNAs bound to the cells (Fig, 2). RNA library and RNA pools after the 6th, 11th, or 16th rounds of selection were tested for binding normal PBMC or Jurkat cells. The purified RNA (200 ng) was incubated with each cell sample (5×10^5 cells/

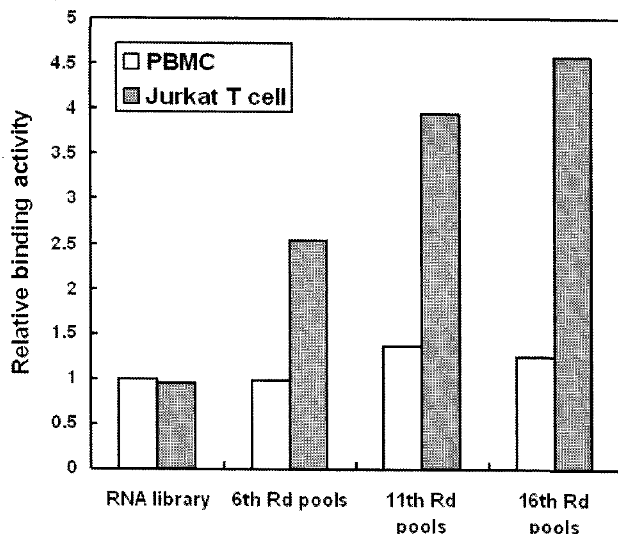

Fig. 2. RNA aptamers, bound to PBMC and Jurkat T cell leukemia, by semiquantitative RT-PCR analysis.
Original RNA library bound to PBMC or Jurkat T cells was eluted and amplified by RT-PCR. RNA pools after 6th, 11th, or 16th round selection were incubated with PBMC or Jurkat cells, and the RNAs bound to the cells were also purified and amplified. The amount of RT-PCR products from the bound RNAs was quantitated relative to the amount of RNA library bound to PBMC.

ml), and the cell-RNA complexes were then isolated from unbound RNAs by centrifugation and washed five times with 0.5 ml of binding buffer, and the target cell-bound RNAs were eluted from the pellets. The RNA was reversely transcribed, and the resulting cDNA was amplified (forward primer, 5'-GGGTAATACGACTCACTATAGGG-ATACCAGCTTATTCATT; reverse primer, 5-AGATTGC-ACTTACTATCT) with 10 thermal cycles [9, 18]. The amplified DNAs were analyzed and quantified on a 3% agarose gel. As a result, no difference was shown between the amount of RNA library bound to PBMC and RNAs to Jurkat cells. In sharp contrast, however, the amount of RNA aptamers bound to the Jurkat cells was higher than that to normal PBMC after the 6th round of selection. Moreover, the amount of RNAs bound to the target cells gradually increased from the subsequent rounds of selection. However, there was no significant difference in the amount of RNAs bound to the normal PBMC during the selection procedure, thus indicating that RNA aptamers specific to the Jurkat T cell leukemia were enriched.

After the 11th or 16th rounds of selection, the amplified cDNAs were cloned, and 20 different clones were sequenced. All of the 20 aptamers identified after the 11th selection round had different sequences (data not shown). However, some RNAs after the 16th selection round had the same or very similar sequences and were found in multiple clones in their amplified cDNAs (Fig. 3). Interestingly, clones I-V were also found in RNA pools at the 11th round of selection. However, the remaining 15 clones in the 11th round were

Sequence (Occurrence)

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I-1    (3)  5' GAACGCGAAUUUGACACUGAUCAGGCGUGUUGAUCGCCAACUGUAAUGUCCUCUGUCUCG 3'
I-2    (1)  5' ---------------------------------------------------C-------- 3'

II-1   (3)  5' GUCGAUGCUUAAGUGAUUUAUCCUCAAUACAACGAGUGGAUCAAUGUUUGUACGCGUGG 3'
II-2   (1)  5' -----------------------------G----------------------------- 3'

III    (2)  5' GGUUGAGGACUAUGAAUUAUUAUCCCAGACUGGUUCCCGAACACUCACAUGCUGCGUGG 3'

IV-1   (1)  5' GAACGAGCAUUAAGUCAUAGCAGCAUUUUUAGAAGUGGGAAUUGUUGUCGCUGUUUGUGUUG 3'
IV-2   (1)  5' ----------------- ------------------------------------------C- 3'

V      (2)  5' CGCCAUUGCUCAAGAGUAGCAUUCGCUGUGACUAAUUCCCAUGCUGGUUGGUGUCGGUGG 3'

VI     (1)  5' GGCGAUGAAUAGUUUUCCACGAGUGUAAUGAGUUUUGTGAAUGCUCAGUACCUGUCCCG 3'

VII    (1)  5' UAGUUGCGCAAACGUAGUAAGACAUGAUUGCUGUUGGUAGUAUUAUUUGUGUGGCUGGUG 3'

VIII   (1)  5' AUCGACAAUUAAACAUUUUCUCUGGCAGUGAUUCCAUAGCGUGUACCCGUUGGCUGGUCG 3'

IX     (1)  5' AACCCGUGGAACGUAGAAGUACAAUUGACUAGUAGUCCUUUUGACCCGUGTACCGCGCG 3'

X      (1)  5' AGCCGUCUGACAUUUACCAUAUAUUAGCGUAAAACGACCUUUACACUGUGUAUCCGGGUGG 3'

XI     (1)  5' GAUCAAUGGCACAAUACAACAGUUCGCUGAAUAUAGCUGUCGUCCUUUGGUAUCCGUGUGG 3'
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Fig. 3. Sequences of selected 2'-fluoro RNA aptamers.
After 16 rounds of *in vitro* selection, selected RNAs were reverse-transcribed, and the resulting cDNAs were PCR amplified and subcloned. Twenty clones were sequenced and found to encode 11 different RNA insert sequences. Several of the RNA sequences were present multiple times (numbers in parentheses). The lines drawn indicate that nucleotides found at these positions are identical. C and U in this figure correspond to 2'-fluoro C and 2'-fluoro U, respectively.

not identified in the 16th round of selection. This could be due to an increase of stringency of the selection procedure: Selection stringency was enhanced with increasing the number of washing step of the cell-RNA complexes and the subtraction step with normal PBMC, and with reducing the amount of incubated RNAs during the selection rounds. Thus, of the RNAs found in the 11th round, aptamers with higher specificity and affinity might be enriched in the 16th round of selection. Semiquantitative RT-PCR analysis of the RNA aptamer clones bound to PBMC or Jurkat T cells showed that all aptamers identified after the 16th round of selection bound specifically to the target Jurkat cells (Fig. 4).

To confirm that the finally selected RNA aptamers bound specifically to the target cells, flow cytometry analysis was performed with FITC-labeled aptamers (Fig. 5). Nonradioactively labeled RNA aptamers were prepared, as previously described [19]. Briefly, *in vitro* transcribed RNA aptamers (121 pmol) were labeled at their 3'-ends by incubation with 5× reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml BSA,

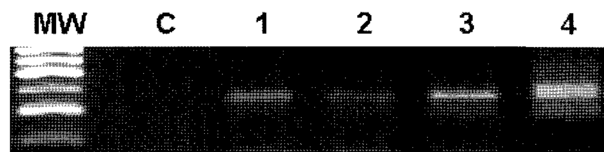

Fig. 4. RNA aptamer clone bound to PBMC or Jurkat T cell leukemia by semiquantitative RT-PCR analysis.
Original RNA library bound to PBMC (lane 1) or Jurkat T cells (lane 2) was eluted and amplified by RT-PCR. One representative RNA aptamer was incubated with PBMC (lane 3) or Jurkat cells (lane 4), and the RNAs bound to the cells were also purified and amplified. The amplified cDNAs were analyzed on a 3% agarose gel. Lane C represents PCR control. All other aptamer clones showed similar binding patterns (data not shown).

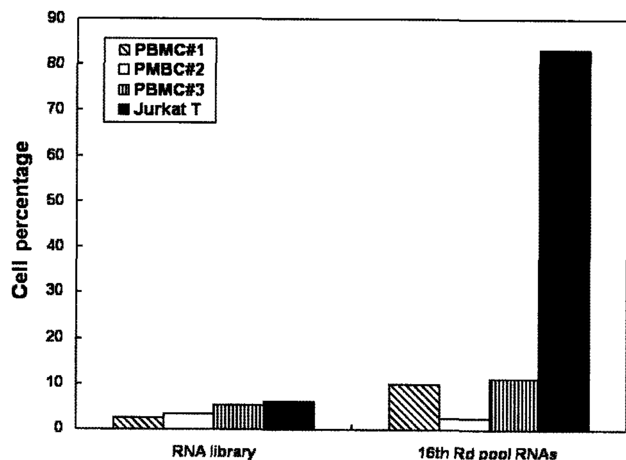

Fig. 5. Flow cytometric analysis of FITC-labeled RNA library or RNA pools after the 16th round of selection bound to PBMC isolated from three different donors or Jurkat T cell leukemia.
FITC-labeled RNA library or 16th round pool RNAs were incubated with each of the cells, and the RNA binding was monitored by fluorescence-activated cell sorting (FACS) analysis. Cell percentage represents the fraction of cells bound to the FITC-labeled RNA.

and 0.5 mM dNTP), 0.5 mM FITC-dUTP (Roche), 5 mM $CoCl_2$, and 20 U of terminal deoxynucleotide transferase (Roche) in a final volume of 30 µl for 30 min at 37°C. A total 1×10^6 cells was incubated with FITC-labeled RNA aptamers (30 pmoles) at 37°C for 1 h in 200 µl of binding buffer. Cells were rinsed 5 times with 0.5 ml of binding buffer and resuspended in 1 ml of PBS containing 1.5 mM $MgCl_2$. FITC fluorescence was then monitored using FACScan apparatus (Becton Dickinson) [14], and results were presented as frequency distribution histograms of logarithmic fluorescence. The cell fraction with positive fluorescence was then represented. In accordance with the semiquantitative RT-PCR analysis of the cell-bound RNAs, the original RNA library did not bind well either to PBMC or Jurkat cells. On the other hand, FITC-labeled RNA pools after the 16th round bound more strongly to Jurkat T cells, with about 10-fold fluorescence intensity, than the FITC-labeled original RNA library. However, no increase of the binding capacity to PBMC was shown with the selected RNA aptamers, compared with the RNA library. Importantly, the selected RNA aptamers could not bind to any normal PBMC from three different donors. These results indicate that the selected RNA aptamers specifically bound to the surface of target Jurkat T leukemia cells.

In conclusion, a subtractive SELEX procedure was developed to identify nuclease-resistant RNA aptamers that specifically bound to Jurkat T leukemia cells. These aptamers could be useful for specifically sorting out the cancer cells from the mixtures of normal and cancer cells. However, it is not clear from this study whether the selected aptamer clones recognize different targets on the surface of Jurkat cells. Identification of target molecules binding to the individual aptamer clones will be the solution to that question. Now, a large amount of efforts are focused on the identification of specific messages associated with a wide range of human diseases including cancers [22]. A conventional SELEX procedure could be performed to identify cancer-specific RNA aptamers with purified proteins after expression of the specific messages. However, an obvious advantage of the cell-based subtractive SELEX performed in this study is that a specific disease such as cancer could be targeted without prior knowledge of any molecular changes associated with the disease state [3]. Therefore, characterization of molecular targets against aptamers selected from the subtractive SELEX might be useful for identification of novel molecular markers of a specific cancer. In the future, specific aptamers against primary cancer cells, but not specific to adjacent normal tissues, will be selected from human patients, using the subtractive SELEX procedure developed in this study. Aptamers from this SELEX could be highly specific to cancer cells, and therefore, they can be applied to tumor diagnosis and/or therapy together with the identification of cancer markers.

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