Original Article

Selection and characterization of single-stranded DNA aptamers against interleukin-5

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Abstract

Asthma as a chronic inflammatory disorder is associated with many cytokines like interleukin-5 (IL-5) which plays essential role in eosinophil differentiation and maturation. Accordingly, blockage of IL-5 using mepalizumab has been considered as a promising therapeutic approach for asthma. Despite the monocolonal antibody advantages, some restrictions provided an acceptable background for alternative agents like aptamers which could replace with antibodies. In the current study, aptamer isolation against IL-5 molecule was intended, according to the valuable benefits of aptamers over antibodies. HEK-293T/IL-5 cell was constructed to select aptamer using cell-systematic evolution of ligands by exponential enrichment (SELEX) method. Integration of the IL-5 fragment to genome of the HEK-293T was verified by polymerase chain reaction on the genomic DNA of the transfected cells. Moreover, IL-5 protein expression on the cell surface was confirmed using flow cytometry analysis. Then, cell SELEX was carried out in 12 rounds and isolated aptamers were evaluated by flow cytometry analysis. The selected clones were then sequenced and assessed for any possible secondary structure. The results of this study led to the selection of 19 different single-stranded DNA clones after 12 rounds of selection which were clustered to five groups based on common structural motifs. In conclusion, the findings revealed the isolation of IL-5-specific single-stranded DNA aptamers, which can further be substituted with mepolizumab.

Keyword: Aptamer; Asthma; Cell-SELEX; HEK-293T; Interleukin-5.

INTRODUCTION

Asthma is a chronic complex inflammatory disease of the whole bronchial tree with increasing incidence and the annual death rate of about 180,000 people in the world (1). Despite a high prevalence of asthma, its pathophysiology is still unclear (2). Due to the high number of eosinophils in people with asthma, the most effective factor in pathophysiology of the disease is attributed to eosinophils (3-5).

Eosinophils have a major role in mucosa secretion, pulmonary contraction, allergic reactions, airway constriction, and also remodeling accompanied with acute eosinophilic inflammation. IL-3, GM-CSF and especially interleukin-5 (IL-5) are main factors for recruitment of these cells (2). Among them, IL-5 is produced by lymphocytes, mast cells, eosinophils, epithelial cells, and smooth muscles of airways and plays a role in the survival, maturation, differentiation, and activation of eosinophils in bone marrow (6,7). The serum level of IL-5 is significantly higher in patients with exacerbation asthma than healthy people and even patients with mild asthma. On the other hand, IL-5 decreases in patients receiving glucocorticoids (6). Hence, the IL-5 inhibition could be a main therapeutic target to improve signs of asthma. There are many new therapeutic and diagnostic tools for disruption and inhibition of target gene or protein at DNA, RNA, and protein level (8-12).

Blockade of IL-5 using mepolizumab (SB-240563; GlaxoSmithKline Co., United Kingdom), an approved monoclonal antibody against IL-5 by Food and Drug Administration (3), leads to decrease blood and lung eosinophils, ameliorate asthma severity in the patients, and improve their life quality (1,2,6,7).

Despite of considerable effects of the antibody against IL-5, there are many disadvantages such as high immunogenicity and thermal instability which led to vast investigations on other agents to overcome the aforementioned problems. One of the most promising and novel methods for this purpose is aptamer. Aptamer, a short single-stranded DNA (ssDNA) or RNA sequences with high affinity and specificity for their target known as a good substitution for monoclonal antibodies (13,14). Moreover, it is possible to select an aptamer molecule with a unique conformation and high binding affinity to target a wide range of specific ligands such as metal ions, small molecules, proteins, cell surface antigens, and whole cells or tissues (15,16).

The most common procedure for aptamer selection is systematic evolution of ligands by exponential enrichment (SELEX). Cell SELEX is the most common SELEX method in which partitioning performs based on aptamer interaction with target protein expressed on the cell surface naturally or synthetically (13).

Given the role of IL-5 in asthma pathophysiology and its potential as a therapeutic target, this study was conducted to design and select an aptamer against IL-5 using cell SELEX method.

MATERIALS AND METHODS

Transformation of TOP10F′ Escherichia coli by pdisplay-IL-5 plasmid

Vector of pdisplay containing human IL-5 fragment (618 bp, pdisplay-IL-5) was ordered to synthesize by GeneCust Company (Luxembourg) and transformed into *TOP10F′ Escherichia coli* (*E. coli*) (Pasteur Institute of Iran, Tehran, I.R. Iran) using $CaCl_2$ chemical DNA transfer method. Subsequently, the transformed cells were isolated using ampicilin as selection marker and the pdisplay-IL-5 plasmids were extracted using commercial kit (SolGent Plasmid Mini Prep kit, SPM01-C200, South Korea). The extracted plasmids were then digested with *BglII* restriction enzyme (Thermo Scientific, USA) to make a linear plasmid.

HEK-293T cell transfection by pdisplay-IL-5

The HEK-293T cell line (Pasteur Institute of Iran, Tehran, I.R. Iran) was grown in dulbecco's modified eagle medium (DMEM) media (Biosera, France) supplemented with 10% fetal calf serum (Gibco, USA), 100 U/mL penicillin (Gibco, USA) and 100 μg streptomycin (Gibco, USA) at 37 °C in a 5% CO2 incubator. The HEK-293 cells were then transfected with the linear pdisplay-IL-5 using TurboFect reagent (Thermo Fisher Scientific, USA) based on manufacturer's instruction. Subsequently, positive cells were selected by exposition to hygromycin B (Roche, Germany) for 21 days. Moreover, pLOX-GFP transfected and untransfected HEK-293T cells were served as positive and negative controls, respectively.

Verification of pdisplay-IL-5 insertion to HEK-293T cells and IL-5 protein expression on their surface

Genomic DNA was extracted from the HEK-293T/IL-5 cells (target cells) using DNA extraction kit (BIONEER, Korea). The IL-5 fragment integration into the transfected cells genome was assessed by polymerase chain reaction (PCR) using universal primers. The PCR products were visualized by agarose gel electrophoresis.

Additionally, the expression of IL-5 protein on the HEK-293T/IL-5 cells was verified with fluorescein isothiocyanate (FITC)-conjugated anti-His6 tag antibody using FACSCalibur™ flow cytometer (BD Bioscience, San Jose, California, USA). Un-transfected cells were used as negative control.

DNA library and primers

An 88-bp DNA library and primers were purchased from Tag Copenhagen A/S (Frederiksberg, Denmark). The DNA library consists of two 18-bp flanks on both sides with a 52-bp randomized region in between.

Cell SELEX for IL-5 aptamer selection

The cell-SELEX process was performed based on previous study conducted by Kouhpayeh *et al.* (12). In brief, the DNA library was resuspended in binding buffer, heated, and then snap-cooled on ice. The DNA library was incubated with HEK293T/IL-5 cells in order to positive selection. The bounded DNA fragments were eluted and used for incubation with HEK-293T cells in negative selection round. *DNase* I digestion (Sigma, USA) solution was applied for further removal of weakly HEK-293T/IL-5 cell bounded DNA sequences, at room temperature for 1 h with strong pipetting. The ssDNA sequences which do not bind to control cells were retrieved.

The selected pool after each round of SELEX was considered as the template for PCR optimization using forward and reverse primers (5′-ATACCAGCTTATTCAATT-3′, 5′-ACACTGTGATTGCACTTACTATCT-3′, respectively).

To produce the sub-library, PCR amplification was performed with the optimized cycle number. Asymmetric PCR was performed after each round to generate ssDNA as template. Asymmetric PCR was divided into two phases of 15 cycles of symmetric PCR in the first phase followed by asymmetric PCR with linear amplification using FITC-conjugated forward primer for 90 cycles.

The positive selection cycle was repeated 12 times, along with counter selection, with a slight increase in annealing temperature to 45 °C. The stringency of selection was gradually increased by changing concentration of library, tRNA, and $MgCl_2$, cell count, and incubation time (Table 1).

SELEX monitoring

In order to verify SELEX progression, asymmetric PCR program was performed using FITC-conjugated forward primer. Approximately 5×10^5 HEK-293T/IL-5 cells were incubated with 50 μL of the ssDNA selected pool diluted in binding buffer. Flow cytometric analysis was performed using BD FACSCalibur™, and the data analyzed by Cell Quest software (BD Bioscience, San Jose, California, USA). Unselected initial DNA library pool was considered as the negative control.

Clustering and prediction of aptamers

The PCR products were TA cloned (SinaClon, Tehran, I.R. Iran) and the positive clones were then selected, amplified, and sequenced (Noor Genetic Lab, Ahwaz, I.R. Iran). Chromas software (Version 1.45, http://www.technelysium. com.au/chromas.html, South Brisbane, Australia) was applied to analyze the sequence data. Subsequently, the sequence alignment was carried out with ClustalX software (Version 2.1, http://www.clustal.org, Dublin, Ireland). The secondary structure of selected sequences was determined using Mfold web server (http://unafold.rna.albany.edu/?q=mfold).

RESULTS

Verification of pdisplay-IL-5 insertion to HEK-293T cells and IL-5 protein expression on their surface

After PCR amplification, a 618 bp band was observed in 1% agarose gel electrophoresis which confirmed the integration of pdisplay-IL-5 into HEK-293T cell genome (Fig. 1A). Flow cytometry analysis revealed 76% expression of IL-5 protein on transfected HEK-293T cell surface as shown in Fig. 1B and C.

Aptamer selection

Monitoring the progress of aptamer selection

Fluorescent emission of cells incubated with 750 nM of the fourth, eighth, and twelfth rounds of SELEX pools showed 11.47%, 51.68%, and 73.70% intensity, respectively, when compared with cells that received same concentration of initial library (Fig. 2A-D).

Clustering and prediction of aptamers

The sequence identity was used to cluster the IL-5 specific aptamers into five main groups (Fig. 3A). ClustalX results illustrated five aptamer families based on shared random sequence motif identity which was over 90% within each family (Fig. 3B and Table 1).

The potential two dimensional structure of each 92 bp-aptamer candidate was generated by Mfold webserver (Fig. 4). All candidate aptamers with the lowest free energy form the secondary structure with three to five small stem loops and a large open loop motif.
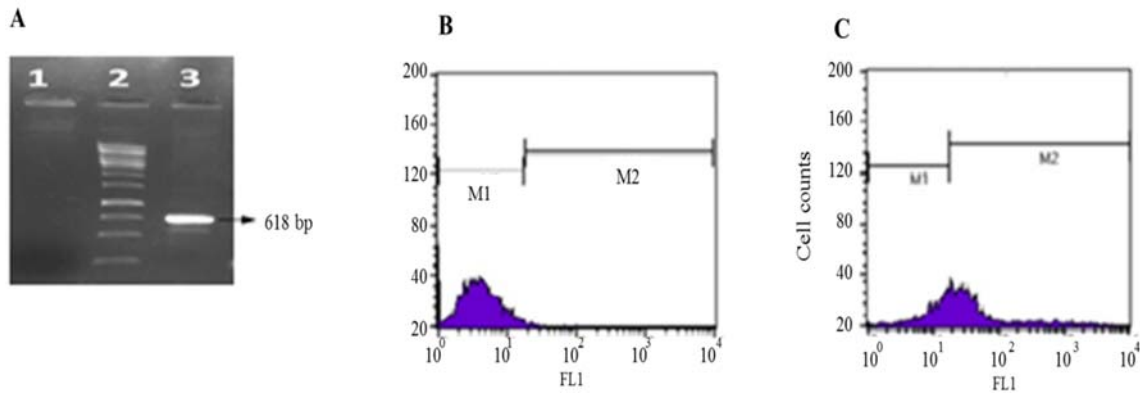
Fig. 1. Verification of pCDNA/IL-5 insertion. (A) Genomic DNA amplification. Agarose gel electrophoresis results illustrated the insertion of IL-5 gene into the genome of transfected cells; Lane 1, untransfected cells; lane 2, DNA marker (1 kb Fermentas); and lane 3, amplified fragment of transfected cells (618 bp). (B and C) Expression level of IL-5 protein on HEK-293T cell surface. Flow cytometric analysis shows 76% expression of IL-5 protein on (C) the transfected cells compared to (B) the un-transfected cells.
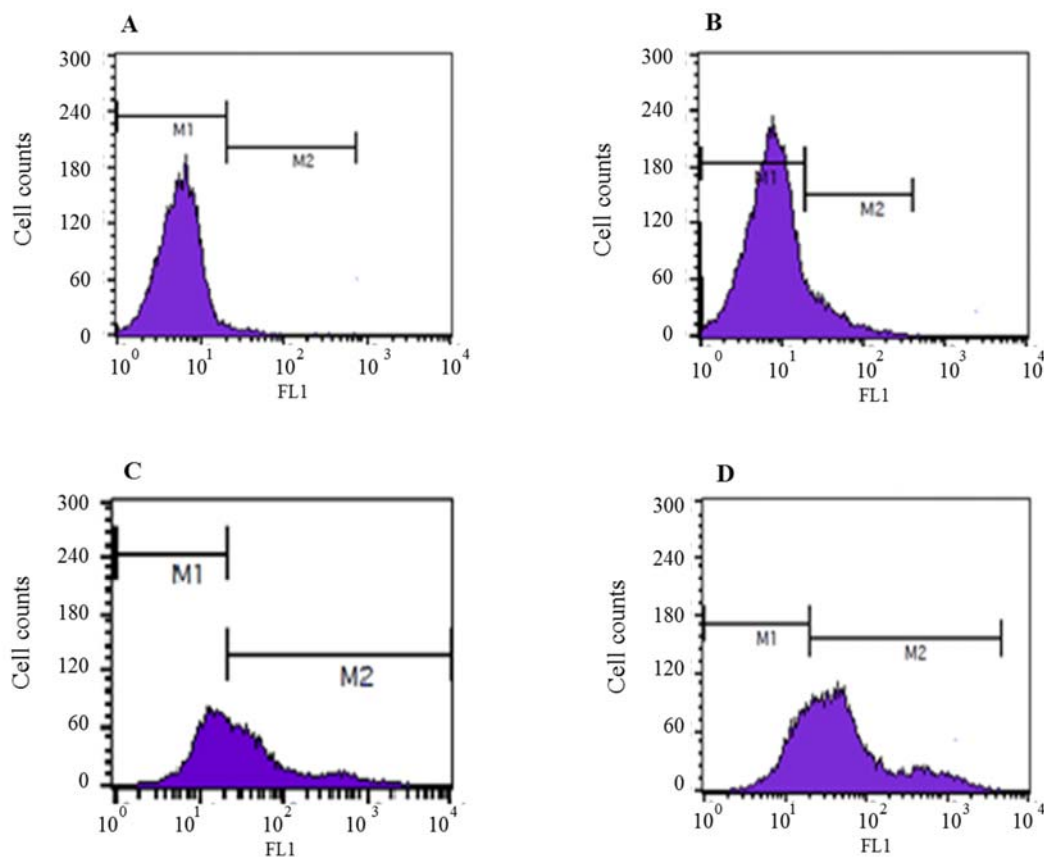
Fig. 2. Cell-SELEX progression monitoring. Cell samples were prepared for the flow cytometry aptamer binding assay with 1×10^5 cells and 750 nM of DNA aptamers. FITC fluorescence emission of the live cell population is presented for the: (A) initial library, (B) forth, (C) eighth, and (D) twelfth rounds of SELEX which have the mean fluorescent intensity more than M1 (initial library), 11.47%, 51.68%, and 73.70%, respectively. FITC, fluorescein isothiocyanate; SELEX, systematic evolution of ligands by exponential enrichment.
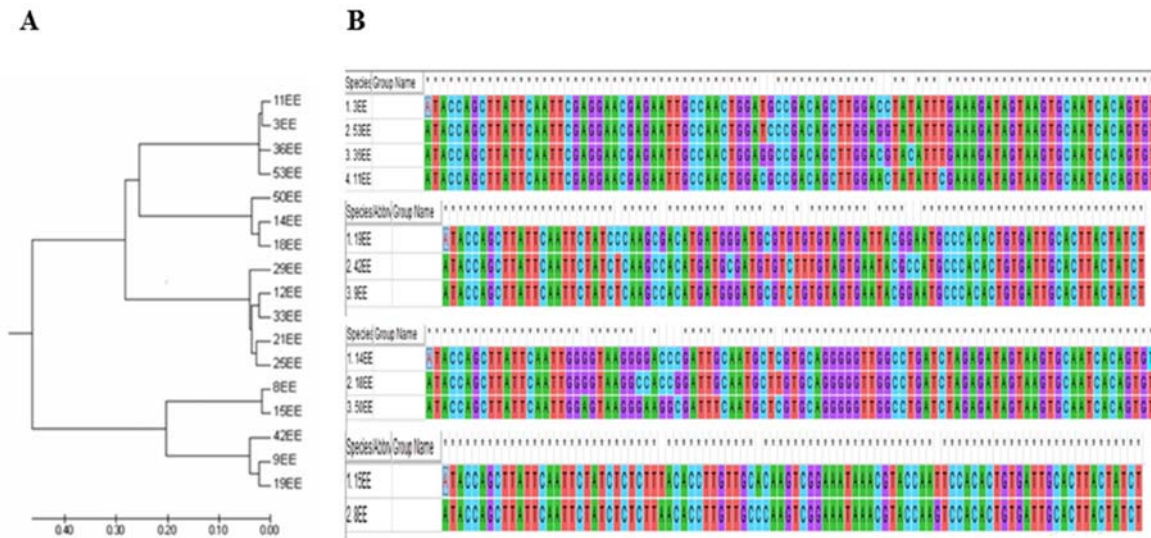
Fig. 3. Phylogenetic tree, alignment and clustering. (**A**) ClustalX phylogenetic tree analysis of the 92 bp DNA sequences from 19 different clones. (**B**) DNA sequence alignment. ClustalX alignment of the 92 bp sequences for the DNA sequences in the 11th SELEX round pool illustrated five families of sequences emerge that have 90% sequence identity within each family. Common nucleotides in all families are denoted with an asterisk. SELEX, systematic evolution of ligands by exponential enrichment.
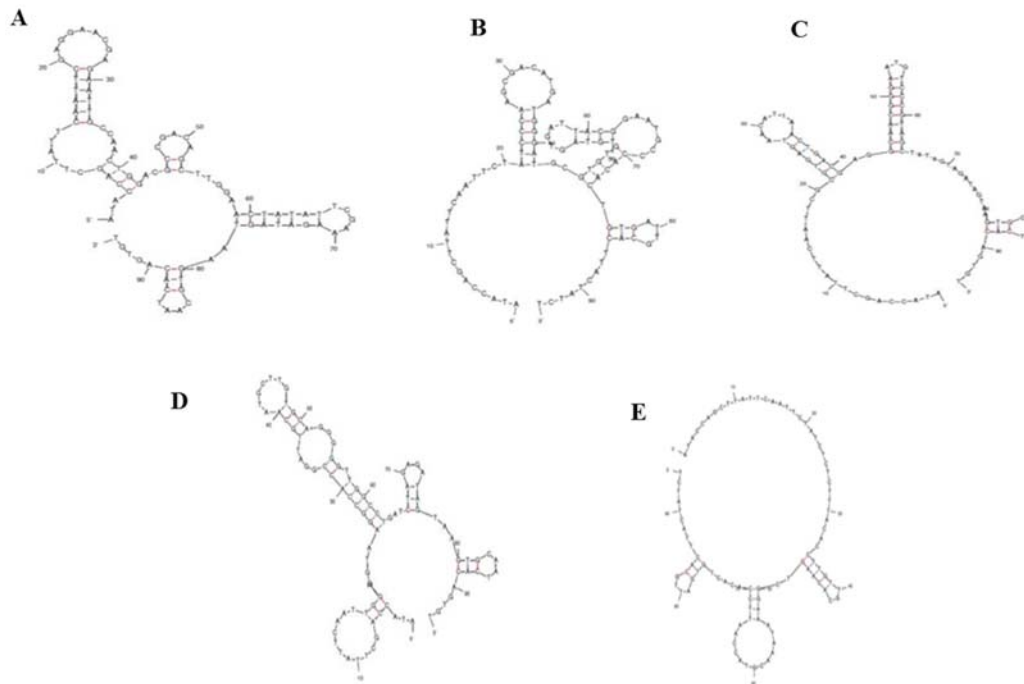
Fig. 4. Secondary structure predictions. Predicted secondary structures for the five families of aptamer candidates that were chosen from the 11th round of SELEX. (A) to (E) are representatives of families 1 to 5, respectively with lowest potential energy structures in each family to bind interleukin-5 expressing cells. Secondary structures were predicted by DNAMAN software. SELEX, systematic evolution of ligands by exponential enrichment

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Table 1. DNA aptamer selected sequences against interleukine 5.

Clone	Family	Sequence
3E	1	ATACCAGCTTATTCAATTCGAGGAACGAGAATTGCCAACTGGATGCCGACAGCTTGGACCTATATTTGAAAGATAGTAAGTGCAATCACAGTGT
11E	1	ATACCAGCTTATTCAATTCGAGGAACGAGAATTGCCAACTGGACGCCGACAGCTTGGAACTATATTCGAAAGATAGTAAGTGCAATCACAGTGT
36E	1	ATACCAGCTTATTCAATTCGAGGAACGAGAATTGCCAACTGGAGGCCGACAGCTTGGACGTACATTTGAAAGATAGTAAGTGCAATCACAGTGT
53E	1	ATACCAGCTTATTCAATTCGAGGAACGAGAATTGCCAACTGGATCCCGACAGCTTGGAGGTATATTTGAAAGATAGTAAGTGCAATCACAGTGT
9E	2	ATACCAGCTTATTCAATTCTATCTCAAGCCACATGATGGGATGCGTCTGTGTAGTGAATACGGAATGCCCACACTGTGATTGCACTTACTATCT
19E	2	ATACCAGCTTATTCAATTCTATCCCAAGCGACATGATGGGATGCGTGTGTGTAGTGATTACGGAATGCCCACACTGTGATTGCACTTACTATCT
42E	2	ATACCAGCTTATTCAATTCTATCTCAAGCCACATGATGCGATGTGTCTTTGTAGTGAATACGCCATGCCCACACTGTGATTGCACTTACTATCT
12E	3	ATACCAGCTTATTCAATTCGCGTGAGTAACATAAACTGTCGAGGCAACGGGAATGTCCCGTAGCTGTTGTAGATAGTAAGTGCAATCACAGTGT
21E	3	ATACCAGCTTATTCAATTCGCGTGAGTAACATTCACTGTCGAGGCAACGGGAATGTGTCGTAGCTGTGGTAGATAGTAAGTGCAATCACAGTGT
25E	3	ATACCAGCTTATTCAATTCGCGTGAGGTACATTAACTGTCGAGCCAACGGGAATGTGTCGTAGCTGTCGTAGATAGTAAGTGCAATCACAGTGT
29E	3	ATACCAGCTTATTCAATTCGCGTGAGTTCCATACACTGTCGACGCAACGGGAATGTCTCGTAGCTGTAGTAGATAGTAAGTGCAATCACAGTGT
33E	3	ATACCAGCTTATTCAATTCGCGTGAGTAACATtAACTGaCGAGGCAACGGGAATGTCCCGTAGCTaTaGTAGATAGTAAGTGCAATCACAGTGT
14E	4	ATACCAGCTTATTCAATTGGGGTAAGGGGACCCGATTGCAATGCTCGTGCAGGGGGTTGGCCTGATCTAGAGATAGTAAGTGCAATCACAGTGT
18E	4	ATACCAGCTTATTCAATTGGGGTAAGGCCACCGGATTGCAATGCTTGTGCAGGGGGTTGGCCTGATCTAGAGATAGTAAGTGCAATCACAGTGT
50E	4	ATACCAGCTTATTCAATTGGAGTAAGGGAAGGCGATTTCAATGCTCGTGCAGGGGGTTGGCCTGATCTAGAGATAGTAAGTGCAATCACAGTGT
8E	5	ATACCAGCTTATTCAATTCTATCTCTCTTAACACCTTGTTGCCCAAGTCGGAAATAAACGTACCAAGTCCACACTGTGATTGCACTTACTATCT
15E	5	ATACCAGCTTATTCAATTCTATCTCTCTTTACACCTTGTTGCACAAGTCGGAAATAAACGTACCAATTCCACACTGTGATTGCACTTACTATCT
13E	-	ATACCAGCTTATTCAATTCTATCTTTCAGGGGATTGGTGTGGATCTGTCGTTGTAGTTCCCGTTCAATTACACTGTGATTGCACTTACTATCT
47E	-	ATACCAGCTTATTCAATTCTATCTTTCAGTTGATTGGTGTGGATCTGCCGTTGTAGTTCGCGTTCATATACACTGTGATTGCACTTACTATCT

DISCUSSION

The role of IL-5 in pathogenesis of allergic diseases like asthma has been largely investigated (6,17), thus blockage of this molecule has been considered as a promising therapeutic approach for allergic diseases. Aptamers have been introduced as appropriate candidates in order to block the molecule of interest. There are various types of aptamer selection method which cell SELEX introduced as the most common technique for aptamer preparation amongst different available strategies. Cell-SELEX method requires both positive and negative cell lines to facilitate the aptamer development against the target of interest (6,13).

In this study, we constructed the HEK-293T/IL-5 cell for positive selection which was followed by the negative selection round to minimize the non-binder sequences. Integration of the IL-5 fragment to genome of the HEK-293T was verified by PCR on the genomic DNA of the transfected cells. Moreover, IL-5 protein expression on the cell surface was confirmed using flow cytometry analysis.

There are some evidences on the use of HEK-293T cell for protein expression (18). As post translational modification and proper folding are necessary for protein biological function, mammalian cells are the best choice for expression and precise assembly of target molecule. Portolano *et al.* showed the importance of using mammalian cells like HEK-293T for expression of target proteins due to having a high rate of post translational modification or supra-assembling (19). Hence, HEK-293T cell has been chosen for over expression of IL-5 on its surface. For instance, a recent study constructed HEK-293T cells overexpressing α4 integrin for further use as a positive cell in SELEX strategy (20). In this study, PCR and flow cytometry methods were similarly used in order to verify α4 integrin integration into genomic DNA and the protein cell surface expression, respectively (20). In addition, the same procedure was applied to produce recombinant cells overexpressing HBsAg (16), rabbit MCP-1 (21,22), human MCP-1 (21),

and TIM-1 (23) for further application in aptamer selection as diagnostic and therapeutic tools. Moreover, Bruun *et al.* generated HEK 293 cell lines expressing envelop protein of HIV-1 on their surface to introduce a technology for affinity-based selection of envelope variants from libraries (24). Recombinant HEK293 cells with germ line V-gene segments on their surface were also produced to apply in therapeutics antibody preparation (25).

There are three general systems for transferring gene into the cells including biological, chemical, and physical methods (26,27). In the present study, a chemical method with polycationic reagent was applied to transfer IL-5 cDNA into the HEK-293T cells. Transfection efficiency of 76% using chemical methods indicates high efficiency of the transfection method. As high similarity of the expressed target with the intact molecule, leads to isolation of the more specific aptamer with less cross-reaction and side effects, the complete IL-5 gene CDS was used in the designed construct.

Isolation of aptamer against adhesion molecules was performed using recombinant proteins of interest in previous studies (28-31). However, application of recombinant proteins for aptamer selection has a few obstacles including internalization of isolated aptamer (32). In addition, commercially available recombinant proteins are infrequently produced in full length and similar to intact protein. Thus, the recombinant protein may not fold properly and as the intact molecule. In this study HEK-293T/IL-5 cells were applied for specific aptamer isolation. In accordance with our study, Kouhpayeh *et al.* constructed a HEK-293T cell line overexpressing α4 integrin (12). Similarly, the HepG2 cell line overexpressing EpCAM was employed for specific aptamer isolation (33).

In the present study a modified-cell SELEX strategy was applied for partitioning IL-5 specific ssDNA aptamers. Various SELEX methods were employed for target-specific oligonucleotide isolation previously, including immuno magnet, enzyme-linked immunosorbent assay (ELISA), combinatorial methods, and cell SELEX.

However, only a few studies employed negative selection step (34,35) which indicated to result in low affinity binder sequences isolation (28,30,31).

In our study, fluorescence-activated cell sorting (FACS) analysis was performed to evaluate the affinity growth and selection completion. Evaluation of enriched selected pool for cytokine-specific aptamers includes variety of techniques including surface plasmon resonance, electrophoretic mobility shift assay, FACS analysis and radioassay (35-40). It is of great importance to evaluate the SELEX progression based on quantitative amounts for a precise and proper monitoring in addition to determining the number of SELEX rounds (41).

The enriched pool of ssDNA aptamers in this study consists of 19 sequences which were clustered in three main groups based on sequence relationship. The selected structures with the lowest potential energy (ΔG) consist of a large open loop and three to five small stem loops. The similarity of the resultants showed the possible structure required for IL-5 binding which is also verified in the earlier related studies (42).

CONCLUSION

The IL-5 specific aptamers were isolated in this study. The isolated sequences, however, need to be further assessed *in vitro* and *in vivo* for affinity, stability, and the capabilities of potential inhibition of IL-5 to be qualified as a substitution for mepolizumab in allergic disease treatment.

ACKNOWLEDGMENTS

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