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Design and Characterization of DNA Aptamer for Breast Tumor Marker by an Advantageous Method

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ABSTRACT: Aptamers are agents able to bind tightly and selectively to disease markers, offering great potential for applications in disease diagnosis and therapy. Functionally they are ssDNA or RNA oligonucleotides which are highly specific and selective to their target along with highest affinity. We have selected DNA aptamer against breast tumor marker (CA 15-3) by using a modified SELEX methodology. For the selection round CA 15-3 were immobilized on magnetic beads and to check the affinity of selected aptamers for their target fluorescent labeled aptamers were used. Aptamers were selected from an initial library containing 25 base long variable regions for their ability to bind to the CA 15-3. After 12 rounds of the selection and amplification we found 90% pool of DNA sequences which are able to bind with CA 15-3. These aptamers were cloned, sequenced and labeled by fluorescent molecule. The binding affinity of these aptamers (K_D value) was quantified by using FAM labeled aptamers. Our results aim to develop new diagnostic assays against breast tumor marker for the early diagnosis of breast cancer.

KEYWORDS: Aptamer; SELEX; Breast Tumor; Magnetic beads; CA 15-3.

I. INTRODUCTION

Since the first appearance of aptamers in the literature in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990) they have been selected for multiple substances, thus the fields of application of aptamers are very wide ranging (Sun, 2000). Aptamers have great potential to function as molecular recognition elements in analytical systems for detection, separation, or purification of target molecules. Aptamers can substitute antibodies or be used in conjunction with antibodies in different assays and play increasingly important role in medical diagnosis and therapy (Brody and Gold, 2000), in cytomic research (Ulrich *et al.*, 2004), and also in the wide area of environmental analysis.

In spite of the very encouraging promises, research on aptamers is still at the beginning. Medical and pharmaceutical basic research as well as clinical diagnostic and therapy offer a big field of application (Klug *et al.*, 1999; Hermann and Patel, 2000; Thiel, 2004). Aptamers are starting to compete with antibodies as therapeutic agents (Nimjee *et al.*, 2005; Ulrich *et al.*, 2006). Aptamers in general are more suitable than antibodies for escorting and in vivo imaging (Hicke and Stephens, 2000). Because of their small size, aptamers can penetrate into cells and tissues more effectively than antibodies (Stoltenburg *et al.*, 2007). If the longer aptamer circulation in the body is required, aptamers can be modified to extend their bloodstream half-life (Yan *et al.*, 2005).

The SELEX process (Tuerk and Gold, 1990) for the selection of target-specific aptamers is a universal process characterized by repetition of the five mentioned main steps (binding, partition elution, amplification and conditioning). The starting point of the SELEX process is a combinatorial library of synthetic oligonucleotide consisting of a multitude of ssDNA fragments with different sequences.

The use of affinity chromatography with immobilization of target on column material like sepharose or agarose is a conventional method for this separation step (Liu and Stormo, 2005; Tombelli *et al.*, 2005a). However, substantial amounts of targets are necessary to obtain a high efficient loading of the column. Another kind of separation method is filtration, for example, using nitrocellulose filters (Tombelli *et al.*, 2005b). The use of magnetic beads offers another

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possibility for target immobilization (Lupold *et al.*, 2002; Kikuchi *et al.*, 2003; Murphy *et al.*, 2003; Stoltenburg *et al.*, 2005). This method requires only very small amounts of target and enables a very simple handling.

Our aim is to develop ssDNA aptamers, especially to design diagnostic assays and to develop biosensors for breast cancer through an easy to handle (enable work in an ordinary biomolecular laboratory), cost effective (avoiding additional expenditure), and environmentally compatible method. This process was started by the selection of specific aptamers against breast tumor marker through an easy to handle modified SELEX process which combines the fluorescence labeling of DNA to check affinity against target. Thus the modifications simplify basic SELEX process, make it very easy to pursue in an ordinary biomolecular laboratory.

Material and Methods

Materials All chemicals until specified were purchased from Sigma -Aldrich, Himedia, CDH, QIAgen and Invitrogen. Glassware's were purchased from Borosil (India). Plasticwares were procured from Tarsons products Pvt. Ltd. (India) and Nunc (Denmark). Breast Tumor Antigen (CA 15-3) from Human Cell Culture was purchased by Sigma –Aldrich. 5' 6FAM labeling of screened aptamers was done by Sigma–Aldrich. ssDNA Library 5′GGGAGACAAGAATAAACGCTCAA-(25N)-TTCGACAGGAGGCTCACAACAGGC, Forward primer 5′GGGAGACAAGAATAAACGCTCAA3′ and Reverse primer 5′AAGCTGTCCTCCGAGTGTTGTCCG3′was purchased by Sigma –Aldrich.

Amplification of single stranded DNA library
An aptamer library was amplified to enrich all DNA aptamer species in the original library. An ssDNA pool was generated from the original synthetic pool by large-scale unidirectional PCR. A 100µL PCR mix was prepared in a thin-walled PCR tube: aptamer library 5µM (5µL), forward primer 0.5µM (50µL), reverse primer 0.1µM (10µL), 10× PCR buffer 10µL, $MgCl_2$ 1.25mM (5µL), dNTP 200µM (8µL), 5U Taq DNA polymerase. Thirty two rounds of amplification were performed at 94°C denaturing (1.5 min), 56 °C annealing (1.0 min) and 72 °C extension (2.0 min) in a thermocycler; a final extension step was included at 72 °C for 10 min and soaking at 4°C.

Coupling of CA 15-3 ligand with magnetic beads
Dynabeads (magnetic beads) were purchased from Invitrogen. To format ligand coupled magnetic beads we have followed the protocol as described by Invitrogen. Washed beads were diluted in 240µL Buffer E [5mM sodium phosphate buffer (pH 7.6) with 0.1% Tween20] to achieve the final bead concentration 20 mg/mL. Magnetic beads are a very useful matrix for immobilization of targets during aptamer selection and enable easy and efficient processing (Stoltenburg *et al.*, 2005).

Screening of CA 15-3 specific aptamers
To separate the relevant DNA strands from the double stranded amplified PCR product of DNA library, all purified PCR product were pooled and precipitated with chilled ethanol. Precipitates were resuspended in 100µL TE buffer (pH 7.4) followed by heated to 90^{o}C for 10 minutes, immediately cooled and kept at 4^{o}C for 15 minutes, incubated 5 minutes at room temperature (Snap Cooling).

For selection of aptamers against CA 15-3 a fresh aliquot of 240µL ligand coupled magnetic beads (final concentration 20mg/mL) was washed 4-5 times with binding buffer (100mM NaCl, 20mM Tris-HCl pH 7.6, 2mM MgCl2, 5mM KCl, 1mM CaCl2, 0.02% Tween20) before each selection round. To initiate screening of CA 15-3 specific aptamers in the first selection round washed ligand coupled magnetic beads were re-suspended in total volume of 500µL binding buffer, containing 100µL previously purified pool of DNA library. The mixture was incubated at 21^{o}C for 30 minutes (mild shaking in incubator) followed by removal of unbound oligonucleotides by five washing steps with 1mL binding buffer using magnetic separation stand. Subsequently, the bound oligonucleotides were eluted by incubating the binding complex with 250µL elution buffer (40mM Tris-HCl pH 8, 10mM EDTA, 3.5M urea, 0.02% Tween20) at 80^{o}C for 10 minutes with mild shaking (in heating block). The elution process was repeated twice and pooled oligonucleotides were precipitated with ethanol. The selected oligonucleotides were re-suspended in a smaller volume of TE buffer (pH 7.4) followed by amplification in 10 parallel PCR reactions (reaction conditions were similar as described for library amplification). Amplified DNA was monitored on 2.5% agarose gel and gel bands were purified

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by QIAEX II Gel Extraction Kit. 12 selection rounds were performed to enrich CA 15-3 specific aptamers. To enhance the specificity of the aptamers last 3 rounds of selection were performed as counter selection rounds. In the 10^{th}, 11^{th}, and 12^{th} round selected aptamer pool was first incubated with uncoupled tosylactivated magnetic beads followed by incubation at 21^{o}C for 30 minutes, supernatant were separated through magnetic separation stand, and subsequently transferred to CA 15-3 coated magnetic beads.

Cloning, Sequencing and binding affinity assay of selected aptamers

The aptamers from 12^{th} selection round were amplified and cloned into pGEM-T Easy Vector. This vector construct were transformed in JM109 Competent Cells. Plasmid was isolated using QIA prep spin mini prep kit (QIAGEN, Germany) followed by sequencing of inserted aptamers of all positive clones by Merck Genei. The binding affinity of selected individual aptamer clones were characterized by fluorescence labeled aptamer based binding assays. The conditions for the binding assay were applied according to the screening steps. To calculate dissociation constant of screened aptamers different dilutions of fluorescence labeled aptamer (5nM-325nM) was prepared. 500µL of binding solution was prepared containing 100µL of each dilution of fluorescence labeled aptamer (5nM-325nM). Binding solution was added in washed ligand coupled beads (20mg/mL) in separate dark color tubes. Tubes were incubated with tilting and rotation at 21^{0}C for 30min followed by removal of supernatant using magnetic stand. After the binding reaction unbound aptamers were removed by several washing steps by 1mL of binding buffer. The bead bound aptamers were eluted with 200 µL elution buffer incubated at 80^{0}C for 10min with mild shaking. Unbound and bound fractions were measured on Fluorescence spectrophotometer and binding constant (K_D) was calculated by non-linear regression analysis using Graph Pad Prism software.

II. RESULTS & DISCUSSION

A modified in vitro selection procedure of DNA Aptamer from ssDNA Library was optimized. In the selection procedure magnetic beads were used as an immobilization matrix for target protein (Stoltenburg *et al.,* 2005) (Figure 1).

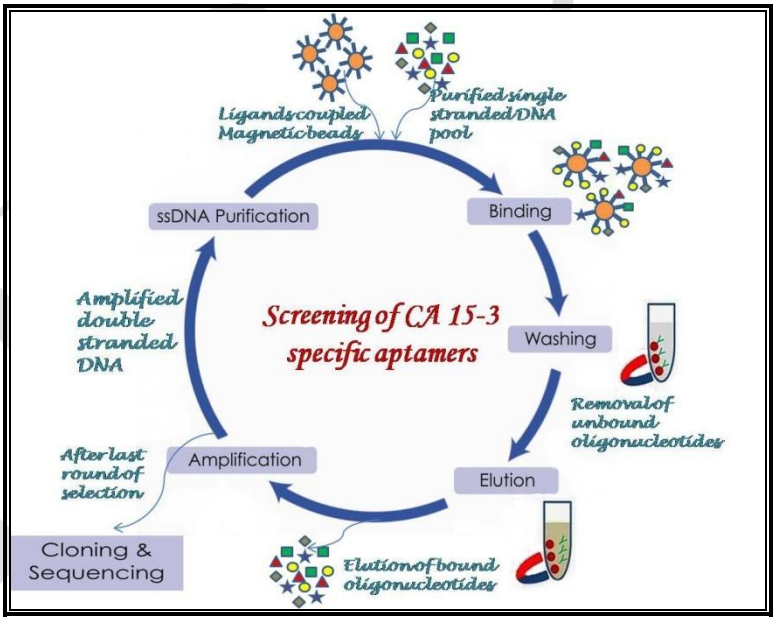

Figure 1. Schematic representation of the screening process for generating DNA aptamers specific for CA 15-3 immobilized on magnetic beads.

Selection procedure was started by amplification of ssDNA library. DNA library was amplified using corresponding forward and reverse primers. CA 15-3 coated magnetic beads were used for the selection of CA 15-3 specific aptamer in the further selection rounds. Magnetic beads were used to achieve highly efficient separation of aptamer specific to CA 15-3. The enrichment of CA 15-3 specific aptamers in the last selection round was monitored on 2.5% agarose gel

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(Figure 2) and an increased amount of aptamer bound to CA 15-3 coated magnetic beads was observed in 9th round of selection process.

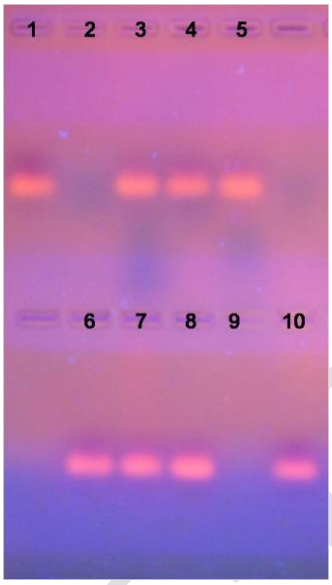

Figure 2. Image shows the enrichment of CA 15-3 specific DNA aptamer eluted from CA 15-3 coated magnetic beads on Agarose Gel Electrophoresis.

Last three rounds of selection process were counter selection rounds, i.e. the enriched eluted aptamers were incubated first with the uncoated tosylactactivated magnetic beads, then were incubated with CA 15-3 coated magnetic beads (Figure 3).

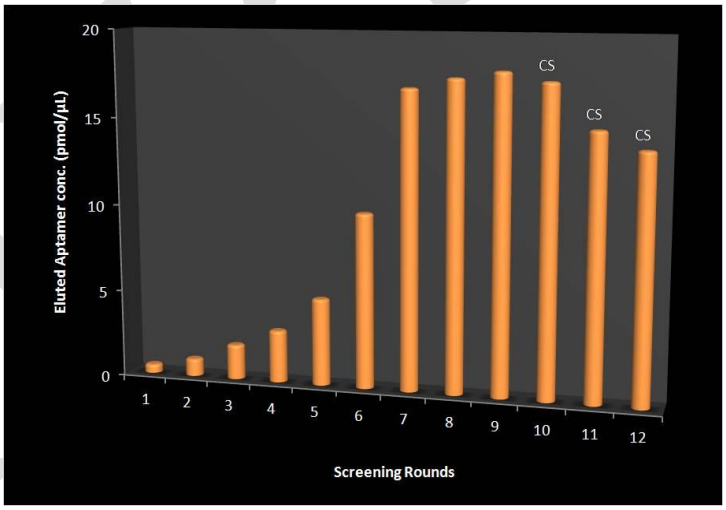

Figure 3. The bar graph shows the concentration of ssDNA aptamer eluted from CA 15-3 coated magnetic beads in each selection round. Round 10th, 11th and 12th were counter selection steps.

Nonspecific aptamer population was not observed. In the last rounds of selection process the concentration of aptamer eluted remains almost constant, possibly it was because the all of the binding sited of the magnetic beads were occupied. After 12th round of selection process, selected aptamer pool was cloned in an *E. coli* strain. Three positive clones were found after whole cell PCR of selected clones (Figure 4).

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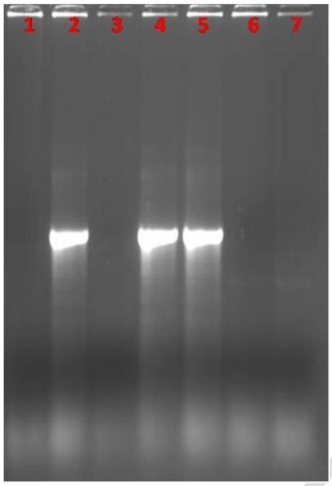

Figure 4. Image shows the results of whole cell PCR reaction of selected clones on Agarose Gel Electrophoresis.

Plasmids were isolated from the positive clones and identified through sequencing. Three aptamer sequences were found highly specific for CA 15-3 (Table 1).

Serial No.	Selected aptamer clone	Sequence	K_D (nM)
1	Clone 2	GAAGTGAATATGACAGATCACAACT	45.47 ± 3.415
2	Clone 4	TACTGCATGCAGACCACATCAACTT	67.1 ± 3.289
3	Clone 5	CATACAATCAATCACCAGTGCGGTG	81.56 ± 4.198

Table 1. The data represents the sequences of selected CA 15-3 specific aptamers and their dissociation constants (K_D) calculated by non-linear regression analysis.

To check the binding affinity of these three screened aptamers, FAM labeled aptamers were used. FAM labeled aptamers was synthesized chemically. The conditions for the binding assay were applied according to the screening steps. All of the eluted aptamers have affinity for CA 15-3. But the best results were obtained with aptamer clone 2 (Figure 5).

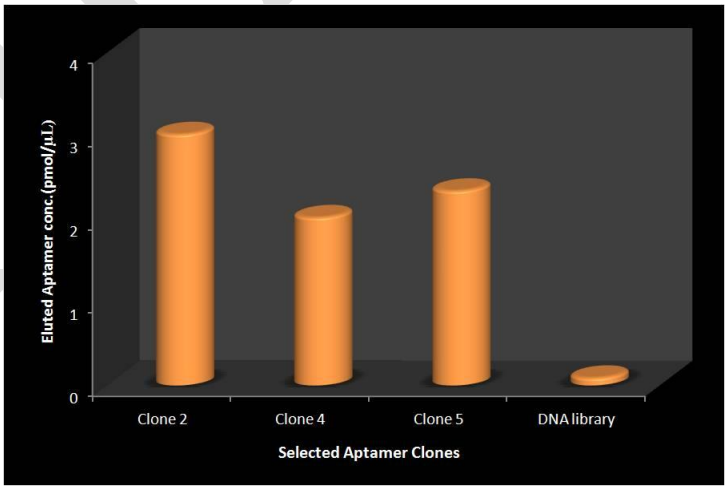

Figure 5. The bar graph shows the concentration of different clones of CA 15-3 specific ssDNA aptamers eluted from CA 15-3 coated magnetic beads. Clone 2 shows the maximum bound concentration of aptamer with CA 15-3 coated magnetic beads.

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To determine the binding affinities of these aptamers for CA 15-3, the dissociation constant (K_D value) were ascertained. A constant number of CA 15-3 coated magnetic beads were incubated with different concentration series of each FAM labeled aptamer (5nM-325nM). After several screening steps eluted aptamers were calculated by fluorescence measurements. The dissociation constant of the three screened aptamers were calculated by the nonlinear regression analysis of the binding data using Graph pad prism software (Figure 6).

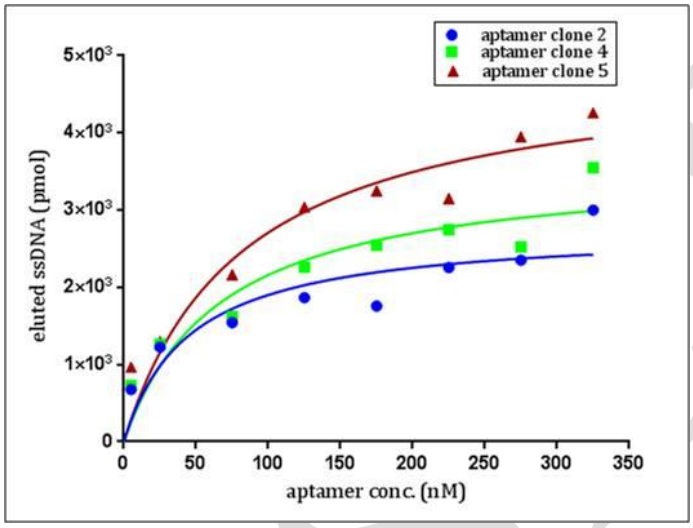

Figure 6. The data represents nonlinear regression analysis of different clones of CA 15-3 specific DNA aptamers eluted from CA 15-3 coated magnetic beads. The assays were performed by using a constant number of CA 15-3 coated magnetic beads in combination with the concentration series (5-325nM) of the FAM labeled aptamers.

The lowest K_D of 45.47 ± 3.415 nmol L^{-1} was obtained for aptamer clone 2 (Table 1). The results obtained were enough to learn that aptamer clone 2 have more binding affinity with CA 15-3 (Breast Tumor Antigen). Once selected the aptamers can be chemically synthesized allowing for different functional groups or labeling molecules to be easily integrated. This property combined with their selectivity and affinity makes them ideal for use in diagnostic assays.

III. CONCLUSION

In this research work we have used an optimized in vitro selection procedure for generating DNA aptamers against CA 15-3 (Breast Tumor Antigen). Enrichment of target specific aptamers was monitored during selection process. For the CA 15-3 immobilization magnetic beads were used as matrix. Magnetic beads based selection process of aptamer enables efficient separation of CA 15-3 specific aptamers and nonspecific aptamers, also this technique was very easy to handle. There was no need of any expensive Robotics. The dissociation constant of selected aptamers was very low i.e. they are having very good affinity with CA 15-3. These aptamers will provide very efficient results in diagnostic assays for Breast Cancer.

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Vol. 3, Issue 10, October 2014

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