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DNA Aptamers Selected as Molecular Probes for Diagnosis of Cancereous Cells

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Abstract: DNA aptamers are small single stranded DNA molecules that possess antibody-like characteristics; they bind with high affinity to specific antigens as a result of their three dimensional structure and chemical properties. Carcinoembryonic antigen (CEA) is expressed on the surface of 95% of adenocarcinomas. Anti-CEA antibodies have been used diagnostically *in vitro* to monitor possible metastases and clinically for the treatment of tumors *in vivo*. The aim of this research was to isolate aptamers against CEA for *in vivo* imaging and diagnosis of cancer cells. Fluorescence polarization assay was used to measure affinity and specificity of the candidate aptamers sequences towards purified CEA and related proteins. The aptamers were labeled with fluorescein at their 5' end during synthesis. The results were read with a Pan Vera Beacon 2000 fluorescence polarization instrument. To detect the binding of our DNA aptamers to CEA on the surface of cells, fluorescence microscopy was used. Our results showed that in the fluorescence polarization assay, the K_d values of BSA, BGG and IgGs were found to be 100 to 1000-fold higher than those obtained for CEA, respectively. The FP experiments revealed two disparate aptamer sequences (primer versus consensus). We showed that these aptamers bind specifically to CEA on the surface of cancerous cell line: MCF7 (human breast adenocarcinoma) by fluorescence microscopy. We also demonstrated that CEA specific DNA aptamer did not bind to the surface of a CEA negative cell line: COLO320DM (human colon adenocarcinoma).

Key words: Aptamer · Carcinoembryonic antigen · Cancer · Fluorescence polarization · Fluorescence microscopy

INTRODUCTION

Advances in biotechnology have led to new techniques for the design, selection and production of ligands suitable for molecular targeting. One promising approach is the production of specific receptor binding molecules based on defined nucleic acid sequences that are capable of recognising a wide array of target molecules. These oligonuclide ligands are known as aptamers [1]. Aptamers are artificial specific oligo-nucleotides, DNA or RNA, with the ability to bind to non-nucleic acid target molecules such as peptides, proteins, drugs, organic and inorganic molecules or even whole cells, with high affinity and specificity comparable to antibodies [2]. Because of the unique characteristics of aptamers and these molecules represent ideal ligands for bioanalytical applications [3]. Aptamers possess a number of desirable properties such as ease of synthesis, stability, robustness and lack of immunogenicity [4]. These molecules are identified through an in-vitro selection protocol called "systematic evolution of ligands by exponential enrichment" (SELEX) that interrogates using large pools of random RNA or DNA sequences [5]. These molecules penetrate tumour readily, reach peak levels quickly and clear from the body rapidly, thus having properties of low toxicity and immunoreactivity [1]. Aptamers offer significant advantages over existing antibody-based recognition procedures in that they offer higher binding affinity and specificity to the target, higher selectivity against mutated protein epitopes and potentially reduced immunogenicity and increased tumour penetration associated with their size [6-8]. Aptamers

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have been a focus of therapeutic research for the last two decades. These have potential use as a biomarker, therapeutics and diagnostics [9]. Techniques involving antibodies or phage displayed antibody fragments are increasingly being replaced by aptamers in different configurations, taking advantage of the unique properties of aptamers [10]. Recently, aptamers have been selected against whole living cells, opening a new path which presents three major advantages: (1) direct selection without prior purification of membrane-bound targets, (2) access to membrane proteins in their native conformation similar to the in vivo conditions and (3) identification of (new) targets related to a specific phenotype [11]. Carcino-embryonic antigen (CEA) is a 180 kDa highly glycosylated, membrane-anchored, protein over-expressed on breast, ovarian, colon and other cancer cells [12]. So far, no anti-CEA aptamers have been reported. CEA is, arguably, the best-studied tumor epitope and present on the largest number of tumors. These and other conditions, lead to an increase in blood CEA; hence, clinically, serum CEA levels may be indicative (but not diagnostic) of the return of active metastatic disease [13]. The goal of this project was to demonstrate convincingly that DNA aptamers bind with high affinity to CEA on the surface of cancerous cell line: MCF7 (human breast adenocarcinoma) *in vitro*.

MATERIALS AND METHODS

Samples: DNA sequences (aptamers) (Table 1) were obtained from Operon (Technologies, Alameda, Ca). The aptamers were also selected from a library of 100-base single stranded DNAs obtained from Operon The 100-base single stranded DNA library consisted of an internal 64 base variable region flanked by two 18 base constant sequences used as PCR primer sites. The constant sequences were chosen due to lack of secondary structure to prevent annealing to each other [14]. CEA, obtained from Calbiochem (La Jolla, Ca). Bovine gamma globulin (BGG) was provided by Pan Vera Corp (Madison WI). Bovine serum albumin (BSA), Horse, Dog and Bovine IgGs obtained from Sigma-Aldrich, Saint Louis, MO, USA.

Fluorescent Polarization: Fluorescence polarization (FP) analysis works by exciting a fluorophore with plane polarized light and measuring the amount of time it takes for molecules to rotate a certain number of degrees by detecting the level of polarization emitted by the fluorophore. A positive test will have more polarized light as molecular rotation is slower. Control experiments were tested for binding of the same DNAs to non-CEA proteins and a random DNA sequence binding to CEA. The FP

Table 1: Oligonucleotides sequences tested in the FP assay. The G-rich consensus was developed from 21 occurrences in a selected library. Note that R= A or G bases deviate from the consensus. Primer sequence is shadowed and consensus sequence is underlined

Consensus Sequences	**Level of conservation (%)**	
GGG..GGGG..G..G...TACCC	60	
GGGGAGGGGG.G..GGGATACCC	50	
GGGGGAGGGGGTGRGGGATACCCC	40	
Oligonucleotides studied		**Name**
AGGGGGTGAAGGGATACCC		consensus sequence
GGGG G AGGGGGTGAAGGGATACCC		G-rich-consensus sequence
ATACCAGCTTATTCAATT GGGGTAGGGGGCGAAGCGATACCCTAATCAGC		c26b1-50
ATACCAGCTTATTCAATT GGGGGAGGGGGCGACGCGATACCC		c22b1-42
GGGGGAGGGGGCGACGCGATACCC		c22b19-42
ATACCAGCTTATTCAATT		b1-18 (5' primer)
CGGGAATTCTGGCTCTGCGACATGA		random sequence

Table 2: The dissociation constants (K_d's) for the oligonucleotide binding to the CEA and control proteins such as BSA, BGG and IgGs

Oligonucleotide	K_d (nM^{-1})			
Name	CEA	BSA	BGG	IgG*
[F] G-rich-consensus sequence	2.63	not done	1500	>1000
[Fl] Clone26bases1-50	2.86	969.3	1229	>1000
[Fl] Clone22bases1-42	2.26	171.9	551.3	>1000
[Fl] Clone22bases19-42	3.04	967.5	167.4	>1000
[Fl] b1-18 (5'-Primer)	0.69	3366	488.4	>1000
[Fl] Random Primer	>1000	>1000	>1000	>1000

*Dog/Horse/Bovine

assay was used to assess binding of the aptamers to control proteins (Table 2) such as BSA, BGG, Dog IgG, Horse IgG and Bovine IgG. For fluorescent polarization measurements, the aptamers were labeled with fluorescein isothiocyanate (FITC) at their 5' end during synthesis. All measurements were made in binding buffer representative of physiological conditions. Experiment was carried out at 25°C. Cancer cells (CEA) and control proteins were incubated for two hours with deferent aptamers in a total volume of 100 µL. The results read with a Pan Vera Beacon 2000 fluorescence polarization instrument.

Fluorescence Microscopy: In another experiment, to detect the binding of our DNA aptamers to CEA on the surface of cells, fluorescence microscopy was used. Data for the microscopy experiments were collected using the camera installed on our UV microscope. Cancer cells (MCF7: human breast adenocarcinoma) were incubated at 37°C with %5 CO_2 on a multi-well slide overnight and then washed 3X with PBS. Cells were incubated for two hours at 4°C with deferent aptamers either: COLO320DM (human colon adeno-carcinoma as negative control), consensus sequence, b1-18 (5' primer) (Table 1) and anti-CEA antibody (as positive control). All concentrations were 1uM in a total volume of 150 µL. Cells were then washed 3X with PBS on shaker for 5 min, fluorsave mounting media was added to the slide and coverslips were placed on the slide. The slide was then imaged immediately using both brightfield (left column) and fluorescence (right column) microscopy. Images were collected and compared to those of the CEA negative control cell line and adjustments in conditions made to optimize aptamer binding. Unless otherwise noted total magnification was 600 X.

RESULTS

Our results showed that in the fluorescence polarization assay, the dissociation constants (K_d's) values of BSA, BGG and IgGs were found to be 100 to 1000-fold higher than those obtained for CEA (Table 2). The fluorescein-labeled random DNA used as a negative control showed no binding to CEA in the 2-800 nM

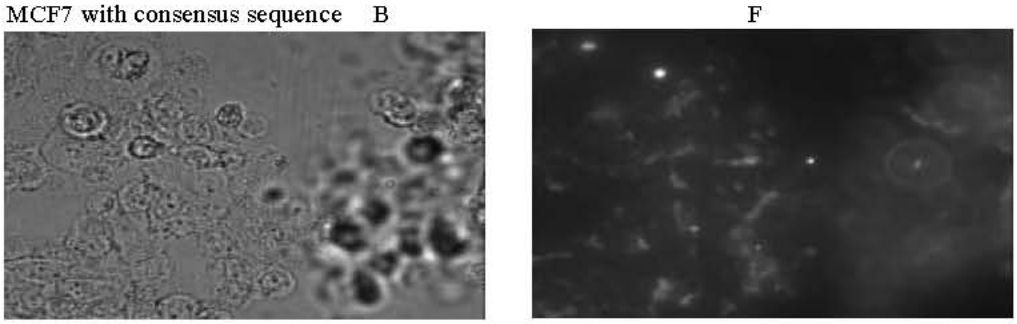

Fig. 1: Photographs were taken of the cells under light microscopy and superimposed on fluorescent images. MCF7 indicates as cancer cell and bound properly to consensus sequence, B and F indicate as bright and flourecense picture with fluorescence microscopy, respectively

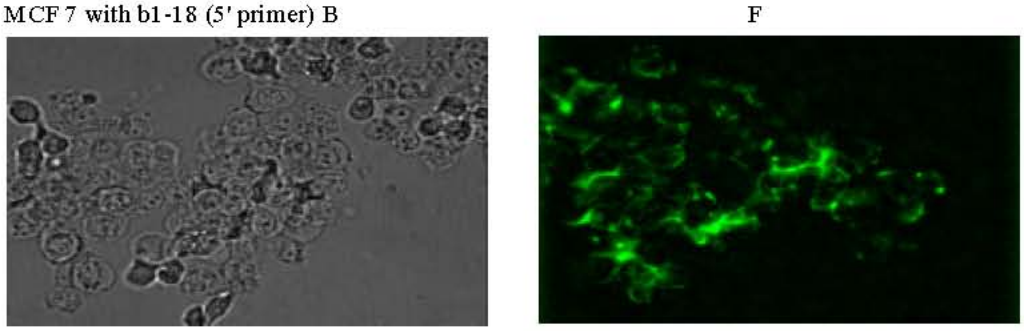

Fig. 2: Photographs were taken of the cells under light microscopy and superimposed on fluorescent images. MCF7 indicates as cancer cell and bound properly to b1-18 (5' primer), B and F indicate as bright and flourecense picture with fluorescence microscopy, respectively

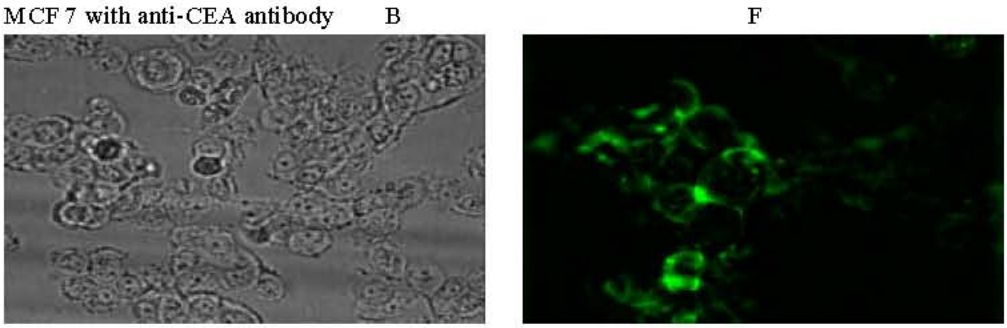

Fig. 3: Photographs were taken of the cells under light microscopy and superimposed on fluorescent images. MCF7 indicates as cancer cell and bound to anti-CEA antibody, B and F indicate as bright and flourecense picture with fluorescence microscopy, respectively.

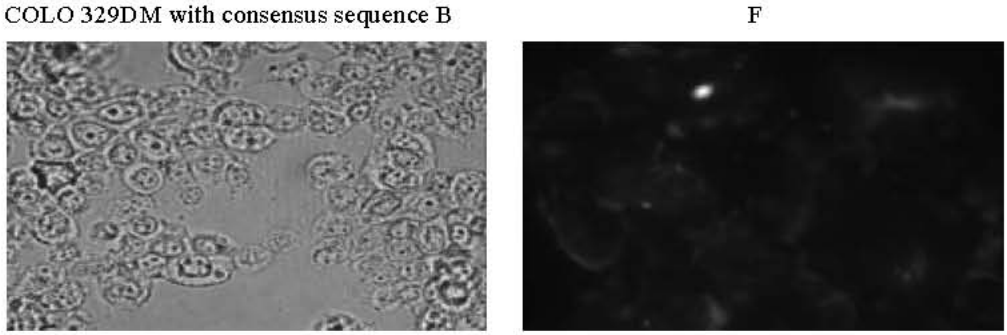

Fig. 4: Photographs were taken of the cells under light microscopy and superimposed on fluorescent images. COLO329DM indicates as negative cancer cell and did not bind to consensus sequence as an aptamer. B and F indicate as bright and fluorescence picture with fluorescence microscopy, respectively.

concentration ranges. The FP experiments revealed two disparate aptamer sequences (primer vs consensus). In fluorescence microscopy experiment, our results showed that the two aptamers [consensus sequence and b1-18 (5' primer)] (Figurr 1 and 2) and also anti-CEA antibody (Figure 3) bound to CEA expressing MCF7 cells. We also demonstrated that CEA specific DNA aptamer did not bind to the surface of COLO320DM (human colon adenocarcinoma), a CEA negative cell line (Figure 4).

DISCUSSION

There are a large number of studies targeting tumor cells *in vivo* using anti-CEA antibodies. Today, several antibodies against CEA have been generated for use as therapeutic or diagnostic reagents. The anti-CEA antibody T84.66, which has a high affinity and specificity for CEA, is suitable for *in vivo* tumor targeting [15]. It has been successfully used for *in vivo* imaging and diagnosis of human colorectal carcinoma [16]. Biparatopic antibodies (BpAbs) having the capability of binding 2 different non-overlapping epitopes on the same target antigen molecule (CEA) have been developed [17]. Because aptamers can be chemically synthesized, manufacturing and the regulatory approval process should be substantially simpler and less costly than for antibodies. Agonistic aptamers could therefore represent a superior alternative to antibodies for the therapeutic manipulation of the immune system. So, it can be concluded that aptamers are able to distinguish proteins that differ by a single amino acid substitution [18]. The developed anti-CEA aptamers may be useful to use these reagents as *in vitro* diagnostic tools. In such products, aptamers can play a key role either in conjunction with, or in place of, antibodies. Also, these experiments also established an FP assay for the detection of CEA in homogenous solutions. The assay was quick, highly selective and can detect CEA in low nM range.

Our results showed that in the fluorescence polarization assay, the K_d values in BSA, BGG and IgG of dog, horse and bovine were higher than those obtained for CEA. The FP experiments revealed two disparate aptamer sequences [b1-18 (5' primer) versus consensus sequence]. Also we showed that two high affinity and

specificity anti-CEA aptamers [consensus sequence and b1-18 (5' primer)] bound to CEA expressing MCF7 cells using fluorescence microscopy that deserve further study. The anti-CEA antibody bound to CEA expressing MCF7 cells as positive control. We also demonstrated that CEA specific DNA aptamer did not bind to the surface of COLO320DM (human colon adenocarcinoma), as a negative cell line control. Aptamers have shown affinity for their targets, comparable to, if not better, than their monoclonal antibody counterparts, with dissociation constants (K_d's) values in the picomolar range achieved [19, 20]. Additionally, the binding specificities of the aptamers have been demonstrated to allow 10,000-fold to 12,000-fold discrimination of aptamers towards their target molecules even in the case of very closely related structures, such as the case of the theophylline aptamer [21]. A protocol for measuring binding of anti-CEA antibodies to cells expressing this protein on their surface has been developed [22]. They tested the binding of three aptamers to cells expressing CEA. Some experiments indicated excellent binding to CEA expressing cells, while other experiment failed to show binding. It has been shown that the selected aptamers can be used as a molecular tool for further under standing surface protein expression patterns on tumor cells and thus providing a foundation for effective molecular analysis of leukemia and its subcategories [23].

In contrast to conventional methods, such as phage display antibody production targeting a previously known specific protein, the novelty of SELEX based protein discovery is rooted in its focus on finding cell surface membrane markers with no prior knowledge of the molecular contents of the cell surface. Also, owing to their easy chemical manipulation and reproducible generation of DNA aptamers by automated synthesis, this method is more universal and technically feasible. Finally, apart from the ability of identification of disease markers that may play key roles in cancer progression, this method can also be useful in early diagnosis, targeted therapy and as molecular tools in recognition as well as mechanistic studies of diseased cells [24]. It has been reported that generated aptamers can serve as high-affinity and specific probes for the identified biomarkers. This will be useful for future diagnostic applications. The cost and complexity of this approach are significantly lower than those of antibody-based techniques. This offers the potential for wider application and may have a very positive impact on the field of biomarker discovery for diseases, as well as for specific cellular functions [25]. This strategy will enable efficient discovery of new malignancy-related bio-markers, facilitate the development of diagnostic tools and therapeutic approaches to cancer and markedly improve our understanding of cancer biology. The high affinity of the aptamers for their target cells is absolutely essential to apply them to disease diagnosis and drug delivery [26].

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