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In vitro selection of RNA aptamers against CA125 tumor marker in ovarian cancer and its study by optical biosensing

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ABSTRACT

Early identification of neoplastic diseases is essential to achieve timely therapeutic interventions and significantly reduce the mortality of patients. A well-known biomarker is the Cancer Antigen 125 (CA125) or 16 mucin (MUC 16), a glycoprotein of the human family of mucins, already used for the diagnostic and prognostic evaluation of ovarian cancer. Therefore, the detection of CA125 to now remains a promising tool in the early diagnosis of this tumor. In this paper, we describe the development of RNA aptamers that bind with high affinity the tumor antigen CA125. We performed eight cycles of selection against CA125 purified protein. The selected aptamers were cloned and sequenced and the binding properties of the most promising sequences were studied by Real Time PCR and Surface Plasmon Resonance (SPR) to evaluate their ability in targeting CA125 protein with perspective applications in aptamer-based bioassays.

1. Introduction

The epithelial ovarian carcinomas represent approximately 90% of all types of ovarian malignant neoplasms [1], and are currently among the most difficult cancers to early diagnose due to the lack of specific signs and symptoms, coupled to the absence of reliable screening strategies. Most patients are diagnosed in the advanced stage of the disease, resulting in low overall cure rates. Ovarian cancer patients are generally treated with surgical resection and subsequent chemotherapy [2]. Although many patients initially respond well to chemotherapy, long term survival remains poor due to eventual tumor recurrence and emergence of drug-resistant disease. Overall, the five years survival rate is 45% [1]. The most widely used tumor marker for ovarian cancer is the CA125/MUC16 antigen. Measurement of serum level of this biomarker has become a gold standard component of routine management of women with ovarian cancer [3,4]. CA125 antigen, identified for the first time in 1981 [5], is a high molecular weight glycoprotein which is raised in 90% of patients with epithelial ovarian cancer. Several studies have confirmed the usefulness of CA125 levels in monitoring the progress of patients and in addition most reports indicated that the rise in CA125 levels precedes clinical detection by about 3 months [6–8]. Unfortunately, a few prospective studies indicate the inadequate sensitivity of CA125 screening in asymptomatic populations, suggesting the need for a more sensitive detection. Therefore, the identification of new tools able to recognize with high efficacy and sensitivity this protein is an important challenge in oncology.

An emerging class of promising targeting molecules is represented by nucleic acid aptamers and their use as bioanalytical tool is impressively increasing. Aptamers are single-stranded RNA or DNA oligonucleotides of 15–60 bases in length, raised to bind with high affinity and specificity to a wide range of molecular targets such as nucleic acids, proteins, small compounds or entire cells [9,10]. These oligonucleotides bind to their targets by folding into complex tertiary structures, thus acting similar to antibodies. Advantages of these molecules *vs* antibodies or other protein-based receptors, have been reported by several authors [11–15]. Indeed, aptamers combine the high binding affinity (K_d in the low nanomolar–picomolar range) comparable to that of antibodies, with relatively simple production by the Systematic Evolution of Ligands by Exponential enrichment (SELEX) technology [16–18] that avoids the use of animal cells. Therefore, aptamers are cost-effective, easier to characterize than antibodies and, once selected,

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can be chemically synthesized with high purity and very low inter-batch variability. In addition, aptamers are not immunogenic and thanks to their small size have improved tissue penetration. Differently from antibodies, they are sufficiently stable, resistant to harsh environments (pH and temperatures) and can be easily modified. Modifications can be done to further improve their stability, bioavailability, and pharmacokinetics or to allow their simple use as biocomponents in diagnostic assays by immobilizing them onto solid supports or by employing several labeling strategies [19]. Impressive examples are Enzyme Linked OligoNucleotide Assays (ELONA), which are now proposed as an alternative to Enzyme Linked Immunosorbent Assays (ELISA) or biosensor-based platforms [20,21]. Moreover, aptamers can be designed to interface electronic detectors for aptasensing applications to point-of-care instrumentation, and their coupling to nanotechnology represents an interesting challenge for molecular disease diagnostics [22]. In order to develop efficient bioanalytical platforms, the aptamer should be selective and bind the target analyte with high affinity [21]. The complex between the aptamer and the target molecule should be stable to allow reliable analytical measurements. In this perspective, it is evident how the evaluation of kinetic parameters of the affinity interaction represents an important task of aptamers development. In this paper, a protein-SELEX approach to generate nucleases resistant aptamers able to specifically recognize CA125 antigen is reported. The strategy was based on the use of a CA125 purified protein as target, modified with a Histidine (His) tag. The His-tag modification was adopted to recover at each cycle bound sequences on Ni^{2+} NTA agarose magnetic beads [23–25]. At each round, the selection step was preceded by one or two counter-selection steps against His-tagged VEGF recombinant protein to avoid the isolation of non-specific binders. Magnetic particles or magnetic separators are used in different SELEX techniques [23–25]. For example, the Capture-SELEX describes the immobilization of the aptamer pool on the magnetic particles or other surfaces for soluble targets [26]. Also different applications include a magnetic separator for both partial automation of the aptamer selection process [23–25,27,28]. The SELEX cycle was repeated for 8 times and the final enriched pool was cloned and sequenced. By such an approach, we identified two aptamers (CA125.1 and CA125.11) that effectively bind to CA125 protein and Surface Plasmon Resonance (SPR)-based approach was performed to characterize their binding kinetics. The calculated dissociation constants serve as a basis for the evaluation of the obtained aptamers and for their further application in aptamer-based assays for point-of care diagnostics [29].

2. Materials and methods

2.1. RNA library and target protein

For the SELEX procedure, a 2′-Fluoro-Pyrimidine (2′-F-Py)-modified RNA library with a variable internal region of 45 base pairs and two fixed regions for the amplification reaction at 5′ and 3′ ends was used. To prepare the library, the correspondent DNA pool was PCR amplified using the following oligonucleotides: forward primer (p10), 5′-TAATACGACTCACTATAGGGAGACAAGAA TAAACGCTCAA-3′ and reverse primer (p20), 5′-GCCTGTTGTGAGC CTCCTGTCGAA-3′. The forward primer p10 contains a T7 promoter sequence for the *in vitro* transcription [30]. The PCR was performed in the presence of 2 μM of each primer, 1 mM dNTPs mix (Amersham Pharmacia Biotech) and 0.05 U/μl Taq DNA polymerase (Roche, Germany). After 3 min initial denaturation at 95 °C, the PCR program consisted of 8 cycles of: 30 s at 94° C, 1 min at 60 °C and 30 s at 72 °C.

Transcription was performed in the presence of 1 mM 2′-F-Py (2′F-2′-dCTP and 2′F-2′-dUTP, TriLink Biotech, San Diego, CA), 1 mM ATP, 1 mM GTP (Amersham Pharmacia Biotech), 10 mM dithiothreitol (DTT) (Sigma, St. Louis, MO), 0.5 U/μL RNAse inhibitors, 1 U μL^{-1} inorganic pyrophosphatase (Roche, Germany), and 2.50 U μL^{-1} of a mutant form of T7 RNA polymerase (T7Y639F RNA polymerase, Epicentre Biotechnologies), that was used to improve yields. 2′F-Py RNAs were used for increasing resistance to the nucleases degradation. After transcription, any leftover DNAs were removed by DNase I (Roche) digestion and the resulting sequences were purified by phenol:chloroform extraction and ethanol precipitation. The RNAs were run on a denaturing 8% acrylamide/7 M Urea gel and the band of expected size was cut out from the gel and eluted with 0.3 M sodium acetate, and 2 mM EDTA for 2 h at 42 °C. The purified 2′F-Py RNA library was quantified with NanoDrop UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA). The complexity of the starting pool was roughly 10^{14}.

Recombinant Human CA125 with a C-terminal 6-Histidine tag was purchased from R&D (Minneapolis, MN) and Human VEGF1 full length protein (His tagged) by Abcam (Cambridge, UK) Ni^{2+} NTA Magnetic Agarose Beads (Qiagen, Milan, Italy) was used to separate aptamer-His-tagged proteins complexes.

2.2. In vitro CA125 protein SELEX

For the SELEX strategy, His-tagged CA125 was used as target for the selection and His-tagged VEGF for the counterselection. Before each cycle of SELEX, 2′F-Py RNA pool was dissolved in MilliQ water and heated at 85 °C for 5 min, cooled on ice for 2 min, and allowed to warm up to room temperature. The RNA–protein incubation was performed in Binding Buffer (BB: 10 mM Tris HCl 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton). At each cycle, in order to avoid selecting for aptamers that non-specifically recognize the target protein, the pool was first incubated (counterselection step) for 30 min with His-tagged VEGF. The RNA–protein complexes were then separated on Ni^{2+} NTA Magnetic Agarose Beads previously washed with BB buffer and the unbound sequences were recovered for the selection step aimed to isolate sequences specifically recognizing CA125. The recovered sequences were thus incubated with His-tagged CA125 at room temperature and the RNA–protein complexes were again isolated by Magnetic Agarose Beads for 30 min. Unbound RNAs were removed and after several washings with the binding buffer, the bound sequences were recovered by total RNA extraction using the TRIzol Reagent (Life Technologies, Carlsbad, CA). Extracted RNAs were reverse transcribed with M-MuLV Reverse Transcriptase (Roche, Indianapolis, IN) and PCR-amplified under low stringency conditions (high $MgCl_2$ and dNTP concentrations) in order to introduce random mutations. PCR program used was: 1 min at 93 °C, 1 min at 53 °C and 1 min at 72 °C. This cycle was repeated for least 10 times (see Table 1). Finally, the DNA template was *in vitro* transcribed. During the selection process, we progressively enhance the selective pressure by increasing the number of washings and by reducing the RNA/protein molar ratio. The *in vitro* counter-selection/selection scheme was repeated for eight cycles (Table 1). The final pool was TOPO-TA cloned, transforming into *Escherichia coli* (TOPO Cloning, Life Technologies, Carlsbad, CA). Isolated clones (69) were subsequently sequenced (www.eurofinsdna.com). Sequences analysis and alignments were carried out using the ClustalW2 software and phylogenetic tree was visualized with TreeVieX program. Secondary structures were predicted by using RNA structure version 5.1.

Sequences of the three most represented clones are displayed in Section 3.