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MINIREVIEW

High-specificity nucleic acid aptamers for detection of ovarian cancer protein biomarkers: Application in diagnostics

Antonija Hanžek[1], Christian Siatka[1, 2] and Anne-Cécile E Duc[1, *]

[1]University of Nîmes - *UPR CHROME*, 7 Place Gabriel Péri, 30000 Nîmes, France

[2]Ecole de l'ADN, 19 Grand Rue, 30000 Nîmes, France

***Correspondence to:** Anne-Cécile Duc, Email: anne-cecile.duc@unimes.fr

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ABSTRACT

Ovarian cancer (OC) is the most deadly gynaecological cancer and the 8th leading cause of cancer-related deaths in women. Unfortunately, screening programs and early-stage diagnostic methods are not available, so late diagnosis remains a major contributor to the poor prognosis. Recently, aptamers have emerged as useful tools in cancer diagnostics. Aptamers are short single-stranded DNA or RNA oligonucleotides that bind with high specificity to diverse targets, including cancer biomarker proteins. Aptamers are developed *in vitro* using different variations of Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Herein, we describe the advances in the development of high-specificity DNA and RNA aptamers for detection for OC protein biomarkers. Here, we present a discussion on their diagnostic application in the detection of two clinically relevant ovarian cancer biomarkers: Cancer Antigen 125 (CA125) and Human Epididymis protein 4 (HE4). Ten promising candidate aptamers (seven for CA125 and three for HE4) have been identified. The stability in human serum and target detection in clinically relevant concentration range demonstrates the utility of aptamers as molecular probes in OC biomarker tests. Aptamer-based diagnostic platforms, such as biosensors and clinical assays could enable easy and rapid protein detection, with potential for earlier diagnosis of OC that could be implemented in clinical practice in the future.

KEYWORDS: Ovarian cancer, CA125, HE4, SELEX, aptamers, biosensors, diagnostics

INTRODUCTION

Ovarian cancer (OC) is the most deadly gynaecological cancer and the 8th leading cause of cancer-related deaths among women globally (Bray et al, 2018). OC is a heterogeneous disease, with 90% of primary tumours arising from the ovarian surface epithelium (Kurman et al, 2014; Berek et al, 2018). Due to the silent, asymptomatic or non-specific nature of the symptoms, most patients are diagnosed in advanced stages, with a 5-year survival rate of 15 - 40%. However, when diagnosed in early stages, the survival raises to 90% (Berek et al, 2014; Siegel et al, 2021). Unfortunately, screening methods are not available (Nash and Menon, 2020), so diagnosis remains one of the most significant barriers for survival (Siegel et al, 2021). Current diagnosis relies on transvaginal ultrasound, pelvic examination and increased serum concentration of Cancer Antigen 125 (CA125), but is ineffective in detecting cancer early (Ledermann et al, 2013). Numerous protein biomarkers have been discovered and evaluated in the last decade, but only CA125 and Human Epididymis protein 4 (HE4) are clinically used in the management of OC (Sölétormos et al, 2016). Both proteins are detected using immunoassays. Development of novel, sensitive detection methods that could detect the presence of cancer in the

early phase would improve the prognosis of OC. Recently, nucleic acid aptamers emerged as molecular probes in diagnostics, holding potential as alternative to antibodies. Aptamers are synthetic single-stranded DNA or RNA oligonucleotides that bind with high-affinity to a wide range of target compounds, including small molecules, proteins or whole cells (Komarova and Kuznetsov, 2019; Kulabhusan et al, 2020). Due to their unique characteristics of high specificity and binding affinity, low toxicity, and easy and reproducible synthesis, they offer advantages over antibodies. Also, the use of animals or cells is not required since the development is *in vitro* (Kulabhusan et al, 2020)" Aptamers in diagnostics offer assay stability, easy regeneration and variety of chemical modifications to detect the target of interest in direct, indirect or sandwich concepts. Herein, we describe the recent advances in development of aptamers for detection of OC protein biomarkers and present applications as recognition probes in various diagnostic technologies.

OVARIAN CANCER PROTEIN BIOMARKERS

Several protein biomarkers are studied in management of OC (Yang et al, 2017). The most relevant, clinically-approved biomarker is CA125 (Sturgeon et al, 2008). CA125 is a heavily glycosylated, high-molecular weight transmembrane mucin overexpressed in 80% of OC (Charkhchi et al, 2020). Serum levels of CA125 are measured routinely to assist in diagnosis, monitoring the response to treatment or disease progression (Sturgeon et al, 2008; Felder et al, 2014). Elevated CA125 levels are found in 50% of patients at the early-stage and in 85 % patients with advanced disease (Ledermann et al, 2013). CA125 can also be elevated during menstruation, pregnancy, benign gynaecological conditions and in other cancers (Meden and Fattahi-Meibodi, 1998; Sölétormos et al, 2016). *Diagnostic tests aim at classifying or predicting the presence or absence of OC. Two measures of quantifying the diagnostic accuracy are sensitivity, ability of a test to detect the OC when it is truly present; and specificity, the probability of a test to exclude OC in patients who do not have the disease (Mandrekar, 2010).*

$$Sensitivity = \frac{True\ Positives}{True\ Positives + False\ Negatives}$$

$$Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positives}$$

The sensitivity of serum CA125 provided by the standard immunoassays is 74%, while specificity is 83% for detection of OC (Zhen et al, 2014). The cut-off value for serum CA125 is 35U/ml (Bast et al, 1983; Donach et al, 2010), which falls within K_d values that can be obtained for aptamer-protein interactions. Another promising biomarker is Human epididymis protein 4 (HE4) (Huang et al, 2018). HE4 is a glycoprotein overexpressed in the majority of OC (Drapkin et al, 2005). HE4 plays a role in the pathogenesis of OC by mediating cell proliferation and tumour growth (Moore et al, 2009; James et al, 2018). It is clinically used in the monitoring of treatment and prediction of relapse (Colombo et al, 2019; Scaletta et al, 2017). Serum level of HE4 in healthy woman ranges from 60-150pmol/l (Sölétormos et al, 2016). The sensitivity of serum HE4 by standard immunoassays is 74%, while specificity is 90% for detection of OC (Zhen et al, 2014). Serum HE4 level can be useful alone or combined with CA125 as part of Risk of Ovarian Malignancy Algorithm (ROMA) algorithm in the preoperative differential diagnosis of pelvic masses (Holcomb et al, 2011; Dochez et al, 2019; Kim et al, 2019). Recently, multiple biomarkers for OC have been identified but, but are still in research phase. One such biomarker is a stress-induced phosphoprotein 1 (STIP1), reported as protein elevated in blood of patients (Wang et al, 2010; Chao et al, 2013). The simultaneous detection with criteria of serum CA125 > 35U/ml and STIP1 > 55ng/ml achieved 100% sensitivity and 95.6% specificity for detecting OC. Using STIP1 appears useful in detecting early-stage OC, suggesting a combined approach for early detection (Wang et al, 2010).

DEVELOPMENT OF THE NUCLEIC ACID APTAMERS FOR DIAGNOSTIC PURPOSE

Aptamers are single-stranded DNA or RNA oligonucleotides that bind with high affinity and specificity to targets, including cancer proteins (Wu et al, 2015). Their unique secondary and tertiary structures enable the recognition of the specific target, thus acting like antibodies (Hori et al, 2018). Aptamers are obtained by *in vitro* selection, using various methods based on *Systematic Evolution of Ligands by EXponential enrichment* (SELEX) (Komarova and Kuznetsov, 2019). High-affinity aptamers are selected from a highly diverse library composed of ssDNA or RNA oligonucleotides pool. The initial aptamer library consists of a random 20-60 nucleotide-long region, flanked by two fixed, primer binding sites for amplification (Vorobyeva et al, 2018; Komarova and Kuznetsov, 2019) be it therapeutics, drug delivery systems or biosensors. It is now generally acknowledged that in vitro selection enables one to generate aptamers to almost any target of interest. However, the success of selection and the affinity of the resulting aptamers depend to a large extent on the nature and design of an initial random nucleic acid library. In this review, we summarize and discuss the most important features of the design of nucleic acid libraries for in vitro selection such as the nature of the library (DNA, RNA or modified nucleotides. Target protein characteristics are important aspects to consider before SELEX. Human protein biomarkers are often heavily post-translationally modified. To ensure proper target recognition, human native proteins or recombinant proteins expressed in mammalian cells should be favored over recombinant proteins from bacterial systems. The experimental overflow of SELEX is displayed in the Figure 1. Aptamers are selected from the library after repeated cycles of (a) incubation of ssDNA or RNA with the OC protein target, which leads to (b) recognition and binding. Specifically-bound sequences are separated from unbound by (c) washing. Washing conditions stringency, including salt concentration and temperature are adapted to increase

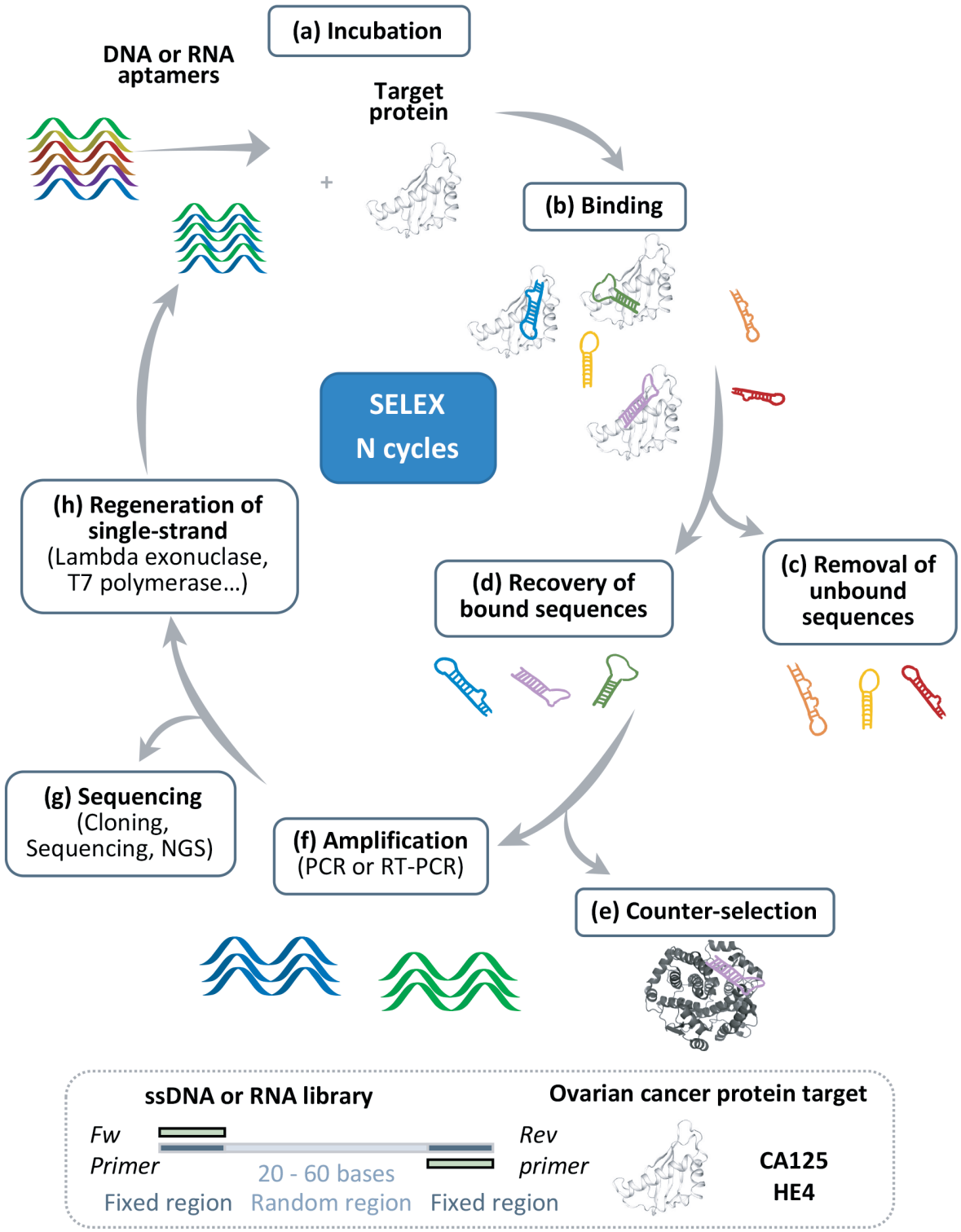


Figure 1. *In vitro* selection of aptamers for ovarian cancer protein biomarkers by Systematic Evolution of Ligands by EXponential enrichment (SELEX). High-affinity aptamers are selected after N cycles from the initial single-stranded DNA or RNA aptamer library. The library consists of a random region flanked by two fixed regions used as primer binding sites. The target ovarian cancer protein biomarker (Cancer Antigen 125 - CA125 or Human Epididymis protein 4 - HE4) are (a) incubated with DNA or RNA aptamers which results in their (b) binding. (c) The Non-specific unbound sequences are removed using multiple steps of washing. The aptamers specific to the ovarian cancer protein target are (d) recovered by elution and/or purification and (f) amplified by Polymerase Chain Reaction (PCR). To remove non-specific aptamers, (e) counter-selection is performed to non-specific protein targets. Aptamer sequence determination can be achieved through (g) cloning followed by Sanger sequencing or Next-Generation Sequencing (NGS) methods. The single-stranded aptamers are (h) regenerated from double stranded PCR products and used as input for the next cycle of SELEX.

binding strength to the target. Specific sequences are then (d) recovered by elution or purification from the aptamer-protein complexes and (f) amplified by polymerase-chain reaction (PCR) for DNA or reverse transcription polymerase chain reaction (RT-PCR) for RNA aptamers. ssDNA or RNA sequences are (h) regenerated, yielding a new oligonucleotide pool used as the input for the next cycle. Regeneration of single-stranded sequences from PCR products is accomplished using streptavidin columns, lambda-exonuclease activity, strand separation by size, nickases for DNA or by *in vitro* transcription for RNA aptamers. Aptamer sequence determination can be achieved through (g) cloning followed by Sanger sequencing or high-throughput methodologies, such as Next-Generation Sequencing (NGS). Non-specific binding is eliminated by selection against non-specific protein target counter-selection (e) and/or components of the matrix (negative-selection) (Komarova and Kuznetsov, 2019).

Further development of the diagnostic aptamers is displayed in Figure 2. Different SELEX methodologies are used for selection of diagnostic aptamers. "Membrane-SELEX" is a technique where the protein or the library is immobilized on the nitrocellulose membrane. "One-pot SELEX" is the approach where selection and amplification are performed within the same PCR tube, to minimize the contamination and loss of binders during transfers. The use of capillary electrophoresis SELEX (CE-SELEX) is growing due to shorter time of selection (even one-round screening) and increased screening-rate efficiency (Zhu et al, 2019). After (1) selection and identification by SELEX, candidate aptamers are chemically synthetized and subjected to (2) characterization of the interaction between the aptamer and protein. The binding affinity is characterized by strength, displayed as K_d (dissociation constant), and the specificity to target (Kaur et al, 2019). K_d is typically expressed in molar concentration (M) or in U/ml, the clinical unit in the standard immunoassays (Scoville et al, 2017). Once the aptamers have confirmed binding affinity, they are applied as (3) recognition probes in diagnostic tests. With a global need for rapid, easy and accurate tests, Point-Of-Care Testing (POCT) industry is a rapidly growing area of the diagnostic sector. Depending on the detection principle, POCT incorporates various assay formats for cancer-related proteins (Dhiman et al, 2017). A variety of aptamer-based diagnostic tests are designed for detection of OC protein biomarkers, including lateral flow assays (FLA), aptamer-antibody sandwich type assays and numerous biosensors. The overview of currently available diagnostic platforms and their analytical characteristics is displayed in Supplementary Table 2. Herein, we present the recent advantages in development of aptamers for the detection of OC biomarkers.

DIAGNOSTIC APTAMERS FOR OVARIAN CANCER BIOMARKERS

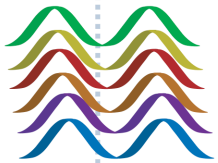
CA125

Most diagnostic aptamers are selected against the gold standard OC biomarker CA125. All selection conditions are available in the Supplementary Table 1. The first reports were RNA aptamers selected from 2'-Fluoro-Pyrimidine (2'-F-Py) RNA library with a 45 bases-long random region, against recombinant 6xHis-tagged CA125 immobilized on Ni^{2+} Nitrilotriacetic acid (NTA) magnetic beads. After 8 cycles of SELEX, including a counter-selection to Vascular Endothelial Growth Factor (VEGF), the oligonucleotide pool was cloned, sequenced and analyzed. Authors used ClustalW2 Software for sequence analysis and alignment, TreevieX for phylogenic tree visualization and RNA structure version 5.1 for secondary structures. Two aptamers, CA125.1 and CA125.11 (Supplementary Table 1) were evaluated for binding to CA125 using Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR) and Surface Plasmon Resonance (SPR). By SPR, CA125.1 appeared a better binder, with a K_d in the nanomolar range (4.13×10^{-9} M) (Lamberti et al, 2016). In Scoville *et al.*, aptamers were selected using "One-Pot" SELEX from a 5'FAM-modified ssDNA library (25-nucleotides random region) to human CA125 from ascites after 4 cycles. The bioinformatic analysis at each cycle was performed with the Illumina data pipeline made available on GitHub (Rebecca Whelan Python Enrichment). Based on fold enrichment, candidate aptamers were selected for characterization by fluorescence anisotropy (FA) and affinity-probe capillary electrophoresis (APCE). Two aptamers, CA125_1 and CA125_12 (Supplementary Table 1) displayed concentration-dependent binding with K_d values of 207±109U/ml by FA and 80±38U/ml by APCE for aptamer CA125_1; and K_d values of 118±123U/ml by FA and 131±93U/ml for aptamer CA125_12, respectively (Scoville et al, 2017). The aptamer CA125_1 was applied in the aptamer-antigen-antibody sandwich-type assay for electrochemical detection of CA125. The diagnostic test was evaluated in spiked blood and serum samples. The analytical performances of the biosensor is within the clinically-relevant detection range from 2U/ml to 100U/ml, with limit of detection (LOD) of 0.08U/ml (Sadasivam et al, 2020). This set-up using magnetic microparticles and HRP detection allows preconcentration, which can enhance detection sensitivity. A limited number of steps are required, but the incubation time remains long at 16hr. Another ssDNA aptamer was selected to recombinant His-tagged CA125 after 10 rounds of SELEX (Gedi et al, 2018). The aptamers were characterized using Enzyme-Linked Immunosorbent Assay (ELISA) and biolayer interferometry (BLI). The aptamer rCAA-8 had the highest affinity to CA125 with a Kd value of 166×10^{-9}M and it was used in on-chip bioassay based on the anti-CA125 aptamer-antibody pair on a three-dimensional network of carbon nanotubes. This diagnostic platform achieved sensitive detection (as low as 10pg/ml) and wide detection range (10pg/ml to 1µg/ml). Significantly, the sensitivity was superior than with ELISA using two antibodies (Gedi et al, 2018). The simultaneous detection of two markers CA125 and STIP1 was achieved using ssDNA aptamers in the resonance light scattering (RLS) biosensor. The RLS biosensor achieved detection in the concentration range from 0.1 to 2U/ml for CA125 and 1-40ng/ml for STIP1. The RLS biosensor was tested in spiked human serum (n=3) and achieved great correlation (Chen et al, 2017). However, the clinical concentration for the presence of OC is CA125 >35U/ml and STIP1 >55ng/ml, so concentration range tested in the study appears to be below cut-off value. Another aptamer target-

1

In vitro identification of the single stranded DNA or RNA aptamers

- Protein SELEX
- Membrane SELEX
- "One-pot" SELEX
- Capillary electrophoresis SELEX



2

Characterization of candidate aptamers

Binding affinity between aptamers and protein

- Surface plasmon resonance
- Fluorescence anisotropy
- Affinity probe capillary electrophoresis
- Differential pulse voltammetry

3

Application in diagnostics

Development of the protein detection methods

- Surface plasmon resonance biosensor
- Fluorescence biosensor
- Resonance light scattering biosensor
- Aptamer/carbon nanotube network platform
- Aptamer-nanozyme lateral flow assay

Figure 2. Overview of the development of the diagnostic aptamers for the detection of the ovarian cancer protein biomarkers. First step in the aptamer development is (1) selection and identification of the candidate aptamers specific for ovarian cancer protein biomarkers, using various formats of Systematic Evolution of Ligands by EXponential enrichment (SELEX). (2) Binding interaction between candidate aptamers and protein target is then characterized using different methods. (3) High-affinity aptamers with desired characteristics are used as recognition probes in the development of aptamer-based diagnostic methods, such as biosensors or lateral flow assays.

ing CA125 has been described in different aptasensors and is described elsewhere (Vandghanooni et al, 2021). A novel DNA aptamer was published by Tripathi and colleagues using a Membrane-SELEX approach. Native CA125 from human ascites was incubated with ssDNA library (30nt long random region). After 5 cycles (and a 6th cycle as negative selection against the membrane), the oligonucleotide pool was cloned and sequenced. Prediction of secondary structures was achieved using Mfold web server. Tertiary structure was analyzed using RNA composer web server and Discovery studio visualizer. The *in-silico* docking of-3D structures were performed with Patchdock and interactions were predicted using Ligplot (where algorithms provide a binding score proportional to the binding affinity). The aptamers were screened for cross-reactivity with Bovine Serum Albumin (BSA), Human Serum albumin (HSA) and Immunoglobulin G (IgG). The aptamer with the best affinity and specificity to CA125, Apt 2.26 (Supplementary Table 1) was selected for the characterization with membrane-based assessment of bound ssDNA to CA125, with K_d value of 166 x 10^{-9}M. The aptamer was stable in human serum, an important aspect when developing serum-based diagnostics (Tripathi et al, 2020a). Using this aptamer, the authors designed Aptamer-nanozyme lateral flow assay (ALFA). The results of the diagnostic test showed specificity (tested with BSA and Ig) and sensitivity (LOD 5.21U/ml). The assay validation was performed by testing in real human serum (n=35) and yielded high correlation with standard CA125 immunoassay, proving the diagnostic performance (Tripathi et al, 2020b). Recently, a biosensor based on ssDNA aptamer and upconversion of luminescence resonance energy transfer was developed for the detection of CA125. The platform had a detection range between 0.01-100U/ml and a LOD of 9.0×10^{-3}U/ml for CA125 in serum. Moreover, the test was evaluated using samples from ovarian cancer patients (n=3), with good correlation with clinical values of CA125 (Zhang et al, 2021), holding a potential for future POCT device for OC.

HE4

A study by Eaton et al (2015) reported aptamers targeting HE4. Capillary electrophoresis with 5 rounds of selection was performed with ssDNA library (25-nucleotides random region) to recombinant glutathione-S-transferase (GST)-HE4 protein. Two rounds of counter-selection were performed against GST, to eliminate protein tag-binders. After PCR amplification, ssDNA oligonucleotides were regenerated using streptavidin columns. Each round was sequenced and analyzed using a bioinformatic data pipeline (Rebecca Whelan Python Enrichment). Based on high-fold enrichment and cluster abundance, candidate aptamers were selected for characterization. Aptamers A1, A3 and B10 (Supplementary Table 1) displayed affinity to HE4, with K_d values of 2.2x10^{-6}M with FA and 390x10^{-9}M with APCE for aptamer A1; and 9.1x10^{-6}M with FA and 500x10^{-9}M with APCE for aptamer A3; and 280x10^{-9}M with FA and 870x10^{-9}M with APCE for aptamer B10, respectively (Eaton et al, 2015). Surprisingly, the A1 aptamer against HE4 has been used as recognition probe in aptasensors targeting CA125, using either multiple-wall carbon nanotube or electrospun-Ag-nanoparticles nanofibers (Mansouri Majd and Salimi, 2018; Farzin et al, 2019). In both cases, CA125 could be detected in serum from OC patients. Recently, the sequence of A1 aptamer was used in the development of the aptasensor for detection of HE4 based on the upconversion luminescence resonance energy transfer. The biosensor achieved HE4 detection in the range of 0.4ng/ml to 7.0ng/ml, with a LOD of 0.021ng/ml in buffer and 0.049ng/ml in 100-fold diluted serum samples (Ma et al, 2021)which shows a significant advantage in a bioassay. Lanthanide-doped upconverting nanoparticles (UCNPs. Aptamers for HE4 detection have potential, but since the clinical concentration of HE4 is in pmolar range, they should be further analyzed in wider concentration range and validated with samples from OC patients for future diagnostic applications.

CONCLUSIONS

In this review, we described the use of aptamers for the detection of two clinically relevant protein biomarkers of ovarian cancer CA125 and HE4.

- Ten nucleic acid aptamers for OC proteins with diagnostic potential have been identified.
- Seven aptamers were developed for the detection of CA125 (one RNA- and six DNA-based aptamers) and three (DNA-based aptamers) for detection of HE4.
- The aptamers for detection of CA125 have good binding characteristics in clinically-relevant concentration range, demonstrating the potential utility in diagnostic tests. Furthermore, aptamers as molecular probes have been shown to be stable and validated in human serum samples, implying their prospective use as alternatives to antibodies in detection of ovarian cancer-related proteins.
- In order to implement the aptamer-based diagnostics in clinics in future, all tests should be validated with both controls and samples from OC patients and compared to standard immunoassays.
- Nucleic acid aptamers can be used as recognition probes in OC biomarkers tests, paving the way for the development of novel POCT tests, including biosensors and lateral flow assays. Utilization of aptamers could enable easy and rapid detection with potential for earlier diagnosis of OC.

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STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

ALFA: Aptamer Lateral Flow Assay

APCE: Affinity Probe Capillary Electrophoresis
AuNPs: Gold nanoparticles
BLI: Biolayer Inferometry
CA125: Cancer /Carbohydrate Antigen 125
DAB: Diaminobenzidine
DPV: Differential Pulse Voltammetry
ELASA: Enzyme-Linked Aptamer Sorbent Assay
ELISA: Enzyme-Linked Immunosorbent Assay
FA: Fluorescence Anisotropy
FAM: Fluorescein amidites
GST: Glutathione S-Transferase
HE4: Human Epididymis protein 4
IgG: Immunoglobulin G
LFA: Lateral Flow Assay
LOD: Limit of Detection
NALFA: Nucleic Acid Lateral Flow Assay
RLS: Resonance Light Scattering
ROMA: Risk of Ovarian Malignancy Algorithm
SPR: Surface Plasmon Resonance
STIP1: Stress-induced phosphoprotein 1
VEGF: Vascular Endothelial Growth Factor

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