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· Expert Forum ·

Developing aptamers into tumor diagnostics and therapeutics

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[**Abstract**] Aptamers are small single-stranded nucleic acid molecules that bind a target protein with high affinity and specificity. Due to their stability, low toxicity and immunogenicity, as well as improved safety, aptamers are attractive alternatives to antibody and are therefore suitable for *in vivo* applications. Aptamers are typically isolated, through a process termed SELEX (systematic evolution of ligands by exponential enrichment), from combinatorial libraries with desired proteins. In the present review, the recent non-conventional aptamer selection process will be discussed together with an overview on the aptamer application in cancer diagnosis and therapy.

[**Key words**] aptamer; systematic evolution of ligands by exponential enrichment (SELEX); neoplasms diagnosis; neoplasms therapy

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1 Introduction

Targeting of tumor cells relies on the development of molecular beacons, suited for *in vivo* application, that are endowed with affinity, specificity and favorable pharmacokinetic property. Using monoclonal antibody as disease diagnostics and therapeutics has been around for more than three decades and made substantial contributions in clinics today. However, antibody-based therapy, especially in the setting of chronic diseases such as cancer requiring repeated administration over a long period of time, poses significant cost, manufacturing, and regulatory challenges. Thus, novel approaches that challenge traditional methods are being discovered.

Aptamers has emerged as a novel approach to evolve highly specialized molecules that can recognize, bind and inhibit a specified target, such as small molecules, peptides and proteins. Aptamers are short single-stranded oligonucleotides, either RNA or DNA, that fold into precise three-dimensional conformations and bind to their ligands by complementary shape interactions[1-2]. Due to the nature of short nucleic acids (20-80 mers), aptamers possess a number of favorable characteristics that rival antibodies in their potential for therapeutic and diagnostic applications. First, aptamers are relatively small (8 to 15 kDa) that are amenable to diverse, targeted, and chemical modification to improve their stability and bioavailability. Second, aptamers are stable and can be repeatedly denature/renature. Third, aptamers are nontoxic and low- or non-immunogenic, a very useful property for reagents being repeatedly administrated as therapeutic compounds. Finally, aptamers are synthesized chemically and therefore offer significantly advantage in term of production cost[3-4].

Aptamers can be isolated from combinatorial libraries by an iterative *in vitro* selection procedure. To date, many therapeutic aptamers targeting a wide array of disease-associated proteins have been developed. In this review, we have focused on those of aptamers that bind cancer-related targets and exhibit potential in cancer diagnosis and therapy. Also, we have summarized recent non-conventional and innovative approaches that are utilized for the development of aptamers.

2 Aptamer identification: SELEX process

A general approach to select aptamers from a completely random nucleic acid library was initially intro-

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duced by two teams, Ellington and Gold, who created the basic concept for SELEX (systematic evolution of ligands by exponential enrichment)[5-6]. Theoretically, a library containing a 40-nucleotide random region is represented by 1.2×10^{24} individual sequences. SELEX typically consists of four sequential steps: (1) incubating the library with the target molecules under conditions favorable for binding; (2) partitioning unbound aptamers from those bound specifically to the target; (3) dissociating the nucleic acid-protein complexes; and (4) amplifying the nucleic acids pool to generate the complexity reduced library for next round of selection. The number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as on the nature of the target. For most targets, affinity is enriched maximally within 8-15 cycles. Once the sequence is identified, an aptamer is produced by chemical synthesis.

As the *in vitro* selection of aptamers is frequently repetitive, time-consuming and poorly adapted to high-throughput applications, a fully automated SELEX process recently was designed by Ellington and his co-workers[7]. The automated workstation has allowed multiple SELEX experiments running on microtiter plates and reduced aptamer selection time from months to days.

2.1 Apply SELEX to purify protein

Binding of aptamers to a specific target molecule is conceptionally more similar to monoclonal antibodies rather than to antisense oligonucleotides or ribozymes which are linear and act to disrupt the expression of proteins at the mRNA level. Since these RNA and DNA aptamers bind their targets with similar and even better affinities than antibodies, and are able to distinguish between isotypes of a molecule, aptamers have been also called synthetic antibodies. Using purified proteins as targets, RNA aptamers have been generated and are capable of discriminating between isoforms of protein kinase C, a potential target involved in cancer cell survival pathways[8]. Also, DNA aptamers have been obtained and they are active against MUC1 tumor marker expressed in a variety of malignant tumors[9].

2.2 Apply SELEX to complex biologic systems

Although, technically, using purified proteins as targets has the advantage of easy control of the conditions to achieve optimal enrichment during the selection process, it drives a risk that these selected aptamers only target purified proteins but not their corresponding ones in the natural environment (such as, cells, biologic fluid, etc.) due to the changed protein conformation. To this purpose, various modified SELEX processes have applied SELEX directly to complex biologic systems.

They include cell-based SELEX running on two relatively homogeneous tumor cells for the selection of panel of target cell-specific aptamers[10]; *ex vivo* SELEX against cells overexpressing the target protein with a native conformation together with counter-selection against its wile type species[11]; combining SELEX and the yeast three-hybrid system to select and map the key binding site of RNA aptamers[12]; using "Toggle" SELEX via alternating rounds of selection between species to generate species cross-reactive aptamers for the convenience of pre-clinical evaluation[13]; and cross-over SELEX from cell SELEX to the recombinant protein to identify aptamers against conserved epitopes between the native and the recombinant protein[14]. Applying SELEX to complex targets, aptamers targeting intracellular (ERK-2, Ras and Raf-1) and extracellular (PSMA, tenascin-c, PDGF and tyrosine kinase) have been generated and exhibited micromolar to picomolar range of binding affinities and potential in tumor diagnosis and therapy[15-19].

3 *in vivo* applications of the RNA aptamers

3.1 Stability of the aptamers

RNA aptamers for therapeutics are severely limited by nuclease sensitivity, poor bioavailability, and fast renal clearance. As such, various modifications on nucleic acid backbone are introduced by replacing ribonucleotides with 2′-amino, -fluoro, or -O-alkyl nucleotides[20]. A dT-cap at the 3′-end can further confer nuclease resistance. Such modifications extend plasma half-lives of RNA aptamers from seconds to 5-15 h. Also to allow for practical chemical synthesis, full-length aptamers that come out of a SELEX with generally 70-80 nucleotides long need to be truncated to fewer than 40 nucleotides, but this unexpectedly results in rapid systemic clearance. In order to overcome this

limitation, Clary and Sullenger group developed a truncated angiopoietin 2 aptamer conjugated to 40 kDa polyethylene glycol moiety (PEG) to enhance its bioavailability. Systemic delivery of this pegylated aptamer efficiently inhibited *in vivo* tumor angiogenesis and growth[21]. By far, the first and only approved aptamer for human therapy is the use of pegylated, nuclease-stabilized VEGF aptamer, macugen for the treatment of age-related macular degeneration, an eye disease that can lead to adult blindness[22].

In addition, due to the cross-linking being a requisite for signal transduction, the bioavailability of aptamer that functions as agonist to cell surface receptors may best be achieved by increasing the avidity of the aptamers to their target. To this aim, Gilboa and Sullenger group developed bivalent and multivalent configurations of the aptamer 4-1BB connected by a double-stranded nucleic acid linker and reported their significantly enhanced costimulation of T cell activation *in vitro* and promoted tumor rejection in the mice employing mastocytoma[23].

3.2 Aptamer expression and delivery

While aptamers have been used primarily to target secreted or membrane bound protein, their use in modulating the function of intracellular proteins has been complicated by the fact that the proper folding of RNA aptamers expressed through vector-based approaches has been interfered in the contex of flanking vector sequence. As such, one group has developed an expression cassette-based SELEX strategy to randomize flanking sequences and select for chimeric tRNA-aptamer transcripts that retained high affinity for transcription factor E2F[24]. Despite the apparent success of this approach, repeated SELEX is technically challenging and may not be universally effective. Another research group also developed a vaccinia virus-based RNA expression system based on double infection with two recombinant vaccinia viruses encoding T7 RNA polymerase and an aptamer against a 2 integrin, respectively[25]. This approach utilized a vector with flanking stem-loop structures as RNA-stabilizing motifs, which were required for correct termination of the T7 transcripts, but it is not clear whether these stem-loop structures would be universally effective for other aptamers.

In order to achieve high-level expression of 'pure' aptamer without spurious sequences, Clary group successfully developed a novel H1 RNA polymerase III promoter-driven adenoviral vector to express the 'pure' RNA aptamer directed against the p50 subunit of NF-B[26]. The advantage of this vector is that RNA transcription starts accurately at the initial base of inserted sequences and terminates with the addition of two uridines (U) at the 3' end, which is verified to posses a negligible effect on the aptamer conformation. They demonstrated that the expressed aptamer effectively inhibited NF- B transactivation and induced apoptosis in both human lung adenocarcinoma cells A549 and murine mammary carcinoma cells 4T1 and also delayed tumor growth in a human tumor xenograft model[27-28].

Since aptamers can be selected to bind to a cell surface marker, aptamer has been recently developed as a tool to mediate cell type-specific siRNA delivery. Levy group used streptavidin as a non-covalent linker to conjugate anti-PMSA aptamer and siRNA, and demonstrated that the aptamer was capable of delivering the functional siRNA into cells expressing the prostate specific membrane antigen (PSMA) and mediated fast inhibition of gene expression[29]. Almost at the same time, Sullenger group reported prostate cancer cell-specific delivery of siRNA by using a chimera RNA transcript, that consists of an aptamer portion (A10) mediating the binding to PSMA and a siRNA portion targeting the expression of the two survival genes PLK1 and Bcl2. The A10 aptamer-siRNA chimeras were proved to bind prostate cancer cells in a PSMA-dependent manner. When directly applied to cells expressing PSMA, these chimeras RNA were internalized and processed by Dicer, resulting in depletion the siRNA target proteins, induction of cell apoptosis and inhibition of a human prostate cancer xenograft model[30].

4 Conclusion

The encouraging data obtained with aptamers combined with their intrinsic properties make them promising candidates for diagnostic and therapeutic applications in cancers. Remarkable progress has been achieved applying SELEX to complex biologic systems to generate aptamers with high *in vivo* binding affinity.

The development of aptamers as specific delivery vehicles for other therapeutics facilitates their translation into clinically useful agents.

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