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RNA aptamers targeting cancer stem cell marker CD133

Sarah Shigdar [a], Liang Qiao [b], Shu-Feng Zhou [c], Dongxi Xiang [a], Tao Wang [a,d], Yong Li [e], Lee Yong Lim [f], Lingxue Kong [g], Lianhong Li [h,*], Wei Duan [a,h,*]

[a] School of Medicine, Deakin University, Pigdons Road, Waurn Ponds, Victoria 3217, Australia
[b] Storr Liver Unit at The Westmead Millennium Institute, Department of Medicine and Western Clinical School of The University of Sydney, Westmead, NSW 2145, Australia
[c] Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, FL 33612, USA
[d] The Nursing College of Zhengzhou University, Zhengzhou 450052, China
[e] Cancer Care Centre, St. George Hospital, and St. George Clinical School, Faculty of Medicine, University of New South Wales, Kensington, NSW 2052, Australia
[f] Laboratory for Drug Delivery, Pharmacy, University of Western Australia, Crawley, WA 6009, Australia
[g] Institute for Frontier Materials, Deakin University, Pigdons Road, Waurn Ponds, Victoria 3217, Australia
[h] Department of Pathology and Forensic Science, Dalian Medical University, Dalian 116044, China

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ABSTRACT

The monoclonal antibody against the AC133 epitope of CD133 has been widely used as a cell surface marker of cancer stem cells in several different cancer types. Here, we describe the isolation and characterisation of two RNA aptamers, including the smallest described 15 nucleotide RNA aptamer, which specifically recognise the AC133 epitope and the CD133 protein with high sensitivity. As well, both these aptamers show superior tumour penetration and retention when compared to the AC133 antibody in a 3-D tumour sphere model. These novel CD133 aptamers will aid future development of cancer stem cell targeted therapeutics and molecular imaging.

1. Introduction

CD133, also known as Prominin-1, is a pentaspan, highly glycosylated, membrane glycoprotein that is associated with cholesterol in the plasma membrane [1,2]. Though this protein is known to define a broad population of cells, including somatic stem and progenitor cells, and is expressed in various developing epithelial and differentiated cells, its exact function is still being elucidated. It has, however, been linked to the Notch-signalling pathway which is critical for binary cell fate, differentiation of intestinal epithelium, and lymphopoiesis [3]. CD133 has gained its prominence in the cancer research field due to its reported role as a marker of cancer stem cells (CSCs) in glioblastomas [4]. Indeed, growing evidence has shown that CD133 is an important cell surface marker for CSCs in a variety of solid cancers, including those of the brain, prostate, pancreas, melanoma, colon, liver, lung and ovarian cancers [5] by virtue of the enhanced tumourigenic potential of $CD133^+$ cells versus their negative counterparts in immunodeficient mice [6].

Most of the work in isolating $CD133^+$ putative cancer stem cell subpopulation from the bulk cancer cells utilises one monoclonal antibody, AC133 [7]. However, there has been some controversy regarding the notion that CD133 can be used as a marker for CSCs, as investigators from independent laboratories showed that the $CD133^-$ cells are also tumourigenic in immunocompromised mice [8–10]. This contention is further complicated by the near ubiquitous expression of CD133 on non-CSCs as well as CSCs, especially in tumours of the colon [11]. A recent study has shed some light on this, with a conformational change postulated to hide an epitope on the second extracellular membrane loop of CD133 during the differentiation process [5]. Kemper and co-workers suggested that the CD133 protein becomes differentially folded as a result of glycosylation, thus masking the AC133 epitope. These results were further supported by later studies [5,12] which suggested that the AC133 epitope, rather than the complete CD133 protein, is the marker for CSCs. As well, a recent report has shown that the AC133 epitope is lost upon cell differentiation, suggesting that this epitope is a marker of primitive cells [5]. While the AC133 epitope has been shown to be a marker for CSCs, not all cells positive for

* Corresponding authors. Addresses: Department of Pathology and Forensic Science, Dalian Medical University, 9 Western Section, Lyshun Sooth Street, Dalian 116044, China. Tel.: +86 411 8611 0050; fax: +86 411 8611 0050 (L. Li), School of Medicine, Deakin University, Pigdons Road, Waurn Ponds, Victoria 3217, Australia. Tel.: +61 3 5227 1149; fax: +61 3 5227 2945 (W. Duan).
E-mail addresses: lilianhong917@126.com (L. Li), wduan@deakin.edu.au (W. Duan).

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the AC133 epitope are CSCs. In fact, the first description of this epitope was by Yin et al. in 1997, who described this as a marker of haematopoietic stem and progenitor cells [13]. However, the expression of AC133 on these cells is approximately 1000-fold lower than that observed in CSCs [14]. Despite the on-going debate of the utility of using AC133 to identify cancer stem cells, a retrospective study on colorectal patients showed that a high level of AC133 expression was associated with a poorer prognosis [15], though the sole use of the AC133 antibody is not recommended as it is thought to underestimate the level of CD133 expression [1,16].

AC133-positive cells have been shown to have an increased resistance to radiation therapy due to activation of the DNA damage checkpoint proteins, and an increased chemoresistance due to an increased Akt/PKB and Bcl-2 cell survival response [17]. These data suggest that a more targeted response is required to eradicate this population of cells, especially given the increasing evidence regarding the roles that CSCs play in the relapse of cancer after initial treatments. Immunotherapy has had a great impact on the treatment of cancer in recent years [18,19]. However, the use of antibodies, even humanised antibodies, can lead to adverse side effects with fatal consequences [20]. This has led to the search for 'bigger and better' options. There have been several attempts to use nucleic acids as therapeutics though these have met with disappointing results, not least because of the failure of these nucleic acids to enter the cell [21]. The reports in 1990 by two separate groups describing the generation of nucleic acids that can bind target molecules in the same manner as antibodies seemed to be the answer [22,23]. These chemical antibodies, termed aptamers, have been increasingly utilised for clinical applications recently. Indeed, one RNA aptamer has been approved by the FDA and several more are in clinical trials [21,24]. The increased interest in these aptamers is due to the fact that they exhibit no immunogenicity, little batch-to-batch variation due to being chemically synthesised, and are more stable than conventional antibodies. Due to their small size, aptamers also show superior tumour penetration. One important feature of these chemical antibodies is their versatility as they can be attached to nanoparticles, drugs, imaging agents or other nucleic acid therapeutics without loss-of-function [25,26]. This functionalisation is leading to new and more targeted therapies, with fewer side effects than current treatment modalities [25]. When compared to conventional treatment which is largely a passive process, targeted delivery systems are much more effective. For an aptamer to be an effective drug delivery agent, the aptamer must be efficiently internalised upon binding to its target on the cell surface [27].

In this study, we performed iterative rounds of an in vitro selection process, known as the systematic evolution of ligands by exponential enrichment (SELEX), to identify RNA aptamers that specifically bind to CD133. Further studies identified one aptamer, CD133-A15, specifically bound to the same epitope as the AC133 antibody, while the other aptamer, CD133-B19, bound to the extracellular domain of the CD133 protein. These aptamers were efficiently internalised into CD133-positive cancer cells and showed superior penetration of three-dimensional tumour sphere.

2. Materials and methods

2.1. Cell lines and cell culture

The cell lines of human origin used in this study were purchased from American type Culture Collection. They are human colorectal cancer HT-29; human hepatocellular carcinoma Hep3B; human glioblastoma multiform carcinoma T98G; human embryonic kidney cells HEK293T; human ductal breast carcinoma, T47D; human lung adenocarcinoma, A549; human ovarian teratocarcinoma, PA-1; human hepatoma, PLC/PRC/5; and human prostate carcinoma, DU145. Cells were grown and maintained in culture with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Victoria, Australia) supplemented with 10% foetal calf serum (FCS) (HEK293T and HT-29), or Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% FCS (Hep3B and T98G). Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

2.2. Protein expression and cell SELEX

Human CD133 cDNA was purchased from Invitrogen and cloned into a mammalian expression vector, pcDNA 3.1/V5-His-TOPO (Invitrogen). The recombinant 6× Histine-tagged CD133 was transiently expressed in HEK293T cells. Briefly, HEK293T cells were seeded in 100 mm or 60 mm dishes to reach 70% confluency after 24 h incubation, and transfected with a total of 24 or 8 μg, respectively, of CD133 using Lipofectamine 2000 (Invitrogen Life Technologies) in antibiotic-free medium according to the manufacturer's instructions. Following a 72 h incubation, the transfected cells were used as the target for cell SELEX. Successful transfection and expression of the recombinant CD133 were confirmed using flow cytometry and the AC133-APC antibody (Miltenyl Biotech) prior to each round of SELEX.

2.3. SELEX selection

A DNA library containing a central 40-nt randomised sequence (5′-TAA TAC GAC TCA CTA TAG GGA GAC AAG AAT AAA CGC TCA A-N40-TTC GAC AGG AGG CTC ACA ACA GGC, with the T7 RNA polymerase promoter sequence underlined) was synthesised (GeneWorks, Australia). The double stranded DNA pool was generated from the original synthetic library via a large scale PCR using primers flanking the randomised sequence, 5′-TAA TAC GAC TCA CTA TAG GGA GAC AAG AAT AAA CGC TCA A-3′ and 5′-GCC TGT TGT GAG CCT CCT GTC GAA-3′. A portion of the large-scale PCR products ($\sim 10^{14}$ sequences) was used as a template for in vitro transcription to produce the initial 2′-fluoropyrimidine modified RNA pool using a Durascribe© T7 Transcription kit (EPICENTRE® Biotechnologies, USA). For SELEX, RNA, at a concentration of 5 μM for initial selection or 1 μM for each iterative rounds, was diluted in 100 μL of wash buffer (Dulbecco's phosphate buffered saline containing 2.5 mM $MgCl_2$) and denatured at 85 °C for 5 min, allowed to cool to room temperature for 10 min, and annealed at 37 °C for 15 min, before incubation with the target protein expressed in HEK293T cells for 1 h at 4 °C. Following incubation and extensive washes, the bound RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen), followed by PCR amplification and in vitro transcription and used for the next round of SELEX. Counter-selection steps were included from round 4, using a His-tagged irrelevant protein expressed in HEK293T cells, to decrease the enrichment of species specifically recognising the His-tag, the HEK293T cells or the tissue culture plate. The number of PCR amplification cycles was also optimised to prevent over-amplification of non-specific "parasite" PCR products. In addition, the stringency of the selection process was enhanced to promote the selection of high-affinity aptamers through adjustments to aptamer concentration, incubation times, and the number of washes. To acquire aptamers of high specificity, the number of cells used was progressively decreased while the washing stringency increased during the progression of SELEX, with negative selections included from round four. Enrichment was monitored using restriction fragment length polymorphism (RFLP) and flow cytometry using live cells.

2.4. RFLP analysis

The enrichment of aptamer candidates during selection was determined by RFLP. Briefly, RFLP was performed as previously described [28,29], with minor modifications. Approximately 5 ng of cDNA from iterative cycles was amplified by PCR for eight cycles. The amplified DNA was digested with four restriction enzymes, *Afa* I, *Alu* I, *Hha* I and *Xsp* I that recognise four nucleotides (frequent cutters) in Buffer T supplied by the manufacturer (Takara) with 0.1% (w/v) bovine serum albumin at 37 °C overnight. Following the overnight digestion, the DNA was heated to 65 °C, cooled on ice, and separated via electrophoresis on a native 20% polyacrylamide gel in TBE buffer. The gel was then stained in GelStar and visualised using a standard gel imaging system.

2.5. Flow cytometry assays

Cells were harvested at 80% confluence with trypsin digestion and resuspended in washing buffer (DPBS with 2.5 mM $MgCl_2$) and enumerated. Following centrifugation (1000*g* for 5 min), the cell pellet was resuspended in DMEM with 10% FCS and diluted to 1×10^6/mL. The cells were allowed to re-establish their cell surface markers for a period of 2 h prior to binding analysis. To confirm aptamer binding to the target protein, RNA from iterative rounds were labelled at the 3′-ends with fluorescein isothiocyanate (FITC) according to a previously described method [30]. Amber tubes were used throughout to minimise photo-bleaching. Briefly, samples were oxidised with sodium periodate. The oxidation was terminated with the addition of 10 mM ethylene glycol, followed by ethanol precipitation. FITC was added at a 30-M excess, and the reaction was completed overnight at 4 °C. 1 μM of FITC-labelled RNA was incubated with trypsinised 5×10^5 HEK293T transfected with CD133 protein or non-transfected HEK293T cells in 100 μL of binding buffer (DPBS with 2.5 mM $MgCl_2$, 0.1 mg/mL tRNA and 0.1 mg/mL salmon sperm DNA) for 1 h on ice, followed by washing three times and resuspension in 300 μL of binding buffer.