

## **Aptamer-Mediated Cancer Gene Therapy**

Dongxi Xiang<sup>1\*</sup>, Sarah Shigdar<sup>1\*</sup>, Greg Qiao<sup>2</sup>, Shu-Feng Zhou<sup>3</sup>, Yong Li<sup>4</sup>, Ming Q Wei<sup>5</sup>,  
Liang Qiao<sup>6</sup>, Hadi Al.Shamaileh<sup>1</sup>, Yimin Zhu<sup>7</sup>, Conglong Zheng<sup>8</sup>, Chunwen Pu<sup>9\*\*</sup> and Wei  
Duan<sup>1\*\*</sup>

<sup>1</sup>School of Medicine, Deakin University, Pigdons Road, Waurn Ponds, Victoria, 3217, Australia.

<sup>2</sup>Department of Chemical and Biomolecular Engineering, Melbourne School of Engineering  
The University of Melbourne, Parkville, Victoria 3010, Australia

<sup>3</sup>Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, FL 33612, USA.

<sup>4</sup>Cancer Care Centre, St George Hospital, Kogarah, NSW2217, and St George and Sutherland Clinical School, University of New South Wales (UNSW), Kensington, NSW2052, Australia

<sup>5</sup>Division of Molecular and Gene Therapies, Griffith Health Institute and School of Medical Science, Griffith University, Gold Coast, QLD 4222, Australia

<sup>6</sup>Storr Liver Unit, at the Westmead Millennium Institute, the University of Sydney at the Westmead Hospital, Westmead NSW, 2145, Australia

<sup>7</sup>Suzhou Key Laboratory of Nanobiomedicine, Division of Nanobiomedicine, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou, Jiangsu, China, 215123

<sup>8</sup>Department of Biology, Medical College, Dalian University, Liaoning, People's Republic of China

<sup>9</sup>The Affiliated Zhongshan Hospital of Dalian University, 6 Jiefang Road, Dalian, Liaoning, The People's Republic of China, 116001.

\* These authors contributed equally.

\*\* Corresponding authors: E-mail: [dahaixiaowen@163.com](mailto:dahaixiaowen@163.com) (C. Pu); or [wduan@deakin.edu.au](mailto:wduan@deakin.edu.au) ([W. Duan](#)).

## **Abstract**

Cancer as a genetic disorder is one of the leading causes of death worldwide. Conventional anticancer options such as chemo- and/or radio-therapy have their own drawbacks and could not provide a cure in most cases at present. More effective therapeutic strategies with less side effects are urgently needed. Aptamers, also known as chemical antibodies, are single strand DNA or RNA molecules that can bind to their target molecules with high affinity and specificity. Such site-specific binding ability of aptamers facilitates the delivery and interaction of exogenous nucleic acids with diseased genes. Thus, aptamer-guided gene therapy has emerged as a promising anticancer strategy in addition to the classic treatment regimen. Aptamers can directly deliver anti-cancer nucleic acids, e.g. small interfering RNA, micro RNA, anti-microRNA and small hairpin RNA, to cancer cells or function as a targeting ligand to guide nanoparticles containing therapeutic nucleic acids. This review focuses on recent progress in aptamer-mediated gene therapy for the treatment of hepatocellular carcinoma and other types of cancers, shedding light on the potential of this novel approach of targeted cancer gene therapy.

## INTRODUCTION

Cancer is a multifaceted disease characterized by inter- and intra-patient genomic and cellular heterogeneity, arising from the long-term accumulation of genetic mutation and/or epigenetic alterations [1]. Cancer is a leading cause of death worldwide, with 1,665,540 new cases and 585,720 deaths predicted in the United States of America alone in 2014 [2]. Thus, the exploration of alternative and more efficacious approaches for eradicating cancer is urgently needed. Cancer gene therapy emerges to be a promising strategy to combat cancer.

Gene therapy aims to introduce therapeutic genetic materials (oligonucleotides, DNAs or RNAs) into the diseased cells/tissues or an organ to correct genetic errors [3, 4]. Gene therapy for cancer has been the most attractive area in this research field with two-thirds of gene therapies trials focusing on cancer therapy [5-7]. Molecularly targeted therapies entail inhibiting oncogenes, restoring the function of tumour suppressor genes or the introduction of suicide genes/prodrug systems [7]. The success of cancer gene therapy largely depends on the selective delivery of sufficient therapeutic dose of nucleic acids into target tumour cells. To achieve this aim, a great number of sophisticated tools have been engineered, which can be divided into two major classes – viral vectors and non-viral vectors (i.e. liposomes, oligonucleotides, DNA or RNA multiplexes) [8, 9]. However, these vectors still face several challenges such as immune response, insertional mutagenesis and lack of specificity of transgene delivery [7].

Aptamers, also known as, chemical antibodies are single-stranded oligonucleotides and bind to their targets through shape complementarity with high affinity and specificity in a wide array of clinical applications [10-12]. Being generated by chemical synthesis, aptamers can be easily modified for desired applications, e.g. attaching to therapeutic drugs, nanoparticles and imaging agents without loss-of-function [13, 14]. In addition, upon binding to their cell surface ligands, aptamers together with the conjugated nucleic acids carrier can rapidly internalise into cells via receptor-mediated endocytosis. After internalisation, aptamers can direct exogenous therapeutic genes to the cytoplasm or nuclei to enhance the efficacy of cancer gene targeting and/or genetic modification [15, 16]. Thus, these attributes make aptamers promising choices for molecular recognition and gene delivery in targeted cancer therapy. The current review will update recent progress of aptamer-guided gene targeting in cancer therapy, with hepatocellular carcinoma (HCC) as a prime example to illustrate the principles and capabilities that can be applied to other types of cancer.

## **AN OVERVIEW on the TARGETED CANCER GENE THERAPY**

Gene therapy is a therapeutic modality in which nucleic acids are used as therapeutic agents and selectively delivered into patient's cells with minimal toxicity [4, 17]. The functionalised therapeutic genes aim to correct genetic errors by inhibiting defective gene expression or repairing the mutated gene. Among genetic diseases, cancer is the most studied case in gene therapy for clinical trials as gene therapy is considered to be a promising and rational approach of combating cancer [7]. Because of the complexity of tumour, several effective gene therapy strategies have been investigated for treatment of malignant tumour. These strategies can be categorised into two approaches: immunologic and molecular avenues [18]. Transfection of immune-stimulant genes such as cytokines (i.e., Interleukin-12) into cancer cells or engineering antigen presenting cells with tumour-associated antigens are commonly adopted approaches in cancer immunologic-based gene therapy [19]. As for molecular approaches in cancer gene therapy, blockage of cellular oncogenes, restoration of tumour suppressor gene and introduction of genes encoding enzymes that can convert non-toxic substances to toxic agent (suicide gene) are three most frequently pursued approaches [20]. Other effective strategies of molecular cancer gene therapy entail the introduction of anti-angiogenic genes into tumour vasculature, expression of suicide genes coupled with otherwise non-toxic chemotherapeutic agent and inhibiting multi-drug resistance genes in tumour cells [20]. In 2003, the first commercially produced gene based cancer therapy was manufactured by Shenzhen SiBiono GeneTech. Gendicine™, a replication-incompetent recombinant human Ad5-p53, was approved by the Chinese State Food and Drug Administration to treat head and neck squamous cell carcinoma [21]. Again in 2006, Shanghai Sunway Biotech, commercially released a conditionally replicative adenovirus therapy, Oncorine™ [22]. However, it is fair to say that cancer gene therapy has yet to realise its full potential.

Successful cancer gene therapy relies on the efficient delivery of therapeutic genes into tumour cells with high specificity. Non-viral gene delivery has several advantages, including relative ease for large-scale production and reproducibility, with much enhanced safety over viral methods. This is because viral delivery remains a safety concern as a viral administration may enhance the risk of host immune response with subsequent adverse effects occurring [8, 9]. Although viral vectors are generally more efficient in gene delivery than their non-viral counterparts, virus can only carry very limited gene. Furthermore, insertional mutagenesis caused by the viral vector poses an additional risk to the patient [23].

Recent technological advances herald new generations of smart non-viral vectors that could yield high gene transfer efficiencies similar to their viral counterparts for liver hepatocellular gene therapy [24]. Among those non-viral carriers, aptamer has emerged as one of the promising non-viral gene delivery systems [25, 26].

The anatomic characteristics of the liver render it as an ideal therapeutic target for gene therapy. Being the only organ with two circulation systems in the body, the liver is extensively vascularized that hepatocytes and liver parenchyma are readily accessible to macromolecules such as DNA fragments or viral particles [27]. These factors are important for gene therapy applications for liver malignancies. Here we focus on recent advances in aptamer-mediated cancer gene therapy for the treatment of hepatocellular carcinoma (HCC), one of the common and aggressive types of liver cancer [28, 29].

## **APTAMER-GUIDED CANCER GENE THERAPY**

### **Aptamer and Advanced Properties**

Aptamers, single-stranded DNA or RNA oligonucleotides, derive their name from the Latin word ‘aptus’ meaning ‘to fit’. They can be selected *in vitro* by a process known as systematic evolution of ligands by exponential enrichment (SELEX) (Figure 1) [30, 31]. The targets for aptamers include nucleic acids, peptides, proteins, organic and inorganic molecules, or even a whole cell [10, 11]. **Due to its nature of *in vitro* selection, the SELEX can be used to select/isolate aptamers with extreme high affinity (e.g. a  $K_D$  of 1 pM) as well as high specificity.** Aptamers fold through intra-molecular interactions to form tertiary conformations that recognize and bind targets via shape complementarity. Once the sequence of an aptamer is confirmed, it can be synthesized with high reproducibility and purity *in vitro* at a relatively low cost, which is a key advantage for drug development [14]. Antibodies differ from aptamers in that they are produced and purified from animals or cell culture with their binding characteristics being influenced by conditions of each animal and cultured cells. In contrast, aptamers are manufactured by chemical synthesis *in vitro* without the need of using animals or cells and thus possess much less batch-to-batch variations. Also, being composed of nucleic acids, aptamers are much more stable at room temperature and in non-physiological conditions. Thus, unlike antibodies, the stability of aptamers allows them to be stored stably across a wide range of storage and temperature conditions without the need of stabilization aids, as aptamers can be easily re-folded to assume their 3-D structure by one cycle of heating and cooling [32, 33]. Furthermore, aptamers have been shown to have low

immunogenicity and are non-toxic [14]. Critically important for the application for cancer therapy, aptamers can penetrate into the core of the tumour much more efficiently than antibodies as they are ~20-25-fold smaller than a full sized monoclonal antibody [34, 35]. Nonetheless, there are several limitations of nucleic acid aptamers when they were functioned *in vivo* through the blood circulation. Being polynucleotides, aptamers are exposed to enzyme degradation by exo- and/or endo-nucleases, resulting in a reduction of *in vivo* circulatory half-life. This problem can be addressed by incorporating unnatural nucleotide bases (locked and unlocked nucleic acids), introducing backbone or chemical modifications to aptamers, thus reducing the susceptibility to the exposure of endonuclease and exonuclease [13, 36, 37]. Limit residence time in blood is another challenge with *in vivo* aptamer applications, which is largely because of fast removal of aptamer by renal filtration as the size of most aptamers is smaller than the renal filtration threshold of 40 kDa [37]. Judicial modification of aptamers with polyethylene glycol (PEG) can increase aptamer's blood resident time and thus enhance tumour delivery [38]. Table 1 summarizes key features aptamers in comparison with antibodies.

### **Principle of Aptamer-Guided Gene Therapy**

Unlike antibodies that can be easily denatured or inactivated because of the rising acidity in the vesicles/organelles down the endocytic pathway, aptamers engineered with chemical modifications remain stable and functional after endocytosis [39-44]. In addition to functioning as drug delivery carriers, aptamers have been actively exploited as a gene delivery vehicle for small interfering RNA (siRNA), micro RNA (miRNA) and short hairpin RNA (shRNA) [13, 45-47]. These aptamer-guided nucleic acids can be delivered *in vivo* via multiple routes such as intratumoural injection, intravenous injection, and percutaneous injection. Once internalized, aptamers interact with the host DNA and/or RNA to alter the gene expression of targeted malignant cells. Aptamer can mediate gene therapy through either the immunologic or the molecular avenues. Intense effort has been devoted to explore aptamers as an effective gene therapy modality for cancer therapeutics [48-50].

Gene targeting is a powerful tool in functional analysis of genes. However, the application of targeted gene modification in gene therapy is hampered by its low frequency, i.e.  $1 \times 10^{-5}$  -  $10^{-7}$ , in many organisms. Recent work from Ruff and colleagues provided an elegant demonstration for the power of aptamers in repairing a genomic locus [51]. They developed a novel aptamer-based gene targeting approach for gene correction, in which a DNA aptamer linked with an exogenous oligonucleotide of 40-54 bases was delivered to the close vicinity

of the genomic locus to efficiently repair the target gene. Specifically, the authors generated an aptamer against the homing endonuclease, site-specific meganuclease I-SceI, to deliver the tethered donor DNA for gene correction close to the site of the I-SceI DNA double-strand break. With the I-SceI aptamer, the donor molecule for gene correction is brought to the vicinity of its target site to enhance the specificity of efficacy of genetic modification. Indeed, this aptamer-guided gene targeting approach has enhanced gene targeting up to 16-fold in human cells [51]. Although this was accomplished *in vitro* using plasmid transfection, this work represents a novel proof-of-principle concept, where aptamers can be used as tools for increasing gene targeting efficiency for oncogenes and modulating overexpressed signalling or regulatory genes in cancer.

### **Free Aptamer-Mediated Inhibition of Hepatocellular Tumour Metastasis**

The failure of cancer treatment and poor prognosis with HCC is often due to tumour recurrence and metastasis after initial surgical resection or chemotherapeutic treatment [28, 29]. To date, sorafenib, also known as nexavar, is the only chemotherapy agent approved for the treatment of advanced primary liver cancer [52]. There are also ongoing clinical trials for HCC treatment such as brivininib (an inhibitor of FGFR1 and VEGFR1), doxorubicin, bevacizumab and ABT-869 (a receptor tyrosine kinase inhibitor of VEGF), but most of them lack specificity and have limited efficacy [53]. A DNA methyltransferase inhibitor, SGI-110, used as epigenetic therapy for cancer has entered Phase 2 clinical trial for advanced HCC patients who failed prior treatment with sorafenib [54]. The final completion date for primary outcome measurement of SGI-110 is expected by October 2014 [54]. We are awaiting novel targeted therapy with improved efficacy.

Osteopontin (OPN), a secreted glycoposphoprotein overexpressed in malignant cells, is a potential prognostic marker and a therapeutic target for HCC invasion, progression and metastasis, it is also capable of promoting epithelial-mesenchymal transition (EMT) for HCC migration [55-57]. Studies from independent groups have shown that increased serum/plasma osteopontin level is highly associated with poor clinical prognosis and treatment failure of HCC after treatment with curative intent, suggesting that blockage of OPN function could significantly reduce HCC growth and progression [53, 55, 57-60]. Being an extracellular protein, OPN is one of the ideal therapeutic targets for aptamer-based therapy. Zhao and colleagues [61] engineered polyethylenimine nanoparticles (M-PEI) to carry a short-hairpin RNA (shRNA) for targeting and reducing OPN expression both *in vitro* and *in vivo* using a HCC-LM3 xenograft model. The nano-encapsulation of shRNA could

circumvent the nuclear degradation and sustain the duration of M-PEI-carried shRNA *in vivo*. The studies confirmed that OPN facilitated tumourigenesis and metastasis by protecting tumour cells from apoptosis. Down-regulation of OPN-induced or/and mitochondria-mediated HCC apoptosis coupled with the improved sensitivity of shRNA-transfected HCC cells to chemotherapeutic drugs resulted in the inhibition of HCC cell growth, adhesion and invasion *in vitro*, as well as suppression of tumourigenicity and lung metastasis *in vivo*. In addition, Talbot's group utilised a 2'-O-methoxy (2'-OMe) modified RNA OPN aptamer of 40 bases (OPN-R3) with a  $K_d$  of 57.2 nM for the blockade of extracellular OPN in HCC to evaluate the antitumour properties [53, 62]. Analysis of anti-tumour function both in *in vitro* and *ex vivo* settings revealed a significant reduction of EMT by this aptamer [53]. Moreover, following the establishment of an *in vivo* xenotransplant model of Sk-Hep-1 HCC, the OPN aptamer or control aptamer (0.5 mg/kg) were administrated i.v. every 2 days from week 3 to week 8. In the course of 8 weeks, OPN aptamer inhibited tumour growth by more than 10-fold compared with that of untreated or control-aptamer treated mice. If the efficacy of OPN aptamers can be confirmed in patient-derived xenografts to demonstrate their ability to significantly decrease EMT marker expression and inhibit the HCC growth, aptamer-guided inhibition of OPN will be a promising therapeutic option for HCC.

Carcinoembryonic antigen (CEA) is overexpressed in the majority of carcinomas including colon, lung, mucinous ovarian and endometrial carcinomas [63]. Being an important tumour marker for colorectal cancer, CEA overexpression is known to promote hepatic metastasis of colon cancer cells [64]. It has been revealed that five amino acids (PELPK) in the hinge region is required for binding to the CEA receptor, heterogeneous nuclear ribonucleoprotein M4, on Kupffer cells and is thus associated with the initiation process of hepatic metastasis [65]. In order to inhibit hepatic metastasis of colon cancer cells, Lee and co-workers have generated an RNA aptamer with 49 nucleotides in length, YJ-1, that specifically binds to the PELPK motif in CEA [41]. Upon binding specifically to CEA-positive cells, the CEA RNA aptamers inhibited interactions between CEA and heterogeneous nuclear ribonucleoprotein M4, homotypic aggregation, migration, as well as invasion by CEA-positive cancer cells, but did not affect adhesion of endothelial cells. In addition, the CEA aptamer induced colon cancer cell anoikis by interrupting the interaction between death receptor 5 and CEA. In a LS174T xenograft colon cancer model, two million of CEA-positive LS174T cells were preincubated with CEA aptamers for 5 min before being splenetically injected. Amazingly, when the animals were sacrificed on the 35<sup>th</sup> day after injection, there was a 91% reduction in total tumour volume of hepatic metastasis in the CEA aptamer-treated group compared with

the control. Thus, the CEA aptamer is a promising lead for future development of an agent capable of inhibiting migration and invasion by colonrectal cancer cells, and preventing hepatic metastasis.

### **Aptamer-siRNA Chimera for Cancer Gene Targeted Therapy**

Small interfering RNA (siRNA) can be incorporated into the RNA-induced silencing complex (RISC), resulting in the degradation of target mRNA, based on the pathway known as RNA interference (RNAi) [66]. Despite the potential of specifically inhibiting gene expression of unlimited targets with high efficiency, the inability to deliver a sufficient dose of siRNA to the target tissue *in vivo* hampers the translation of this Nobel prize-winning technology to the clinic. Aptamer-based targeted siRNA delivery merges as a promising approach for cancer-targeted RNAi therapy. By forming a chimera, *i.e.* the direct link of siRNA with an aptamers, the aptamer acts as a targeting moiety to guide the siRNA to the target cells. Upon binding to the cell surface marker protein, the chimera enters the cells via receptor-mediated endocytosis. Through a yet-to-be elucidated route/mechanism, the siRNA is delivered to the cytoplasm and recognized and processed by Dicer to release the mature siRNA followed by the degradation of target mRNA [16] (Figure 2).

McNamara *et al.* were among the first to have developed A10-aptamer-siRNA chimera against prostate-specific membrane antigen (PSMA) fused with siRNA to pro-survival genes polo-kinase 1 (PLK1) or B-cell lymphoma-2 (BCL2) [50]. This chimera maintained the aptamer's binding ability and silenced mRNA expressions of Plk1 and Bcl2 with an efficiency of approximately 80% and 90% *in vitro*, respectively. The chimera is internalized into cells via endocytosis where the siRNA is subject to the endogenous Dicer enzyme processing following its incorporation into the RISC, which subsequently targets the antiapoptotic genes and promotion of cell death [50]. After administration of the chimera to mice bearing PSMA-positive xenografts of prostate cancer (200 pmol/mouse every other day for 3 weeks), tumour volumes in control-treated mice increased 3.63-fold, compared to a 2.21-fold reduction in the chimera-treated mice. Also, no obvious morbidity or mortality was observed following the treatment, suggesting that this aptamer-siRNA chimera is not toxic to the animals in these experiments. Subsequently, second generation of A10-aptamer-siRNA chimeras were devised to facilitate chemical synthesis and for better systemic administration by reducing the size of the aptamer to 32-40 nucleotides and the addition of a 20 kDa polyethylene glycol (PEG) to increase blood residence time [67, 68]. These optimized second-generation chimeras exhibited the same knockdown efficacy at a 100-fold lower

dosage (4 nM) compared to the first generation chimera. About 70% PSMA-positive prostate tumours treated with the chimera (250 pmol/mouse, every other day for a total five injections) was regressed and there was a statistically significant reduction in size compared to controls at the end of the treatment. These studies suggest that the aptamer-siRNA chimera system could be a promising approach for targeted delivery of siRNAs for cancer therapeutics.

In addition to the direct fusion of siRNA molecules to aptamers for the specific delivery of RNAi-based therapeutics, siRNAs also can be non-covalently conjugated to aptamers via adapter linkages [69]. Chu and colleagues have non-covalently coupled biotinylated A9 anti-PSMA aptamer with functional siRNAs against lamin A/C mRNA or GAPDH through a modular streptavidin linkage [70]. Delivery of aptamer-streptavidin-siRNA conjugates has been found to be an efficient and successful means of knockdowning target gene expression following cellular internalisation into LNCaP cells expressing the PSMA antigens, but not the control PC-3 cells, indicating selective and specific delivery of siRNA to target cells mediated by non-covalently aptamer-siRNA conjugates. Bridging a specific aptamer with a functional siRNA through the non-covalent interaction such as biotin-streptavidin for targeted therapeutics is widely used primarily due to its ease of use and modularity [71, 72]. However, the elicitation of immunogenicity by streptavidin as a non-covalent conjugates limits their usage as for siRNAs delivery in the clinic. As a result, other forms of less immunogenic non-covalent linkages, such as hydrogen bonding, are also being explored [70, 73, 74]. Nonetheless, there are some merits of utilising a non-covalent linkage for delivery of non-coding RNAs (siRNA, shRNA). For instance, prior to entrance to the RISC complex, siRNA should be separated from the conjugated moiety including streptavidin that could be beneficial to further processing of siRNAs [70]. Table 2 summarizes cell-specific aptamers for targeted delivery of siRNA.

### **Aptamer-miRNA Delivery System**

MiRNA, a small RNA with 20-22 nucleotides, regulates cellular functions such as differentiation, proliferation, apoptosis as well as tumour suppression [81, 82]. It is speculated that modulation of multiple genes simultaneously via manipulating miRNA functions constitutes a promising next generation of anti-cancer therapeutics [83, 84]. However, the possibility of interacting with multiple genes at multiple tissues by a single miRNA demands specific delivery of miRNAs to their targets, in order to minimize unexpected side effects [83]. Therefore, the development of safe and effective strategies for specific delivery of miRNAs is currently under intense investigation. As illustrated in Figure

2, the aim is to conjugate an aptamer with a miRNA so that the miRNA can be specifically targeted to the cells expressing the ligand recognized by the aptamer. The miRNA gains entry into the cancer cells via [aptamer](#)-dependent endocytosis, followed by processing by Dicer and loading of the miRNA duplex into an Argonaute protein in the miRNA-induced silencing complex.

Esposito and colleagues developed a multifunctional aptamer-miRNA conjugate by bridging the tumour suppressor let-7g miRNA to the GL21.T aptamer that specifically binds to the oncogenic receptor tyrosine kinase Axl [85]. They demonstrated that this conjugate could selectively deliver functional let-7g miRNA to Axl-expressing A549 cancer cells and silence let-7g target genes mediated by the RNAi machinery both *in vitro* and *in vivo* (1.6 nmol/mouse, three injections a week for 3 weeks), resulting in inhibition of tumour cell proliferation, migration and survival *in vitro* and reduction of tumour growth in a xenograft model of lung adenocarcinoma. In addition, Dai *et al.* explored a MUC1 aptamer-miR-29b chimera (Chi-29b) for increasing chemosensitivity in an OVCAR-3 ovarian cancer model [86]. This is based on the known functions of miR-29b in inducing global DNA hypomethylation and tumour suppressor gene re-expression by directly targeting DNA methyltransferase (Dnmt) 3a and 3b expression and indirectly targeting Dnmt1 expression [87]. As PTEN methylation underlies the inactivation of the PTEN gene in ovarian endometrioid carcinoma and clear cell carcinoma of the ovary [88], gene therapy by overexpression miR-29b may confer enhanced anti-tumour effect. Indeed, intraperitoneal injection (15 µg/mouse, once every two days for 4 weeks) of the chimera to the tumour-bearing mice significantly promoted apoptosis in the stepwise developed paclitaxel-resistant OVCAR-3-Taxol cells and achieved a 238-fold reduction in IC<sub>50</sub> value compared to normal OVCAR-3 cells with increased apoptosis in the tumour cells. Mechanistically, anti-chemoresistant function the MUC1 aptamer-miR-29b chimera was attributed to the elevated PTEN expression, downregulation of Akt phosphorylation and upregulation of Bax proapoptotic protein levels.

### **Aptamer-mediated shRNA Delivery for Cancer Therapy**

ShRNA is an alternative approach to RNAi. Instead of being chemically synthesized as in the case of siRNA, shRNA can be introduced by a number of forms of DNA construct: DNA plasmid, linear template, or packaged retroviral/lentiviral vectors [89, 90]. shRNAs possess several intrinsic advantages compared to siRNA, as generated in host cells, shRNA elicits a more durable gene silencing than that of siRNAs, which are normally degraded around 48 h

after the *in vivo* administration [91]. However, the requirement of the use of gene transfection or viral vector limits the application of shRNA in the clinical settings.

In order to develop vector-free RNAi using shRNA, Ni *et al* fused 56 nucleotides RNA A10-3 aptamer targeting PSMA to a 50 nucleotide DNA-activated protein kinase, catalytic polypeptide (DNAPK) shRNA, as DNAPK is a radiosensitisation target [47]. After incubating LNCaP cells with 400 nM chimera for 48 hours, the aptamer-shRNA caused a significant reduction in DNAPK, BRCA2 and ATM mRNA levels. It was confirmed that the A10-3 aptamer-shRNA was internalized into cells and processed by RNAi machinery to yield mature siRNA. Treating subcutaneously implanted LNCaP tumour with radiation plus two intratumoural injections of 200 pmol of A10-3 aptamer-DNAPK shRNA resulted in a dramatically extended time to reach quadruple tumour volume by ~10 weeks compared with one week in tumour treated with control. Thus, aptamer-targeted delivery of shRNA resulted in targeted knockdown of DNAPK, enhanced radiosensitivity and suppressed tumour growth.

### **Aptamer-functionalised Nanoparticles for Targeted siRNA/miRNA/shRNA Delivery**

Following specific binding to tumour cell receptors and the internalization of aptamer-siRNA chimera, siRNA-mediated silencing of target oncogenes could elicit growth arrest and tumour cell death [70]. However, how such chimeras escape from endosomes/lysosomes after cell entrance remains unclear, which might account for the phenomena that high concentrations of siRNA related chimeras need to be administrated for cancer treatment [50, 75, 92]. To overcome this limitation, recent advances in bionanotechnology enables the conjugation of aptamer-siRNA to nanocarriers such as liposome, gold or other magnetic nanoparticles with endosome-rupturing capabilities [93, 94]. The nanoparticle based siRNA delivery strategy remarkably increased the siRNA payload and held the potential for multifunction improvement.

Bagalkot and Gao have developed a novel two-step approach for linking siRNA-aptamer chimeras to nanoparticles-bearing endosome rupturing capability for conferring siRNA functions [95]. This non-covalently engineering not only assists siRNA removing from the surface of nanoparticles after internalisation but also reduces some of the positive charges of nanoparticle. The later subsequently weakened the interaction between nanoparticles and aptamers, which conferred flexibility to aptamer, improved binding affinity and made it more accessible to target molecules. The approach resulted in a remarkable 34% improvement in efficiency of gene silencing, compared to the conventional approaches that was based on

simple mixing of siRNA-aptamer with nontargeted complexes. Another example of non-covalently fusion between aptamer-siRNA with nanoparticles was reported by Zhao *et al* in that they developed an efficient ALCL cell/tissue-specific carrier system by formulating a functional RNA nanocomplex comprised of the CD30-targeting aptamer and an anaplastic lymphoma kinase (ALK)-targeted siRNA within nano-sized polyethyleneimine (PEI) polymer carriers [96]. Through western blotting analysis and immunocytochemical staining, Karpas 299 cells treated with nanocomplexes displayed an efficient knockdown of the nucleophosmin- ALK (NPM-ALK) fusion protein, but did not affect the  $\beta$ -actin expression. Consistently, the percentage of apoptotic cells was 14.1% after 24 h treatment with nanocomplex, showing a ~7-fold increase in cell death compared to the control ( $P < 0.05$ ). Combining siRNAs into nanoparticles increases their size that could prevent the rapid clearance of siRNA from the blood circulation. This system is able to join various types of siRNAs and/or small therapeutic agents to create synergistic effects on cancer therapy. In addition, the positively charged nanocarrier is beneficial for siRNA immobilization and escape from organisms (endosome and lysosome).

In addition to the non-covalent conjugation, RNA aptamers also can be chemically modified to achieve the covalent conjugation to nanovehicles. Kim *et al.* covalently assembled a 50-NH<sub>2</sub>-modified PSMA aptamer with a polymer containing branched PEI and PEG (PEI-PEG) for co-delivery of Bcl-xL shRNA and doxorubicin [97]. In this study, doxorubicin was directly intercalated into the double-stranded stem of aptamers and the anti-apoptotic factor Bcl-xL shRNA was linked with PEI polymer to form aptamer-conjugated nanoparticles. This combinatorial design synergistically caused LNCaP cell death selectively, compared to the treatment in which two individual therapeutics were simply mixed. In addition to targeted delivery of siRNA/shRNA, miRNAs also can be covalently conjugated to an aptamer functionalised nanoparticle for miRNA-mediated silencing to their target messenger RNAs, resulting in the inhibition of mRNA translation and tumouricidal efficacy [98].

### **Clinical Trials of Aptamer-mediated Cancer Therapy**

With the encouraging results from preclinical development of aptamer-mediated gene therapy, two aptamer-based cancer therapeutics have progressed to clinical trial stages (Table 3).

#### **AS1411 Aptamer**

AS1411 is a 26-nucleotide phosphodiester DNA aptamer that targets to the external domain of nucleolin on the membrane, a nuclear matrix protein over-expressed on cell surface of various cancers such as lung cancer, breast cancer, pancreatic cancer, colon cancer, ovarian cancer and leukemia and lymphoma [99]. The nucleolin protein mediates cellular adhesion, endocytosis, and signal transduction and plays important roles in progression of malignant transformation and invasion [99, 100]. The AS1411 aptamer gains the intracellular access when nucleolin is transported from the plasma membrane to the inside of the cell [101]. Blocking of nucleolin by AS1411 contributes to the inhibition of tumour progression both in preclinical and clinical anticancer trials. AS1411 was able to block the activation of canonical nuclear factor- $\kappa$ B signalling which closely correlates to the chemoresistance of cancers and decrease the binding of nucleolin to Bcl-2 mRNA, resulting in the reduction of Bcl-2 and enhanced anticancer effects [67, 102-104]. AS1411 was the first aptamer approved in clinical trials for treatment of various types of cancer. The phase I clinical trial of AS1411 in patients with various solid tumours revealed promising antitumour activity and a lack of significant systemic toxicity [100, 105, 106]. After a continuous intravenous infusion with a dose escalation from 1 mg/kg/d up to 10 mg/kg/d in patients with advanced solid tumours, no serious toxicity of AS1411 was observed in 15 patients and 2 patients who received a 4-day and a 7-day infusion, respectively. In addition, no significant clinical toxicity was reported after administration of AS1411 to rats and dogs with IV bolus dosages of up to 100 mg/kg and 10 mg/kg/d, respectively [105]. Multi-institutional phase II clinical trials of AS1411 in refractory or relapsed acute myeloid leukaemia (at a dose 40 mg/kg/day or 80 mg/kg/day) and non-small cell lung cancers (NSCLC) are now under way [100, 106, 107]. Most recently, Rosenberg and colleagues examined the efficacy and safety of AS1411 in the anti-cancer treatment of patients with advanced renal cell carcinoma (RCC) who had failed tyrosine kinase inhibitor therapy in a phase II trial [107]. The extensive clinical trial data available so far suggest that AS1411 is a promising candidate for targeted anticancer gene therapy.

### **NOX-A12 Aptamer**

NOX-A12 is a 45 nucleotide-long L-RNA aptamer with a 40 kDa PEG linkage at the 3-terminal developed by Spiegelmer technology on the basis of classic SELEX against the chiral mirror image of the target [108]. NOX-A12 was produced to target stroma cell-derived factor-1 ligand 12 (CXCL12), a chemokine involved in promotion of stem cell migration, tumour metastasis, vascular angiogenesis and tissue regeneration [100, 109]. NOX-A12 aptamers have been shown to possess various intrinsic advantages when used in clinical trials:

1) they are small molecules and thus facilitate biopharmaceutical manufacture; 2) as L-nucleotides, they are extremely resistant to nuclease mediated degradation and hybridization in cells even without the need for further chemical modification; 3) Spiegelmers are non-immunogenic and do not activate the cellular immune response; 4) NOX-A12 has therapeutic potential for hematopoietic stem cell transplantation, multiple myelomas and non- Hodgkin's lymphoma. Recently, A Vater and colleagues [110] evaluated white blood cells (WBCs) mobilization in animals (mice and monkeys) and first-in-human clinical study to ascertain safety, pharmacokinetics, and pharmacodynamics of NOX-A12 in healthy volunteers. Both in mice and cynomolgus monkeys, NOX-A12 was superior to the CXCR4 antagonist plerixafor in mobilization of WBCs, hematopoietic stem cells (HSCs) and progenitor cells into peripheral blood (PB) by targeting the chemokine CXCL12. Single dose administration of NOX-A12 (0.05 mg/kg to 10.8 mg/kg) had a benign safety profile in healthy volunteers and also dose-dependently mobilized WBCs and HSCs into PB. At the highest dose (10.8 mg/kg), NOX-12 had a plasma half-life of 38 h and a prolonged duration of WBC and HSC mobilization for more than 4 days. HSC peak mobilization reached a plateau at five times the original level at a 5.4 mg/kg intravenous injection. In this study, NOX-A12 has exhibited a safe and well tolerated profile and demonstrated efficacy *in vivo* through antagonism of CXCL12 signalling as evident from WBC and CD34<sup>+</sup> cell mobilization under different dose regimen over several days. Studies by independent groups revealed that disruption of CXCL12/CXCR4/CXCR7 signalling pathways could improve cancer therapies [110-113]. Through modulating CXCL12-CXCR4 interactions, NOX-A12 aptamer mobilizes malignant cells away from healthy tissues and functions as a novel anticancer therapeutics for not only hematological cancers but also for a number of malignant cancers. Therefore, NOX-A12 aptamer may provide fresh opportunities to advance conventional chemotherapy or facilitating gene therapy.

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

Cancer is still one of the leading causes of death world-wide. The outcome of current treatment options for cancer, including surgery, chemo- and/or radio-therapy, is still far from ideal in many cases. Aptamers as a chemical antibody possess great attributes for clinical application both in cancer diagnostics and targeted cancer therapy. In particular, aptamers are emerging as a promising targeting agent for vector-free delivery of therapeutic genes (i.e. siRNA, miRNA and shRNA) for the next generation of cancer gene therapy. Future developments will focus on the selection and/or engineering shorter aptamers for a better

cost-benefit ratio. Tumour penetration is a vastly under- appreciated and under-studied aspect area in cancer gene therapy, but the extent of tumour penetration throughout the entire tumour is one of the key aspects underlying failure of current cancer drug therapy in solid tumours [114]. The selection of effective aptamers with the smallest possible size through careful post-selection screening and engineering plus the fine tuning of blood residence time and tumour penetration will assist the future development of aptamer-guided gene therapy modalities with superior tumour penetration and thus better clinical outcome. The combination of traditional anticancer therapies along with single or multiple gene therapy regimens may offer more promising outcomes than either of these anticancer approaches alone. With further reduction of potential immune responses and side effects, the vector-free form of aptamer-guided cancer gene therapy will become one of the effective weapons in the anticancer arsenal in the near future.

#### **CONFLICT OF INTEREST**

The author(s) confirm that this article content has no conflicts of interest.

#### **ACKNOWLEDGEMENT**

Work at the authors' laboratory was supported by grants from National Health and Medical Research Council of Australia, Australia-India Strategic Research Fund, Discovery Project grant (DP140100002) from Australian Research Council and The CASS Foundation.

#### **PATIENT CONSENT**

Declared none.

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**Table 1 Comparison of Key Features between Aptamer and Antibody**

	<b>Aptamer</b>	<b>Antibody</b>
<b>Size</b>	~ 2 nm	~ 15 nm
<b>Generation</b>	<i>In vitro</i> SELEX	<i>In vivo</i> biological system
<b>Affinity</b>	High	High
<b>Specificity</b>	High	High
<b>Chemical modification</b>	Easy and straightforward	Limited
<b>Targets</b>	Wide range: nucleic acids, peptides, proteins, organic and inorganic molecules, a whole cell, entire organ	Limited to immunogenic molecule
<b>Immunogenicity</b>	No or low	Yes
<b>Batch to batch</b>	No or low	Significant
<b>Cost</b>	Becoming low with new technology	Low
<b>Stability</b>	Stable across a wide range of storage and temperature conditions	Low

**Table 2 Application of Cell-specific Aptamers for Targeted Delivery of SiRNA**

<b>Aptamer</b>	<b>Target gene</b>	<b>Cancer</b>	<b>Research Group</b>
<b>RNA aptamer</b>	PLK1 and BCL2	Prostate cancer	McNamara <i>et al.</i> 2006 [50]
<b>RNA aptamer</b>	lamin A/C mRNA	Prostate cancer	Chu <i>et al.</i> 2006 [70]
<b>RNA aptamer</b>	PLK1	Prostate cancer	Liu <i>et al.</i> 2013 [26]
<b>RNA aptamer</b>	PLK1 or BCL2	Prostate cancer	Dassie <i>et al.</i> 2009 [75]
<b>RNA aptamer</b>	EGFRvIII	Glioblastoma	Zhang <i>et al.</i> 2014 [76]
<b>RNA aptamer</b>	mTORC1	Melanoma, Breast cancer	Berezhnoy <i>et al.</i> 2014 [77]
<b>RNA aptamer</b>	SLUG and NRP1	Lung cancer	Lai <i>et al.</i> 2014 [78]
<b>RNA aptamer</b>	BRAF	Melanoma	Li <i>et al.</i> 2014 [79]
<b>DNA aptamer</b>	MUC1	Adenocarcinomas	Yoo <i>et al.</i> 2014 [80]

BCL2, B-cell lymphoma-2; BRAF, proto-oncogene B-Raf; EGFRvIII, epidermal growth factor receptor variant III; mTORC1, mammalian target of rapamycin complex 1; MUC1, cell surface associated; NRP1, neuropilin 1; PLK1, pro-survival genes polo-kinase 1; SLUG, snail family zinc finger.

**Table 3 Clinical Trials of Aptamer-Based Anti-Cancer Therapies**

NCT number	Drug (Target)	Condition	Status	Phase	Eligibility			Locations	Sponsor
					Ages (years)	Genders for Study	Accepts Healthy Volunteers		
NCT01034410	AS1411 (Nucleolin)	AML	Completed	2	18-70	Both	No	U.S, Australia, New Zealand, Taiwan	Antisoma Research
NCT01194934	NOX-A12 (CXCL12/SD F-1)	HSCT	Completed	1	18-55	Both	Yes	Germany	Noxxon Pharma AG

AML: Acute Myeloid Leukemia, HSCT: Hematopoietic Stem Cell Transplantation

## Figures Legends:

**Fig. (1). Schematic Representation of RNA Aptamers Generation by SELEX.** Specific targets of interest (cells or proteins) are incubated with a pool of single-stranded RNA oligonucleotides with random sequences, followed by the extraction of bound sequences and removal of unbound RNAs. The selected sequences can be subjected to rounds of negative selection by incubating with negative control cells or proteins. The unbound oligonucleotides are eluted and amplified by RT-PCR, followed by next rounds of selection. After the last round of selection (typically round 6-10), the remaining RNA aptamers are converted to DNA and subjected to next generation sequencing or conventional cloning followed by characterization and further engineering.

**Fig. (2). Aptamer-Mediated Cancer Gene Therapy Pathways.** Therapeutic nucleotides such as siRNA, miRNA and anti-miRNA can be directly conjugated with aptamers or encapsulated within nanoparticles followed by aptamer functionalization, in which aptamer acts as targeted-delivery modalities. Following the specific binding of aptamers to the cell surface targets, both of aptamer conjugated therapeutic nucleotides or nucleotides-loaded nano-carriers internalise into target cancer cells via receptor-mediated endocytosis. After the entrance and escape from endosomes, the released siRNA/miRNA/anti-miRNA in the cytoplasm are cleaved by Dicer into mature siRNA/miRNA/anti-miRNA. These mature nucleotides are unwound and subsequently incorporated into the RNA-induced silencing complex (RISC) system. The guide strand enables the mature siRNA/miRNA/anti-miRNA to recognise target sequences of mRNA, leading to mRNA degradation or translation promotion or repression of specific RNA targets. Part of mature miRNAs are transported into nuclei and interact with their target [nucleic acids](#) or genomic DNAs to elicit translation promotion/repression or DNA binding activation/repression.