Expert Opinion

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Strategies for tumor-directed delivery of siRNA

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Introduction: Current treatment of malignant tumors relies predominantly on chemotherapy delivering a single antineoplastic drug or a combination of two or more drugs intravenously. Problems with such treatments can include the killing of healthy cells, adverse side effects and chemoresistance. As cancer basically results from different types of mutation leading to the overexpression or suppression of the signaling cascades responsible for cancer cell survival and proliferation, tailor-made approaches capable of interfering precisely with those pathways are the potential revolutionary tools that could pave the way for highly effective cancer therapy.

Areas covered: This review summarizes recent progress in the identification and validation of the target genes for cancer gene therapy using small interfering RNA (siRNA) technology and, more importantly, the delivery strategies that have been designed and implemented for tumor-directed delivery of siRNAs.

Expert opinion: Cancer-targeted delivery of a gene in order to produce a particular protein (such as a tumor-suppressor or a nucleic acid sequence that can silence the expression of a specific gene, such as an oncogene or an antiapoptotic gene) is the most promising concept for cancer treatment in the future. siRNA has the ability to recognize and cleave a specific mRNA, thus inhibiting the expression of a particular protein. The success of targeted gene silencing as a potential cancer therapeutic demands the development of more effective delivery devices and the removal of siRNA off-target effects.

Keywords: apoptosis, cancer, gene silencing, small interfering RNA

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1. Introduction

Fighting cancer has become one of the top priorities in pharmaceutical industries and clinical medicine, with huge budgets as well as enormous efforts being committed to developing effective strategies to cure various types of cancer. Clinical applications of current chemotherapeutic drugs are often limited owing to their toxic effects on normal cells, causing the patients to be able to tolerate a level of the drug that is therapeutically insufficient, with the final outcome of chemoresistance and subsequent tumor recurrence [1]. As cancer is the result of overexpression or suppression of certain molecular signaling pathways affecting cancer cell survival and proliferation, approaches of interfering with those pathways would be the potential treatment options that could render cancer cells more sensitive to cytotoxic chemotherapy. Several strategies have been developed for specific silencing of gene expression, such as triple helix forming, or decoy transcription factor binding, oligodeoxynucleotides to disrupt gene expression at the level of transcription in the cell nucleus and antisense oligonucleotides (ODN), small interfering RNA (siRNA) or short hairpin RNA (shRNA) to disrupt expression at the level of translation in cytoplasm [2].

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Article highlights.

- Intracellular delivery of an siRNA designed against the mRNA of a specific gene results in the inhibition of target protein expression.
- Complexation of siRNA with delivery vehicles prevents degradation of siRNA by nucleases and facilitates targeted delivery of siRNA to the tumor.
- Silencing of the oncogenes and the genes regulating the cell cycle, apoptosis, cellular senescence, tumor angiogenesis, metastasis and muti-drug resistance has potential implications for cancer therapy.
- The synthetic biomaterials used at present for siRNA delivery in cancer model animals include SNALPs, LPD nanoparticles, neutral liposome, cationic liposome, immunoliposome, immunonanoplex, PEI, protamine, cyclodextrin and atelocollagen.
- The major barriers to the implementation of siRNA technology in cancer therapy are the inefficiency of the delivery systems to carry sufficient siRNA into the cancer cells, non specific silencing of the cellular genes other than the target one and the stimulation of immune response.

This box summarizes key points contained in the article.

siRNA, a double-stranded RNA of ~ 21 – 28 nucleotides with 3′-overhangs, that selectively degrades mRNA, blocking production of a particular protein, has evolved as a potential tool for the study of functional genomics, drug target validation, and even for targeted therapy. The high level of gene-silencing specificity confers an extra advantage to siRNA technology over other gene-targeting approaches [3]. The concept of siRNA emerged with the finding that the introduction of a foreign double-stranded RNA (dsRNA) into cytoplasm induced sequence-specific degradation of the endogenous mRNAs bearing homology to the dsRNA in a cascade where dsRNAs are cleaved by Dicer (a cellular ribonuclease III) into siRNA duplex, which subsequently incorporated into a multiprotein RNA-induced silencing complex (RISC) and unwound into single-stranded RNAs by Argonaute 2, a multifunctional protein within the RISC, forming antisense strand-associated RISC that finally guides and selectively degrades the complementary mRNA with the help of Argonaute-2 [4-10]. Perfect hybridization between the antisense (guide) strand of siRNA and the target mRNA induces degradation of the mRNA near the center of the target–siRNA duplex, whereas several mismatches cause translation arrest [11,12]. The antisense strand of siRNA is protected within the RISC complex and thus preserved as a catalyst to degrade extra copies of the target mRNA [13]. Silencing by synthetic siRNA is more advantageous than plasmid-encoded shRNA partly owing to the difficulty of constructing shRNA expression systems before the selection and verification of the active sequences and the requirement of the expression system to cross the nuclear membrane for shRNA expression [14].

2. Potential gene targets for silencing in cancer therapy

Many cancers are characterized by abnormal gene expression patterns. The major cellular genes that are involved in initiation, survival and progression of cancer include the oncogenes, cell cycle regulatory genes, apoptosis- and cellular senescence-associated genes and the genes influencing angiogenesis, metastasis, multi-drug resistance and immune evasion of tumor [13,15].

2.1 Silencing of oncogenes

Among the oncognes, receptor protein tyrosine kinases (PTKs), which are frequently found mutated in human malignancies [16], play a critical role in the development and progression of many types of cancer [17]. Normally the receptor PTK pathway is tightly regulated; however, chromosomal translocation can produce an oncogenic fusion protein including a PTK catalytic domain and an unrelated protein that provides constitutive tyrosine kinase activity [18]. When specific siRNA was allowed to silence the mRNA of Bcr-Abl, a fusion protein in chronic myeloid leukemia where the *ABL1* gene of a PTK is translocated within the *BCR* (breakpoint cluster region) gene, Bcr-Abl overexpressing cells became more sensitive to the chemotherapeutic drug imatinib [19]. Another example of a chromosomal translocation-mediated fusion protein in chronic myelomonocytic leukemia is TEL/PDGFbetaR, and silencing of the mRNA with sensitized siRNA transformed cells to the PDGFbetaR inhibitor imatinib [20]. Gain-of-function mutations can also cause oncogenic transformation of PTKs, as seen in some non-small-cell lung cancers (NSCLCs) where mutations in the catalytic kinase domain of epidermal growth factor receptor (EGFR) cause the constitutive kinase activation. Consequently, knockdown of the mutant EGFR with siRNA led to extensive apoptosis of NSCLC cells expressing mutant EGFRs [21]. Gene amplification can also cause oncogenesis by overexpressing PTK in breast and ovarian carcinomas, leading to constitutive kinase activity of the HER2/Neu, a receptor PTK. Silencing of the Her2/neu gene expression with siRNA resulted in the apoptosis of Her2/neu-positive cell lines [22,23]. Other examples of PTKs that are overexpressed in various cancers and where siRNA-mediated silencing of the expression leads to the induction of apoptosis include ErbB3 [24], insulin-like growth factor-I (IGF-I) receptor (IGF-IR) [25,26], colony stimulating factor 1 receptor (CSF1R) [27,28], FMS-like tyrosine kinase 3 (FLT3) [29], c-Met receptor [30], EphA2 [31] and c-SRC [32].

The other oncogenes commonly involved in tumorigenesis and investigated as potential therapeutic targets by siRNA silencing technology include beta-catenin [33,34], c-Myc [35], K-ras [36-38], C-Raf [39], phosphatidylinositol 3-kinase (PI3K) [40,41], AKT [42], IKK-β [43], NF-κB [44] and EWS/FLI-1 [45,46].

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2.2 Silencing of cell cycle regulatory genes

Rb tumor suppressor protein (pRb) and p53 are the most important regulators for cancer cell cycle. One major function of pRb is to associate with transcription factor E2F and prevent it from activating the expression of cyclins E and A, leading to the inhibition of cell-cycle progression [47]. Malignant transformation of human papilloma virus (HPV)-infected cells takes place when E7, an oncogenic protein of HPV, binds and inactivates pRb [48]. Silencing of HPV-16 E7 mRNA with siRNA induces apoptosis in HPV-16-associated cervical cancer cell lines [49]. E2F4, another molecule of the Rb pathway, has also been targeted by siRNA to prevent p130/E2F4 complex formation, with the consequence of sensitizing cells to irradiation-induced apoptosis [50]. On the other hand, the tumor suppressor protein p53, which is normally activated in response to a stress signal leading to cell-cycle arrest, cellular senescence and apoptosis, is found in an inactivated form in almost half of all human cancers [51]. Interfering RNA-mediated reduction of Hdmx, which is a key p53-negative regulator and overexpressed in many tumors, markedly inhibited the growth potential of MCF-7, a breast cancer cell line harboring wild-type p53 [52]. Similarly, silencing the expression of Notch-1, Delta-like 1 or Jagged-1 invloved in the p53 pathway induces apoptosis in multiple glioma cells [53]. siRNA-induced downregulation of the viral oncogene E6, which is also constitutively expressed like E7 in HPV-associated neoplasms and promotes the degradation of p53, causes massive apoptotic cell death in HPV-positive cells [48,54]. Other cell-cycle regulatory molecules that have have been targeted by silencing technology include cyclin B1/cdc2 [14,55], cyclin D1 [56,57], cyclin D3 [56,58] and Checkpoint kinase [59].

2.3 Silencing of apoptosis- and cellular senescence-associated genes

Many cancers express antiapoptotic proteins, rendering the cells chemo- and/or radioresistant [51]. Restoration of apoptosis by gene silencing of the antiapoptotic molecules is potentially useful for therapeutic intervention. The antiapoptotic proteins whose roles in cancer progression have been validated through knockdown by siRNA include Fas-associated death domain-like interleukin-1β-converting enzyme-like inhibitory protein (FLIP) [60,61], Bcl-2 [62,63], Bcl-xL [64], Mcl-1 [65], survivin [66-69] and X chromosome-linked IAP (XIAP) [62,63,70], clusterin [71], stem cell antigen-2 (Sca-2) [72], glycogen synthase kinase-3β (GSK-3β) [73] and protein kinase casein kinase II (CK2) [74].

Cellular senescence, a phenomenon by which normal cells lose their dividing capacity owing to the shortening of telomere repeats, is reversed in rapidly dividing human cancers through the synthesis of new repeats by telomerase. Silencing the expression of telomerase reverse transcriptase (hTERT), the protein component of telomerase, could successfully inhibit telomerase activity in several cancer cell lines [75-81]. Also, siRNA-mediated knockdown of mammalian heterogeneous nuclear ribonucleoparticulate A1 and A2 proteins, which bind to the single-stranded DNA tails (G-tails) of telomeres with high affinity, induces apoptosis in cervical, colon, breast, ovarian and brain cancer cell lines [82].

2.4 Silencing of the genes regulating tumor angiogenesis, metastasis and multi-drug resistance

Vascular endothelial growth factor (VEGF) and VEGF receptor are responsible for pathological angiogenesis in cancers. Introduction of the siRNAs against VEGF inhibited the secretion of VEGF in human prostate cancer cells (PC-3) [83] and suppressed cell proliferation in gallbladder cancer (GBC) cells [84] and human colorectal cancer HCT116 cells [85]. Similarly, knockdown of VEGF receptor expression resulted in inhibition of the growth of tumors [86,87].

Tumor progression and metastasis require the enzymatic degradation of extracellulat matrix (ECM) by cysteine protease, serine protease and matrix metalloprotease (MMP). siRNAs targeting urokinase-type plasminogen activator (u-PA) (a serine protease) [88-90], cathepsin B (a cysteine protease) [91,92] and MMP-9 [92,93] reduced the invasiveness of tumor cells. Other molecules that are involved in tumor cell invasion or metastasis and have been subjected to knockdown studies include small GTPases such as RhoA and RhoC [94,95], CXC chemokine receptor-4 (CXCR4) [96,97], insulin-like growth factor-binding protein 2 (IGFBP2) [98], EphA2 [31], $\alpha_6\beta_4$ integrin [99] and epithelial cell adhesion molecule (EpCAM) [100].

Whereas the antiapoptotic molecules enable cancer cells to resist chemotherapy by blocking the apoptotic pathways (described in Section 2.3), multi-drug resistance (MDR) proteins contribute by effluxing the anticancer drugs. Silencing the genes of the MDR genes, such as *ABCB1* (*MDR 1*) [101-106], *ABCB4* (*MDR 3*) [106] and *ABCB5*, [107] could sensitize cancer cells to chemotherapy drugs.

3. *In vivo* delivery of siRNA for cancer therapy: challenges and prospects

siRNAs have clear advantage over the traditional anticancer drugs owing to their specificity in silencing particular mRNAs and thus preventing formation of the targeted proteins that are uniquely responsible for cancer, whereas the chemotherapy drugs usually have multiple cellular targets, causing adverse effects in normal healthy cells in addition to the effects exerted in cancer cells. However, the success of siRNA technology largely depends on the delivery of siRNA because siRNA, being anionic and hydrophilic, cannot penetrate the anionic cell membrane through passive diffusion. Moreover, siRNA can be degraded by the nuleases in plasma and even subjected to renal elimination owing to its small size (< 50 kDa and 6 nm) before reaching the target site [108,109]. Although chemical modifications within siRNAs by replacement of the phosphodiester group with phosphothioate at the 3′-end or

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introduction of an *O*-methyl group, a fluoro group or a 2-methoxyethyl group resulted in prolonged half-lifes of the siRNAs [110-114], modified siRNAs could be inefficient at silencing activity [115] and susceptible to glomerular filtration in the kidney following systemic administration [116]. Development of siRNA delivery systems is, therefore, essentially required to overcome the obstacles. The following devices have been established so far for tumor-directed delivery of siRNA.

3.1 Stable nucleic acid lipid particles

A stable nucleic acid lipid particle (SNALP) consists of a lipid bilayer having cationic lipids to facilitate complexation with anionic siRNA and subsequently cellular uptake of SNALP–siRNA complex, and fusogenic lipids to enable ensomal escape of siRNA following endocytosis of the complex (**Table 1**). The surfaces of SNALPs are coated with a PEG, which provides a neutral and hydrophilic exterior [117]. Recently, SNALPs have been used successfully for siRNA delivery in experimentally induced tumors [118]. 2′-*O*-methyl-modified siRNAs targeting the essential cell-cycle proteins polo-like kinase 1 (PLK1) and kinesin spindle protein (KSP) were formulated separately in SNALPs and administered by standard intravenous (i.v.) injection through the lateral tail veins of mice having induced hepatic and subcutaneous tumors [118]. SNALP-mediated siRNA delivery effectively cleaved the target mRNAs and caused extensive mitotic disruption and tumor cell apoptosis without induction of measurable immune responses [118]. Recently, PEGylated siRNA-loaded lipid particles were formulated by hydration of a freeze-dried matrix with high siRNA entrapment efficiency (> 90%) and high gene-silencing efficiency [119,120]. Using these particles, on systemic delivery of the siRNA to target E6/7 oncogenes that are expressed in cervical cancer, a 50% reduction in tumor size was observed, with the level of tumor growth suppression being comparable to that achieved with cisplatin at the clinically used dose [119,120].

3.2 PEGylated liposome-polycation-DNA nanoparticles

Liposome-polycation-DNA (LPD) particles are usually composed of a cationic lipid, a cationic polymer (protamine) and DNA (calf thymus DNA). A mixture of siRNA and DNA is complexed with protamine and subsequently coated with cationic liposomes consisting of1,2-dioleoyl-trimethylammonium-propone (DOTAP) and cholesterol to form LPD. A PEGylated LPD nanoparticle having asparagine-glycine-arginine (NGR) peptide that can target aminopeptidase N(CD13) usually expressed in the tumor cells or tumor vascular endothelium was fabricated by incubation of LPD with a PEGylated ligand lipid, for systemic and specific delivery of c-myc siRNA into solid tumors in mice [121]. LPD-PEG-NGR could efficiently deliver siRNA into the cytoplasm of HT-1080 xenograft tumor 4 h after i.v. injection. Three daily i.v. injections (1.2 mg/kg) of LPD-PEG-NGR-formulated c-myc siRNA effectively suppressed c-myc expression and induced tumor cell apoptosis, resulting in a partial tumor growth inhibition [121]. However, when siRNA and doxorubicin were co-formulated in LPD-PEG-NGR particles, enhanced tumor growth inhibition was observed [121]. In another study, synthesized LPD particles were modified with PEG and a ligand, anisamide, which has moderate affinity for sigma receptors on prostate and lung cancer cells [122]. Four hours after i.v. injection of the LPD particle formulation carrying an siRNA into a xenograft model of NCI-H460 (human lung cancer cells), 70 – 80, ~ 10 and ~ 20% of injected siRNA/g was detected in the tumor, liver and lung, respectively [122]. Three daily injections (1.2 mg/kg) of EGFR siRNA formulated in the targeted and PEGylated LPD silenced the EGFR in the tumor and induced ~ 15% tumor cell apoptosis [122].

3.3 Neutral liposome

Neutral liposome consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) was successfully used for efficient *in vivo* siRNA delivery. Fluorescence-labeled siRNA was shown to be distributed in the tumor as well as the major organs following i.v. injection of siRNA/DOPC liposomes into breast xenograft mouse model of HeyA8 or SKOV3ip1 cells [123]. Also, DOPC-encapsulated siRNA targeting EphA2, an oncoprotein overexpressed in ovarian and other human cancers, was highly effective in downregulating EphA2 expression into the tumor 48 h after a single-dose i.v. administration of the DOPC-associated siRNA [123]. Intravenous delivery of EphA2-targeting siRNA-DOPC (150 μg/kg twice in a week) reduced tumor growth of the ovarian cancer and further tumor growth inhibition was observed when EphA2-targeting siRNA-DOPC was administered intraperitoneally (i.p.) with paclitaxel (100 μg) [123]. In a similar study, i.p. injection of DOPC carrying siRNA against FAK, a protein overexpressed in ovarian cancer with roles in cell survival and metastasis, was found to reduce FAK expression into the tumor of the same breast cancer model for up to 4 days. The mean tumor weight was reduced by 44 to 72% following i.p. delivery of FAK siRNA-DOPC (150 μg/kg twice weekly) in the breast xenograft mouse model of HeyA8, A2780-CP20 and SKOV3ip1 cells. When FAK siRNA-DOPC was combined with docetaxel or cisplatin, there was even greater reduction in mean tumor weight in the models [124]. In another study, DOPC nanoliposomes carrying both EphA2 and FAK-targeted siRNAs were administered i.p. in orthotopic models of ovarian carcinoma. In the HeyA8 model, whereas EphA2 siRNA-DOPC resulted in a 67% ($p < 0.02$) and FAK siRNA-DOPC in a 62% decrease in tumor growth, the combined EphA2 + FAK siRNA-DOPC treatment resulted in a 90% reduction in tumor growth [125]. In the SKOV3ip1 model, whereas EphA2 and FAK siRNA-DOPC resulted in a 50 – 61% decrease in tumor growth, the combination therapy showed 76% reduction in the growth.

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Table 1. Summary of different delivery systems and their components, the genes targeted for siRNA-mediated silencing and the cancer models investigated for tumor regression following siRNA delivery.

Delivery systems	Components	Targeted genes	Tumor models	Ref.
SNALP	Cationic lipid with fusogenic lipid and PEG coating	*PLK1, KSP, E6/7*	Hepatic	[118,119]
			Cervical	[120]
PEGylated LPD	Cationic lipid with DNA, cationic polymer, PEG coating and ligands	*c-myc, EGFR*	Fibrosarcoma	[121]
			Lung	[122]
Neutral liposome	DOPC (a neutral lipid)	*EphA2, FAK and IL-8*	Ovarian	[123,124]
			Breast	[125]
			Ovarian, breast	
Cationic liposome	Cationic lipid (LIC- 101)	*bcl-2*	Liver, prostate	[127]
		RecQL1	Liver	[128]
	Cardiolipin	*Raf- 1*	Prostate	[129]
	Cationic lipids and PEG	*Integrin alphaV*	Prostate	[130]
	Cationic lipid, AtuFETOT and DSPE-PEG	*CD31*	Prostate	[131]
	Cationic lipid, folate, PEG and DSPE	*Her-2*	Cervical	[132]
Immunoliposome	DOTAP, DOPE, TfRscFv and HoKC	*Her-2*	Breast,pancreatic	[135]
Polyplex	PEGylated PEI and RGD	*VEGF R2*	Neuroblastoma	[143]
	Poly(ester amine)	*Akt1*	Lung	[145]
	PEI	*PTN*	Glioblastoma	[146]
	Stearic acid-modified PEI	*STAT3*	Melanoma	[147]
	Fab-conjugated protamine	*c-myc, MDM-2 and VEGF*	Melanoma	[148]
	Cyclodextrin, PEG and transferrin (ligand)	*EWS-FLI1*	Ewing's sarcoma	[30]
		RRM2	Ewing's sarcoma	[152]
	Atelocollagen	*VEGF*	prostate, bone metastases, testicular	[153-156]

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Moreover, the combination of EphA2 and FAK siRNA-DOPC produced the most significant decreases in tumor metastasis in both of the models [125]. DOPC-based neutral liposome was also used for i.p. delivery of the siRNA targeting IL-8, a proangiogenic cytokine overexpressed in many human cancers (including breast cancer) [126]. In all three mouse models described above, treatment with IL-8 siRNA-DOPC plus taxane docetaxel reduced the tumor growth dramatically [126].

3.4 Cationic liposome

A cationic liposome (LIC-101) containing 2-*O*-(2-diethylaminoethyl)-carbamoyl-1,3-*O*-dioleoylglycerol and egg phosphatidylcholine was used to deliver human bcl-2 mRNA-specific siRNA in a mouse model of liver metastasis (A549 cells) by bolus intravenous injection and in a mouse model of prostate cancer (PC-3 cells) by subcutaneous injection near the tumor [127]. In both cases, significant tumor growth inhibition was observed [127]. In a similar study, subcutaneous injection of human RecQL1 DNA helicase-targeted siRNA formulated in LIC-101 in a mouse model of liver cancer (Hep3B cells) clearly prevented tumor growth [128]. Cationic liposomes based on cardiolipin carrying an siRNA against Raf-1 were shown to inhibit tumor growth after i.v. administration into a xenograft model of human prostate cancer in mice [129]. Intratumoral administration of anti-integrin alphaV siRNA formulated into a cationic liposome composed of dipalmitoylethylphosphocholine, dioleoylphosphoethanolamine, dipalmitoylphosphoethanolamine and polyethylene glycol in xenograft models of human prostate cancer (PC-3 cells) was associated with increased apoptosis in tumor cells [130]. siRNA targeting CD31 was complexed to cationic liposomes composed of AtuFECT01, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine and DSPE-PEG. Intravenous injection of the complexes into mice with an established prostate tumor resulted in the reduction of tumor growth and metastases [131]. A folate-linked nanoparticle consisting of of cholesteryl-3beta-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol), Tween 80 and folate-poly(ethylene glycol)-distearoylphosphatidylethanolamine conjugate (f-PEG(2000)-DSPE) was used for complexing with and subsequently delivering anti-Her-2 siRNA by an intratumoral route, resulting in significant tumor growth inhibition of KB xenografts [132].

3.5 Immunoliposome

For systemic delivery of siRNA, a tumor-specific, nanosized immunoliposome complex consisting of DOTAP and dioleoyl phosphatidylethanolamine (DOPE) with the surface being decorated with an anti-transferrin receptor single-chain

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antibody fragment (TfRscFv) was developed [133-135] for binding to the epitope of the TfR overexpressed in various types of cancer cell [136].

Elevated TfR levels also correlate with the aggressiveness or proliferative activity of tumor cells [136]. As the scFv (f28 kDa) is much smaller than the Tf molecule (80 kDa) or the parental mAb (155 kDa), the scFv-liposome-DNA complex may have better penetration into small capillaries characteristic of solid tumors. For enhanced endosomal escape of siRNA, one formulation of the targeted immunoliposome complex carries a small linear pH-sensitive histidine-lysine peptide peptide (HoKC) that possesses a cysteine residue at the end, enabling it to be conjugated to the liposome through a maleimide group [135]. When i.v. administered, both forms of the complex (with and without inclusion of the HoKC peptide) delivered the fluorescently labeled siRNA specifically and efficiently to both large primary prostate tumors [134] and in two metastasis models of human pancreatic cancer and human melanoma MDA435/LCC6 [134,135]. Moreover, tumor-specific delivery of anti-HER-2 siRNA by means of the HoKC nanocomplex resulted in virtually complete knockdown of HER-2 expression in the induced breast tumors (MDA-MB-435 cells) of mice [135]. Furthermore, i.v. administration (thrice weekly) of HER-2-targeted siRNA with the HoKC nanocomplex either alone or in combination with gemcitabine (a chemotherapeutic agent used at present for pancreatic cancer) caused significant growth inhibition of established human pancreatic (PANC-1) xenograft tumors [135].

3.6 Immunonanoplex

An antibody-coupled nanocomplex was developed [137] by conjugating an oligo-9-arginine peptide, a cell-penetrating peptide (CPP), to an antibody specific to JL1, a unique antigen of leukemia cells, but not mature hematopoietic cells [138-140] for siRNA targeting to T leukemic cells [137]. The anti-JL1 immunonanoplexes were effectively targeted to JL1-positive cells (CEM) inoculated in the mouse bone marrow of an immunocompromised mouse. FACS analysis of bone marrow cells indicated that the anti-JL1 immunonanoplexes could deliver FITC-labeled siRNA to 7.32% of total CEM cells, suggesting that the anti-JL1 immunonanoplex is a powerful siRNA delivery system for human leukemia therapies, as T leukemic cells are reluctant to be transfected by non-viral vectors [141]. Another immunonanoplex consisting of a streptavidin-monoclonal antibody of anti-transferrin receptor associated with mono-biotinylated luciferase siRNA was i.v. injected once at a dose of 0.27 mg/kg to rats bearing intracranial tumors of luciferase-expressing glial cells, and the treatment caused a 69 – 81% decrease in luciferase gene expression in the intracranial brain cancer *in vivo* [142].

3.7 Polyethyleneimine

Polyethyleneimine (PEI) is a linear or branched cationic polymer widely used for cellular delivery of DNA, oligonucleotide and also siRNA. PEGylated PEI having at the distal end of the PEG an Arg-Gly-Asp (RGD) peptide with specificity towards the $\alpha_V\beta_3$ integrin expressed in tumor vasculature was used for complexatation with antivascular endothelial growth factor receptor-2 (VEGF R2) siRNA. Subsequent i.v. administration of VEGF R2-targeted siRNA (40 μg) into nude mice with established tumors of N2A murine neuroblastoma cells led to the inhibition of both tumor angiogenesis and growth rate [143]. In similar research, a new branched low-molecular-mass PEI F25 was introduced to deliver siRNA for selective knowdown of VEGF in subcutaneous tumor xenograft mouse models, resulting in the antitumor effects synergistically with Bevacizumab, a humanized anti-VEGF monoclonal antibody [144]. An aerosol of anti-Akt1 siRNA complexed with poly(ester amine) (a PEI derivative) was delivered into K-ras(LA1) and urethane-induced lung cancer models through a nose-only inhalation system (twice weekly for 4 weeks), resulting in the downregulation of Akt-related signals and inhibition of tumor progression in the lung cancer model of K-ras(LA1) mice [145]. PEI-mediated subcutaneous or intraperitoneal delivery of the siRNAs specific for the secreted growth factor pleiotrophin (PTN) into subcutaneous tumor xenografts significantly inhibited tumor growth without a measurable immunostimulation of the siRNAs. Moreover, injection of the PEI-PTN siRNA complexes into the CNS exerts antitumoral effects in a clinically more relevant orthotopic mouse glioblastoma model with U87 cells growing intracranially [146]. *In vivo* delivery of the siRNA targeting signal transducer and activator of transcription 3 (STAT3) with the help of stearic acid-modified PEI induced tumor regression accompanied with an increase in IL-6 levels and Caspase 3 activity along with a decrease in VEGF level and STAT3 activity in the tumor tissue [147].

3.8 Protamine

Like cationic liposome and PEI, protamine, being a cationic polyamine, can complex with DNA, oligonucleotides or siRNA. Recently, FITC-labeled siRNA was selectively delivered (intratumorally or intravenously) to the B-16 melanoma tumors modified to express HIV *env* using protamine conjugated with a fragment antibody (Fab) specific for the HIV-1 envelope protein gp160 [148]. Moreover, a cocktail of siRNAs against c-myc, MDM-2 and VEGF was administered with the Fab-conjugated protamine to mice bearing subcutaneous B-16 HIV *env*-expressing xenografts, resulting in tumor growth inhibition [148]. It was also shown that an ErbB2 single-chain antibody fused with protamine delivered siRNAs specifically into ErbB2-expressing cancer cells [148]. In a different study, a cell-penetrating peptide derived from natural protamine, termed low-molecular-mass protamine, was demonstrated to carry and localize siRNA inside tumors and inhibit the expression of VEGF through systemic administration of the siRNA/peptide complex, thereby suppressing tumor growth without having any measurable immunostimulatory effect [149].

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3.9 Cyclodextrin

Cyclodextrins with short polycations as required for self-assembly with siRNA are stabilized for use in biological fluids by surface decoration with PEG containing transferrin as the targeting ligand [150,151]. Tail vein delivery of the targeted, anti-luciferase siRNA-containing polyplexes in mice with luciferase-producing metastasized EFT (Ewing's family of tumors) showed a strong decrease (> 90%) in luciferase signal 2 – 3 days after the injection [151]. Also, three consecutive daily injections of the targeted polyplexes formulated with the siRNA specific for siRNA against *EWS-FLI1*, a chimeric fusion gene found in 85% of EFT patients, resulted in growth inhibition of metastasized EFT in mice [30]. Using the same targeted nanoparticle system, the first in-human Phase I clinical trial is now being conducted with the administration of siRNA specific for RRM2, an established anticancer target, to patients with solid cancers refractory to standard-of-care therapies on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion [152]. Tumor biopsies from the patients obtained after the treatment showed the presence of intracellularly localized nanoparticles in amounts that correlate with dose levels of the nanoparticles administered. Furthermore, a reduction was found in both the specific messenger RNA (M2 subunit of ribonucleotide reductase (*RRM2*)) and the protein (RRM2) [152].

3.10 Atelocollagen

Atelocollagen, which is derived from pepsin-treated type I collagen and unique in being a liquid at 4°C and a gel at 37°C, was shown to increase cellular uptake, nuclease resistance and prolonged release of the siRNA administered systemically and locally in tumor models [153-156]. *In vivo*, this polymer was able to deliver effectively siRNA targeting VEGF to tumor vasculature in a xenograft model of prostate cancer [153], to bone metastases [154], and to an orthotopic model of human testicular cancer [156].

4. Conclusion

The discovery of siRNA as an efficient tool for selective inhibition of gene expression has revolutionized the molecular biology research and pharmaceutical research for validation of potential gene targets in cancer therapy. Concurrently, intensive efforts have been made for tumor-targeted *in vivo* delivery of siRNAs by different routes (intratumoral, intravenous, intraperitoneal and intranasal) using lipid-, polymer-, peptide- and protein-based nanomaterials, with the consequence of tumor regression in preclinical trials with cancer models. Several clinical trials are underway, with siRNA therapeutics targeting the M2 subunit of ribonucleotide reductase, VEGF and kinesin spindle protein [152,157]. However, there are still challenges to minimize the siRNA-mediated off-target effects and immune stimulation. Moreover, in most of the *in vivo* siRNA delivery studies, pharmacokinetic analysis has been ignored and therefore the efficacy level of a delivery system for tumor-directed delivery of siRNA has not been evaluated properly.

5. Expert opinion

Cancer is a complex disease arising from several genetic changes in the same cell over a period of time. Although the classical anticancer drugs have multiple targets and can therefore kill cancer cells effectively, normal healthy cells are also highly vulnerable to those cytotoxic effects because of the ability of the cancer drugs to escape blood capillaries immediately after the injection and subsequently penetrate the cancer cells as well as the normal cells through passive diffusion. Treatment of cancer with siRNA therapeutics has clear advantages over conventional chemotherapy drugs for treating a variety of cancers partly because siRNAs, being anionic, can easily be complexed with cationic lipids, polymers or peptides, thus restricting their passive diffusion across the blood capillaries and cell membrances. By controlling the sizes of the siRNA complexes, selective transport of the complexes across the endothelial gaps of tumor vasculature is feasible as a result of the enhanced permeability and retention effect [158]. More specific delivery to the tumor tissue could be done by coating the surface of the complex with a tumor-specific ligand along with a hydrophilic molecule in order to prevent nonspecific interactions with blood components and normal cell membrane. On the other hand, the traditional cancer drugs are mostly hydrophobic and small in size, presenting a physical barrier for stable association with the existing carriers. Moreover, unlike the cancer drugs, siRNA can precisely block a protein function by silencing the respective gene, and a combined delivery of multiple siRNAs can simultaneously silence a group of genes that are responsible for a particular cancer.

The principal obstacle to siRNA delivery into the tumor of an immunocompromised animal is the inefficieny of the current devices at transporting a sufficient amount of siRNA into the cytoplasm of most of the tumor cells; and the intravenous route should be the best option for siRNA delivery study in cancer models considering the applicability in future clinical trials. However, most of the studies performed until now have used the intratumoral, intranasal, or intraperitoneal route, probably because delivery through any of these routes has fewer barriers compared with the intravenous route. siRNA that survives in the plasma following intravenous injection in carrier-associated form must extravasate through the tight vascular endothelial junctions and subsequently diffuse through the dense extracellular matrix consisting of fibrous proteins and carbohydrates surrounding a cell before being internalized by the cell through endocytosis [159]. Also, the post-delivery effects of siRNAs have not shown sustainable growth inhibition of tumors, demonstrating that more intensive efforts should be made to establish the key regulatory genes critically responsible for cancer development, and to develop a

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superior nanocarrier system for tumor-specific delivery of the siRNAs for targeted cleavage of those genes. Moreover, to avoid the off-target effects of siRNA as a result of hybridization with unwanted target mRNA sequence(s) and the induction of interferon responses through either double-stranded RNA-activated protein kinase [160] or toll-like receptor 3 [161], siRNA should be designed and validated perfectly following the synthesis so that it can precisely recognize the target mRNA without stimulating an immune response.

Declaration of interest

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