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APPLICATION OF RNAI TO CANCER RESEARCH AND THERAPY

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1. ABSTRACT

The introduction of double-stranded RNA (dsRNA) into cells can effectively and specifically lead to the degradation of corresponding mRNAs in a gene-dependent manner, which is defined as RNA interfering (RNAi). This powerful technology has been widely employed to manipulate gene expression in mammalian and human cells, elucidate signal pathways and identify gene functions in a whole-genome scale. Simultaneously, many pharmaceutical companies are very interested in the research and development of RNAi-based drugs for various diseases, especially in cancers. In present review, we attempt to recapitulate the potential application of this breakthrough technology in many aspects of cancer gene therapy such as genome-scale screens, target identification and validation, functional analysis and animal models for diverse diseases.

2. INTRODUCTION

Cancers are a group of genomic functional disease with the features of cancer gene activation, tumor suppressor inactivation and stability gene dysfunction (1). Oncogene activation can be related to point mutations, oncogene amplification, or gene fusions (1-3). Numerous alterations in these cancer-susceptibility genes are involved in different stages of tumor genesis such as tumor initiation, progression, angiogenesis and metastasis (4). Over the past decades, investigations of the molecular mechanisms of cancer have revealed a number of oncogenes (5). With the tremendous efforts in identification, cloning, sequencing and functional analysis (6-8) of oncogenes, novel preventive and therapeutic strategies are being developed to conquer cancers. Of them, RNA-mediated gene regulation system is a powerful gene-silencing arm within cells (9). This approach has been used to identify genes critical for the growth, differentiation and death of cells [10], to protect cells from invasion of viruses (11-13), to inhibit cancer genesis (6, 14-16), and to investigate molecular mechanisms of other diseases (6, 9). Naturally, when these genomic functional RNA molecules are used as targets, small fragments of their cognate sequences can be one of the most ideal drugs. The drug discovery based on this approach will have huge potentials to facilitate the identification and characterization of specific target mRNAs for cancers with unique modes of action, lower the cost of research and development, and make a personalized drug possible in the future. In fact, new types of gene drugs such as antisense oligonucleotides (17-19), ribozymes (20-22), 2',5'-A oligonucleotides (23-24), and siRNA duplexes (25-29) are converging a powerful arm to overcome genomic functional disorders such as different cancers.

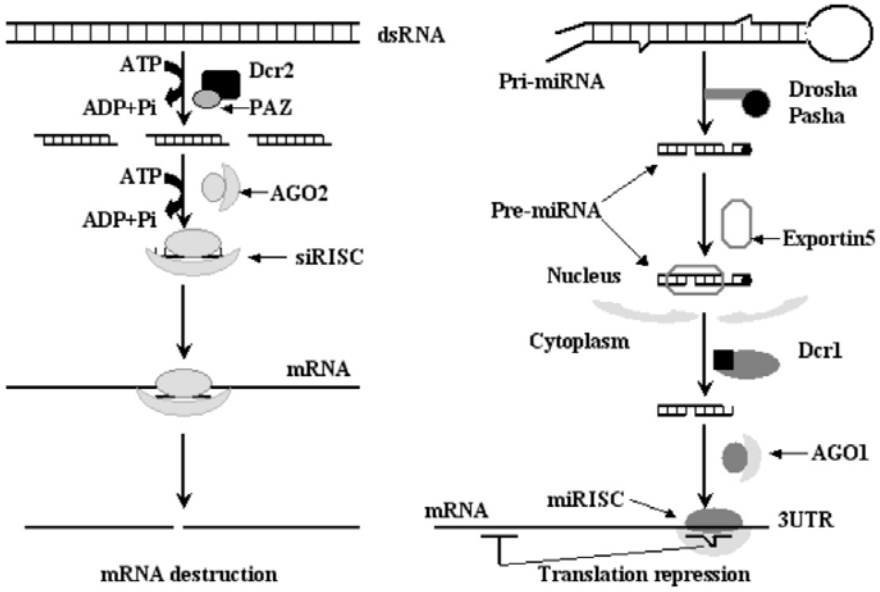

Figure 1. The molecular mechanisms of RNAi. Two small RNAs, siRNAs and miRNAs are processed from long dsRNA and stem loop precursors by Dicer, and then are incorporated into RISCs for the guidance of mRNA cleavage and translation repression.

A number of experimental studies have made it clear that different types of mRNAs can be efficiently, specifically and rapidly inactivated by powerful RNAi technology. Using stable production of siRNAs in numerous cell lines, Paddison and his colleagues (30) have demonstrated that continuous expression of dsRNA can induce stable homology-dependent suppression of the target gene. Phenotypic changes induced by complete knock-down of specific sequences can be used as reliable indications for genomic functional study and new gene identification. If over-expression of oncogenes is involved in uncontrolled growth and proliferation of cells, the specific suppression of oncogene activities can reverse the phenotype of tumor cells and control their growth and proliferation. This notion has been tested by a number of experiments. It has been discovered that the long-term and complete silencing of oncogenes in tumor cells may be a new and powerful way for the prevention and treatment of cancer. Researchers worldwide have used RNAi for functional analysis and target validation (15-16,25,29). In the resent couple years, RNAi has been put into the creation of different classes of potential therapeutic agents in cancer therapy, and it has promised an excellent model for the prevention and treatment of human cancer (7).

3. RNAi MACHINERY

It has been well studied how the length of dsRNA influences the silencing of homologous target RNAs (26). There are two small RNAs in the RNAi pathway: small interfering RNAs (siRNAs) and microRNAs (miRNAs) that are generated via processing of longer dsRNA and stem loop precursors (Figure 1) (6,28,31). Dicer enzymes play a critical role in the formation of these two effectors of RNAi (31). They can cleave long dsRNAs and stem loop precursors into siRNAs and miRNAs in an ATP-dependent manner, respectively. Dicer is a member of the RNase III family of dsRNA-specific ribonucleases with ~200kD and multiple domains including an N-terminal DEXH-box RNA helicase domain, a domain of unknown function, a PAZ domain, one or two dsRNA binding domain, two RNaseIII catalytic endonuclease domains (7,32-33). In fly cells, there are two clearly different Dicers: DCR1 is vital in the production of mature miRNAs, DCR2 is the major enzyme in the generation of siRNAs. Now it is clear that siRNA is transferred from Dicer to RNA-induced silencing complex (RISC) through the bridge mediated by R2D2 (34). R2D2 is a protein associated with DCR-2. It binds nascent siRNA and facilitates its loading onto RISC.

The main component of miRNP and RISC is Argonaute protein. It is a 100-kD highly basic protein that includes two common domains such as PAZ and PIWI domains (35-37). The PAZ domain, consisting of 130 amino acids, has been identified in Argonaute and Dicer proteins. Several lines of evidence have revealed that PAZ is involved in specifically recognizing and binding 2-nucleotide (nt) 3_ overhang and the base-paired terminus of siRNA duplexes characteristic of RNaseIII processing. The PIWI domain is at the C terminus of Argonaute (residues 545 to 770), whose fold is similar to RNase H, with two conserved active-site residues. Its domain core also has a tertiary structure belonging to the RNase H family of enzymes. Recent results indicate that Argonaute contributes "Slicer" activity to RISC, providing the catalytic engine for RNAi. Studies on the four different Ago complexes furthermore demonstrate that only Ago2 is related to target RNA cleavage (38-40). Both endogenous siRNAs and transfected synthetic siRNAs enter Ago2 to guide target RNA cleavage. In Drosophila, Argonaute2 (AGO2) is identified as a part of the siRISC complex, and Argonaute1 (AGO1) is required for the biogenesis of mature miRNA (41).

Table 1. Comparison of RNAi and other methods.

Parameters	RNAi	Other strategies
Target site selection Efficiency	Easier Only substoichiometric amounts	Difficult Higher dose
Specificity	Higher	Low to high
Stability	Long-term expression of vector-driven small RNAs	Transitory and generally no more than 24h
Cost	High	Low to high
Safety	Natural RNA chemistry	Induction of many side-effects

This table summarizes the advantages of RNAi compare to other RNA-based or DNA-based therapies. Other strategies such as antisense oligodeoxyribonucleic acids [53], ribozymes [54], peptide nucleic acids[55], morpholino phosphorodiamidates[56], DNAzymes [57] and RNAi used for inhibiting gene expression by sequence-specific targeting of mRNAs have been developed in the hope of creating therapeutic agents.

In addition, many other proteins such as Exportin-5 and Pasha participate in miRNA maturation and miRNA-directed gene regulation (Figure 1). Exportin-5 is responsible for exporting pre-miRNAs and short hairpin RNAs from the nucleus (42-43). Pasha, a partner of Drosha, can bind a double-stranded RNA (28). So these proteins also are important for processing pri-miRNA into mature miRNA, and necessary for miRNP suppressing activity (44-45).

4. MOLECULAR MECHANISMS

Preliminary studies have showed that siRNAs are 21- to 26-nucleotide dsRNA duplexes with 2-nucleotide 3′ overhangs and 5′ phosphate and 3′ hydroxyl termini (17). 21-22 nt, has been implicated in mRNA degradation, and a longer size class, 24-26 nt, in directing DNA methylation and in systemic silencing. A component of RISC, the PAZ domain of Argonaute, facilitates recognition of the siRNAs with 3' single-stranded overhangs generated by the Dicer enzyme. It has been shown that duplex siRNAs are incorporated in RISC precursors, and then ATP-dependent unwinding of siRNAs converts RISC precursors into active RISC. The ratio of RISC containing the antisense or sense strands of siRNAs is determined by the thermodynamic stability of the 5′ terminal base pairs of siRNA duplex (25). Bases near the 5′ end contribute to target RNA-binding energy, whereas base pairs formed by the central and 3′ regions of siRNAs provide a helical geometry required for catalysis (24). The PIWI domain of Argonaute in RISC is similar to ribonuclease H, with a conserved aspartate-aspartate-glutamate motif. The phosphate between nucleotides 11 and 12 from the 5'end of the mRNA falls near the active cleavage center of RISC (40-41).

Mature miRNAs are endogenous ~22-nt RNAs. They are important to guide mRNAs for cleavage or translational repression in animals and plants (44-46). These short RNA molecules are generated in cytoplasmic Rnase III Dicer from the hairpin-shaped pre-miRNAs that are processed by nuclear Rnase III Drosha (45-46). The first 2-8 residues of miRNAs should perfectly pair to the 3′ untranslated region (UTR) elements of target RNAs (47). Endogenous siRNAs and miRNAs are so similar that these two classes of RNAs cannot be distinguished by either their chemical composition or mechanisms of action. In mammals, siRNAs can function as miRNAs by repressing expression of target mRNAs with partial complementarity to multiple sites of 3′ UTR (48). The 5′ region of siRNAs and miRNAs all plays analogical roles in target recognition and the binding of RISC to RNA targets (26, 46, 48). However, distinctions on their origin, evolutionary conservation, and types of genes that they silence have been elucidated clearly (48-50).

The potential of RNAi in functional genomics has already been demonstrated (51). RNAi technology has become a preferred approach for target identification and validation as well as disease prevention and therapy (52). In comparison with other RNA-base therapies, RNAi has many advantages shown in the table 1. It is out of question that this powerful technology will exercise great influences on cancer therapy.

5. RNAi In Target Discovery

With the complete sequence of the human genome in hand, researchers can identify almost all genes that are responsible for biological processes (58). The long-term expression of small RNAs makes it possible to generate stable mammalian cells carrying specific sets of inactive genes. In this way, specific function can be assigned to human cancer-susceptibility genes, and cancer–specific molecular targets can be identified. Therefore, RNAi-based gene drugs can be developed.

5.1. RNAi in tumor initiation and progression

Cells with cancerous lesions often resemble their normal counterparts except for difference in the presence of a series of genetic alterations (3). RNAi has become a loss-of-function genetic screen tool that is employed to identify gene function through inactivation of corresponding mRNA and elucidate molecular mechanisms of tumor genesis and progression. It has been widely accepted that gatekeeping mutations of replication-competent cells give rise to tumorigenesis. However, subsequent mutations are also necessary for tumor progression (1). There are many genes involved in the tumorigensis and progression. They can be divided into the following three classes: cellular oncongenes (*c-onc*), viral oncogenes (*v-onc*), tumor-suppressor genes (TSGs).

5.1.1. *c-onc*

Cellular oncogenes are normal cellular genes with the potential to aid cell growth and tumor formation. Numerous proteins such as growth factors and their receptors, signal transducers and transcription factors associated with tumor initiation and progression are encoded by *c-onc*. R*as* genes are frequently mutated in human cancers, particularly in pancreatic and colon carcinoma. The proteins (K-RAS, H-RAS, and N-RAS) encoded by *ras* genes are guanine nucleotide binding proteins that are associated with the inner plasma membrane and transduce external signals to the interior of cells. The down-regulation of K-RAS protein by *RNAi* in human pancreatic cell line CAPAN-1 leads to loss of anchorage-independent growth and tumorigenesis (59). In 2003, Yin *et al.* successfully transfected HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, and melanoma cells by using single siRNA or combinatorial siRNAs with cationic lipid complexes. The results showed that these siRNAs could not only specifically knockdown their cognate targets such as *bcl-2*, *cdk-2*, *mdm-2*, *pkc-α*, *tgf-β*1, *H-ras*, *vegf*, and *gfp* mRNAs, but also effectively suppressed the proliferation of cancer cells to different extents (60). So, it is no exaggeration to say that all *c-onc* might become potential molecular targets for human cancer treatment by the wide application of RNAi.

5.1.2. *v-onc*

Many DNA and RNA viruses have been shown to induce malignant transformation. These viral genomes can integrate randomly into the host chromosomal DNA. When they are cloned and transfected into normal cells in culture, the cells will undergo malignant transformation. Hepatitis B virus (HBV) infection substantially increases the risk of chronic liver disease and hepatocellular carcinoma in humans. By cotransfecting with plasmids expressing short hairpin RNAs (shRNAs) homologous to HBV mRNAs in cultured cells and mammalian liver, McCaffrey *et al.* (61)showed that HBV replication initiation was effectively inhibited. Beside viral mRNAs, viral oncogenes are usually employed as the target to treat cancers. Human papillomavirus (HPV) is a causative agent of cervical cancers with *E6* and *E7 v-onc*. Yoshinouchi *et al*. (62) demonstrated that SiHa cells treated with *E6* siRNAs formed the tumors significantly smaller than in those treated with control siRNAs in NOD/SCID mice. Moreover, E6 was defined as a most promising therapeutic target to eliminate HPV-positive tumour cells (63). These results showed that either viral mRNAs or single viral oncogene could be selected as candidates for viral-related cancers.

5.1.3. Tumour suppressor genes

Tumour suppressor genes (TSGs) inhibit cell proliferation and tumor formation. Loss-of-function or mutation of TSGs drives normal cells toward cancer. Retinoblastoma (Rb) gene is the prototype of this category. The somatic inactivation of Rb gene leads to tumor growth. By using RNAi technology, Dimova *et al.* (64) detected a new program of dE2F/*RBF*- dependent transcription, in which dE2F2/RBF complexes repressed oncogene expression in SL2 cells. Similarly, the *INK4A* locus has also been studied by RNAi. It is often inactivated in human cancer. *INK4A* encodes p14ARF and p16INK4A that inhibit cell growth through *p53* and *pRb*, respectively. Voorhoeve and Agami (15) firstly delineated the function of the human *INK4A* genes in normal and tumorigenic growth. They showed that the suppression of p16INK4A expression did not affect cell proliferation but promoted their growth and transformation with *p53* loss.

Taken together, the knowledge of the functions and molecular mechanisms of tumor initiation and progression can not only permit us to search more targets for cancer treatment, but also direct us to prevent cancers. The identification of gatekeeping mutations will provide more insights into the tumorigenesis.

5.2. RNAi in cancer genome stability

It is well known that genetic, chromosomal or/and telomere instabilities are characteristics of most human cancers and one of the most active research areas in cancer biology (1). DNA damage is a relatively common event in the life of normal cells, which can induce several cellular responses leading to mutation, cancer, and cell or organism death (64). Recent reports indicated that RNAi could improve the efficacy of chemotherapy and radiotherapy by blocking transcription factors implicated in aberrant signaling pathways triggered by DNA damage (65-66).

rad-51 gene is a DNA repair gene. Its mutations often confer on yeast cells enhanced sensitivity to genotoxic agents, reduction in mitotic recombination, and impaired meiosis. Upon the suppression of *rad-51* by the RNAi method, Takanami *et al.* (67)found that *rad51* played some roles after both endogenous and exogenous double-strand breaks (DSB) formation during meiosis, but not as 'pairing centers' for meiotic synapsis. If DSB was prevented by *spo-11* or *mre-11* mutation, *rad-51* RNAi did not affect chromosome appearance. However, *rad-51* silencing in oocytes that lack MSH-5 led to chromosome fragmentation (68). This is a novel trait distinct from that seen in *msh-5* mutants and in *rad-51* RNAi oocytes, indicating new potential roles for the *msh-5* gene.

Chromosomal DNA degradation is a key point in cell death execution and a hallmark of apoptosis. Although there are many potential molecular targets for cancer therapy in this aspect, yet little is known about its molecular mechanisms. Recently, scientists in University of Colorado have identified seven additional cell death-related nucleases by utilizing RNAi-based functional genomic approach (69). Among these nucleases, CPS-6 and NUC-1 comprise at least two independent pathways that contribute to cell death provoked by degrading chromosomal DNA.

Besides these genes, others involved in the genome stability were also studied (70,71). The utility of RNAi could not only elucidate the mechanism of genome instability, but also explore some molecular targets for clinic application. It is promising a new efficient and potential strategy to treat cancer.

5.3. RNAi in tumor-cell invasion and metastasis

Tumor cells possess a broad spectrum of invasion and metastasis mechanisms. These include both single cell locomotion and cohort migration strategies (72). The knowledge of the cellular and molecular basis of tumor cells invasion and metastasis will help us develop new strategies to treat cancer.

Endogenous CXC chemokine receptor-4 (CXCR4) gene expression is significantly up-regulated in the highly invasive MDA-MB-231 human breast cancer cells. The high expression of CXCR4 may play a role in the tissue preferential metastasis of breast cancer cells. Using siRNAs expression system, Chen *et al* (73) showed that inducible knockdown of endogenous *CXCR4* gene expression in breast cancer cells resulted in significant inhibition of breast cancer cell migration *in vitro*.

Matrix metalloproteases (MMP)-9 and cathepsin B have been shown to participate in the processes of tumor invasion and metastasis of gliomas by influencing matrix degradation. It was revealed that when *MMP-9* and *cathepsin B* gene expression was suppressed by hairpin RNAs in SNB19 cells, the cell-cell interaction of human microvascular endothelial cells was significantly reduced (74). Direct intratumoral injections of plasmid DNA expressing hairpin RNAs for *MMP-9* and *cathepsin B* significantly inhibited established glioma tumor growth and invasion *in vivo*. Further intraperitoneal injections completely regressed pre-established tumors for almost 4 months, showing no indication of these tumor cells (75). It is the first to demonstrate that the simultaneous RNAi-mediated suppression of *MMP-9* and *cathepsin B* genes is potential for the treatment of human gliomas.

Tumor-cell invasion and metastasis was the most difficult problem in conquering the cancer. However, RNAi-based approach is a very useful tool for suppressing the invasion and metastasis of cancers for a long time (almost 4 month). It is optimism when the whole process of tumor invasion and metastasis was delineated by RNAi and other methods, the cure of cancer is not far from us.

5.4. RNAi in tumor angiogenesis

Tumor angiogenesis is mediated by the balance change between angiogenesis factors and angiogenesis inhibitors (76). For tumor cells, the appropriate alteration between positive and negative controls resulted in the constant growth of new tumor blood vessels.

It is known that histone deacetylase inhibitors (HADC) can suppress angiogenesis of tumors and RECK belongs to HADC. RECK is a membrane-anchored glycoprotein that negatively regulates matrix metalloproteinases (MMPs) and inhibits tumor metastasis and angiogenesis. Recently, Liu *et al.* (76)found that inhibition of RECK by siRNAs abolished the inhibitory effect of trichostatin A (TSA) on MMP-2 activation. This study revealed a detailed mechanism by which HDACs suppress tumor invasion and angiogenesis and provided a new strategy for cancer therapy.

Another good example is Tie-2 that is a small molecule inhibitor of endothelial cell specific tyrosine kinases. When Tie-2 signaling is interrupted via RNAi, the viability of endothelial cells lost, even in the presence of serum. Investigation indicated that this was linked to the blocking of Akt signal pathway and related to an increase in thrombospondin expression (77). Thrombospondins are endogenous anti-angiogenic proteins known to regulate tumor growth and angiogenesis. These data demonstrated that angiopoietin signaling through Tie-2 activated PI3Kinase/Akt, which in turn repressed thrombospondin expression. Thus, the blocking of Tie-2 signal pathway in endothelial cells might be efficacious against angiogenesis.

5.5. RNAi in tumor cell apoptosis

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of apoptosis. Evading apoptosis is therefore a prerequisite for tumor formation, and the ability of cancer cells to disrupt this process can be considered as its lifeline (3,78). Naturally, apoptosis research is also a pop field in cancer therapy.

The *livin* (*ML-IAP*, *KIAP*) gene expressed in a subset of human tumors has been demonstrated as antiapoptotic factors. The inhibition of its function may provide a rational basis for the development of novel therapeutic strategies. Butz's team designed a vector-based *livin*-siRNAs plasmid to silence endogenous *livin* gene expression. They discovered that the silencing of livin was associated with caspase-3 activation and a strongly increased apoptotic rate in response to different proapoptotic stimuli (79). Their work provided direct evidence that the *livin* gene could be taken as a promising molecular target for cancer therapy.

Bcl-2 or xIAP are apoptosis suppressors that may be responsible for resistance to apoptosis induced by cytotoxic drugs. The down-regulation of bcl-2 or xIAP by RNA interference (RNAi) could sensitize MCF-7 cells to etoposide and doxorubicin (80). Both siRNAs reduced the number of viable cells and increased cellular apoptosis. Treatment with siRNAs following etoposide or doxorubicin further reduced the number of viable cells, when compared to either of the treatments alone. Therefore, the study describes an attractive diagram of how RNAi worked with the traditional chemotherapeutic agents and inspires a new combined cancer treatment strategy.

Though acquisition of the ability to evade apoptosis is one of the master switches that contribute to cellular transformation and ultimately to invasive cancer. The identification and validation of targets for apoptosis-based cancer therapy have been a great challenge. As a breakthrough technology, RNAi is an emerging strategy to understand how these abilities of cancer cells interfere with the treatment of cancers.

5.6. RNAi in the tumor immunology

The magnitude and specificity of immune responses to pathogenic organisms have inspired ways to aim at patients' own metastatic cancer (81). With the

Table 2. Potential targets for cancer therapy.

Class	Target	Cell system	References
Genome stability	RAD51	Oocyte	68
	ERCC1	COS-7	150
	ERCC2	Drosophila S2 cell	151
	ATR	NBS1 and FA	71
Tumor initiation and progression	K-RAS	CAPAN-1	59
	EGFR	A431	152
	C-MYC	COS-1	153
	HBV mRNA	HuH-7	61
	E6	SiHa	62
	Rb	SL2	64
	INK4A	MCF-7, HeLa	15
	Mdm-2	MCF-7, HCT116	94
Tumor-cell invasion and metastasis	CXCR-4	MDA-MB-231	73
	FOS	HCT116	154
	MMP-9	SNB19	74
	EphA2	PANC1	155
Tumor angiogenesis	RECK	CL-1	75
	Tie-2	Endothelial cells	77
Tumor-cell apoptosis	Livin	HeLa, MeWo, H1299	79
	Bcl-2, XIAP	MCF-7	80
Tumor immunology	IL-6	(animals)	82
	PKB	Hep3B	83

RNAi is an effective and fast tool for loss-of-function study. Based on large-scale genetic screens, more and more valuable targets have been identified, and new therapeutics might be developed upon these discoveries. This table showed only a small part of targets involved in cancer gene therapy.

knowledge of basic theory of tumor immunology, researches have increased their abilities in manipulating host immunity and specifying target tumor cells for immune destruction.

Gliomas are the most common primary brain tumors in humans. Owing to their diffuse infiltrative nature, most gliomas are not curable by surgery alone. Their obstinacy to radio- and chemotherapy makes them extraordinarily lethal. Treatment modalities based on a sketchy conception of the mechanisms that drive gliomagenesis have not changed markedly for decades. Recently, Weissenberger *et al* (82)validated the functional role of cytokine interleukin-6 (IL-6) in gliomagenesis. They found that the ablation of IL-6 by RNAi prevented tumor formation in animals with spontaneous astrocytoma, but did not affect preneoplastic astrogliosis. Their results indicated the important role of IL-6 in the development and malignant progression of astrocytomas and provided a clue to delineate the precise mechanism of gliomagenesis.

Tumor immunotherapy to date is focused on the priming of immune response to fight cancer. In addition, the issue of immune tolerance should be considered when developing an immune-based strategy for cancer. Transforming growth factor β (TGF-β) that is known to down-regulate immune function and favor tumor escape has been studied. TGF-β has a major role in cell proliferation, differentiation and apoptosis in many cell types. Remy *et al.* (83)reported a new mechanism for the integration of signals arising from growth-factor- and TGF-beta-mediated pathways, mediated by a physical interaction between the serine-threonine kinase PKB (protein kinase B)/Akt and the transcriptional activator Smad3 by applying RNAi. PKB inhibited Smad3 by preventing its phosphorylation, resulting in a decrease in Smad3-mediated transcription and the protection of cells against TGF-beta-induced apoptosis (84). Consistently, the knockdown of the endogenous *PKB* gene with siRNAs had the opposite effect.

Rapid progress in understanding the role of host immune system by RNAi will ultimately impact on cancer therapy strategies. RNAi technology might be combined with clinic strategies for systemic immune stimulation to improve and enhance the scope and efficacy of cancer gene therapy. It is trustfully that the employment of RNAi will accelerate the basic and clinic research of tumor biology and disclose more molecular target for the treatment of cancer (Table2).

5.7. Multidrug resistance of cancer therapy

Multidrug resistance (MDR) is a cross-resistant phenomenon concerning the ability of human cancer cells to resist several unrelated drugs with wide differences in molecular structure and target specificity. Resistance to antineoplastic drugs may take the main charge of why traditional chemotherapy-based treatment of malignant tumors failed (85).

P-glycoprotein (P-gp), a member of the superfamily of ATP binding cassette (ABC) transporters over-expressed in tumor cells is the main cause conferring MDR phenotype. Recently, two siRNAs were designed to

two regions of the P-gp-encoding mRNA to treat human pancreatic carcinoma (EPP85-181RDB) and gastric carcinoma (EPG85- 257RDB) cells. The results showed that the resistance against daunorubicin was decreased to 89% (EPP85-181RDB) or 58% (EPG85-257RDB) (86). It was also true for K562 cells with MDR (87). These data indicated that RNAi might be used for the treatment of patients with MDR tumors as a specific means.

Besides P-gp, environmental factors, such as the extracellular matrix, tumor vasculature or tumor geometry, might be involved in drug resistance. They could impair drug delivery systems, result in poor absorption of orally administered drugs, increase drug metabolism and excretion, and reduce the diffusion of drugs from blood into tumor mass. Much remains to be learned about the drug resistance and its role in clinical oncology. Multiple mechanisms such as increase of drug efflux or decrease of influx by transporters, activation of detoxifying systems, activation of DNA repair systems and evasion of apoptosis that may operate singly or in concert are believed to be involved in drug resistance. It is certain that the working mechanisms of MDR may be elucidated by RNAi in the near future. Meanwhile, *MDR* gene sequences can be integrated into gene therapy vectors with potential clinical application.

5.8. RNAi in tumor signal pathway

The tumor signal pathways are apparent different from those of normal cells. Many cancer-susceptibility genes can interference normal signal pathway to get tumorigenesis and progression. To identify signaling pathway components is a potential way to discover new therapy targets for human cancers (88-90). The clearest pathways identified by RNAi include Hedgehog pathway, Fas-induced apoptosis pathway and *p53* signal pathway (90-92,94).

The secreted signal protein, Hedgehog (Hh), elicits cellular proliferation and differentiation responses during normal embryonic development. The inappropriate activation of this pathway contributes to tumorigenesis (90). Beachy' group synthesized dsRNA molecules corresponding to roughly 5700 genes, and then revved down the Hedgehog pathway in cultured cells for identifying whether the pathway needed a specific protein to function. Two gene products were identified as Hh pathway components. One of them, casein kinase 1alpha, may be a tumor suppressor that normally blunts the pathway's activity in humans (95).

Fas-induced apoptosis is implicated in a broad spectrum of cancers. The best model is liver failure that is a dramatic clinical syndrome with high tumorigenesis rates. In the process of liver failure, signals released from the cell membrane of hepatocytes trigger suicide pathways, leading to the activation of caspase cascades that subsequently execute apoptotic death of hepatocytes. *In vivo* silencing *Fas* gene protects mice from liver failure and fibrosis in autoimmune hepatitis by intravenous injection of *Fas* siRNAs (91). In addition, the down-regulation of *caspase* 8 gene expressions by siRNAs protects hepatocytes from acute liver damage induced by agonistic Fas antibody or by adenovirus expressing Fas ligand (92). Taken together, these data throw lights into the application of RNAi in classifying the Fas-induced apoptosis pathway.

Probably, the most frequent genetic abnormality in human cancer is mutations in *p53*. Over 90% of small-cell lung cancers, and about 50% of breast and colon cancers have been shown to be associated with mutations in p53 pathway. Many oncogenes are involved the *p53* signal pathway. The activation of the *p53* pathway has been investigated by eliminating the p53-hdm2 interaction through the degradation of *hdm2* mRNA molecules. *Hdm2* silencing directly resulted in enhanced MCF-7 cell apoptosis and decreased cell proliferation (91). Recently, Berns *et al.* (93)developed a set of retroviral vectors encoding 23,742 shRNAs targeting 7,914 different human genes for stable suppression. They identified one known and five new molecules of p53 signal pathway by applying this RNAi library. These gene silencing enhanced resistance to p53-dependent proliferation arrests. Obviously, RNAi may greatly facilitate the identification of components of cellular signal pathways. With this technology, researchers will identify more targets in a specific and rapid manner, and step out in cancer therapy research.

5.9. RNAi in large-scale genetic screens

Large-scale genetic screens have also been widely used in the target discovery of cancer treatment. Some laboratories have utilized the RNA libraries to identify genes in cell invasion (96,97). The penetration of RNAi in the large-scale genetic screens permits researchers to identify and validate more molecular targets efficiently. As RNAi was defined in *C. elegans* (10), large-scale genetic screens by this technology were also first used in nematode. Fraser and his colleagues used RNAi to target nearly 90% of predicted genes on *C. elegans* chromosome I. They assigned function to 13.9% of the genes analyzed, and increased the number of genes with known phenotypes on chromosome I (98). Furthermore, RNAi was also used in large-scale genetic screens of genes involved in fat regulation, longevity and genome stability in *C. elegans* (99,101-102).

Large-scale genetic screens by RNAi were quickly employed in cancer therapy. In 2003, Brummelkamp *et al.* (102)designed RNAi vectors to suppress 50 human de-ubiquitinating enzymes, and used these vectors to identify de-ubiquitinating enzymes in cancer-relevant pathways. They firstly reported that the familial cylindromatosis tumour suppressor gene (CYLD) could enhance the activation of transcription factor NF-kappaB. Another noteworthy work in this field was finished by Aza-Blanc and his colleagues (103). They synthesized a group of siRNAs targeting 510 human kinases for identifying genes involved in TRAIL-induced apoptosis in HeLa cells. They uncovered that DOBI and MIRSA were modulators of TRAIL-induced apoptosis pathway. At the same time, their data also suggested that the MYC and WNT pathways play biological roles in maintaining the susceptibility to TRAIL, and provided

some insights on how TRAIL components mediate the selective killing of tumor cells.

cDNA microarray is a effective tool that was usually used in RNAi-based large-scale genetic screens. It has been used to investigate systematically genes that involved in cancer cells. Zhou *et al.* (104)constructed a 23k human cDNA microarray to screen TNFα-inducible genes in HeLa cells to investigate the stimulation effects of TNFα and the role of NF-κB in regulating this response. Inflammatory, proapoptotic, and antiapoptotic genes regulated by NF-κB were also shown by using RNAi screens. In addition, RNAi-based cDNA microarrays were employed to profile gene expression changes in colorectal tumors, identify new targets and design approaches for the management of this disease. Several over-expressed genes were disrupted by RNAi in a colon tumor cell line. The results showed similar patterns of gene expression as those in the patient tumors (105). The combined use of RNAi and cDNA microarray analysis provides an excellent system for large-scale genetic screens to define gene function *in vitro* and *in vivo as well as* an effective and fast way to discover more molecular targets for cancer treatment.

Although not yet used in the whole genome, screens for other types of genetic interaction should prove useful in cancer therapy (90). Of course, a whole genome-wide screen typically includes thousands of genes. A unique molecular bar-code should be included for each gene to allow highly parallel screens to be done in one culture vessel. The bar-code system, developed in studies of yeast (106), was successfully introduced into RNAi-based system screens by Paddison *et al.* (107)and Berns *et al.* (93) Berns *et al.* used the shRNA sequence itself, while Paddison *et al.* employed an independent bar-code by linking a unique 60-nuclotide DNA bar code to each shRNA vector. Bar-code system remains to be in its infancy but has great potential for analyzing RNAi selection screens (108). The improvement of bar-code in the RNAi-based large-scale libraries will become a valuable resource for function analysis and target discovery.

5.10. miRNAs and cancers

Up to now, endogenously expressed siRNAs have not been found in mammals. However, another small RNAs, miRNAs, have been identified from various organisms and cell types (109-111). Accumulated evidence has showed that miRNAs can abnormally express in several kinds of human cancers include chronic lymphocytic leukemia (112), colorectal neoplasia (113) and Burkitt's lymphoma (114). A several lines investigation indicated that human miRNAs usually located at fragile sites and genomic locus involved in cancers (115).

Because miRNA expression is restricted to specific environmental conditions, some miRNAs might be missed in cloning efforts (7-8). Fortunately, with the improvement of bioinformatics tools, people have developed many methods to fill up the gap of experiment approaches to miRNAs identification. Several miRNAs-finding approaches have been developed on the basis of homology and proximity to known genes (116) while some other approaches are also established by scoring candidate miRNA stem loops and pairing known miRNAs genes (110, 117). By applying these methods, it is estimated that there are over 175 miRNAs in human beings (118).

After the establishment of miRNA-finding, the next difficult step is to validate their mRNA targets. Most miRNAs regulate gene expression by base pairing with miRNA-recognition elements (MREs) found in their mRNA targets and direct either target RNA degradation or translational repression (8). Accordingly, the computational identification of mRNA targets was dependent on by the complementarity between miRNAs and their mRNA targets[119,120]. However, most animal miRNAs are thought to recognize their mRNA targets by partial complementarity (8). Recently, Kiriakidou *et al.* (121) developed a combined computational-experimental approach to predict human miRNAs targets by the incorporation of rules derived from experiments that guide single miRNA:MREs recognition in computational algorithms.

Recently, Liu *et al*. (96) identified and selected a total of 281 miRNA precursor sequences by using oligonucleotide microchip. It was not only for the detection of the pattern of miRNAs expression, but also for expression quantification. Accordingly, this approach could be utilized to decipher the miRNAs expression in normal or disease states and disclose new pathogenetic pathways in human tumorigenesis.

Small RNAs have permeated through almost all aspects of cancer treatment. The absorbability of RNAi in this field will recognize more targets for the prevention and treatment of diseases while the exploration of miRNAs in cancer-susceptibility genes will provide another decipherment for cancer evolution and another feasible strategy for cancer therapy.

6. RNAi IN DRUG DESIGN OF CANCER THERAPY

Small RNAs are such a powerful tool that it can be applied to solve the most cureless diseases such as HIV and cancer in human society. Nowadays, it is very difficult to find one biopharmacological company without interests in this technology. Small RNAs are now being utilized for therapeutic purposes (Figure 2).

6.1. Design of small RNAs

Although RNAi has become a preferred modality for loss-of-function studies, there are some drawbacks of this technology. Jackson *et al.* (122) revealed that some siRNA-specific signatures led to the silencing of non-target genes. Transfection of siRNAs and shRNA vectors could trigger an interferon response (123-124). By the employment of genechip, Persengiev *et al.* (125) showed that more than 1000 genes involved in diverse cellular functions were nonspecifically stimulated or repressed in a dose-dependent manner. In addition, certain siRNAs might induce an innate immune response, silence chromatin,

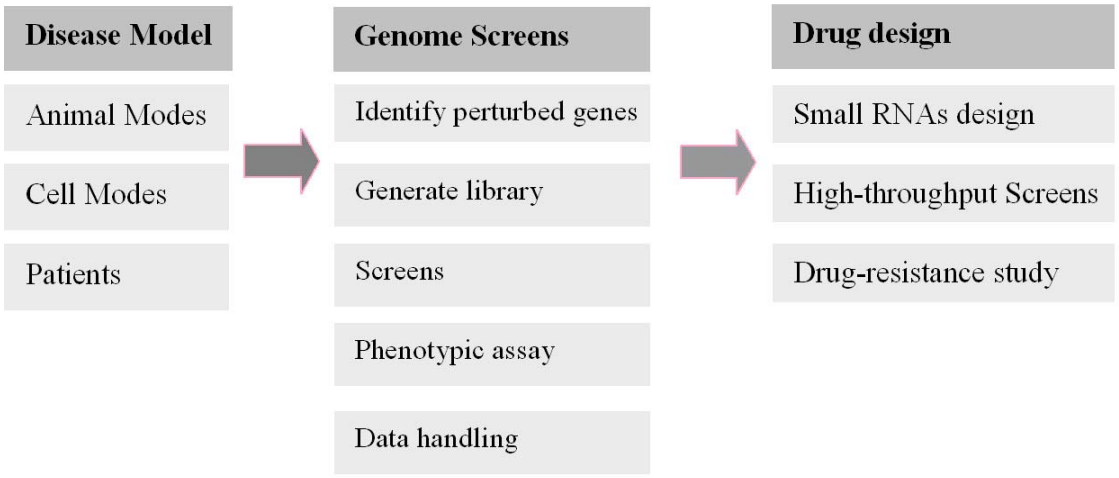

Figure 2. There are three aspects of application in RNAi-based cancer therapy. Firstly, RNAi is used in the study of disease models and underlying mechanisms. Secondly, RNAi-based genome screens are employed to identify and validate targets for cancer therapy. Thirdly, siRNAs are developed for the drugs to treat cancers.

display poor tissue distribution, and lead to false conclusions on end points. Obviously, it is essential to test the specificity of a siRNA prior to a full phenotypic analysis and the further clinical application of RNAi (126). To this end, researchers have developed many approaches so that the specificity of RNAi technology can be ensured (127-129). So, effective and specific small RNAs should be carefully designed and selected.

The secondary structure of mRNA target greatly influences the efficiency of small RNAs silencing. On the basis of the analyses of a small number of target genes, several groups proposed a set of guidelines to design small RNAs (107, 130-131). The guidelines were based on sequence composition, G/C content, thermodynamic flexibility and some other standard parameters for selecting effective small RNAs (130). Bioinformatics were also employed to small RNAs design. Cui *et al.* (132) developed a computational tool, OptiRNAi, which used the criteria proposed by Elbashir *et al.* (130) to predict appropriate target sequences for siRNAs production. Nowadays, many websites provide the service to pick siRNAs, but the veracity is not very high. Our laboratory has applied a patent to design siRNAs. The success rate of gene silencing is more than 90%.

To optimize the effectiveness of RNAi-based gene therapy *in vivo*, the following parameters should be considered: stability of siRNAs within body, bioavailability to different tissues, affinity to the blood proteins, and specific delivery to the given position. So, the chemical modifications are necessary in the increasing stability of small RNAs while maintaining good silencing efficiency (133). Generally, siRNAs tolerate mutations in the 5' end, while the 3' end exhibit low tolerance. The blockade of the 5'-hydroxyl terminus of antisense strand leads to a dramatic loss of silencing activity, whereas the alteration of the 3' terminus had no negative effect. It was shown that phosphorothioate modifications and 2'-O-methylation and 2'-O-allylation could stabilize the persistence of siRNAs silencing (134). 2'-O-methyl modifications at specific positions in siRNAs protected the siRNA molecules against serum-derived nucleases and improved the stability of siRNAs in serum (135). Collectively, the chemical modifications of siRNAs may extend the longevity of RNAi and make them more suitable for therapeutic application *in vivo*.

6.2. High-throughput Screens of small RNAs

Based on the large-scale genetic screens, plentiful molecular targets have been revealed. Simultaneously, with the employment and improvement of the combinatorial chemistry, thousands of new chemical entities (NCE) can be synthesized at one time. The numerous targets and NCE make the high-throughput screens (HTS) become vital in the application of RNAi-based drugs to clinic trails.

HTS has been widely conducted by the technologies such as 96- or 384-well plate formats, cationic transfection, robotics and automated imaging in mammalian tissue culture cells (136). HTS for small RNAs have been developed by using siRNAs spotted on glass slides and reverse transfection in order to allow for rapid screening of mammalian cells (137). Most recently, Hannon' team described the use of shRNA-based live-cell microarray that allowed HTS of phenotypes caused by the silencing of small RNAs. In their experiments, mammalian cells were cultured in a 384-well plate and then transfected with different shRNAs. Trasfection mixes were spotted on GAPS II glass. After incubation with medium, groups of transfected cells could be detected inside the spotted transfection mixes (138). These microarray studies might ultimately be utilized to filter effective small RNA drugs for clinic experiments.

At the present, the screen efficiency was greatly improved by the substituted HTS for ultra-high throughput screens (UHTS) which used 1536-well plates on the whole in industry (139). The appearance of system biology and the application of computational biology have increased the screen quality enormously, and the conception of high-

Table 3. Approaches for generation of siRNAs, and vectors for RNA interference

Approaches		Vectors	
In vitro	Chemical synthesis	DNA-vector-mediated RNAi	U6-promotor-based vector
	In vitro transcription		H1-promotor-based vector
	RNase III digestion of dsRNAs		7SK-promotor-based vector
In vivo	siRNA expression vector	Virus-vector-mediated RNAi	Oncoretrovirus vectors
			Lentivirus vectors
			Adenovirus vectors
	PCR expression cassette		Adeno-associated viral vectors

content screens (HCS) is in process of shaping (140). The advance of HTS might be used in the screens of small RNA drugs to boost the therapy of cancer.

6.3. Delivery of small RNAs

RNAi-based drugs have displayed a bright and fascinating future in the cancer therapy. Many methods have been developed to generate and delivery small RNAs (Table 3). Recombinant RNAi vectors could not only transfect dividing but also non-dividing mammalian cells with a long-term expression (59). However, how to deliver *in vivo* small RNAs effectively remains to be the main obstacles of the clinic applications. In laboratory, two *in vivo* delivery approaches have been established for animal experiments.

6.3.1. Electroporation

Electroporation is a rapid and convenient method for both gain- and loss-of function studies *in vivo* and *in vitro*. By using electroporation of plasmids encoding shRNAs against double cortin gene (*DCX*), Bai *et al.* (141)successfully down-regulated the DCX protein of rat neocortex *in utero*. Electroporation is very efficient and stable. The expression of genes can even persist for a long time. When plasmid DNA carrying retinal cell type-specific promoters was introduced into the retina *in vivo* by high-voltage pulses, transfected GFP exhibited an appropriate expression pattern with little damage to the operated pups, where the expression persisted for more than 50 days (142).

6.3.2. Injection

Along with the rapid development on eletroporation, there is emerging evidence that both local and systemic injections may represent a novel therapeutic modality for cancer treatment. Local injection has displayed its potency in the therapy of neural diseases. When *TH*-shRNAs were injected into 9-week-old C57BL/6J mice, a substantial reduction in tyrosine hydroxylase was observed in *TH*-shRNAs-infected cells of the substantia nigra compacta 12 days after the injection (143). Upon injection, recombinant adeno-associated virus (AAV) vectors expressing shRNAs profoundly improved motor coordination restored cerebellar morphology and resolved characteristic ataxin-1 inclusions in Purkinje cells of spinocerebellar ataxia type 1 (SCA1) mice (144). Systemic injection is more promising than local injection in the application of gene therapy. High-pressure tail vein injection, a kind of systemic injection, was the first procedure to successfully deliver small RNAs into highly vascularized mouse tissue, causing up to 90% reduction in target gene expression in the liver and, to a lesser extent, in the lung, kidney, spleen and pancreas (145). Intravenous injection of *Fas*-siRNA via hydrodynamic tail vein specifically down-regulated the expression level of Fas protein in mouse hepatocytes. The effects persisted without diminution for 10 days (91). On the other hand, the gene silencing caused by injection is transient and the levels of gene suppression are different because there is significant animal-to-animal variation and some other uncertain factors (146) Accordingly, it is very important to develop effective delivery systems of siRNA.

7. RNAi IN THE ANIMAL MODELS OF CANCER

Cancers are a group of diseases with uncertain mechanisms. RNAi could be used not only in the therapy of cancers, but also in the basic research of cancers with model animals and animal models. Many model animals such as nematode, drosophila, zebrafish and mouse have been developed over the past decades. They have made an indelible contribution to the exploitation of cancers. With the better understanding of comparative medicine, animal models that have similar phenotypes for various diseases are playing more and more important roles in cancer therapy (147). At present, RNAi has been employed into the establishment of animal models to mimic tumorigenesis and development.

It has been found that the tumor suppressor p53 is up-regulated in a wide variety of transformed cells. It can inhibit cell growth through activation of cell cycle arrest and apoptosis. Most cancers lack active p53 protein that promotes apoptosis in response to hyperproliferative signals, suggesting a therapeutic intervention. Hemann et al. (148) investigated whether RNAi applied to p53 could stably suppress gene expression in hematopoietic stem cells and produce detectable phenotypes in mice. Deletion of the p53 tumor suppressor gene by p53-shRNAs greatly accelerated Myc-induced lymphomagenesis, resulting in highly disseminated disease. In addition, intrinsic differences between individual shRNAs expression vectors targeting the same gene might be used to create an epi-allelic series for dissecting gene function in vivo.

Taken together, RNAi can stably suppress gene expression in stem cells and animals, and produce various phenotypes. The application of this technology to investigating molecular mechanisms of tumor initiation and progression will greatly benefit the cancer therapy.

8. PERSPECTIVE

As the most excited discovery over the past two decades, RNAi has exhibited its bright future in the

research and therapy of cancers. For applying RNAi to clinic therapeutics, some side-affects are under correction by the elucidating of RNAi mechanisms. It's worth mentioning that the preliminary phase I/II clinical trial, which targeted Ras oncogene with anti-sense directed therapeutics, has failed. The enhanced delivery methods and potential gene therapy applications of RNAi need to be further and deeply investigated (149). Large-scale genetic screens to identify genes involved in tumorigenesis and progression, and seek miRNAs/siRNA for cell proliferation and differentiation as well as development of long-circulation vectors to specifically deliver RNAi-based agents are essential steps in RNAi-based cancer therapy. Therefore, it is beyond all doubt that the improvement of this landmark technology will play a critical role in the process of cancer treatment.

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