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Use of RNA interference technology for cancer specific gene silencing

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Abstract

RNA interference (RNAi) can be applied to human cancer cells by using small interfering RNAs (siRNAs) for inhibition of activated oncogenes' expression with the amelioration of several malignant phenotypes such as uncontrolled cell growth, resistance to apoptosis or invasive ability. siRNAs with high specificity to active oncogenes (e.g. missense mutation, gene rearrangement or over-expression) could be alternatives for molecular target drugs although several issues such as *in vivo* siRNA delivery methods, off-target effects, interferon responses require to be solved.

Key Words: RNA interference, siRNA, cancer gene therapy

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Introduction

RNA interference (RNAi) is a mechanism of posttranscriptional gene silencing followed by an introduction of double-stranded RNA (dsRNA) into cells, in which specific mRNAs homologous to the introduced dsRNAs are degraded. Although RNAi was originally discovered in Caenorhabditis elegans (*C. elegans*)[1], this mechanism was found to be conserved in a wide variety of species, including insects, plants and mammalian cells including a human. Since RNAi utilizes the endogenous mechanism for mRNA degradation, the efficacy of posttranscriptional gene silencing is much superior to other methods such as antisense technology or ribozymes. However, application of RNAi to mammalian cells appeared to be difficult due to a global gene silencing caused by interferon responses. In 2001, Tuschl *et al*. reported that synthetic 21-nt siRNAs could specifically inhibit target gene expressions without affecting irrelevant gene expressions[2]. Since then, many applications of RNAi to mammalian cells have emerged, in which many activated oncogenes in cancer cells were considered as the targets for siRNAs. In this review, I would like to focus on the basic mechanism, delivery methods, factors determining RNAi effects, potential problems upon application of RNAi, and to introduce previous application of RNAi to cancer gene therapy.

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Mechanism of RNAi

After the introduction into cells, long dsRNAs are cleaved in the cytoplasm by Dicer, a family of RNAse III, yielding duplexes of about 21-23 nt in length called small interfering RNA (siRNA). siRNA has a characteristic 2-3 nt overhangs in its 3'-ends and 5'-phosphates[3]. After the duplex of siRNA is unwound, siRNA is incorporated into a protein complex called RNA-induced silencing complex (RISC), and the antisense strand in siRNA guides RISC to a target mRNA, resulting in the cleavage of the target mRNA[4]. In plants or *C. elegans*, RNA-dependent RNA polymerase (RdRP) produces new dsRNAs between a target mRNA as a template and an antisense strand of siRNA as a primer[5]. The new dsRNA will enter another cycle of RNAi, and the RNAi effects will continue and expand through the tissues. However, RdRP dose not exist in mammalian cells or Drosophila[6].

RNAi in mammalian cells

Although the mechanism of RNAi is conserved among species, dsRNAs longer than 30nt will activate interferon responses in mammalian cells, resulting in the induction of dsRNA-dependent PKR and 2'-5'-oligoadenylate synthase (2-5 AS). The activated PKR phosphorylates eIF2α, leading to the inhibition of protein translation. 2-5 AS produces 2-5 A that activates RNase L, leading to the non-specific degradation of mRNAs[7]. Hence, under this condition, specific posttranscriptional gene silencing effects by RNAi cannot be observed.

In 2001, Tuschl *et al.* reported that synthetic 21-nt siRNAs could specifically suppress the homologous genes without inducing toxic interferon responses[2]. From this discovery, extensive application of RNAi to mammalian cells has emerged. In 2002, several groups reported siRNA expression vector systems including plasmid[8-14] and viral vectors[15-19]. Most of the siRNA expression vectors utilize RNA polymerase III (Pol III) promoters such as U6, H1 or tRNA. Two different siRNA expression vector systems have been reported. One is a tandem type, in which two Pol III promoters transcribe sense and antisense RNAs independently. Then, the transcribed RNAs will hybridize within the cells, making an siRNA. Another system is a hairpin type, in which one Pol III promoter transcribes a sense-linker-antisense RNA. After the transcription, the RNA will make a fold-back stem loop structure with a linker sequence as a loop, and with the sense and antisense RNAs as a stem. This short hairpin RNA (shRNA) will be then exported from nucleus to cytoplasm, and the linker sequence will be degraded by Dicer, making an siRNA. In high copy numbers, both types of vectors work equivalently; however, in low copy numbers, a hairpin type is superior to a tandem type, probably due to the higher efficiency in the formation of dsRNAs within one RNA molecule. In fact, lentiviral vectors, that can express transgenes integrated from a viral genome within the host genome (referred to as "provirus"), have very limited copy numbers of transgenes within the cell. Hence, tandem types of lentiviral siRNA vectors do not work while hairpin types of lentiviral siRNA vectors work efficiently[19].

Parameters for RNAi effects

Two important parameters are required for the efficacy of siRNAs. The first one is a copy number of siRNAs introduced into cells. In general, higher copy numbers result in better effects. The second is the selection of target sequences. Many algorithms predicting the efficacy of siRNAs have been developed based on the systematic analysis of siRNAs targeting certain mRNAs (Reynolds *et al.,* 2004; Yoshinari *et al.,* 2004). Several important features for effective RNAi are, 1) low G/C content (30-50%), 2) base preferences at sense strand; A or U at position 19, U at position 10, and 3) absence of internal repeats. The predicting algorithms for RNAi effects combining many parameters probably reflect strand selection during siRNA-RISC assembly and activation, which determines the overall efficiency of RNAi.

Specificity or RNAi

Several groups have reported successful targeting of siRNAs specific for the mRNAs with only one base difference[8,20,21]. Alleles with point mutations occurring in several oncogenes such as K-ras^{V12} or BRAF^{V599E}, were specifically suppressed with siRNAs having the mutant nucleotide located around the central positions. Although siRNAs have such stringent specificity, some reports indicated that siRNAs could also suppress some mRNAs with partial homology to siRNAs, based on cDNA microarray profile of the cells transfected with siRNAs. The suppressed genes had homology with nine nucleotides at the 5'-ends of antisense strands of siRNAs. This so-called "off-target effect" requires special consideration for the application of siRNAs. Multiple siRNAs with different target sequences for one gene should be simultaneously examined for the phenotypic changes to exclude the possible off-target effects.

Table 1 siRNA targets against cancer.

Cancer	*siRNA target*	*Cellular function*
CML	bcr-abl	tyrosine kinase
Leukemia	bcl-2	anti-apoptotis
Melanoma	BRAF^{V599E}	MAPKKK
Small-cell lung cancer	Skp2	ubiquitin ligase
Prostate cancer	PI3KC α, β	PI3-kinase catalytic subunit
Colon cancer	β-catenin	Wnt signaling
	stat-3	transcription factor
Cervical cancer	E6, E7	HPV oncogene
Pancreas cancer	K-ras^{V12}	small GTP-binding protein

Interferon responses with siRNAs

siRNAs with 21-23 nt were originally considered to induce RNAi without toxic interferon responses. However, Sledz *et al.* reported that several interferon-related genes could be induced even with siRNAs, without any toxic effects[22]. The mechanism of this interferon responses with siRNAs has not been fully revealed. Several chemical modifications or introduction of mutations in the sense strands of siRNAs could suppress such interferon responses (personal communication).

Application of siRNAs to cancer gene therapy

There have been several reports targeting at activated oncogenes in cancers with RNAi strategy (Table 1). These activated oncogenes could be specific molecular targets relating with the malignant phenotype of cancers. RNAi against them resulted in the amelioration of uncontrolled cell growth, celluar invasive ability, resistance to chemotherapeutic agents or apoptosis. For instance, point mutation-specific siRNAs such as K-ras^{V12} [8] or BRAF^{V599E} [21], rearranged oncogenes such as bcr-abl fusion mRNAs[23], or over-expressed genes such as Skp2[24], could become specific and safe targets of siRNAs without affecting normal tissues.

Delivery systems of siRNAs

There are several *in vitro* delivery methods of siRNAs; tranfection of siRNAs or shRNA plasmid vectors by using cationic liposomes or by electroporation, and infection of shRNA viral vectors (retroviral, lentiviral, adenoviral, adeno-associated). For *in vivo* delivery, systemic targeting methods are very limited. Most of the present viral vector systems could be applied only locally. Hydrodynamic transfection method for introduction of siRNA to livers revealed to be effective[25]. Recently, researchers from Alnylam Co. reported that systemic administration of chemically modified siRNAs could successfully result in the specific inhibition of the target genes in the organs[26]. However, this delivery system is not tissue-specific, and it requires a large amount of modified siRNAs with high costs. At present, the development of effective delivery systems is a major obstacle to siRNA therapeutics.

Conclusions

There are many issues to be overcome for the clinical applications of siRNAs, including safety such as off-target effects and interferon responses, delivery methods and acquired resistance. Numerous works are now ongoing to solve these issues, and therapeutic application of siRNAs is quite promising and may become real in near future.

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