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Commentary



Ge-tu Zhaori, *professor of pediatrics. He is currently the editor-in-chief of Chinese Medical Journal English Edition, deputy editor-in-chief of Chinese Journal of Pediatrics and a member of the Editorial Board of The New England Journal of Medicine (N Engl J Med, since 2007). His fields of interest include infectious diseases, pediatrics, pediatric infectious diseases, clinical virology, and certain molecular biologic techniques.*

Editor's note: *The conversion of basic research innovation to clinical application is limited by multiple factors, of which the safety is extremely important for both patients and researchers. Based on literature searching, the author takes the RNA interference (RNAi) technique in clinical application as an example to address the safety issues in transferring of basic discoveries into clinical trials, which is a rigorous scientific project in translational medicine research. .*

More studies on safety issues are needed for clinical application of RNA interference technique

Ge-tu Zhaori *

The Editorial Office, Chinese Medical Journal English Edition, Chinese Medical Association, Beijing 100710, China

It is exciting to see published articles on phase I and II clinical studies on RNA interference (RNAi), a technique that can selectively (sequence-specifically) silence a targeted gene, for application in treatment of a few human diseases although the technique is believed to have the potential of being applied for thousands of various diseases. Publication of the original articles on 6 clinical trials on 4 potential RNAi products signals that this fascinating gene-silencing technique is very close to clinical application and therefore, clinicians will soon have some entirely new weapons in the battles against diseases like malignant tumors, viral infections, certain age-related degenerative diseases and potentially many diseases that are associated with mutated or over-expressed genes. This commentary aimed to have an overview on the current status of studies on safety issues of RNAi technique, based on literature published in international medical journals, to try to understand the status of safety issues in clinical as well as animal in vivo studies, and to make certain suggestions for safe application of the technique in humans.

Safety is extremely important to any new therapeutic agents that is clinically used for treatment of human diseases. Without confirmed safety, even a very effective therapy should never be used clinically, and especially for the first-in-human studies, even though for small-scale phase I or II trials, extremely cautious considerations should be made before the trial is initiated. Clinicians need to understand what safety concerns exist for a new type of therapeutic agent, from what aspects of an agent the safety problem arises, how the safety issues have been studied, and if there are sufficient evidences showing the safety of a new agent before it is administered to patients, so that the patients safety is ensured

* Correspondence: Ge-tu Zhaori E－mail: zhaorig@126.com

without causing any unnecessary harm to the clinical trial participants as well as millions of patients who would be treated with the new agents.

1 Few reports on studies focusing on safety of RNAi were available on a literature search

A literature search at PubMed by using the search terms "RNAi OR siRNA AND treatment AND in vivo" with limits including "recent 5 years", "Enlish language", "journal articles" retrieved 1739 articles. While "safety" was added into search terms preceded by "AND", 31 articles were obtained. It was found after reading abstracts of each of these articles that 18 were original articles and 13 were review articles. It was further found that in only 3 of the 18 original articles safety issues were substantial components of the study while in all the remaining 15 articles, the word "safety" only appeared once or twice, safety issues were not a major part of the studies. These results clearly show that most of the published studies on RNAi did not concentrate on the safety issues of this extremely important technique that potentially applicable in clinical treatment of various diseases. Reading abstracts of a part of the 1739 articles on RNAi suggested that most of the articles did not focus on or even did not mention safety issues of the siRNAs or their vectors or delivery systems.

2 Possible factors associated with safety of RNAi

RNAi is a sequence-specific targeted gene silencing technique that applies synthesized small or short interfering RNA (siRNA) or a vector that can express a short hairpin RNA (shRNA) which can finally be converted into siRNA which is in sequence complementary to the messenger RNA (mRNA) of the target gene. Specifically designed siRNAs, if delivered into cells where the target gene's mRNA exists, can bind to the target sequence and interfere with the mRNA expression and thereby silence the gene. For treatment of some diseases, the siRNA may be directly injected into the tissues, while for many other diseases, certain vector or delivery system are needed to administer the siRNA systematically or via other routes to the target organ or tissues or cells where the siRNAs can be released to act with the target mRNA. The vectors or delivering systems can be classified into viral and non-viral, the latter include cationic polymers, cationic lipoplexes and/or liposomes, nanoparticles[1-3] and even bacteria (nonpathogenic)[4], etc. The safety concerns arise from both siRNA itself and its vector or delivery system.

Safety concerns from siRNA itself are mainly associated with seed-sequence (the small region of the small RNA that mediates target binding) -dependent off-target effects and activation of innate cellular immune response (gamma-interferon response). The former is related to design of siRNA, if certain particular sequences are avoided for the siRNA, such off-target effect can be prevented from occurring, but attention to the safety of an siRNA should be paid at preclinical cellular studies for possible cytotoxic effect or functional disturbance[5].

On the other hand, siRNAs, although may exist in normal human body at a very low amount, do not result in serious problems. However, those given at high doses for prolonged time, may activate innate immune response since the immune system may "regard" them as foreign RNAs. Expressed shRNA is believed to be able to prevent the activation of the innate immune response. Nevertheless, it was pointed out that abundant shRNA expression can be toxic, and that high level expression of shRNA using U6 promoter proved to be lethal in about half of mice tested because of saturation of the endogenous silencing pathway. Using a weaker H1 promoter or in the case of liver-targeted delivery, a pol-II promoter, may prevent the toxic effect[5,6].

The molecular sizes, structure and other features of vectors or delivery system for siRNA are associated with safety issues. The most frequently studied viral vectors include nonpathogenic adenovirus, lentivirus, etc. Viral vectors have the advantages of certain cell-specificity, highly efficient expression of the shRNA in cells after infecting the cells, and the expression of shRNA can be prolonged and stable. However, there are still concerns on pathogenicity although the viruses used as vectors for RNAi are genetically modified to eliminate their pathogenicity. Activation of innate and acquired immune response is another major concern, which, if develops, may reduce the efficacy and efficiency of delivery, can be toxic, may induce inadvertent gene expression changes following random integration into the host genome[7] and possibly cause problems associated with immune complex or unexpected cell-mediated immune response or inflammatory reactions.

Nonviral delivery systems may also have some safety concerns although they are believed to be safer as compared with viral vectors. Akhtar and Hollins suggest that cationic lipids and cationic polymers can directly induce changes in gene expression that might have an impact on siRNA activity and specificity[8,9]. Polyethylenimine (PEI) was demonstrated to be safe and effective in deliv-

ery of siRNA[10]. However, its branched form induced gene changes in vivo and had toxicity [7,8]. Systemic administration of lipidprotamine-DNA nanoparticles, once proved to be a good delivery system for siRNA, was found to increase various inflammatory or immune cytokines, including interleukins 6, 12 and interferon-gamma dose-dependently. [11,12]

3 Safety issues associated with the published clinical studies

A literature search for clinical trials on RNAi at PubMed could retrieve 45 articles, but after reading the abstracts of all these articles, only 6 of them were found to be original articles published in journals reporting clinical trials. These articles were on 6 clinical trials of 4 potential siRNA products designed to silence genes for treatment of 4 diseases (Tab. 1), i. e., respiratory syncytial virus (RSV) infection[13-15], neovascular age-related macular degeneration[16], an inherited skin disorder known as *pachyonychia congenita* (congenitally abnormal thickening of the fingernails or toenails)[17] and brain tumor (glioblastoma multiforme)[18]. The authors of these articles should be congratulated for their successes in treatment of these diseases by using the completely new therapeutic approaches although the studies are very preliminary phase I or phase II clinical trials. Two of the diseases, RSV infection and the brain tumor, can often be severe enough to threaten the patients life, and there are no satisfactory treatments for both diseases as well as the other 2 diseases studied. These studies demonstrated that the siRNAs were effective and safe, well tolerated, without causing any serious adverse events. The successful knockdown of the target genes could significantly reduce or alleviate symptoms and signs, prevented or reduce occurrence of some of the diseases, and improved the patients conditions. However, all the clinical trials except for study No. 3 listed in table 1 seemed not to have sufficiently concentrated on safety of the siRNAs. Interestingly, all the 6 clinical trials applied the siRNAs locally, either by inhalation or by injection, i. e., the therapeutic agents used were naked siRNAs, and none of the trials used any kind of vector or delivery system. Therefore, there was no chance to evaluate safety and efficacy of any vector or delivery system in human subjects.

When the author tried to track retrospectively the preclinical safety studies on the potential siRNA products listed in table 1, no published systematic safety studies, neither in vivo, nor in vitro, were found for most of the 6 studies. Part of the possible reasons may be that the siRNAs had different names, therefore, when only the acronyms listed in the table 1 were used as search terms, some of the published articles might have been missed. But it seems to be clear that there are limited safety studies not only on the 4 clinically tested potential siRNA products, but also on numerous potentially applicable siRNAs that are in the process of research and development. In addition to predictable safety problems, a new therapeutic agent may result in absolutely unpredicted severe and life-threatening adverse events even the candidate product was well tested in different species of animals,

including non-human primates. The "cytokine storm" caused by a monoclonal antibody prepared for direct activation of regulatory T cells in a phase I clinical trial unexpectedly resulted in refractory shock and multiple organ system failure [19]. This is an astonishing and striking example of unexpected severe adverse effect of a study drug seen in a first-in-human trial. The lesson from this trial is extremely important and meaningful. In the preclinical studies of the test drug, a temporary lymphocytosis was observed; in contrast, severe lymphopenia was seen in the 6 healthy volunteers after administration of the test drug. The authors of the report pointed out that this meant that entirely opposite phenomenon may occur in preclinical and clinical studies, which makes estimation over safety of a study drug more difficult and complicated. However, such a striking difference in safety-related phenomena may be related with the genetic difference between humans and the species of animals that were chosen for the preclinical studies. [19] If we look at the animal species used in preclinical studies on the 4 potential products and many other siRNAs in development, we would find that most were conducted in small animals (rats and mice). Use of animals that are closer to human being in genetics should be seriously considered. Table 2 may provide some relevant information concerning the similarities of gene DNA sequences between non-human organisms and humans[20].

Large mammals have rarely been used for siRNA studies although Tang et al have made a good paradigm with rhesus macaque model and found that siRNA at larger or smaller doses did not show any sign of siRNA induced toxicity [21].

Tab. 1 Basic information and safety issues related to siRNA preparations in published clinical trial reports

No.	Author name and reference	No. of case #	Acronym of siRNA	Target of the siRNA	Expected therapeutic effect	Vector	Method of delivery	Description of safety	Published preclinical study on safety
1	Zamora MR [13]	16	ALN-RSV01	Respiratory syncytial virus (RSV) N protein gene	Reduce or prevent development of RSV infection in lung transplant patients	None, Aerosol	Inhalation	Well tolerated, with no drug-related serious adverse events or post-inhalation perturbations in lung function	Not available
2	DeVincenzo J [14]	43	Ditto	Ditto	Ditto	None	Intranasal spray	Well tolerated. AEs were similar in nature and severity in groups, few moderate and no serious AEs occurred	Ditto
3	DeVincenzo J [15]	101	Ditto	Ditto	Ditto	None	Ditto	Well tolerated, safety profile similar to saline placebo	Ditto
4	Kaiser PK [16]	26	Sirna-027	VEGFR-1	Improve neovascular age-related macular degeneration	None	Local (intravitreal) injection	No dose-response or dose-limiting effects were noted	Not available, 2 animal studies did not include safety
5	Leachman SA[17]	1	TD101	keratin 6a (K6a) N171K mutant mRNA	Regression of callus	None	Local (intradermal) injection	No adverse events occurred during the trial	None of 5 available references was about safety
6	Wyszko E[18]	46	ATN-RNA	tenascin-C, an extracellular matrix glycoprotein *	Delayed growth of tumor or lack of its recurrence symptoms, improvement of overall survival	None	Local (intratumoral) injection	Not mentioned	Not available

Note: # The numbers listed in this column are those of the cases who received the siRNA. * This glycoprotein contributes to tumor cell adhesion, invasion, migration and proliferation. RSV denotes respiratory; VEGFR-1 denotes vascular endothelial growth factor receptor-1; AE denotes adverse event.

Tab. 2 DNA sequence similarities between non-human organisms and humans

Non-human organisms	Proportion of gene DNA sequence identical to human	Fields of use in previous studies
Banana	50%	NDA
Fruit fly	60%	NDA
Mice	~85%	Gene transfer
Pigs	71%	Induced diseases, cardiovascular disease and arthitis
Dogs	75%	NDA
Cattle	83% *	NDA
Rabbit	(All but "Y" chromosome paints)	NDA
Chimpanzee	98.8%	NDA

Note: This table was made according to the data from the reference 20 and the website www.genome.gov. NDA denotes no data available.

4 Suggestions for safety issues related to siRNA or its vectors or delivery system

Since safety issues are extremely important for research and development of any types of novel therapeutic agents for protecting the subjects who participate in clinical trials and the patients who would possibly receive treatment with the new therapeutic agents, there should be relatively strict rules and regulations for initiation of first-in-human clinical trials. The rules and regulations should be mainly science-based. The preclinical safety studies should be comprehensive, with sufficient transparency, and the results should be published in peer-reviewed journals. The preclinical safety studies should include acute, subacute and chronic toxicity tests, carcinogenicity, teratogenicity, toxicity on reproductivity, immunotoxicity, and should be safe in children of different age groups. There should be requirements for animal species, especially requirements for safety study in non-human primates.

In conclusion, for clinical application of the RNAi technique, safety should always be emphasized. Strict rules and regulations on safety of new therapeutic agents, in this context, of siRNA or other agents for RNAi may slow down the processes of research, development and approval by authorities on one hand, but may better protect the health and wellbeing of clinical trial participants and millions of patients on the other hand.

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