RNAimmuno: A database of the nonspecific immunological effects of RNA interference and microRNA reagents

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ABSTRACT

The RNAimmuno database was created to provide easy access to information regarding the nonspecific effects generated in cells by RNA interference triggers and microRNA regulators. Various RNAi and microRNA reagents, which differ in length and structure, often cause non-sequence-specific immune responses, in addition to triggering the intended sequence-specific effects. The activation of the cellular sensors of foreign RNA or DNA may lead to the induction of type I interferon and proinflammatory cytokine release. Subsequent changes in the cellular transcriptome and proteome may result in adverse effects, including cell death during therapeutic treatments or the misinterpretation of experimental results in research applications. The manually curated RNAimmuno database gathers the majority of the published data regarding the immunological side effects that are caused in investigated cell lines, tissues, and model organisms by different reagents. The database is accessible at <http://rnaimmuno.ibch.poznan.pl> and may be helpful in the further application and development of RNAi- and microRNA-based technologies.

Keywords: RNA interference; nonspecific effects; toxic effects; siRNA; interferon response

INTRODUCTION

RNA interference (RNAi) technology takes advantage of the natural pathways and protein factors to silence selected genes with the use of various exogenous reagents (Olejniczak et al. 2010). The RNAi process is efficiently triggered by short interfering RNAs (siRNAs) (Caplen et al. 2001; Elbashir et al. 2001; Yang et al. 2001; Kim et al. 2005; Amarzguoui et al. 2006), synthetic (McManus et al. 2002; Siolas et al. 2005) or vector-based short hairpin RNAs (shRNAs) (Paddison et al. 2002; Boudreau et al. 2008), or pri-miRNA-based expression cassettes (sh-miRs) (Zeng et al. 2002; Silva et al. 2005; Chang et al. 2006).

Deregulation of the cellular miRNA level has been shown to be associated with different types of diseases, including cancer. Therefore, the use of the spectrum of miRNA-based tools (Olejniczak et al. 2010) may allow for the increase of underexpressed miRNAs (e.g., miRNA mimetics) (Tsuda et al. 2009) or the decrease of overexpressed miRNAs (e.g., anti-miRs [Krützfeldt et al. 2005], miRNA sponges [Ebert et al. 2007; Ebert and Sharp 2010], decoys [Haraguchi et al.

2009], or target protectors [Choi et al. 2007]) in tumor tissues.

The tools of RNAi and microRNA technologies have been used successfully both in basic research to study the functions of specific proteins and miRNAs and in the experimental treatment of many human diseases. However, in addition to triggering sequence-specific effects, the reagents used in both technologies often stimulate cellular sensors of foreign RNA and DNA, which recognize patterns associated with pathogens (pathogen-associated molecular patterns, PAMPs) (Fig. 1; Marques et al. 2006; Reynolds et al. 2006; Robbins et al. 2008, 2009; Olejniczak et al. 2010). This effect depends mostly on the reagent length, structure, chemical modification, concentration, and cellular localization rather than on its specific sequence, which may be involved in off-target effects (Jackson et al. 2003; Fedorov et al. 2006). The first identified cellular sensors of foreign RNA were the IFN-inducible dsRNA-activated protein kinase (PKR) (Roberts et al. 1976) and 2'-5'-oligoadenylate synthetase (OAS) (Farrell et al. 1978). It was reported that the full activation and dimerization of PKR is undertaken after binding ~33 bp of helical RNA, whereas PKR monomers are able to bind 16–18 bp of RNA (Manche et al. 1992; Bevilacqua and Cech 1996; Zhang et al. 2001). Additionally, blunt-ended dsRNAs with 5'-triphosphate were shown to activate PKR (Nallagatla et al. 2007). Activa-

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FIGURE 1. Reagents of RNAi and miRNA technologies delivered to cells either by transfection or released from expression vectors may be recognized by specific cellular sensors of foreign RNA and DNA. After the activation of cytoplasmic (RIG-I, MDA5, PKR, and OAS1-3) and endosomal (TLR3, 7, and 8) sensors, a signal is transduced through specific adaptor proteins to transcription factors which, in turn, stimulate production of proinflammatory cytokines and IFN. The activation of other sensors may induce cell death by pyroptosis (AIM2, NLRP3). TLR3 localization (denoted by asterisk) is cell type-dependent.

tion of the PKR and OAS signaling pathways by long dsRNA results in the general inhibition of protein synthesis and the degradation of cellular RNA, respectively. The other cytoplasmic sensors of foreign RNA in human cells are two helicases, retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene-5 (MDA5). RIG-I recognizes blunt-ended dsRNA molecules with a 5' triphosphate (5' ppp) that are over 20 bp long (Schlee and Hartmann 2010), and MDA5 is activated by long dsRNA (Kang et al. 2002). Widely used reagent carriers composed of cationic lipids localize RNA to endosomal compartments, where it can be recognized by Toll-like receptors (TLR3, 7, and 8) (Alexopoulou et al. 2001; Heil et al. 2004; Gantier and Williams 2009). Some of these receptors are sensitive to specific RNA sequences; however, chemical modification of these immunostimulatory motifs may abrogate this effect (Sioud 2009). The other group of cellular sensors (TLR9, AIM2, and ZBP1) recognizes foreign DNA that has been introduced into cells either as a plasmid or through a viral vector. Less is known about the NLRP3 (Kanneganti et al. 2006) and NOD2 (Sabbah et al. 2009) sensors that belong to the nucleotide oligomerization domain (NOD)-like family of proteins (NOD-like receptors, NLRs) and recognize dsRNA and ssRNA, respectively.

The activation of cytoplasmic or endosomal sensors results in signal transduction through specific adaptor proteins to transcription factors, which, in turn, stimulate production of proinflammatory cytokines and interferons (IFNs). IFNs regulate the transcription of ~2000 genes in an IFN subtype-,

dose-, cell type-, and stimulus-dependent manner (Samarajiwa et al. 2009). Changes in the cellular transcriptome and proteome, however, can lead to the inhibition of cell division and growth and eventually to apoptosis. In research applications, an additional undesired effect may be the misinterpretation of experimental results (Robbins et al. 2008). Because the data regarding the nonspecific effects of RNAi and miRNA technology are dispersed among numerous papers, we decided to gather all these data in the form of a widely accessible and comprehensive database. The RNAimmuno database provides an opportunity to analyze and compare the results of different studies and may help develop safer RNAi technology reagents. The name of the database is a combination of the words “RNAi” and “immunology” and simply reflects the contents of RNAimmuno. To our knowledge, this is the first database concerning the non-sequence-specific effects of RNAi and miRNA reagents.

RESULTS

Database contents and web interface

The goal of RNAimmuno is to collect the published data regarding the nonspecific immunological effects of reagents (e.g., siRNAs, shRNAs, sh-miRs, and anti-miRs) observed in investigated cell lines and model organisms. RNAimmuno is also designed to be the comprehensive source of knowledge regarding different aspects of these immunological off-target effects. Currently, the database contains more than 2000 records, most of which describe the effects caused by various siRNA reagents (Supplemental Fig. 1A). The lower number of records regarding other reagent types simply reflects the underrepresentation of the relevant data in published papers. The detailed statistics of the records that represent different categories of advanced search is shown in the Statistics section.

Searching options

RNAimmuno may be searched either by entering a keyword in the “quick search” window or by choosing specific records from panels in the “advanced search.” As a keyword, the user may enter a gene or protein symbol (e.g., PKR, TLR3, or IFN), reagent type (e.g., siRNA or shRNA), reagent carrier (e.g., Lipofectamine, DOTAP, or lentivirus), experimental model (e.g., HEK, HeLa, or mice), target gene, reagent ID number, or the name of the first author of a

relevant publication. When using the “advanced search” option, the user specifies the reagent type, reagent carrier, experimental model, sensor or responder name, level of activation/induction, reagent sequence, immunostimulatory motif, or reagent modifications. As responders, we mean proteins from the activated pathway (e.g., transcription factors, cytokines, or chemokines) functioning downstream from cellular sensors recognizing foreign RNA or DNA. Additionally, there is the possibility to search records derived from selected papers by entering the name of the first author of the relevant publication in the “advanced search” panel. The main table of results contains data concerning reagent ID and its original name, experimental model, reagent carrier, level of induction/activation of foreign RNA sensors and responders, and the chemical modification of the reagent when specified. Particular records contain drop-down detailed information which includes reagent type, its sequence, length, end structure, origin, concentration, target gene, observed effects (raw data), and comments. Data which need additional comment are marked with asterisks.

We found it difficult to apply a universal scale of activation effects due to the use of different experimental conditions, methods of analysis, and biological systems by different authors (e.g., in vivo systems vs. in cell lines). Therefore, the scale we used to describe the observed effects, i.e., no activation/induction (–/mock), activation/induction (+/mock),

and strong activation/induction (++/mock), generally refers to the results described in specific publications (obtained in specific experimental conditions). Additionally, the activation status of the sensor or responder is presented as an activation/induction relevant to a specific control (up/specific control, down/specific control, similar/specific control), and, wherever possible, the fold-change is also indicated. To avoid any misinterpretation of the results, the raw data from the original publications are also included. An example of possible search results is shown in the screenshot in Figure 2.

RNAimmuno tools

The database provides the user with tools that may be helpful in designing safer RNAi reagents. The “simple tool” option allows scanning the reagent of interest for the presence of the most important molecular patterns known to activate the cellular sensors of foreign RNA (immunostimulatory motifs, reagent’s length, blunt end, and 5’ triphosphate). A more advanced “sequence alignment” tool uses the BLASTN algorithm (Altschul et al. 1990) to align input sequence with reagents collected in the RNAimmuno database. The results of a search are summarized in a simple tabular format of hits followed by alignments of the query sequence against each hit sequence. Detailed information

SEARCH RESULTS - found 1293 records							
<input type="button" value="Show only 'checked'"/> <input type="button" value="Show All"/> <input type="button" value="Go back"/> <input type="button" value="Help"/>							
Add ID	Reagent name	Experimental model	Reagent carrier	Sensor (Induction or activation/control)	Responder (Induction or activation/control)	Modifications	References
☐ si00724	si3589/3584	In Vivo: Black Swiss mice	nanoparticles*	—	IFN-α (–/mock) TNF-α (–/mock)	2'-OMe, PS	Nguyen DN et al. (2009)
☐ si00720	si3583/4747	In Vivo: Black Swiss mice	nanoparticles*	—	IFN-α (+/mock) TNF-α (+/mock)	PS	Nguyen DN et al. (2009)
☐ si00044	si4746/4747	In Vivo: Black Swiss mice	nanoparticles*	—	IFN-α (+/mock) TNF-α (+/mock)	—	Nguyen DN et al. (2009)
☐ si00075	siE6/E7	PBMC	DOTAP	—	IFN-α (–/mock) IFN-γ (+/mock) TNF-α (+/mock) IL-1β (+/mock) IL-12 (+/mock)	—	Gantier MP et al. (2010)
☐ si00076	siE6/E7-U	PBMC	DOTAP	—	IFN-α (+/mock) IFN-γ (+/mock) TNF-α (+/mock) IL-1β (+/mock) IL-12 (+/mock)	—	Gantier MP et al. (2010)
☐ si00729	dsLamin 3'17 - TI03 5'17 T989*		Lipofectamine 2000	OAS 2 (+/mock)	IFIT1 (p56) (+/mock) IFN-β (+/mock)	—	Chang C et al. (2009)
Sequence: ► UUCUUCUGGAAGUCCAGGUCACAGGCGUUCUCUA ► UAGAGAACGCGUGAGAC CCUGGA CUUCCAGAGAGAA							
Type: dsRNA Length: 34 bp, End structure: blunt/blunt, Origin: chemically synthesized by Bioneer Concentration: 10 nM, Target: Lamin A/C and TI03, Comment: Dual - target siRNA (si00727), *similar results in HeLa cells, Reference: Chang C et al., Dual-target gene silencing by using long, synthetic siRNA duplexes without triggering antiviral responses. Mol Cells. 2009 Jun 30;27(6):689-95.							
☐ si00730	dsLamin 19 - TI03 19 dTdT	T989*	Lipofectamine 2000	OAS 2 (–/mock)	IFIT1 (p56) (–/mock) IFN-β (–/mock)	—	Chang C et al. (2009)

FIGURE 2. An example of search results. Detailed information about the selected reagent is available after clicking on a reagent (e.g., reagent si00729). The specified reagent sequence is highlighted in red.

about the effects generated by reagents showing a high degree of similarity to the query sequence is accessible via specific links in the table.

Pathways and sensors

RNAimmuno also contains the most important information about the known cellular sensors of foreign RNA and DNA (SENSORS button) and the pathways these sensors mobilize (PATHWAYS button). This information is presented in tables with links to external public databases. These pages also provide information such as the gene and protein name, with aliases, HGNC ID, NCBI gene page, OMIM ID, NCBI transcript page, gene expression data, protein data, cellular localization of the sensor, type of ligand and adaptor protein, and the signal transduction pathway. For each sensor, the “references” link redirects the user to the PubMed database.

Delivery/uptake

Existing data have shown that the reagent carriers are not neutral to cells and may induce immune responses. Therefore, we gathered the published data regarding the toxicity of the transfection reagents frequently used in RNAi experiments (DELIVERY/UPTAKE button). Currently, this page includes information on 22 carriers used in the experiments described in the RNAimmuno database. The reagent carriers are characterized by their type and source and also by the type of reagent and cell line dedicated to transfection with the use of each specific carrier. In addition, the page contains links to the publications where the toxic effects of the reagent carriers were examined.

DISCUSSION

The RNAimmuno database was created to provide information regarding the nonspecific immunological effects of RNAi and miRNA reagents. Because data of this nature are dispersed among the numerous papers where RNAi and miRNA reagents are used, there is a need to gather these data sets in one location. In addition, there is a lack of clear guidelines on how to study immunological side effects, and the selection of immune response markers is left to researchers' decisions. RNAimmuno provides researchers with an opportunity to analyze and compare all these data. Statistical insight into RNAimmuno records shows that nonspecific immunological effects are predominantly analyzed through the measuring of the cytokine levels (ELISA and RT-PCR). Among them IFN α , TNF α , IFN β , and IL-6 account for more than 70% of all records (Supplemental Fig. 1B). OAS1, PKR, and RIG-I are the most frequently studied sensors, but their induction is typically analyzed with the use of the qRT-PCR method and microarrays. Since most of the sensors belong to the group of Interferon Stimulated Genes (ISGs), the observed increase of mRNA

level may result from the secondary effect of IFN stimulation and should not be considered as the effect of direct activation of a specific sensor protein. We have also performed a more detailed analysis of types of sensors and responders studied in different experimental systems, classified into: immune cells (mostly PBMC and pDCs); nonimmune cells (mostly HEK and HeLa cells); and in vivo models (Supplemental Fig. 1C). Immunological side effects of RNAi triggers in vivo and in immune cells are mostly studied by measuring cytokine levels (IFN α , TNF α , and IL-6), whereas in nonimmune cells more than 20 different inflammatory markers were studied. In addition to simple viability tests, IFN β , OAS1, IL-8, IL-6, STAT1, and IFIT1 analysis are also often performed (Supplemental Fig. 1C). Careful inspection of the gathered data clearly demonstrates that the analyses of the immunological side effects in nonimmune cells are often carried out in an incoherent way, and the current knowledge concerning sensors and their ligands is rarely taken under consideration.

A sample use of this database would be to check whether or not a reagent of interest, or similar reagents, delivered with the use of a selected carrier, activated the immune response in a specific experimental model. By the use of specific filtering options, it is possible to analyze the influence of various parameters (e.g., reagent type, sequence, concentration, and chemical modifications) on the activation/induction of specific cellular sensors and on cell viability. Users may also be able to find a less toxic reagent and experimental conditions (e.g., reagent concentration or delivery method) to silence the selected target gene. Among the delivery methods, cationic lipids account for the vast majority of records (e.g., Lipofectamine—38 papers, and DOTAP—22 papers) (Supplemental Fig. 1A). DOTAP is very often used as a reagent carrier in papers where TLR7/8 recruitment by RNAs containing immunostimulatory motifs was studied. Because reagent carriers may influence the expression level of many genes (Delivery/Uptake page), this possibility should be taken into account when interpreting experimental results.

The RNA reagents that strongly activate the immune system may also be desired in some studies (e.g., in antiviral and cancer therapy), and the database may be helpful in identifying such reagents. The RNAimmuno database also provides an opportunity to find appropriate control reagents that were characterized with regard to the induction of non-sequence-specific effects. The problem of using improper control siRNA in RNAi studies was already discussed (Robbins et al. 2008; Rossi 2009; Shukla et al. 2010). For example, siRNA targeting GFP, widely used as a negative control in RNAi studies, has extremely low immunostimulatory properties, whereas most other unmodified siRNAs can stimulate the innate immune response. There is a possibility that observed therapeutic effects may result from the activation of an immune response rather than from the RNA interference mechanism, as reported previously

(Kleinman et al. 2008). Therefore, it is necessary to evaluate the contribution of the immune response in the final effect, especially in in vivo and clinical studies. RNAimmuno also contains ranking of the most commonly used siRNAs. The most frequently used EGFP siRNA was studied in 11 different papers and generated 144 records (due to different experimental conditions and reagent modifications).

RNAimmuno is targeted to a wide community of biologists, primarily bench scientists, and may be helpful in both the design of experiments and the interpretation of results. As new data are incorporated, RNAimmuno will provide opportunities for more comprehensive analysis of the immunological side effects of specific reagents. Currently, the database contains mostly data from in vitro experiments, but we anticipate an increased number of records with in vivo applications of RNAi and miRNA technologies in the future. We believe that widespread access to this database will hasten efforts to develop safer gene silencing technologies.

MATERIALS AND METHODS

Implementation

The RNAimmuno database v. 1.0 runs in a Linux environment, and it has been developed as a relational database in MySQL. The search engine is served by the Apache http daemon along with the PHP scripts. The interface component consists of the web pages designed and implemented in HTML/CSS. It has been tested in many web browsers, including Mozilla Firefox, Internet Explorer, Opera, Safari, and Google Chrome. The service is hosted and maintained by the Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland.

Submission of new data

The usefulness of RNAimmuno depends on the amount of published data describing immunological side effects of RNAi and miRNA reagents. Therefore, it is very important to keep the database up to date with new data. Currently, it is not possible to update the database automatically; all relevant papers have to be carefully analyzed before new data can be introduced into the database. Data can be submitted to the database by contacting the database manager either through the submit form available for download or by e-mail. Detailed instructions for completion are contained in the form, which requires Excel or another compatible spreadsheet program. The data will then be evaluated and will be added to the database during the next update.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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