Review

Application of RNAi library to oncology investigation

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Key words: RNA interference, RNAi library, neoplasm

RNA interference (RNAi) library was pioneered in *C. elegans* in a broad range of organism-based screens. During the past few years, RNAi has become a powerful tool to silence the expression of genes and analyze their loss-of-function phenotype in mammalian cells. There are two types of RNAi library, synthetic oligonucleotide library and vector library, and two screening strategies, high throughput and selective screenings. Short hairpin RNA (shRNA), which is processed intracellularly into short duplex RNAs and has siRNA-like properties, can mediate persistent gene silencing after stable integration of the vector into the host-cell genome. In combination with the shRNA vector library and suitable screening strategies, much greater depth will be added to the functional understanding of therapeutic applications of potential targets in oncology.

With in-depth study on the mechanism of RNAi, loss-of-function genetic screens in cells of higher animals have become a very promising research method. In 2000, Fraser and Gonczy reported for the first time that a large scale RNAi screening technique was applied to identify embryonic lethal allele on individual chromosome of nemic genome, which was considered to be the embryonic form of RNAi library technique. Now, RNAi library has been applied in mammal studies as an effective method to investigate functioning genome. RNAi library can sequentially silence the expression of a series of genes in the genome to validate whether a certain gene is necessary for a specific phenotype. In ontological study, a protein will become a good target for the treatment of tumor if its gene is necessary for the persistent expression of a certain phenotype. There are two types of RNAi library, synthetic oligonucleotide library and vector library, and there are two screening strategies, high throughput and selective screenings.

Chemically synthetic double strand RNA (synthetic oligonucleotide), which is stable in nature, was used in early studies. In addition, it can be chemically modified to improve stability and transfection efficiency, and thus the transfection dose can be accurately controlled. However, siRNA is easily degraded intracellularly, so it can only temporarily inhibit gene expression. Furthermore, the transfection efficiency in primary cells is very low, and the cost for siRNA synthesis in high throughput genome screening is very high.

Recent studies have found that short hairpin RNA (shRNA) can be processed intracellularly into short duplex RNAs having siRNA-like properties. Numerous research groups are also engaged in the construction of vectors that can express shRNA in eukaryocytes, since they can mediate persistent gene silencing after stable integration into the host-cell genome. shRNA expression frame can be inserted into retrovirus, lentivirus and adenovirus vector, and thereby transfected into many kinds of cells. At present, shRNA vectors that can be expressed under certain regulation have been constructed and successfully applied in gene screening.

High throughput screening aims at observing the change of phenotype after the silencing of a single gene. In selective screening, during which multiple genes can be silenced at the same time, the required phenotype can only appear when a certain gene is silenced. Then RNAi sequence of the silenced gene is obtained by detecting positive clones. The present paper will review the application prospect of RNAi library formed by shRNA vectors in oncology investigation.

**Design Principle for shRNA**

The design principle for short hairpin RNA (shRNA) sequence is similar to that for siRNA. Recent design principle for high performance RNA interference sequence is as follows: low G + C content (30–50%), low internal stability of 5’ end antisense strand, high internal stability of 3’ end sense strand, no palindrome inside the strand, no thelix between target mRNA and siRNA, the third, tenth and nineteenth position of sense strand is A, U and A, respectively, the thirteenth position of sense strand can not be G and the nineteenth position can not be G or C, no high homology with other gene sequences.

**ShRNA Vectors**

The selection of a suitable shRNA vector is a very important step in RNAi library. It was reported that RNAi library can be
introduced into the cell via retrovirus particles and DNA transfection.15,16 Berns et al.15 used pRETRO SUPER retrovirus vector to load H1 RNA promoter, which can be identified by RNA polymerase III. The so expressed shRNA sequence consisted of a 19 bp gene specific double strand RNA and a 4 nt ring. The vector had puromycin resistance. Three shRNA sequences were designed for each gene and a total of 7,914 human genes were covered.15

Paddison et al.16 used a different pSHAG MAGIC retrovirus vector to load U6 RNA promoter, which can also be identified by RNA polymerase III to initiate shRNA expression. Its shRNA sequence consisted of a 29 bp gene specific RNA and a 4 nt ring. In addition, a 27 nt U6 RNA leading sequence was added to improve silencing efficiency. The vector had puromycin resistance. A unique 60 nt “barcode” sequence was added when designing the vector to facilitate amplification of shRNA sequence from the vector at a later period of screening for sequencing identification. The library covered 9,610 human genes and 5,563 mouse genes. Furthermore, almost all genes had three targeting shRNAs.

Several other RNA interference vectors can be used for library construction except for those mentioned above. Loading of GFP onto the vector can help to determine transfection efficiency of the library. Many companies can provide vectors with this function, such as pRETRO SUPER by Oligoengine, the pSIRENs series from Takara Biosciences Clontech, and the GeneSilencers series from Gene Therapy Systems, Inc., Different virus vectors can also be used, such as RNAi adenovirus vector20 provided by Takara Biosciences Clontech, Ambion and Invitrogen, and lentivirus vector,7,21 provided by Invitrogen. Expression efficiency of shRNA is higher when using adenovirus vector, while nonproliferating cells can be transfected when using lentivirus vector.

Inducible vector is the new development direction of vector mediated RNA interference library. Some research groups have constructed tetracycline inducible RNAi plasmid vector and lentivirus vector12,22,23 and ecdysone inducible RNAi vector.24 Further investigation is required to demonstrate whether they can be effectively applied in large scale screening without bias.

Application of Vector Library in High Throughput Screening

It is very important to mark transfected cells when vector library is applied in high throughput screening, since the transfection efficiency of plasmid is obviously lower than that of oligonucleotide. Only by this means can we measure the transfection efficiency of whole cell colony to realize standardized process, and clearly observe the behavior change of transfected cells to differentiate them from the un-transfected ones. Vector RNAi library was applied in a study focusing on protease function by Paddison et al.16 in which single shRNA plasmid, a plasmid coding for DsRed fluorescent protein, a plasmid coding for ZsGreen fluorescent protein and a fused with one coding for mouse guanine nucleotide decarboxylase degron PEST sequence were co-transfected into 293 cell. In negative control, PEST sequence was able to ensure the degradation of ZsGreen fluorescent protein by protease so as to increase the ratio of red fluorescence to green. Any shRNA plasmid that was able to inactivate protease can decrease that ratio. In this study, subunits of most known proteases were screened by a library containing 7,000 shRNA vectors.

In another study, a familial cylindroma inhibiting gene product was screened. It was found to be a ubiquitin degrading enzyme and to have an important regulatory effect on NFκB. This small scale screening covered known ubiquitin degrading enzymes and their homologous sequences. U2-OS cell was transiently transfected with NFκB luciferase report gene vector and 50 targeting human ubiquitin degrading enzyme gene shRNA vectors, followed by TNFα treatment. It was demonstrated that the activity of NFκB had a positive correlation with the fluorescence intensity of luciferase. In addition, silencing of CYLD gene can increase the fluorescence intensity of luciferase, suggesting that NFκB mediated enhancement of anti-apoptosis signal may be one of the pathogenesis of benign familial cylindroma.14

Observation of cell death rate via co-transfection of oncogene and siRNA is a feasible way to apply RNAi library in searching for treatment target of cancer. In short, RNAi library is used to transfect normal cells and oncogene transformed cells to observe cell death or growth retardation. During tumorigenesis, the survival of the tumor cell is called an “oncogene dependent” process,25 since it depends on the signal produced by oncogenes. Therefore, RNAi library can be used to find targets that lead to selective death of tumor cells.

Application of Vector Library in Selective Screening

Selective screening is another screening method using RNAi library, in which negative cells die and are growth inhibited or removed from the culture medium via some selection pressure, while cells transfected with RNAi library survive and form clones under the selection pressure. It is required that one or some genes sensitive to the selection pressure are silenced by the RNAi sequence. The surviving clones are amplified and the RNAi sequence can be identified by PCR amplification and sequencing. Vector shRNA library rather than synthetic oligonucleotide siRNA library can be used in this screening strategy, since RNAi sequences obtained by long-term screening and clone amplification are required.

Berns et al. used this screening method to search for genes that can help human fibroblast escape from p53 mediated cell aging.15 Temperature sensitive SV40 large T antigen can immortalize human BJ primary fibroblast. Cell aging would occur under improper temperature, while it would not if the signaling pathway was blocked by a certain intracellularly transfected RNAi sequence. It was found in this study that six genes participated in p53 mediated cell aging.

Treatment of the cells with a mixture of lots of RNAi vectors to decrease workload is a main advantage of selective screening, but also a limitation. After treatment of the cells with mixed RNAi vectors, it is ideal that few rather than many vectors are contained in most of the cells. At present, the ideal result can be realized only by virus mediated transfection rather than direct transfection. High grade strict operating condition is required for treatment of human cells with virus, which is difficult to realize in common laboratories. Berns et al. used a retrovirus packaging system, which is only able to infect murine cells, to screen pretreated human...
cells that can express murine retrovirus receptor\textsuperscript{15} and successfully solved the safety problem.

Determining the capacity of RNAi library is another important point of selective screening, since the treatment of cells with different numbers of mixed vectors leads to different false positive rates. High false positive rates will bring trouble to subsequent identification work, leading to unnecessary waste. It has been determined by NKI library that mixed RNAi vectors targeting 100 genes can be used to treat cells at one time. Based on this design principle, three retroviruses containing different RNAi sequences have been constructed for each gene, so approximately 300 different retroviruses are used to treat the cells at the same time. We can also decrease false positive rates by multiple repetitive screening so as to obtain reliable conclusions.

Results obtained from selective screening in tumor cells by RNAi library usually deviate from the initial aim of investigators. Tumor cells survive more easily than normal cells, in that they can survive in serum-free or non-adherent environments. An ideal screening intends to screen loss of some transformation phenotype, which is difficult to realize in normal cells. For this reason, many investigators turn to a reverse strategy, such as screening for genes which can promote malignant transformation of the cells at low expression level or after being silenced. Most such genes belong to tumor suppressor genes. Tumor suppressor genes can not be used as direct targets for tumor treatment. However, it can serve as a diagnosis index. So if further investigation on such genes can find enzymes or other analogues that are able to inhibit its gene activity, it will become a new target for drug treatment of tumor.

Problems Existing in RNAi Library

Redundant genes. There are a great number of redundant genes existing in mammalian cells, making it very tedious work to construct RNAi vectors targeting all genes. When using virus RNAi vector to perform selective screening, the number of viruses that can be held in each cell is limited. Therefore there is little chance to obtain recombinant and the achievement of expected results is a problem.

Low silencing efficiency. With ceaseless improvement of the design principle for RNAi sequence, the effect of RNAi silencing is expected to improve. However, even after stable screening, the silencing effect of RNAi vector is still not as good as that of transient transfection by RNAi oligonucleotide. It is difficult to obtain expected results if the false positive rate is high during screening.

Off-target effect. There are many papers discussing off-target effect and their conclusions are often disputed. Off-target effect is indeed a very serious problem. Currently, known off-target effect can be classified into two categories, sequence specific effect and sequence independent effect. It has been reported that off-target effect can occur as long as 11 bases match.\textsuperscript{26} Currently, sequence specific off-target effect is solved mainly by designing several specific off-target effect is solved mainly by designing several different RNAi sequences for each gene, so approximately 300 different retroviruses are used to treat the cells at the same time. We can also decrease false positive rates by multiple repetitive screening so as to obtain reliable conclusions.

With the wide application of RNAi technique in mammalian studies, RNAi library will receive long-term development. One of its outstanding characteristics will be its application in finding new components in tumor signaling pathway, which may become a potential target for drug treatment. However, RNAi library is still not mature. The current two vector RNAi libraries are still embryonic forms, and therefore need further improvement. In-depth investigation on the specificity of RNAi sequence will help to increase the silencing effect of vector library and oligonucleotide library significantly. In addition, technical development, which changes with every passing day, especially the development in fluorescence microscope, will offer great help to vector screening. Of the two screening strategies, the extensive applicability of high throughput screening makes it suitable for screening for multiple phenotypes, especially screening for the main characteristics of multiple tumors. On the contrary, the applicability of selective screening is relatively narrow due to the strict selection condition required by positive clones. However, new components in tumor signaling pathway, especially molecules resistant to phenotype transformation, can be found by this screening strategy if the design is appropriate. In conclusion, RNAi library has incomparable advantages over previous methods in studies aimed at finding new targets for tumor treatment and therefore has extensive application prospects in tumor investigation.

Future Prospects

With the wide application of RNAi technique in mammalian studies, RNAi library will receive long-term development. One of its outstanding characteristics will be its application in finding new components in tumor signaling pathway, which may become a potential target for drug treatment. However, RNAi library is still not mature. The current two vector RNAi libraries are still embryonic forms, and therefore need further improvement. In-depth investigation on the specificity of RNAi sequence will help to increase the silencing effect of vector library and oligonucleotide library significantly. In addition, technical development, which changes with every passing day, especially the development in fluorescence microscope, will offer great help to vector screening. Of the two screening strategies, the extensive applicability of high throughput screening makes it suitable for screening for multiple phenotypes, especially screening for the main characteristics of multiple tumors. On the contrary, the applicability of selective screening is relatively narrow due to the strict selection condition required by positive clones. However, new components in tumor signaling pathway, especially molecules resistant to phenotype transformation, can be found by this screening strategy if the design is appropriate. In conclusion, RNAi library has incomparable advantages over previous methods in studies aimed at finding new targets for tumor treatment and therefore has extensive application prospects in tumor investigation.

Acknowledgements

Grants: National Natural Science Foundation of China (No. 30571950, No. 30528012); “973” Program of China (No. 2002 CB513100).

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