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Prediction of Human MicroRNA Targets

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Summary

MicroRNAs (miRNAs) are small, nonprotein-coding RNAs that regulate gene expression. Although hundreds of human miRNA genes have been discovered, the functions of most of these are unknown. Computational predictions indicate that miRNAs, which account for at least 1% of human protein-coding genes, regulate protein production for thousands of or possibly all of human genes. We discuss the functions of mammalian miRNAs and the experimental and computational methods used to detect and predict human miRNA target genes. Anticipating their impact on genome-wide discovery of miRNA targets, we describe the various computational tools and web-based resources available to predict miRNA targets.

Key Words: MicroRNA; microRNA targets; gene regulation; gene silencing; translational repression; computational prediction.

1. Introduction

miRNAs are small (~22 nucleotides) RNAs that mediate posttranscriptional silencing of genes by base pairing with target messenger RNAs (mRNAs) *(1)*. Animal miRNAs are known to either inhibit translation initiation *(2)* or direct the target mRNA degradation *(3,4)*. The study of miRNAs may revolutionize our view of genes and open up a new world of molecular networks. To date, approx 300 miRNAs have been identified in the human genome *(5)* and hundreds more have been predicted computationally *(6–8)*. Computational predictions of miRNA target genes reveal the potential scope and scale of gene regulation by miRNAs. In particular, miRNAs are predicted to regulate translation of thousands or possibly all of human genes. The regulation of genes by miRNAs is likely to generally involve the modulation of multiple mRNAs by a given miRNA ("multiplicity") and cooperative interactions of multiple miRNAs ("cooperativity") to regulate a given gene *(9–12)*. The emerging complexity of miRNA-mediated gene regulation is reminiscent of transcriptional regulation in promoter regions of DNA in which one-to-many and many-to-one relations exist between regulatory

From: *Methods in Molecular Biology, vol. 342: MicroRNA Protocols*
Edited by: S. Ying © Humana Press Inc., Totowa, NJ

factors and regulated genes *(13,14)*. The combinatorial relationships influence the methods to determine the likelihood that a particular gene is a target of a given miRNA.

Differential expressions of mammalian miRNAs have been shown to occur in specific cell types, tissues, and embryonic stem cells *(15–26)*. Distinct miRNA expression profiles are associated with diseases, such as human B-cell chronic lymphocytic leukemia *(27)*, lung cancer *(28)* and diabetes mellitus *(29)*. Recently, miRNAs were also identified in human pathogens such as, Epstein–Barr virus *(30)*, HIV *(31)*, cytomegalovirus *(32)*, and Kaposi's sarcoma virus *(32,33)*. The observed dynamic range of expression of miRNAs underscores their functional importance and suggests that their expression profile could be useful to predict their function.

Although the molecular mechanisms behind miRNA-mediated gene regulation are largely unknown, the principles of miRNA target recognition are emerging. Experimentally verified miRNA target sites indicate that the 5' end of the miRNA tends to have more bases complementary to the target than its 3' end *(34)*. The 5' end of an miRNA: mRNA duplex does not seem to generally tolerate G:U wobble basepairs *(12,34)*. However, some miRNA target sites with G:U basepairs are also known to be functional *(3,10,35)*. Interestingly, the regions near the miRNA-binding site may also be important for miRNA target recognition. In *lin-41*, a 27-nucleotide intervening sequence between two consecutive *let-7* sites is necessary for its regulation *(36)*. We anticipate that other regions in the target mRNA (e.g., RNA sequence motifs that bind protein complexes) may also be important for the function of miRNAs.

In this chapter, the known functions of mammalian miRNAs are briefly discussed, followed by the experimental and computational methods used to detect human miRNA targets. In general, computational methods have been a key factor in the identification of miRNA-regulated genes in several species *(34,37–42)*. Therefore, we provide a detailed description of the steps involved in the prediction of human miRNA targets.

1.1. The Known Functions of Mammalian miRNAs

Genetic and molecular studies in mammals have identified the biological functions of several mammalian miRNAs. Experiments in mouse have shown that *miR-181a* modulates hematopoietic differentiation *(43)*; *miR-196* negatively regulates the expression of *Hoxb8* *(3,44)*; *miR-375* inhibits glucose-stimulated insulin secretion by targeting the pancreatic endocrine protein, myotrophin *(39)*; *miR-1* downregulates a transcription factor (*Hand2*) that promotes ventricular cardiomyocyte expansion *(45)*; and *miR-122a* reduces the expression of the germ cell transition protein 2 (*Tnp2*) via the cleavage of the *Tnp2* mRNA transcript *(46)*. Additionally, *miR-122a* is thought to negatively regulate *CAT-1*, an essential gene that encodes an endogenous ecotropic murine retrovirus receptor, which is associated with leukemia *(47)*. Experiments conducted using human cell lines and tissues indicate that the *let-7* miRNA family negatively regulates the *NRAS* and *KRAS* oncogenes *(48)*. The expression of *let-7* inversely correlates with expression of the NRAS protein in lung cancer tissues, suggesting a possible causal relationship *(48)*. In HeLa cells, *miR-17-5p* and *miR-20a*, which are located within a 500-nucleotide region (*miR-17-92* cluster) and are under the transcriptional control of *c-Myc*, negatively regulate the transcription factor, *E2F1* *(49)*, possibly buffering the mutual posi-

tive activation of *E2F1* and *c-Myc* **(50)**. The overexpression of *miR-17-19b*, a member of the *miR-17-92* cluster, accelerates *c-Myc*-induced lymphomagenesis in mice **(51)**. Current evidence indicates that miRNAs could act as potential human oncogenes or tumor suppressors. In cultured human preadipocytes, *miR-143* promotes adipocyte differentiation, possibly by increasing the level of its suggested target gene, *ERK5* **(52)**. Human miRNAs are also thought to have antiviral potential **(53)**. For instance, the accumulation of the retrovirus primate foamy virus type 1 in human cells is effectively restricted by *miR-32* **(53)**.

A number of studies have linked specific miRNAs to cancer **(17,27,54–61)**, Fragile X syndrome **(10,62–65)**, and neurodegenerative diseases **(18)**. Recent analysis of the genomic location of known miRNA genes suggests that 50% of miRNA genes may be located in cancer-associated genomic regions or in fragile sites **(58)**. For instance, the miRNAs *miR-15* and *miR-16* are located within a 30-kb region at chromosome 13q14, a region deleted in 50% of B-cell chronic lymphocytic leukemias, 50% of mantle cell lymphoma, 16 to 40% of multiple myeloma, and 60% of prostate cancers **(54,55,66)**. This raises the possibility that loss of *miR-15* and *miR-16* contributes to tumorigenesis. Similarly, *miR-143* and *miR-145* are downregulated at the adenomatous and cancer stages of colorectal neoplasia **(56)**. Additionally, *miR-155* is upregulated in children with Burkitt lymphoma **(57)**. The clinical isolates of several types of B-cell lymphomas, including diffuse large B-cell lymphoma, have 10- to 30-fold higher copy numbers of *miR-155* than do normal circulating B-cells. Interestingly, the mRNA of the transcription factor PU.1, which is required for late differentiation of B-cells, contains potential *miR-155* regulatory sites that are conserved in human, mouse, rat, dog, and chicken **(10,59)**. A recent study of global expression levels of miRNAs in normal and tumor-tissue samples, using beads marked with fluorescent tags, suggested that miRNA profiles reflect the developmental lineage and differentiation state of the tumors **(60)**. The study also showed that miRNA expression patterns may be generally more accurate than mRNA profiles for the classification of poorly differentiated tumors.

Several miRNAs are also known to be important factors in the development or maintenance of the neoplastic state. In adult differentiated cancer cells, depletion of *miR-125b* profoundly decreases cell proliferation **(67)**, and the knock-down of the strongly overexpressed *miR-21* in cultured glioblastoma cells triggers the activation of caspases, which leads to increased apoptotic cell death **(68)**. Additionally, several miRNAs that mediate the regulation of cell growth and apoptosis were identified in HeLa cells **(69)**. A growing amount of evidence suggests that miRNAs are associated with a multitude of biological processes. However, the details of the regulation by miRNAs are still largely unknown. Although a few miRNA target genes have been experimentally identified, the details of their precise binding sites are largely unknown. We anticipate that many more miRNA-regulated genes will be identified in the near future.

1.2. Experimental Methods to Identify miRNA Functions

The genes regulated by hundreds of human miRNAs must be experimentally identified. Most experimental protocols that probe a specific miRNA–target interaction assess the regulatory capacity of the 3' untranslated region (UTR) that contains the predicted

miRNA binding sites. Such protocols fuse the regions of the 3' UTR from predicted targets to a reporter construct *(38,41,48)*. The predicted UTR targets tested by the reporter constructs generally seem to confer significant repression on the reporter, with respect to a control UTR *(38,41)*. However, the reporter-based assays fall short of establishing whether the observed regulation would occur with the full-length UTR in vivo or whether other endogenous miRNAs regulate the candidate gene of interest. In addition, if the miRNA is exogenously introduced, its concentration may exceed the physiological levels in the cell. The abnormal miRNA levels may cause aberrant cellular functions, which may result in the indirect regulation of the gene. The problem can be partially overcome if the effects of point mutations in the target site are assessed using an assay that makes use of the full-length 3' UTR.

Overexpression of endogenous miRNAs may be used to provide additional evidence to validate a potential miRNA target *(28,39,45)*. Recent evidence suggests that miRNAs can affect transcript levels, as well as protein levels of target genes *(4)*. Therefore, microarray-based profiling of miRNA-transfected cells may also be useful for the identification of the functions of miRNAs *(4)*.

Loss-of-function-based experiments are among the definitive tests to validate miRNA-mediated gene regulation. A given miRNA loss-of-function mutant must cause corresponding deregulation of its genuine targets, and the mutation of miRNA-binding sites must at least partially phenocopy the corresponding miRNA loss of function *(70)*. The loss of function of a miRNA can be achieved by small interfering RNA knockdown, in which the small interfering RNA is targeted to the loop region of the precursor miRNA *(71)*. Loss of function may also be achieved by inhibiting miRNAs via chemical inhibitors, such as 2'-*O*-methyl oligoribonucleotides *(72)*, locked nucleic acids *(73,74)*, and morpholinos *(75)*.

1.3. Computational Methods to Identify miRNA Functions

Experimental identification of miRNA-regulated genes is mainly dependent on computationally predicted miRNA targets or on conjectures based on miRNAs associated with specific biological pathways. Most computational methods for the prediction of miRNA targets are based on models of the nature of the pairing between the miRNA and the target gene. Computational predictions indicate that miRNAs may regulate protein production for thousands of human genes. Because of the importance of computational methods for the identification of miRNA targets, we describe them in greater step-by-step detail (**Subheading 2.**).

2. Methods

The three main steps involved in most computational approaches to predict miRNA targets are (**Fig. 1A**):

1. To obtain miRNA and 3' UTR sequences.
2. To scan for potential miRNA target sites.
3. To analyze evolutionary conservation of the target sites.

We next describe these steps in detail.

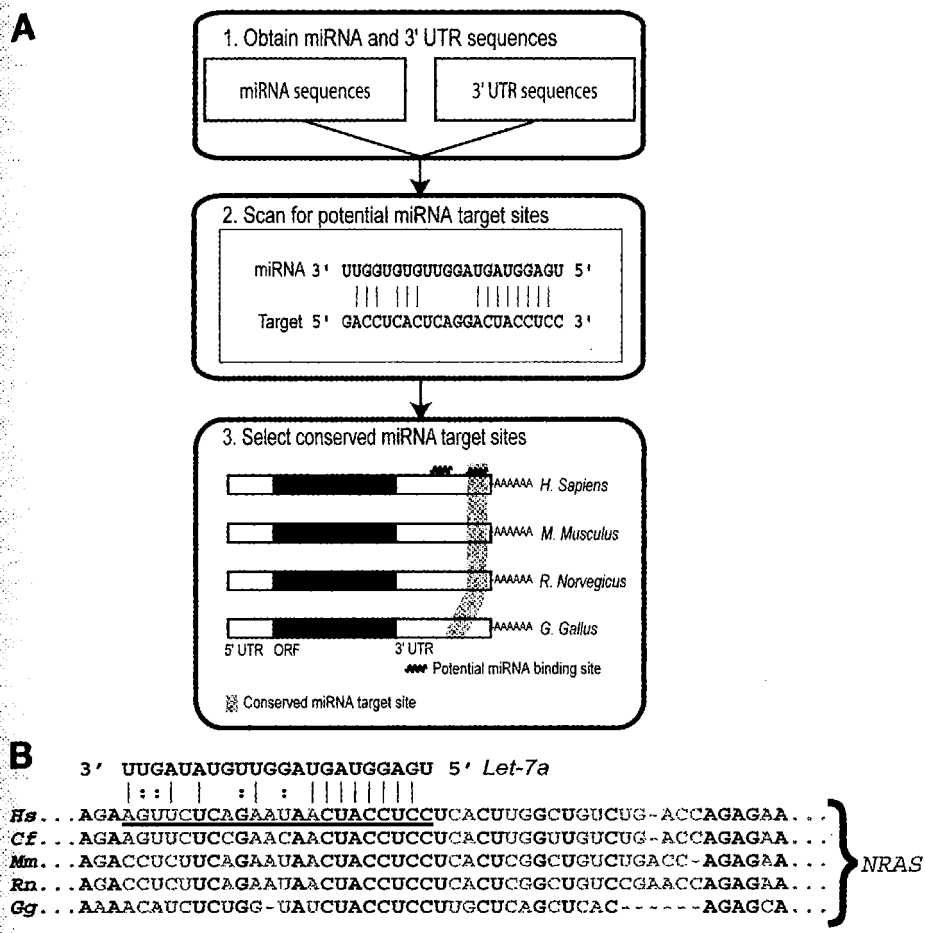

Fig. 1. Prediction of human microRNA (miRNA) targets. Steps involved in computational prediction of miRNA targets and an example of the conservation pattern of an miRNA target site in distantly related species. (**A**) The mature miRNAs are used to scan the 3' untranslated regions (UTRs) of human genes using a computational method (**steps 1** and **2**). The candidate miRNA target sites are then analyzed for their conservation in distantly related species (**step 3**), such as *Canis familiaris* (dog), *Mus musculus* (mouse), *Rattus norvegicus* (rat), and *Gallus gallus* (chicken). (**B**) An example for a conserved miRNA target site; a *let-7* target site (underscore) in the 3' UTR of the protooncogene, *NRAS*, is conserved between human, mouse, rat, and chicken. The 5' end of the miRNA-binding site has more completely conserved nucleotides (solid black) than at the 3' end of the miRNA. The predicted mode of binding between *let-7* and the human *NRAS* 3' UTR is also indicated.

2.1. Obtain the miRNA and 3' UTR Sequences

The known human miRNA sequences (*see* **Note 1**) can be obtained from public data repositories, such as RFAM *(5)*. The 3' UTR sequences of human genes can be extracted using the Entrez nucleotide database *(76)*. If a 3' UTR sequence of a given human gene

is unknown, the genomic region downstream of the stop codon of the gene may be used instead. The Ensembl *(77)* web interface provides a simple way to extract downstream genomic regions (*see* **Note 2**). The use of 3' UTR sequences to scan for miRNA binding sites assumes that the miRNA-binding sites are located in the 3' UTR region. However, the observation that binding sites in the coding region *(78)* can also mediate translational suppression leaves it unclear whether this assumption is warranted.

2.2. Scan for Potential miRNA Target Sites

Recently, several computational methods were developed to predict animal miRNA target sites *(9–11,37,41,42,79–84)*. The computational methods, such as DIANA-microT *(41)*, MicroInspector *(85)*, miRanda *(9)*, and RNAhybrid *(80)*, are readily available to the scientific community (**Table 1**). In general, computational methods that predict miRNA targets are based on the complementarity between the miRNAs and the 3' UTRs of their potential targets, with an emphasis on the pairing around positions two to eight at the 5' end of the miRNA, and on the thermodynamics of the association between the miRNA and its target. The principles behind the various computational approaches are similar and are based on experimentally observed pairing patterns between animal miRNAs and their target genes. However, the methods differ in important details, specifically in the scoring schemes that are used to detect, score, and rank the target sites. For instance, methods such as DIANA-microT, MicroInspector, miRanda, and RNAhybrid allow occasional mismatches and G:U basepairs between the complete miRNA sequence to the 3' UTR sequence *(9,41,80,85)*. PicTar and TargetScanS require a perfectly base-paired stretch of 6 to 7 nucleotides ("seed") at the 5' end of the miRNA and its target site *(40,84)*. The cooperative regulation of genes by multiple miRNA target sites is also incorporated by miRanda and PicTar in their cumulative scoring schemes. In addition, miRanda makes use of a weighted scoring scheme to detect target sites that require binding at the 3' end of the miRNA *(86)* and target sites that require perfect base pairing at the seed region *(34)*.

Among these methods, the miRanda software was the first open-source software made available. It is one of the commonly used tools to scan for miRNA target sites *(9,10,30, 33,86,87)*. The miRanda (v1.9) algorithm searches for stretches of complementary nucleotides that correspond to a double-stranded antiparallel duplex between a given miRNA and its target sequence. The complementarity scores at positions two to eight are multiplied by a scaling factor (currently set to 4.0), so as to reflect the observed 5' to 3' asymmetry in miRNA–target interaction. The method also calculates the free energy of duplex formation using the Vienna package *(9)*. miRanda may be used through a graphical user interface on Mac OS X via the command line on UNIX-based systems, and via a web browser. The software also provides ways to adjust the parameters used for detecting candidate target sites.

2.3. Select Conserved Target Sites

Biologically relevant animal miRNA regulatory sites generally do not have a statistically significant level of pairing between the miRNA and its target. Therefore, additional information, such as evolutionary conservation of the candidate target sites in

Table 1

Resources for the Prediction of Human MicroRNA Targets[a]

Resource	Availability	Targets	Program	Server	References
DIANA-MicroT	http://diana.pcbi.upenn.edu/DIANA-microT			✓	*(41)*
MicroInspector	http://mirna.imbb.forth.gr/microinspector			✓	*(85)*
miRanda	http://www.microrna.org	✓	✓	✓	*(9,10,30)*
MovingTargets	Program is made available on request		✓		*(95)*
PicTar	http://pictar.bio.nyu.edu	✓			*(40)*
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid		✓	✓	*(80)*
TargetScan	http://genes.mit.edu/targetscan/index.html	✓			*(38,84)*

[a]The checked lists of resources are freely accessible. Targets, predicted mammalian miRNA targets; program, a downloadable program to scan for miRNA target sites in 3' UTRs; and server, a computational server to identify candidate miRNA targets.

distantly related species, could be useful to limit the list of candidate targets. For instance, 12 of the 22 nucleotides of the *let-7* target site in human *NRAS* are conserved in dog, mouse, rat, and chicken (**Fig. 1B**). The use of evolutionary conservation may aid in indirectly incorporating other unknown requirements for miRNA–target recognition.

To analyze the evolutionary conservation of the human miRNA–target interaction in other species, such as mouse or chicken, the orthologous target gene and the orthologous miRNA must be identified. The Ensembl web interface *(77)* and the National Center for Biotechnology Information HomoloGene *(76)* are valuable resources to identify homologs of human genes in several other species (*see* **Note 3**). The orthologs of human miRNAs in other genomes may be obtained from RFAM *(88)* or identified *(89)* by an alignment search program, such as BLAST, the basic local alignment search tool *(76,90)*. Once the ortholog of a candidate target human gene is identified, the simplest approach to analyze the evolutionary conservation of the target site is to check whether the 3' UTR of the orthologous gene also contains target sites for the given miRNA ortholog (**Subheading 2.2.**). More stringently, multiple sequence alignment programs, such as MAVID *(91,92)*, can be used to align the 3' UTR sequences (**Fig. 1B**), or precomputed multiple sequence alignments may be used (e.g., University of California, Santa Cruz genome browser *[93]*). The multiple sequence alignment tool can then be used to analyze the positional conservation of the target sites in the 3' UTR sequences.

Current methods for prediction of miRNA targets generally rely on conservation filters to enhance the significance of their predictions. However, some of the biological processes that require a specific miRNA–target interaction may be specific to humans *(8)*; such miRNA–gene interactions are evolutionarily not conserved in other species. Therefore, other considerations are essential to add confidence to the list of candidate miRNA–target interactions (*see* **Notes 4–7**).

3. Notes

1. Distinct strategies (e.g., cloning of miRNAs) used to clone a given miRNA may cause variations in the reported sequence composition of the miRNA at its terminal ends. The computational prediction methods are sensitive to sequence variations at the 5' end of the miRNA. Hence, the sequences of mature miRNAs must be carefully selected, preferably based on the most frequently observed mature form.
2. The average length of mammalian 3' UTRs is approx 1000 nucleotides. Therefore, when 3' UTR sequences are arbitrarily assigned by extracting downstream sequences of the stop codon (**Subheading 2.1.**), it is prudent not to extend them far beyond 1000 nucleotides, so that the number of false predictions is minimized.
3. In some cases, for a given gene, computational tools (**Subheading 2.3.**) may not identify the functionally equivalent orthologous gene or may predict several orthologs. In such cases, additional information must be used to identify orthologs.
4. The miRNA and the gene must be coexpressed to interact physically. Therefore, the coexpression pattern of the miRNA and the potential target gene may be useful to increase the accuracy of the predictions. However, if the miRNA directs the degradation of the target mRNA, their expression patterns will be anticorrelated.
5. A number of experimentally validated miRNA targets contain multiple predicted miRNA-binding sites. Thus, in addition to evolutionary conservation of the target sites, the presence

of multiple sites in a 3' UTR may also be used as a confidence measure to evaluate target genes. However, single miRNA-binding sites are also known to confer regulation of mRNAs in vivo, hence, they should not be disregarded.

6. The predicted regulation of multiple mRNAs by a given miRNA in a common biological process is a useful feature for identifying compelling miRNA target candidates. For example, *miR-196* is thought to repress the expression of several of the *HOX* genes during vertebrate development ***(3,44)***.
7. The secondary structure of the mRNA can be calculated using RNA folding programs ***(94)***. The information regarding accessibility of the target site to the miRNA based on the mRNA secondary structure may be used to eliminate false predictions ***(42,45)***.

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