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Computational methods to identify miRNA targets

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Abstract

MicroRNAs (miRNAs) are short RNA molecules that regulate the post-transcriptional expression of their target genes. This regulation may take the form of stable translational repression or degradation of the target transcript, although the mechanisms governing the outcome of miRNA-mediated regulation remain largely unknown. While it is becoming clear that miRNAs are core components of gene regulatory networks, elucidating precise roles for each miRNA within these networks will require an accurate means of identifying target genes and assessing the impact of miRNAs on individual targets. Numerous computational methods for predicting targets are currently available. These methods vary widely in their emphasis, accuracy, and ease of use for researchers. This review will focus on a comparison of the available computational methods in animals, with an emphasis on approaches that are informed by experimental analysis of microRNA:target complexes.

Keywords

miRNA; miRNA Target Prediction; computational methods

1. Introduction

MicroRNA (miRNA) genes are transcribed much like their protein-coding counterparts, and undergo additional processing steps in the nucleus and cytoplasm to produce a short (17–22 nucleotide) single-stranded “mature” RNA molecule¹. Mature miRNAs are embedded within a larger RNA-protein complex collectively known as the RNA-induced Silencing Complex (RISC). Within RISC, miRNAs provide the specificity that selects for individual gene targets through partially complementary base-pairing between the miRNA and the mRNA transcript of its target gene. This target selection then brings the mRNA transcripts within range of the RISC effector proteins, the principle components of which are a miRNA-specific Argonaute protein and a GW182 protein, although there may be many other players². Binding of a miRNA to its target usually results in repression of target mRNA expression through translational inhibition or mRNA degradation. These are not mutually exclusive outcomes, and the mechanisms that determine the outcome of target repression remain unclear³. Finally, in specialized cellular contexts, studies suggest that miRNAs can enhance the expression of their targets⁴, indicating that the nature and potency of any particular microRNA:mRNA interaction may be governed by factors that have yet to be identified.

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MiRNA-mediated regulation plays an important role in animal development and disease. In particular, it has become clear that miRNAs are key components of diverse regulatory networks in animals⁵. This importance was reinforced by the finding that mutations disrupting miRNA biogenesis result in early embryonic lethality in many organisms. Moreover, it has been less than a decade since miRNA genes were found to be expressed in all higher animals and, in that short time, the number of human diseases linked to misregulation of miRNAs has ballooned⁶.

To fully understand the importance of miRNAs in animal development and disease, it is necessary to get an accurate list of the target genes that undergo miRNA-mediated regulation *in vivo*. The set of experimentally validated miRNA targets is steadily growing but still represents what is likely a small fraction of the total number of genes regulated by miRNAs⁷. While computational target prediction methods are improving in their accuracy, the lack of overlap between the different methods⁸ suggests that the field of target prediction still has a long way to go before converging on the set of rules necessary to identify all functional miRNA targets. This review will focus on the parameters of functional miRNA targets that have been illuminated by experimental studies of miRNA interactions, shown in Figure 1, with reference to those target prediction methods that emphasize this parameter.

2. Parameters of Functional miRNA binding Sites

2.1 Topology of binding sites: Primacy of the seed

A fundamental challenge to computational prediction of microRNA targets is the incomplete base-pairing of microRNAs to their target sites. Early studies focused on the miRNA seed region, defined as miRNA positions 2–7 and shown in Figure 1a, as the primary determinant of miRNA target recognition. This “seed rule” for target recognition could be seen for the first identified microRNA target⁹ as well as in early computational analyses that showed a greater signal for preferential conservation of motifs that are perfectly complementary to miRNA seeds as opposed to imperfectly complementary motifs¹⁰. Experiments to directly test the seed rule began with two studies showing that mismatches introduced into the miRNA seed recognition elements resulted in weaker regulation of miRNA reporters as compared to reporters with perfectly paired seeds^{11,12}. Finally, recent studies of the crystal structure of an archeal Argonaute protein suggest that the miRNA 5' seed region is constrained to lie along one surface of Argonaute, presenting the seed for binding to complementary targets and preventing large bulges that would cause the duplex to deviate from a helical structure¹³. Collectively, these studies argue that miRNA target prediction methods should employ specific rules for the number and type of imperfections allowed within the 5' seed region. However, the determination of optimal seed rules has become a major point of contention within the field of target prediction. Many methods have chosen to constrain their target predictions to perfectly matched seeds, as stringent seed rules radically reduce the total number of predicted miRNA target sites. However, requiring perfect seed matches will miss the many genetically identified miRNA targets that are regulated through binding sites with imperfectly matched seed recognition elements. Functional targets with imperfectly matched seeds have been validated both for endogenous miRNA binding sites (reviewed in Smalheiser & Li¹⁴), and for endogenous targets that normally have perfect sites, but where reporters have been designed to introduce imperfections in the seed region¹⁵.

The first published target prediction methods, PicTar¹⁶, TargetScan¹⁰, and Miranda¹⁷, emphasized the identification of conserved matches to miRNA 5' seeds in the 3' UTRs of target transcripts. These methods varied from strict requirements of a perfectly matched seed (TargetScan), toleration of a single mismatch in the seed region (PicTar), or toleration of multiple imperfections in the seed (Miranda). Nearly every miRNA target prediction method

now includes some seed rule constraints that fall in a spectrum between the TargetScan and Miranda extremes. One notable exception is StarMir¹⁸, which will be discussed later.

While seed matching is the dominant topological constraint in miRNA target recognition, it is not the only constraint. It is clear that miRNA targets preferentially contain loops or bulges just after the 5' seed, presumably to avoid presenting an siRNA-like conformation to the miRNA Argonaute. RNA interference in animals is mediated through siRNAs that recognize perfectly complementary targets. In particular, siRNA targets contain long un-interrupted pairing that continues beyond small RNA positions 2–13. The siRNA target mRNAs are consequently degraded and, perfect matching to siRNA positions 9–13 appears to be crucial for catalyzing this reaction¹⁹. In contrast, miRNA targets in animals preferentially contain bulged regions opposite miRNA positions 9–13, just where an siRNA-like slicing reaction might occur. Accordingly, some methods (DIANA-microT20, FindTar21, and SBM22) score the quality or identity of nucleotides within this bulge or “loop” region.

In addition to providing structural support for mandatory seed pairing, the Argonaute crystal structures also suggested that the 3' end of the miRNA is relatively unconstrained, and free to base-pair with target sequences. Uninterrupted base pairing to the 3' part of the miRNA could theoretically strengthen a miRNA:target hybrid, but any bulges or wobbles in this region should not present a conformational challenge to the Argonaute portion of RISC. Accordingly, there is experimental evidence that a well-paired 3' region can compensate for weaker binding of the 5' seed region in synthetic miRNA reporter targets¹². Taking all of these topological considerations into account, some groups score the entire miRNA binding site topology (DIANA-microT20 and SBM22).

2.2 Phylogenetic Conservation of Binding Sites

Next to seed matching, conservation of miRNA binding sites in the UTRs of orthologous genes is the most commonly employed parameter in target prediction. The rationale for employing this parameter is to identify functional miRNA:target relationships that are important to animals, such that positive selection is acting upon the miRNA target sequences. Calculations to distinguish purifying selection from neutral evolution of a given sequence can be quite complex. So, for miRNA target predictions, conservation filters often take the form of a simple search for perfect miRNA seed matches within aligned blocks of pre-computed multiple sequence alignments. However, not all functionally important miRNA targets are conserved within aligned blocks. This is most prominently displayed in worms, where many targets of the *C. elegans let-7* miRNA were identified genetically; 40% of these do not have binding sites that lie within aligned blocks, but do show conservation of a functional interaction through sites for the *let-7* miRNA elsewhere in the 3' UTRs of orthologous genes²³. Similar results were found through a meta-analysis of experimentally verified targets in mammals, which showed that about 30% of mammalian targets are not conserved in alignment²⁴.

The methods that emphasize phylogenetic conservation in their predictions vary greatly in how this parameter is implemented. Many methods require conservation of the seed match within an aligned block in pre-computed multi-species alignments (DIANA-microT²⁰, miRanda¹⁷, PicTar¹⁶, TargetRank²⁵, and TargetScanS²⁶). Others employ more elaborate calculations that consider evolutionary distance and branching when scoring the degree of conservation (Stark et al., BLS²⁷ and EIMMO²⁸). Notable exceptions include the miRanda-Mirbase²⁹, mirWIP²³, and rna22³⁰ target prediction methods, which simply require that suitable binding sites for a miRNA pass filters in orthologous genes, but do not require that these binding sites lie within aligned blocks.

2.3 Thermostability of the binding sites

The formation of a stable miRNA:target duplex *in vivo* must be governed, at least in part, by thermodynamic considerations. One simple view is that stable miRNA:target duplexes will remain paired longer than unstable duplexes, giving time for the RISC proteins to carry out their enzymatic activities. An alternate view of the importance of stable hybrids states that conditions are favorable for forming many miRNA:target hybrids if the system gains more energy by forming the duplex than it expends on the creation of that duplex. A highly stable (or favorable) RNA duplex is represented as having a very low minimum free energy of hybridization (MFE). There are many software packages available for calculating the MFE of a miRNA:target duplex (e.g., FASTH31, RNAfold32, and RNAhybrid33). While any calculated negative MFE represents a theoretical net gain in energy for the system, the actual energetics of the reaction will be governed by parameters that are usually unavailable for these calculations, including: temperature, the relative concentration of miRNA and mRNA molecules, and the presence of proteins that facilitate or impede the reaction. Despite these caveats, it is clear that the predicted stability of miRNA:target duplexes is a relevant parameter for identifying functional binding sites 23,31,33. There are five currently available methods that focus their prediction schemes primarily on thermodynamic considerations: FastH³¹, MicroTar³⁴, PITA³⁵, RNAhybrid³³, and StarMir¹⁸. Other methods that include thermodynamic considerations, among other parameters, include MicroInspector³⁶, miRanda¹⁷ and miRanda-Mirbase²⁹, mirWIP²³, and PicTar¹⁶.

2.4 Accessibility of target binding sites

In order to form a miRNA:target duplex, RISC-associated miRNAs must gain access to the binding sites on the mRNA transcript. This access could be accomplished through structural constraints on the target mRNA transcript that act to maintain the local availability of the miRNA binding sites. Alternatively, there could be active disruption of local 3' UTR structure by the RISC complex or other proteins. Computational analysis of RNA secondary structures around miRNA binding sites supports both models. Many groups have shown that miRNA binding sites tend to reside in regions of the 3' UTR that are predicted to be structurally accessible^{2,18,35,37–39}. For miRNA binding sites in regions that are not predicted to be structurally accessible, MFE calculations that include the energetic cost of disrupting local pairing within the binding site can often distinguish functional miRNA binding sites from non-functional sites^{18,35}.

While accessibility is clearly an important parameter for many miRNA targets, recent reports have raised an important exception. There may be a class of targets for which the predicted mRNA transcript secondary structure does not correspond well with the presence of functional binding sites *in vivo*. Analysis of miRNA targets identified by immunoprecipitation of RISC components, which preferentially identifies stable miRNA:target duplexes, shows a strong preference for binding sites in regions of the UTR predicted to be structurally accessible^{23,39,40}. However, miRNA targets identified by changes in target mRNA level, following knockdown of RISC components or mis-expression of miRNAs, do not show an enrichment for local structural accessibility^{39,40}; this “transcriptomics” method preferentially identifies destabilizing miRNA:target interactions. Whether or not the binding sites are predicted to be structurally accessible, the miRNAs must somehow gain access to their targets. Therefore, it is likely that the mRNA-regulated targets, which are not predicted to be structurally accessible, contain other elements in their UTRs that alter the local secondary structure in a way that is difficult to predict based on sequence information alone. This could take the form of local cooperative binding with other miRNAs or RNA binding proteins. Alternatively, a global change in UTR secondary structure might result if, for instance, a sufficiently large number of post-transcriptional regulators are bound to the transcript.

The target prediction methods that include structural accessibility as a miRNA binding site parameter vary widely in the size of the sequence window used for calculating that accessibility, in the way those calculations are performed, and in the way those calculations are included in the predictions. TargetScanS²⁶ and TargetRank²⁵ look at the percent of A/U nucleotides adjacent to the seed match of the binding site, which correlates with the local accessibility. StarMir¹⁸ and PITA³⁵ both calculate the accessibility of the binding site, incorporating this predicted accessibility into an energy parameter referred to as ΔG_{total} or $\Delta\Delta G$, respectively. mirWIP²³ uses the ΔG_{total} calculation from StarMir, and also scores the accessibility of a 25 nucleotide window upstream of the binding site.

2.5 Clustering and Position of Binding Sites within UTRs

The parameters discussed above have all focused on characteristics of individual binding sites. The identification of functional individual binding sites is a key goal in target prediction, but this goal is best served by recognizing that some information about functional miRNA targets may lie outside of the binding sites. One example of this includes clustering of binding sites for the same miRNA in the 3' UTRs of target genes. Specifically, many targets contain tandem binding sites, spaced within about 50 nucleotides of each other, which seem to act synergistically to regulate their targets (e.g., *cog-115*). Experimental assays have shown that tandem binding sites appear to have a synergistic effect on target regulation^{25,26,41}, even when the binding sites respond to distinct miRNAs⁴². The methods that include a boosted score for cooperative regulation include TargetScanS²⁶, TargetRank²⁵ and PicTar⁴². Most other methods include the notion of clustering for miRNA binding sites by simply combining the scores of multiple individual binding sites into a total score for miRNAs on a target.

In addition to clustering of miRNA binding sites near each other, some groups have reported increased efficacy of miRNA binding sites positioned at either end of the 3' UTR²⁶. The proposed explanations include: interference with translational machinery that needs access to the transcript stop codon and to its poly-adenylated 3' end, or a proposed likelihood that the ends of the 3' UTR would be more structurally accessible. Importantly, this position effect was also seen in recent high throughput HITS-CLIP⁴³ assays that sequenced thousands of individual endogenous miRNA binding sites. So far, TargetScanS²⁶ and MirTarget2⁴⁴ include scores for relative UTR position of the binding sites. Lastly, recent efforts to extract and sequence individual miRNA binding sites show evidence that many miRNAs bind to regions in the 5' UTR or coding sequence of the transcript⁴³. Because there were few prior reports of miRNA targets outside of 3' UTRs^{30,45,46}, very few target prediction methods report predictions in the 5' UTR or coding sequence in published databases. An exception is rna22³⁰. Future refinements to miRNA target prediction methods will need to include the potential for binding sites outside of the 3' UTR.

2.6 Interaction of miRNAs with RNA-binding proteins

The above section dealt with the possibility that multiple miRNAs could function cooperatively on the same target mRNA. It is also possible that miRNAs could interact with other forms of post-transcriptional regulation. A few examples from recent studies are highlighted here. The RNA binding protein DND1 binds to sequence elements adjacent to miRNA binding sites, antagonizing miRNA-mediated regulation of these transcripts⁴⁷. Poly(A) binding protein has been shown to both antagonize miRNA-mediated regulation⁴⁸ and to promote miRNA-mediated de-adenylation⁴⁹. This theme of context-dependent changes in activity of RNA binding proteins has also been seen in interactions between miRNAs and AU-rich element (ARE) binding proteins. Several groups have reported synergistic interactions between miRNAs and ARE binding proteins^{50,51}; in contrast, one ARE binding protein was shown to mediate stress-related de-repression of a miRNA target⁵². Finally, computational analyses of experimentally identified miRNA targets have shown a significant enrichment for ARE binding

elements in the UTRs of targets that are degraded in response to miRNAs^{39,53}, suggesting that the co-occurrence of miRNA binding sites and ARE sequences may be indicative of degraded miRNA targets. Collectively, these studies suggest a complex interplay between many different modes of post-transcriptional regulation. However, It remains to be seen whether the existence of predicted RNA binding protein response elements can bolster the predictions for miRNA response elements. As such, an analysis of RNA-binding protein motifs has not been included in any miRNA target prediction method.

2.7 Inferring Parameters from Machine-Learning Tools

Rather than enumerate specific rules for predicting miRNA targets, many groups employ machine-learning algorithms to identify the parameters of functional targets based on a list of gold standard true and false positive miRNA targets. Machine learning algorithms automatically search through multiple binding site criteria to identify the parameters most predictive of true miRNA targets. The database of validated interactions most often used to train these learning tools is TarBase⁵⁴, a web searchable database of targets manually curated from the literature. The machine learning algorithms most often employed include Bayesian classifiers (e.g., NBmirTar⁵⁵), genetic programming algorithms (e.g., TargetBoost⁵⁶), or support vector machines (e.g., miRTif⁵⁷, MirTarget²⁴⁴, MiTarget⁵⁸, and TargetMiner⁵⁹). Typically, these machine learning tools are used on top of other miRNA prediction methods, as a secondary filter. While an automatic determination of important miRNA parameters sounds ideal, these algorithms suffer from an important drawback. Depending on the size, diversity, and quality of the database used in training the algorithm, it is possible for these methods to overfit their parameter set, such that characteristics are identified among a subset of validated targets that are not relevant to general target identification.

2.8 Co-expression of miRNAs and their Targets

One recent development in miRNA target prediction involves analyzing the expression patterns of miRNAs and their predicted targets to filter out unlikely interactions. Typically, these methods begin with a dataset of target predictions from one or more of the methods discussed above. They then use experimental evidence of co-expression of miRNAs with their targets to select the set of interactions that are more likely to occur in cells. The general rationale behind this approach is that prediction methods produce many false predictions that look like strong miRNA target candidates, but the interactions do not take place *in vivo* simply because the miRNA and its predicted target are never present in the same cell. Accordingly, there have been recent experimental methods to identify co-expressed miRNAs and targets^{60–62}.

In contrast, if the miRNA causes destabilization of its mRNA targets, the level of its target mRNAs might show an anti-correlation with the abundance of that miRNA. That is, when the miRNA is abundant in a particular tissue or at particular timepoints, the target mRNAs will be efficiently degraded and will show decreased relative expression. This idea of miR/target expression anti-correlation, originally shown by Stark et al.⁶³, has been the most widely incorporated into miRNA prediction methods, since these can rely on published data of miRNA and mRNA expression levels. Methods that maintain online databases of these predictions include: GenMir++⁶⁴, MMIA⁶⁵, HOCTAR⁶⁶, and miRGator⁶⁷.

3. Availability of target prediction data and software

The variety of techniques employed for miRNA target prediction is mirrored by the wide variation in the availability of the predictions themselves. While searchable web interfaces for target prediction in multiple genomes would be the easiest way for most users to access the target predictions, this is not available for many of the target prediction methods. Indeed some methods are only available upon request from the authors of the original publication – these

methods will not be discussed here. The methods maintaining a searchable online database of their predictions in multiple species are listed in Table 1. There are many more methods with searchable online databases, also listed in Table 1, which do not include predictions for most species.

4. Conclusions

Experimental analysis of miRNA targets appears to be converging on a set of parameters that are important for functional targets. This includes seed topology, accessibility of the binding sites, thermodynamic stability of the binding sites, clustering of the binding sites with each other and other RNA binding proteins, and co-expression of the regulating miRNAs with their targets. At the same time, the large number of target prediction methods (over 30 are reviewed here, with only those maintaining searchable online databases listed in Table 1) and the large differences in how each method chooses to implement parameter sets into their scoring schemes results in very little overlap in the predicted target lists⁸. It may be that each of these different methods is selecting a subset of real miRNA targets such that different algorithms are better at picking particular classes of targets. This is consistent with recent reports that miRNA binding site parameters are differentially enriched in stable versus unstable interactions^{39,40}, although it contrasts with two other reports that simultaneously measured protein and mRNA levels following manipulation of miRNA levels and found no significant differences^{68,69}. The recent development of high throughput methods to extract and sequence individual miRNA binding sites, HITS-CLIP⁴³, implies that we will soon have extremely large datasets of individual miRNA binding events from which to discover new parameters important for miRNA targeting specificity. Presumably, these large, high quality experimental datasets will lead to a rapid convergence of the miRNA prediction methods. However, these datasets will not render computational target prediction obsolete, as there are likely to be many targets that will not be amenable to high-throughput methods for technical or biological reasons.

Currently, the best target prediction method will likely depend on the interests of the researcher. While most researchers would prefer a short list of high-confidence targets to select for experimental testing, there are drawbacks to using only the most selective target prediction methods. Biologically important targets may not reside among the prediction lists of the more stringent methods (*let-7* targets *lin-41* and *let-60/RAS* are examples of deeply conserved, biologically important targets that do not appear on most prediction lists). Once there is experimental evidence to suggest an interaction, such as co-expression of the miRNA and target or evidence of UTR-mediated regulation of a transcript, it may be wiser to look through many prediction lists or to use methods that have more inclusive rules for seed matching and conservation.

Once high confidence miRNA binding site parameters have been identified, the next important goal for miRNA target prediction might involve predicting the outcome of miRNA-mediated regulation. Early computational analyses show some progress in identifying parameters that are associated with particular outcomes of miRNA mediated-repression^{39,40}. However, the complicated, context-dependent interplay between miRNAs and other RNA binding proteins hints at a network of post-transcriptional regulators that may require detailed individual study rather than simple generalizations. For instance, there may be many factors that are indirectly bound or only bound in specific cellular contexts, which may alter the outcome of any particular interaction. These additional factors may alter the accessibility of target binding sites, as in the case of DND1, discussed above. Other factors might alter the efficacy and/or nature of miRNA-mediated regulation, as seems likely for the TRIM-NHL proteins, the first identified miRNA co-factors^{70,71}.

Abbreviations

CLIP	cross-linked immuno-precipitation
microRNA	miRNA
RISC	RNA-induced Silencing Complex
UTR	untranslated region

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Figure 1. Parameters of functional microRNA Targets

Shown in panel (A) is a cartoon of microRNAs and RNA binding proteins interacting with a target mRNA. The parameters listed here have experimental motivation for their importance in functional microRNA targets. These include (1) parameters of the individual binding sites detailed in panel B, (2) the presence of cooperating miRNAs, and (3) the presence of interacting proteins. Panel (B) shows a detailed inset of a particular miRNA binding site including: (1a) the seed region, (1b) the thermostability of the duplex (MFE), and (1c) the structural accessibility of regions around the miRNA binding site. Parameters not shown in this figure include conservation of binding sites and the relative expression level of miRNA to target mRNA.

Methods with Searchable Web interfaces

Table 1

The methods listed in this table make their predictions easily accessible by providing a searchable web interface of genome-wide predictions in at least one species. The first column gives the prediction method and a website address for accessing the predictions. Checks in the following columns indicate the binding site parameters incorporated into that microRNA target prediction method. The last column indicates the species for which targets are made available. An asterisk (*) in the column labeled "Expression/Pathway" indicates that these parameters are not included in the main target prediction method, but links are provided on the group website to include expression or pathway analysis. Finally, the miRGator algorithm begins with pre-computed prediction lists downloaded from the miRanda, PicTar and TargetScan websites rather than performing de novo predictions, so the parameters used by this "method" will be reflected by the parameters of those three methods.

Method	Seed	Accessibility	Energy	Conservation	Clustering/Position	Expression/Pathway	species
DIANA-microT http://diana.cslab.ece.ntua.gr/microT	✓			✓		*	human, mouse
EIMMO http://www.mirz.unibas.ch/EIMMO2	✓			✓		*	human, mouse, fish, fly, worm
miRanda/miRbase http://www.microna.org http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5	✓		✓	✓			human, mouse, fish, fly, worm
miRGator http://genome.ehwa.ac.kr/miRGator		(miRanda, PicTar, TargetScan)				✓	human, mouse
mirWIP http://www.mirtargets.org	✓	✓	✓	✓			worm
MirTarget2 http://mirdb.org/mirDB/	✓		✓	✓	✓		human, mouse
PicTar http://pictar.mdc-berlin.de	✓		✓	✓		*	human, mouse, fly, worm
PITA http://genie.weizmann.ac.il/pubs/mir07/index.html	✓	✓	✓	✓			human, mouse, fly, worm
TargetBoost https://demo1.interagon.com/targetboost	✓		✓				worm
TargetRank http://genes.mit.edu/targetrank	✓			✓	✓		human, mouse
TargetScanS http://www.targetscan.org	✓			✓	✓		human, mouse, fly, worm