Biochemical and Biophysical Research Communications 445 (2014) 780–784

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Refining microRNA target predictions: Sorting the wheat from the chaff

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ARTICLE INFO

Article history:
Available online 10 February 2014

Keywords:
Bioinformatics
microRNA target prediction
gene regulation

ABSTRACT

microRNAs are short RNAs that reduce gene expression by binding to their targets. The accurate prediction of microRNA targets is essential to understanding the function of microRNAs. Computational predictions indicate that all human genes may be regulated by microRNAs, with each microRNA possibly targeting thousands of genes. Here we discuss computational methods for identifying mammalian microRNA targets and refining them for further experimental validation. We describe microRNA target prediction resources and procedures and how they integrate with various types of experimental techniques that aim to validate them or further explore their function. We also provide a list of target prediction databases and explain how these are curated.

1. Introduction

microRNAs (miRNAs) are short, ~22 nucleotide long RNAs that reduce gene expression, usually by binding to the 3′ untranslated region of target mRNAs. miRNAs guide a protein complex called RNA-induced silencing complex (RISC) to specific mRNA target sites called microRNA responsive elements (mREs). miRNAs were first discovered in 1993 during an analysis of larval developmental timing in the worm *Caenorhabditis Elegans*, where a 22-nucleotide RNA regulated protein abundance of LIN-14 [1]. First regarded as a Nematode-specific RNA family, it was only in 2000 that another microRNA, let-7, was characterized and identified in other species. In 2002, Eric Lai compared the sequences of 11 microRNAs to the K box and Brd Box motifs that were known to mediate post-transcriptional regulation in Drosophila. He demonstrated that the first eight nucleotides, now called the seed region, of miRNAs, were perfectly complementary to these motifs and concluded that this complementarity may be essential in post-transcriptional regulation by microRNAs [2]. This simple bioinformatics analysis established one of the strongest predictive features used in target prediction to date and was the basis for numerous algorithms that enabled the explosion of miRNA functional characterization.

To date over 1000 miRNAs have been identified in Humans, hundreds of which are associated with major biological processes including cell proliferation and differentiation, development and disease. As such miRNAs are arguably one of the most important classes of functional RNAs. However, the rules governing miRNA target recognition are not fully understood and may vary for each miRNA–mRNA pair. Computational approaches that can test various models of miRNA binding and predict target sites are therefore essential to understanding the function of microRNAs.

In this review, we will outline the major concepts underlying the most popular target prediction algorithms, discuss the context in which these algorithms are optimal and provide simple techniques to refine them. A list of readily available miRNA target prediction algorithms and databases is given in Table 1.

2. Materials and methods

All the techniques and approaches discussed here are available online.

3. Results

An ouroboric relation has always existed between the computational prediction of miRNA targets and their experimental validation. Experimental approaches are often designed and interpreted by using computational predictions and these in turn have been created and refined based on experimental output. It is therefore difficult to obtain an independent experimental dataset to compare the efficiency of target prediction algorithms and until recently, experiments were not designed to discover novel targets that were not predicted by the algorithms.

Experimental approaches can be divided into two categories based on whether they directly discover mRE sites or whether they

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http://dx.doi.org/10.1016/j.bbrc.2014.01.181
0006-291X/

Table 1
MicroRNA target prediction tools and databases.

Algorithm	Approach	References	Resource
miRWalk	Database	Dweep et al. (2011)	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html
Stark et al.	Complementarity	Stark et al. (2003)	http://www.russell.embl.de/miRNAs goes to www.russelllab.org/miRNAs/
miRanda MiRBase	Complementarity	Enright et al. (2003)	http://microrna.sanger.ac.uk goes to www.mirbase.org/
miRanda	Complementarity	[10]	http://www.microma.org
DIANA microtest	Thermodynamics	Kirakidou et al. (2004)	http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi/
RNAHybrid	Thermodynamics & statistical model	[9]	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid
Target scan	Seed complementarity	[7,17]	http://www.targetscan.org
PicTar	Thermodynamics	[20]	http://pictar.mdc-berlin.de/
TarBase	Experimentally validated targets	Sethupathy et al. (2006), Verguvlis et al. (2012)	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index
miRGen++	Bayesian analysis	Huang et al. (2007b)	http://www.psi.toronto.edu/genmir
GenMiR++	Expression anti-correlation	Huang et al. (2007)	www.psi.toronto.edu/genmir/
PITA	Complementarity	[11]	http://genie.weizmann.ac.il/pubs/mir07/
MiRtage2 MiRDB	Support vector machine	Wang and El Naqa (2008)	http://mirdb.org
mimiRNA	Expression anti-correlation	[3,18]	http://mimirna.centenary.org.au/mep/formulaire.html
Lasso-mir.R	Expression anti-correlation	Lvetal (2011)	http://biocompute.bmi.ac.cn/CZlab/alarmnet
multiMitar	Support vector machine	Mitra et al. (2011)	http://www.isical.ac.in/~bioinfo_miu/multimitar.htm
ComiR	Combined seed algorithms	Coronnello et al. (2012)	http://www.benoslab.pitt.edu/comir/
TaLasso	Expression anti-correlation	Muniategui (2012)	http://talasso.cnb.csic.es/

indirectly discover genes regulated by miRNAs. Indirect experimental approaches usually consist in observing the effect of perturbed miRNA expression on mRNA or protein output as measured by high throughput techniques such as microarrays or mass spectrometry. These indirect approaches suffer from knock-on effects where the miRNA of interest directly targets a gene that in-turn regulates the expression of numerous other genes that do not contain mREs [3]. Direct experimental approaches establish the existence of a miRNA–mRNA binding site either by isolating and characterizing the miRNA–mRNA duplex or by introducing an mRE into a reporter gene and measuring how the reporter protein levels are affected by changes in miRNA expression. For any given miRNA, These approaches are generally restricted to a dozen of mREs that have already been predicted computationally and are of restricted use in elucidating novel mechanisms of interaction between miRNAs and their mREs. Recently progress has been made on genome-wide direct experimental approaches that use high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) [4] and photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) [5]. These techniques can identify novel sites of interaction between miRNAs and their mRNA targets but lack sufficient resolution to perfectly define the mRE. Despite sometimes contrasting results between these different experimental approaches, the wealth of data they have produced converge on three fundamental characteristics of the miRNA-target interaction described below and in Fig. 1A.

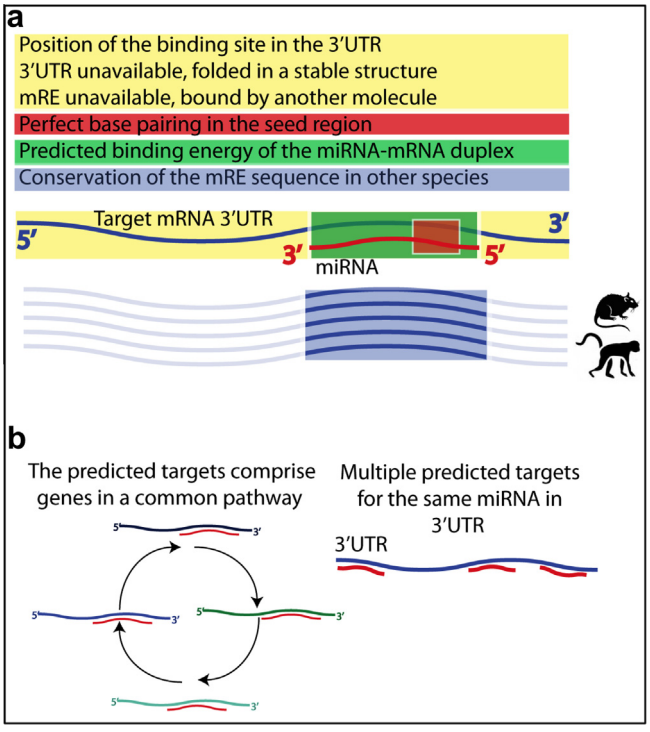

Fig. 1. Commonly used criteria for predicting miRNA targets. (A) Criteria based on the binding properties of miRNAs and their targets (colours match corresponding text in figure). (B) Two criteria based on the biological function of a miRNA.

3.1. Seed pairing

The first fundamental characteristic extracted from experimental data was the requirement for perfect Watson–Crick pairing between nucleotides 2 and 8 at the 5′ end of the miRNA and the target mRNA. This region is called the 'seed' sequence and is the starting point for many current target prediction algorithms. Different types of seeds sites have been identified based on their length and number of complementary bases (Fig. 2). The seed region is so central to our current understanding of miRNA targeting that regions of the miRNA–mRNA duplex are defined as either in the seed region or part of the out-seed region. Base complementarity in the out-seed region is thought to compensate for incomplete seed pairing, making the duplex more stable [6]. Recent studies have challenged the dogma that seed pairing is sufficient and necessary for miRNA targeting of mRNAs. For example, an extensive study of targets from CLIP-seq data have shown the existence of mREs with imperfect seed complementarity. A seven-nucleotide seed sequence that matches perfectly with the exception of a bulged-out G nucleotide corresponding to positions 5–6 of the miRNA was frequently found in mREs of neuronal microRNA 124 [4]. Interestingly, this study also showed that 27% of the identified

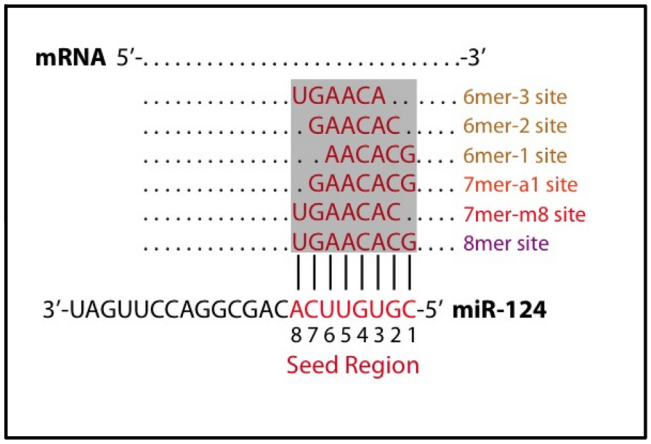

Fig. 2. Types of seed sequence matches. Positions in the seed region are numbered from 1 to 8 starting from the miRNA 5′ end. In the annotation of seed types (right), the 6, 7 or 8-mer refers to the number of complementary bases between the miRNA and its target; the 1, 2 or 3 site refers to the position at which the match starts in the seed. 7-mer m8 sites are composed of the seed match supplemented by a Watson–Crick match at seed position 8. 7-merA1 sites comprise the seed match with an additional adenine in the mRNA opposite seed position 8. 8-mer sites comprise the seed match supplemented by both the m8 and the A1 nucleotide matches.

mREs did not possess seed complementarity to the expressed miRNAs. Many miRNA–mRNA interactions are therefore not mediated through seed complementarity.

3.2. Sequence conservation of the target site between multiple species

Evaluating sequence conservation of predicted mREs between distantly related species can efficiently reduce the number of false positive predictions. Simply searching for conserved 7 nt matches in aligned regions of vertebrate 3′UTRs could successfully detect miRNA targets well above the noise threshold [7]. Because the majority of human coding genes have a high degree of conservation in the 3′UTR region [8], this approach can be used for discovering a large fraction of human miRNA targets. TargetScan for example initially searches for conserved seed pairing regions in 3′UTR alignments between 28 vertebrate species. This set of putative targets is then refined using a context score based on the target position in the 3′UTR and surrounding sequence composition. This approach is of little use in detecting species-specific binding sites or binding sites of species-specific miRNAs. TargetScan also provides non-conserved targets on their website.

3.3. Thermodynamic stability of the microRNA:mRNA duplex

Recent advances in the computational prediction of RNA secondary structures have allowed multiple groups to successfully predict stable RNA interactions. These algorithms have also been used to predict the stability of miRNA binding sites. Algorithms such as RNAhybrid [9] combine empirical data on RNA interactions with thermodynamic modelling to evaluate the probability that a given duplex exists. The stability of interactions are represented by Minimal Free Energy (MFE) calculations. This algorithm has been incorporated into miRNA target prediction algorithms to refine their results. miRanda [10], the first freely-available miRNA target prediction program, measures the thermodynamic stability between a miRNA and its putative target to increase prediction accuracy. Different scores for the C:G, A:U, and G:U pairs are used to measure stability with a requirement for more stable energy scores at the 5′ end of the miRNA. A user-defined threshold can then be set to eliminate potentially unstable duplexes. The PITA algorithm [11] also uses thermodynamic stability of a miRNA:mRNA duplex but compares it to the stability of local structures within the 3′UTR of the target mRNA. If the duplex is predicted to occur within a region of the 3′UTR that is already involved in a stable structure, the miRNA is less likely to bind to its target. This approach is limited by the accurate prediction of stable secondary structures, which becomes unreliable when considering long distance interactions and therefore larger RNA structures.

Other algorithms also consider the environment of the miRNA–mRNA duplex to assess target likelihood. The position of the target site in the 3′UTR as well as AU enrichment around the target can contribute to the accessibility of miRNA targets. The presence of binding motifs for proteins or other non-coding RNAs that overlap a predicted target may also affect a miRNAs ability to bind to it however no consideration is currently given to proteins which may potentiate interactions or colocalisation of duplexes. The efficiency of miRNA repression as well as targeting ability is affected by its environment in the 3′UTR. Certain mREs are more efficient when another mRE is located in close proximity, providing a multiplicative effect on mRNA repression [12]. The TargetScan algorithm analyses the 3′UTR environment of predicted targets and displays a context score that models their accessibility.

3.3.1. Using expression data to discover miRNA targets

Because miRNAs can inhibit gene expression by destabilizing mRNAs [13], a high level of endogenous expression or artificially induced expression of a miRNA should lead to observable inhibition of mRNA target expression. Certain approaches successfully combine mRNA expression with computational target prediction to increase prediction accuracy. For example, correlating miRNA and mRNA expression across numerous samples and selecting those pairs that are negatively correlated can successfully detect target genes [3]. Because this method is independent of any sequence analysis, it can be used to filter predictions made by any of the computational approaches discussed above. Another advantage of this approach is that it is not restricted to targets located in the 3′UTR. Although there are fewer published examples of miRNA targets in other regions of mature mRNAs, there may be numerous targets in the coding region that have been overlooked because the high level of sequence conservation in exons prohibits the use of sequence conservation-based techniques. However, the use of expression-based data alone cannot distinguish between direct targeting effects and knock-on or indirect effects. Moreover, the presence of a miRNA does not always induce observable changes in mRNA levels. The availability of Argonaute, one of the core protein components that contribute to miRNA stability and function can influence the efficiency of miRNA target regulation [14] and numerous studies have demonstrated that miRNAs can inhibit translation with little effect on mRNA stability [15]. In these cases, a high level of miRNA expression will not affect expression of bona fide targets.

3.3.2. Prediction refinement through meta-analysis

In addition to techniques aimed at identifying individual targets based on their sequence and binding context, a more global analysis that takes into account the biological function or pathway of miRNA targets can be used to enhance predictions (Fig. 1B). mirBridge [16] starts with a set of genes with a known common function and searches for enrichment of putative targets based on sequence analysis amongst this gene set. This approach is useful for experiments where a specific function or pathway is being dissected but may prove limiting in studies where a specific miRNA or mRNA is being analysed with no prior knowledge of its function.

Previous studies have demonstrated that neighbouring mREs can have a multiplicative effect on repression if they are sufficiently close to each other in the 3′UTR [12]. Because 3′UTRs have evolved to accommodate or avoid miRNA sites [17], it is very likely that multiple targets for the same miRNA could be favoured in a

context where strong repression is required. Recent analysis demonstrates that numerous mRNAs are targeted by the same miRNA at different sites within their 3′UTR [18]. This multi-targeting occurs at a significantly higher rate than expected. Focusing therefore on mRNAs that have more than one predicted site for the same miRNA in the 3′UTR can increase the signal to noise ratio for different algorithms [18]. Although this approach will eliminate numerous true target sites it has the advantage of producing a list of high confidence gene targets. This method requires the user to first select one or more target prediction programs and subsequently refine their results for multi-targeting. This last step can be performed on the mimiRNA website [19] (http://mimirna.centenary.org.au). The PicTar [20] algorithm uses a combinatorial approach that not only accounts for multiple binding sites of the same miRNA but also computes the likelihood that a sequence is bound by a combination of input miRNA sequences. Filtering predictions based on multi-targeting drastically reduces the number of predicted targets and, because they increase the probability of discovering true target genes, they are useful for studies where experimental validation of miRNA targets is necessary.

3.3.3. Target databases

Recent benchmarking of target prediction algorithms showed that a large portion of confirmed miRNA-target interactions could not be identified even by the most sensitive approaches [21]. Other studies have demonstrated that many algorithms produce conflicting results not only because of the differences in how miRNA–mRNA targets are modelled but also because different online algorithms use different reference gene and 3′UTR databases [18]. For these reasons, curated repositories of experimentally verified targets have become necessary to evaluate existing algorithms but also to develop novel approaches to detect targets. The curation of these databases is tedious because numerous experimental techniques can be used to validate targets and each of these techniques has its own variants. The luciferase reporter assay for example can be used to demonstrate repression of a mRE in a specific genomic and cellular context but rigorous controls are essential. These controls include whether mutagenesis of the binding site is performed to identify the seed region, whether the 3′UTR context of the original target was conserved or whether the cellular context is similar to the *in vivo* interaction. Moreover, publishers do not require that experimentally validated miRNA targets be uploaded to a common online resource prior to publication. Each database must therefore extract these data from articles published online. Here we give a brief description of some of the most popular databases.

3.4. mirRecords

The first version of miRecords [22] was released in 2008 and was last updated in 2013. miRecords contains manually curated experimental evidence for 2705 records of interactions between 644 miRNAs and 1901 target genes in 9 animal species. It also hosts predicted miRNA target results produced by 11 miRNA target prediction programs (including all algorithms discussed in this chapter). The database provides information regarding the type of experimental validation performed, the pubmed ID and a short excerpt from the original article describing the validation approach. Results cannot be filtered on a specific type of experimental evidence.

3.5. StarBase

StarBase [23] version 2.0 was released in 2013. This database takes advantage of recent high-throughput experimental technologies such as CLIP-Seq (HITS-CLIP and PAR-CLIP) and degradome sequencing across six organisms. StarBase integrates 108 datasets from 37 different studies with miRNA–mRNA target prediction from 6 different algorithms to produce ~500,000 targets. StarBase also provides a Genome Browser and functional annotation tools linked to 5 different gene ontologies to discover enriched biological functions or pathways associated with their targets.

3.6. TarBase

Tarbase [24], first introduced in 2005, is currently in its 6th version. TarBase hosts a collection of over 65,000 manually curated experimentally validated miRNA–gene interactions. TarBase integrates data from high-throughput techniques as well as individual miRNA studies. These studies provide either direct or indirect evidence of miRNA–mRNA pairs. The user can filter results based on the type of evidence and the experimental approach used. Users must sign up to use the software and an e-mail request is necessary to obtain the entire dataset.

As different techniques emerge to evaluate miRNA function and verify their targets, the requirement for new data structures and databases emerges. Extracting the information from online articles has been a bottleneck in this field. Advances in the field of natural language parsing can allow a certain level of automation for this task but these introduce a considerable amount of error when defining the type of interaction and experimental evidence, ultimately requiring manual curation.

4. Discussion

Experimentalists using miRNA target prediction software for the first time will invariably observe two apparent shortfalls. The first is that target prediction algorithms produce large lists of candidates, many of which cannot currently be validated experimentally. These false positives are generally attributed to a poor modelling of the miRNA–mRNA interaction. This may not always be the case. Recent studies have demonstrated that miRNAs, their guide, and their effector proteins are tightly controlled by their relative stoichiometry [14]. Changes in the relative abundance of miRNAs and critical proteins such as Argonaute alter the efficiency with which a miRNA binds its targets. Certain bona-fide targets predicted by an algorithm may therefore be impossible to validate in specific cells even though they are functional for example, due to an insufficient quantity of Argonaute. A recent study even suggested that most predicted targets could in fact be functional. Seitz proposed that mRNA targets may actually be competitive inhibitors of miRNA function, preventing miRNAs from binding their authentic targets by sequestering them. Under this hypothesis, the efficiency of miRNA inhibition (on which experimental validation relies) could depend on the transcriptomic environment of the cell.

The second apparent shortfall of target prediction is the lack of coherence between lists of targets produced by different algorithms. Again, biological variation may be at play here. Each algorithm integrates different criteria for selecting targets with a varying amount of emphasis. For example, certain algorithms require perfect base pairing in the seed region where others will allow one mismatch or even a bulge in the duplex formed by miRNA–mRNA pairs. Recent studies have demonstrated that different types of seed bindings are functional [25] and therefore none of the algorithms uses an incorrect model but rather each one is modelling a different type of binding site. Moreover, miRNAs have different modes of targeting with different effects on gene regulation. A recent study in the *Arabidopsis* plant showed that the AMP1 gene was necessary for translational inhibition of genes but not for mRNA cleavage [15]. Because miRNAs can use both of these modes

of regulation, algorithms trained on one may not be suited for detecting the other. As the repertoire of validated miRNA targets increases, it is our belief that algorithms will be optimized for a specific biological function or type of mRNA–miRNA duplex rather than attempting to win the endless race for fractional increases in sensitivity and specificity across the entire range of possible miRNA–mRNA interactions.

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