Systematic identification of microRNA functions by combining target prediction and expression profiling

Xiaowei Wang* and Xiaohui Wang

Ambion, Inc., 2130 Woodward Street, Austin, TX 78744, USA

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ABSTRACT

Target predictions and validations are major obstacles facing microRNA (miRNA) researchers. Animal miRNA target prediction is challenging because of limited miRNA sequence complementarity to the targets. In addition, only a small number of predicted targets have been experimentally validated and the miRNA mechanism is poorly understood. Here we present a novel algorithm for animal miRNA target prediction. The algorithm combines relevant parameters for miRNA target recognition and heuristically assigns different weights to these parameters according to their relative importance. A score calculation scheme is introduced to reflect the strength of each parameter. We also performed microarray time course experiments to identify downregulated genes due to miRNA overexpression. The computational target prediction is combined with the miRNA transfection experiment to systematically identify the gene targets of human miR-124. miR-124 overexpression led to a significant downregulation of many cell cycle related genes. This may be the result of direct suppression of a few cell growth inhibitors at the early stage of miRNA overexpression, and these targeted genes were continuously suppressed over a long period of time. Our high-throughput approach can be generalized to globally identify the targets and functions of other miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are a new family of small RNA molecules (~22 nt) that control the expression levels of their target genes (1,2). Hundreds of miRNAs have been identified in recent years and miRNA functional identification has become one of the most active research fields in biology. miRNAs function through suppressing the expression of their targeted genes. They are known to be involved in many diverse functions such as regulation of cell proliferation, differentiation, apoptosis, carcinogenesis and viral infection (1–8).

Unfortunately most of the hundreds of newly identified animal miRNAs have unknown functions (9). One major obstacle is to identify the targets regulated by miRNAs. Target prediction in plants is relatively straightforward because of the near-perfect alignment between a miRNA and its target sequence (10). In contrast, prediction of animal miRNA targets is very challenging because there are only partial miRNA pairing to the targets. In addition, only a limited number of predicted targets have been experimentally validated and the miRNA mechanism is poorly understood. All these factors make it difficult to develop rational target prediction algorithms based on experimental results. Although multiple approaches have been suggested from bioinformatics (11–18), accurate target prediction and validation are still major obstacles facing miRNA researchers.

In plants, most miRNAs regulate target gene expression via mRNA degradation (19,20). In animals, a general model has been that miRNAs suppress target gene functions by translational repression (2). The mRNA level has generally not been altered if the target-binding sites have only partial sequence complementarity to the miRNA. However, recent studies have convincingly demonstrated that animal miRNAs can also reduce mRNA expression level via mRNA degradation (21); another study has demonstrated that miR-16 can mediate mRNA degradation although the binding site has only partial sequence match (22); a few miRNAs in Drosophila have led to the downregulation of their predicted target mRNAs (23,24); more recent analyses suggested the mutually exclusive tissue expression of miRNAs and their predicted targets (25,26). These findings indicate that expression regulation at the mRNA level may be a common mechanism for miRNA function (20). This has broad implications for miRNA target validation because, comparing to protein expression changes, it is more convenient to monitor
transcriptional changes using a high-throughput experimental approach. Overexpression of miRNA in cells has been reported to lead to the downregulation of a large number of transcripts as revealed by microarrays (27). There is a significant enrichment of miRNA complementary sequences in these downregulated genes, implying they could be directly regulated by miRNA. Thus, it might be possible to use microarrays to simultaneously identify a large number of miRNA targets.

Here we present a novel algorithm for animal miRNA target prediction. The algorithm combines relevant parameters for miRNA target recognition and heuristically assigns different weights to these parameters according to their relative importance. A raw score calculation scheme is introduced to reflect the strength of each parameter. We have also performed microarray time course experiments to identify downregulated genes due to miRNA overexpression. The computational target prediction is combined with the miRNA overexpression experiments to systematically identify the gene targets and functions of human miR-124.

MATERIALS AND METHODS

Sequence retrieval

The 3′-untranslated region (3′-UTR) sequences were downloaded from ENSEMBL website http://www.ensembl.org for five organisms: human, mouse, rat, dog and chicken. The orthologous gene clusters were built using ENSEMBL ortholog gene index to map UTR sequences across different organisms. NCBI Gene IDs were used to map ENSEMBL IDs and GenBank accessions. miRNA mature sequences were retrieved from Sanger miRBase version 7.0 (29).

Gene Ontology (GO) annotations

The annotations were downloaded from http://geneontology.org/. The index file mapping GO IDs to NCBI Gene IDs were downloaded from ftp://ftp.ncbi.nlm.nih.gov/gene/. Since only the lowest level in the GO tree were specified for a Gene ID, a recursive search for all the parent GO IDs based on the GO hierarchical data structure was performed to identify all the parent GO IDs associated with each Gene ID.

miRNA transfections

miR-124 RNA duplex (Pre-miR) molecule and negative control miRNA duplex were transfected into HepG2 cell line using the Reverse Transfection protocol recommended by Ambion. In brief, siPORT NeoFX Transfection Agent (Ambion) was diluted in optiMEM medium (Invitrogen). miR-124 RNA duplex, Pre-miR-124, was also diluted in optiMEM medium for a final concentration of 30 nM. The miR-124 RNA duplex (Pre-miR) molecule and negative control miRNA transfections were performed to generate RNA samples for real-time RT–PCR validations.

Microarrays

Total RNA was amplified with MessageAmp II (Ambion), labeled and hybridized to Affymetrix human U133Plus2 chips following the manufacturer’s protocols (30,31) (http://www.affymetrix.com/support/technical/technote/30_02_08_03.pdf). Array signals were normalized using the RMA method from the BioConductor package (http://www.bioconductor.org/). A gene was considered to be downregulated if the expression reduction was at least 50% when compared with both negative control and the 4 h miR-124 reference time points.

Real-time RT–PCR

All PCR primer sequences were retrieved from PrimerBank website http://pga.mgh.harvard.edu/primerbank (32). The primers were synthesized at the Integrated DNA Technologies.

RT reaction was carried out with RETROscript System under conditions suggested by the manufacturer (Ambion). A 20 μl RT reaction contained 0.5 μg of total RNA, 2 μl of 50 μM random hexamers, 2 μl of 10× RT, 4 μl of dNTP mix (25 mM each dNTP), 1 μl of MMLV-RT (100 U/μl), 1 μl of RNase Inhibitor (10 U/μl) and Nuclease-free water. After incubation at 25°C for 30 min and 37°C for 1 h, the reaction mixture was incubated at 92°C for 10 min.

Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with the following conditions: 50°C for 2 min and then at 95°C for 10 min followed by 35 cycles of amplification (95°C for 15 s; 60°C for 30 s; 72°C for 30 s). PCR specificity was checked by melting curves and agarose gel electrophoresis.

RESULTS

miRNA target prediction algorithm

Our miRNA target prediction algorithm is summarized in Figure 1. The algorithm was implemented as a Perl program, MirTarget, running on a Linux platform. As the first step of target prediction, the miRNA seed sequence (positions 2–8) was considered as an extended seed-binding site in 3′-UTR sequences are important for target recognition (14). Thus limited seed extension was evaluated for pairing to miRNA positions 1, 9 and 10. The longest stretch of perfect matches (including positions 2–8) was considered as an extended seed for raw score calculation.
Raw scores were calculated separately for the screening filters. One example is given in Supplementary Figure 1. The cross-species conservation filter raw score was calculated with the following equation, where \( n \) is the number of species with miRNA seed pairing.

\[
S_i = \frac{9 + 2^{n-1}}{10},
\]

where \( n > 2 \) to pass the cutoff threshold. Raw score value increases exponentially as \( n \) gets larger. In this way, high degree of seed pairing conservation was exponentially rewarded. Raw scores for limited seed extension (positions 1–10) and binding free energy were calculated in a similar way by first converting the parametric values into relative ranks. Based on literature mining of validated targets, A/T terminal match is often observed in miRNA target sites (14). Thus MirTarget assigns a positive raw score 1 to A/T terminal match.

The target prediction score is computed based on the raw scores from individual filters as well as the total number of binding sites. The presence of multiple binding sites may enhance miRNA regulation (24).

\[
S = \sum_{j=1}^{n} \sum_{i=1}^{4} W_i \times S_i,
\]

where \( i \) represents four screening filters; \( S_i \) represents the raw score for each filter and \( W_i \) represents the raw score weight; \( j \) represents the number of binding sites in one UTR sequence. Different weights were assigned to the filters in the following order to differentiate their relative importance: seed conservation > limited seed extension > duplex binding stability > terminal base match. A score is recorded if it is no less than the threshold value 30.

MirTarget was used to predict the potential gene targets of all known human miRNAs. Overall, 8810 gene targets were predicted for 319 miRNAs (Supplementary Table 2). The prediction result and more details about the MirTarget algorithm will be available at http://www.ambion.com. To estimate the level of false positives, the miRNA sequences were shuffled 100 times and the total numbers of predicted targets were recorded for these shuffled sequences. On average one round of shuffling only produced 317 \( \pm \) 72 predicted targets for 319 human miRNAs, indicating the false positive rates of the target prediction are well contained (\( P = 0 \) by Z-test). As to miR-124 target prediction, 131 candidate genes received prediction scores and 85 gene targets were predicted (score \( \geq 30 \), Supplementary Table 1). In contrast, one shuffled miR-124 sequence has only 1.7 predicted targets on average.

**miR-124 transfection microarrays**

miR-124 RNA duplex (Pre-miR) was transfected into HepG2 cell line. The Pre-miR RNA duplex mimics the miR-124 precursor. miR-124 is highly expressed in brain and kidney (34), and it does not express in the cell line we studied. miR-124 overexpression profiles were examined at 4, 8, 16, 24, 32, 72 and 120 h post transfections. A negative control RNA duplex was also transfected as reference for each of these time points. Expression profiles were first examined at 72 h post transfection, and hundreds of genes were shown to be downregulated by miR-124 overexpression. These genes were
analyzed in the context of GO annotations to identify significantly affected GO functional categories. As shown in Table 1, the most significant changes were observed in categories related to cell cycle/proliferation. K-means clustering for downregulated genes also resulted in a cluster highly enriched in genes involved in cell cycle control (data not shown). This suggested miR-124 may be involved in cell growth control.

Potential miR-124 targets were computationally predicted by MirTarget. Of the 85 predicted targets 76 were represented on the arrays. The downregulated genes at different time points were examined to count how many genes were predicted to be miR-124 targets. We also performed real-time RT–PCR to validate all predicted targets downregulated at early stages of transfections. Figure 2 is a summary of the real-time RT–PCR validation results. The expression levels at 24 and 48 h were presented as percentages of the expression levels at 4 h. Among the validated targets, the microarray results indicated that genes 8801 and 8992 were first downregulated at 8 h; genes 8763, 3915, 6566, 9341, 10 449 and 10 634 were first downregulated at 16 h; genes 6836, 60 481 and 84 061 were first downregulated at 24 h; genes 27 230 and 55 225 were first downregulated at 32 h (downregulation is defined as at least 50% reduction of gene expression).

To identify significant enrichment of genes in each GO category, the P-values were calculated by hypergeometric test using all 14 485 genes represented by U133Plus2 arrays as background.

| GO ID          | GO description     | Enrichment P-value |
|---------------|--------------------|--------------------|
| GO:0000278    | Mitotic cell cycle | 3.1E-14            |
| GO:0000075    | Cell cycle checkpoint | 1.5E-12          |
| GO:0051301    | Cell division      | 5.2E-08            |
| GO:0051325    | Interphase         | 9.9E-08            |
| GO:0005819    | Spindle            | 1.3E-06            |
| GO:0006260    | DNA replication    | 3.5E-06            |

As shown in Table 2, there were very significant enrichments of predicted targets at all the time points we examined. There was a rapid accumulation of downregulated targets at early stages of miR-124 overexpression. However, the rate of accumulation slowed down dramatically at later time points. The rate showed a good fit to a logarithmic relationship (Figure 3A). Because the number of downregulated genes increased dramatically at later transfection stages, the percentages of predicted targets among downregulated genes decreased rapidly (Figure 3B), following a power law distribution.

The score distribution of the downregulated predicted targets at 24 h was compared with that of all predicted miR-124 targets in human. The two target lists are significantly different (P = 0.002 by Wilcoxon rank-sum test). The median score of all predicted targets is 33 while the downregulated predicted targets have median score of 54 (Figure 4).

Characterization of the downregulated miR-124 targets

As shown in Table 2, the most affected GO categories after miR-124 transfection for 72 h were first downregulated at 32 h (downregulation is defined as at least 50% reduction of gene expression by array measurement). Thus, the real-time RT–PCR results agreed well with the microarray results.

| Transfection time (h) | Downregulated genes | Predicted miRNA targets | Enrichment P-value |
|------------------------|----------------------|-------------------------|--------------------|
| 8                      | 6                    | 2                       | 4.0E-04            |
| 16                     | 37                   | 8                       | 1.3E-11            |
| 24                     | 134                  | 11                      | 9.2E-11            |
| 32                     | 159                  | 13                      | 1.6E-12            |
| 72                     | 583                  | 20                      | 1.1E-11            |
| 120                    | 46                   | 8                       | 8.6E-11            |

\[P\]-values were used to assess whether there was a significant enrichment of predicted miR-124 targets in total downregulated genes at each time point. The P-values were calculated by hypergeometric test using all genes represented by U133Plus2 arrays as background.

Eight miR-124 targets were downregulated at 16 h, which represents 22% of all downregulated genes (Figure 3). These genes can be classified into four functional categories (Table 3). Three of the genes are associated with cell growth arrest: (i) CD164 is a potent signaling molecule and functions to suppress hematopoietic cell proliferation (35); (ii) the mouse homolog of GAS2L1 is an actin-associated protein highly abundant in growth-arrested cells. GAS2L1 mouse homologue is negatively regulated by serum and growth factors (36); (iii) SLC16A1 mediates the transport of butyrate across the colonocyte luminal membrane. SLC16A1 is able to induce cell cycle arrest and differentiation and its expression is significantly downregulated during colon carcinogenesis (37). Therefore, the downregulation of these gene

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Table 1. The most affected GO categories after miR-124 transfection for 72 h

Table 2. Downregulation of predicted miRNA targets after miR-124 transfection
targets by miR-124 can profoundly affect the regulation of cell proliferation.

After 5 days of miR-124 transfection, the number of downregulated genes was significantly reduced compared with that at 72 h (Table 2). When the data at 16 and 120 h post transfection were compared, the numbers of downregulated genes were similar; however, only 13 genes were found to be downregulated at both stages. It is interesting to notice that most predicted targets downregulated at 16 h were among these overlapping genes (Figure 5). Thus, gene targets downregulated at the early stage of transfection were more likely to be continuously suppressed over a long period of time.

DISCUSSION

miRNA target prediction

Because of the scarcity of experimentally validated miRNA targets, target prediction rules have not been clearly defined to date. Recent analyses have suggested the importance of perfect seed (miRNA positions 2–8) pairing to target-binding sites (13,38). To exemplify this, one popular prediction algorithm considers only seed pairing and its evolutionary conservation in target prediction (14). Other studies have also indicated that most target sites align perfectly to miRNA seed sequences. Therefore, perfect seed pairing is likely to be the most important filter for target recognition in our algorithm. In other settings, this strategy has been shown to greatly reduce the false positive rate (14). In the case of target prediction for miR-124, 92% of all gene candidates would have been excluded if the seed pairing filter were applied alone. It has been known that a small number of validated targets have imperfect matches to miRNA seed sequences (5). Thus, our algorithm will miss these targets because perfect seed pairing is required.

Cross-species conservation of seed pairing is another important parameter for target prediction (14,39). However, genome annotations are still a work in progress and the risk of omitting valid target candidates will increase if we place too much emphasis on incompletely annotated genomic information (16). For example, many gene 3’-UTR sequences have not been identified in non-human genomes. In our algorithm, a balanced approach is adopted to require seed pairing conservation in at least three out of five orthologs. This strategy was chosen to minimize the effect of insufficient cross-species conservation as well as poor genome quality on target prediction.

Unlike most existing prediction algorithms, MirTarget uses a combinatorial heuristic approach to consider the relative importance of each filter for score computation. The contribution of each filter to the prediction score may vary depending on its filter weight and strength. For example, the seed pairing conservation filter has a higher weight than the terminal base pairing filter. And seed pairing across five species will contribute more than seed pairing conserved in only three species. In this way, a ‘weak’ filter may be compensated by another ‘strong’ filter so that the score can pass the cutoff threshold.

Target validation by mRNA profiling

Recent studies have indicated it is common for animal miRNAs to downregulate target mRNA expression. This
The downregulated targets at 16 h post transfection were examined for their functions. Three of them are known to be negative regulators of cell proliferation and/or abundantly expressed in growth-arrested cells (Table 3). Therefore, we hypothesize that miR-124 may be a master positive regulator of cell proliferation by suppressing the expression of cell growth inhibitors.

In summary, we have presented a novel algorithm for miRNA target prediction using a combination of weighted selection filters. The computational predictions were combined with microarray expression profiles to identify downregulated miR-124 gene targets. miR-124 overexpression led to a significant downregulation of many cell cycle related genes. This may be the result of direct suppression of a few cell growth inhibitors at the early stage of miR-124 overexpression, and these targeted genes were continuously suppressed over a long period of time. Our high-throughput approach can be generalized to globally identify the targets and functions of other miRNAs.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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