Large-Scale Screens of miRNA-mRNA Interactions Unveiled That the 3’ UTR of a Gene Is Targeted by Multiple miRNAs

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Abstract

Animal microRNA (miRNA) target prediction is still a challenge, although many prediction programs have been exploited. MiRNAs exert their function through partially binding the messenger RNAs (mRNAs; likely at 3’ untranslated regions [3’UTRs]), which makes it possible to detect the miRNA-mRNA interactions in vitro by co-transfection of miRNA and a luciferase reporter gene containing the target mRNA fragment into mammalian cells under a dual-luciferase assay system. Here, we constructed a human miRNA expression library and used a dual-luciferase assay system to perform large-scale screens of interactions between miRNAs and the 3’UTR of seven genes, which included more than 3,000 interactions with triplicate experiments for each interaction. The screening results showed that the 3’UTR of one gene can be targeted by multiple miRNAs. Among the prediction algorithms, a Bayesian phylogenetic miRNA target identification algorithm and a support vector machine (SVM) presented a relatively better performance (27% for EIMMo and 24.7% for miRDB) against the average precision (17.3%) of the nine prediction programs used here. Additionally, we noticed that a relatively high conservation level was shown at the miRNA 3’ end targeted regions, as well as the 5’ end (seed region) binding sites.

Introduction

MicroRNAs (miRNAs) are a class of small single-strand non-coding RNAs with a common length of about 22 nucleotides (nt) [1]. MiRNAs usually play a role in posttranscriptional regulation of coding genes by partially complementing with targeting miRNAs [1,2]. The miRNA target site has been considered to be the 3’ untranslated region (3’UTR) of a mRNA, however, recent studies have shown that miRNAs may also bind the coding regions or the 5’ untranslated regions (5’UTRs) [3,4]. In animals, when a miRNA binds to its target mRNA, it usually inhibits gene translation and sometimes degrades the mRNA [5,6]. MiRNAs widely exist in plants and animals and the number of hairpin precursor miRNAs was updated to 21,264 in miRBase 19, which was made public in August 2012 [7]. The functions of miRNAs are involved in most biological processes (e.g., development [8,9]) and in disease pathogenesis (e.g., cancer [10,11]). Discovery of the miRNA target genes is urgently needed for functional and mechanical study of these small RNAs.

MiRNA target prediction is often used to determine the candidate target genes for experimental verification. Unlike plant miRNAs, which are always perfectly complementary to their target genes [12], animal miRNAs are often partially complementary to the target miRNAs, which makes it more difficult to predict miRNA-mRNA interactions. Many prediction programs have been developed since miRNA was discovered [1]. The first generation of miRNA target prediction programs were designed based on a hypothesis (e.g., seed complementary, binding free energy and site conservation), such as TargetScan [13,14], DIANA_microT [15] and miRanda [16,17]. Since each program contains different features, the overlap between each prediction result has been quite low [18]. To get a better prediction result, several bioinformatic methods were introduced into the second generation of prediction programs, such as the hidden Markov model (HMM) [19], support vector machine (SVM) classifier [20,21] and the Bayesian phylogenetic model [22]. In addition, the number of predicted target genes has been increased.

It is important to experimentally evaluate the performance of the prediction programs and to choose the correct prediction programs. A commonly used method for validation of predicted interactions is a dual-luciferase assay through co-transfection of the luciferase reporter gene containing the target 3’UTR and synthetic miRNA mimics or a miRNA expression vector, which has been used to confirm predicted interactions in small scale studies [1,23,24,25]. However, there is no report on using this...
approach in large-scale or genome-wide studies. Recently, several new approaches have been developed to identify the miRNAs and targets on large-scale, including proteomic methods, co-IP based experiments and miRNA transfection methods [26]. In the proteomic approach, the capability of mass spectrometry to identify and quantify proteins from complex mixtures depends on the level of accuracy and sensitivity [27]. For co-IP based methods, an antibody that recognizes a protein (usually an Argonaute protein) is used to profile the target miRNA [28]. In addition, miRNA transfection that combined with other approaches including transcriptomic or proteomic analysis has been widely used to identify miRNA targets [26].

In the present study, we presented large-scale screens for 3′UTRs in seven genes, which included more than 3,000 interactions with triplicate experiments individually, using a co-transfection and dual-luciferase assay system approach. The gene 3′UTRs cloned into the multiple cloning regions were located at 3′ end to the Renilla luciferase gene. If a miRNA targeted its binding site in the 3′UTR of the gene, the activity of Renilla luciferase would be decreased. We tested 1,018 interactions predicted by computer programs and 2,433 interactions screened by a genome-wide miRNA expression library. We demonstrated that the 3′UTR of a gene can be targeted by multiple miRNAs and many of them cannot be predicted using popular prediction programs.

Materials and Methods

Vector Construction

To express miRNA, a pLL3.7 vector was first modified by the insertion of an overlap extension PCR (OE-PCR) product containing multiple cloning sites, the miRNA transcriptional stop sequence 5′-TTTTT-3′ and the puromycin resistant gene driven by the SV40 early promoter, downstream of the human U6 promoter. The SV40 early promoter was PCR amplified from pDNA3.1 using the forward primer (primer 1, 5′-CTCGAGAAGCACC GGGAATCTTATTTTATTTAAGC GAATTC-3′) with XhoI and BamHI double digestion. The total RNA of 293T cells that were transfected with the modified psiCHECK-2 reporter vector was used as a control and transfections were performed for each plate. The cells were harvested 48 h after transfection and assayed using the Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instructions. Each transfection was repeated in triplicate.

The ratio of Renilla luciferase activity to firefly luciferase activity in each well was normalized to the average ratio of the control wells in each plate in which the ratio was designed as 1. The data for the same 3′ UTR reporter collected from different plates were integrated by combination of the normalized ratios. All experimental data are presented as the mean ± S.D. from three independent transfection experiments. Significant differences from the control value analyzed with Student’s t-test are indicated by P<0.05.

RNA Isolation and Quantitative Real-time PCR

The total RNA of 293T cells that were transfected with a miRNA empty vector, pre-miR-24 or pre-miR-27a was isolated by Trizol (Invitrogen). One microgram of RNA was used to synthesize the first strand complementary DNA (cDNA) with Rever-Tra-Ace-α- (TOYOBO) and random primers. Quantitative real-time PCR (qPCR) was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Premix ExTaq II (TaKaRa) to detect miR-24-3p, miR-27a-3p and internal control U6 RNA. The primers were purchased from GenePharma. Each sample was tested in triplicate and the expression fold change was determined using the ΔΔCT method [31].
Immunoblotting Analysis

293T cells were transfected with a miRNA empty vector or various miRNA expression vectors. After 72 h, cells were harvested and lysed by RIPA lysis buffer (Biotek) with 1 mM protease inhibitor PMSE. Total proteins were separated by 10% SDS-PAGE gels, and subsequently transferred onto Polyvinylidene fluoride (PVDF) membranes (Millipore). The membrane was blocked with 5% nonfat dry milk in TBS-T buffer for 1 hour and incubated overnight at 4°C with antibodies against MXI1 (1:500, Santa Cruz) or β-actin (1:5000, Abcam). Followed by washing in TBS-T, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, respectively. Finally, the signal was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Data Analysis

Triplicate experiments were performed for each interaction. Significant differences between each interaction and the control group were determined with a Student t-test. P<0.05 was considered as statistically significant. The inhibition effect was shown by counting the percentage of each luciferase-activity ratio taken in the control groups. A modified SSMD method was used for statistical analysis of the genomic miRNA screening results [32]. Interactions that produced a value equal to or less than -2 were considered as strong downregulation effects, except as specifically indicated.

miRNA Prediction

Nine programs were chosen to predict the miRNAs that targeted the 3’UTRs of seven genes (MXI1, TP53, PTEN, CYP24A1, FSCN1, POT1 or TRF2). For TargetScan, TargetScanS, miRanda, miRDB and PicTar, predictions were determined using their web servers with default parameters. For microT_v3.0, the threshold 7.3 was used to predict miRNAs; for EIMMo, the cutoff score for medium precision. The miRNA expression vectors, with the length of the cloned pre-miRNA fragments ranging from 287–456 bp, could produce miRNA with high efficiency. Even the constructs with a cloned size of 647 bp for miR-128-5p or 1011 bp for miR-27a-3p could produce miRNA at a reasonable level (Figure 1A and 1E). For those miRNAs with more than one locus in the genome, 3 sensors for miR-128-5p, -138-5p and -199a-5p, each of which has two loci, were used to measure the miRNA expression levels with cloning both genomic loci, respectively. The genomic DNA of each miRNA could produce the miRNA quite efficiently (Figure 1D and 1E). The quantitative RT-PCR (qRT-PCR) results for miR-24-3p and miR-27a-3p also showed that the expression vector could generate mature miRNA efficiently (Figure 1F). Collectively, our results demonstrated that the miRNA expression library we constructed could efficiently be processed into mature miRNAs.

Measurement of Interactions between miRNAs and Target 3’UTRs

The target site of miRNA is often located at the 3’UTR [3]. In order to establish a reliable large-scale method to test the interactions between miRNAs and target genes, we first constructed Renilla luciferase reporter vectors containing the 3’UTRs to examine two reported interactions (Figure 2A). Co-transfections with the miRNA expression vector plus the 3’UTR report vector into 293T cells were performed to detect the interactions. It has been reported that the human enhancer of zeste homolog 2 gene (EZH2) is targeted by miR-101-3p in SKBr3 breast epithelial cells, DU145 prostate carcinoma cells and benign immortalized H16N2 breast epithelial cells [35] and homeobox D10 (HoxD10) is targeted by miR-10b-5p in SUM149 primary breast carcinoma cells [36].
Our recent study also showed that HoxD10 is a functional target gene of miR-10b-5p in human microvascular endothelial cells (HMEC-1) involved in heparin inhibition of angiogenesis [29]. In the present study, we showed that the miR-101-3p produced by the constructed vector could inhibit the EZH2 3'UTR reporter.
gene and that miR-10b-5p could inhibit the HoxD10 3'UTR reporter gene (Figure 2B and 2C).

Large-scale Screens of Predicted Interactions Revealed Multiple miRNAs Targeting the 3'UTR of Each Gene

To assess the efficiency of miRNA target prediction programs, we performed systematic experiments to validate miRNA-mRNA interactions using our human miRNA expression library and the miRNA-mRNA interaction detecting system. Nine miRNA prediction programs were chosen to perform predictions, including TargetScan [37], TargetScanS [13,14], PITA [38], DIANA-microT_v3.0 (microT_v3.0) [15], PicTar [19,39], miRanda [16,17], miRDB [20,21], NBmiRTar [40] and EIMMo [22]. These are often used to stand for rule based algorithms and machine learned algorithms (Table S4).

Seven genes, including MXI1, TP53, PTEN, CYP3A4, FSCN1, POT1 and TRF2, were chosen for miRNA prediction with the above mentioned programs. We chose these genes in order to exclude the effect that genes of similar function share similar miRNA-target profiling. The prediction parameters were described in the methods section and the numbers of predicted miRNAs for each gene were summarized in Table 1. The predicted miRNAs from the nine software programs for each gene were combined together to obtain a final list of predicted miRNAs (Table S3). These prediction results showed that there was a significant difference for miRNAs and miRNA numbers predicted by the different software programs (Tables 1 and S3). Subsequently, 18-75% of predicted interactions for these 7 genes were randomly chosen for large-scale experimental validation using a dual luciferase assay with a co-transfected miRNA expression vector and cognate 3'UTR reporter vector. Totally, 1,018 predicted interactions were examined. The positive interactions were defined as the data that had statistically significant difference compared to the control experiments that replaced the miRNA expression vector with a miRNA empty vector. The other tested interaction data were classified as negative. The luciferase results indicated that each gene was regulated by multiple miRNAs (Figure 3; data not shown), with 3'UTRs of CYP3A4, TP53, PTEN, TRF2, FSCN1, MXI1 and POT1 bound by 9 miRNAs, 10 miRNAs, 18 miRNAs, 23 miRNAs, 24 miRNAs, 31 miRNAs and 12 miRNAs, respectively (Table 1). The total number of positive interactions for the 1,018 predicted interactions of the seven genes was 127 (Figure 3E).
To examine whether the positive interactions was reliable, some of these interactions were chosen for further confirmation using mutation and immunoblotting analyses. 12 interactions for POT1, TP53, PTEN and MXI1, with 3 interactions for each gene, were randomly chosen for mutation analysis of miRNA target sites. When the complementary sequences of the miRNA seed region in the target sites were mutated, the miRNAs did not interact with the 3'UTRs or the inhibition effect was attenuated (Figure 4A, B and C, data not shown). For the further confirmation, we choose half positive interactions of MXI gene using immunoblotting analysis. When the miRNA vectors expressing miRNAs, which had positive interactions with the MXI1 gene 3'UTR, were transiently transfected into the 293T cells, the endogenous MXI1 protein level decreased significantly (Figure 4D). These data, as well as many unpublished target site mutation results and immunoblotting results performed in our laboratory, might suggested that the identified positive interactions were reliable.

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Our analysis showed that EIMMo had the highest prediction precision (27%), and the second was miRDB (24.7%). The lowest precision was observed from PITA (4.3%). The precisions of other prediction tools were 16.2% for TargetScan, 17% for TargetScanS, 20.6% for DIANA-microT_v3.0, 18.8% for PicTar, 16% for miRanda and 10.9% for NBmiRTar (Table 1).

**Figure 3. Validation of the predicted interactions for seven genes.** (A–C) Co-transfection of 0.15 μg of the pre-miRNA expression vector and 0.05 μg of the luciferase reporter vector containing the full length 3’UTR of PTEN (A), TRF2 (B) and POT1 (C) into 293T cells to validate the predicted interactions, with co-transfection of the empty vector of the pre-miRNA and the luciferase reporter containing the same 3’UTR as control. The data were normalized to the ratio of Renilla and firefly luciferase activities measured at 48 h post transfection. Values represent the mean ± S.D. from triplicate transfection experiments. (D) Summary of the results for each gene based on the tested interactions. Inhibitive miRNAs with p<0.05 were chosen as positive. (E) The cumulative curve showed the distribution of fold changes for all the predicted interactions. Fold changes were calculated according to the negative controls in each experiment.

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**Genome-wide Screens of MXI1, POT1, TRF2 and FSCN1 Revealed Unpredicted miRNAs Targeting the 3’UTR of Each Gene**

To test whether there were unpredicted miRNA-mRNA 3’UTR interactions, we performed genome-wide screens of MXI1, POT1, TRF2 and FSCN1 genes, with the constructed miRNA expression library. For each gene, every miRNA expression vector (about 600 in total), whether predicted to bind the gene 3’UTR or not, were co-transfected with the luciferase reporter.
reporter containing the full-length 3' UTR of the gene (an empty miRNA expression vector or rno-miR-344a-3p expression vector expressing mature miRNA with no human homology was used as a negative control) in 293T cells. Among 2,430 tested interactions, 193 positive interactions were found with a significant difference (P < 0.05), accounting for 7.9% of all tested interactions (Figure 5A, upper pie chart). Among 795 predicted interactions, 90 (11.3%) were positive (Figure 5A, lower left pie chart), while 103 (6.3%) of 1,638 unpredicted interactions were positive (Figure 5A, lower right pie chart). These results demonstrated that one gene's 3' UTR can be targeted by multiple miRNAs. For MXI1, individual miRNAs were shown by SSMD-fold change dual-flashlight dots (Figure 5B). Forty-two miRNAs were found to have SSMD values under 2, indicating a strong interaction [32]. Five unpredicted miRNAs, which down-regulated the MXI1 3' UTR in the screening results, were chosen for further confirmation by immunoblotting and all significantly decreased the level of endogenous MXI1 protein (Figure 5C). Collectively, our results showed that there were many positive miRNA-mRNA interactions that were not predicted by 9 commonly used programs.

**Distribution and Conservation of miRNA Target Sites**

miRNA target sites were validated using conservation analysis to characterize the miRNA target elements (MREs). The predicted miRNA target sites were divided into positive group and negative group. Randomly chosen sequences of the human genome were used as control group. The average PhastCons score of positive group and negative group was 0.57 and 0.49 respectively, while that of the random group was only 0.12 (Figure 6A). The sequences of MREs in positive group were more conserved than those in the other two groups (Wilcoxon test, P < 0.05).
A

Total Tested
(2430)

- Positive 7.93%
- Negative 92.07%

Total Predicted and Tested
(795)

- Positive 11.32%
- Negative 88.68%

Total Non-predicted and Tested
(1635)

- Positive 6.29%
- Negative 93.71%

B

MXI1

SSMD

Average log₂ fold change

C

| CTRL | mir-167 | mir-379 | mir-516b-1 | let-7a-2 | mir-508 |
|------|---------|---------|------------|----------|---------|
| MXI1 |         |         |            |          |         |
| β-actin |       |         |            |          |         |

1.0  0.50  0.28  0.27  0.42  0.32
conservation level of each single nucleotide was higher in positive group than in the other two groups. Two conserved regions contained in positive group that were not found in the negative or random group. One was miRNA seed region target sites, 2–8 nucleotides, and the other was in the 3′ end of miRNA target region, 14–25 nucleotides. However, the region between them was less conserved (Figure 6B).

Discussion

In the present study, using the dual luciferase assay in large-scale screens, many miRNA-mRNA interactions were identified, including predicted and unpredicted interactions. Some genes were found to have been regulated by multiple miRNAs. The results of this study also demonstrate that, in addition to the complementary sequences of the miRNA seed region, a relatively high conservation region (i.e., the miRNA 3′ end targeted region) is also important to miRNA targets.

Large-scale Methods for the Identification of miRNA Targets

We showed that EIMMo had the highest prediction precision (27%) and miRDB had secondly highest precision (24.7%), and the positive interaction ratio was 6.3% in the unpredicted group. Co-IP based methods are widely used to identify miRNA targets, which are often combined with miRNA transfection experiments [41]. Transcriptome-wide identification of RNA-binding proteins (RBPs) and miRNA target sites by photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) allows high-resolution mapping of binding sites of cellular RBPs and microRNA-containing ribonucleoprotein complexes (miRNPs) across the transcriptome [42]. Co-IP based methods have some flaws although they are effective to identify miRNA targets. For example, in the sequence clusters, it is unable to identify each miRNA’s direct target. In addition, these co-IP based methods require a stable association between AGO, miRNA and targets. Some miRNA-mRNA interactions cannot be identified probably because a subset of miRNAs and/or targets do not survive during the washing steps of miRNP complexes [26]. We tested every miRNA with its target miRNA using only a full-length 3′ UTR cloned in a luciferase reporter gene, which could partially rule out some indirect effects. However, we observed that several miRNAs led to increase luciferase activity. The cause of this is probably because microRNAs and their associated protein complexes (microribonucleoproteins or microRNPs) could function to posttranscriptionally stimulate gene expression by relief of repression where the action of a repressive microRNA or microRNP is abrogated. Although there are some issues just as we are not sure whether every vector could produce mature miRNA for the library, our approach could generally identify each miRNA’s target and avoid the interactions that were unpredicted using other methods.

One Gene Targeted by Multiple miRNAs

MiRNA target prediction provides a clue for experimental identification of miRNA-mRNA interactions. Ideally, high-throughput experiments would give concise answers in simple over-expression experiments. Unfortunately, this is not entirely the case [43]. To identify the miRNA target genes, we performed a systematic experiment to understand miRNA-RNA interactions. We chose 4 genes for a large-scale screening experiment. For each gene, about 600 miRNA expression vectors were transfected individually with luciferase reporter into the cells. It has not been previously reported partially because of the large scope of the task. We found many miRNAs could target one gene’s mRNA. It has been reported that a single miRNA can target many genes, while a
single gene can be regulated by multiple miRNAs [44,45]. In a study of miRNAs targeting p21/Waf1, 20 of 266 miRNAs were reported to target p21/Waf1. These miRNAs can substantially inhibit p21/Waf1 expression, particularly at the translational level. The results were then verified by a series of mutational analyses and luciferase assays [44]. In another study, 7 of 43 miRNAs were found to repress a CyclinD1 3' UTR luciferase reporter [46]. It has been suggested that, in particular tissues or developmental stages, these miRNAs might have both spatial and/or temporal specificities [44]. Moreover, the potential additive effect of 2 (or more) miRNAs targeting the same 3’UTR would be also interesting. For example, in the study of Hand2, the authors found that mir-1 and mir-133a can act together on the 3’UTR of Hand2 to produce additive or synergistic effects on protein production [45]. To our knowledge, so far, it is the first systematic large-scale screening with the task of large scope.

Evaluation of Prediction Software
Although many miRNA prediction programs have been developed, they are insufficient for use as efficient and powerful tools. An appropriate database is the essential tool for this purpose. Alexiou et al. [47] compared some prediction programs with analysis of a proteomic dataset obtained by Selbach et al. [27]. Although the dataset was based on the changes in protein levels with specific miRNA overexpression or endogenous miRNA knockdown, we know less about the direct relationship of the changes between protein levels and miRNA expression levels [49]. In this study, our dataset was obtained from the interactions between the miRNAs and the full-length 3’UTRs of coding genes. The only full-length 3’UTR cloned in the luciferase reporter gene is capable of ruling out some indirect effects, such as the change in protein levels that arise from endogenous miRNA-target transcriptional factors, which may regulate many target genes. Although every miRNA was tested with one gene’s 3’UTR, indirect effects could not be completely ruled out in our interaction validation system. The dataset from this system is already much closer to direct interaction, which was demonstrated by the MRE site mutation analysis and immunoblotting experiments.

All 9 miRNA prediction programs used in this study showed low prediction efficiencies. Among them, EIMMo and miRDB gave relatively higher precision than the others. EIMMo was designed based on a Bayesian phylogenetic miRNA target identification algorithm, which could predict miRNA target sites, and each site could be ranked by a posterior probability [22]. miRDB was designed based on a support vector machine (SVM) algorithm and the rules, such as seed conservation, seed match type, 3’UTR base composition, the secondary structure of the miRNA binding and the target site location in the 3’UTR are the classifier features [20,21]. Although most of these features have been used by other prediction programs, miRDB appears to have a better performance than others. Our results indicated that these kinds of algorithms may be more suitable for exploitation of miRNA prediction.

Pattern of MREs
Sequence conservation is a well-known character of miRNAs, especially in the seed region and the 13–16 nt positions of miRNA [4,13]. We found that the MREs of the positive interactions were relatively more conservative than the MREs of the negative and random interactions (Figure 6A). Our results demonstrated two relatively conserved regions in the miRNA target sites. One corresponds to the seed region and the other is the 3’ end of the miRNA targeting segment. The conservation pattern is found only among the positive MREs, but not in the other two groups. The finding for the 3’ end of the miRNA targeted region is consistent with the results from C. elegens, in which the artificial 3’UTR containing only the miRNA seed site was totally out of control of the miRNA [49]. Some single mutations introduced in the positions of the miRNA 3’ end targeted 3’UTR can also impact regulation [50]. Several studies indicated that the RNA folding of both local segment and global 3’UTR could affect the prediction precision [51,52], which implicated that the second structure of miRNA binding region as well as surrounding region could be contributed to the authentic miRNA target. This may be the reason that the higher conservation level in that region extends after the end of the miRNA binding sequence. Collectively, the features of miRNA targeted sequences may shed some light on the development of new prediction tools.

In summary, we have performed a large-scale screening method to identify miRNA targets and provided an option to evaluate the prediction programs. Meanwhile, we demonstrated that miRNA of one gene could be targeted by multiple miRNAs. This is an important improvement in the study of miRNAs in human disease for the future.

Supporting Information
Table S1 Primers used for amplifying 3’UTRs. (XLS)
Table S2 Synthesized oligos. (XLS)
Table S3 Prediction data for seven genes. (XLS)
Table S4 Prediction software. (XLS)

Author Contributions
Conceived and designed the experiments: QX WL ZS SJ. Performed the experiments: PX WX ZL JC XZ XW. Analyzed the data: PZ. Contributed reagents/materials/analysis tools: PZ. Wrote the paper: PZ XC SJ.

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