Computational Characterization of Exogenous MicroRNAs that Can Be Transferred into Human Circulation

Jiang Shu  
*University of Nebraska-Lincoln*, jshu2@unl.edu

Kevin Chiang  
*University of Nebraska-Lincoln*, t3-kchiang@unl.edu

Janos Zempleni  
*University of Nebraska-Lincoln*, jzempleni2@unl.edu

Juan Cui  
*University of Nebraska-Lincoln*, jcui@unl.edu

Follow this and additional works at: [http://digitalcommons.unl.edu/csearticles](http://digitalcommons.unl.edu/csearticles)

Part of the [Biomedical Commons](http://digitalcommons.unl.edu/biomedicalcommons), [Computational Engineering Commons](http://digitalcommons.unl.edu/computationalengineeringcommons), [Other Biomedical Engineering and Bioengineering Commons](http://digitalcommons.unl.edu/otherbiomedicalengineeringandbioengineeringcommons), and the [Systems and Integrative Engineering Commons](http://digitalcommons.unl.edu/systemsandintegrativeengineeringcommons)

Shu, Jiang; Chiang, Kevin; Zempleni, Janos; and Cui, Juan, "Computational Characterization of Exogenous MicroRNAs that Can Be Transferred into Human Circulation" (2015). *CSE Journal Articles*. 115.  
[http://digitalcommons.unl.edu/csearticles/115](http://digitalcommons.unl.edu/csearticles/115)

This Article is brought to you for free and open access by the Computer Science and Engineering, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in CSE Journal Articles by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Computational Characterization of Exogenous MicroRNAs that Can Be Transferred into Human Circulation

Jiang Shu¹, Kevin Chiang¹, Janos Zempleni², Juan Cui¹*

¹ Department of Computer Science and Engineering, University of Nebraska-Lincoln, Lincoln, NE, United States of America, ² Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE, United States of America

* jcui@unl.edu

Abstract

MicroRNAs have been long considered synthesized endogenously until very recent discoveries showing that human can absorb dietary microRNAs from animal and plant origins while the mechanism remains unknown. Compelling evidences of microRNAs from rice, milk, and honeysuckle transported to human blood and tissues have created a high volume of interests in the fundamental questions that which and how exogenous microRNAs can be transferred into human circulation and possibly exert functions in humans. Here we present an integrated genomics and computational analysis to study the potential deciding features of transportable microRNAs. Specifically, we analyzed all publicly available microRNAs, a total of 34,612 from 194 species, with 1,102 features derived from the microRNA sequence and structure. Through in-depth bioinformatics analysis, 8 groups of discriminative features have been used to characterize human circulating microRNAs and infer the likelihood that a microRNA will get transferred into human circulation. For example, 345 dietary microRNAs have been predicted as highly transportable candidates where 117 of them have identical sequences with their homologs in human and 73 are known to be associated with exosomes. Through a milk feeding experiment, we have validated 9 cow-milk microRNAs in human plasma using microRNA-sequencing analysis, including the top ranked microRNAs such as bta-miR-487b, miR-181b, and miR-421. The implications in health-related processes have been illustrated in the functional analysis. This work demonstrates the data-driven computational analysis is highly promising to study novel molecular characteristics of transportable microRNAs while bypassing the complex mechanistic details.

Introduction

Mature microRNAs (miRNAs) are a class of short non-coding RNAs, 21–25 nucleotides in length and endogenously transcribed in animals, plants, and viruses. These small molecules often regulate gene expression post-transcriptionally via base paring with complementary sites.
in target messenger RNAs (mRNAs) and either promote the degradation of mRNA or inhibit
the translation of the mRNAs into proteins [1, 2]. In human, 2,588 known miRNAs (according
to miRBase v21 [3]) have been estimated to target ~60% of human genes and regulate a vast
array of fundamental cellular processes in different cell types [4].

Since miRNAs have been long considered to be synthesized endogenously, little has been
studied on miRNA cross-species transportation during the past decade. It was very recently
discovered that humans absorb a meaningful amount of certain exosomal miRNAs from cow’s
milk, e.g., miR-29b and 200c; the endogenous miRNA synthesis does not compensate for die-
tary deficiency [5]; the biogenesis and function of such exogenous miRNAs are evidently health
related [5–8]. While the evidence in support of milk-miRNA bioavailability is unambiguous, a
recent report that mammals can absorb plant miRNAs (e.g. miR-168a) from rice [9], however,
was met with widespread skepticism [10–13]. Based on these evidences, challenging questions
may be raised regarding how human pick up miRNAs from dietary intake, why some exoge-
nous miRNAs can be transferred into human circulation while others cannot, and what are the
broader functional roles played by exogenous miRNAs in human disease processes.

A bioinformatics study is herein introduced to characterize the cross-species transportation
of miRNA computationally where the following procedures have been employed. Firstly,
through a comparative analysis across a large set of species, we systematically assessed the
sequence conservation among all available miRNAs in the public databases. Current knowl-
edge related to this issue is that miRNAs are well conserved in sharing common mature
sequences, biosynthetic pathways and reaction mechanisms throughout evolution [14], while
there is a large proportion newly evolved in each species and are considered to be species-spe-
cific [15]. Likewise, in this study, significantly different sequence profiles with some overlap are
expected among species. Secondly, we applied a data mining strategy to identify discriminative
molecular features that can classify miRNAs into different groups, e.g. different kingdom
groups or circulating miRNAs versus the rest. Our initial list under evaluation covers the
sequence features such as nucleotide composition, %G+C content and palindromic properties;
the secondary structure of precursor miRNAs (pre-miRNAs); and the physicochemical prop-
teies, e.g., minimum free energy of the secondary structure. The rationale behind this collection
is that functional study of miRNA has been largely depending on the target identification
where sequences information is needed for identifying the complementary sites; and that
miRNA gene recognition is mostly based on the prediction of pre-miRNA-like hairpin second-
ary structures that are conserved in closely related genomes. For example, current miRNA pre-
diction methods have shown that sequential features, such as %G+C content and several
normalized dinucleotide frequencies (%UA, %AA, %GC), are critical for detecting miRNAs
from other types of non-coding RNAs [16–19]. In this study, all sequential and structural fea-
tures that possibly capture the commonality and differentiation among miRNAs have been
taken into account.

In addition, we know that extracellular miRNAs are found in circulation in two different
forms: 1) associated with exosomes (also known as vesicles or microparticles) [20, 21], whose
detailed molecular mechanism remains to be elucidated. Current studies show that microparti-
cles exhibit highly distinct binding patterns with miRNAs, suggesting that there is a selection
of miRNAs to be transported out of cells [22]. Hence the binding and transport mechanism
may play a pivotal role in determining whether a miRNA will be excretory or not; 2) indepen-
dent from exosomes/microversicles, but instead bound to Argonaute (Ago) proteins as part of
the RNAi silencing complex. Evidences suggest that the Ago-bound miRNAs may be the major
form of miRNAs in blood circulation and their stability could be due to the binding with the
Ago2 complex, which protects them from the RNAse degradation [23, 24], although the mech-
anism of miRNA-Ago2 complex secretion remains to be understood.
As there is a lack of prior knowledge of the secretory mechanism of miRNA to circulation, we plan to heavily rely on experimental data to identify features that can differentiate secreted miRNAs from the rest. Instinctively, the secretory features should be highly associated with the intake and release mechanisms through transporting vesicles or the association with Ago proteins. In addition to the mature form of miRNA, we also include precursor sequences to possibly capture the editing associated features. Both structure- and sequence-based features are generated, including those related to the presence of branching and helical structure in pre-miRNAs and those describing the sequences with respect to their compositions of monomers and dimers, the existence of palindrome sequences, and the sequence length. While the precise effect of each feature on distinguishing secretory miRNAs from others is unclear, it is possible that these features could possibly contribute in recognizing whether the miRNAs are transportable by microvesicles, or measuring the strength of the miRNA-Ago2 complex formation.

The binding strength between the miRNA and these proteins may inversely correspond to the likelihood of secretion. Based on the aforementioned features, we have conducted feature selection, followed by Manifold ranking analysis to infer the potential of exogenous miRNAs, particularly dietary miRNAs, being transported into human circulation. Experimental data was provided for validation.

Materials and Methods
A full description of the methods is provided in S1 Methods while a brief synopsis follows.

Data sets
The miRNA sequence and annotation data were downloaded from miRBase (Version 21) [3], which contains 34,612 mature miRNAs expressed from 28,421 stem-loop precursor sequence in 194 species. We first categorized the miRNAs into five kingdoms including Animalia, Plantae, Fungi, Protista and Viruses (detailed statistics is shown in Table 1). With the goal to find secretory miRNAs in human blood circulation, we adopted 360 human plasma miRNAs uncovered by Weber in 2010 [25].

For assessment purpose, we have compiled a comprehensive collection of dietary miRNAs from literatures, a total of 5,217 miRNAs from 15 types of common food species such as cow’s milk, breast milk, tomato, grape, and apple fruit. All dietary miRNA information is accessible through our Dietary microRNA databases (DMD) [26]. In addition, annotation data also include exosome-associated information from ExoCarta and EVpedia [27, 28] for another dimension of assessment.

| Types               | Animal | Plantae | Fungi | Viruses | Protista | Total   |
|---------------------|--------|---------|-------|---------|----------|---------|
| Mature miRNAs       | 26,705 | 7,645   | 84    | 152     | 26       | 34,612  |
| Precursor miRNAs    | 21,257 | 6,990   | 53    | 91      | 30       | 28,421  |
| Species             | 111    | 71      | 5     | 5       | 2        | 194     |

Various annotation information are collected from the following resources
1. miRBase [3], which complies the species/kingdom information of 34,612 mature miRNAs included in this study
2. DMD [26], which contains dietary species information of 5,217 miRNAs
3. Weber et al. [25] provides a list of 360 human circulating miRNAs
4. ExoCarta and EVpedia [27, 28] provide 370 exosomal miRNAs in human and mouse.

doi:10.1371/journal.pone.0140587.t001
Feature collection

All features can be categorized into two classes: sequential features and secondary structural features. For each mature miRNA, a total of 1,102 features were generated including:

1. 1,031 features calculated based on following sequences:
   a. extend seed region sequence (first 8 nucleotides on 5’ end of mature miRNA sequence);
   b. mature miRNA sequence;
   c. corresponding precursor stem-loop sequence.

2. 71 structural features identified based on the predicted secondary structure of precursor stem-loop sequence.

We note the key deciding factor of transportability might be related to the interaction between protein and miRNA, e.g., mature miRNAs may be associated with Ago proteins in cells [29], and the binding strength may inversely correspond to the likelihood of secretion. Hence, features that possibly associated with miRNA binding capabilities were examined, including the existence of palindromic sequences [30], sequence length and the compositions of monomers and dimers.

Secondary structural features were calculated based on the stem-loop structure of pre-miRNA. For example, RNAfold was employed to predict secondary structure and calculate Minimum Free Energy (MFE) [31]. Subsequently, 32 triplet features and 11 base-pairing features were calculated, such as $A()$ (frequency of 3 paired nucleotides leading by A) and $\% pairGC$ (length-normalized frequency of G-C pairing). NOBAI was utilized to compute Shannon Entropy ($Q$) and Frobenius Norm ($F$) [32]. The detailed descriptions and the references of each feature are given in Table A in S1 File.

Classification-based feature selection

Based on all aforementioned features, a support vector machine (SVM)-based feature elimination strategy was developed to identify features that can discriminate miRNAs of a certain class from others. The recursive feature elimination (RFE) based strategy has been employed to remove features irrelevant or negligible to the classification results in an iterative fashion [33–35]. Specifically, each iteration eliminates features with the lowest scores given by RFE. This process continues until a minimal subset of features is obtained while maintaining an acceptable level of classification performance.

We noted a major problem with our experimental dataset was its imbalance. For example, in the Plantae-against-Others case, the positive set that represents all Plantae miRNAs (7,645) was significantly outnumbered by the negative set (all miRNAs from other kingdoms, 26,967). To overcome the imbalance that presented challenges for SVM-based classification [36], synthetic minority over-sampling technique (SMOTE) [37] was utilized to produce a balanced dataset for each kingdom separation (Details in S1 Methods). We also grouped three minority kingdoms, namely, Fungi, Protista, and Viruses, into one virtual kingdom denoted as FPV.

Based on 5-fold cross validation, we evaluated the overall classification performance by calculating sensitivity, specificity, accuracy, and the Matthews correlation coefficient (MCC) [38]. It should be noted that, for each SVM-training and testing, we re-estimated the parameters by grid searching [39] and ensured optimized models were achieved for each classification. Last, the SVM-based feature elimination produced the minimal set of features that yields the best separation of one kingdom against others, and similarly, for the separation of circulating miRNAs against others miRNAs in human.
Manifold ranking to infer the miRNA transportability

Considering a large number of exogenous miRNAs might be transported into human circulation but have not been detected yet, which leads to a problem without well-defined negative sets, a different classification strategy, so-called ranking approach [40–42], can be alternatively employed. Here we built a model based on the identified discriminative features to rank miRNAs according to their potential of getting transported into circulation instead of predicting them to be transportable or not. The essence of such algorithms is as follows: the problem is defined on two datasets, a positive set, e.g. known secreted miRNAs, and a background set (an undetermined set which may include both positive and negative data); and the goal is to rank the individual members of the whole dataset according to their relevance to the positive data. A weighted graph is used to represent the whole dataset, with each data represented as a node, each pair of nodes as an edge and a weight defined as the similarity between the two nodes in the (to be identified) feature space. Then each positive data propagates its presence (as evidence) to its neighboring nodes to increase their relevance to the positive dataset, where this relevance is valued proportionally to the corresponding edge weight in the graph. An overall relevance score of each node is the sum over all the scores propagated to it from all the related positive data. One way to assess a ranking method is by checking the percentage of the positive training data that is ranked among the top X% of all the training data. Generally the higher the percentage is for each fixed X, the better the trained ranking algorithm is.

It has been well documented that Manifold Ranking algorithm (MR) helps in finding the most relevant samples from background to true positive datasets [43, 44]. In this study, we used all 360 human blood-detectable miRNAs as the positive set, and all other 34,252 miRNAs as background set in this experiment. The detailed description of MR can be found in the S1 Methods.

Functional inference through target analysis

The top-ranked miRNAs that are highly transportable were subject to further stratification according to their origins and if they are known exosomal miRNAs. As the functions of miRNA can be inferred based on its gene targets, we extracted the known human gene targets from CLASH dataset [45], miRTarBase [46] and DIANA-TarBase [47] if the dietary miRNA has identical sequences with human miRNA; otherwise, we predicted their targets in human using TargetScan [48] and miRDB [49]. Last, Gene ontology (GO) and pathway enrichment analysis [50] was carried out to infer the biological processes and functional pathways that the miRNA may get involved.

MiRNA-sequencing analysis on milk feeding study

A miRNA-sequencing analysis was conducted based on the archived human blood samples collected from a previous milk-feeding study [5]. These samples are from five health adult participants at four time points (0, 3, 6, 9 hours) after they consumed 1-liter bovine milk. In this study, both mRNA and microRNA were extracted from each blood samples at the BGI (Hong Kong, China) and the pooled miRNA was subject to small RNA sequencing analysis by using Illumina-HiSeq2000. For bioinformatics analysis, the CAP-miSeq [51] was applied to identify both human and bovine microRNAs and calculate the expression. The miRBase (Version 21) [3] was used as reference library. We have carefully filtered out the low quality reads and strictly mapped the qualified reads to all known mature sequences, precursor sequences and the genomes of human and cow.
Results

MiRNA sequence conservation across species

A total of 34,612 miRNA sequences from 194 species and five kingdoms are used for the initial comparative analysis. Although miRNA sequences have 21-25bps in length in general, skewed length distributions were shown with respect to the different kingdoms (Fig 1A). For example, compared to animal miRNA, the majority of viral miRNAs tend to have longer sequences.

We doubt if the miRNA sequence conservation could be a feature contributing to the cross-species transportation. To test this, we compared all collected miRNA sequences across species using CD-HIT [52]. In total, 16,458 highly conserved clusters were derived (sequence identity higher than 0.98 with length variation no more than 1bp). We found most of species have miRNA homologs in other species within the same kingdom (Fig 1B, purple), e.g. 96 animal species share significant number of identical miRNA sequences with human (Fig 1B, blue). On the contrary, there are 18,154 (~52%) miRNAs that still lack of homologs in any other species (Fig 1B, gray), indicating each species gains specific miRNAs during evolution.

It seems to be quite rare that different kingdoms share identical mature sequences, which may partially explain why cross-kingdom transferring is challenging. For instance, among 7,645 plant miRNAs, none has identical or similar sequences in human, even using loose criteria allows up to 2 mismatches. In Fig 2, we illustrated the sequences conservation using a phylogenetic tree built on the precursor sequences of miR-190 and -171 families. It showed, among three miRNA gene clusters (miR-190a, miR-190b, miR-171), human miR-190a and -190b are close to many animal species, e.g. cow and mouse, within their respective clusters. However, a different gene cluster of plant miR-171 is closer to miR-190b, compared to miR-190a (Fig 2A). Specifically, human miRNA, hsa-miR-190b, show sequence identity of 79% and 77% with sly-miR-171a (tomato) and miR-190a (human), respectively (alignments shown in Fig 2B). It indicates while miRNA genes are often conserved among species or even across kingdom during evolution, the derived mature sequences, however, may vary from each other.

Data access

All the data and programs used in this analysis can be found at http://sbbi.unl.edu/publications/microrna.
A close look at the 2,588 human miRNAs shows that 930 of them share identical sequences with orthologs in other species. We suspect the exogenous miRNAs with identical sequences, if possibly getting into circulation, might be able to regulate the same gene targets in human; moreover, they might regulate the same homolog targets in their own species if other criteria are met, e.g. 3' UTR of mRNAs are conserved across species.

**MiRNA features related to cross-species transportation**

Since sequence conservation alone cannot fully explain the miRNA cross-species bioavailability and molecular actions, we examined the aforementioned 1,102 features based on the sequence, structure and physicochemical properties to identify important features that can differentiate each kingdom group or distinguish human circulating miRNAs from the rest.

For each kingdom, we trained an SVM-based classifier wrapped by recursive feature elimination to select discriminative features associated with that kingdom. Based on 5-fold cross-
validation, we discovered a set of features that yields the best performance for each kingdom-against-others classification (Table 2). For example, in the Plants-against-other separation, we detected 147 features that produce a classifier with overall accuracy of 93.28% (Sensitivity = 89.71%, Specificity = 96.86%, MCC = 86.79%). Table 3 listed 21 features that contributed

### Table 2. Performance summary for kingdom-wise classification and human secreted miRNA prediction.

| Classification       | Selected Features | Accuracy | Sensitivity | Specificity | MCC    |
|----------------------|-------------------|----------|-------------|-------------|--------|
| Animalia             | 166               | 93.29%   | 96.46%      | 90.11%      | 86.75% |
| Plantae              | 147               | 93.28%   | 89.71%      | 96.86%      | 86.79% |
| FPV                  | 126               | 89.79%   | 87.39%      | 92.19%      | 79.68% |
| Human secretion      | 96                | 90.03%   | 84.68%      | 95.37%      | 80.51% |

The classification results are obtained through 5-fold cross validation with respect to different feature subsets selected.

"FPV" denotes the virtual kingdom of Fungi, Protista and Viruses.

doi:10.1371/journal.pone.0140587.t002

### Table 3. Examples of overlapped discriminative features chosen by three kingdom-wise classifications and the human blood secretory prediction.

| Features                | Details                                      | A  | P  | F  | H  | adj-P     |
|-------------------------|----------------------------------------------|----|----|----|----|-----------|
| Ensemble Free Energy (EFE) | Binding energy in kcal/mol                   | 2  | 8  | 8  |    | 3.81E-02  |
| Pairs                   | Number of pairing on stem-loop structure     | 1  | 2  | 19 |    | 9.43E-14  |
| Minimum Free Energy (MFE)| Precursor sequence fold with least thermodynamic free energy in kcal/mol  | 7  | 9  | 7  |    |           |
| %pairGC                 | Normalized frequency of G-C pairing on stem-loop structure. | 10 | 24 | 5  |    |           |
| Length_P                | Length of precursor sequence                 | 9  | 10 | 57 |    |           |
| G(((                    | Frequency of 3 paired nucleotides leading with G | 15 | 17 | 31 |    |           |
| C(((                    | Frequency of 3 paired nucleotides leading with C | 12 | 77 | 11 | 1.14E-03 |
| freqUUCC_seed           | Normalized frequency of UUCC in the seed region | 29 | 33 |    |    |           |
| freqCCA_seed            | Normalized frequency of CCA in the seed region | 27 | 35 |    |    |           |
| freqCG_P                | Normalized frequency of CG on precursor sequence | 41 | 12 |    |    |           |
| %G+C content_P          | Normalized frequency of G and C on precursor sequence | 16 | 12 | 57 | 5.14E-16|
| Max–stem-length         | Longest stems block on stem-loop structure   | 39 | 20 |    |    |           |
| A(((                    | Frequency of 3 paired nucleotides leading with A | 26 | 1  |    |    |           |
| freqGA_m                | Normalized frequency of GA on mature miRNA   | 105| 18 |    |    |           |
| freqC_m                 | Normalized frequency of C on mature miRNA    | 126| 94 | 30 |    |           |
| freqCUG_P               | Normalized frequency of CUG on precursor sequence | 24 | 58 | 98 | 80 |           |
| freqCUGG_P              | Normalized frequency of CUGG on precursor sequence | 122| 120| 92 |    |           |
| freqGU_P                | Normalized frequency of GU on precursor sequence | 137| 101| 52 | 5.78E-08|
| freqGU_m                | Normalized frequency of GU on mature miRNA   | 51 | 103| 5  | 2.27E-04 |
| freqU_m                 | Normalized frequency of U on mature miRNA    | 114| 53 | 127| 48 | 6.89E-08 |
| freqUGU_m               | Normalized frequency of UGU on mature miRNA  | 101| 98 | 93 |    |           |
| Palindromes             | Number of palindromes with length greater 3 occurring on precursor sequence | 11 | 16 | 79 | 68 | 2.22E-16 |
| freqC_seed              | Frequency of C in the seed region             | 82 | 26 | 70 |    |           |
| %G+C content_m         | Frequency of CG that occurs on mature miRNA  | 93 | 70 | 37 | 3.90E-09|

The numbers in the table represents the ranks of the selected feature in the corresponding classifiers (A: Animalia versus others, P: Plantae versus others, F: FPV versus others; H: human blood secretory miRNAs versus other human miRNAs) and the unselected ranks are not shown. The last column of “adj-P” shows that the adjusted p-value when analyzing the corresponding feature in the blood secretory prediction using Wilcoxon signed-rank test (insignificant p-values are not shown). Complete list was given in Table B in S1 File.

doi:10.1371/journal.pone.0140587.t003
Predicted transportable miRNAs

Since only 360 blood-detectable miRNAs (positive class) have been reported in previous study [25], we naturally assume that all other miRNAs may also possibly enter in human circulation. We performed a manifold ranking analysis on all 34,612 mature miRNAs based on the 221 selected features to rank miRNAs according to their transportable potential.

The final ranking list is given in Table C in S1 File. As expected, the query set of 360 known human plasma miRNAs were ranked among the top of the list. A close look at this list shows the top ranked entries are dominated by Animalia origin (Table 5). For example, 962 animal-borne miRNAs are ranked among top-1000 while 2812 are among the top-3000. Considering
the percentages of miRNAs from *Animalia, Plantae and Viruses* in the original dataset are 77.16%, 22.09% and 0.44%, respectively, it indicates *Animalia* and *Viruses* miRNAs are highly enriched among the predictions of transportable miRNAs in blood circulation compared to others.

There are 14 dietary miRNAs were ranked among top 500 and five of them have identical sequences in human including three bovine miRNAs (bta-miR-487b, -miR-421 and miR-216) and two chicken miRNAs (gga-miR-29a-3p and –miR-20b-5p). The identical sequence may indicate a higher chance that the exogenous miRNA will regulate human genes after transportation into circulation. As seen in Table 5, the number of dietary miRNAs scattered in the ranking list indicating the different likelihood of transportation. In particular, bta-miR-29b, a cow milk miRNA, which we have previously validated in human blood circulation [5], is ranked as the 345th among all dietary miRNAs, which indicates there might be many other dietary targets to be explored in blood as a large screening is available. Among the top 345 dietary miRNAs including bta-miR-29b, there are 117 entries showing identical sequences with their homologs in human and 97 are exosome related. Intuitively, all exosomal miRNAs are highly likely to get into human blood circulation since exosomes are widely present in most of biological fluids.

In contrast, the brassica-specific miR-824 and miR-167a were ranked at the bottom of list, as the 31,502th and 29,669th, respectively, which is consistently with our previous discovery that they are the least detectable in circulation [5].

### Validation of predicted transferrable miRNAs

From the prediction, the experimental data from cow milk study validated 9 transportable milk miRNAs in human blood, including bta-miR-487b, miR-181b, miR-421, miR-215, let-7c, miR-301a, miR-432, miR-127, and miR-184. The first three are highly-ranked in the dietary category and their functions are listed in Table 6.

In addition, the top-ranked 9 Epstein–Barr virus (EBV) miRNAs (ebv-miR-BART9-5p, BART8-5p, BART9-3p, BART8-3p, BART14-3p, BART14-5p, BART15, BART13-5p, and BART13-3p) have been reported in [54]. These miRNAs show meaningful abundances in human B cells and they may cooperatively regulate several human genes in ebv-infected samples. Moreover, ebv-miR-BART13 and BART9 were proven to be involved in WNT signaling and cell cycle control in human [54], partially consistent with our analysis in Table 6.

Similarly, 14 miRNAs from *Rhesus lymphocryptovirus* (rLCV) (rLCV-miR-L1-16-3p, RL1-16-5p, RL1-7-3p, RL1-7-5p, RL1-33-5p, RL1-33-3p, RL1-2-5p, RL1-24-3p, RL1-2-3p, RL1-24-5p, RL1-10-3p, RL1-10-5p, RL1-1-5p, and RL1-1-3p) that are highly transportable in our prediction have been reported in [55] where Raily et al. have found these rLCV miRNAs detectable in B cells of infected mammilla samples.

Based on all internal evaluation evidences, we provide a list of 368 exogenous miRNAs (23 viral miRNAs and 345 dietary miRNAs) as highly transportable miRNAs. The complete list can be found in Table D in S1 File. http://jvi.asm.org/content/84/10/5148.full.pdf

---

**Table 5. Statistics of the top miRNA entries in the ranking list with respect to their origins.**

|                | Animalia | Plantae | Viruses | Fungi | Protista | Dietary miRNAs |
|----------------|----------|---------|---------|-------|----------|----------------|
| **Original**   | 26705 (77.16%) | 7645 (22.09%) | 152 (0.44%) | 84 (0.24%) | 26 (0.08%) | 5217 (15.07%) |
| **Top-500**    | 499 (99.8%) | 1 (0.02%) | 0 | 0 | 0 | 14 (2.8%) |
| **Top-1000**   | 962 (96.2%) | 30 (3%) | 8 (0.8%) | 0 | 0 | 62 (6.2%) |
| **Top-3000**   | 2812 (93.7%) | 163 (5.43%) | 25 (0.87%) | 0 | 0 | 273 (9.1%) |
| **Top-5000**   | 4678 (93.56%) | 295 (5.9%) | 27 (0.54%) | 0 | 0 | 519 (10.38%) |
| **Top-10000**  | 9269 (92.69%) | 670 (6.7%) | 55 (0.55%) | 4 | 2 | 1024 (10.24%) |

*doi:10.1371/journal.pone.0140587.t005*
MiRNA-mediated gene regulations in human

For each miRNA that is potentially transferred into human circulation, 208 to 4,000 targets were collected through database search and computational prediction. The function and pathway enrichment analysis indicated that the 368 exogenous miRNAs may regulate human genes participating in immune development, metabolism and cancer. The detailed information for 9 exogenous miRNAs is provided in Table 6 while the full list is given in Table D in S1 File.

Table 6. Gene targets and functional analysis of the three top predictions of the transportable miRNAs in cow's milk, EBV, and rLCV.

| Dietary miRNAs | Human homologs with identical sequences | Number of targets | Related functional processes | Enriched pathway examples | Enriched GO term examples |
|----------------|----------------------------------------|------------------|-----------------------------|----------------------------|---------------------------|
| **Cow**        |                                        |                  |                             |                            |                           |
| bta-miR-487b   | hsa-miR-487b-3p                        | 46               | 468                         | Axon guidance (4.2E-2);    | Cell migration (8.0E-3);  |
|                |                                        |                  |                             | Regulation of actin       | Positive regulation of    |
|                |                                        |                  |                             | cytoskeleton (4.2E-2);    | locomotion (9.8E-3);     |
|                |                                        |                  |                             | Butanoate metabolism (5.2E-2); | Localization of cell    |
|                |                                        |                  |                             | MAPK signaling pathway (3.4E-2). | (1.2E-2); Protein    |
| bta-miR-181b   | hsa-miR-181b-5p                        | 1077             | 2084                        | Glioma (5.8E-3); Melanoma (8.1E-3); | Organelle lumen (7.3E-9); Nuclear envelope (9.5E-5); Intracellular non-membrane-bound Organelle (1.4E-4). |
| bta-miR-421    | hsa-miR-421                           | 793              | 808                         | Huntington's disease (3.5E-2); Hypoxia and p53 in the Cardiovascular system (4.6E-2); Apoptotic Signaling in Response to DNA Damage (3.6E-2). | Endomembrane system (4.6E-5); Nuclear lumen (7.8E-5); Establishment of protein localization (1.6E-4); Intracellular transport (2.8E-4). |
| **EBV**        |                                        |                  |                             |                            |                           |
| ebv-mir-BART13-3p | -                                      | -                | 208                         | Long-term potentiation (3.3E-2); Neurotrophin signaling pathway (3.9E-2); ErbB signaling pathway (6.1E-2); Melanogenesis (8.3E-2). | Phosphate metabolic process (1.2E-4); Wnt receptor signaling pathway (4.7E-2); phosphorylation (1.3E-3); Enzyme linked receptor protein signaling pathway (2.6E-3). |
| ebv-mir-BART8-3p | -                                      | -                | 549                         | Aldosterone-regulated sodium reabsorption (6.5E-3); Growth factors Survival factors Mitogens (2.3E-2); Prostate cancer (4.5E-2); Renal cell carcinoma (5.9E-2). | Nuclear lumen (5.6E-5); Transcriptional repressor complex (6.2E-5); Synapse (8.6E-5); Nucleoplasm (8.8E-5). |
| ebv-mir-BART9-3p | -                                      | -                | 568                         | Adherens junction (1.4E-2); Endocytosis (2.0E-2); Signaling Pathway from G-Protein Families (6.4E-2); Cell cycle (5.9E-2). | Membrane raft (1.5E-4); Regulation of transcription (1.8E-4); Regulation of transcription from RNA polymerase II promoter (3.4E-4); Positive regulation of macromolecule metabolic process (3.8E-4). |
| **rLCV**       |                                        |                  |                             |                            |                           |
| rLCV-mir-r11-16-3p | -                                      | -                | 466                         | Fc gamma R-mediated phagocytosis (5.6E-4); Ubiquitin mediated proteolysis (6.0E-4); Endocytosis (6.6E-4); Melanoma (1.9E-3). | Regulation of endocytosis (1.0E-4); protein modification by small protein Conjugation or removal (1.4E-4); Extrinsic to membrane (5.7E-4). |
| rLCV-mir-r11-16-5p | -                                      | -                | 403                         | Regulation of actin cytoskeleton (5.6E-4); Ca++/ Calmodulin-dependent Protein Kinase Activation (7.9E-3); Vascular smooth muscle contraction (9.3E-3); Focal adhesion (9.9E-3). | Positive regulation of cell migration (1.2E-4); Cytoskeleton (2.0E-4); Positive regulation of locomotion (2.6E-4). |
| rLCV-mir-r17-3p | -                                      | -                | 228                         | Axon guidance (6.9E-3); Adherens junction (3.5E-2); Metabolism of Anandamide (5.7E-2). | Intrinsic to membrane (1.8E-4); Alkali metal ion binding (9.0E-4); Plasma membrane part (1.6E-3); Metal ion transport (1.7E-3). |

The experimentally validated targets are collected from CLASH, MirTarBase and DIANA-TarBase; the complete list of the enriched pathways and GOs are listed in Table D in S1 File.

doi:10.1371/journal.pone.0140587.t006
Theoretically, when human absorb meaningful amount of exogenous miRNAs from food, these confounders must successfully bind to human genes in order to make subsequent regulatory impacts on certain biological processes in human. To further assess this binding potential, we examined the sequence conservation among the targets in human and other species. Specifically, we collected the 3'UTR sequence of the target genes from different organisms and performed multiple sequence alignment based on the binding sites reported in TargetScan [48] and DIANA-TarBase [47]. For example, the top ranked cow-milk miRNA, bta-miR-487b, was confirmed in our validation and it shows identical sequence with hsa-miR-487b in human circulation. We compared the sequences of 15 predicted bovine target genes of bta-miR-487b and 46 experimentally validated targets of hsa-miR-487b in human. As shown in Fig 3, three
Conserved alignment blocks were observed among miRNA-mRNA binding regions in human and bovine. The consistency may provide more confidence if such exogenous miRNAs enter into human circulation, they may be able to play regulatory roles in human pathways by interacting with human genes. Based on our analysis, hsa-miR-487b targets 464 human genes targets and may be able to regulate human pathways related to MAPK signaling, actin cytoskeleton regulation, axon guidance, and Butanoate metabolism (Fig 4).

Another example is bta-miR-29b, which has also been experimentally validated in human blood [5]. Based on the 301 predicted mRNA targets, miR-29b is found to be involved in leukocyte transendothelial migration, cancer, and bone development. Overall, the transportable exogenous miRNAs predicted in this study are involved in many major biological processes including development, differentiation, cell proliferation, and metabolism [56], e.g. miR-27b, miR-34a, miR-106b, and miR-130 that are related to immune or development [6–8].

Discussion

While our knowledge of miRNAs secretion and circulation is still limited, compelling evidences has indicated there is an selective intake and release mechanism involved in these processes. Our study has followed this line to explore the mechanistic features that may contribute in miRNA cross-species transfer and gene regulation in human using an integrative approach.
Through sequence comparison, miRNAs from different species show moderate conservations among mature sequences throughout phylogeny. Subsequently, various sets of features related to sequence, structure and physicochemical properties are found to be discriminative for miRNAs in different kingdom groups and blood secretory group. The selected feature contributing to blood secretion may reflect molecular mechanism related to selective package and exportation [57], carrier-mediated transport realized by its encapsulation in exosomes and microvesicles or Ago2-bound complexes, and the microparticles exhibit highly distinct binding patterns with miRNAs [22] in which, intuitively, involved certain molecular sequence, structure, or physicochemical properties.

Selected features may bring new insights of transposable miRNAs. For example, the length of pre-miRNAs and %G+C content of mature miRNAs show different patterns between human circulating miRNAs and the rest of human miRNAs (shown in S1 Fig), suggesting human blood miRNAs are produced by longer pre-miRNAs and often show higher percentage of C, G nucleotides. In the kingdom-wise classifications, several selected features were related to the frequency of nucleotide G in the first segment of miRNAs, i.e., the 6–7 nucleotides of 5’ end of miRNAs. This could result from the following. For target recognition by two groups of miRNAs, each recognizes its mRNA targets by 5’ or 3’ end complementary pairing. The first 6 or 7 nucleotides on the 5’ end are known to be used for target recognition with little or no support from the 3’ miRNA end [58]. This suggests that 5’ end and its nucleotide composition are important factor in determining the fate of miRNAs. A recent study showed that strand bias selection exists for miRNAs in incorporation into the RISC complex; and highly expressed strands tend to have nucleotide G-bias and U-bias at 5’ end [59]. All these clues suggest that miRNAs enriched with G and U nucleotides at 5’ end are more likely to bind to the Ago2 protein, forming a RISC complex.

Within the top-1000 ranked prediction, 96.1% miRNAs are from animal origin and only 3% are from plant, which is consistent with our intuition that animal-borne miRNAs are subject to more significant absorption in human compared to plant miRNAs. However, it should be noted the bioavailability of milk miRNAs has not been investigated at a large scale, and the uptake mechanism is still ambiguous regarding which and how miRNAs enter blood circulation. In contrast, it was shown that rice miR-168a (osa-miR-168a) is also detectable in human and animal sera, and it decreases the expression of low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA [60]. Nonetheless, the low concentration reported by multiple follow-up studies seems to exclude any impact of these miRNAs on gene expression. For example, the levels of osa-miR-168a in human plasma were only about 3% of the bta-miR-29b levels observed in our preliminary studies. It is possible that the miRNAs from plant have sequential or structural features that prevent their secretion into blood, or that the methylation of the 3’-terminal ribose in position C2 in plant miRNAs by the methyltransferase HEN1 [61], impairs the intestinal transport of miRNAs, but this hypothesis is currently untested. We also expect the interaction between exosome and host intestinal cells may influence the transport. An in-depth investigation of transport mechanisms and kinetics of milk-borne miRNAs was beyond the scope of this study, but is currently pursued in the investigator’s lab.

Another critical challenge for uncovering the diverse biological roles of miRNAs lies in the efficient identification of targeting genes where current computational methods are still at a very early stage of focusing on static miRNA target prediction [62], while new observations have revealed the dynamic nature of miRNA-mRNA interactions that may vary in different phenotypic conditions [63–66]. Our on-going efforts are focused on the integration of gene expression information into target prediction toward identifying the real regulatory events under a pathway context. Empowered by the next-generation sequencing technology, we can study miRNA existence and expression in different specifics. However, sequencing based
analysis on cross-species transportation study still encounter challenges in terms of the sensitivity of detecting exogenous miRNAs with low abundance and differentiation of the sources when identical sequences are involved. With that has been said, such computational study is important to provide an efficient tool that can facilitate a targeted search for exogenous miRNAs in human circulation rather than profiling in the old fashion.

Conclusion
Here we presented an integrative study where comparative analysis and computational prediction have been applied to assess the cross-species transportation of miRNAs, particularly focusing on inferring the likelihood of exogenous miRNA in human circulation. Given the limited understanding about miRNA circulation, this study will contribute substantially in overcoming the aforementioned scientific limitations and dramatically reducing the extensive lab-load in miRNA biology research by using a revolutionary systems-driven strategy to study this complex problem. Specifically, this bioinformatics-driven study enables bypass the following key issues: (1) Lack of supporting information to discern between endogenous miRNA synthesis or dietary miRNA absorption in the miRNA expression change in human blood test subjects; (2) Inference from endogenous miRNA synthesis [67] that might compensate for dietary miRNA deficiency; (3) potential distinct metabolism of dietary miRNAs in the intestinal mucosa. Substantial follow-up studies will be conducted to extend the analysis and clarify in greater detail the information generated by this study in revealing information on miRNA exchange and functional regulation in human disease prevention. We anticipate the novel computational tools developed for characterizing miRNA circulation and targeting will be useful for other miRNA and nutrigenomics research areas.

Supporting Information

S1 Fig. Distributions of three example features. (A) Number of palindromic sequences in precursor sequence in each kingdom. The x-axis contains five kingdoms and the y-axis represents the number of palindromic sequences. (B) Length of pre-miRNA (left) and %G+C content on mature miRNAs (right) over the miRNAs found in blood and the other human miRNAs. The x-axis contains the value of the corresponding feature, and the y-axis represents the frequency.

S1 File. Supplementary Tables. Table A. Detailed descriptions of all 1102 miRNA features; Table B. All overlapped discriminative features chosen by three kingdom-wise classifications and the human blood secretory prediction; Table C. Final manifold ranking list of all 34,612 miRNAs; Table D. Gene targets and functional analysis of 368 predicted transferrable exogenous miRNAs.

S1 Methods. Supplementary Methods.

Acknowledgments
The authors would like to thank all the individuals who have participated in this study for their helpful discussions and technical assistance. In particular, we thank Dr. Scott Baier for his assistance in preparing RNA samples for NGS analysis. The Holland Computing Center at UNL has provided us the computational facilities for data analysis.
Author Contributions
Conceived and designed the experiments: JS JC. Analyzed the data: JS KC JC. Contributed reagents/materials/analysis tools: JS KC JZ JC. Wrote the paper: JS KC JZ JC.

References
1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136(2):215–33. doi: 10.1016/j.cell.2009.01.002 PMID: 19167326; PubMed Central PMCID: PMC3794896.
2. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annual review of biochemistry. 2010; 79:351–79. doi: 10.1146/annurev-biochem-060308-103103 PMID: 20533884.
3. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic acids research. 2014; 42(Database issue):D68–73. doi: 10.1093/nar/gkt1181 PMID: 24275495; PubMed Central PMCID: PMC3965103.
4. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome research. 2009; 19(1):92–105. doi: 10.1101/gr.082701.108 PMID: 18955434; PubMed Central PMCID: PMC2612969.
5. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. The Journal of nutrition. 2014; 144(10):1495–500. doi: 10.3945/jn.114.196438 PMID: 25122645; PubMed Central PMCID: PMC4162473.
6. Izumi H, Kosaka N, Shimizu T, Sekine K, Ochiya T, Takase M. Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions. Journal of dairy science. 2012; 95(9):4831–41. Epub 2012/08/25. doi: 10.3168/jds.2012-5489 PMID: 22916887.
7. Arnold CN, Pirie E, Dosenovic P, McInerney GM, Xia Y, Wang N, et al. A forward genetic screen reveals roles for Nfkbid, Zeb1, and Ruvbl2 in humoral immunity. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(31):12286–93. Epub 2012/07/05. doi: 10.1073/pnas.1209134109 PMID: 22671313; PubMed Central PMCID: PMC3411946.
8. Liu R, Ma X, Xu L, Wang D, Jiang X, Zhu W, et al. Differential microRNA expression in peripheral blood mononuclear cells from Graves’ disease patients. The Journal of clinical endocrinology and metabolism. 2012; 97(6):E968–E72. doi: 10.1210/jc.2011-2982 PMID: 22456620.
9. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell research. 2012; 22(1):107–26. doi: 10.1038/cr.2011.158 PMID: 21931358; PubMed Central PMCID: PMC3351925.
10. Snow JW, Hale AE, Isaacs SK, Baggish AL, Chan SY. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA biology. 2013; 10(7):1107–16. Epub 2013/05/15. doi: 10.4161/rna.24905 PMID: 23669076; PubMed Central PMCID: PMC3849158.
11. Dickinson B, Zhang Y, Petrick JS, Heck G, Ivashuta S, Marshall WS. Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nature biotechnology. 2013; 31(11):965–7. Epub 2013/11/12. doi: 10.1038/nbt.2737 PMID: 24213763.
12. Chen X, Zen K, Zhang CY. Reply to Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nature biotechnology. 2013; 31(11):967–9. Epub 2013/11/12. doi: 10.1038/nbt.2741 PMID: 24213764.
13. Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, Huang D, et al. The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PLoS ONE. 2012; 7(12):e51009. Epub 2012/12/20. doi: 10.1371/journal.pone.0051009 PMID: 23251414; PubMed Central PMCID: PMC3519536.
14. Lee CT, Risom T, Strauss WM. Evolutionary conservation of microRNA regulatory circuits: an examination of microRNA gene complexity and conserved microRNA-target interactions through metazoan phylogeny. DNA and cell biology. 2007; 26(4):209–18. doi: 10.1089/dna.2006.0545 PMID: 17465887.
15. Mor E, Shomron N. Species-specific microRNA regulation influences phenotypic variability: perspectives on species-specific microRNA regulation. BioEssays: news and reviews in molecular, cellular and developmental biology. 2013; 35(10):881–8. doi: 10.1002/bies.201200157 PMID: 23864354.
16. Brameier M, Wulf C. Ab initio identification of human microRNAs based on structure motifs. BMC bioinformatics. 2007; 8:478. doi: 10.1186/1471-2105-8-478 PMID: 18088431; PubMed Central PMCID: PMC2238772.
17. Ru Y, Kechris KJ, Tabakoff B, Hoffman P, Radcliffe RA, Bowler R, et al. The multiMiR R package and database: integration of microRNA-target interactions along with their disease and drug associations. Nucleic acids research. 2014; 42(17):e133. doi: 10.1093/nar/gku631 PMID: 25063298; PubMed Central PMCID: PMC4176155.
18. Ding J, Zhou S, Guan J. MiRenSVM: towards better prediction of microRNA precursors using an ensemble SVM classifier with multi-loop features. BMC bioinformatics. 2010; 11 Suppl 11:S11. doi: 10.1186/1471-2105-11-S11 PMID: 21772046; PubMed Central PMCID: PMC3024864.

19. Batuwita R, Palade V. microPred: effective classification of pre-miRNAs for human miRNA gene prediction. Bioinformatics. 2009; 25(8):989–95. doi: 10.1093/bioinformatics/btp107 PMID: 19233894.

20. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of miRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007; 9(6):654–9. doi: 10.1038/nccb1596 PMID: 17486113.

21. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PloS one. 2008; 3(11):e3694. doi: 10.1371/journal.pone.0003694 PMID: 19002258; PubMed Central PMCID: PMC2577891.

22. Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovasc Res. 2012; 93(4):633–44. doi: 10.1093/cvr/cvs007 PMID: 22258631; PubMed Central PMCID: PMC3291092.

23. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic acids research. 2011; 39(16):7223–33. doi: 10.1093/nar/gkr254 PMID: 21609964; PubMed Central PMCID: PMC3167594.

24. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(12):5003–8. doi: 10.1073/pnas.1019055108 PMID: 21383194; PubMed Central PMCID: PMC3064324.

25. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clinical chemistry. 2010; 56(11):1733–41. doi: 10.1373/clinchem.2010.147405 PMID: 20847327.

26. Chiang K, Shu J, Zempleni J, Cui J. Dietary MicroRNA Database (DMD): An Archive Database and Analytic Tool for Food-Borne microRNAs. PloS one. 2015; 10(6):e0128089. doi: 10.1371/journal.pone.0128089 PMID: 26030752; PubMed Central PMCID: PMC4451068.

27. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic acids research. 2012; 40(Database issue):D1241–4. doi: 10.1093/nar/gkr828 PMID: 21989406; PubMed Central PMCID: PMC3245025.

28. Kim DK, Lee J, Kim SR, Choi DS, Yoon YJ, Kim JH, et al. EVpedia: a community web portal for extracellular vesicles research. Bioinformatics. 2015; 31(6):933–9. doi: 10.1093/bioinformatics/btu741 PMID: 25388151; PubMed Central PMCID: PMC4375401.

29. Meister G, Landthaler M, Patkaniovska A, Dorsett Y, Teng G, Tuschi T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Molecular cell. 2004; 15(2):185–97. doi: 10.1016/j.molcel.2004.07.007 PMID: 15260970.

30. Mathelier A, Carbone A. Large scale chromosomal mapping of human microRNA structural clusters. Nucleic acids research. 2013; 41(8):4392–408. doi: 10.1093/nar/gkt112 PMID: 23444140; PubMed Central PMCID: PMC3632110.

31. Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flam C, Stadler PF, et al. ViennaRNA Package 2.0. Algorithms for molecular biology: AMB. 2011; 6:26. doi: 10.1186/1748-7188-6-26 PMID: 22115189; PubMed Central PMCID: PMC3319429.

32. Knudsen V, Caetano-Anolles G. NOBAI: a web server for character coding of geometrical and statistical features in RNA structure. Nucleic acids research. 2008; 36(Web Server issue):W85–90. doi: 10.1093/nar/gkn220 PMID: 18448468; PubMed Central PMCID: PMC2447726.

33. Keerthi SKS S., Bhattacharyya C., Murthy K. R. K. Improvements to Platt's SMO Algorithm for SVM Classifier Design Neural Computation. 2001; 13:637–49.

34. Platt JC. Fast Training of Support Vector Machines using Sequential Minimal Optimization. Advances in kernel methods: support vector learning. Cambridge, MA, USA: MIT Press 1999. p. 185–208.

35. Tang ZQ, Han LY, Lin HH, Cui J, Jia J, Low BC, et al. Derivation of stable microarray cancer-differentiating signatures using consensus scoring of multiple random sampling and gene-ranking consistency evaluation. Cancer Res. 2007; 67(20):9996–10003. Epub 2007/10/19. doi: 10.1158/0008-5472.CAN-07-1601 PMID: 17942933.

36. J. Brank MG, N. Milić-frayling, D. Mladenić. Feature selection using support vector machines. In Proc of the 3rd Int Conf on Data Mining Methods and Databases for Engineering, Finance, and Other Fields. 2002.

37. Nitesh V. Chawla KWB, Hall Lawrence O., Kegelmeyer W. Philip. SMOTE: Synthetic Minority Over-sampling Technique. Journal of Artificial Intelligence Research. 2002.
38. Matthews BW. Comparison of the predicted and observed secondary structure of T4 phage lysozyme. Biochim Biophys Acta. 1975; 405(2):442–51. PMID: 1180967.
39. Chih-Chung Chang C-JL. LIBSVM: a library for support vector machines. ACM Transactions on Intelligent Systems and Technology. 2011.
40. Zhou D, Weston J, Gretton A, Bousquet O, Scholkopf B, editors. Ranking on Data Manifolds 2004: Bradford Book.
41. He J, Li M, Zhang HJ, Tong H, Zhang C, editors. Manifold-ranking based image retrieval 2004: ACM New York, NY, USA.
42. He J, Li M, Zhang H, Tong H, Zhang C. Generalized Manifold-Ranking-Based Image Retrieval. IEEE TRANSACTIONS ON IMAGE PROCESSING. 2006; 15(10):3170. PMID: 17022278
43. Liu Q, Cui J, Yang Q, Xu Y. In-silico prediction of blood-secretory human proteins using a ranking algorithm. BMC bioinformatics. 2010; 11:250. doi: 10.1186/1471-2105-11-250 PMID: 20465853; PubMed Central PMCID: PMC2877692.
44. Zhao YF, He LY, Liu BY, Li J, Li FY, Huo RL, et al. Syndrome classification based on manifold ranking for viral hepatitis. Chinese journal of integrative medicine. 2014; 20(5):394–9. doi: 10.1007/s11655-013-1659-4 PMID: 24174345.
45. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell. 2013; 153(3):654–65. doi: 10.1016/j.cell.2013.03.043 PMID: 23622248; PubMed Central PMCID: PMC3650559.
46. Hsu SD, Tseng YT, Shrestha S, Lin YL, Khaleel A, Chou CH, et al. miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. Nucleic acids research. 2014; 42(Database issue):D78–85. doi: 10.1093/nar/gkt1266 PMID: 24304892; PubMed Central PMCID: PMC3965058.
47. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic acids research. 2015; 43(Database issue):D153–9. doi: 10.1093/nar/gku1215 PMID: 25416803.
48. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Molecular cell. 2007; 27(1):91–105. doi: 10.1016/j.molcel.2007.06.017 PMID: 17612493; PubMed Central PMCID: PMC3800283.
49. Wang X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. RNA. 2008; 14(6):1012–7. doi: 10.1261/rna.965408 PMID: 18426918; PubMed Central PMCID: PMC2390791.
50. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(43):15545–50. doi: 10.1073/pnas.0505680102 PMID: 16199517; PubMed Central PMCID: PMC1298996.
51. Sun Z, Evans J, Bhaqwate A, Middha S, Bockol M, Yan H, et al. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. BMC genomics. 2014; 15(10):1512–4. doi: 10.1186/1471-2164-15-1423 PMID: 24894665; PubMed Central PMCID: PMC4070549.
52. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics. 2012; 28(23):3150–2. doi: 10.1093/bioinformatics/bts565 PMID: 23060610; PubMed Central PMCID: PMC3516142.
53. Xuan P, Guo M, Huang Y, Li W, Huang Y. maturePred: efficient identification of microRNAs within novel plant pre-miRNAs. PloS one. 2011; 6(11):e27422. doi: 10.1371/journal.pone.0027422 PMID: 22110646; PubMed Central PMCID: PMC3217989.
54. Riley KJ, Rabinowitz GS, Yario TA, Luna JM, Darrell RB, Stelitz JA. EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J. 2012; 31(9):2207–21. doi: 10.1038/emboj.2012.63 PMID: 22473208; PubMed Central PMCID: PMC3343464.
55. Riley KJ, Rabinowitz GS, Stelitz JA. Comprehensive analysis of Rhesus lymphocryptovirus microRNA expression. J Virol. 2010; 84(10):5148–57. doi: 10.1128/JVI.00110-10 PMID: 20219930; PubMed Central PMCID: PMC2863793.
56. Neilson JR, Sharp PA. Small RNA regulators of gene expression. Cell. 2008; 134(6):899–902. doi: 10.1016/j.cell.2008.09.006 PMID: 18805079.
57. Zhang Y, Liu D, Chen X, Li J, Li L, Bian Z, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. Mol Cell. 2010; 39(1):133–44. doi: 10.1016/j.molcel.2010.06.010 PMID: 20603081.
58. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol. 2005; 3(3):e85. Epub 2005/02/22. doi: 10.1371/journal.pbio.0030085 PMID: 15723116; PubMed Central PMCID: PMC1043860.

59. Hu HY, Yan Z, Xu Y, Hu H, Menzel C, Zhou YH, et al. Sequence features associated with microRNA strand selection in humans and flies. BMC Genomics. 2009; 10:413. Epub 2009/09/08. 1471-2164-10-413 [pii] doi: 10.1186/1471-2164-10-413 PMID: 19732433; PubMed Central PMCID: PMC2751786.

60. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell research. 2012; 22 (1):107–26. Epub 2011/09/21. doi: 10.1038/cr.2011.158 PMID: 21931358; PubMed Central PMCID: PMC3351925.

61. Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, et al. Methylation as a crucial step in plant microRNA biogenesis. Science. 2005; 307(5711):932–5. Epub 2005/02/12. doi: 10.1126/science.1107130 PMID: 15705854.

62. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120(1):15–20. Epub 2005/01/18. S0092867404012607 [pii] doi: 10.1016/j.cell.2004.12.035 PMID: 15652477.

63. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2):281–97. Epub 2004/01/28. S0092867404000455 [pii] PMID: 14744438.

64. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. Nat Genet. 2005; 37(5):495–500. Epub 2005/04/05. doi: 10.1038/ng1536 PMID: 15806104.

65. Seitz H. Redefining microRNA targets.Curr Biol. 2009; 19(10):870–3. Epub 2009/04/21. doi: 10.1016/j.cub.2009.03.059 PMID: 19375315.

66. Cannell IG, Kong YW, Bushell M. How do microRNAs regulate gene expression? Biochem Soc Trans. 2008; 36(Pt 6):1224–31. Epub 2008/11/22. doi: 10.1042/BST0361224 PMID: 19021530.

67. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome research. 2004; 14(10A):1902–10. doi: 10.1101/gr.2722704 PMID: 15364901; PubMed Central PMCID: PMC524413.