RESEARCH ARTICLE

Alternative miRNAs? Human sequences misidentified as plant miRNAs in plant studies and in human plasma [version 1; referees: 2 approved]

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Abstract

Background: A 2017 study reported that "Plant miRNAs found in human circulating system provide evidences of cross kingdom RNAi". Analysis of two human blood plasma sequencing datasets was said to provide evidence for uptake of plant miRNAs into human plasma. The results were also purportedly inconsistent with contamination.
Methods: Sequences from public datasets and miRNA databases were compared with results downloaded from the website of the reporting journal.
Results: Only one putative plant miRNA ("peu-MIR2910) mapped consistently above background, and this sequence is found with 100% identity in a human rRNA. Several other rarer but consistently mapped putative plant miRNAs also have 100% or near 100% matches to human transcripts or genomic sequences, and some do not appear to map to plant genomes at all.
Conclusions: Reanalysis of public data suggests that dietary plant xenomiR uptake is not supported, but instead confirms previous findings that detection of rare plant miRNAs in mammalian sequencing datasets is artifactual. Some putative plant miRNAs, including MIR2910 and MIR2911, may represent human sequence contamination or other artifacts in plant studies, emphasizing the need for rigorous controls and data filtering strategies when assessing possible xenomiRNAs.

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Introduction

Reports of plant or other dietary miRNAs, or xenomiRs, entering mammalian circulation through the diet[1–4] generated initial excitement for the xenomiR transfer hypothesis, yet negative results of replication and reproduction studies have cast doubt on xenomiR transfer as a general mechanism[5–11]. A prominent claim of xenomiR function[1] has also failed rigorous reproduction[7], unmasked as the result of an uncontrolled variable in the original experiment. Analyses of public datasets have revealed that studies of xenomiRs and other foreign-origin nucleic acids are fraught with artifacts: combinations of contamination, amplification or sequencing errors, permissive analysis pathways, and batch effects[8,10,12–16]. A particularly comprehensive study recently found that foreign miRNAs in human biofluids and tissues do not match human food consumption, are marked by batch effects, and are thus most parsimoniously explained as artifacts[13]. Studies of organisms with no exposure to plants have also found evidence of the same types of apparent plant contamination that plague some measurements of human samples[8,17]. Liu *et al.*[18] mapped sequencing data from two studies of human plasma and other samples[19,20] to various plant genomes using a 2010 plant miRNA database, PMRD[21], concluding that previous reports of dietary xenomiR transfer are supported. In this brief report, these results are examined critically.

Methods

Plant mapping results from Liu *et al.*[18] (total mapped counts) were downloaded from the BMC Genomics website. Accession numbers of sequencing datasets were checked against the publications of Ninomiya *et al.*[19] and Yuan *et al.*[20], as well as the Sequence Read Archive (SRA). Data were sorted and analyzed in Microsoft Excel for Mac 2011, Version 14.7.1. Plant miRNA sequences were obtained from miRBase[22]. Because certain plant sequences have been removed from miRBase because they have been identified as ncRNA degradation artifacts, the plant microRNA database (PMRD)[21] was consulted; however, repeated attempts to access the site were unsuccessful, so information was retrieved instead from miRMaid[23] or miRNEST 2.0[24]. Supplementary File 1 contains the relevant count data from the Ninomiya *et al.*[19] and Yuan *et al.*[20] studies.

An earlier version of this article can be found on bioRxiv (https://doi.org/10.1101/120634).

Results

Data evaluation

A cross-check of the source files and articles shows that the plasma data evaluated by Liu *et al.* were from 198 plasma samples, not 410 as reported. Ninomiya *et al.* sequenced six human plasma samples, six PBMC samples, and 11 cultured cell lines[19]. Yuan *et al.* sequenced 192 human plasma libraries (prepared from polymer-precipitated plasma particles)[20]. Each library was sequenced once, and then a second time to increase total reads. Counts were presented as reads per million mapped reads (rpm)[20]. In contrast, Liu *et al.* appear to have reported total mapped reads in their data table[18]. Yuan *et al.* also set an expression cutoff of 32 rpm (log2 rpm of 5 or above). With an average 12.5 million reads per sample (the sum of the two runs per library), and, on average, about half of the sequences mapped, the 32 rpm cutoff would translate to around 200 total reads in the average sample as mapped by Liu *et al.*[18].

Only one putative plant miRNA above background levels

Consulting the Liu *et al.* mapping table[18] and the SRA, results from duplicate sequencing runs from the Yuan *et al.* dataset were combined, and two samples without reliable replicates were eliminated. A total of 1294 putative plant miRNAs had at least one mapped read in at least one of the remaining 190 samples. However, many of these miRNAs were identical orthologs or paralogs, and most were mapped at one or fewer rpm on average, and in only a small minority of samples. Across all samples, only one putative plant miRNA mapped above a median 200 read cutoff, roughly corresponding to the 32 rpm cutoff of Yuan *et al.* (Table 1). All other RNAs, including previously reported xenomiRs such as MIR159a, MIR168a, and the plant ribosomal degradation fragment MIR2911[25–27], were thus below the level of background noise established by the original investigators. Indeed, previously reported xenomiRs were mapped in few samples and below 1 rpm. The absence of these RNAs is confirmed by Liu *et al.*'s analysis of the Ninomiya, *et al.* study[19], where MIR159a, MIR168a, and MIR2911 mapped in none of the plasma samples. The single putative plant miRNA that mapped above background levels in this study was, again, peu-MIR2910 (Table 2).

Lowering the threshold: still only a handful of possible xenomiRs

Since only one plant miRNA appeared to map consistently above background, the inclusion threshold of Yuan *et al.* was relaxed to include all miRNAs with three or more mapped reads (Liu *et al.* data) in 10% or more of the samples from either study. These are rather permissive criteria but may at least screen out some false positives due to amplification and sequencing errors. All samples from the Ninomiya study were included, despite the fact that most were not plasma. 11 miRNAs satisfied these criteria for the Yuan *et al.* data (Table 1). (One low-mapping miRNA was excluded because its sequence could not be found in miRBase[22,28], miRMaid[23], miRNEST 2.0[24] or indeed through any searches attempted.) 10 satisfied the criteria from the Ninomiya study (Table 2), including one sequence that was part of another (compare ath-MIRf10046-akr and ath-MIRf10045-ak, Table 3). However, if only the plasma samples from the latter study are considered, three miRNAs remain (Table 2). In total, 15 putative miRNAs satisfied the permissive inclusion criteria, including five (Yuan only), four (Ninomiya only), and six (both) (Table 3).

To miR or not to miR

As miRNA discovery, validation, and annotation has advanced, numerous reported miRNAs have been reclassified as degradation fragments of other noncoding RNAs (ncRNAs). A classic example is MIR2911, a plant rRNA degradation fragment that has been misidentified as a microRNA. Interestingly, only 2 of the 15 miRNAs identified as plant miRNAs in this study are annotated in miRBase. Although some of these sequences may represent rare or unusually structured miRNAs, several are part of non-miRNA ncRNAs or other sequences that seem unlikely, at least at first

Table 1. Summary of the most frequently mapping putative plant miRNAs in the Liu *et al.* analysis of datasets from Yuan *et al.* Here, data from only 190 of 192 plasma samples were included, since all but the excluded 2 samples were successfully sequenced twice. miRNA inclusion criteria were: 1) Three total mapped reads according to Liu *et al.*'s data in at least 10% of the samples and 2) discoverable putative mature sequence through miRBase, miRMaid, or miRNEST 2.0. An "estimated median rpm" value was calculated based on median total counts, average reads, and the midoint of the reported mapping percentage range. miRNAs with perfect human matches are in red. Note that only MIR2910 consistently exceeds the rpm threshold set by Yuan *et al.*

Putative miRNA	Samples w/ reads ≥3	Average total counts	Median total counts	Max	Est. median rpm in avg sample
peu-MIR2910	190	1143.4	1072.5	2020	180.6
peu-MIR2916	190	119.7	115	315	19.4
tae-MIR2005	178	8.7	9	23	1.5
peu-MIR2914	169	14.3	9	348	1.5
tae-MIR2018	167	5.9	6	14	1.0
ath-MIRf10482-akr	161	7.3	6	29	1.0
ppt-MIR896	147	4.6	4	15	0.7
ptc-MIRf12412-akr	47	2.7	2	18	0.3
peu-MIR2911	42	2.6	2	10	0.3
ppt-MIR894	39	2.3	2	18	0.3
ptc-MIRf12524-akr	28	1.8	1	5	0.2

Table 2. Putative plant miRNA mapping from the Ninomiya *et al.* dataset. This dataset consisted of both cellular and plasma miRNA. Here, all results are shown in the left half of the table, and plasma results on the right. miRNA inclusion criteria were: 1) Three total mapped reads according to Liu *et al.*'s data in at least 10% of the samples (cells and plasma together) and 2) discoverable putative mature sequence through miRBase, miRMaid, or miRNEST 2.0. "Avg rpm" is calculated from the total mapped reads and total reads per sample (not mapped reads). Putative miRNAs that met inclusion criteria in the Yuan *et al.* study are italicized, and sequences with perfect human matches are in red.

	Cells (n=17) and plasma (n=6)					Plasma only (n=6)				
Putative miRNA	Samples w/reads ≥3	Average	Median	Max	avg rpm	Samples w/ reads ≥3	Average	Median	Max	avg rpm
peu-MIR2910	21	370	41.5	5369	21.3	6	1210	480.5	5369	83.2
ptc-MIRf10804-akr	17	26	26	60	1.2	0			0	0
ptc-MIRf12412-akr	17	63	61	156	2.8	0			0	0
tae-MIR2018	17	39	29	163	1.7	0			0	0
ptc-MIRf12524-akr	10	13	10.5	31	0.4	2	16	16	31	0.4
tae-MIR2005	7	17	11	69	0.4	0			0	0
ath-MIRf10045-akr	4	5	4	10	0.1	0			0	0
ath-MIRf10046-akr	4	5	4	10	0.1	0			0	0
peu-MIR2914	3	26	11	81	0.3	3	33.7	19	81	1.2
peu-MIR2915	3	5	4.5	8	0.0	0			0	0

Table 3. Putative plant miRNAs mapped by Liu *et al.* from the Yuan *et al.* or Ninomiya *et al.* studies. Inclusion criteria were: 1) Three total mapped reads according to Liu *et al.*'s data in at least 10% of the samples in the respective studies and 2) discoverable putative mature sequence through miRBase, miRMaid, or miRNEST 2.0. miRNA status was considered unlikely if miRBase listed the miRNA as a non-miRNA or if the sequence mapped to non-miRNA regions. Human matches were exact (with an example given), "partial" (at least 15 nt stretches with 100% identity), or as otherwise described. Note that the ath-MIRf10046-akr is found within the ath-MIRf10045-akr sequence.

Putative miRNA	Yuan *et al.* or Ninomiya *et al.*	Sequence	miRBase	miRNA status in plant	Human matches?	Example match
peu-MIR2910	Both	UAGUUGGUGGAGCGAUUUGUC	No	rRNA fragment	18s rRNA	NR_003286.2
peu-MIR2916	Yuan	UGGGGACUCGAAGACGAUCAUAU	Yes	Possible, but 20 nt maps to rRNA	Partial	
tae-MIR2005	Both	GGGUGUAUAGCUCAGUUGG	No	Unlikely: plant mitochondrial genome	Partial	
peu-MIR2914	Both	CAUGGUGGUGACGGGUGACGGAG	No	rRNA fragment	Partial	
tae-MIR2018	Both	GCCCGUCUAGCUCAGUUGGU	No	Unlikely; maps to many plant transcripts	Partial	
ath-MIRf10482-akr	Yuan	UCUACUCGACUAGGUGGUCGAGUGG	No	Maps to *Arabidopsis*	Partial	
ppt-MIR896	Yuan	GUCAAUUUGGCCGAGUGGUUAAGGC	No	tRNA fragment	Partial	
ptc-MIRf12412-akr	Both	GCUGGGAUUACAGGCGUGAGCCACC	No	Does not map to *Populus*	Many exact	XR_001736898.1
peu-MIR2911	Yuan	GGCCGGGGGACGGGCUGGGA	No	rRNA fragment	1 mismatch in a 20-nt stretch	NC_018921.2
ppt-MIR894	Yuan	CGUUUCACGUCGGGUUCACC	Yes	Yes, but other transcriptome matches	No	
ptc-MIRf12524-akr	Both	CCUGUAAUCCCAGCUACUCGGG	No	Does not map to *Populus*	Many exact	NG_053018.1
ath-MIRf10045-akr	Ninomiya	UCUACUCGACCUGGUGGUCGAGUGGU	No	Unlikely; chromosomal region	Partial	
ath-MIRf10046-akr	Ninomiya	CUCGACCUGGUGGUCGAGUGGU	No	Part of above sequence	Partial	
peu-MIR2915	Ninomiya	CCCGUCUAGCUCAGUUGGUA	No	tRNA fragment; sequence found in many transcripts	Partial	
ptc-MIRf10804-akr	Ninomiya	CCUGUAAUCCCAGCACUUUGG	No	Unlikely; microsatellite sequence	Many exact, also hsa-miR-3929 precursor (1 mismatch)	XR_001736898.1 (antisense)

glance, to give rise to microRNAs. Among the apparently misidentified miRNAs is MIR2910, the most abundant plant miRNA identified by Liu *et al.* The MIR2910 sequence, UAGUUGGUGGAGCGAUUUGUC, is found in the highly conserved and expressed large subunit (LSU) rRNA of plants, and has been specifically removed from miRBase as a non-miRNA. Even the two identified miRNAs that remain in miRBase, MIR2916 and MIR894, are not above question. A 20 nucleotide stretch of MIR2916 map to rRNA, while the full MIR894 sequence appears to be found in a variety of plant transcripts.

Human sequences in the plant database and vice-versa

Curiously, several sequences did not map to the species to which they were ascribed by the PMRD[21]. Unfortunately, the PMRD could not be accessed directly during this study; however, other databases appear to provide access to its contents. Specifically, ptc-MIRf12412-akr and ptc-MIRf12524-akr did not map to *Populus* or to other plants. The poplar tree is also not a common dietary staple of human populations. In contrast, both sequences mapped with 100% identity and coverage to numerous human sequences (Table 3). ptc-MIRf10804-akr had numerous 100% identity human matches, plus a 1-mismatch alignment to the human miR-3929 precursor. Other miRNAs, including MIR2911, also displayed some lesser degree of matching to human transcripts or the genome. Strikingly, the putative MIR2910 sequence is not only a fragment of plant rRNA; it has a 100% coverage, 100% identity match in the human 18S rRNA (see NR_003286.2 in GenBank; Table 3). These matches of putative plant RNAs with human sequences are difficult to reconcile with the statement of Liu *et al.* that BLAST of putative plant miRNAs "resulted in zero alignment hit"[18], suggesting that perhaps a mistake was made, and that the BLAST procedure was performed incorrectly.

Discussion

In mammalian studies, mapping of MIR2910 and other dubious plant miRNAs is best explained as mapping of human degradome fragments to plant RNAs that are in some cases genuine sequences but not miRNAs, and in other cases, human sequences that have contaminated plant RNA samples and databases. Re-analysis of the results of Liu *et al.*[18] thus echoes the recent findings of Kang, Bang-Berthelsen, and colleagues[13], as well as previous negative findings surrounding dietary xenomiRs, summarized above. A stringent data analysis procedure, such as filtering all reads against the ingesting organism genome/transcriptome with one or two mismatches, then requiring perfect matches of remaining reads against plant or other foreign organisms, would engender higher confidence that "foreign" RNAs are not simply amplification or sequencing artifacts. Indeed, pre-mapping to the ingesting organism's genome may not be sufficient; as shown[13], the largest number of xenomiRs in some human studies are from rodents, likely because of proximity in research laboratories. Therefore, it may be best to screen against mammalian sequences in general, and perhaps also against widespread microbe contaminants. Of course, even the most stringent analysis procedures cannot distinguish a physical contaminant from a "real" read; therefore strict process controls are also needed to assess possible contamination. In general, such controls have not been done in existing studies.

This report underlines the danger in assuming that xenomiRs in mammalian material originate from the diet. When the species and roles are reversed—for example, with the finding of human sequences in a list of poplar tree miRNAs—few analysts would conclude that poplar trees consume humans. The simplest explanation is that the sequenced plant material was contaminated with human nucleic acid. In the same way, the extremely low-level, variable, and batch-effect prone concentrations of several plant sequences in human plasma and tissue could be due to uptake from the diet, albeit at levels far too low to affect physiologic processes. However, artifact remains the simplest explanation.

Data availability

Data used in these analyses were downloaded from the supplementary materials of Liu *et al.*[18] and are summarized in Supplementary File 1. Source data for the Ninomiya, *et al.* study[19] are under DRA002550, DRX021324–DRX021346, and DRR023270–DRR023292 (DNA Data Bank of Japan), and, for the Yuan, *et al.* study[20], available as GSE71008 (Gene Expression Omnibus).

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Supplementary material

Supplementary File 1: Tabs for the miRNA counts from the Yuan *et al.* and Ninomiya *et al.* datasets.

Click here to access the data.

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✔ **Claus H. Bang-Berthelsen**
Microbial Biotechnology and Biorefining, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark

K. W. Witwer presents some interesting findings in his brief report: Alternative miRNAs? Human sequences misidentified as plant miRNAs in plant studies and in human plasma. I consider the brief report scientifically good as well as well written.

Additionally, I would like to recognize the effort made by K. W. Witwer on examination of the methodology and critical reviewing a recent publication by Liu and colleagues in 2017[1]. Here Liu et al. report plant cross kingdom RNAi in two human sRNA plasma sequencing studies. Here Witwer identify a number of methodology issues in the Liu et al. paper by reanalyzing the public available data that were used in their paper. Witwer concludes that the most likely explanation for the Liu et al. reported plant RNA results, detected in the human plasma datasets, are traces of human degradome fragments and not of plant origin.

The central conclusion in the manuscript are line with the findings that Kang et al. 2017[2] reported based on a massive analysis of public available sRNA datasets comprising 10 billion sequencing reads. All in all supporting the null hypothesis that presence of xenomiRs are technical/contamination artifacts.

Based on the overall impression of the paper it is scientifically sound and I can fully support the conclusions.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: RNA biology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 08 March 2018

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Jens Allmer
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The article 'Alternative miRNAs? Human sequences misidentified as plant miRNAs in plant studies and in human plasma' by Kenneth W. Witwer addresses the recurring claim of plant xeno-microRNAs found in human plasma. Such xenomiRs are allegedly ingested and enriched in human; and this time Liu et al. claim evidence for cross kingdom RNAi via this route. Thus far, similar claims could not stand up against thorough validation as, for example, we (Bagci and Allmer, 2016[1]) and Kenneth W. Witwer (Auerbach et al. 2016[2]) have shown before.

Here, Witwer has a closer look at the article 'Plant miRNAs found in human circulating system provide evidence of cross kingdom RNAi' by Liu et al. 2017[3]. Liu et al. 2017 claim that the plant miRNAs are not contamination, but only checked whether they contain sequencing adapters. Other sources of contamination were not considered. Additionally, Liu et al. 2017 reported to have used BLAST to check the plant miRNAs they found against the human genome without any similarities. Conversely, Witwer was able to find full-length identical alignments for these sequences in the human genome. For example, peu-MIR2910 is claimed by Liu et al to be conserved within comestible plants but a quick check on miRBase reveals that it is dead entry and not considered a miRNA anymore (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0012633). Witwer correctly finds the mature sequence for this miRNA (UAGUUGGUGGAGCGAUUUGUC) in human while Liu et al. used the precursor sequence (UAGUUGGUGG AGCGAUUUGU CUGGUUAAUU CCGUUAACGA ACGAGACCUC AGCCUGCUA) for their search. I repeated the latter and found that the first 50 nucleotides are identical with several human RNAs (e.g.: NR_046235) which supports Witwer's findings. It is of note, that PMRD is still not reachable and that the sequences I used were provided by Liu et al. in their commentary on Witwer's article in biorxiv[4].

Witwer points out that mapping results were not normalized which is supported by the original manuscript by Liu et al. However, such normalization is of essence especially when the number of observations is very low (spurious even?).

I fully support the conclusions Kenneth W. Witwer draws in this article.

References

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.