Reply: Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs

Dear Mrs. Hughes:

Thanks a lot for your email and your information. I am happy that you are interested in our discovery[1]. Actually, I have reviewed the manuscript and I am familiar with the study by Ken Witwer at Johns Hopkins[2]. Since our finding of cross-kingdom regulation of exogenous plant microRNA (miRNA) in food[1] is so important to lots of fields in biology and life sciences, many scientists in the world are trying to reproduce and to extend our study. Although Witwer et al. have showed negative results of five plant miRNAs in only two experimental animals, there is a report that exogenous miRNAs are able to be detected in human plasma[3]. As the authors of this manuscript themselves have pointed out "carefully controlled studies with larger groups of subjects are needed to answer the many questions surrounding the xenomiR hypotheses"[2], it is not appropriate to draw conclusion "that seems to claim that your 2012 study in *Cell Research* was likely based on a false positive".

There are several critical issues of detecting exogenous miRNAs in the serum or plasma samples from human and animals, which I think the authors of this manuscript have not addressed. 1) It is important to use deep-sequencing technique to screen the circulating exogenous miRNAs. There are thousands of miRNAs in plants. However, only 30 known plant miRNAs were detected in human plasma[1]. Thus, the abundance of miRNAs in plant may not accurately reflect the distribution of miRNAs in animal tissues. Therefore, the authors, selecting miRNAs based on their high levels in the plant, might detect wrong miRNAs. 2) An internal control or reference gene should be employed in the qRT-PCR assay to normalize miRNAs in plasma. Proper normalization is critical for quantitative analysis of extracellular miRNAs, as variations in the amount of material, sample collection, RNA extraction and enzymatic efficiency can introduce potential bias and contribute to quantification errors. There is no internal control being used in their study. 3) Considering the limitation of qRT-PCR sensitivity, it needs to select "right" miRNAs which concentrations in the circulation should be within the linear working range of qRT-PCR. If the level of miRNA is too low, it would be outside the working range of qRT-PCR and no difference should be detected. Finally, the sample size is too small in their study to make any conclusion. The soy- and fruit-substance was only administered to two pigtailed macaques.

High-throughput sequencing is widely regarded as a preferable method for detecting plant miRNAs in human plasma because it can directly provide small RNA sequence. In our study, we detected plant miRNAs in the serum of human and mouse directly by deep sequencing[1]. In separate experiment, we also treated samples with oxidization and then analyzed miRNAs by deep sequencing[1]. Our results showed that these exogenous

miRNAs were successfully sequenced after oxidation, suggesting that they are genuine plant miRNAs containing 2'-O-methylated 3' ends. Witwer and co-workers mentioned that a survey of the animal small RNA datasets showed no significant plant-derived miRNA accumulation in animal samples, except for MIR168[4]. They suggested that the detection of plant miRNA, particularly MIR168, could be due to technical artifact or cross-contamination of sequencing experiments. However, after carefully surveying the publicly available sequencing data, we found that the presence of plant miRNAs in animal samples had been reported. For example, a considerable amount of MIR156 was detected in human peripheral blood mononuclear cell (Accession number: GSM494809) and liver (Accession number: GSM531978). In fact, there are some tricks in sequencing plant miRNAs in plasma samples of human and animals. A common step in high-throughput sequencing is the ligation of adapter oligonucleotides using RNA ligase. Because plant miRNAs are 2'-O-methyl modified at the 3' terminal nucleotide and the 2'-O-methyl modification of small RNA 3'-ends can result in decreased ligation efficiency[5], the sequencing procedure is biased against plant miRNAs compared to non-modified mammalian miRNAs. In other words, if sample is a mixture of 2'-O-methyl and 2'-OH small RNA, ligations would favor capture of the 2'-OH small RNAs. Thus, the convenient sequencing platform may result in low copy number of plant miRNAs in human and animal plasma. To efficiently capture small RNA from both animals and plants, we should optimize reaction parameters (e.g., extending the ligation time).

We have also conducted qRT-PCR assay to validate the deep sequencing data of plant miRNAs in animal systems. We have performed numerous qRT-PCR experiments to assess the accurate level of serum/plasma miRNAs since the discovery of circulating miRNAs in serum and plasma. We calculated that some plant miRNAs, such as MIR168, are present in mammalian cells and tissues at concentrations similar to other endogenous miRNAs (e.g., miR-25). Recently, we conducted a kinetic study of plant miRNAs in human plasma after oral administration of fresh fruits (1 kg per person). The results showed that many plant miRNAs were detectable in the plasma after the volunteers eating fresh fruits. The raw C_T values were generally lower than 30, and were clearly discriminated from background (the detailed results will be published soon).

In order to assess the prevalence and robustness of the phenomenon of uptake of plant miRNAs via food, our follow-up study using a Chinese herb has found a rapid uptake of a specific plant miRNA derived from this herb in human and animals (the detailed results will be published soon). In brief, Solexa sequencing result indicated that the Chinese herb contains abundant plant miRNAs and a specific plant miRNA was enriched in the soup made from this herb. Interestingly, this specific plant miRNA was readily detected in human serum after drinking the soup made from this herb. In addition, as mentioned by Witwer et al., "artificial miRNAs designed to target disease-related transcripts could be overexpressed in plants for dietary administration", we have also generated transgenic Lactuca sativa L. that expresses artificial miRNAs against HBV. Our preliminary data showed that this artificial anti-HBV miRNA could detected in the plasma and liver of HBV gene knock-in transgenic mouse model after feeding the mice with transgenic Lactuca sativa L. Taken

together, these results strongly support our claim that uptake of food-derived plant miRNAs is a true phenomenon.

The understanding of the biological significance of the uptake of plant-derived miRNAs via food and the molecular mechanism by which plant miRNAs are absorbed and processed is just at its beginning. Future studies with improved designs, careful controls and large samples are required to answer the questions surrounding the hypothesis of dietary uptake of plant miRNAs and their potential biological functions.

References

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