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SUMMARY

PHD THESIS

THE TRANSFER OF PLANT GENETIC MATERIAL – A NEW MECHANISM OF ACTION FOR HERBAL MEDICINE

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INTRODUCTION

MicroRNAs (miRNAs) are a class of small, endogenous post-transcriptional RNAs of 21-25 nucleotides. They play an important role in post-transcriptional regulation in animals and plants by targeting specific mRNAs for degradation or translation repression. A single miRNA can target several hundred genes. In addition, one target gene often contains binding sites for multiple miRNAs, allowing them to play an important role in every aspect of biology.

Plant miRNAs were first discovered in 2002 in *Arabidopsis*. Their methylation at their 3′ ends by HEN1, distinguishes plant miRNAs from those of the animals because most animal miRNA genes are not methylated. The major differences between the two kingdoms are related to target recognition. In animals, the complementary sites are located at the 3-UTR of genes, but in plants they can exist anywhere within the gene. Animal miRNAs bind with mismatches, while plant miRNAs present a lower number of mismatches**.** A central mismatch facilitates the translational repression mechanism but disregards degradation, allowing small animal RNAs to regulate gene expression by translational repression and transcriptional silencing, while in plants the regulation occurs mostly by direct cleavage of target mRNAs due to the high degree of complementarity**.** In other words, the level of miRNA-mRNA complementarity plays an important role in the regulatory process.

The plants have played a vital role in the treatment of diseases for centuries. Natural active products such as polyphenols, alkaloids, saponins and tannins have been highly focused on as an important tool for production of less side effect drugs.

A new interest in plant molecular mechanism of action was brought forward in 2012, when Zhang et al. published their results suggesting that exogenous plant miRNAs in food can regulate the expression of target genes in mammals through food intake.

This study focuses on the investigation of medicinal plant genetic material transfer into animals by oral gavage. Therefor, we identified novel potentials miRNAs in marigold (Calendula officinalis) so we could investigate the putative intestinal absorption of one of the most abundant and stable small RNAs in plants, after gavage administration of total RNA extracts.

RESEARCH MAIN OBJECTIVES

1. The assessment of plant microRNA transfer into animals (mice)
2. microRNA de novo identification from medicinal plant Calendula officinalis
3. Summarize for the first time the findings of miRNA in medicinal plants as well as their possible influence on mammal metabolism.

STUDY no. 1

Detection of plant miR166a in liver of mice after oral feeding

INTRODUCTION

Here we investigate the hypothesis that if surviving digestion, free microRNAs can cross the gut barrier into portal circulation. For this purpose, we fed adult male mice by gavage with miR-166a (one of the most stable and abundant microRNA in plants) either purified (together with other miRNAs) from flax, either synthesized using LNA (locked nucleic acids) nucleotides (resistant to nuclease action).

MATERIAL AND METHODS

- Plant materials and total RNA extraction - We used commercially available brown flax seeds (Linum usitatissimum L., family Linaceae) that were germinated at room temperature for 24 h. Total RNA was extracted using miRVana miRNA isolation kit from Ambion.

- All animals (3-months old male mice of a mixed genetic background) were kept on a 12:12 hours light/dark cycle and fed ad libitum a regular chow (Cantacuzino Institute, Bucharest). We designed three experimental lots: control lot – animals gavaged 12ul scrambled LNA, total RNA lot - animals gavaged 14ul total RNA extracted from Linum usitatissimum (flax), and miR-lot – animals gavaged 10ul synthetic LNA-miR-166a.

- Twelve hours after gavage, mice were euthanized by cervical dislocation and liver samples (approximate 15mg) harvested.

- For miR-66a quantification, cDNA was synthesized from 10ng of RNA using the cDNA Synthesis kit for RT-PCR (Thermofisher) and PCR amplification was performed using a dedicated Taqman microRNA assay (Thermofisher). The relative fold changes of miR-166a expression was calculated using the delta delta Ct method using U6 snRNA as endogenous control.

RESULTS

Based on available data, we selected the highly conserved plant miRNA, miR166a, which is highly expressed in various plants, including Linum usitatissimum (flax), usually consumed for its health benefits. In order to test the effect of intestinal digestion of microRNAs upon intestinal transfer, we also used synthetic LNA-miR-166a, known for their resistance to degradation due to the extra bridge connecting oxygen in 2’ with carbon in 4’.

We reasoned that if a naked microRNA would escape degradation, it might be absorbed, then reach portal circulation and get filtered by the liver. To

verify our hypothesis, mice liver samples were collected 12 hours after gavage intervention and RNA extracted.

All liver tissues were analyzed for miR-166a expression using Taqman qRT-PCR and delta delta Ct method. All reactions were performed in triplicates and changes in expression levels assessed for significance by Student heteroscedastic, twin-tails t-test. Our experiment demonstrated a significant difference in microRNA recovery between samples.

DISSCUTION

To date, microRNAs from plant based-diets have been difficult to detect in blood and tissues of mammals after a single serving.

In the present study, we chose to focus on naked microRNAs and selected miR-166a, a highly conserved plant miRNA and strongly quasi-ubiquitously expressed in plants. We chose to work with Linum usitatissimum (flax), a medicinal plant known for its possible influence on mammal metabolism and for its high content of miR-166a in all the plant tissues.

We asked whether exogenous plant miRNAs can survive digestion and enter the blood stream. For better intake, all RNA probes were given by oral-gavage. Twelve hours after a single dose-feeding of total plant RNA, liver levels of mir166a in mice were significantly elevated by 1,6-fold ($p=0.015$) compared to control lot. Next, we aimed to evaluate the impact of digestive enzyme upon miRNA and gavaged the mice with synthetic, LNA-based miR166a. Interestingly, the liver expression of miR166a was again unregulated but at a lower level (fold change = 1.4) and insignificant from a statistical point of view when compared to control lot ($p=0.282$). This suggests that either the synthetic, rigid miR-166a is less absorbed in the intestine compared to the native form, or the native plant microRNAs is better protected from digestion than their corresponding synthetic forms.

We also noticed that the difference between total RNA group and LNA-miR groups is highly significant statistically ($p < 0.001$), even though the fold

changes are very close. The rather high expression (Ct around 32) of miR-166a in the control group might suggest that either the qRT-PCR assay is not specific, or, the food is contaminated with unprocessed/low processed plant diet. We favor the second hypothesis, the cross-contamination from diet, since our mice chow is rich in soy (Glycine max), where gma-miR166a (sharing the same sequence with miR166a from flax), is one of the most abundant microRNA. It is thus plausible that the detection of plant miRNA in the liver is due to the prolonged contact with this specific miRNA.

Contamination by sources such as non-dietary environmental plant matter also presents a risk of false positives. This hypothesis is sustained by the fact that the level of LNA synthetic mir166a was significantly lower, when in fact methylene bridge between the 2'-O and the 4'-C atoms makes them resistant to endo- and exonucleases. The LNA backbone theoretically confers a higher sensitivity for detection by in situ hybridization or qRT-PCR. Also, these synthetic oligonucleotides can be detected in low doses in plasma for weeks after administration making them suitable to be long-acting modulators.

Moreover, to verify whether a physiological dose of total plant RNA was achieved in our experiment, we compared the dose given to mice with the equivalent in humans. Based on estimated exposures to plant-derived RNAs from food consumption, an individual (daily intake should be somewhere in between 30-40g) would have to consume 600g of flaxseed, in order to achieve these results. This suggests that the presence of exogenous miRNAs in mammals' circulation upon oral ingestion is a highly dose-dependent phenomenon.

CONCLUSION

Our results suggest that when administered in high quantity through gavage, plant miR166a may survive in the GI tract of mice, get absorbed and enter the portal circulation.

STUDY no. 2

Identification of microRNAs in Calendula Officinalis: new insights into herbal medicine

INTRODUCTION

Plant miRNAs play a role in root initiation, leaf morphology, flower development or stress response, but they could also have an impact on animals/humans. In the last decade, a large number of experimental and computational studies have been made to identify miRNAs from plants. Today, a high amount of miRNAs is deposited in different databases, but still no miRNA from Calendula officinalis (marigold), a very well known medicinal plant, has been discovered.

MATERIAL AND METHODS

- Total RNA was extracted from the flowers and petals of marigold by phenol/chloroform using miRVana miRNA isolation kit from Ambion.

- New Generation Sequencing (NGS) of small RNAs in marigold using two different platforms: Ion Torrent and Illumina NextSeq500

- Data analysis

RESULTS

Due to the absence of the complete genome or transcriptome sequences and with no information regarding Calendula officinalis miRNAs, we used only the non-redundant mature plant miRNA sequences from miRBase against our massive dataset to predict miRNAs in Calendula using similarity searches. The small RNA sequences from the raw dataset were considered as miRNA candidates only if they correspond to the following criteria: at least 18 nt length and to have 0 mismatches in sequences with the known mature plant miRNAs.

A total of 4 miRNAs with 100% homology rate were identified in the flowers of marigold: ath-miR166a, osa-miR166h, ppt-miR894 and ath-miR817. The miR166 family is one of the most conserved family in the plant kingdom due to its important regulatory role. These are the first miRNAs ever identified in Calendula officinalis.

The conservancy of miR166 family found in marigold shown high similarity with their homologs in other medicinal plants. We choose BLASTN to search for miR166a and miR166h homologs within miRBase. We selected only the miRNA candidates from plants used in herbal medicine.

DISSCUTION

Without the genome sequence, identification of miRNA in marigold is a great challenge. High-throughput sequencing technologies provide an efficient and inexpensive approach for identifying small RNAs in many plant species.

In the present study, using Next Generation Sequencing, we were able to identify 4 potential conserved miRNAs from the fresh flowers of marigold. We chose this specific part of the plant because only the inflorescence has significant value in herbal medicine. We identified 2 members of the miR166 family, a very well conserved family in other plant species. Interestingly, we found out that many medicinal plants have a large number of miRNAs from the miR166 family.

Moreover, in a recent research article, one of the miRNA identified in marigold, ath-mir166a, had one of the highest abundance level in mammalian breast milk, showing that this specific plant miRNA has a role in inflammation and immune response by inhibiting Interleukin1 Receptor-like 1 protein. The anti-inflammatory and immunomodulatory actions are among the main benefits of using marigold.

Here, we raise a question regarding a new aspect of plant biology that might have a powerful impact on understanding herbal medicine. The

microRNA expressed in medicinal plants might act as a new bioactive compound capable to interact with the mammalian system.

CONCLUSION

In this study, using high-throughput sequencing technologies, we have identified 4 potential miRNAs in marigold, one of the best known medicinal plants. This is, to our knowledge, the first report on Calendula miRNA identification.

STUDY no. 3

New insights of medicinal plant therapeutic activity - the miRNA transfer (a metanalysis)

Since the discovery of plant miRNA in human tissue and sera after ingestion, the connection between the two kingdoms is presented under a new perspective. Yet, a very small number of studies were conducted to determine whether medicinal herbs are possibly inducing miRNAs in humans or if plant miRNAs are currently linked to pathologies in mammals.

Public datasets such as MirBase and Pubmed were consulted to summarize the published miRNA sequences from medicinal plants, included domestic crop plants with medicinal values. There are a total of 36 plants with identified miRNAs (conserved+novel) used in traditional medicine according to The Encyclopedia of Medicinal Plants. To our knowledge, this is the first time that such a summary has been made.

This study summarizes for the first time the findings of miRNA in medicinal plants as well as their possible influence on mammal metabolism. This may lead to the foundation of a public miRNA database for plants with medical value.

GENERAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

- This study represents the first attempt in Romania to investigate a new mechanism of action regarding the role of herbal medicine. Because botanical compounds have been used in the treatment of different pathologies, we are trying to cover the gap and underline the necessity of further studies between plant exogenous genetic material and the changes in mammal upon oral ingestion.
- Only after ingesting high quantities of exogenous microRNA, the systemic levels of plant miRNA may reach the mammal tissue concentrations that could be relevant for gene regulation.
- In this study, using high-throughput sequencing technologies, we have identified 4 potential miRNAs in marigold, one of the best known medicinal plants. This is, to our knowledge, the first report on Calendula miRNA identification. Further studies are yet necessary to identify more Calendula miRNAs, but also to validate their targets.
- Besides reporting the latest findings regarding the cross-kingdom transfer of miRNA and its therapeutic application, this study can inform further investigations that could lead to a modern definition of herbal medicine.
- Although the knowledge of cross-kingdom miRNA effects is at a very early stage, it is adding new dimensions to herbal medicine and could have a number of applications in the pharmaceutical industry such as potential biomarkers or drug- delivery systems in a non-cytotoxic manner.