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LETTER TO THE EDITOR

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A novel chemopreventive strategy based on therapeutic microRNAs produced in plants

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Dear Editor,

MicroRNAs (miRNAs) are small non-coding RNAs that play a critical role in regulation of gene expression in nearly all eukaryotic organisms, including mammals. In humans, an estimated 60% of all protein-coding genes are targeted by miRNAs, affecting virtually every physiological process in the body [1]. In addition, a diverse array of human diseases is associated with dysregulation of miRNAs [2]. In many forms of cancer, for example, certain miRNAs, termed tumor suppressor miRNAs, are downregulated in diseased cells. Restoration of the downregulated tumor suppressor miRNA has been shown to block one or more steps in oncogenesis in animal models and cell culture systems. Thus, the therapeutic potential of tumor suppressor miRNAs has been experimentally confirmed and is now widely recognized. However, systemic delivery of such therapeutic small RNAs in humans is challenging and numerous delivery options are currently under investigation.

We have investigated the possibility of an effective oral delivery system for therapeutic miRNAs. It has long been known that ingested RNA from food sources is taken up by the digestive system in nematodes and insects and can control the expression of genes in those organisms [3]. More recent evidence suggests that a similar phenomenon might occur in humans and other mammals [4]. These data indicate that plant miRNAs from foods are absorbed by cells of the mammalian digestive tract and packaged into microvesicles, which protect them from degradation. The miRNAs are then trafficked via the bloodstream to a variety of tissues, where they are capable of regulating the expression of mammalian genes. Such work has generated considerable excitement because it raises the possibility of bioengineering edible plants to produce therapeutic miRNAs that could then be delivered to affected tissues by ingestion. However, the work has also generated controversy as several groups have subsequently reported being unable to detect ingested plant miRNAs in mammalian tissues at levels significantly above background [5]. We addressed this controversy in experiments designed to both detect a therapeutic effect of ingested miRNAs and to demonstrate their uptake.

Here we report that oral administration of a cocktail of tumor suppressor miRNAs reduced tumor burden in the well-established $Apc^{Min/+}$ mouse model of colon cancer. The cocktail contains three validated tumor suppressor miRNAs (miR-34a, miR-143, and miR-145), synthesized with the exact nucleotide sequence of the mouse miRNAs, but with a methyl group on the 2′ position of the ribose of the 3′ terminal nucleotide, which is a characteristic of miRNAs made by plants [6]. These three miRNAs are all downregulated in colon cancer, and block tumorigenesis in animal models when their levels are restored [7-9]. Three groups of seven mice each were tested in a preventive regimen (gavage starting at 5 weeks and continuing for 28 days) to determine the effect of treatment on tumor burden. The experimental group received total plant RNA spiked with the three tumor suppressor miRNAs. Two negative control groups received either total plant RNA alone or water. To assess the effect of the treatments on the overall health of the mice, their weights were monitored daily. All mice remained healthy and gained weight during the course of the experiment, indicating no obvious toxicity. On day 28, the tumor burden in each mouse was determined. Additional details of the methods and analyses are described in Supplementary information, Data S1.

To establish whether the miRNA-treated group had significantly fewer tumors than the control groups, we used a nonparametric statistical analysis, the Kolmogorov-Smirnov (K-S) test. Figure 1A shows the number of tumors for each mouse in the three different groups and a plot of those numbers for the miRNA-treated and water-treated groups. A one-sided K-S test shows a highly significant reduction in tumor burden in the miRNA-treated mice compared to the water-treated mice ($P = 0.0058$). The K-S plot highlights a striking feature of our data, which is that six of the seven mice in the tumor suppressor miRNA-treated group had fewer tumors than the mouse with the fewest tumors in the water-treated group.

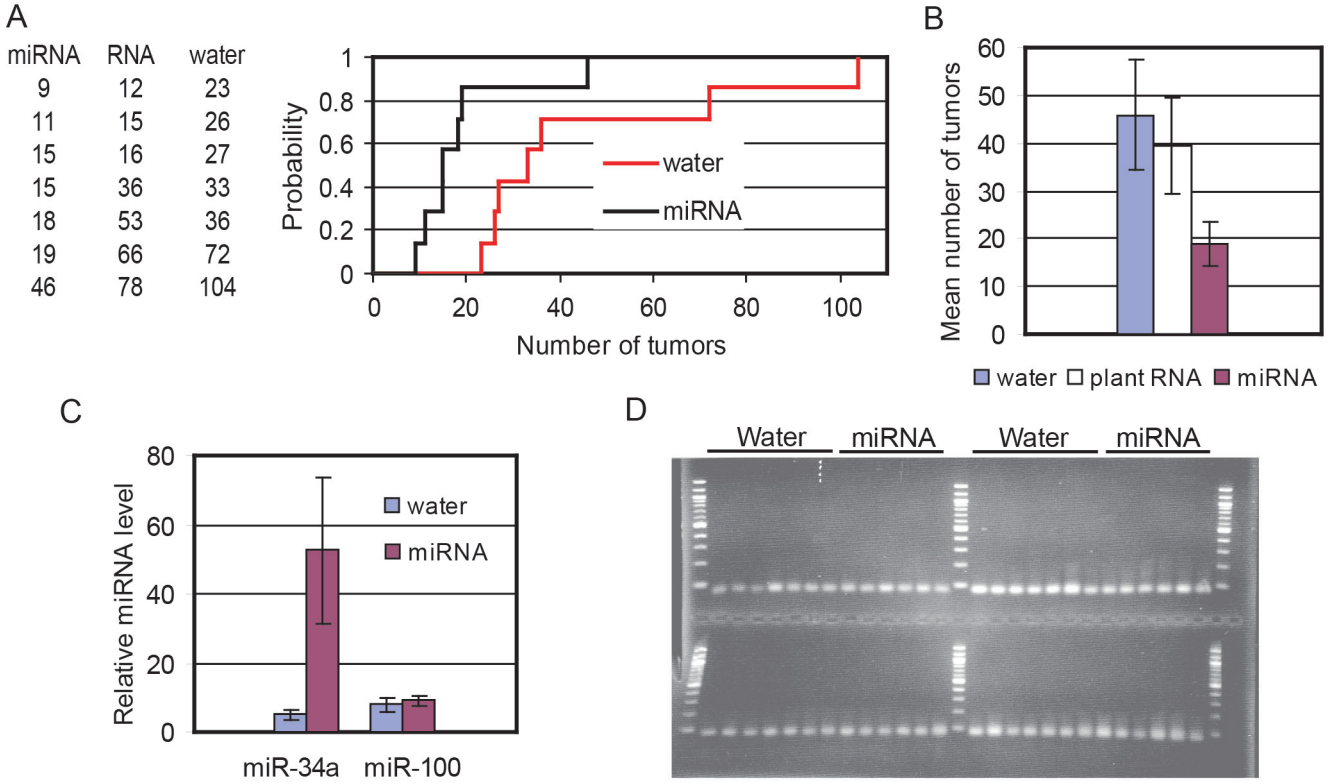

Figure 1 Orally administered tumor suppressor miRNAs reduce tumor burden in *Apc*$^{Min/+}$ mice and are detectable in intestinal tissue. **(A)** Reduction in tumor burden in miRNA-treated compared to water-treated mice. The table shows the number of tumors in each mouse in the three groups listed in ascending order within each group: miRNA = tumor suppressor miRNA cocktail + total plant RNA; RNA = total plant RNA alone; water = only water. The number of tumors from mice in the miRNA-treated and water-treated groups were plotted to show the distributions that are the basis of the K-S statistical analysis. **(B)** Mean number of tumors in each treatment group. Error bars show the standard error of the mean. **(C**, **D)**. Total RNA isolated from intestinal tissue of the mice was oxidized with sodium periodate, and miRNAs were assayed using the miScript-PCR system (Qiagen), which involves polyadenylation of the RNA, reverse transcription, and quantitative real-time PCR (RT-qPCR). Because periodate oxidizes all endogenous RNA controls in the samples, methylated *C. elegans* miR-39 was added to all samples before oxidation to serve as the normalization control. MiScript-PCR system analysis of samples before oxidation showed that the concentration of the spiked-in miR-39 was the same in all samples. RT-qPCR results showing higher relative miR-34a concentration in miRNA-treated mice compared to water-treated mice. The mean relative miR-34a and miR-100 concentrations in the miRNA-treated and water-treated mice are shown. Error bars are the standard error of the mean **(C)**. Visualization of RT-qPCR products to confirm that a single product of the expected size was amplified in the reactions that assayed miR-34a, miR-100, and miR-39 in intestinal RNA from miRNA-treated and water-treated mice. The RT-qPCR reactions were performed in triplicate, and an aliquot from one of each triplicate was loaded on a 2.5% agarose gel. Each lane represents a sample from an individual mouse: miRNA, samples from miRNA-treated mice; water, samples from water-treated mice. The samples were all electrophoresed on one gel that had been poured using two combs. MiR-100 and control miR-39 reactions from the same PCR run were loaded into the upper wells, with the miR-100 reactions in the left half of the gel. Similarly, miR-34a and miR-39 reactions done in another RT-qPCR run were loaded into the lower wells, with the miR-34a reactions in the left half of the gel. A 100-bp ladder provides the size standards. Ethidium bromide was used to stain the gel **(D)**.

The tumor burden in the mice treated with total plant RNA alone was less than that in the water-treated mice, suggesting that plant RNA alone may have a therapeutic effect. However, these two groups were not statistically different (P = 0.28), and a larger sample size would be required to evaluate the therapeutic potential of plant RNA. Figure 1B shows the average number of tumors in the three groups.

To determine the levels of administered tumor suppressor miRNAs in mouse intestine, we exploited the fact that the synthesized miRNAs used in our experiment, like plant-produced miRNAs, are methylated at the 2′ position of the ribose of the 3′ terminal nucleotide, whereas endogenous mouse miRNAs are not. Periodate oxidation of unmethylated miRNA breaks the bond between the 2′ and 3′ carbons of the 3′ terminal ribose [10], preventing subsequent *in vitro* polyadenylation. In contrast, methylated miRNAs are resistant to periodate

oxidation, providing a robust method to reduce the background mouse miRNAs and thereby allowing specific quantitation of the orally administered methylated miRNAs. We used periodate oxidation followed by polyadenylation, reverse transcription, and quantitative real-time PCR (RT-qPCR) to assay the level of miR-34a in intestinal RNA isolated from the mice. Relative miRNA concentrations were calculated from the RT-qPCR results, using the ΔΔCt method. This method is based on differences in the amplification curves of the reactions during the exponential phase of amplification, when starting miRNA concentrations can be accurately measured. The analysis showed with high statistical significance that the level of miR-34a in the miRNA-treated group was higher than that in the water treated-control mice (K-S test, P = 0.0082), with an average increase of about 10-fold (Figure 1C). In contrast, the level of miR-100, an endogenous mouse miRNA that had about the same background level in the intestine as miR-34a, but was not fed to the mice, was not significantly different between the miRNA-treated and water treated-control groups (Figure 1C). To rule out the possibility that the observed difference in miR-34a level between miR-treated and water treated-control groups was due to spurious amplification of unrelated molecules, we analyzed the RT-qPCR products at the end of the reactions using gel electrophoresis (Figure 1D). Because all the amplification curves had reached essentially the same plateau level at this point, the gel does not reflect differences in starting concentrations of the miRNAs. However, it shows that a single product of the expected size was produced in all reactions, indicating that amplification was specific. Unfortunately, specific detection of the administered miR-143 and miR-145 in mouse intestine failed because the background levels of endogenous miR-143 and miR-145 after periodate oxidation were too high.

Our results suggest that tumor suppressor miRNAs designed to mimic small RNAs produced in plants were taken up by the digestive tract of *Apc*$^{Min/+}$ mice upon ingestion, as evidenced by their higher concentration in the miRNA-treated animals (Figure 1C), and were functional, as evidenced by the reduction in tumor burden (Figure 1A and 1B). These results are consistent with a previous study [4] showing that plant miRNAs are taken up by the mammalian digestive tract and can function to target mammalian genes. Our results raise the intriguing prospect of using edible plants engineered to produce mammalian tumor suppressor miRNAs as an effective, nontoxic, and inexpensive chemopreventive strategy in humans. The initial experiments presented here used synthetic mammalian tumor suppressor miRNAs designed to mimic plant-produced miRNAs and tested their uptake and anti-cancer function in the intestine, a tissue with high exposure to dietary RNAs. However, the same basic idea could extend to other miRNA-associated diseases and to the use of novel miRNAs designed for specific targets, such as viral RNAs. Bioengineering of plants to produce miRNAs of any desired sequence is a well-established technology currently used for research purposes in diverse food crops [11]. Thus, using edible plants to produce therapeutic miRNAs is highly feasible and has significant potential in basic, translational, and clinical applications to provide a cost-effective alternative to currently available synthetic RNA production and delivery methods.

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Conflict of interest

VV has founded a start-up biotechnology company, "MicroRNA Meds, LLC", that has interests related to this research, and is also primary co-inventor on patents to bioengineer plants to make microRNAs. There are two issued patents and one pending patent application, all entitled "Compositions and Methods for the Modulation of Gene Expression in Plants": US patent application no. 13/282,680, Canadian patent no. 2,276,233 and Australian patent no. AU 2003/254052 B2.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)