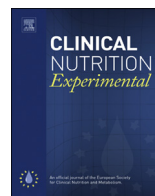




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Regulation of microRNA expression in humans following ingestion of the whole food FFC[®] Pairogen – A preliminary, pilot study

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SUMMARY

Background: Some natural substances may affect the expression of microRNAs (miRNAs) in humans. However, it is unclear whether this effect can be induced by the ingestion of whole foods containing these natural substances.

Objective: We investigated whether ingestion of the whole food FFC[®] Pairogen affects the expression of miRNAs in humans.

Design: This was a pilot study and part of a randomized controlled trial. Four subjects ingested the test material daily for 8 weeks (three times 180 ml bottles a day, morning, noon and evening; a total of 540 ml per day). The study did not include control subjects. **Results:** Several miRNAs showed differential expression. The expression of two miRNAs, hsa-let-7c and hsa-miR-92a-2-5p, changed in three of the four subjects, who had lower concentration of serum total antioxidant status. The expression of hsa-let-7c increased and that of hsa-miR-92a-2-5p decreased.

Conclusions: The expression of hsa-let-7c and hsa-miR-92a-2-5p has been linked with lung cancer manifestation. The increased expression of hsa-let-7c and the decreased expression of hsa-miR-92a-2-5p suggest possible oncogene suppressive functions of these miRNAs. The ingestion of FFC[®] Pairogen may have a positive effect on cancer prevention.

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1. Introduction

To achieve a long life, people are increasingly being encouraged to lead active and healthy lifestyles. Many health and nutritional supplements have been marketed with phrases such as 'enhancing your immune system' and contain antioxidant components including resveratrol, polyphenols, lycopene and vitamins A, B9, D, and E. For most people, it would be impossible to take all of these separate health supplements. Therefore, whole foods or beverages with the potential for immune system enhancement would be a more convenient choice. In this context, cancer prevention is the most common concern because the mortality rate from cancer in people over 60 years old dramatically increases with age [1]. Food is vital for human health and can reduce the risks of various illnesses. These functions of food have been evaluated by analysis of various biomarkers, through hemanalysis, urinalysis and physical examination. In recent years, attempts have been made to evaluate human health status and/or contraction of diseases in terms of microRNAs (miRNAs), which are small, non-coding, single-stranded RNAs that negatively regulate gene expression either by translational inhibition or exonucleolytic mRNA decay. Experiments using rats and mice and *in vitro* studies have demonstrated that the modulation of miRNAs by dietary and pharmacological agents can induce cell growth, cell cycle regulation and mutation, as well as inhibit stress responses, angiogenesis, cell proliferation and inflammation [2].

MicroRNAs are classified into various families or clusters and specific physiological features of some have been investigated. For example, let-7c family miRNAs act as tumor suppressor genes by targeting cell cycle related genes and inhibiting cell division and the progression of lung cancer [3,4]. The miRNA17-92 cluster is oncogenic [4], its over-expression contributes to the manifestation of lung cancer [5], it is involved with mastocarcinoma through the down-regulation of estrogen receptors [6] and it is up-regulated in patients with ovarian epithelial carcinoma [7]. Furthermore, Dhar et al. reported in their *in vitro* study that resveratrol, a component of various berries and red wine, up-regulated tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) in prostate cancer cells [8], indicating that miRNA expression related to tumorigenesis or tumor suppression can be changed by food ingestion. However, most studies that have discussed the relationship between miRNAs and food ingestion investigated a single nutrition element such as a vitamin, polyphenol or isoflavone [9]. Furthermore, very few miRNA studies using whole foods or beverages have been performed, despite evaluation of nutrition through ingestion of a whole food or beverage being more realistic of human diet. In addition, most studies discussing the relationship between food ingestion and miRNAs have focused on effects on cell lines [10] and very few have discussed effects on the human body. Therefore, these relationships require evaluation *in vivo*.

In our previous study, we found that the beverage FFC® Pairogen Special Three had potential as an immune activator and, therefore, could have a great influence on human health and disease prevention [11]. Now we used FFC® Pairogen that has similar ingredients to FFC® Pairogen Special Three (Table 1).

Table 1
Test material ingredients (in 180 ml).

Material name	FFC® Pairogen	
Form	Liquid	
Ingredients	Glucose-fructose syrup, rice vinegar, honey, apple vinegar, Japanese persimmon vinegar, Japanese apricot vinegar, apple juice, substances extracted from Japanese apricot, galactooligosaccharide, citric acid, Vitamin C, malic acid, stevia, flavors, Vitamin B2, Vitamin B6	
Nutrition facts	Protein	0 mg
	Lipid	0 mg
	Carbohydrate	9200 mg
	Sodium	59.5 mg
	Energy	37 kcal

This study, as a pilot study, aimed to investigate the relationship between FFC[®] Pairogen, and the expression of miRNAs that have possible anti-oxidation effects. In this report, we discuss whether the effect of a food or beverage on human physiology can be evaluated by the expression of various miRNAs.

2. Subjects and methods

2.1. Study design

This study was designed as a case study as part of a randomized controlled trial using FFC[®] Pairogen provided by the Akatsuka Corporation (Table 1). This study was registered in the Japanese University Hospital Medical Information Network as UMIN000012608.

2.2. Participants

The four subjects were 50.3 ± 2.6 years old selected from among those enrolled in our previous antioxidant study (Hiroe et al. unpublished) that adhered to the following protocol.

A total of 30 healthy volunteers between 40 and 60 years old were recruited to undergo a screening test held at the Seishin-kai Medical Association Inc., Takara Medical Clinic. Participants included in the trial were individuals who felt fatigued daily. People were excluded for the following reasons: previous history of cardiac arrest or cardiac infarction; presence of arterial fibrillation, cardiac arrhythmia, hepatic disorder, renal disorder, cerebrovascular disorder, rheumatism, diabetes, dyslipidemia, hypertension, and other chronic conditions; use of medications, including Chinese medicines and dietary supplements; an allergy to medicines and foods related to the test materials of the trial; pregnancy, lactation, or desire to become pregnant during the test period; being enrolled in another clinical study; and judgment by the trial physician to be unsuitable for this trial. Twenty-two female participants were selected based on the concentration of serum total antioxidant status (STAS) in the screening test. No men had passed the screening test. In fact, only 2 had been eligible for the trial, so they were not included because of the possibility of biased results compared to the 22 women.

In this present study, blood samples in 4 participants were collected for microarray analysis while those in other 18 participants were not collected (Fig. 1). The sample size was determined as much as the budget of this trial limited. Four subjects were included from FFC[®] Pairogen group of 11 subjects at first allocation for our previous study (unpublished), and had relatively lower STAS in the group (Fig. 1).

This trial was conducted from 20 May 2013 to 23 October 2013. All the participants gave written informed consent before participating in the screening test. This trial was conducted according to the Declaration of Helsinki and approved by the ethics committee of the Seishin-kai Takara Medical Clinic before start of the trial.

2.3. Study products

The participants consumed 1 pack of FFC[®] Pairogen 3 times daily. Each pack of FFC[®] Pairogen contained 180 ml of the product; therefore, daily consumption was 540 ml of FFC[®] Pairogen. The FFC[®] Pairogen used in this trial was the product of the Akatsuka Co., Ltd., with which the first and second authors are affiliated. The participants were not restricted to take any waters or drinks other than their trial beverages, except for dietary supplements.

2.4. RNA extraction and miRNA expression profiling

We measured the expression of miRNAs. RNA was extracted from the blood serum using 3D-Gene RNA extraction reagent from liquid sample (Toray, Kamakura, Japan) according to the manufacturer's

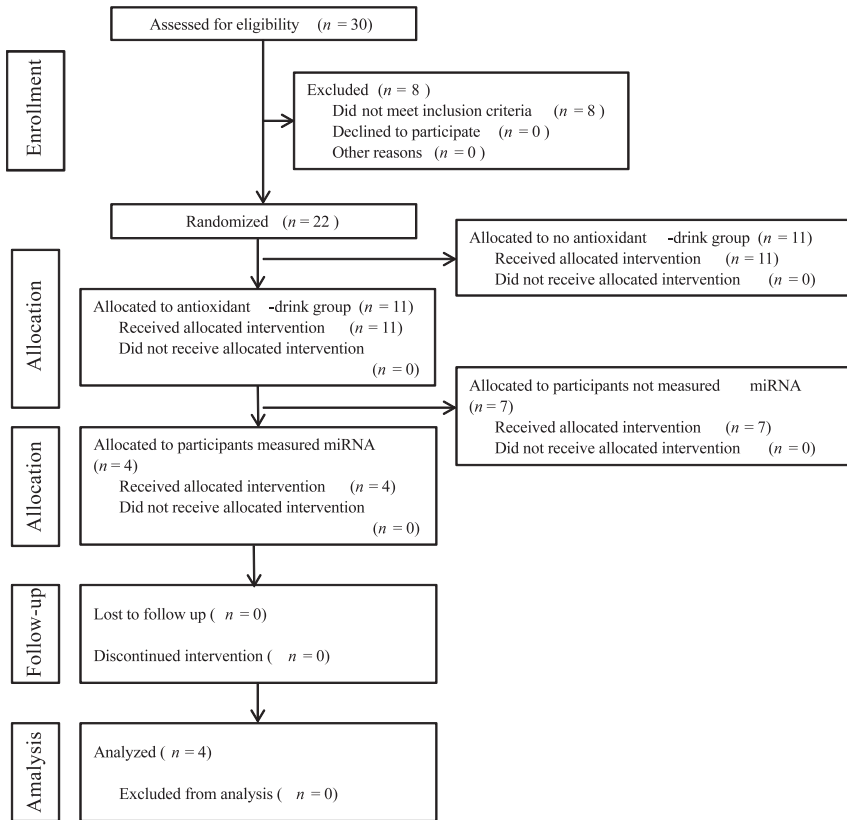


Fig. 1. Subject flowchart. Four participants who showed low STAS score had FFC® Pairogen in the intake period for that evaluate miRNA expressions were investigated.

instructions. Extracted total RNA was labeled with Cy5 using the 3D-Gene miRNA labeling kit (Toray, Kamakura, Japan). Labeled RNAs were hybridized onto 3D-Gene Human miRNA Oligo chips (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes were conformed to the miRBase miRNA data base (<http://microrna.sanger.ac.uk/sequences/>). After stringent washes, fluorescent signals were scanned with the 3D-Gene Scanner (Toray Industries) and analyzed using 3D-Gene Extraction software (Toray Industries).

The raw data of each spot was normalized by substitution with a mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. Measurements of spots with the signal intensities greater than 2 standard deviations (SD) of the background signal intensity were considered to be valid. A relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. The Normalized data were globally normalized per array, such that the median of the signal intensity was adjusted to 25.

The miRNAs in which abundance had changed in the same direction in three out of the four subjects were selected. A miRNA was defined as 'up-regulated' when its abundance was more than twice that before intake, and when the abundance was less than half that before intake, the miRNA was defined as 'down-regulated'. We then defined the physiological significance of each miRNA that had clear

'Community annotation' [13] and functional data through PubMed searches of the miRNA gene name, and then the name of the gene in which the miRNA gene was located.

3. Results

The expression levels of miRNAs for all four subjects are shown in Fig. 2. The increased and decreased expression of miRNAs between Exam 1 (before FFC[®] Pairogen ingestion) and Exam 2 (after ingestion) are illustrated for each subject in Fig. 3. Among the 362 miRNAs detected, eleven showed significant \log_2 fold-changes after ingestion, and were chosen for further analysis. Table 2 shows the levels of miRNA expression after FFC[®] Pairogen ingestion. Although none of the miRNAs showed augmentation of expression in all four subjects, the expression of hsa-let-7c and hsa-miR-4755-3p was increased in three of the four subjects while the expression of hsa-miR-1238-3p and hsa-miR-6717-5p was decreased in all four subjects. The expression of seven miRNAs—hsa-miR-671-5p, hsa-miR-92a-2-5p, hsa-miR-1238-3p, hsa-miR-3131, hsa-miR-4298, hsa-miR-642a-3p and hsa-miR-652-5p—was decreased in three of the four subjects.

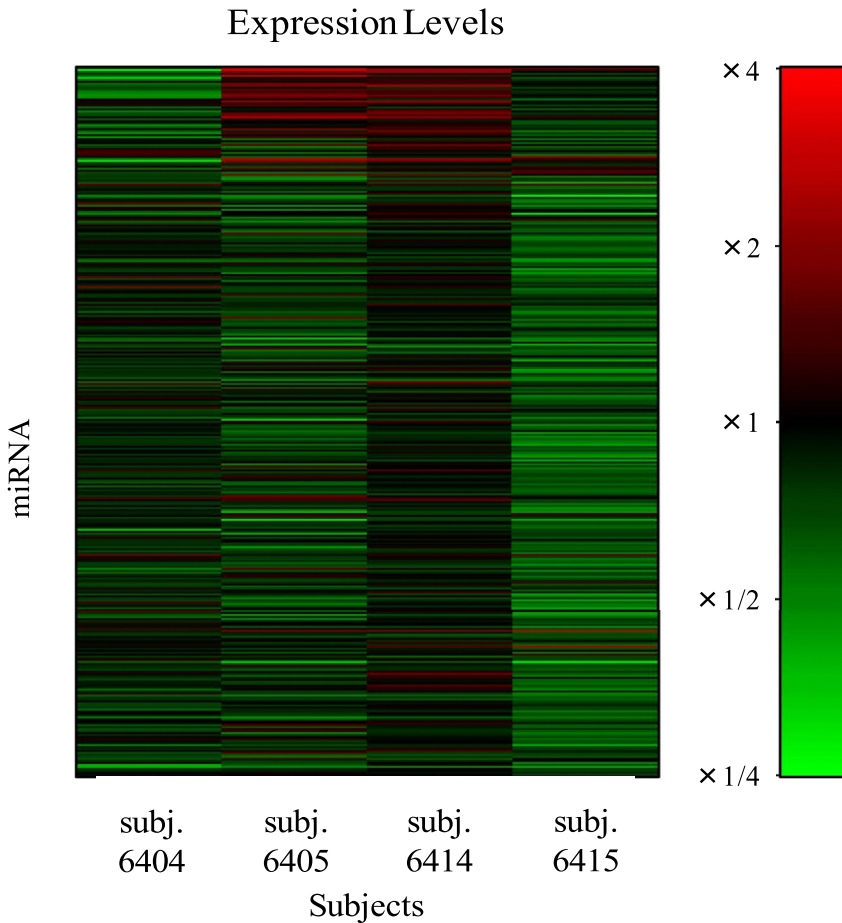


Fig. 2. \log_2 fold-change in the expression of the 362 microRNAs analyzed pre- and post-test in all subjects. Green and red indicate enhancement and suppression of expression compared with that at week-0, respectively.

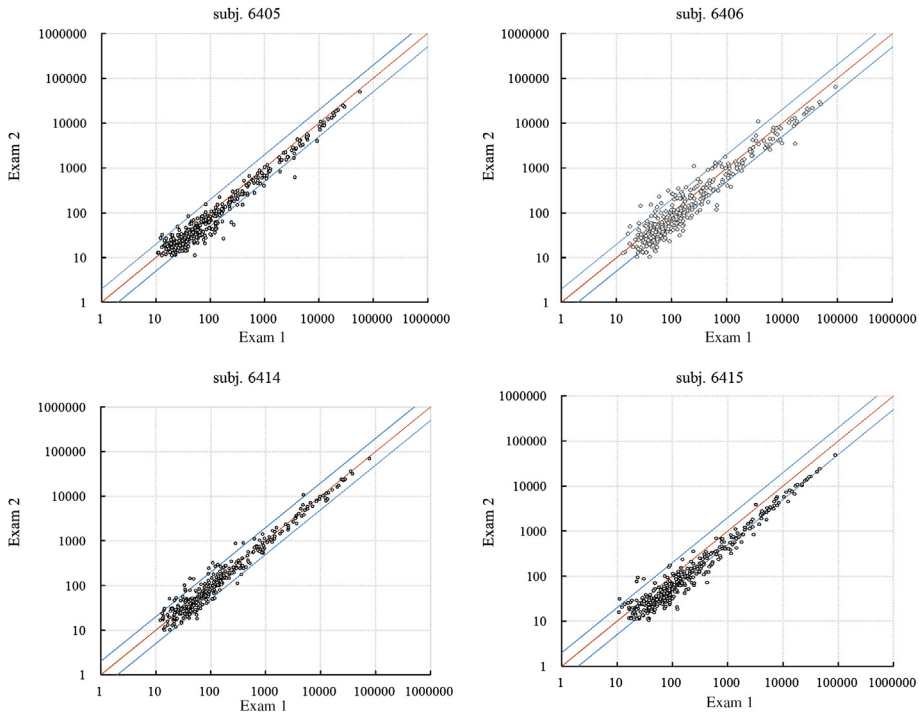


Fig. 3. Comparison of expression for all miRNAs between Exam 1 and Exam 2 for each subject. The horizontal axis shows miRNA expression on Exam 1 and the vertical axis shows expression on Exam 2. The red line indicates the value in Exam 1 was equal to that in Exam 2. If a point is under the red line, the miRNA was induced on Exam 2. Otherwise, the miRNA was suppressed on Exam 2. The two blue lines show double or halved expression levels.

Table 2
Results of miRNA examination.

miRNA	Increase rate of regulation after ingesting for all subjects					Annotation	GenBank code
	subj. 6405	subj. 6406	subj. 6414	subj. 6415	Mean		
Increased in all subjects							
None	–	–	–	–	–	N/A	N/A
Increased in three subjects							
hsa-let-7c	0.92	5.44	3.16	2.95	3.12	Yes ^a	MI0000064
hsa-miR-4755-3p	1.43	2.39	2.50	4.02	2.59	N/A	MIMAT0019896
Decreased in all subjects							
None	–	–	–	–	–	N/A	N/A
Decreased in three subjects							
hsa-miR-671-5p	0.35	0.33	0.50	0.15	0.33	N/A	MIMAT0003880
hsa-miR-92a-2-5p	0.44	0.34	0.90	0.35	0.51	Yes ^a	MIMAT0004508
hsa-miR-1238-3p	0.41	0.45	0.73	0.33	0.45	N/A	MIMAT0005593
hsa-miR-3131	0.51	0.24	0.47	0.40	0.41	N/A	MI0014151
hsa-miR-4298	1.09	0.35	0.42	0.39	0.56	N/A	MI0015830
hsa-miR-642a-3p	0.49	0.23	0.51	0.17	0.35	N/A	MIMAT0020924
hsa-miR-652-5p	0.50	0.40	0.49	0.43	0.46	N/A	MIMAT0022709

^a Community annotation (Ref. [13]).

4. Discussion

The results of this study suggest that after the intake of FFC[®] Pairogen for 8 weeks, some miRNA expressions changed, especially hsa-let-7c and hsa-miR-92a-2-5p.

hsa-let-7c targets cell cycle related genes, inhibits cell division [3,12], suppresses the manifestation of lung cancer [3] and functions as a tumor suppressor gene [4]. In contrast, the miR-17-92 cluster and hsa-miR-92-q-2-5p act as oncogenes when over-expressed [4], contribute to the manifestation of lung cancer through over-expression [5], are involved with the manifestation of mastocarcinoma through the down-regulation of estrogen receptors [6] and show increased expression in patients with ovarian epithelial carcinoma [7].

The concurrent over-expression of hsa-let-7c and under-expression of hsa-miR-92-a-2-5p, could be considered preventative for cancer [3,7] and, therefore, could serve as disease preventive genes.

The daily intake of FFC[®] Pairogen may affect the systematic regulation of miRNAs correlated with diseases and carcinomata. In addition, intake of another whole food could affect the expression of miRNAs.

These results suggest that effects of whole foods ingested by humans could be evaluated through analysis of miRNAs. However, the small sample of only four participants and the lack of control subjects are limitations of this trial. And participants ingested FFC[®] Pairogen also with regular meals at each day so that the miRNA expression changes did not directly determine by the ingestion. Moreover, this preliminary study did not include any control groups thereby further studies could resolve this problems including the number of subjects.

In conclusion, the functional effect of a whole food ingested by humans can be evaluated by the comprehensive analysis of miRNAs.

Conflict of interest

This trial was contract research. Akatsuka Co. Ltd. entrusted this research to Orthomedico Inc. Akatsuka Co., Ltd. supplied FFC[®] Pairogen, the material that was tested.

Disclaimers

None.

Clinical trial registry

University Hospital Medical Information Network Clinical Trials Registry (<http://www.umin.ac.jp/ctr/index.htm>). Registration code: UMIN000012608.

Acknowledgments

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The authors' responsibilities were as follows: TH, AK, SY, NS, and KY designed the research; NS and TT conducted the research; WD performed the statistical analysis; NS, and WD wrote the paper; NS had primary responsibility for the final content. All authors read and approved the final manuscript.

Orthomedico Inc. undertook this trial as contract research from Akatsuka Co Ltd. The test material, FFC[®] Pairogen, was supplied by Akatsuka Co., Ltd. There are no other potential conflicts of interest.

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