Circulating microRNAs and life expectancy among identical twins

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Summary

Human life expectancy is influenced not only by longevity assurance mechanisms and disease susceptibility loci but also by the environment, gene-environment interactions, and chance. MicroRNAs (miRNAs) are a class of small noncoding RNAs closely related to genes. Circulating microRNAs have been shown as promising non-invasive biomarkers in the development of many pathophysiological conditions. However, the concentration of miRNA in the circulation may also be affected by environmental factors. We used a next-generation sequencing platform to assess the association of circulating miRNA with life expectancy, for which deaths are due to all causes independent of genes. In addition, we showed that miRNAs are present in 41-year archived plasma samples, which may be useful for both life expectancy and all-cause mortality risk assessment. Plasma miRNAs from nine identical male twins were profiled using next-generation sequencing. The average absolute difference in the minimum life expectancy was 9.68 years. Intra-class correlation coefficients were above 0.4 for 50% of miRNAs. Comparing deceased twins with their alive co-twin brothers, the concentrations were increased for 34 but decreased for 30 miRNAs. Identical twins discordant in life expectancy were unlike in the majority of miRNAs, suggesting that environmental factors are pivotal in miRNAs related to life expectancy.

INTRODUCTION

Human life expectancy is influenced not only by longevity assurance mechanisms and disease susceptibility loci but also by the environment, gene-environment interactions, and chance (Brooks-Wilson, 2013; Cournil & Kirkwood, 2001). A recent laboratory experiment has discovered that microRNAs (miRNAs) regulate lifespan of the nematode Caenorhabditis elegans, suggesting that miRNAs play a role in life expectancy (Smith-Vikos & Slack, 2012). MiRNAs are a class of small (18-24 nucleotides) noncoding RNAs that can induce gene silencing by targeting mRNA either by translational repression or mRNA cleavage (Lau & Lai, 2005; Matzke & Birchler, 2005). The most intriguing feature of miRNAs is their unusual stability in the extracellular environment. For example, circulating miRNAs are stable through multiple freeze-thawing cycles (Fichtlscherer, Zeiher and et al., 2011), RNase-mediated degradation (Fichtlscherer, Zeiher and et al., 2011), extreme pH conditions, high temperatures and prolonged storage (van Empel, De Windt and et al., 2012). The concentration of miRNAs are measurable with a greater sensitivity than proteins (Su, Han and et al., 2011). Circulating miRNAs have been shown as promising biomarkers in the development of cardiovascular disorders (Su, Han and et al., 2011; Zampetaki, Willeit and et al., 2012), cancers (Grady & Tewari, 2010; He, Lin and et al., 2015) and other pathophysiological conditions. Studies have shown that extracellular miRNAs can be taken up by cells and affect target gene expression profile in the recipient cells (Fichtlscherer, Zeiher and et al., 2011). Peripheral blood samples are easy to obtain with little risk to individuals compared to biopsies and surgical procedures. Therefore, we thought that circulating

miRNAs could be promising less-invasive biomarkers for understanding of the life expectancy.

Genetic makeup can affect phenotypes including longevity and disease susceptibility loci (Brooks-Wilson, 2013; Cournil & Kirkwood, 2001), and the expression of miRNAs (Geeleher, Huang and et al., 2012). A previous study of 56 trios from the HapMap project showed that genetic factors affect 124 miRNAs in lymphoblastoid cell lines with an average heritability of 30% (Geeleher, Huang and et al., 2012). Identical twins are ideally a natural setting to control for germline (including genes and inherited epigenetic modifications) that cannot be controlled for in the traditional singleton population study. As identical twins share 100% genetic materials, any difference in miRNA in the circulation between co-twins might be caused by environmental factors such as diet (Ryu, Langkamp-Henken and et al., 2011) and exercise (Gomes, Kim and et al., 2015).Therefore, understanding of plasma miRNA composition that is free of germline influence is pivotal to assess environmental effects on the spectrum of circulating miRNAs. In this study, we first showed that miRNAs present in long-term archived plasma samples were measurable with a next-generation sequencing platform, and then we assessed the association of circulating miRNA with life expectancy, for which deaths are due to all causes.

MATERIALS AND METHODS

Subjects

The National Heart, Lung, and Blood Institute (NHLBI) Twin Study has been widely described (Dai, Krasnow and et al., 2013; Reed, Carmelli and et al., 1993; The U.S.National Heart Lung and Blood Institute (NHLBI)., 2005). The NHLBI Twin Study was designed to prospectively investigate the genetic and environmental role in cardiovascular risk through inclusion of 514 middle-aged, white male, veteran twin pairs born between 1917 and 1927 at baseline (1969-1973) (Dai, Krasnow and et al., 2013; Reed, Carmelli and et al., 1993). Based on zygosity ascertained by 8 red blood cell antigen groups (serotyping 22 erythrocyte antigens) in the 1960s and variable number of tandem repeat DNA markers in the 1980s (Reed, Carmelli and et al., 1993), the baseline parent cohort included 253 monozygotic (MZ) and 261 dizygotic twin pairs (Reed, Carmelli and et al., 1993). The reported study was approved by the Institutional Review Boards of Vanderbilt University.

At baseline, blood was drawn from the forearm vein after an overnight fast into EDTA tubes and immediately placed on ice. After centrifugation, plasma aliquots were frozen at -70 °C (Dai, Krasnow and et al., 2013; Mikulec, Holloway and et al., 2004; Reed, Tracy and et al., 1994). In this exploratory study, the inclusion criteria were identical twin pairs in which co-twins were discordant for age at death (excluding accidental causes), had a minimum difference in age at death of at least 5 years, and had available baseline plasma samples that were collected 41 years ago. We originally included 5 discordant MZ twin pairs. As one plasma sample yielded an insufficient amount of RNA, we present data from 9 twin subjects (four discordant MZ pairs and one unpaired twin) in this report.

Assessment of endpoints

We previously described the assessment of endpoints (Dai, Krasnow and et al., 2013; Wu, Neale and et al., 2014). Vital status as well as the cause and date of death through December 31, 2010 were ascertained from medical records and death certificates in four follow-up examinations (exam 2, 1981-1982; exam 3, 1986-1987; exam 4, 1995-1997; and exam 5, 1999-2000), and through death certificates or the National Death Index after exam 5 (Mikulec, Holloway and et al., 2004). Criteria used for ascertaining outcomes in four follow-up examinations were standardized, and a panel of investigators made decisions regarding disease diagnosis: at examinations 2 and 3 two independent physicians reviewed medical records; at examinations 4 and 5 one physician reviewed medical records. Physicians assigned corresponding International Classification of Diseases Ninth Revision codes. Death certificates or the National Death Index with the Ninth Revision codes was obtained for decedents. The endpoint was death from all causes. Twins were considered lost to follow-up if a death certificate or coding from the National Death Index could not be traced.

miRNA profiling using next-generation sequencing (NGS)

Blood was drawn from the forearm vein after an overnight fast into EDTA tubes and immediately placed on ice. After centrifugation, plasma aliquots were frozen at -70 °C (Mikulec, Holloway and et al., 2004; Reed, Tracy and et al., 1994). We performed miRNA profiling using NGS at the Vanderbilt Center for Genomic Research. The sequencing libraries were constructed using the New England Biolabs (NEB) small RNA

sequencing kit and were analyzed on an Illumina HiSeq 2000 platform with single end 50 nucleotide read length. Post sequencing data analysis was performed with an in-house small RNA sequence analysis pipeline as described (Wang, Li and et al., 2012). The miRNA mapping results were normalized with reads per million then log2 transformed before statistical analysis. The data was deposited in the GEO database with an accession number "GSE80730".

Statistical analysis

All mapped reads were normalized to reads per million then converted to log 2 values. If a log 2 value of a miRNA was above the average value of all miRNAs, we defined this miRNA as detectable. A detectable miRNA that was present in at least 70% of samples analyzed was defined as “present miRNA” in the sample set. A total of 167 present miRNAs were observed from the primary analyses. The miRNA levels were compared by the paired t-test.

For surviving twins, we calculated the minimum life expectancy as age at the last follow up date. For deceased twins, we calculated the life expectancy using age at death. As all twin pairs were discordant for vital status (i.e. one co-twin was deceased and his co-twin was still alive), we compared the minimum life expectancy for surviving twins with the life expectancy of deceased twins.

Intraclass correlation coefficient (ICC) was calculated to estimate the similarity of the measurement between the co-twins in a twin pair. ICC values greater than 0.75 represent “excellent similarity” in miRNAs between co-twins and values between 0.4 and 0.75 represent “fair to good similarity” (Lexell & Downham, 2005). Fold changes

were the ratio of log2 transformed normalized read counts of deceased twins to their alive co-twins. Using a 2-fold expression difference as a cutoff point for dysregulated miRNAs (SGC, 2004). We only showed miRNAs present in at least 70% of samples to determine the concentration variation of miRNAs. The between-subject variation of the coefficient (CV) for each miRNA was estimated to quantify the concentration variation among the 9 studied subjects.

A *P* value below 0.05 was considered significant without control for multiple testing. The Bonferroni corrected significance level of alpha that controlled multiple testing was 0.0003 as 167 miRNAs were tested (Dunn, 1961). All statistical tests were two-sided and were performed using SAS statistical software, version 9.3 (SAS Institute, Cary, NC).

RESULTS

Participants

The twins were middle-aged at baseline with an average age of 46 years and ranged from 44 to 51 years. The characteristics of the twins are shown in **Table 1**. Baseline plasma samples were collected 41 years ago. During the 41-year follow-up for all-cause mortality in each of the four pairs studied, one co-twin was deceased (**Figure 1**). The average years of follow-up to death was 32.5. The average life for deceased twins was 74.8 years and the minimum life expectancy for the still alive twins was 81.4 years. The sole unpaired twin died at the age of 77.5 (**Table 1**). The average absolute difference in

the minimum life expectancy between age at the last follow-up date of alive co-twins and the age at death of their co-twins was 9.68 years (SD 2.24 years). Since identical co-twins differed in life expectancy, environmental factors must be pivotal factors affecting the life expectancy.

Detectability and presence of plasma miRNA

The number of detectable miRNAs for each individual is shown in **Figure 2**. A total of 866 miRNAs were detected in the 41-year old plasma collected from nine twins (709 miRNAs in deceased twins and 781 miRNAs in living twins); 276 miRNAs were detected only in one individual. Of the 866 miRNAs, 167 miRNAs were observed in at least of 70% samples and were defined as “present miRNAs” in the sample set.

ICC: the similarity of the measurement between the co-twins in one twin pair

Table 2 shows the ICCs of miRNAs present in at least 70% of samples between co-twin brothers. ICCs for 47% of miRNAs were in the category of “excellent similarity” or “fair to good similarity between co-twins”. ICCs for the remaining 53% of miRNAs were not similar (ICC <0.4). About 4% of the miRNAs have ICCs that are greater than 0.75, which indicate excellent similarity between co-twins for those miRNAs; about 43% of miRNAs have ICCs between 0.4 and 0.75, indicating a fair to good similarity.

Concentration difference between deceased twins and alive co-twin brothers

Using a 2-fold change as a cutoff point, we identified 34 (20%) miRNAs with increased concentrations and 30 (18%) miRNAs with decreased concentration in alive co-twins compared to deceased twin brothers (**Table 2**).

Among the 4 twin pairs that were discordant for life expectancy, at alpha 0.05, the concentrations of hsa-miR-3615 and hsa-miR-619 that were present in at least 70% of the plasma samples were significantly higher in deceased twins than living twins in the primary analysis. The concentration of hsa-miR-423-5p and hsa-miR-4305 detectable in less than 70% of the plasma samples were significantly higher in deceased twins than alive twins in a secondary analysis. However, none of miRNAs were statistically significant after multiple-testing adjustment. **Table 3** shows the top 20 miRNAs with the smallest p-value for differences in miRNA levels between long-lived and short-lived co-twins for both miRNAs present in at least 70% and <70% of plasma samples respectively (**Table 3**).

Among the 34 miRNAs having higher concentrations that were present in at least 70% of samples, 29% had between-subject CVs less than 10%, 6% had between-subject CVs ranging in 10-15%, 3% in 15-20%and 62% had between-subject CVs of at least 50%. Among the 30 miRNAs showing lower concentration in alive twins, 53% had between-subject CVs less than 10%, 7% varying in 10-15%, 3% in 15-20%, 7% in 20-30% and 30% had between-subject CVs of at least 50% (**Table 2**).

Abundance of miRNAs

The five miRNAs having highest concentrations in the samples were miR-1224, miR-518d-3p, miR-518a-3p, miR-320 and miR-4532 (**Figure 3**). The sum of read counts from these five miRNAs accounted for 23.9% of the total number of all miRNAs present in the samples. Of the top five miRNAs, miR-4532 had the highest average level.

DISCUSSION

This study detected 866 miRNAs among the plasma samples that were collected 41 years ago from nine male monozygotic twins. Comparing deceased twins with their living co-twin brothers, 34 miRNAs had higher concentrations and 30 miRNAs had lower concentration in deceased twins. Among the miRNAs showing concentration differences, 40.4% had between-subject CVs less than 20% for miRNAs which concentration were higher in deceased twins and 55.6% had CVs less than 20% for miRNAs which concentration were lower in deceased twins .

This is the first report to profile plasma miRNAs in relation to all-cause mortality by next generation sequencing. Circulating miRNAs have been shown as promising non-invasive markers in various pathological conditions including cardiovascular disorders (Su, Han and et al., 2011; Zampetaki, Willeit and et al., 2012) and cancers (Grady & Tewari, 2010) which were the two top causes of death in the United States (Yoon, Bastian and et al., 2014). Previous studies have reported the characterization of miRNA spectrum in plasma, serum, and whole blood by NGS (Moldovan, Batte and et al., 2014). For example, using NGS to profile plasma miRNAs, one study found that low plasma concentrations of **miR-483-5p** and **miR-103** had better prognosis while low plasma **miR-29a** and let-7c concentrations had poorer prognosis for 5-year survival than those with high concentrations for Chinese patients with nasopharyngeal carcinoma (Wang, Yan and et al., 2014). It is interesting that the current study also found the **miR-483-5p**, **miR-103a** and **miR-103b** concentrations in plasma were inversely associated with all-cause mortality in male monozygotic twins. A study from the US found that serum miR-146b, miR-221, miR-155, miR-17-5p, **miR-27a** and miR-

106a concentrations were significantly reduced in lung cancer patients using real-time PCR (Heegaard, West-Norager and et al., 2012). Consistently, our study also found that **miR-27a** was inversely associated with all-cause mortality. Although the design and subjects for these two studies are different, the consistent results may be useful for all-cause mortality risk detection.

Monozygotic twins are an ideal natural setting to control for germline contribution (including genes and inherited epigenetic modifications) that otherwise cannot be controlled for in traditional population studies. As monozygotic twins share 100% of their germline, any difference in miRNA levels between co-twins most likely are caused by environmental factors. Therefore, characterizing the spectrum of circulating miRNA free of germline influence is pivotal to understanding the influence of environmental factors on miRNAs in circulation. Changing environmental factors are more feasible to alter compared to genomic sequence. The identification of some miRNA concentration differences associated with all cause of mortality in our twin study encourages the exploration of the possible association of specific miRNAs in circulation related to all-cause mortality, which in turn would help clinicians to identify individuals that may have a higher risk for death. This information may assist public health professionals to assess possible interventions.

One limitation of our study is that our results were not validated by RT-qPCR. If any method could be considered a single gold standard among miR detection techniques, it would be RT-qPCR as it offers a good balance between cost, precision, and sample size along with a large functional dynamic range. It is used to validate results from whole-genome screening methods, such as NGS, and in the screening of

clinically relevant subsets of miRs (Hunt, Broyles and et al., 2015). However, the NGS method is not constrained to previously reported miR sequences logged in miRBase (as is the case with RT-qPCR), and although this means novel miR sequences may be discovered, not every small RNA read obtained will be a functional miR.

Our study has a number of interesting findings. The NGS technology is not hindered by variability in melting temperatures, co-expression of nearly identical miRNA family members, or post-transcriptional modifications (Git, Dvinge and et al., 2010). NGS is ideal for the identification of novel miRNAs, and provides unprecedented quantitative and qualitative accuracy. However, recent findings as well as our own study indicate different profiling methods, for example qPCR vs. NGS or even a different library construction method will generate different miRNA profiles (Raabe, Tang and et al., 2014). Therefore, one needs to be cautious when compare results from different studies. Plasma samples used in our study have been stored for 41 years. Globally it is not common to have more than 30-year old plasma samples from identical twins. These samples are extremely precious, limited, and non-replaceable. However, other limitations in our study need to be acknowledged. Our twins were Caucasian males, thus our results may not be generalized to women and other ethnic groups. Our study was not able to collect plasma from more twins and from dizygotic twins. Further study with a larger sample size is warranted. We could not completely rule out the possibility of residual confounding.

In conclusion, this exploratory study provides information on circulating miRNAs that may be related to life expectancy and all-cause mortality. This finding might provide

clinicians and public health professionals with additional information to identify an individual with higher risk for death, and facilitate the decision on the use of appropriate intervention methods. In addition, our study also suggests non-genetic factors might have stronger influence on miRNAs related to life expectancy compared with genetic factors.

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Author contributions include study design: JD; data collection: TK, XW, KS, DB, KW, RK, TR, JD; data analysis: SW, KW; manuscript preparation: SW, JD, and KW.

Conflict of interest

None declared.

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Table 1. Characteristics of nine monozygotic twins

Pair	ID	Vital Status*	Follow-Up Years	Baseline Age (years)	Age at death or last follow-up date (years)
P0001	V0001	1	32.5	45	77.5
P0002	V0002	0	37.4	45	82.4
P0002	V0004	1	25.5	45	70.5
P0003	V0005	0	37.4	44	81.4
P0003	V0006	1	30.8	44	74.8
P0004	V0007	0	37.2	51	88.2
P0004	V0008	1	26.7	51	77.7
P0005	V0009	0	37.2	46	83.2
P0005	V0010	1	27.5	46	73.5

*0=alive, 1=deceased

Table 2. Fold change, ICC, between-subject CV of plasma miRNAs presented in at least 70% samples from nine monozygotic twins

MiRNA	Fold change†	ICC‡	CV
Detectable in all samples (n=78)			
hsa-miR-4306	2.46¶	-0.20	9.59%
hsa-miR-425	2.25¶	-0.67	15.32%
hsa-miR-4290	1.92	-0.14	10.11%
hsa-miR-2861	1.85	-0.23	7.38%
hsa-miR-3928	1.82	-0.12	11.26%
hsa-miR-3960	1.76	-0.84	9.85%
hsa-miR-3648	1.66	-0.19	6.67%
hsa-miR-4419a	1.63	0.77	9.15%
hsa-miR-3181	1.54	0.12	9.26%
hsa-miR-619	1.53	0.58	7.58%
hsa-miR-1292	1.46	0.26	9.73%
hsa-miR-940	1.46	-0.06	11.91%
hsa-miR-203	1.42	-0.08	6.09%
hsa-miR-671	1.40	0.36	7.29%
hsa-miR-1273	1.38	0.52	8.45%
hsa-miR-185	1.36	0.43	7.76%
hsa-miR-4511	1.36	0.89	10.18%
hsa-miR-3175	1.35	0.14	10.61%
hsa-miR-3192	1.33	0.55	9.66%
hsa-miR-518e	1.31	-0.52	13.53%
hsa-miR-762	1.28	-0.46	8.67%
hsa-miR-1469	1.25	-0.62	19.52%
hsa-miR-4651	1.24	0.12	7.21%
hsa-miR-3127	1.20	-0.52	7.21%
hsa-miR-370	1.20	0.97	6.64%
hsa-miR-3180-5	1.20	0.57	9.87%

hsa-miR-92b	1.18	0.13	7.09%
hsa-miR-125b	1.15	0.69	11.17%
hsa-miR-4741	1.13	0.06	7.58%
hsa-miR-3195	1.07	0.67	8.27%
hsa-miR-22	1.06	-0.66	11.26%
hsa-miR-4259	1.04	0.13	6.87%
hsa-miR-296-3p	1.03	-0.48	9.87%
hsa-miR-572	1.02	-0.64	6.97%
hsa-miR-3125	1.02	0.23	10.27%
hsa-miR-1237	1.02	0.22	4.61%
hsa-miR-5095	1.01	-0.79	9.89%
hsa-miR-518a-3p	1.01	-0.53	9.59%
hsa-miR-518d-3p	1.01	-0.53	15.32%
hsa-miR-3939	1.00	0.49	6.54%
hsa-miR-4497	-7.69§	-0.88	8.22%
hsa-miR-4508	-2.86§	-0.81	10.85%
hsa-miR-378c	-2.63§	0.07	17.86%
hsa-miR-378	-2.44§	0.00	18.44%
hsa-miR-658	-2.27§	-0.83	9.43%
hsa-miR-320a	-2.04§	-0.21	12.15%
hsa-miR-451	-2.04§	0.53	16.59%
hsa-miR-320b	-2.04§	-0.13	9.99%
hsa-miR-100	-1.82	0.39	12.84%
hsa-miR-636	-1.79	-0.97	9.82%
hsa-miR-3665	-1.69	-0.98	15.55%
hsa-miR-4532	-1.61	-0.78	14.67%
hsa-miR-1224	-1.61	-0.71	13.43%
hsa-miR-639	-1.56	-0.89	10.98%
hsa-miR-432	-1.45	0.17	8.56%
hsa-miR-657	-1.43	-0.01	8.36%
hsa-miR-9	-1.43	0.37	6.93%

hsa-miR-4674	-1.39	-0.73	8.47%
hsa-miR-4488	-1.39	-0.59	13.41%
hsa-miR-128	-1.32	0.47	8.35%
hsa-miR-3196	-1.23	-0.78	10.53%
hsa-miR-3178	-1.20	-0.12	7.57%
hsa-miR-4463	-1.20	0.23	7.34%
hsa-miR-3653	-1.20	0.28	8.29%
hsa-miR-1908	-1.20	0.34	7.83%
hsa-miR-184	-1.19	0.98	6.62%
hsa-miR-662	-1.18	-0.70	6.57%
hsa-miR-4660	-1.16	0.81	8.54%
hsa-miR-4784	-1.15	0.66	6.63%
hsa-miR-3972	-1.12	0.44	7.59%
hsa-miR-26a	-1.11	0.46	7.97%
hsa-miR-1231	-1.10	-0.31	9.21%
hsa-miR-1289-2	-1.09	-0.22	8.56%
hsa-miR-2110	-1.05	0.47	9.56%
hsa-miR-627	-1.04	0.52	5.53%
hsa-miR-103a-2	-1.03	0.72	7.01%
hsa-miR-103b-2	-1.03	0.72	7.02%
hsa-miR-935	-1.02	0.55	9.39%
hsa-miR-3615	10.20¶	0.17	36.26%
hsa-miR-760	9.16¶	-0.14	36.44%
hsa-miR-944	8.82¶	-0.19	37.44%
hsa-miR-617	8.35¶	-0.34	38.02%
hsa-miR-411	7.45¶	-0.27	37.50%
hsa-miR-212	6.45¶	0.43	36.05%
hsa-miR-3925-5p	6.35¶	0.44	>100%
hsa-miR-3180-4	6.17¶	-0.04	35.78%
hsa-miR-3940	5.97¶	0.58	36.65%
hsa-miR-26a-1	5.93¶	0.32	36.92%

hsa-miR-665	5.38¶	0.38	36.84%
hsa-miR-3619	5.37¶	0.35	36.97%
hsa-miR-1247	5.11¶	-0.09	36.32%
hsa-miR-4785	4.96¶	0.13	35.81%
hsa-miR-4767	4.94¶	0.08	36.43%
hsa-miR-765	4.68¶	0.57	36.34%
hsa-miR-4459	4.65¶	0.09	36.54%
hsa-miR-1249	4.38¶	0.56	36.08%
hsa-miR-4313	4.03¶	-0.16	36.20%
hsa-miR-99a	3.95¶	0.28	36.62%
hsa-miR-1273e	3.02¶	0.60	36.48%
hsa-miR-4538	3.01¶	0.06	36.28%
hsa-miR-4492	-16.67§	-0.06	38.46%
hsa-miR-3941	-7.69§	0.05	36.38%
hsa-miR-3656	-7.14§	-0.03	36.31%
hsa-miR-143	-6.25§	0.14	37.27%
hsa-miR-3168	-6.25§	-0.16	36.29%
hsa-miR-7	-5.88§	0.27	37.75%
hsa-miR-920	-5.56§	-0.17	35.95%
hsa-miR-4285	-4.76§	0.10	36.27%
hsa-miR-326	-4.35§	0.14	35.98%
hsa-miR-551a	-4.00§	0.21	36.46%
hsa-miR-584	-3.85§	-0.03	36.00%
hsa-miR-483-5p	-3.23§	0.23	36.80%
hsa-miR-4458	-2.94§	0.08	36.25%
hsa-miR-4754	-2.86§	-0.04	36.12%
hsa-miR-197	-2.44§	0.03	36.25%
hsa-miR-4449	-1.35	-0.56	37.78%
hsa-miR-320e	27.86¶	0.26	54.38%
hsa-miR-638	25.33¶	0.22	54.10%
hsa-miR-4258	22.09¶	0.13	53.61%

hsa-miR-526a-1	19.49¶	-0.04	53.70%
hsa-miR-3916	5.19¶	0.31	55.00%
hsa-miR-3545	4.93¶	-0.12	53.75%
hsa-miR-4778-5p	3.27¶	0.28	58.40%
hsa-miR-549	3.24¶	-0.25	56.73%
hsa-miR-4453	3.05¶	0.15	54.31%
hsa-miR-1302-5	2.25¶	-0.51	54.87%
hsa-miR-647	1.82	-0.16	53.93%
hsa-miR-4329	1.78	0.05	54.78%
hsa-miR-4484	1.57	-0.08	54.01%
hsa-miR-936	1.48	-0.58	55.91%
hsa-miR-4283-1	1.46	-0.32	54.00%
hsa-miR-4283-2	1.46	-0.32	54.00%
hsa-miR-4763	1.40	0.03	54.15%
hsa-miR-484	1.31	0.18	54.32%
hsa-miR-4308	1.29	-0.02	53.60%
hsa-miR-182	1.27	-0.07	53.75%
hsa-miR-4632	1.25	-0.31	53.67%
hsa-miR-518e*	1.21	-0.30	54.15%
hsa-miR-519a*	1.21	-0.30	54.15%
hsa-miR-519b-5p	1.21	-0.30	57.44%
hsa-miR-519c-5p	1.21	-0.30	57.44%
hsa-miR-522*	1.21	-0.30	54.15%
hsa-miR-523*	1.21	-0.30	54.15%
hsa-miR-1181	1.18	0.21	53.87%
hsa-miR-1250	1.18	-0.39	54.52%
hsa-miR-4535	1.15	-0.49	53.57%
hsa-miR-448	1.15	-0.31	54.23%
hsa-miR-525-3p	1.10	-0.34	54.27%
hsa-miR-524-3p	1.09	-0.34	54.26%
hsa-miR-4311	1.06	0.23	54.43%

hsa-miR-1273d	1.04	0.05	54.13%
hsa-miR-519a-2	1.04	0.01	53.70%
hsa-miR-1827	1.02	0.03	54.44%
hsa-miR-135b	-33.33§	-0.12	53.67%
hsa-miR-4707-3p	-25.00§	-0.18	>100%
hsa-miR-761	-20.00§	-0.19	54.01%
hsa-miR-27a	-16.67§	-0.13	54.01%
hsa-miR-524	-5.88§	-0.13	53.96%
hsa-miR-219-1	-4.55§	-0.39	54.04%
hsa-miR-378d	-2.33§	-0.25	56.01%
hsa-miR-1254	-1.96	-0.09	53.98%
hsa-miR-27b	-1.35	0.97	54.69%
hsa-let-7a	-1.22	-0.19	54.49%
hsa-miR-193a	-1.16	-0.19	53.77%
hsa-miR-29a	-1.08	1.00	54.01%
hsa-miR-200b	-1.03	-0.19	54.41%
hsa-miR-298	-1.02	-0.10	54.51%

Abbreviation: ICC: Intraclass correlation coefficient; CV: variation of coefficient.

†Fold change of plasma miRNAs presented in at least 70% samples was the ratio of log2 transformed normalized read counts of deceased twins to their co-twin brothers alive. ¶over-expressed (increased concentration) miRNA with a fold change ≥ 2; §under-expressed (decreased concentration) miRNA with a fold change ≤-2.

‡ICCs of plasma miRNAs present in at least 70% samples from co-twins in twin pairs.

Note: the bold miRNAs were mentioned in the discussion.

Table 3 Top 20 plasma miRNAs with the smallest p-value*

miRNAs (present at least 70%)				miRNAs (present less than 70%)			
miRNA ID	Short-lived co-twins†	Long-lived co-twins†	*P*	miRNA ID	Short-lived co-twins†	Long-lived co-twins†	*P*
hsa-miR-3615	10.52(0.8)	9.51(0.47)	0.02	hsa-miR-423-5p	2.25(4.5)	9.74(1.19)	0.03
hsa-miR-619	11.49(1)	10.68(0.8)	0.04	hsa-miR-4305	2.26(4.52)	9.72(0.28)	0.04
hsa-miR-4419a	10.96(0.85)	10.34(1.22)	0.09	hsa-miR-661	0(0)	6.53(4.35)	0.06
hsa-miR-1273e	9.2(0.59)	9.86(1.05)	0.09	hsa-miR-1273c	6.73(4.49)	0(0)	0.06
hsa-miR-135b	4.98(5.75)	10.08(0.41)	0.15	hsa-miR-192	0(0)	6.54(4.36)	0.06
hsa-miR-2861	10.33(0.93)	9.45(0.28)	0.17	hsa-miR-4764-5p	0(0)	6.59(4.4)	0.06
hsa-miR-184	9.71(0.66)	9.61(0.78)	0.20	hsa-miR-4303	6.78(4.53)	0(0)	0.06
hsa-miR-370	10.57(1.24)	10.35(1.18)	0.20	hsa-miR-4288	6.93(4.65)	0(0)	0.06
hsa-miR-3648	11.09(0.77)	10.32(0.55)	0.21	hsa-miR-4721	2.35(4.71)	9.29(1)	0.07
hsa-miR-1254	6.56(4.37)	9.93(0.33)	0.21	hsa-miR-3609	2.16(4.33)	7.47(5.08)	0.11
hsa-miR-761	5.11(5.9)	9.88(0.69)	0.21	hsa-miR-4647	4.37(5.05)	9.34(0.43)	0.14
hsa-miR-4707-3p	4.9(5.67)	9.39(0.4)	0.22	hsa-miR-93	4.94(5.71)	10.02(0.87)	0.15
hsa-miR-1249	9.89(0.83)	10.37(0.65)	0.23	hsa-let-7g	2.99(5.99)	9.05(0.65)	0.15
hsa-miR-4306	11.68(1.18)	10.73(0.53)	0.26	hsa-miR-641	7.5(5.12)	2.56(5.11)	0.15
hsa-miR-4660	11.19(1.08)	11.56(0.88)	0.27	hsa-miR-194-2	4.59(5.32)	9.25(0.35)	0.16
hsa-miR-212	10.04(0.75)	9.57(0.65)	0.27	hsa-miR-1470	7.27(4.91)	2.14(4.27)	0.16
hsa-miR-3941	7.55(5.08)	10.75(0.81)	0.27	hsa-miR-1238	2.24(4.49)	7.04(4.7)	0.16
hsa-miR-665	10.57(1.47)	7.89(5.32)	0.28	hsa-miR-127	2.34(4.68)	7.12(4.75)	0.17
hsa-miR-27a	5.1(5.91)	9.01(0.19)	0.29	hsa-miR-485	7.43(5.05)	2.54(5.08)	0.17
hsa-miR-3168	7.41(4.98)	10.63(0.46)	0.31	hsa-miR-1283-2	7.2(4.88)	2.65(5.31)	0.17

*The comparison of the log 2 transformed normalized miRNA levels between long-lived and short-lived co-twins were performed using paired t-test from the 167 present in at least 70% of samples and 699 miRNAs that were present in less than 70% of samples (i.e. non-present miRNAs).

†mean (SD)

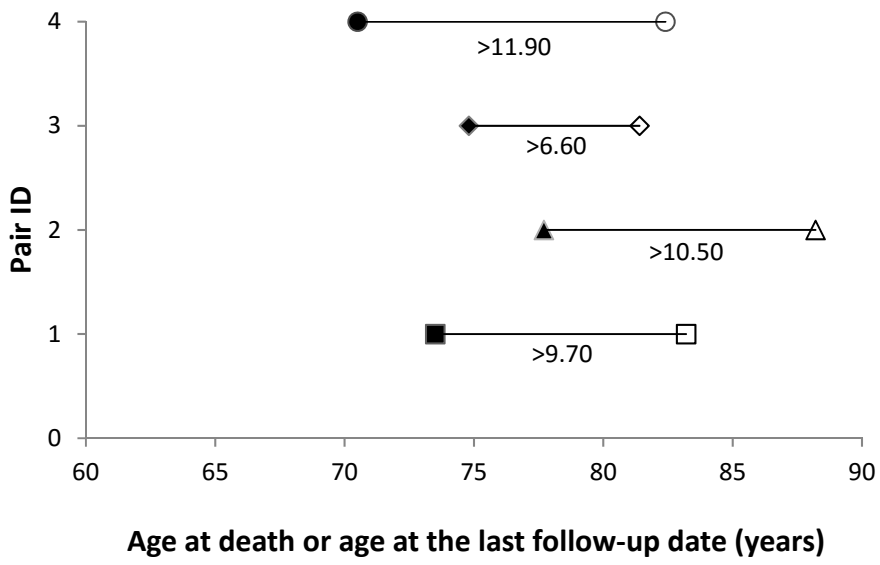

Figure 1. Age at death or age at the last follow-up date (years) in four monozygotic twin pairs

Solid symbol: deceased; Hollow symbol: alive

The number below the horizontal bar inside the figure indicated minimum differences in the life expectancy in years between deceased twins and their alive co-twin brothers

Pair ID: 1: P0005; 2: P0004; 3: P0003; 4: P0002

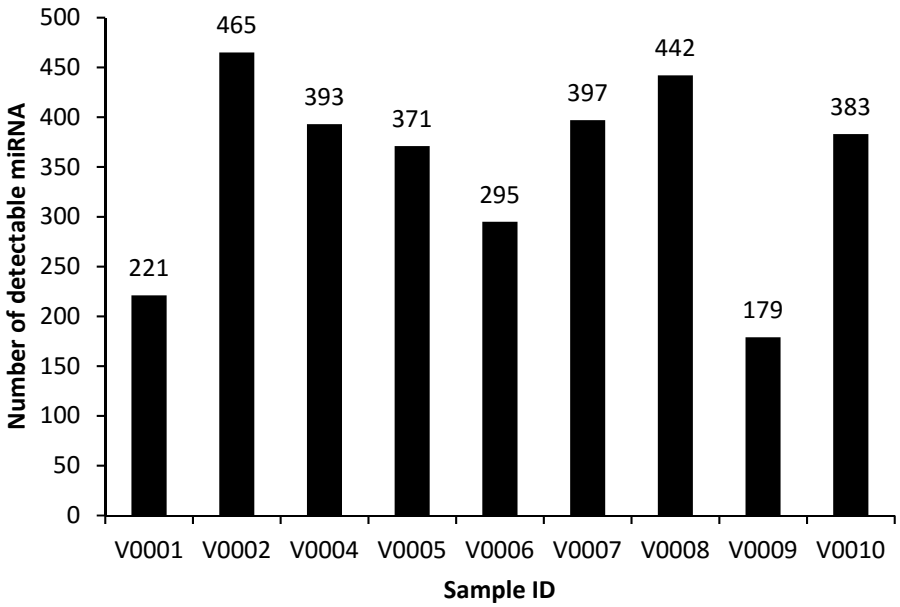

Figure 2 The Number of miRNA presented in the plasma from nine monozygotic twins

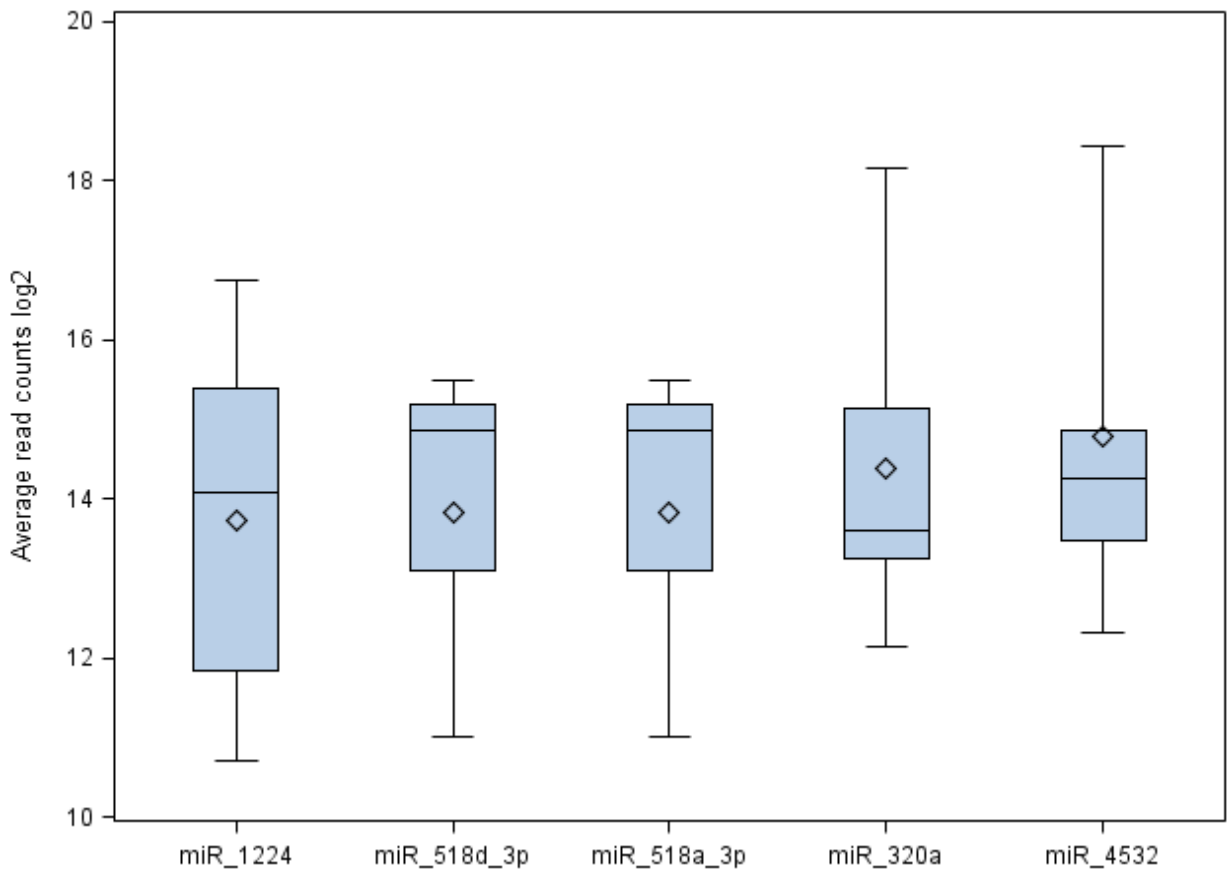

Figure 3. Boxplot of the five most abundant miRNAs

Differential expression of the five most abundantly expressed miRNAs in our monozygotic twins. The total numbers of miRNA reads are log2 transformed.

The length of the box represents the interquartile range (the distance between the 25th and 75th percentiles).

◊ The symbol inside the box represents the group mean.

The horizontal line inside the box represents the group median.

The vertical lines from the box extend to the group minimum and maximum values.