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Brief Report

Investigation of MicroRNA Expression in Human Serum During the Aging Process

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Background. Although serum microRNAs (miRNAs) play essential roles in the diagnosis of various diseases, little is known about circulating miRNAs in the aging process.

Methods. Solexa sequencing technology was used for an initial miRNA screening of serum samples pooled from 21 healthy Chinese subjects with an average age of 22 years, 10 subjects with an average age of 40 years, 10 subjects with an average age of 59 years, and 9 subjects with an average age of 70 years. Other serum samples were obtained from 123 normal people with approximately 31 samples in each age period. A stem-loop quantitative reverse transcription–PCR assay was conducted to confirm the concentrations of the miRNAs altered in the aging process.

Results. Solexa sequencing demonstrated 10 markedly altered miRNAs in the aging process. Quantitative reverse transcription–PCR analysis identified five downregulated miRNAs (miR-29b, miR-106b, miR-130b, miR-142-5p, and miR-340) and three upregulated miRNAs (miR-92a, miR-222, and miR-375) with age. Their target genes, related diseases, molecular and cellular functions, and participated pathways were further analyzed.

Conclusions. The measurement of miRNAs in serum provides a novel, noninvasive approach for the identification of the aging process. Our bioinformatic analyses could form a useful knowledge base for the potential future development of novel therapeutic treatments.

Key Words: MicroRNA—Aging—Serum—Biomarker.

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MICRORNAS (miRNAs) are a family of small, noncoding single-stranded RNAs with a length of 18–25 nucleotides which mainly function in regulating gene expression through base-pair binding to the 3′-UTR of their target mRNAs at the posttranscriptional level (1). They are involved in a wide range of biological and pathological processes, including the cell cycle, differentiation, development, metabolism, patterning, aging, etc. (2).

In 1993, Lee and coworkers had demonstrated for the first time that the *lin-4* gene and its target *lin-14* regulate the lifespan of *Caenorhabditis elegans* (3). Several downregulated miRNAs have been found in aged mice brain and liver tissues (4,5), whereas there are no obvious miRNA changes identified in lung tissues with age. Another group used liver from different time points to screen miRNA expression and revealed that some miRNAs are involved in liver function while others are associated with regeneration ability (6). miRNA microarrays were used to screen specific miRNA expression in the livers of Ames dwarf mice, which are well known for their remarkable delay in the onset of aging, indicating that miRNA-27a is a key posttranscriptional control (7). Additionally, these aging-related miRNAs with altered expression during the aging process also showed tissue-specific characteristics (8). A group examined the differential expression of miRNAs in young and aged human skeletal muscle before and after anabolic stimulation (9). Furthermore, the comparative profiling of genes and miRNAs showed differences between newborn, young adult, and aged human epididymides, and few miRNAs showed an age-enriched expression pattern in the adult and

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aged epididymides (10). Moreover, mononuclear cells from peripheral blood were used as a model to evaluate miRNA expression in samples from young and old individuals, and they identified nine miRNAs that were significantly lower in older individuals (11).

Here, we combined the high-throughput Solexa sequencing screening with a stem-loop quantitative reverse transcription–PCR (qRT–PCR) assay that uses a hydrolysis probe to systematically and comprehensively evaluate miRNA expression profiles in the sera of healthy individuals at different steps of the aging process. Finally, the targeted genes, related diseases, molecular functions, and related pathways of these miRNAs were further analyzed.

Methods

Normal Individual Serum Samples From Four Aging Periods

All participants were recruited from a large pool of individuals seeking a routine health checkup at the Healthy Physical Examination Centre of Jinling Hospital. Participants who showed no evidence of disease were selected. The cohort consists of Chineses residing in Jiangsu province, China. All participants gave written informed consent and the Research Ethics Committee of Jinling Hospital approved this investigation. A total of 173 serum samples were collected and divided into four groups based on the average age difference; these groups are 22 years old, 40 years old, 59 years old, and 70 years old.

RNA Isolation

Total RNA was extracted from 100 µL serum and used in a phenol/chloroform purification protocol for further analysis. For detailed methodology, see Supplementary Methods.

Solexa Sequencing, Reverse Transcription, and qRT–PCR Analysis

Solexa sequencing was performed as previously described (12). A TaqMan probe-based qRT–PCR assay was performed according to the manufacturer's instructions. For detailed methodology, see Supplementary Methods.

Bioinformatic Analysis

Targeted genes for each age-altered miRNA were predicted using the web-based software miRWalk. We obtained the protein class from the PATHER analysis results and then clustered the same functional class of protein with the top 10 classes. The web-based functional annotation tool Database for Annotation, Visualization and Integrated Discovery (DAVID) has key components for disease analysis, gene ontology analysis, and pathway analysis. For each miRNA, the top two items were listed out for each analysis.

Data Analysis

Student's t-test and Kruskal–Wallis test were used to compare continuous variables among two and more than two groups, respectively. The multiple comparison p values were corrected by Bonferroni correction. A p value less than .05 was considered statistically significant.

Results

Solexa Sequencing of Serum miRNAs in Four Age Groups

We employed the Solexa sequencing method to identify the expression levels of different miRNAs in pooled serum samples from four groups of healthy Chinese individuals with different average ages. The demographic features of all participants are listed (Table 1). A miRNA was considered altered if Solexa sequencing detected 100 copies in one of these groups and the miRNA showed at least twofold difference (Figure 1). Among the total 853 serum miRNAs scanned by Solexa sequencing, 7 miRNAs downregulated with aging were detected in females, while 15 miRNAs were detected in males (Figure 2A and 2B). Conversely, six miRNAs upregulated with aging were detected in females, while seven miRNAs were upregulated in males (Figure 2C and 2D). A total of 10 common miRNAs in both the female and male groups were found to be altered in the aging process, with 4 upregulated (let-7b, miR-92a, miR-222, and miR-375) and 6 downregulated miRNAs (miR-19b, miR-29b, miR-106b, miR-130b, miR-142-5p, and miR-340) during the aging process.

Confirmation of the Solexa Results by qRT–PCR Analysis

As shown in Figure 1, only miRNAs with a p value of less than .05 among the four groups were included, and a C_q value of greater than 35 for either group led to exclusion. We used these criteria to generate a list of five downregulated miRNAs (miR-29b, miR-106b, miR-130b, miR-142-5p, and miR-340) and three upregulated miRNAs (miR-92a, miR-222, and miR-375) that showed different patterns among the four groups (Figure 3), while let-7b and miR-19b showed no significant difference (Supplementary Figure 1). Only the concentrations of miR-29b and miR-92a were significantly different for all four periods of the aging process.

Target Analysis of Age-Altered miRNAs

To explore which targets and pathways may be regulated by these age-altered miRNAs, we used the miRWalk database

Table 1. Demographic Features of the Participants*

Groups	1	2	3	4
N	52	41	40	40
Mean age (y)	22.2 ± 0.3	39.8 ± 0.6	59.3 ± 0.7	70.3 ± 1.0
Gender (male/female)	25/27	27/14	28/12	18/22

*Data are presented as mean ± *SD*.

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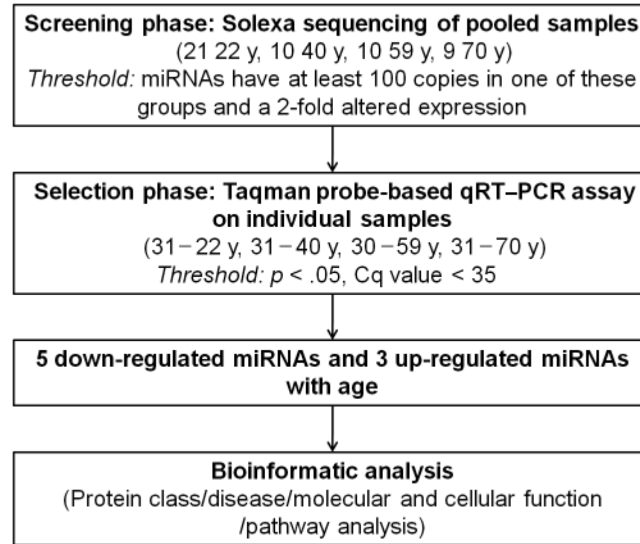

Figure 1. Overview of the design strategy.

Figure 2. Altered serum miRNAs in the aging process, as determined by Solexa sequencing. Of all serum samples, 50 samples were sequenced by Solexa. (**A**) Downregulated miRNAs with aging in females, (**B**) upregulated miRNAs with aging in females, (**C**) downregulated miRNAs with aging in males, and (**D**) upregulated miRNAs with aging in males.

to analyze all potential targets for each age-altered miRNA (AA-miRNA). Protein functions are indicated in Figure 4. A vast number of these targeted genes could encode transcription factors that might control the translation processes

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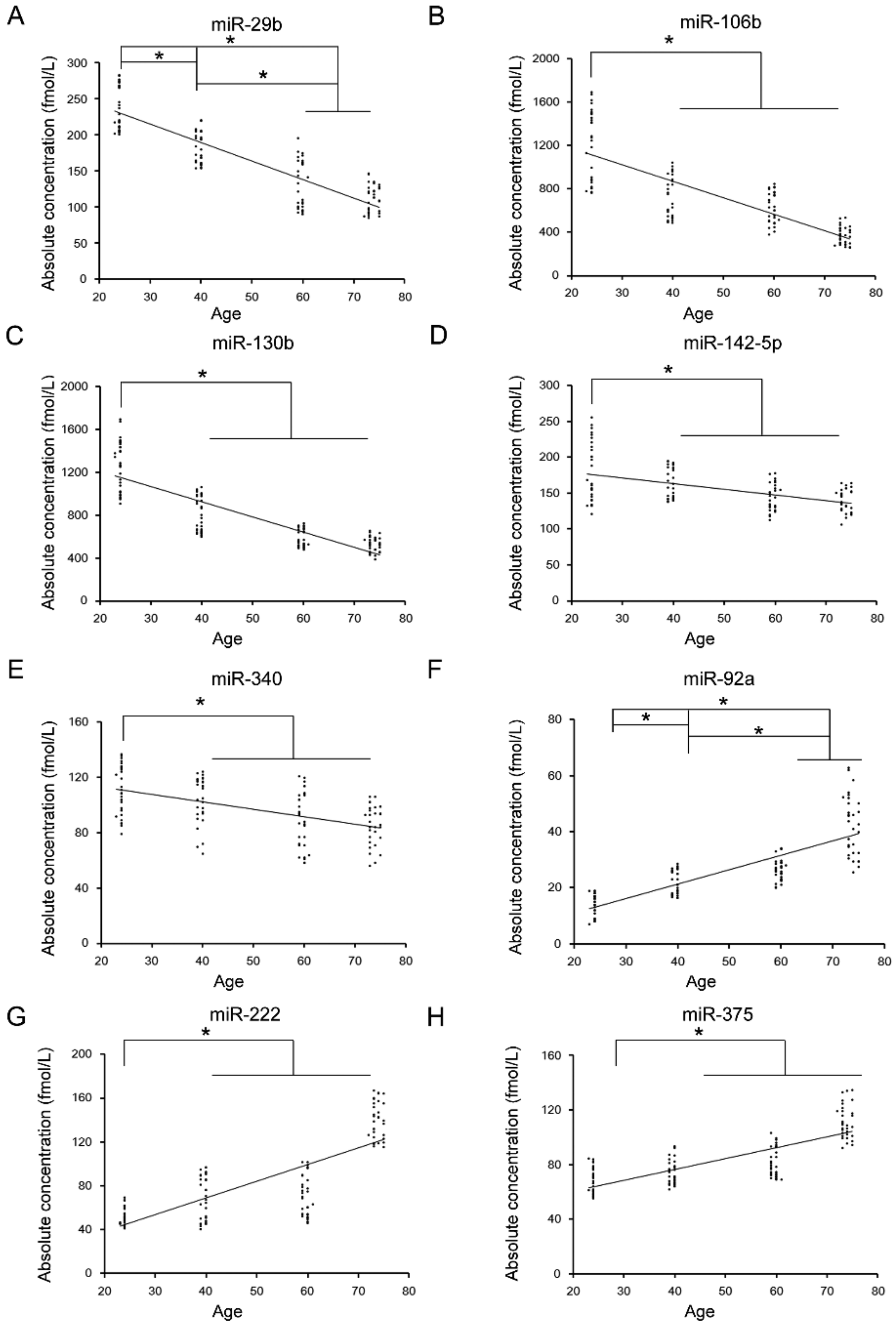

Figure 3. Detection of down- and upregulated miRNAs using qRT–PCR analysis. Of all serum samples, 123 samples were detected by the TaqMan probe-based qRT–PCR assay. C_q values were converted to absolute values from the calibration curves (*p < .05). **A**, miR-29b; **B**, miR-106b; **C**, miR-130b; **D**, miR-142-5p; **E**, miR-340; **F**, miR-92a; **G**, miR-222; **H**, miR-375.

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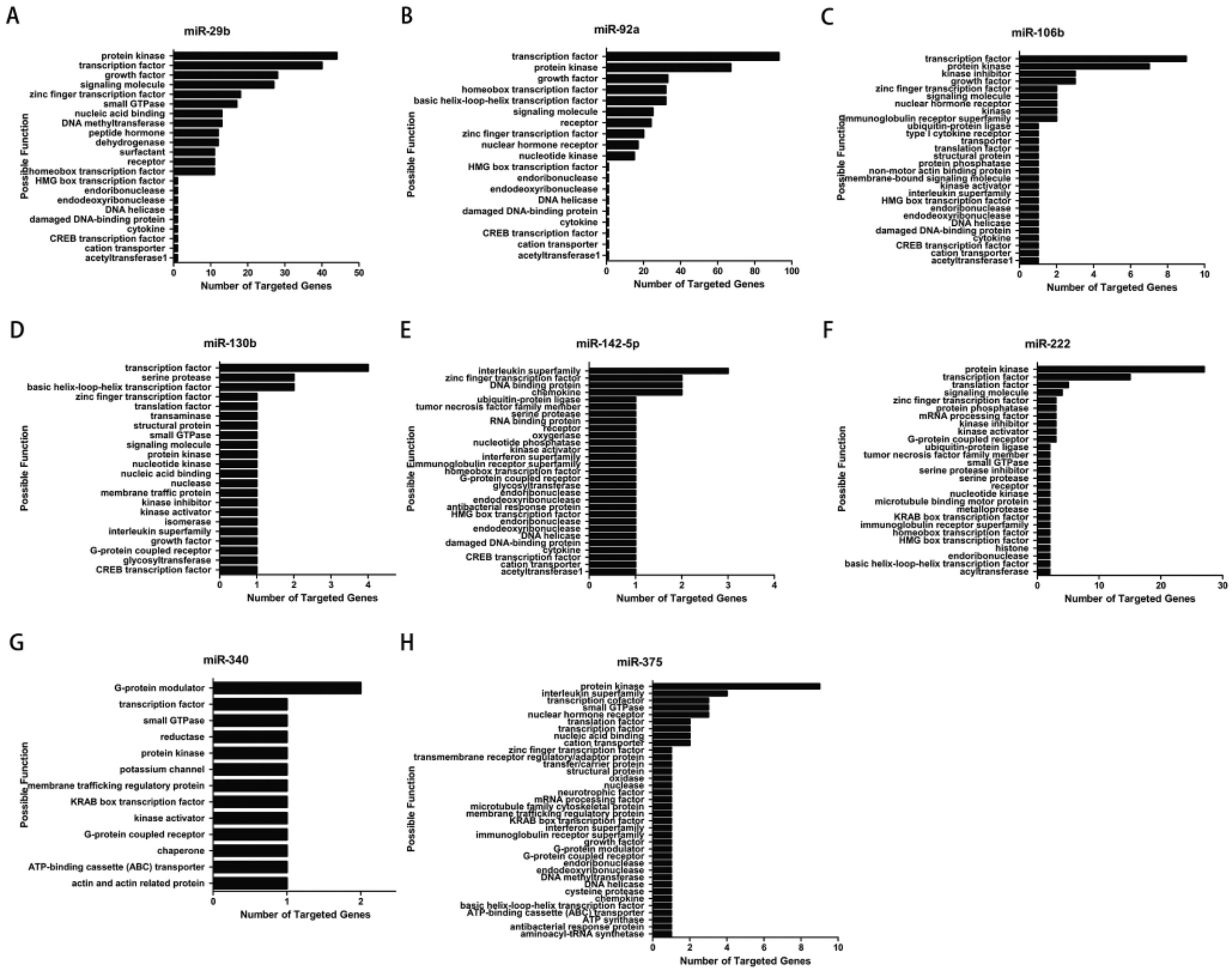

Figure 4. Major protein classes of potential targets of the age-altered miRNAs. **A**, miR-29b; **B**, miR-92a; **C**, miR-106b; **D**, miR-130b; **E**, miR-142-5p; **F**, miR-222; **G**,miR-340; **H**, miR-375.

of nearly all human genes, which was in accordance with our previous results (13). Consistent with previous results (2), the DAVID analyses identified cancer as the most common disease among these miRNAs (Table 2). The targets for the AA-miRNAs were most prominently predicted to function in the regulation of cell proliferation, the cell cycle, and transcription (Table 3). Of note, very little overlap was observed between the pathways for these miRNAs, suggesting that they may regulate multiple pathways that influence diseases (Table 4), as reported previously (14).

Discussion

Human serum from healthy individuals contains abundant quantities and species of circulating miRNAs. They can be readily detected with almost all routine RNA analysis techniques, including qRT–PCR, and can be used to identify aging. A combination of solexa sequencing and qRT–PCR assay used in this study is widely accepted (12). The Solexa sequencing is a high-throughput technique for initial miRNA screening which eliminates potential contamination by other small RNA and DNA fragments. Because of the individual variation of samples, Solexa results obtained from pooled serum samples were further validated by qRT–PCR using a number of individual serum samples. Here, a total of 173 samples were collected and analyzed to minimize election bias produced by small sample size.

MiRNAs are responsible for regulating cell-type–specific functions in the adult organism and are involved in aging process (15). In this study, there were eight miRNAs that changed significantly with the aging process, with five miRNAs (miR-29b, miR-106b, miR-130b, miR-142-5p, and miR-340) that decreased and three miRNAs (miR-92a, miR-222, and miR-375) that increased. Interestingly, these miRNAs are inconsistent with previous study of mononuclear cells, which is caused by significantly differential miRNAs expression levels in different organs or cell types (16,17). Apparently, serum miRNA biomarker is the best choice for the identification of aging or other diseases without the need for biopsy, surgery, and other invasive procedures. Our identified miRNAs have all been shown to have important relationships with aging. Ugalde and coworkers

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Table 2. Related Disease Analysis of Age-Altered miRNAs

AA-miRNA	Diseases and Disorders	% Regulated by AA-miRNAs	*p* Value
miR-29b	Cancer	34.90	1.50E-09
	Metabolic diseases	24.60	9.90E-03
miR-92a	Cancer	27.30	2.00E-07
	Cardiovascular diseases	18.00	1.50E-02
miR-106b	Cancer	36.40	4.30E-05
	Osteoporosis	5.50	1.10E-03
miR-130b	Vision diseases	19.20	1.10E-02
	Cancer	34.60	1.90E-02
miR-142-5p	Malaria	13.30	1.00E-04
	Appendicitis	10.00	1.40E-04
miR-222	Cancer	29.70	1.10E-08
	Infection	10.90	7.20E-03
miR-340	Developmental disorders	2.60	8.90E-04
	Juvenile arthritis	0.60	6.80E-03
miR-375	Immune disorders	19.70	4.00E-02
	Diabetes	13.10	7.10E-03

Table 3. Molecular and Cellular Function Analysis of Age-Altered miRNAs

AA-miRNA	Molecular and Cellular Functions	% Regulated by AA-miRNAs	*p* Value
miR-29b	Regulation of cell proliferation	28.60	9.70E-16
	Regulation of cell cycle	19.00	1.80E-14
miR-92a	Positive regulation of macromolecule metabolic process	31.10	9.40E-25
	Regulation of cell proliferation	29.00	4.10E-23
miR-106b	Transcription factor binding	21.80	3.70E-06
	Protein dimerization activity	21.80	6.30E-06
miR-130b	Transcription regulator activity	46.20	1.10E-05
	RNA polymerase II transcription factor activity	19.20	6.80E-04
miR-142-5p	Cytokine activity	23.30	1.90E-06
	Double-stranded RNA binding	13.30	3.70E-05
miR-222	Non-membrane spanning protein tyrosine kinase activity	9.40	2.10E-15
	Protein tyrosine kinase activity	13.80	2.10E-14
miR-340	Ion binding	28.80	6.70E-05
	DNA binding	17.50	8.10E-06
miR-375	Nucleotide binding	27.90	2.90E-02
	ATP binding	23.00	1.00E-02

has proposed that miR-29 is involved in progeroid and normal aging using the mouse model of Hutchinson-Gilford progeria syndrome, and they have found that aging and

Table 4. Canonical Pathway Analysis of Age-Altered miRNAs

AA-miRNA	Canonical Pathways	% Regulated by AA-miRNAs	*p* Value
miR-29b	Pathways in cancer	27.80	8.00E-23
	Cell cycle	11.90	8.50E-10
miR-92a	P53 signaling pathway	4.40	1.10E-05
	TGF-β signaling pathway	5.50	2.50E-05
miR-106b	P53 pathway feedback loops 2	14.50	1.00E-06
	ErbB signaling pathway	12.70	2.70E-05
miR-130b	Signaling by NGF	15.40	9.10E-03
	Toll-like receptor signaling pathway	11.50	2.30E-02
miR-142-5p	Toll-like receptor signaling pathway	16.70	1.30E-04
	Cytokine-cytokine receptor interaction	20.00	4.80E-04
miR-222	ErbB signaling pathway	9.40	1.40E-08
	P53 pathway feedback loops 2	8.70	4.30E-08
miR-340	TGF-β signaling pathway	1.40	1.30E-05
	MAPK signaling pathway	2.60	5.40E-04
miR-375	T-cell receptor signaling pathway	9.80	5.00E-04
	Neurotrophin signaling pathway	9.80	9.40E-04

chronic DNA damage response activate a regulatory pathway involving miR-29 and p53 (18). miR-92a has been identified as a regulator of aging on human tissue or organism level or of cellular senescence and may be an important regulator at the cross-roads between aging and cancer (19). It is widely reported that the expression of all members of the miR-106 family is decreased in senescent human diploid fibroblasts and trabecular meshwork cells by targeting p21 (20). miR-106b in miR-106b~25 cluster regulate mouse neural stem cell function and is part of a network involving the insulin/IGF-FoxO pathway, which may have important implications for the homeostasis of the neural stem cells pool during mouse aging (21). Also, miR-106 was suggested as a novel biomarker of human cellular aging (22). miR-130b prevents Ras-induced growth arrest of human mammary epithelial cells, which is a crucial step in the process of cellular senescence (23). miR-142 expression was changed in senescent human endothelial progenitor cells induced by remnant-like lipoproteins and in women blood (24,25). As transcriptional target of EGFR, the miRNA-222 plays a key role in EGFR signaling pathway and age-related diseases (26). The upregulation of miR-222 was observed in the rat vascular walls with neointimal lesion formation and the effect of age on miRNA expression might be a potential reason for the change (27). A study based on *Ercc1* knockout and hypomorphic mice has demonstrated that miR-340 expression was altered (28), and the phenomenon was also found in peripheral blood in young adult women (25). miR-375 is one of the most abundant miRNAs in pancreatic islets and β cells and regulates insulin secretion (29). In the field of cellular senescence, the increase of miR-375 has been reported to be implicated in a chemotherapeutically induced senescence of human acute myelocytic leukemia cells by targeting 14-3-3ζ and SP1 genes (30,31).

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Significant amounts miRNA have been detected in serum and other biological fluids (32). Many research groups have revealed that extracellular miRNAs originated from various apoptotic bodies and microvesicles can be transferred to recipient cells, influence target gene expression to mediate cell–cell communication, and donor and recipient cells could be same or different cell types (33). Expression profiles of miRNAs in microvesicles and their donor cells correlate poorly, indicating selective package of miRNA (34–36). Even though extracellular circulating miRNAs serve as biomarkers for various pathological conditions, the mechanism of the downregulation or upregulation of age-related miRNAs expression and extracellular circulating miRNAs-mediated cell-to-cell communication still require further studies.

In summary, this study defines a distinctive circulating miRNA signature during the aging process using Solexa sequencing followed by qRT–PCR validation. In particular, we have demonstrated that the profiles of two circulating miRNAs (miR-29b and miR-92a) gradually changed with the aging process and might serve as novel biomarkers for the identification of human physiological age. Furthermore, we analyzed the potential functions, aging-related diseases, and pathways of these AA-miRNAs. Finally, these results could provide additional information about the molecular mechanisms of human aging and the clinical value of miRNAs in the diagnosis of early senescence.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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