RESEARCH ARTICLE

hsa-miR-376c-3p in The Circulating Plasma is Upregulated in The Elderly Javanese Male When Compared to Their Younger Counterparts

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Abstract

BACKGROUND: MicroRNA (miRNA), short non-coding RNA, plays role in various physiological process such as aging through epigenetic regulation of gene expression. miRNA present intracellular as well as extracellular in body fluids. miRNA that present in blood circulatory system is often referred as circulatory miRNA (c-miRNA). A number of studies trying to identify c-miRNAs as biomarker for ageing have been reported, but majority did not yield results that corroborate one with another. This study reports the identification of a differentially expressed c-miRNAs between elderly and youth groups of individuals, the first step in tracking specific miRNAs that play role in physiologic ageing.

METHODS: The miRNA expression profiles of grandfathers and grandsons from 2 Javanese families were compared to select 5 miRNA candidates with widest expression difference. The 5 candidates were subjected to validation using quantitative polymerase chain reaction (qPCR) in 11 elderly men and 9 young men of the same ethnicity to identify differentially expressed miRNA between elder and younger male groups in the represented population.

RESULTS: Amongst 5 selected c-miRNA candidates, the hsa-miR-376c-3p was validated to be upregulated in the elderly group when compared to the young individuals. Bioinformatic analysis using miRTarBase 7.0, miRTargetLink Human and GeneCards® Human Gene Database suggest the involvement of hsa-miR-376c-3p in pathways relevant with cellular ageing.

CONCLUSION: This study showed that hsa-miR-376c-3p in the circulating plasma to be significantly upregulated in a group of elderly Javanese males compared to their younger counterparts. The results of this study warrant further study to elucidate the specific role of hsa-miR-376c-3p in physiologic ageing mechanism.

KEYWORDS: circulating, microRNA, miR-376c-3p, elderly, Javanese, male

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Introduction

Aging is a global problem in both developed and developing countries. In 2017, people aged 60 year old and above comprise 13% of the global population, and the number (percentage) is growing at a rate of about 3% per year. The elderly population in Indonesia in 2012 had reached more than 7% of the total Indonesian population and it is predicted to increase in the higher speed than its counterparts in other Asian region and globally after 2050.(1,2)

The aging process is very complex mechanism, takes place throughout one's lifespan. Although aging can be influenced by environmental factors, recent data show that genetic and epigenetic factors also play a definitive role in aging regulation and lifespan. One of the epigenetic factors is microRNA (miRNA) that influence the modulation of gene expression, firstly known in *Caenorhabditis elegans*. (3,4)

miRNA is a group of single stranded non-coding small RNA (17-25 nucleotides), that controls gene expression in animals, plants and eukaryotic organisms by mean of negative regulation (repressing) of post transcriptional gene expression or its target mRNA. The 2-8 nucleotides of the 5' miRNA, if partially binds to the target mRNA's 3' untranslated region (UTR) it will inhibit the translation, and if binds perfectly it will cause the target messenger RNAs (mRNAs) to be deadenylated and degraded, therefore, no translation takes place and there is no gene expression products. miRNA can also bind to 5' UTR and the coding region sequences (CDS) of their target mRNAs.(5,6)

The ability of miRNA to regulate multiple target genes simultaneously makes the miRNA an important regulator in various physiological conditions, especially in complex tissues and aging processes involving several interconnecting signalling pathways.(7) Several studies have also shown that miRNA participates in regulating cell cycle stages, cell proliferation, gene expression and stress responses. However, the role of miRNA in aging has not been comprehensively known.

Generally miRNA is intracellular, but studies have showed that miRNA also can be found in various extracellular fluids, including peripheral blood plasma (8) saliva (9), urine (10), breast milk (11) and culture cell supernatant (12). To date, the origin and the functional role of circulatory miRNA (c-miRNA) are still incompletely known. Of significance, concentration of extracellular miRNA in certain body fluids correlates strongly with various pathological conditions including cancer, diabetes and tissue injury.(13,10) This has led to the development of prognostic and diagnostic tests based on the c-miRNA quantification with advantages that include minimally invasive sampling, high stability, easy and fast quantification of miRNA with quantitative polymerase chain reaction (qPCR) devices. (14,15) The use of miRNA quantification as a biological marker is not limited to pathological conditions, but it has extended to physiological condition such as ageing process.

There have been at least 7 studies, including 2 studies that used either plasma or serum, trying to identify c-miRNAs that differentially expressed in young and old age groups. (15-21) However, there was no common miRNA that had been validated by two or more independent studies. One common thing shared amongst the 7 studies was that all the 7 studies recruited donor subjects of unrelated individuals to make the group of young and the group of old. The genetic heterogeneity amongst the subjects might underlie the failure in identifying certain miRNAs that consistently shows the same pattern of differential expression across the studies. Hence, this study used a unique and stringent approach *i.e.*, profiling the c-mRNAs expression in subjects that represent young age and old age groups within same families, grandson and grandfather, aiming to identify specific c-miRNAs that differentially express in the different age groups.

Methods

Sampling of Blood Plasma from Donors

The inclusion criteria of donor sample were: 17-25 years old for the younger age group and >65 years old for the older age group, male descendant of Java-Indonesia, not smoking, not consuming alcohol, generally healthy (not being sick and/or under long medications for various diseases such as diabetes, heart failure, kidney failure, cancer, abnormal liver function) as evidenced by clinical laboratory test, and are willing to participate in the study. Additional inclusion criteria for the 4 subjects (2 pairs of old and young individuals) for miRNA profiling, were that each pair were grandfather and grandson from the same family.

This study was approved by the Ethics Committee in the School of Medicine and Health Sciences, Universitas Katolik Indonesia Atma Jaya (No. 12/08/KEP-FKUAJ/2018). Before the sample collection, all the research subjects were given some explanation about the study before signing the informed consent.

Ten mL of peripheral blood was taken and collected in tubes containing ethylenediaminetetraacetic acid (EDTA)

as anti-coagulant. The blood sample was immediately centrifuged, then the plasma was stored at -80ºC until further analysis.

RNA Extraction

RNA extraction was performed by Exiqon (Vedbaek, Denmark). Total RNA from blood plasma was isolated using miRCURY™ RNA Isolation Kit-Biofluids (Exiqon), following the instruction from the kit manufactory. RNA and DNA spike-in were used to ensure the quality of the RNA extraction, real time and qPCR steps. Three types of RNA spike-ins were used. First was the RNA isolation control: UniSp2, UniSp4, UniSp5 (were added to the purification to detect any differences in extraction efficiency). Second was the cDNA synthesis control: UniSp6 (was added in the reverse transcription reaction giving the opportunity to evaluate the RT reaction). And lastly was DNA spike-in (UniSp3) is present on all panels. The DNA spike-in consists of a premixed combination of DNA template and primers. Deviations in this reaction indicated inhibitions at the qPCR level.

Profiling of c-miRNA Using the Real-Time qPCR Method

The c-miRNAs' expression profile from two pairs, of samples from 2 families were analyzed. miRNA profiling using the Real Time qPCR miRNA method and the circulatory DNA formation by reverse transcriptase using miRCURY LNA™ Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit according to the protocol for miRCURY LNA™ Universal RT PCR miRNA (Exiqon). Each miRNA was tested in triplicates with real time qPCR on the miRNA Ready-to-Use PCR, the Human panel I + II ExiLENT SYBR® Green master mix that covers 752 miRNA. Amplification was carried out in Light Cycler® 480 Real-Time PCR System (Roche, Mannheim, Germany) in a plate of 384 wells. The amplification curve was analysed using Roche LC software to determine both the Cq (with the second method derivative) and the melting curve. To monitor haemolysis two miRNAs are used, one that is expressed in red blood cells (miRNA-451) and one that is relatively stable in serum and plasma and not affected by haemolysis (miRNA-23a). Samples with ratios above 7.0 have an increased risk of being affected by haemolysis. (22) Samples with lower ratios are generally not affected by haemolysis. A ratio of 7.0 has been experimentally validated only for humans.

For normalization of the data, the average of the assays detected in all samples (n=4 samples) was used as this was found to be the most stable normalizer. The RNA extraction and miRNA profiling were done by Exiqon.

Selection of c-miRNA Candidates and Validation Using the Real Time qPCR

The results of the c- miRNAs' expression profiling in young group and old group, for each family were listed as a table in order from the top to bottom as from the largest to the smallest fold change of their differential expression. The miRNAs from the profiling data were listed in a table with the order from the largest difference of absolute ΔΔCq at the top, to the smallest of absolute ΔΔcq at the bottom. The first top 5 miRNAs that consistently appeared in both miRNA lists from the 2 families were handpicked to be miRNA candidates for further validation in samples from a group of elderly men and samples from a group of younger men using real time qPCR. The validation of the selected miRNAs expression was carried out using the mSMRT-qPCR miRNA method patented by miRXES. RNA, U6 Small Nuclear (RNU6) and hsa-miR-486-5p were used as reference genes. The data of the validation output were analysed using the GenEx Professional 5.0 program (MultiD Analyses AB, Gothenburg, Sweden) and t-test was performed for the statistical analysis.

Prediction of miRNA Targets and Enrichment Pathway Signaling

The three public available database use for this analysis, there are: miRTarBase 7.0 (*http://mirtarbase.mbc.nctu.edu.tw/php/index.php*), miRTargetLink Human (*https://ccb-web.cs.uni-saarland.de/mirtargetlink/*), and GeneCards® Human Gene Database (*https://www.genecards.org/*) to predict target genes and signalling pathways that are affected by selected miRNA candidates.

Results

Study Participants and Biological Samples

The recruitment criteria stringency had limited this study in recruiting larger number of participants within the designated time frame. Two pairs of blood plasma samples, each pair were donated by a biologically related grandfather and a grandson within a family, were obtained for miRNA expression profiling. Additionally, 9 samples from elderly men (average age=70.5 year old; SD=5.57) and 11 samples from young men (average age=18.5 year old; SD=1.97), none are biologically related, were obtained for the miRNA expression validation.

The Profile of Differentially Expressed c-miRNA between Young and Old Individuals

The qPCR profiling of miRNA expression in 4 samples identified at least 126 miRNAs in each sample, with an average of 189 miRNAs per samples. The steady level of spike in assays indicated that extraction, reverse transcription and qPCR were successful. The steady level of the RNA spike-ins, which was comparable to the blank purification, also shows that none of the samples contain inhibitors (Figure 1A). The haemolysis assessment of the samples showed no sign of red blood cell contamination (Figure 1B). The profiling results were presented to show the differential expression between the young individual and an elderly individual. Shown in Table 1 was the list of top 90 differentially expressed miRNA in family 1 with the lowest cut of absolute ΔΔcq at least 1.6, with the order from the largest difference of absolute ΔΔcq at the top, to the smallest of absolute ΔΔcq at the bottom. The table listed, from top to bottom, from the most to the least differentially miRNAs expressed between the younger and elder individual in family 1. Forty-three miRNAs were upregulated while 47 miRNA are downregulated in the grandfather when compared to those of the grandson.

Data from both families showed high similarity by sharing 18 % of total miRNAs in the top 90. Figure 2 showed the heatmap presentation of the top 50 from the list to show the contrasting differential expression between the young and old individuals in family 1.

The Selected c-miRNA Candidates and The Validated c-miRNA That Differentially Expressed in Young and Old Individuals

Based on the profiling data from 2 families, 5 miRNAs, namely hsa-miR26a -5p, hsa-miR-214-3p, hsa-miR-376c-3p, hsa-miR345-5p, and hsa-miR-574-3p, were selected for further validation using qPCR in 20 samples, 9 of elderly and 11 of young men. Out of 5 candidates, hsa-miR-376c-3p was validated to be upregulated in the elderly group (average of Δcq=1.83; SD=2.02) when compared to the young men group (average Δcq=0.012 ; SD=1.09). The expression of hsa-miR-376c-3p in older me group is 3.5 times more than that their younger counterpart with significant value of 0.01 (Figure 3).

Prediction of miRNA Targets and Enrichment Pathway Signalling

Analysis using miRTarBase 7.0 showed 7 genes as direct targets of miR-376c-3p evidently confirmed by either reporter gene assay, qPCR, or Western Blot, or their combination (Table 2). The 7 genes were Activin A

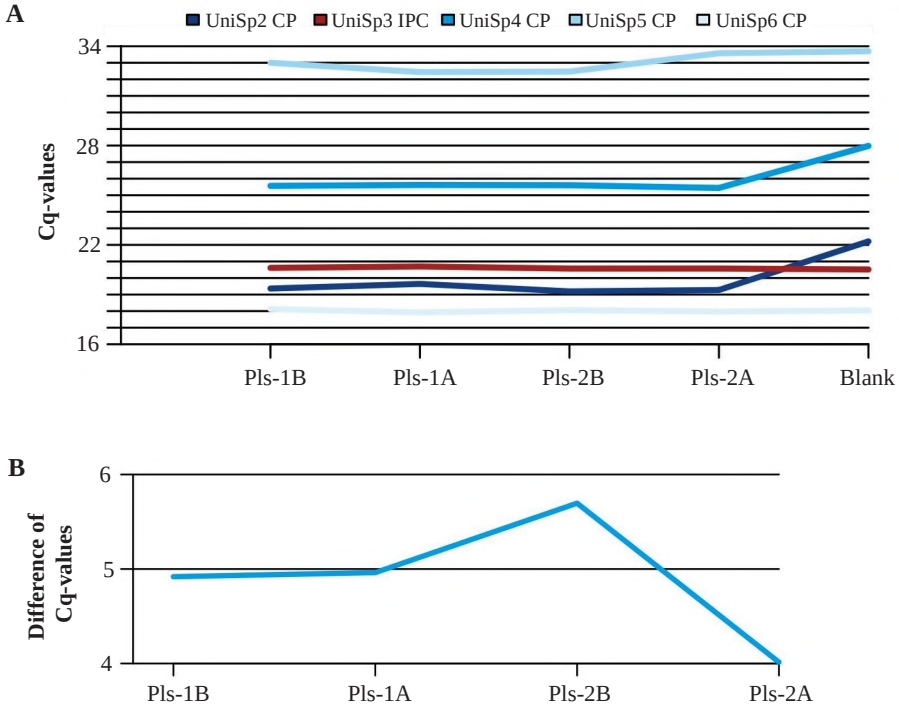

Figure 1. Spike-in raw Cq-values and the difference of Cq-values. A: The raw Cq-values obtained from the control assays. B: The difference of Cq-values between hsa-miR-23a-3p and hsa-miR-451a (ΔCq 23a-451) ratio from the samples. The hemolysis assessment of the samples in this study showed no sign of red blood cell contamination. Pls-1A: elder man from family 1; Pls-1B: young man from family 1; Pls 2A: elder man from family 2; Pls-2B: younger man rom family 2.

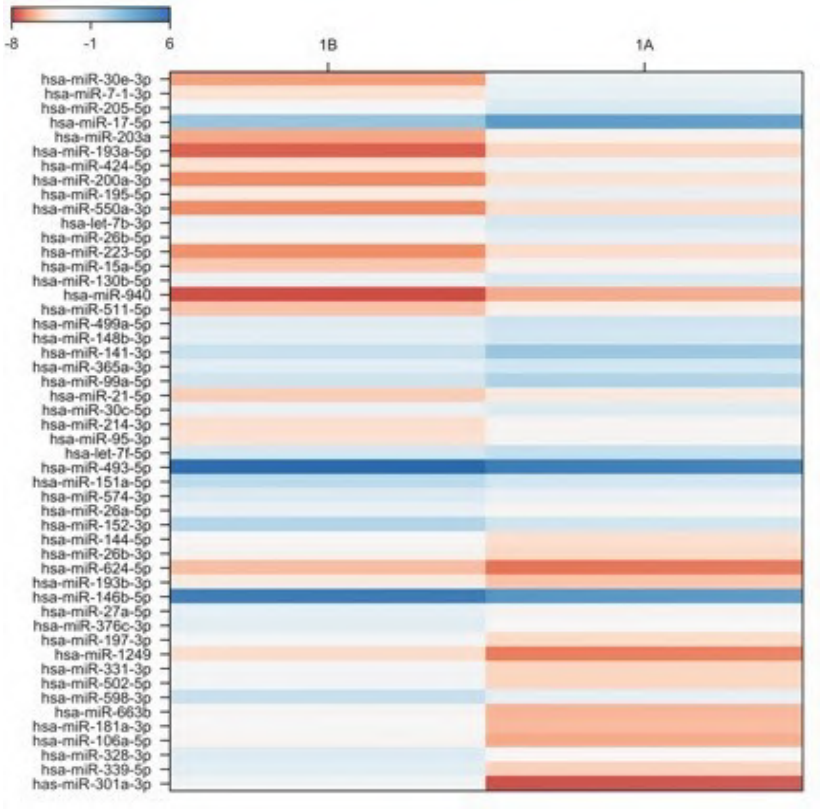

Figure 2. Heatmap presentation of the top 50 c-miRNAs, contrasting comparison between c-miRNAs expression grandfather (A) and grandson (B) and in family 1. The strength of expression can be seen from the intensity of the colour, the weak expression is shown in dark red and increases to pink, bluish white, light blue and the strongest expression is shown in dark blue. Twenty-seven (54%) miRNAs are upregulated and 23 (46%) miRNAs are downregulated in the grandfather when compares to those of the grandson.

Receptor Type 1C (ACVR1C), Growth Factor Receptor Bound Protein 2 (GRB2), Insulin Like Growth Factor 1 Receptor (IGF1R), Transforming Beta Receptor Growth Factor 1 (TGFBR1), Transforming Growth Factor Alpha (TGFA), BMI1 Proto-Oncogene (BMI1), and Run Related Transcription Factor 2 (RUNX2). Signalling pathways that were affected include insulin, mTOR, apoptosis, autophagy, and cellular senescence.

Discussion

C-miRNA has initially been studied for its biological property as a marker of the presence and/or progressive of diseases such as cancer, Alzheimer Disease, neurodegenerative diseases, diabetic and other ageing-associated diseases. Recently the research on miRNA in ageing has developed into deeper area including to explore the usefulness of miRNA as biomarker that can shed light on the biological process of ageing, to predict whether someone will likely lead a healthy long life or, in opposite, soon will develop age related diseases leading to the death (23), and even to predict one's chronological age, similar to the property owned by DNA methylation (24).

So far, there were 10 studies on c-miRNA in relevance with ageing that used different specimens such as whole blood, peripheral blood mononuclear cells (PBMC), plasma or serum.(14-21,23,24) Of note, 7 out of the 10 studies (15-21), all aimed to identify differentially expressed miRNA in different age groups, yielded no corroborating results, or in other words there was no overlapping result amongst the 7 studies. Some of the studies even presented confusing results, for instance one study reported miR-376 to be upregulated in older people (25) while another study reported miR376 to be downregulated in older people (23). Only the latest 3 studies (14,23,24), showed 3 miRNAs that appeared in more than one studies, namely miR-24, miR340-3p and miR-130b-5p. Amongst those 3 miRNAs only miR340 that appeared in 4 studies (14,16,23,24). Such random results may be partly due to the use of blood, which in fact contains various elements.(24)

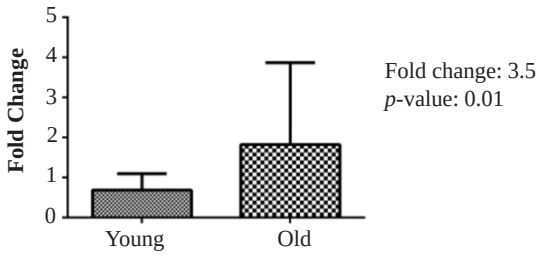

Figure 3. Result of the miR376c-3p validation across 20 individuals shown in expression profile.

Table 1. Top 90 differentially expressed miRNA between grandfather (A) and grandson (B), data of profiling in Family 1.

No.	miRNA Name	1B Average ∆cq	1A Average ∆cq	1A-1B ∆∆cq	Fold Change $2^{-\Delta\Delta cq}$	Absolute ∆∆cq
1	hsa-miR-30e-3p	-3.035	-7.305	-4.27	0.052	4.27
2	hsa-miR-301a-3p	-6.181	-2.763	3.418	10.69	3.418
3	hsa-miR-7-1-3p	-1.917	-5.249	-3.332	0.099	3.332
4	hsa-miR-339-5p	-4.623	-2.318	2.306	4.944	2.306
5	hsa-miR-328-3p	-3.406	-1.230	2.176	4.52	2.176
6	hsa-miR-205-5p	-1.430	-3.605	-2.175	0.221	2.175
7	hsa-miR-17-5P	-3.851	-5.993	-2.143	0.226	2.143
8	hsa-miR-106a-5p	1.213	3.318	2.104	4.3	2.104
9	hsa-miR-203a	-3.642	-5.695	-2.053	0.241	2.053
10	hsa-miR-181a-3p	-5.969	-3.94	2.03	4.083	2.03
11	hsa-miR-193a-5p	-3.756	-5.754	-1.998	0.25	1.998
12	hsa-miR-424-5p	-0.165	-2.122	-1.957	0.258	1.957
13	hsa-miR-200a-3p	-3.181	-5.124	-1.943	0.26	1.946
14	hsa-miR-663b	-7.098	-5.171	1.928	3.804	1.928
15	hsa-miR-195-5p	-3.304	-5.159	-1855	0.276	1.855
16	hsa-miR-598-3p	-4.629	-2.786	1.843	3.587	1.843
17	hsa-miR-550a-3p	-4.811	-6.613	-1.802	0.287	1.802
18	hsa-miR-502-5p	-6.422	-4.658	1.764	3.396	1.764
19	hsa-miR-331-3p	-5.166	-2.412	1.754	3.372	1.754
20	hsa-let-7b-3p	-3.220	-4.943	-1.724	0.303	1.724
21	hsa-miR-1249	-6.445	-4.867	1.578	2.986	1.578
22	hsa-miR-197-3p	-2.631	-1.071	1.56	2.948	1.56
23	hsa-miR-26b-5p	-1.972	-3.531	-1.558	0.34	1.558
24	hsa-miR-223-5p	-2.351	-3.887	-1.536	0.345	1.536
25	hsa-miR-376c-3p	-3.791	-2.29	1.501	2.83	1.501
26	hsa-miR-27a-5p	-6.376	-4.88	1.497	2.822	1.497
27	hsa-miR-146b-5p	-5.369	-3.882	1.486	2.802	1.486
28	hsa-miR-193b-3p	-2.756	-1.332	1.424	2.684	1.424
29	hsa-miR-624-5p	-7.268	-5.852	1.416	2.669	1.416
30	hsa-miR-26b-3p	-5.460	-4.057	1.403	2.645	1.403
31	hsa-miR-144-5p	-1.773	-0.435	1.338	2.529	1.338
32	hsa-miR-152-p	-2.244	-0.915	1.329	2.512	1.329
33	hsa-miR-26a-5p	-0.293	1.024	1.317	2.491	1.317
34	hsa-miR-15a-5p	4.807	3.535	-1.272	0.414	1.272
35	hsa-miR-547-3p	-2.018	-0.763	1.255	2.387	1.255
36	hsa-miR-130b-5p	-4.226	-5.466	-1.24	0.423	1.24
37	hsa-miR-940	-5.561	-6.772	-1.211	0.432	1.211
38	hsa-miR-511-5p	-3.845	-5.017	-1.173	0.444	1.173
39	hsa-miR-499a-5p	-3.627	-4.778	-1.151	0.45	1.151
40	hsa-miR-148b-3p	0.368	-0.767	-1.135	0.455	1.135
41	ha-miR-141-3p	-2.601	-3.727	-1.126	0.458	1.126
42	hsa-miR-151a-5p	-0.646	0.477	1.123	2.179	1.123
43	hsa-miR-365a-3p	-1.519	-2.62	-1.101	0.466	1.101
44	hsa-miR-493-5p	-5.272	-4.209	1.063	2.089	1.063
45	hsa-let-7f-5p	-2.649	-1.633	1.016	2.022	1.016

No.	miRNA Name	1B Average ∆cq	1A Average ∆cq	1A-1B ΔΔcq	Fold Change $2^{-\Delta\Delta cq}$	Absolute ΔΔcq
46	hsa-miR-99a-5p	0.074	-0.924	-0.998	0.501	0.998
47	hsa-miR-95-3p	-4.778	-3.789	0.989	1.985	0.989
48	hsa-miR-214-3p	-4.667	-3.704	0.963	1.949	0.963
49	hsa-miR-21-5p	5.607	4.651	-0.956	0.515	0.956
50	hsa-miR-30c-5p	-1.068	-0.12	0.948	1.929	0.948
51	hsa-miR-20b-5p	-5.205	-6.153	-0.948	0.518	0.948
52	hsa-miR-223-3p	5.068	6.014	0.945	1.926	0.945
53	hsa-miR-32-5p	0.68	-0.247	-0.928	0.526	0.928
54	hsa-miR-30a-5p	-2.333	-3.247	-0.915	0.531	0.915
55	hsa-miR-7g-5p	1.128	2.043	0.914	1.885	0.914
56	hsa-miR-132-3p	-2.364	-3.266	-0.902	0.535	0.902
57	hsa-miR-200c-3p	-3.152	4.051	-0.899	0.536	0.899
58	hsa-miR-199a-5p	-1.728	-0.843	0.885	1.847	0.885
59	hsa-miR-874-3p	-2.289	-3.164	-0.875	0.545	0.875
60	hsa-miR-103a-3p	1.397	2.26	0.863	1.819	0.863
61	hsa-miR-148a-3p	-0.398	-1.258	-0.86	0.551	0.86
62	hsa-miR-1260a	-1.78	-2.626	-0.846	0.556	0.846
63	hsa-miR-125a-5p	-0.348	-1.193	-845	0.557	0.845
64	hsa-miR-10a-5p	-6.379	-7.219	-0.84	0.559	0.84
65	hsa-miR-660-5p	1.182	0.359	-0.824	0.565	0.824
66	hsa-miR-139-5p	-1.423	-2.246	-0.823	0.565	0.823
67	hsa-miR-29c-3p	1.717	0.898	-0.819	0.567	0.819
68	hsa-miR-133a-3p	-2.2	-3.008	-0.809	0.571	0.809
69	hsa-miR-221-3p	0.432	1.235	0.803	1.744	0.803
70	hsa-miR-18b-5p	-1.381	-0.587	0.794	1.734	0.794
71	hsa-miR-423-5p	0.505	-0.286	-0.79	0.578	0.79
72	hsa-miR-20a-5p	3.328	4.111	0.783	1.72	0.783
73	hsa-miR-766-3p	-3.784	-3.002	0.782	1.719	0.782
74	hsa-miR-362-5p	-7079	-6.302	0.777	1.714	0.777
75	hsa-miR-33b-5p	-5.835	-5.076	0.759	1.693	0.759
76	hsa-miR-128-3p	-3.818	-3.066	0.752	1.684	0.752
77	hsa-miR-185-5p	3.331	4.069	0.738	1.668	0.738
78	hsa-miR-194-5p	-0.563	-1.3	-0.736	0.6	0.736
79	hsa-miR-215-5p	-0.919	-0.183	0.735	1.665	0.735
80	hsa-miR-33a-5p	-4.623	-5.344	-0.721	0.607	0.721
81	hsa-miR-199a-3p	-0.167	0.553	0.72	1.647	0.72
82	hsa-miR-210-3p	-0.527	-1.242	-0.715	0.609	0.715
83	hsa-miR-7c-5p	-2.119	-2.829	-0.71	0.611	0.71
84	hsa-miR-362-3p	-1.777	-2.486	-0.709	0.612	0.709
85	hsa-miR-345-5p	-0.582	0.102	0.684	1.607	0.684
86	hsa-miR-150-5p	1.785	2.463	0.678	1.6	0.678
87	hsa-miR-144-3p	4.095	3.422	-0.673	0.627	0.673
88	hsa-miR-425-3p	-2.642	-3.312	-0.67	0.628	0.67
89	hsa-miR-122-5p	1.733	1.081	-0.653	0.636	0.653
90	hsa-miR-374b-5p	-2.499	-1.849	0.65	1.569	0.65

Table 2. List of the genes that have methodologically been proven as strong targets of hsa-miR-376c-3p (miRTarBase 7.0).

ID	Target	Description	Strong Evidence			Less Strong Evidence				Sum	Number of Papers
			Reporter Assay	Western Blot	qPCR	Micro-array	NGS	pSILAC	Other		
MIRT005775	ACVR1C	Activin A receptor type 1C	√	√	√					3	2
MIRT006964	IGF1R	Insulin like growth factor 1 receptor	√	√	√					3	1
MIRT053350	GRB2	Growth factor receptor bound protein 2	√	√	√					3	1
MIRT245244	BMI1	BMI1 proto-oncogene, polycomb ring finger	√	√	√		√			4	2
MIRT437849	TGFBR1	Transforming growth factor beta receptor 1	√	√	√					3	1
MIRT437890	TGFA	Transforming growth factor alpha	√	√						2	1
MIRT735369	RUNX2	Runt related transcription factor 2	√	√	√	√				4	1

Other possible factors contributing to the non-corroborating results are (i) non stringent criteria for subject recruitment, *e.g.*, inclusion subjects with medical condition or under medication, (ii) difference in age grouping or cut off of young age - old age, (iii) different technology platform used to measure miRNA expression, (iv) different way of measurement, e.g. a study that pooled RNA from multiple subjects to be run sequencing as one sample.(14)

Because of all the above, aiming to obtain more reliable data, this reported study set unique criteria for the subjects whom the specimens would be collected from. This includes (i) subjects of being biological grandfather and grandson from whom plasma specimens were collected for miRNA expression profiling. The comparison analysis was performed separately for each family. All the above to avoid any bias caused by interfamily genetic heterogeneity, (ii) Very stringent inclusion and exclusion criteria for subjects whom plasma specimens were collected for validation so that certain pathological conditions, as well as external condition such as cytotoxic medication, long medication, and smoking, would not add noise to the data. The rather small number of the biological samples is indeed a limitation of this study.

However, the study was able to show convincing results both in miRNA expression profiling, and reaffirmed in the validation. Hence, this study demonstrates a rational approach, particularly by the efforts to minimize factors other than the age that may compromise the nature of the miRNA expression.

The miRNA expression profiling in this study showed interesting results. Among the top 90 list we identified a group of miRNAs where the regulation are in accordance with previous studies, and a group of miRNAs where the regulation are in contradictory with the previous studies. In the first group, we identified the downregulation of miR-21-5p that previously was reported as potential biomarker for trajectory ageing (23), as well as upregulation of miR30c-5p, miR-103a-3p, and downregulation of miR-17-5p that previously reported as part of important miRNAs that together could reverse ageing markers in mice (26). In the second group, we identified the downregulation of miR-181a-5p, miR-106a-5p, and upregulation of miR30a-5p that have previously been reported as the opposite in some previous studies.(15,21,27)

The qPCR validation on the samples from unrelated individuals clearly validated hsa-miR-376c-3p to be upregulated amongst elderly Javanese male compared to their younger counterparts. As far as we are aware of, this is the first time for hsa-miR-376c-3p being identified to be c-miRNA upregulated in the elder person. Previously there was one study showed the upregulation of hsa-miR-376 in older persons when compared to younger persons (25), however it was not specifically identified whether the miRNA was produced from which genetic locus and whether it was from 5' or 3' arm. Another study, identified hsa-miR-376c-3p, amongst other miRNAs, to be downregulated in persons who likely to live a longer healthy life compared to those who lived shorter life.(23) However, we can't directly compare our study with that study as it compared the serum hsa-miR-376c-3p expression of persons at their age of 50 year old, 55 year old, and 60 year old.(22) In our study the elderly group participants were males of 65 year old and older, while the younger group were males of age 17 to 25 year old.

The miR-376c-3p has been studied quite widely in various cancer (28-31), there have not been much study on the role of hsa-miR-376c-3p in physiologic ageing. Bioinformatic analysis on the hsa-miR-376c-3p and its strong targets, including Cyclin D1 (CCND1) that just recently demonstrated (29), showed affected signalling pathways such as insulin, mTOR, apoptosis, autophagy, and cellular senescence. These suggest that hsa-miR-376c-3p may play role in cellular ageing.

Conclusion

This study showed that, for the first time, the upregulation of hsa-miR-376c-3p in the circulating plasma of the elderly Javanese males when compared to their younger counterparts. This finding, together with the accumulating knowledge on its direct target genes, warrant further study to elucidate the specific roles of hsa-miR-376c-3p in physiological ageing mechanism.

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