

Profile of microRNAs associated with aging in rat liver

SHIMA MIMURA¹, HISAKAZU IWAMA², KIYOHITO KATO¹, KEI NOMURA¹, MITSUYOSHI KOBAYASHI¹, HIROHITO YONEYAMA¹, HISAAKI MIYOSHI¹, JOJI TANI¹, ASAHIRO MORISHITA¹, TAKASHI HIMOTO¹, AKIHIRO DEGUCHI¹, TAKAKO NOMURA¹, TEPPEI SAKAMOTO¹, KOJI FUJITA¹, EMIKO MAEDA¹, KUNIHIKO IZUISHI³, KEIICHI OKANO², YASUYUKI SUZUKI² and TSUTOMU MASAKI¹

Departments of ¹Gastroenterology and Neurology, ²Signal Transduction Sciences, and ³Gastroenterological Surgery, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan

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Abstract. Recent studies suggest that small non-coding microRNAs (miRNAs or miRs) play an important role in the regulation of genes involved in various cellular and developmental processes. However, the expression of miRNAs during the aging process remains largely unknown. The aim of the present study was to analyze miRNA expression profiles in rat livers during the aging process. The livers of male Wistar rats at different stages of development (fetal, aged 3 days, and 1, 2, 4, 8 and 36 weeks of age) were used. Total RNA was extracted from the livers. We analyzed the expression levels of 679 rat miRNA probes. In addition, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed. Several up- and downregulated miRNAs were identified in the rat livers at 7 different fetal developmental stages and at 36 weeks of age. We observed the upregulation of miR-29a, miR-29c, miR-195 and miR-497, whereas miR-301a, miR-148b-3p, miR-7a, miR-93, miR-106b, miR-185, miR-450a, miR-539 and miR-301b were downregulated in the aging rat livers. The number of PCNA-positive hepatocytes was decreased with age. In conclusion, our findings suggest that these up- and downregulated miRNAs play an important role in aging by regulating cell cycles that are involved in liver senescence. Further investigation is required to reveal additional target genes of the miRNAs expressed in the liver and the roles of miRNAs in the developmental process of aging in the liver.

E-mail: shimamimura@hotmail.com

Key words: microRNA, fetal liver, cell cycle, aging

Introduction

MicroRNAs (miRNAs or miRs) are small non-coding RNAs that regulate both the mRNA and the protein expression of target genes (1). miRNAs play a crucial role in the regulation of genes involved in the control of development, cell proliferation, apoptosis and stress response (2). The expression profile analysis of miRNAs is essential for understanding the complex regulation of gene expression that involves miRNAs in addition to characterizing miRNAs themselves (3). Some miRNAs exhibit tissue-specific expression (4). The liver is a crucial organ in which miRNAs may be involved in the regulation of hepatocyte growth and development. The expression profiles of miRNAs in fetal liver are different from those in the adult liver; fetal miRNA expression has shown specificity in the developmental stage (5). Global gene and miRNA expression in embryonic and adult human livers has been analyzed, revealing multiple regulated genes and demonstrating a change in the expression patterns during the developmental process (6). A number of studies have focused on accelerated aging and miRNAs, such as miRNAs in the aging mouse brain (3,7). Previous studies have analyzed the expression of miRNAs in the livers of young and elderly humans (6) and rats (8), as well as in mice with delayed aging (9,10). However, the association between miRNA expression and the aging of the liver remains poorly understood (11).

In the present study, we identified a gradual up- and downregulation of miRNAs in rats at 7 different developmental stages (fetal to 36 weeks old). We found that miR-29a, miR-29c, miR-195 and miR-497 were gradually upregulated and that miR-301a, miR-148b-3p, miR-7a, miR-93, miR-106b, miR-185, miR-450a, miR-539 and miR-301b were gradually downregulated in livers as the rats aged.

Materials and methods

Animals. Inbred Wistar rats were bred under specific pathogen-free conditions at the Institute for Animal Experimentation of the Kagawa University School of Medicine Kagawa, Japan. Eight male Wistar rats from the ages of postnatal day 3, and post-natal week 1, 2, 4, 8 and 36 were used in this study. Eight fetal livers were also obtained from pregnant

Correspondence to: Dr Shima Mimura, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Abbreviations: miRNA, microRNA; PCNA, proliferating cell nuclear antigen; ABC, avidin-biotin complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HGF, hepatocyte growth factor

rats at day 20. We performed hepatectomy under ether anesthesia. For immunohistochemistry, small sections of the liver tissue were preserved in 10% formalin. The remaining sections of the liver tissue were rapidly placed in liquid nitrogen, transferred individually to pre-weighed tubes containing RNAlater (Ambion, Tokyo, Japan) and stored at -80°C until analysis.

RNA isolation. The frozen tissue samples were thawed on ice and dissolved in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was extracted from the tissues using an miRNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 700 μ l of TRIzol reagent containing samples was mixed vigorously with 140 μ l of chloroform, incubated at room temperature for 3 min, followed by centrifugation at 12,000 x g for 15 min at 4°C. The upper aqueous phase was transferred to another tube containing 525 μ l of 100% ethanol. The mixture was loaded into RNeasy Mini columns, followed by serial washing with solutions provided with the kit. Finally, RNA was collected into RNase-free water for further experiments. All RNA samples used in this study showed A260/280 ratios between 2.0 and 2.1. The integrity of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). The quality of total RNA was determined using the RNA Nano 6000 chips on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Osaka, Japan), and all RNA samples used for microarray analyses had RIN values >8.2. These RNA samples were stored at -80°C.

miRNA arrays. Total RNA was labeled with Hy3 dye using the array labeling kit (Exiqon, Vedbæk, Denmark). Total RNA $(2 \mu g)$ was incubated with a spike for 30 min at 37°C and then at 95°C for 5 min. Hy3 dye and labeling enzyme were added to the samples. The enzyme was then heat-inactivated at 16°C for 1 h and 65°C for 15 min, protected from light and then received an addition of hybridization buffer. The sample was loaded onto the arrays by capillary force using 3D-Gene miRNA oligo chips (version 16; Toray Industries, Inc., Kanagawa, Japan). The chips enabled the examination of the expression of 679 miRNAs printed in duplicate spots. The arrays were incubated at 32°C for 16 h, then briefly washed in a 30°C wash buffer solution (0.5X SSC, 0.1% SDS), rinsed in wash buffer solution (0.2X SSC, 0.1% SDS) followed by a wash in another buffer solution (0.05X SSC), according to the manufacturer's instructions (Toray Industries, Inc.). The arrays were spun for 1 min at 1,000 rpm for drying, followed by immediate scanning using a Toray 3D-Gene 3000 miRNA microarray scanner (Toray Industries, Inc.). The relative expression level of each miRNA was calculated by comparing the average signal intensities of the valid spots with their mean value throughout the microarray experiments following normalization to their adjusted median values.

Heatmap. To illustrate the alterations in the expression levels of the 13 upregulated or downregulated miRNAs, we created a heatmap in which each cell represents the expression level of each of the 13 miRNAs for each of 8 individual rats at different stages of development, namely the fetal period, at post-natal day 3 and post-natal week 1, 2, 4, 8 and 36. The heatmap was color-coded according to the log2-transformed

expression level. The center level of the color code is set as the median value over all of the values used in the heatmap. Briefly, in the heatmap, white color represents mean values, red indicates gains and blue represents losses.

Immunohistochemistry. The avidin-biotin complex (ABC) immunohistochemical method was used. Following formalin fixation, the tissues were dehydrated in a graded series of ethanol and embedded in paraffin. The serial sections were mounted onto glass slides. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed using the following procedure: the sections were deparaffinized, hydrated and quenched for endogenous peroxidase with 0.3% hydrogen peroxide in PBS at room temperature for 30 min. The sections were permeabilized in PBS with 0.3% polyoxyethylene (10) octyl phenyl ether (Triton X-100) (Wako, Osaka, Japan) for 1 h before quenching the endogenous peroxidase activity. The sections were then incubated overnight at room temperature with a mouse anti-PCNA antibody (1:100 dilution; Dako, Glostrup, Denmark). Antibody binding was detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine as a chromogen. After staining, all sections were counterstained with Mayer's hematoxylin. The specificity of immunostaining was examined using non-immune mouse IgG as a negative control for the primary antibody. Images were captured using an Olympus BX51 microscope and Olympus DP72 camera (Olympus, Tokyo, Japan). The nuclear labeling index for the PCNA-positive cells (positive nuclei/total counted) was determined by evaluating at least 500 hepatocytes at random in the microscopic field by 2 observers (T. Masaki and S. Mimura).

Western blot analysis. Frozen liver tissue samples were homogenized in a protein extraction solution (PRO-PERP™; Intron Biotechnology, Inc., Gyeonggi, Korea), and cell lysis was then induced by incubating the samples for 20 min on ice. The samples were prepared by centrifugation at 13,000 rpm for 5 min at 4°C. The supernatant was then collected. The protein concentration was measured by a dye-binding protein assay performed according to the Bradford method (12). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (13), and western blot analysis was performed as previously described by Towbin et al (14) using anti-\beta-actin monoclonal antibody (Sigma-Aldrich; A5442, used at 1:3,000) and cyclin D1 (Thermo Fisher Scientific, Waltham, MA, USA; RB-9041, used at 1:1,000) as primary antibodies and horseradish peroxidase-inked anti-mouse and anti-rabbit IgG secondary antibodies (GE Healthcare UK, Buckinghamshire, UK; used at 1:2,000).

Results

Quality assessment of the total RNA of the sample using a miRNA array chip. Total RNA (20 μ l) from all liver tissues used in this study was loaded into each lane, and the bands of 18S and 28S ribosomal RNA in the gel were detected using the 2100 Bioanalyzer (Fig. 1). Based on these results, RNA samples extracted from various liver tissues were shown to be of adequate quality.





Figure 1. Quality of total RNA of developmental liver tissues using microRNA (miRNA) array chip. The bands of 18S and 28S ribosomal RNA in the gel were detected using a 2100 Bioanalyzer. Total RNA in all liver tissue samples used in the miRNA chip analysis was of high quality. Equal amounts of total RNA obtained from developmental liver tissues were used for miRNA analysis.



Figure 2. MicroRNA (miRNA) expression in rat livers at 7 different stages of development (fetal, post-natal day 3 and post-natal weeks 1, 2, 4, 8 and 36) in miRNA chips. Spot numbers 1-4 (1, miR-29a; 2, miR-29c; 3, miR-195; 4, miR-497) were miRNAs upregulated with liver development. Spot numbers 5-13 (5, miR-301a; 6, miR-148b-3p; 7, miR-7a; 8, miR-93; 9, miR-106b; 10, miR-185; 11, miR-450a; 12, miR-539; 13, miR-301b) were miRNAs downregulated with liver development.

Developmental study of miRNA expression in liver tissue. Using a custom microarray platform, we analyzed the expression levels of 679 rat miRNA probes. Four miRNAs were found to be gradually upregulated with age in the rat livers. The 4 upregulated miRNAs were miR-29a (spot no. 1), miR-29c (spot no. 2), miR-195 (spot no. 3) and miR-497 (spot no. 4) (Figs. 2 and 3). By contrast, 9 miRNAs were gradually down-regulated. These downregulated miRNAs were miR-301a (spot no. 5), miR-148b-3p (spot no. 6), miR-7a (spot no. 7), miR-93 (spot no. 8), miR-106b (spot no. 9), miR-185 (spot no. 10),



Figure 3. MicroRNA (miRNA) expression analysis. (A) Upregulated miRNAs. Fold change levels are reported relative to the levels of the fetal liver. miR-29a, miR-29c, miR-195 and miR-497 upregulation was confirmed. A significant (p<0.01) upregulation was found in the livers of post-natal week 4 and 8 rats for each upregulated miRNA. (B) Downregulated miRNAs. The expression levels of miR-301a and 301b were downregulated by more than half at post-natal day 3.

miR-450a (spot no. 11), miR-539 (spot no. 12) and miR-301b (spot no. 13). The heatmap (Fig. 4) clearly demonstrates increasing trends for 4 miRNAs and decreasing trends for 9 miRNAs in the expression levels among the age groups. It also showed that the trends in miRNA expression were fairly consistent among individual rats and were constant in both increasing and decreasing directions.

Immunohistochemical study of PCNA in developing livers. The typical immunohistochemical staining pattern of PCNA at various stages of development in the rat livers, including fetal liver (Fig. 5A), post-natal day 3 liver (Fig. 5B), post-natal week 1 liver (Fig. 5C), post-natal week 2 liver (Fig. 5D), postnatal week 4 liver (Fig. 5E), post-natal week 8 liver (Fig. 5F) and post-natal week 36 liver (Fig. 5G). The labeling index of PCNA in the hepatocytes in the developing livers decreased with age (Fig. 5H).

Western blot analysis of aging rat liver. Western blot analysis was used to examine the cell cycle of the aging rat liver by using an antibody against cyclin D1. The cyclin D1 level was hardly detectable in the fetal liver (Fig. 6). However, cyclin D1 was detected in the liver at post-natal day 3. The highest level was detected in the post-natal week 1 liver and the level slightly decreased with age. The amount of β -actin (an internal control for protein loading) was almost the same in all age groups.



Figure 4. A heatmap showing increasing and decreasing trends of the microRNA (miRNA) expression levels in rats during different stages of development. The expression level of each miRNA for each individual rat is shown for each age group as a color-coded cell. A vertical bar beside the heatmap indicates the scale of the color code of the log2-transformed expression levels. The center level of the color code is set as the median value over all of the values used in the heatmap. White color represents mean values, red indicates gains and blue represents losses.

Discussion

The dysregulation of miRNA expression may contribute to numerous alterations present in very complex and multifactorial processes, namely, proliferation, differentiation, inflammation, carcinogenesis and aging (11). The profiles of miRNAs have been reported by developmental studies in various organs, such as the brain (15-18), kidneys (19), pancreas (20) and liver (21,22). However, the alterations in miRNA expression in the rat liver during the process of aging remain poorly understood. In the present study, in order to detect miRNAs associated with aging in the rat liver, we investigated miRNAs with a gradually increasing or decreasing expression in the livers of rats at different stages of development. Such miRNAs appear to play an important role in aging. To the best of our





Figure 5. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA). The immunohistochemical staining pattern of PCNA in various stages of development in the rat livers, including (A) fetal liver, (B) post-natal day 3 liver, (C) post-natal week 1 liver, (D) post-natal week 2 liver, (E) post-natal week 4 liver, (F) post-natal week 8 liver and (G) post-natal week 36 liver. (H) The labeling index of PCNA of the hepatocytes in developing livers decreased with age.



Figure 6. Western blot analysis. The cyclin D1 level was almost undetectable in the fetal liver. However, the cyclin D1 level was detectable in the liver at post-natal day 3. It was detected at the highest level in post-natal week 1 liver, and slightly decreased with age. β -actin was used as a loading control.

knowledge, to date, no studies have established an association between miRNAs and aging of the liver in a similar manner as we have done. In the present study, we demonstrated that miR-29a, miR-29c, miR-195 and miR-497 were upregulated with age, whereas miR-301a, miR-148b-3p, miR-7a, miR-93, miR-106b, miR-185, miR-450a, miR-539 and miR-301b were downregulated with age in the rat livers. These data suggest that specific miRNAs are associated with aging in the liver.

miR-29a and miR-29c were upregulated during early development in the liver (rats at post-natal week 4 and 8) and during late development in the liver (post-natal week 36). Notably, the expression levels of miR-29a and miR-29c were >10-fold higher in the livers from the 36-week-old rats than the fetal rat livers. miR-29a and miR-29c have been shown to be age-related in various organs, such as the aorta (23,24), the lungs (25,26), the kidneys (19) and the liver in mice (21,22). The upregulation of miR-29a and miR-29c with age is consistent with a recent report showing an increased expression of miR-29 family members in a model of accelerated aging in mice (11).

Name	Genome context		Target gene (Ref.)
Upregulated			
rno-miR-29a	4: 58107760-58107847 [-]	4q22	 p53 (11), hepatocyte growth factor (31), Bcl-2 (32), nuclear autoantigenic sperm protein (33), TGF-β1 (34) p42.3 (35), Arpc3 (36), monocarboxylate transporter 1 (37), maternally expressed gene 3 (38), Tcl1 (39), DNA methyltransferase 3 (40), phosphatase and tensin homolog (41)
rno-miR-29c	13: 110968048-110968135 [+]	13q27	p53 (11), MCT-1 (37), Bcl-2 (32), TGF- β 1 (34), tumor necrosis factor α-induced protein 3 (42), β-site APP cleaving enzyme 1 (43), cyclin E (44)
rno-miR-195	10: 57074170-57074256 [+]	10q24	Cyclin-dependent kinase 4 (45), Bcl-2 (46-48), ethanol-mediated inhibition of hepatic sirtuin 1 (47), cyclin E1 (49), cyclin D1 (50,51), CDK6 (50), E2F3 (50,52) Raf-1 (52)
rno-miR-497	10: 57073846-57073914 [+]	10q24	Bcl-2 (53,54), cyclin D2 (54)
Downregulated			
rno-miR-301a	10: 75386838-75386937 [+]	10q26	Plasminogen activator inhibitor-1 (55)
rno-miR-148b-3p			AMPKα1 (56), ITGA5, ROCK1, PIK3CA/p110α, NRAS, CSF1 (57)
rno-miR-7a			Barx1 (58)
rno-miR-93	12: 17608173-17608259 [-]	12q11	Sirtuin 1 (8), S-transferase 1 (8), integrin-β8 (59), FUS1 (60), p21 (61)
rno-miR-106b	12: 17608382-17608463 [-]	12q11	p21 (61-64), RB (65)
rno-miR-185	11: 84658785-84658864 [+]	11q23	DNA methyltransferase 1 (66), Rho, Cdc42 (67), Six1 (68)
rno-miR-450a	X: 139994947-139995037 [-]	Xq36	DNA methyltransferase 3a (69)
rno-miR-539	6: 134408917-134408992 [+]	6q32	Holocarboxylase synthetase (HCS) (70,71)
rno-miR-301b	11: 85885248-85885325 [+]	11q23	TP63 (72)
miRNA, microRNA.			

Table	I.	Chromosomal	locations ar	nd target	genes of the up	p- and	downregulated	miRNAs.
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Several studies have indicated that the cellular replication capacity (proliferative activity) declines in various tissues and may compromise the immune system during aging (27-29). In addition, the number of senescent cells increases in various tissues with aging (30). Indeed, in this study, we also demonstrated that the expression of PCNA in hepatocytes decreased with age. These data suggest that the number of senescent cells in the liver increased with age.

In the present study, the 4 miRNAs with a gradually upregulated expression (miRNA-29a, miRNA29c, miRNA-195 and miR-497) possess target genes to promote proliferative activity, whereas the 4 miRNAs (miR-148-3p, miR-93, miR-106b and miR-185) whose expression was gradually reduced, possess genes to inhibit proliferative activity, such as tumor suppressor gene and the cyclin-dependent kinase inhibitor, p21WAF1 (Table I). Hepatocyte growth factor (HGF), cyclin E, Cdk4, Cdk6 and cyclin D1 play important roles in the progression of the cell cycle, and their molecules are the targets of upregulated miRNAs (Table I). By contrast, p21 (also known as p21CIP1/WAF1), sirtuin and fused in sarcoma (FUS)1 play important roles in the arrest of the cell cycle, and their molecules are targets of downregulated miRNAs (Table I). Therefore, these data suggest that numerous up- and down-regulated miRNAs play an important role in the decline of the proliferative activity of hepatocytes with age.

In conclusion, in the present study, we identified the upregulation of miR-29a, miR-29c, miR-195 and miR-497, and the downregulation of miR-301a, miR-148b-3p, miR-7a, miR-93, miR-106b, miR-185, miR-450a, miR-539 and miR-301b in the rat liver with age. Our data also suggest that important changes in miRNA expression occur during development, and one result of aging is likely to be changes in miRNA expression. Our findings suggest that these up- and downregulated miRNAs play important roles by regulating cell cycles that are related to liver senescence. Further studies are required to clarify additional miRNA targets, as well as the roles of miRNAs in the development of the complex process of aging in the liver.



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