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Original Article

Quantitative analysis of miRNA expression in several developmental stages of human livers

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Aim: miRNAs have been found to regulate gene expression at a posttranscriptional level in cells. Studies have shown that expression of miRNAs is tissue-specific and developmental stage-specific. The mechanism behind this could be explained by miRNA pathways.

Methods: We introduce the identification of miRNAs from two human fetal liver cDNA libraries by a cloning protocol. The miRNAs detected were then analyzed in a chorionic villus tissue and four liver tissues using real-time polymerase chain reaction.

Results: After sequencing and database searching, a total of 42 miRNAs in two fetal livers were detected. Quantitative analysis showed that they have higher general expression in the livers of the fetal period than the adult period, and furthermore the expression levels of the miRNAs during the fetal period were dynamically changed.

Conclusion: Our results indicate that a special group of miRNAs may play an important role in human fetal liver development, while their roles in the adult livers are limited.

Key words: miRNA, expression, development, human liver.

INTRODUCTION

THE FUNCTIONAL REPRESSION of genes can be triggered by endogenous microRNAs (miRNAs). The miRNAs are non-coding RNAs which repress translations of target mRNAs. The primary miRNAs (pri-miRNAs) are encoded by miRNA genes in the genome. These pri-miRNAs are trimmed into approximately 70 nucleotides of hairpin structures, called precursor miRNAs (pre-miRNAs), by the RNase III type protein, Drosha, in the nucleus. The pre-miRNAs are then transported to the cytoplasm by Exportin-5 and are cleaved to approximately 22 nucleotides of mature miRNAs by Dicer enzymes, another RNase type III.[1]

Recent studies show that miRNAs play an important role in the biological processes. Some miRNAs, for example let-7 and lin-4, can regulate the timing of early and late larval developmental transition in *Caenorhabditis elegans*.[2,3] In plants, some miRNAs regulate flowering, leaf development and embryonic patterning.[4–6] Furthermore, miR-14 and bantam are found to regulate apoptosis, growth and fat metabolism in *Drosophila*.[7,8]

It has been shown that the miRNAs are involved in development and differentiation of human cells.[8–12] Furthermore, miRNAs exhibit tissue-specific and developmental stage-specific expression.[13,14] The liver is a crucial human organ that has multiple functions, such as processing digested food from the intestine; controlling levels of fats, amino acids and glucose in the blood; combating infections in the body; clearing the blood of particles and infections including bacteria; neutralizing and destroying drugs and toxins; manufacturing bile; storing iron, vitamins and other essential chemicals; breaking down food and turning it into energy; manufacturing, breaking down and regulating numerous hormones including sex hormones; and making enzymes and proteins which are responsible for many chemical reactions in the body, for example those involved in blood clotting and repair of damaged tissues. It can be

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speculated that a special group of miRNAs may be involved in regulation of function and dysfunction, differentiation, growth and development of the liver organ.

Hitherto, only two articles have reported miRNA identification in human fetal and adult livers, respectively.[15,16] More work is necessary to gain an overview of expression of the miRNAs during the process of liver growth and development. Here we identified 42 miRNAs from the livers of two human fetuses of 27 and 35 weeks by a cloning method. Of these miRNAs, some were variants of those described previously. We then quantified the 42 miRNAs in a chorionic villus tissue and four liver tissues (three fetal livers at different stages of development and one adult liver) using real-time polymerase chain reaction (PCR).

METHODS

Ethics statement

THIS RESEARCH HAS been approved by the review board of Huazhong University of Science and Technology. We obtained tissue samples with informed consent in writing from the participants involved in the study. The ethics committee specifically approved the procedures.

Samples

The chorionic villus tissue was obtained from a 35-day pregnant woman in Tongji Hospital, Wuhan, Hubei, China. The liver tissues were obtained from three fetuses of 18, 27 and 35 weeks, respectively, delivered due to severe high blood pressure syndrome in the mothers, also in Tongji Hospital, Wuhan, Hubei, China. The fetuses died shortly after delivery. The mothers of the fetuses had no other diseases than high blood pressure. The adult liver tissue was obtained from a man aged 18 years, who had died due to severe injury, at the Department of Forensic Science, Tongji Medical College, Huazhong University of Science and Technology.

Isolation of small RNA

Small RNAs (≤200 nt) were isolated from the chorionic villus tissue and liver tissues using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Approximately fifty milligrams of tissue were used and the small RNA was eluted in 100 μL RNAse-free water. The RNA concentration was tested by ultraviolet absorbance at 260 nm.

Establishment and screen of cDNA library

The small RNAs were polyadenylated at 37 °C for 30 min in 50 μL reaction volume using approximately 1 μg RNAs and 5 U poly(A) polymerase (New England Biolabs, Ipswich, MA, USA). Then the poly(A)-tailed small RNAs were purified through phenol/chloroform extraction and ethanol precipitation. A 5′ linker (5′-GGA CAC UGA CAU GGA CUG AAG GAG UAG AAA-3′) was ligated to poly(A)-tailed RNAs using T4 RNA ligase (New England Biolabs) and the ligation products were recovered by phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription (RT) was conducted using the entire poly(A)-tailed RNAs and 1 μg RT primer (5′-CGC TAC GTA ACG GCA TGA CAG TG(T)24-3′) with 200 U of SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The cDNA was amplified for 35 cycles at an annealing temperature of 50 °C using primers 5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′ and 5′-CGC TAC GTA ACG GCA TGA CAG TG-3′. The PCR products were run on 3% agarose gel with GoldView staining. Then, gel slices with DNA sized approximately 110 bp were excised and purified using an EasyPure quick gel extraction kit (TransGen Biotech, Beijing, China). The DNA fragments were directly cloned into pEASY-T1 vector (TransGen Biotech). Colony PCR was performed using 5′ and 3′ primers, and the clones with PCR products of approximately 110 bp in length were sequenced.

Validation of the miRNAs by RT–PCR and sequencing

Reverse transcription was carried out using approximately 1 μg poly(A)-tailed small RNAs and 1 μg of the same RT primer as above with 200 U of SuperScript III (Invitrogen) according to the method described above. Amplification of miRNAs was conducted for 35 cycles at an annealing temperature of 55 °C using miRNA-specific forward primers (Table 1) and reverse primer (5′-CGC TAC GTA ACG GCA TGA CAG TG-3′). The internal control was U6 small nuclear RNA. The PCR products were separated on 3% agarose gel with GoldView staining. Then, the PCR bands were excised and purified. The purified PCR products were cloned into pEASY-T1 vector (TransGen Biotech), and clones with PCR products were sequenced.

miRNA quantitative analysis using real-time PCR

Real-time PCR was carried out in a Stratagene MX-4000 system with the same primers as detection of miRNA

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Table 1 Forty-two miRNA specific primers for reverse transcription polymerase chain reaction (RT–PCR) and real-time PCR

miRNA	P primer (5′–3′)
hsa-let-7a	TGAGGTAGTAGGTTGTATAGT
hsa-let-7b	TGAGGTAGTAGGTTGTGTGGTT
hsa-let-7c	TGAAGTAGTACGTTGTAGGGTT
hsa-let-7d	AGAGGTAGTAGGTTGCATAGTT
hsa-let-7f	TGAGGTAGTAGATTGTATAGTT
hsa-let-7g	TGAGGTAGTAGTTTGTACAGTT
hsa-miR-15a	TAGCAGCACATAATGGTTTGT
has-miR-15b	TAGCAGCACATCATGGTTTAC
hsa-miR-19a	TGTGCAAATCTATGCAAAACTG
hsa-miR-19b	TGTGCAAATCCATGCAAAACTG
hsa-miR-21	TAGCTTATCAGACTGATGTTG
hsa-miR-23a	ATCACATTGCCAGGGATTCC
hsa-miR-24	TGGCTCAGTTCAGCAGGAACAG
hsa-miR-26a	TTCAAGTAATCCAGGATAGG
hsa-miR-26b	TTCAAGTAATTCAGGATAG
hsa-miR-27a	TTCACAGTGGCTAAG TTCC
hsa-miR-27b	TTCACAGTGGCTAAGTTCTGC
hsa-miR-30d	TGTAAACATCCCCGACTGGAAG
hsa-miR-30e*	CTTTCAGTCGGATGTTTAC
hsa-miR-98	TGAGGTAGTAAGTTGTATTG
hsa-miR-99a	GACCCGTAGATCCGATCTTGTG
hsa-miR-101	TACAGTACTGTGATAACTGAA
hsa-miR-103	AGCAGCATTGTACAGGGCTATG
hsa-miR-122	TGGAGTGTGACAATGGTGTTTG
hsa-miR-125a-5p	TCCCTGAGACCCTTTAACCTGT
hsa-miR-125b	TCCCTGAGACCCTAACTTGT
hsa-miR-126	TCGTACCGTGAGTAATAATG
has-miR-130a	CAGTGCAATGTTAAAAGGGCAT
hsa-miR-136	ACTCCATTTGTTTTGATGAAGG
hsa-miR-144	TACAGTATAGATGATGTACT
hsa-miR-148a	TCAGTGCACTACAGAACTTTGT
hsa-miR-192	CTGACCTATGAATTGACAGC
has-miR-193a-3p	AACTGGCCTACAAAGTCCAA
hsa-miR-194	TGTAACAGCAACTCCATGTGG
hsa-miR-345	GCTGACTCCTAGTCCAGGGCT
hsa-miR-376a*	GTAGATTCTCCTTCTATGAGT
hsa-miR-410	AATATAACACAGATGGCCTGT
hsa-miR-451	AAACCGTTACCATTACTGAGTT
hsa-miR-483-3p	TCACTCCTCTCCTCCCGTCTT
hsa-miR-483-5p	AGACGGGAGGAAAGAAGGGAGTGG
hsa-miR-495	AAACAAACATGGTGCACTTCT
hsa-miR-885-5p	TCCATTACACTACCCTGCCTAT

expression by RT–PCR and TransStart SYBR green qPCR supermix (TransGen Biotech) following the manufacturer's instructions. The 20 μL reactions including 0.5 μL of RT products, 1× TransStart SYBR green qPCR supermix and 0.5 μM forward and reverse primers were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 20 s. Melting curves for each PCR were carefully monitored to avoid non-specific amplification. The U6 small nuclear RNA was used as internal control. Each miRNA was analyzed in duplicate. The relative level (RL) of each miRNA expression was calculated by the $2^{-\Delta Ct}$ method, and the data were presented as $\log_2$ of RL of target miRNAs. Results were visualized with GENESIS (Alexander Sturn, Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria).

Biological software analysis

The small RNA sequences were analyzed using BLAST analysis against the human genome and miRNA databases.

Statistical analysis of miRNA expression

A two-tailed Student's *t*-test was used to assess the miRNA expression level difference at the five stages. The stage of lowest expression level was compared to the others.

Target gene prediction of each miRNA

Target genes for each miRNA were predicted using miRBase Targets ver. 5 program (http://microrna.sanger.ac.uk/targets/v5/).

RESULTS

Detection of 42 miRNAs from the two cDNA libraries

THE CLONES WERE subsequently examined by DNA sequencing and database searching. More than 20% of the cloned RNAs were degraded products of abundant RNAs such as rRNA and mRNA. A total of 181 clones were identified as miRNAs (Table 2). As regards the previously known miRNAs, some were detected as variants of miRNAs in the miRNA database (Table 2).

Confirmation of miRNAs by RT–PCR and sequencing

The miRNAs identified above were confirmed by using RT–PCR (Fig. 1) and sequencing (Fig. 2), suggesting that they indeed existed in the tissues.

Comparison of miRNA expression using real-time PCR

In order to understand whether the expression level of the miRNAs detected differs between stages of

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Table 2 Forty-two miRNAs and their variants identified in two fetal livers

miRNA	Sequence (5′–3′)	No. of clones		
		27-week	35-week	Total
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGU	1	0	1
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU	1	0	1
hsa-let-7c	UGAAGUAGUACGUUGUAGGGUU	1	0	1
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU	1	0	1
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU	2	0	2
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU	1	0	1
hsa-miR-15a	UAGCAGCACAUCAUGGUUUGU	2	0	2
has-miR-15b	UAGCAGCACAUCAUGGUUUAC	0	1	1
hsa-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	1	0	1
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	2	0	2
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	1	0	1
	UAGCUUAUCAGACUGAUGUUGAC	1	0	1
	UAGCUUAUCAGACUGAUGUUG	0	1	1
hsa-miR-23a	AUCACAUUGCCAGGGAUUCCC	1	0	1
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG	2	0	2
	UGGCUCAGUUCAGCAGGAAC	1	0	1
	UGGCUCAGUUCAGCAGGAACAGAU	1	0	1
	UGGCUCAGUUCAGCAGGAACAGU	0	1	1
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGC	1	0	1
hsa-miR-26b	UUCAAGUAAUUCAGGAUAG	1	0	1
	UUCAAGUAAUUCAGGAUAGGU	1	0	1
hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC	1	0	1
hsa-miR-27b	UUCACAGUGGCUAAGUUCUGC	1	0	1
hsa-miR-30d	UGUAAACAUCCCCGACUGGAAG	1	1	2
hsa-miR-30e*	CUUUCAGUCGGAUGUUUAC	1	0	1
hsa-miR-98	UGAGGUAGUAAGUUGUAUUG	1	0	1
hsa-miR-99a	GACCCGUAGAUCCGAUCUUGUG	1	0	1
hsa-miR-101	GUACAGUACUGUGAUAACUGAA	1	0	1
hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA	1	0	1
hsa-miR-122	UGGAGUGUGACAAUGGUGUU	4	1	5
	UGGAGUGUGACAAUGGUGUUU	7	4	11
	UGGAGUGUGACAAUGGUGUUUG	39	7	46
	UGGAGUGUGACAAUGGUGUUCG	0	1	1
	AGGAGUGUGACAAUGGUGUUU	0	1	1
	GUGGAGUGUGACAAUGGUGUUUG	1	0	1
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGU	1	0	1
hsa-miR-125b	UCCCUGAGACCCUAACUUGU	0	1	1
hsa-miR-126	UCGUACCGUGAGUAAUAAUGCG	2	0	2
has-miR-130a	CAGUGCAAUGUUAAAAGGGCAU	0	1	1
hsa-miR-136	ACUCCAUUUGUUUUGAUGAAGGA	1	0	1
hsa-miR-144	UACAGUAUAGAUGAUGUACU	1	0	1
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU	2	0	2
	UCAGUGCACUACAGAACUUUGUC	1	0	1
hsa-miR-192	CUGACCUAUGAAUUGACAGCC	1	0	1
	CUGACCUAUGAAUUGACAGC	2	0	2
has-miR-193a-3p	AACUGGCCUACAAAGUCCAA	0	1	1
hsa-miR-194	UGUAACAGCAACUCCAUGUGGA	3	0	3
	UGUAACAGCAACUCCAUGUGG	0	2	2
hsa-miR-345	GCUGACUCCUAGUCCAGGGCU	0	1	1
has-miR-376a*	GUAGAUUCUCCUUCUAUGAGU	0	1	1

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Table 2 *Continued*

miRNA	Sequence (5′–3′)	No. of clones		
		27-week	35-week	Total
hsa-miR-410	AAUAUAACACAGAUGGCCUGU	1	0	1
hsa-miR-451	AAACCGUUACCAUUACUGAGU	1	5	6
	AAACCGUUACCAUUACUGAGUU	1	12	13
	AACCGUUACCAUUACUGAGUUU	1	0	1
	AAACCGUUACCAUUACUGAGUUU	4	24	29
	ACACCGUUACCAUUACUGAGUUU	0	1	1
	AAACCGUUACCAUUACUGAGUUUAG	1	0	1
	AAACCGUUACCAUUACUGAGUUUAGU	2	0	2
	GAACCGUUACCAUUACUGAGUUU	0	2	2
hsa-miR-483-3p	UCACUCCUCUCCUCCCGUCUUC	1	0	1
	UCACUCCUCUCCUCCCGUCUU	0	1	1
hsa-miR-483-5p	AGACGGGAGGAAAGAAGGGAGUGG	1	0	1
	AACACGGGAGGAAAGAAGGGAG	0	1	1
has-miR-495	AAACAAACAUGGUGCACUUCU	0	1	1
hsa-miR-885-5p	UCCAUUACACUACCCUGCCUAU	1	0	1
Total	/	109	72	181

development, a quantitative PCR was performed. The relative expression level was calculated and compared between stages (Fig. 3). The miR-122 exhibited moderate to high levels across the five stages, the highest level being seen at the stage of 35 weeks ($P = 0.001$), and the lowest level at the stage of 18 years. For miR-148a, the higher level was detected at the stages of 35 days ($P < 0.001$), 35 weeks ($P = 0.001$) and 18 weeks ($P = 0.001$). The miR-192 was highly expressed at the stages of 35 weeks ($P = 0.004$) and 18 weeks ($P = 0.008$), and moderately expressed at the stage of 35 days ($P = 0.013$). Interestingly, the miR-194 revealed an expression pattern similar to miR-192. The miR-451 was highly expressed at the stages of 35 weeks ($P < 0.001$), 18 weeks ($P = 0.001$) and 35 days ($P = 0.007$). The higher level of miR-21 was also seen at the stages of 35 days ($P < 0.001$), 35 weeks ($P = 0.001$) and 18 weeks ($P = 0.01$). Moreover, a moderate level of let-7a was identified at the stages of 35 days ($P = 0.009$), 18 weeks ($P = 0.002$) and 35 weeks ($P = 0.003$).

The total expression level of the miRNAs at each stage was calculated, and they were compared (Fig. 4). The total level at the stages of 35 days ($P < 0.001$), 18 weeks ($P < 0.001$), 27 weeks ($P < 0.001$) and 35 weeks ($P < 0.001$) was significantly higher than at the stage of 18 years.

Analysis of potential targets for the miRNAs

The miRBase Targets ver. 5 was used to predict the target genes for the miRNAs identified. Table 3 shows the target genes for those miRNAs.

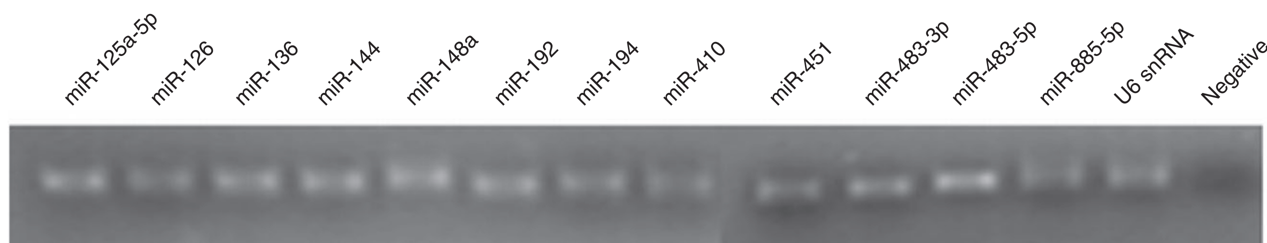

Figure 1 Reverse transcription polymerase chain reaction (RT–PCR) for some miRNAs selected from a fetal liver of 35 weeks. The PCR products were separated on 3% agarose gel with GoldView staining. Positive control is U6 small nuclear RNA. H_2O serves as negative control.

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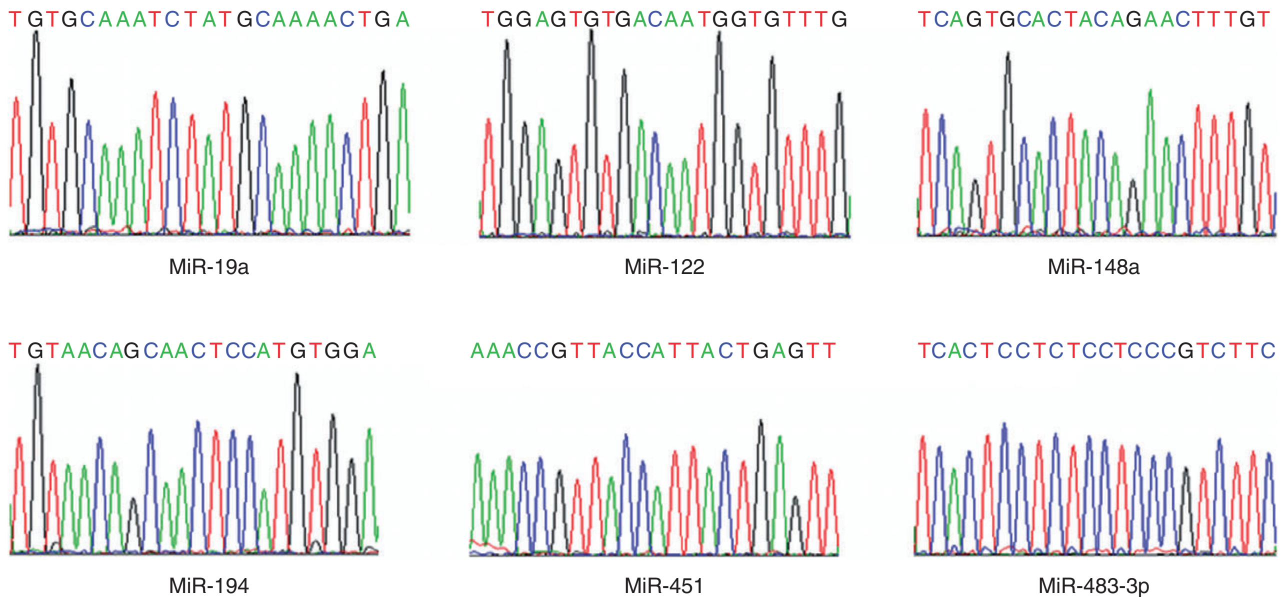

Figure 2 Sequencing analysis of reverse transcription polymerase chain reaction (RT–PCR) products for six miRNAs selected. The RT–PCR bands for miRNAs were excised and purified. The purified PCR products were cloned into vectors, and then the recombinant vectors were sequenced by ABI 3100 DNA Sequencer.

DISCUSSION

GENE EXPRESSION EXHIBITS developmental stage specificity. The mechanism behind this could be that epigenetic events, including methylation modification and miRNA actions, regulate transcription and translation of genes at certain developmental stages. In this study, we analyzed miRNA expression in five human tissues. These tissues were from five different stages of development: four were liver tissues (18-week, 27-week, 35-week fetuses and 18 years) and one was chorionic villus tissue (35 days). At the stage of 35 days of pregnancy, because the liver was not developed and the embryo was too small, we analyzed only the chorionic villus tissue.

We used a cloning method to identify miRNAs that function in the 27- and 35-week human fetal livers. In total, 181 clones were analyzed, of which 109 were from the 27-week liver and 72 from the 35-week liver. After careful database searching, 42 miRNAs and their variants were recognized from the 181 clones (Table 2). A larger number of clones were detected in the 27-week liver compared to the 35-week liver, as well as larger number of identified miRNAs (Table 2). Variants of some miRNAs were also detected. It may be speculated that the variants have different target genes compared to the wild-type miRNAs. Moreover, miR-122 and miR-451 were more frequently identified than other miRNAs in the two livers (Table 2). This is in accordance with the expression analysis (Fig. 3).

Due to the high number of miRNAs identified in the 27- and 35-week livers, we did not seek additional miRNAs in the 18-week and 18-year livers. Instead we analyzed the expression levels of the 42 miRNAs detected in the four stages of human livers: 18 weeks, 27 weeks, 35 weeks and 18 years. In addition, miRNA expression was examined in the chorionic villus tissue at the stage of 35 days. Surprisingly, the total expression level of the 42 miRNAs was higher in the chorionic villus tissue than in the four liver tissues (Figs 3,4). This indicates that the target gene functions are greatly repressed in the chorionic villus tissue. At this stage, the embryo cells start to differentiate. It is conceivable that the highly expressed miRNAs could be involved in embryo differentiation. Furthermore, we found that the miRNA expression level was very low in the stage of 18 years compared to other stages (Figs 3,4). The mechanism behind this difference in expression of miRNAs between fetal and adult liver is unknown, but one explanation could be higher gene methylation status in the adult.[17] In addition to transcriptional control and other mechanism of gene regulation, it can be inferred that at the fetal stage the inhibition of gene functions in the liver is

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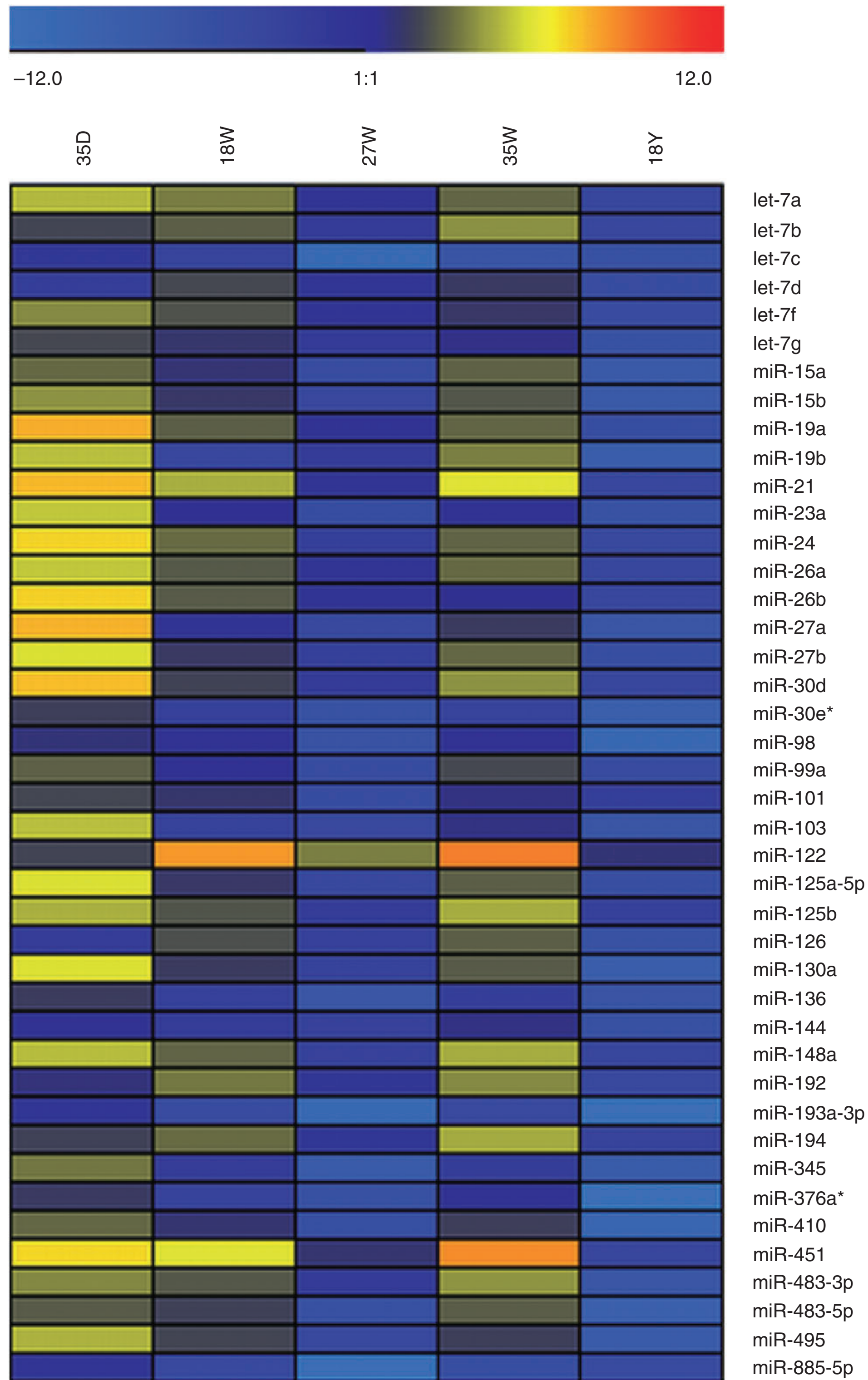

Figure 3 Expression profiles of 42 mature miRNAs at five developmental stages (35 days, 18 weeks, 27 weeks, 35 weeks and 18 years). Samples and miRNAs are displayed in rows and columns, respectively. The relative expression values are ranged from $+12 \log_2$ to $-12 \log_2$, which are exhibited with different colors. The bright red colors represent the highest expression levels, and the light blue colors represent the lowest expression levels.

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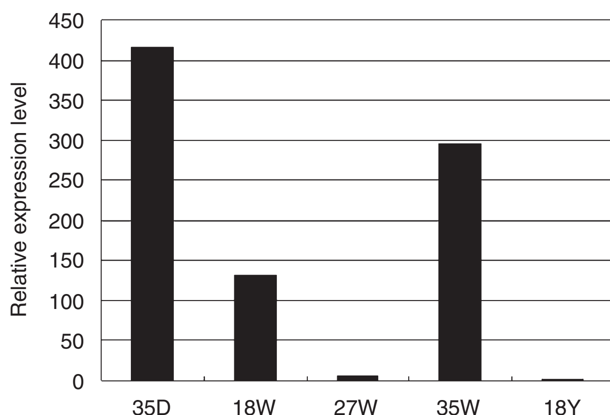

Figure 4 Comparison of the total expression levels (addition of the relative expression level for each miRNA) of all 42 miRNAs in the five tissues for stages of 35 days, 18 weeks, 27 weeks, 35 weeks and 18 years, respectively. The level of the stage of 18 years was set at 1 and relative levels for other four stages were calculated. The levels in the fetal stage are significantly higher than in the adult stage.

mainly induced by the miRNAs, while in adults gene methylation plays a bigger role. A previous study reported a moderate to high expression level for some of the miRNAs detected in this study.[16] However, the method used in that study was oligonucleotide microarrays, and hence we cannot make a direct comparison with our data. Furthermore, in the fetal stage, the level of the miRNA expression was dynamically altered; the level at the stage of 27 weeks was lower than at the other two stages (Figs 3,4). The mechanism behind this is unclear. However, it reveals that hepatocyte growth, development and differentiation are very active at the stage of 27 weeks compared to other fetal stages. This is in line with a discovery that the growth rate of liver was maximum at 26–30 weeks of gestation.[18] The dynamic changes of miRNA expression may play a role in the liver development at the fetal stage.

The let-7 family was identified at a low to moderate level (Fig. 3). The let-7 family can act as a tumor suppressor and plays a critical role in cell-cycle control with respect to differentiation and tumorigenesis.[19] The low to moderate expression of the let-7 family detected across the five stages suggests a certain level of negative regulation of cell growth.

The liver-specific miRNAs (miR-122, miR-148a, miR-192 and miR-194) displayed moderate to high expression levels in the four liver tissues (Fig. 3). The target genes of miR-122 include *PINX1_HUMAN* (Table 3).

Table 3 Prediction of target genes of the 42 miRNAs identified

miRNA	Target genes in human liver
hsa-let-7a	*PRDX5*
hsa-let-7b	*PRDX5, NP_060880.3, CPT1A*
hsa-let-7c	*NP_060880.3, CPT1A*
hsa-let-7d	*GJB1*
hsa-let-7f	*CPT1A, TMEM59*
hsa-let-7g	*PFKFB1, PRDX5*
hsa-miR-15a	*GLS2, PFKFB1*
hsa-miR-15b	*GLS2, PFKFB1*
hsa-miR-19a	*PSG1, DLC1*
hsa-miR-19b	*PSG1*
hsa-miR-21	*PHKA2, CCL20, GJB1, PYGL*
hsa-miR-23a	*PTP4A2, ARG1*
hsa-miR-24	*PHLDA2, DNASE1L3, PSG5, PINX1_HUMAN*
hsa-miR-26a	*TMEM59*
hsa-miR-26b	*TMEM59*
hsa-miR-27a	*PFKL, PFKFB1, COX8A, NP_060880.3*
hsa-miR-27b	*PFKL, PFKFB1, COX8A, NP_060880.3*
hsa-miR-30d	ND
hsa-miR-30e*	*PRDX5*
hsa-miR-98	*PRDX5, Q6SA06_HUMAN*
hsa-miR-99a	*STARD13, COX6A1*
hsa-miR-101	*PFKFB1*
hsa-miR-103	*PHKG2, Q86U10_HUMAN, GLS2*
hsa-miR-122	*PKLR, ALPL, PINX1_HUMAN, PSG5*
hsa-miR-125a-5p	*NR5A2, STARD13*
hsa-mir-125b	*PYGL, NR5A2*
hsa-miR-126	*CSTB*
hsa-mir-130a	*TMEM59, PBLD, STARD13, SLCO1B1*
hsa-miR-136	*PBLD, PYGL, STARD13, TMEM59*
hsa-miR-144	*ALDOB, CCL20*
hsa-miR-148a	*PKLR, PHKG2*
hsa-miR-192	*PTP4A3, TM4SF4, HAMP, COX7A2*
hsa-mir-193a-3p	*GLS2, PHLDA2, DDEFL1*
hsa-miR-194	ND
hsa-mir-345	*PHLDA2, STARD13*
hsa-mir-376a*	*GYS2, DNASE1L3*
hsa-miR-410	*PSG1, TMEM59, CLEC4M*
hsa-miR-451	*TMEM59*
hsa-miR-483-3p	*OSGIN1, GJB1, DLC1*
hsa-miR-483-5p	*PTP4A3, HAMP, GFER*
hsa-mir-495	*DLC1, PTP4A3*
hsa-miR-885-5p	*Q86U10_HUMAN*

ND, no data.

This gene may inhibit cell proliferation and act as a tumor suppressor.[20] It has been suggested that inhibition of this gene may promote hepatic growth. The miR-148a acts on the *PHKG2* gene, whose mutations can cause glycogen storage disease type IX.[21] The *PTP4A3* is a

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target gene of miR-192. A study showed that expression of the *PTP4A3* gene was upregulated in liver cancers, suggesting that it could promote hepatocyte growth.[22] However, the target genes of miR-194 have not yet been determined.

The miR-451 showed similar expression patterns to the liver-specific miRNA (Fig. 3). Its target gene in the livers is *TMEM59*, which encodes for liver membrane-bound proteins. But the function of the protein is unknown. The miR-21, whose target gene is *PYGL* that encodes for glycogen phosphorylase in the liver, was highly expressed at the stages of 35 days and 35 weeks (Fig. 3). The *PYGL* mutations cause glycogen-storage disease type VI (Hers disease; Mendelian Inheritance in Man 232700).[23]

Interestingly, some miRNAs act on the same target genes (Table 3), suggesting a synergy between these miRNAs during hepatic development. Moreover, miR-15a and miR-15b displayed similar expression patterns (Fig. 3), and they also shared two target genes: *GLS2* and *PFKFB1*.

Our results showed that a special group of miRNAs have a significantly higher expression level in the fetal livers than in the adult liver. The discovery indicates that the miRNAs may play roles mainly in the period before birth.

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