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# Identification of human fetal liver miRNAs by a novel method

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**Abstract MicroRNAs (miRNAs) are short 20–25 nucleotides RNA molecules that have been shown to regulate gene expressions in a variety of eukaryotic systems. miRNAs are widespread in eukaryotes and several hundred of miRNAs have been identified, but still a lot of miRNAs have not been detected in various eukaryotic organisms. However, it is not an easy work to clone miRNAs by traditional methods. Here, we describe the identification of 27 miRNAs from a human fetal liver cDNA library by a novel cloning method. Low molecular weight RNA fraction (⩽200 nt) from fetal liver tissue was extracted, and polyadenylated by poly(A) polymerase. A 5′ RNA adaptor was ligated to poly(A)-tailed RNA using T4 RNA ligase. After reverse transcription, the cDNA was amplified by PCR with two adaptor primers. The PCR product with a size about 109 bp was recovered and cloned into T vector. After sequencing, database searching, and expression profiling, 5 novel miRNAs were discovered among other 22 known miRNAs in human fetal liver. These finding indicate that a large diverse population of miRNAs may function to regulate gene expression in hepatocyte.**


*Keywords:* microRNA; Liver

## 1. Introduction

MicroRNAs (miRNAs) ranging in size from 20 to 25 nt represent a new family of non-coding RNAs that are processed from hairpin precursor transcripts by Dicer. Studies show that they can function as the negative regulators of gene expression/downregulate the expression level of many genes [1]. miRNAs are transcribed as long primary transcripts (termed pri-miRNAs) [2]. These primary transcripts are first trimmed into approximate 70 nt stem–loop forms (called pre-miRNAs) by the RNase III type protein, Drosha, in the nucleus [3]. Following this initial processing, pre-miRNAs get exported to the cytoplasm by Exportin-5 and are processed to generate approximate 22 nt mature miRNAs by Dicer, another RNase type III [4,5].

Recent studies have suggested that miRNAs play important roles in development and differentiation. MicroRNA can regulate gene expression by essentially two modes. In plants, miRNAs complement with corresponding mRNA targets precisely, resulting in cleavage and destruction of the target mRNAs through a mechanism involving RNA interference (RNAi) machinery. In animals, some miRNAs including miR-196 also inhibit gene expression through this mechanism, however, most miRNAs, including lin-4 and let-7, are imprecisely complementary to their corresponding mRNA targets and inhibit synthesis and function of proteins [6–8].

Although the first miRNA are identified in 1993 [9], it is not until 2001 that the breadth of the miRNA gene class is recognized with cloning and sequencing of more than one hundred miRNAs from worms, humans, and mice [10–12]. Since then, hundreds of miRNAs have been reported in diverse animals and plants [13]. Studies reveal that miRNAs have critical roles in central biological processes. lin-4 and let-7 are the first characterized endogenous miRNAs. Studies show that they can regulate the timing of early [14,15] and late [16] larval developmental transition and left/right asymmetry of chemoreceptor expression in *Caenorhabditis elegans* [17]. In *Drosophila*, bantam and miR-14 are shown to regulate apoptosis, growth control, and fat metabolism [18,19]. In plants, some miRNAs are shown to regulate flowering and leaf development [20,21], and embryonic patterning [22]. In addition, miRNAs found within the homeobox clusters suggest that they have roles in regulating the highly ordered expression pattern of homeobox genes, which are involved in many aspects of embryonic development [23,24].

Although many miRNA genes have been identified using computational procedures in vertebrates, *C. elegans* and *Drosophila*, the majority of miRNA genes were discovered through cDNA cloning with size-fractionated RNA samples. And more miRNAs remain to be detected in a variety of organisms. Some miRNAs in animals exhibit tissue or developmental-stage-specific expression [10]. miR-375 is expressed only in MIN6 and TC1 cells, and mouse pancreatic islets, but not in other tissues, including exocrine pancreas, liver, lung, intestine, brain, kidney, spleen, heart, testis and fat tissue. It is shown that overexpression of miR-375 suppresses glucose-induced insulin secretion, and on the contrary, inhibition of endogenous miR-375 enhances insulin secretion [25]. Furthermore, a set of six clustered mouse miRNAs (miR-290–miR-295) are expressed specifically in embryonic stem (ES) cells [26]. It is impossible to predict by computational methods whether a miRNA is expressed in certain specific tissue or developmental stage. To study the functions of such miRNAs, we should identify them by cDNA cloning methods. Traditional miRNA cloning methods must run denaturing polyacrylamide gel and

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recover the RNA fraction several times. Therefore, it is not an easy work for those who have less RNA manipulation experience to clone miRNAs by this method. Liver is a crucial human organ, in which, miRNAs should play principal roles. To study their functions in liver, it is necessary to uncover the subset of liver-expressed microRNAs. Here, we identified 27 miRNAs from human fetal liver by a novel simple cloning method. Of these miRNAs, 22 were identical or similar to those reported previously, 5 are novel and conserved in vertebrates.

## 2. Materials and methods

### 2.1. Cell culture

HepG2, BEL-7402 and HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 mg/mL penicillin/streptomycin.

### 2.2. Small RNA extraction

Small RNA (⩽200 nt) was extracted from human liver tissue and cells using mirVana™ miRNA Isolation kit (Ambion, Austin, TX) according to the manufacturer instructions. Thirty milligram tissue was used and the small RNA fraction was dissolved in 30 μl RNA-free water. The concentration of RNA was measured by the UV absorbance at 260 nm.

### 2.3. Construction and screen of cDNA library of small RNA

Small RNAs were polyadenylated at 37 °C for 30 min in 50 μl reaction volume with 1.5 μg RNA and 5 U poly(A) polymerase (Takara) [27]. Poly(A)-tailed small RNA was recovered by phenol/chloroform extraction and ethanol precipitation. A 5′ adapter (5′-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA-3′) was ligated to poly(A)-tailed RNA using T4 RNA ligase (Invitrogen) and the ligation products were recovered by phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription was performed using 1.5 μg RNA and 1 μg of RT primer (ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)30 (A, G, or C) (A, G, C, or T)) with 200 U of SuperScript III reverse-transcriptase (Invitrogen). Poly(A)-tailed small RNA (10 μl total volume) was incubated with 1 μl of RT primer and 1 μl dNTP mix (10 mM each) at 65 °C for 5 min to remove any RNA secondary structure. The reactions were chilled on ice for at least 1 min and the remaining reagents [5× buffer, dithiothreitol (DTT), RNaseout, SuperScript III] were added as specified in the SuperScript III protocol and the reaction proceeded for 60 min at 50 °C. Finally, the reverse transcriptase was inactivated by a 15 min incubation at 70 °C. The cDNA amplification was carried out for 25 cycles at a final annealing temperature of 50 °C using primers 5′-ATT CTA GAG GCC GAG GCG GCC GAC ATG T-3′; 5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′. The PCR product was separated on 12% polyacrylamide gel with EtBr staining. Then the gel slices containing DNA with a size about 109 bp were excised and the DNA were eluted into elution buffer (0.5 M $NH_4Ac$, 10 mM $Mg(Ac)_2$, and 1 mM EDTA) at 37 °C and recovered by phenol/chloroform extraction followed by ethanol precipitation. The DNA fragment was directly subcloned into pGEM-T vector(Promega). Colony PCR was performed using 5′ and 3′ primers, and the clones with PCR products about 109 bp in length were sequenced.

### 2.4. Northern blotting

Northern blotting of miRNAs was performed as described previously [11]. Briefly, 10 μg of small RNA from human fetal liver, HepG2, BEL-7402 and HeLa cells was separated on a denaturing 15% polyacrylamide gel, and then transferred to Hybond N+ membranes (Amersham) by using a Trans-Blot Electrophoretic Transfer (Pharmacia LKB). After electroblotting, the RNAs were fixed to the membrane by UV cross-linking (125 mJ, GS GENE LINKER, Bio-Rad) and baking at 80 °C for 1 h. The 5′-ends of the DNA oligonucleotide probes complementary to miRNA sequences were labeled with [$\gamma$-$^{32}$P] ATP (Yahui) using $T_4$ polynucleotide kinase (NEB) and purified according to the standard laboratory protocols. The membrane was pre-hybridized in ExpressHyb (CloneTech) solution at 37 °C for at least 1 h and hybridized with $^{32}$P-end-labeled oligonucleotide probes in fresh ExpressHyb solution at 37–38 °C for 16 h. The blots were washed thrice with 2× SSC/0.1% SDS and once with 0.5× SSC/0.1% SDS at room temperature. Finally, the blots were exposed to X-film at −70 °C for 48 h.

### 2.5. Detection of miRNA expression by poly(A)-tailed RT-PCR

Reverse transcription was performed using 1.5 μg poly(A)-tailed small RNA of human fetal liver and 1 μg of RT primer (ATT CTA GAG GCC GAG GCG GCC GAC ATG-d$(T)_{30}$ (A, G, or C) (A, G, C, or T)) with 200 U of SuperScript III (Invitrogen) according to the method described above. The amplification of miRNA was carried out for 25 cycles at a final annealing temperature of 60 °C using miR-18* specific primers (5′-ACT GCC CTA AGT GCT CCT TC-3′) and primer (5′-ATT CTA GAG GCC GAG GCG GCC GAC ATGT-3′). The PCR product was analyzed on 12% polyacrlyamide gel with EtBr staining. Then the gel slices containing DNA fragments of about 80 bp were excised and the DNA were eluted into elution buffer (0.5 M $NH_4Ac$,10 mM $Mg(Ac)_2$, and 1 mM EDTA) at 37 °C and recovered by phenol/chloroform extraction followed by ethanol precipitation. The DNA fragments were directly subcloned into T vector and sequenced.

### 2.6. Computational analyses

RNA sequences were subjected to BLAST analyses against the human genome (http://www.ncbi.nlm.nih.gov/blast) and miRNA database [28] (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). Secondary structures of RNA precursors were predicted by using longer genomic sequences of cloned RNAs and the Mfold program [29] (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi).

## 3. Results

### 3.1. Identification of 27 miRNAs from a fetal liver cDNA library

Small sized RNAs (⩽200 nt) were extracted from human fetal liver and polyadenylated with poly(A) polymerase, and a 5′ RNA adapter was ligated to poly(A)-tailed RNA using T4 RNA ligase. RNAs were reverse-transcribed and amplified by RT-PCR as described previously with some modifications [10]. The DNA approximately 109 bp was recovered from 12% polyacrylamide gel and subcloned into T-vector. Total 205 clones were subsequently characterized by DNA sequencing and database searching. Results indicated that there are several kinds of cellular RNA fragments (Table 1). More than 60% of the cloned RNAs represented breakdown products of abundant non-coding RNAs such as tRNA, rRNA, snRNA, and snoRNA. Some of them represented known miRNAs. The remaining novel RNA sequences were searched against

Table 1
Composition of small RNA populations cloned from fetal liver

| RNA class | Number |
|---|---|
| miRNA | 50 |
| mRNA | 3 |
| rRNA | 35 |
| tRNA | 85 |
| sn/sno-RNA | 5 |
| Match with genome[a] | 4 |
| Mitochondrial | 4 |
| Unknown[b] | 19 |
| Total | 205 |

[a]Sequences that do not form miRNA-specific hairpin precursors but match with genomic sequences.
[b]Sequences that do not match with any genomic sequences.

Table 2
Summary of miRNA information

| miRNA | Sequence (5′–3′) | No. clones[a] | Size (nt) | Northern blotting | Hairpin precursors | Location |
|---|---|---|---|---|---|---|
| miR-483 | UCACUCCUCUCCUCCCGUCUUCU | 5 | 22–23 | + | H.s[b] | Ch[c]11 |
| miR-484 | UCAGGCUCAGUCCCCUCCCGAU | 1 | 22 | + | H.s M.m[d] | Ch16 |
| miR-485 | GUCAUACACGGCUCUCCUCU | 1 | 20 | + | H.s M.m | Ch14 |
| miR-486 | UCCUGUACUGAGCUGCCCCGAG | 1 | 22 | + | H.s M.m | Ch8 |
| miR-487 | AAUCAUACAGGGACAUCCAGUU | 1 | 22 | + | H.S | Ch14 |
| miR-18* | ACUGCCCUAAGUGCUCCUUCU | 1 | 21 | – | H.s M.m | Ch13 |
| miR-17-5p | CAAAGUGCUUACAGUGCAGGUAGU | 2 | 24 | NA[e] | H.s M.m | Ch13 |
| miR-18 | UAAGGUGCAUCUAGUGCAGAU | 1 | 21 | NA | H.s M.m | Ch13 |
| miR-19b | UGUGCAAAUCCAUGCAAAACUGA | 1 | 23 | NA | H.s M.m | Ch13,X |
| miR-20 | UAAAGUGCUUAUAGUGCAGGUA**G** | 3 | 23 | NA | H.s M.m | Ch13 |
| miR-21 | UAGCUUAUCAGACUGAUGUUGAC | 1 | 23 | NA | H.s M.m | Ch21 |
| miR-22 | AAGCUGCCAGUUGAAGAACUGU | 2 | 22 | NA | H.s M.m | Ch17 |
| miR-24 | UGGCUCAGUUCAGCAGGAACAG | 3 | 22 | NA | H.s M.m | Ch9,19 |
| miR-26a | UUCAAGUAAUCCAGGAUAGGCU | 3 | 22 | NA | H.s M.m | Ch3,12 |
| miR-30c | UGUAAACAUCCUACACUCUCAGC | 2 | 23 | NA | H.s M.m | Ch1,6 |
| miR-92 | UAUUGCACUUGUCCCGGCCUGU**U** | 6 | 22–23 | + | H.s M.m | ChX |
| miR-93 | **C**AAAGUGCUGUUCGUGCAGGUAG | 1 | 23 | NA | H.s M.m | Ch7 |
| miR-99b | CACCCGUAGAACCGACCUUGCG | 1 | 22 | NA | H.s M.m | Ch19 |
| miR-106b | UAAAGUGCUGACAGUGCAGAU | 1 | 21 | NA | H.s M.m | Ch7 |
| miR-122a | UGGAGUGUGACAAUGGUGUUUG | 3 | 21–22 | + | H.s M.m | Ch18 |
| miR-125b | UCCCUGAGACCCUAACUUGUG | 2 | 21 | NA | H.s M.m | Ch11,21 |
| miR-145 | GUCCAGUUUUCCCAGGAAUCCCUU | 1 | 24 | NA | H.s M.m | Ch5 |
| miR-148a | UCAGUGCACUACAGAACUUUGU | 1 | 22 | + | H.s M.m | Ch2,7 |
| miR-151 | CUAGACUGAAGCUCCUUGAGG | 1 | 21 | + | H.s M.m | Ch8 |
| miR-199a | CCCAGUGUUCAGACUACCUGUUC | 2 | 23 | NA | H.s M.m | Ch1,19 |
| miR-345 | GCUGACUCCUAGUCCAGGGCUC | 2 | 22 | + | H.s | Ch14 |
| miR-410 | AAUAUAACACAGAUGGCCUGU | 1 | 21 | + | H.s M.m | Ch14 |

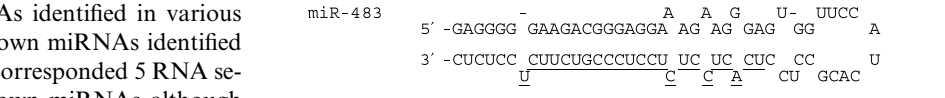
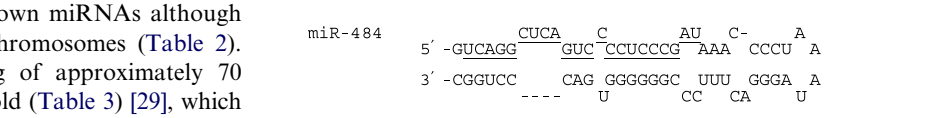
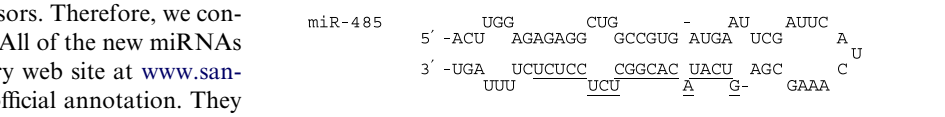
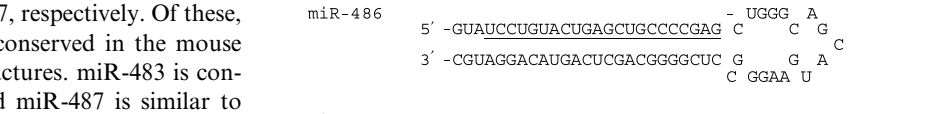
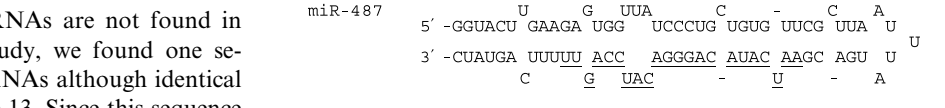
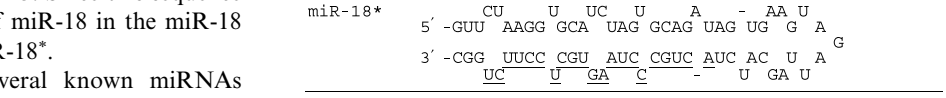
The new miRNAs identified in this study were designated as miR-483 to miR-487, respectively. Sequences listed represent the full length sequence of each miRNA identified in this study. All of the previously uncharacterized miRNAs were examined by Northern blotting with small RNA isolated from human fetal liver, HepG2, BEL-7402 or HeLa cell lines, and those detected by Northern blotting are indicated with (+). Variations between miRNA sequences cloned by us and those reported previously are denoted in bold.
[a]Number of clones.
[b]*Homo sapiens*.
[c]Chromosome.
[d]*Mus musculus*.
[e]Not assayed.

human genome sequence, and then their flanking sequence were used to predict secondary structure by means of Mfold [29].

As shown in Table 1, 50 out of 205 clones from fetal liver were identified as miRNAs. Among them, 29 clones correspond to 14 species of known miRNAs identified in various human cells, 11 clones contained 7 known miRNAs identified in various mouse tissues, and 9 clones corresponded 5 RNA sequences which did not match any known miRNAs although identical sequences exist in human chromosomes (Table 2). They have typical hairpin consisting of approximately 70 nucleotides in length as folded by Mfold (Table 3) [29], which is the characteristic for miRNA precursors. Therefore, we concluded that they are 5 novel miRNAs. All of the new miRNAs were submitted to the miRNA Registry web site at www.sanger.ac.uk_Software_Rfam_mirna for official annotation. They were designated as miR-483 to miR-487, respectively. Of these, miR-484, miR-485 and miR-486 are conserved in the mouse sequence and have typical hairpin structures. miR-483 is conserved between human and horse and miR-487 is similar to miR-376b. However, these novel miRNAs are not found in chicken and fish genomes. In this study, we found one sequence did not match any known miRNAs although identical sequences exist in human chromosome 13. Since this sequence is derived from the strand opposite of miR-18 in the miR-18 precursor-13, it was designated as miR-18*.

Interestingly, the sequences of several known miRNAs cloned by us have a few differences with those reported by

Table 3
Foldback precursor sequences of novel miRNA

| Name | Stem–loop structures of putative miRNA precursors |
|---|---|
| miR-483 |  |
| miR-484 |  |
| miR-485 |  |
| miR-486 |  |
| miR-487 |  |
| miR-18* |  |

Underlined bases represent the mature miRNA sequences.

others. miR-20 cloned by us has an extra G at 3′ end than known sequence, and miR-93, newly cloned has an extra C at 5′ end than known sequence. Sequences of all miRNAs cloned and precursor structures of novel human miRNAs are shown in Tables 2 and 3, respectively.

### 3.2. Positive detection of miRNAs

The expression of novel and some known miRNAs was examined by Northern blotting with small RNAs (⩽200 nt) isolated from fetal liver, HepG2, BEL-7402 and HeLa cells. Northern blotting confirmed that all novel miRNAs and 3 predicted human miRNAs were stably expressed in fetal liver (Fig. 1A). The sequences of two miR-92 clone separated by us have an extra U at their 3′-ends than reported sequence. The expression of miR-92 was investigated by Northern blotting with DNA oligonucleotides complementary to miR-92, two adjacent bands were detected (Fig. 1C), suggesting that miRNAs might wobble at their 3′-end [30]. The results of northern blotting show that the novel miRNAs were all expressed in fetal liver, HepG2 and BEL-7402 cell lines. miR-410, miR-485 and miR-487 accumulated at a higher level in fetal liver, but they were expressed at very low level in two hepatoma carcinoma cell lines (Fig. 1A). This result suggests they could have some relationship with tumor. To determine if these novel miRNAs exhibit fetal liver-specific expression, we detect their expression in fetal and adult liver by Northern blot. As we can see on Fig. 1B, their expression have no notable difference between fetal and adult liver. miR-486 comes from a perfect duplex region (Table 3), to determine if miR-486* is also accumulates in fetal liver cells, we detected it's expression by Northern blots. However, there is no hybridization signal on the membrane. Some miRNAs (such as miR-122a, miR-148, miR-192,miR-194) are liver-specific [24], but they are expressed at similar level in fetal and adult liver (Fig. 1B).

We could not detect the expression of miR-18* with Northern blotting. However, it can be detected by a PCR assay, which is named as poly(A)-tailed RT-PCR. cDNA was prepared from fetal liver poly(A)-tailed small RNA of human fetal liver, PCR reaction was performed using 5′ partial sequence of miR-18* and 3′ adaptor primer according to the method described above. PCR product was analyzed on 12% polyacrlyamide gel with EtBr staining. An approximate 80 bp band was detected and sequenced (Fig. 1D). The result showed that the full length of mature miR-18* sequence was amplified.

## 4. Discussion

Several hundred miRNAs have been verified, however thousands of miRNA genes in various genomes still needed to be identified [31]. Even in mammalian, there are still many miRNAs need to be detected. For the miRNAs expressed in specific tissue or at specific developmental stage, we can

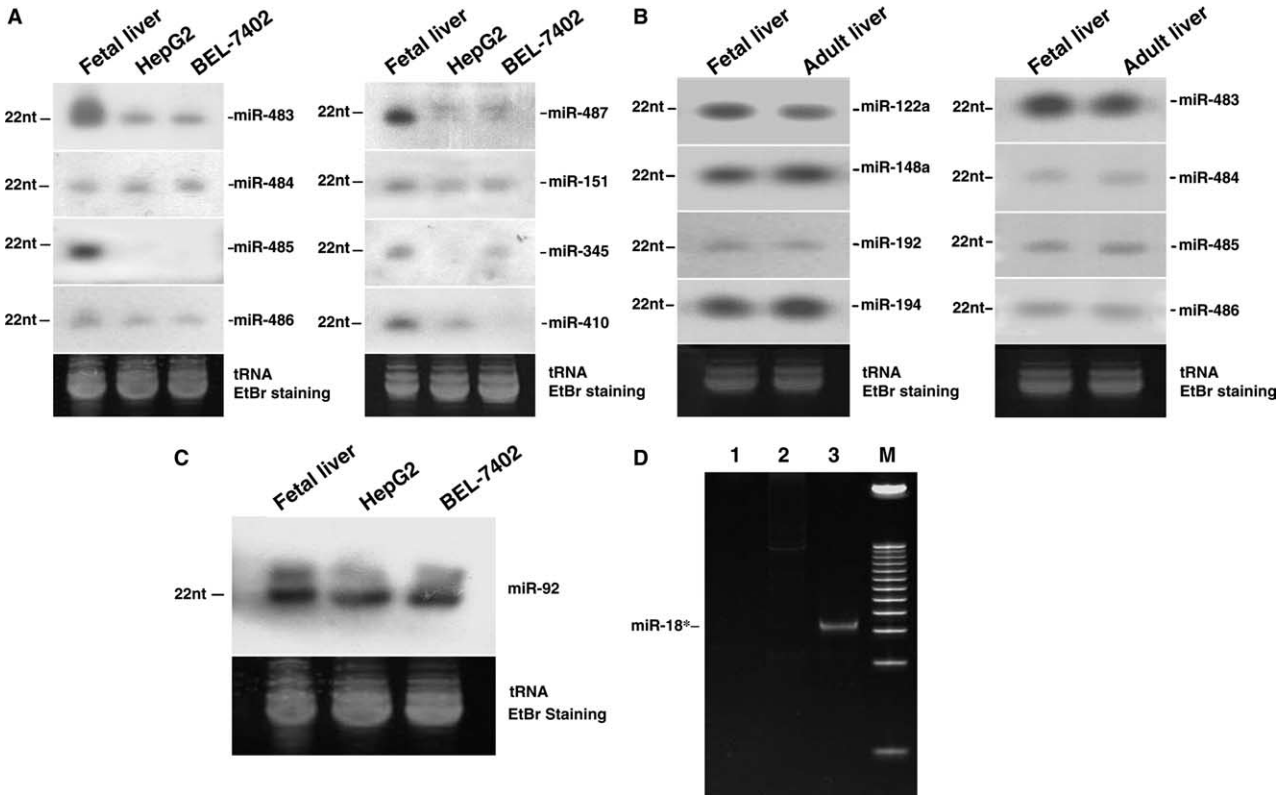



Fig. 1. Detection of miRNAs by Northern blotting and poly(A)-tailed RT-PCR. (A) Expression levels of the indicated miRNAs in human fetal liver, HepG2 and BEL-7402 cells were investigated by Northern blotting. As a loading control, tRNAs were stained with ethidium bromide (EtBr). (B) Expression levels of the indicated miRNAs in human fetal liver and adult liver were investigated by Northern blotting. As a loading control, tRNAs were stained with ethidium bromide (EtBr). (C) Detection of miR-92 by Northern blotting with oligonucleotide probes complementary to miR-92 sequences. (D) Detection of miR-18* by poly(A)-tailed RT-PCR, 1. minus RT, 2. cDNA from small RNA, 3. cDNA from poly(A)-tailed small RNA, M. 25 bp DNA Ladder.

discover them only by miRNA cloning method. However, it is inconvenient and time consuming to detect miRNAs by traditional cloning methods. Here, we have developed and applied a novel method to clone 27 miRNAs, which included 22 known miRNAs and 5 novel miRNAs. miR-122a, miR-148, miR-192, and miR-194 are liver-specific miRNAs [24], but these miRNAs are not cloned frequently in our experiments. In fact, only 50 clones from fetal liver were identified as miRNAs in our study, the number of miRNAs clones cannot correspond to their expression level. Maybe that is the reason why these miRNAs are not cloned frequently.

Only 1–2 μg small RNA (⩽200 nt) are needed to clone miRNAs by this novel method, which could be easily achieved from small piece of tissue or $10^6$ cells using appropriate commercial RNA extraction kit. More important, the new method eliminated several RNA denaturing polyacrylamide gel extraction steps, so that less chance for the RNA to be degraded. Maybe that is why one extra G at the 3′-terminal of miR-20 and one extra C at the 5′ end of miR-93 were obtained comparing with the publish sequences which were cloned by traditional method. Moreover, this method can be expanded to clone other small RNAs (including siRNA).

In summary, this method has several advantages in comparison with the traditional protocol. First, a small quantity of tissue or cells is enough to clone miRNAs by this method, for it is not necessary to recover small size RNA fraction from total RNA. Second, it is an easier and quicker method, especially for those who have little RNA manipulation experience. Third, it can reduce RNA degradation because of less manipulation steps. Finally, radioactive isotope is no need for DNA marker takes the place of labeled RNA marker. We should get more small RNA sequences per colony if the PCR products were concatermerized instead of being ligated into T vector directly.

Some miRNAs may be expressed at low level, it is very difficult to detect them by Northern blotting. We have to load more total RNA to get hybridization signal. For miRNAs expressed at levels below the detection threshold for Northern blotting [32], they can evade detection by Northern blotting. A twice PCR approach [33,34] has also been used to determine the expression levels of mature miRNAs. This method, while useful to clone miRNAs, is impractical for routine gene expression studies since it involves twice PCR, gel isolation of small RNAs and ligation to RNA linkers [35]. With poly(A)-tailed RT-PCR method developed by us, after cDNA is prepared from poly(A)-tailed small RNA, not only the expression of miRNAs can be detected, but the precise 3′-end of miRNAs can be mapped. Here, the expression of miR-18* is detected by this novel methods.

A prominent characteristic of animal miRNAs is that their genes are often organized in tandem, and closely clustered on the genome. In many cases, such clustered miRNAs are probably processed from a same polycistronic precursor transcript (a single mRNA molecule produced from the transcription of several tandemly arranged genes) [6]. Here, we found that miR-410, miR-485 and miR-487 all locate in the human imprinted 14q32 region. The distance between the genes of miR-410 and miR-485 is 10 478 bp, the distance between the genes of miR-485 and miR-487c is 3089 bp. These miRNAs are all expressed at a high level in fetal liver but at very low level in two hepatoma cell lines. These results suggest that they may originate from a same transcript and are regulated as one unit at the transcriptional level. However, they have no similar sequences, suggesting that they are coordinately deployed towards their corresponding targets. In fact, there are many more miRNAs in this region, and they are organized as a large cluster. In the mouse, this miRNA gene cluster is conserved at the homologous distal 12 region [36]. The gene of miR-345 also resides in chromosome 14, but its location is not close to those of miR-410, miR-485 and miR-487.

Liver is one of the most important organs in human body, which can build and store many nutrients that body needs, break down harmful substances, remove waste products from blood. miRNAs are widespread in various organs and play important roles. For example, miR-375 can regulate insulin secretion in pancreatic islet [25]. Now, we know many miRNAs are expressed in liver and a few miRNAs (such as miR-122a,miR-148,miR-192,miR-194) are liver-specific [24]. However, their functions in liver remain to be deciphered. To study the functions of miRNAs, it is very important to identify miRNA target genes. However, the imprecise base pairing between the typical animal miRNA and a target mRNA makes it difficult to identify miRNA target genes. Although exciting progression has been made in this aspect [6], more work remains to be done.

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