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MicroRNA Profiling of Human Intrahepatic Cholangiocarcinoma Cell Lines Reveals Biliary Epithelial Cell-specific MicroRNAs

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Abstract

Intrahepatic cholangiocarcinoma (ICC), which arises in the small bile ducts of the liver, is the second most common liver malignancy. Although modulation of microRNA (miRNA) expression has been shown to be a potent sign of malignant tumors, miRNA profiles of ICC remains unclear. We performed sequencing analysis of the small RNA libraries of 2 ICC cell lines (HuCCT1 and MEC) and one normal intrahepatic biliary epithelial cell line (HIBEpiC) to produce the miRNA profiles of ICC *in vitro*. Furthermore, by means of the real-time polymerase chain reaction (PCR) we validated the differential expression of miRNAs cloned exclusively or predominantly from each of the cell lines. A total of 35,759 small RNA clones were obtained from the 3 cell lines. We identified 27 miRNAs that were expressed exclusively or predominantly in each cell line. Subsequent validation with the real-time PCR confirmed that the miRNAs *hsa-miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424* were expressed specifically in HIBEpiC but were downregulated in the ICC cell lines. Our study provides important information for facilitating studies of the functional role(s) of miRNAs in carcinogenesis of the hepatobiliary system. The biliary epithelial cell-specific miRNAs identified in this study may serve as potential biomarkers for ICC.
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Key words: microRNA, human intrahepatic cholangiocarcinoma, cloning, biomarker

Introduction

MicroRNAs (miRNAs) are noncoding RNAs of roughly 22 nucleotides (nt) that play important roles in the regulation of gene expression at a translational level by binding to the target sites of mRNAs[1–3]. Although some miRNAs display distinctive tissue distribution patterns, their tissue- and organ-specific functions remain largely unknown. On the other hand, several miRNAs have been reported to be involved in developmental regulation[4]. Moreover, miRNAs are believed to be important for carcinogenesis. For example, the absence or downregulation of *miR-15* and *-16* in most cases of chronic lymphocytic leukemia leads to the

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upregulation of anti-apoptotic B cell lymphoma 2 (Bcl-2) protein, the target of *miR-15* and *-16*. The upregulation of Bcl-2 averts apoptotic cell death of leukemia cells and thereby promotes their survival[5]. Similarly, in a human glioblastoma cell line, *miR-21* serves as an anti-apoptotic molecule, the expression of which promotes cancer cell proliferation[6]. Furthermore, miRNAs have been investigated as promising biomarkers for the development of clinical diagnostic and prognostic tools[7]. Indeed, differences in the miRNA expression profiles of cancerous and normal tissues have been reported on the basis of microarray studies[8–11]. bead-based flow cytometric analyses[12]. and miRNA cloning[13,14].

Cholangiocarcinomas (CCs) are classified as intrahepatic or extrahepatic tumors. Intrahepatic CC (ICC) arises in the small bile ducts (i.e., the biliary epithelial cells) of the liver. Although ICCs comprise only 5% to 10% of all CCs, they are the second most common liver malignancy[15]. The incidence and mortality rate of ICC are increasing worldwide, although the reason is unclear[15,16]. Despite advances in operative techniques and chemotherapies, patients with ICC still have a poor prognosis because of the late presentation of the disease. Even after resection, the prognosis for patients with advanced ICC is extremely poor[15,17,18]. The profiles of miRNA expression have been used to accurately identify specific types of cancer[12]; thus, expression profiles of the miRNAs involved in ICC are expected to offer valuable insight into the molecular and clinical basis of carcinogenesis and the progression of ICC. Recently, an miRNA microarray analysis identified specific miRNAs involved in the regulation of growth and the response to chemotherapy in 3 human CC lines, including KMCH, an intrahepatic mixed cholangiocellular-hepatocellular carcinoma[19]. However, few studies have addressed miRNA expression in ICC.

In this study, we performed small RNA sequencing, using 2 ICC cell lines (HuCCT1 and MEC) and one normal intrahepatic biliary epithelial cell line (nonmalignant human intrahepatic biliary epithelial cell line: HIBEpiC), to determine miRNA expression profiles for ICC *in vitro*. Furthermore, by means of the real-time polymerase chain reaction (PCR), we validated the differential expression of the miRNAs cloned from the cell lines.

Materials and Methods

Cell Culture and RNA Preparation

The HIBEpiC cell line was obtained from ScienCell Research Laboratories (Carlsbad, CA, USA), HuCCT1 was obtained from the American Type Culture Collection (Manassas, VA, USA), and MEC, TFK-1, and IHGGK were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). HuCCT1, MEC, and IHGGK are derived from ICCs, whereas TFK-1 is an extrahepatic bile duct carcinoma cell line[20]. All of the cell lines were maintained in the media recommended by the suppliers at 37℃ in a humidified incubator with 5% CO_2. Total RNA was extracted from each sample using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions.

miRNA Cloning and Sequencing

The small RNA libraries were constructued as described previously[21,22]. Briefly, 50 μg of total RNA from HIBEpiC, HuCCT1, and MEC were used. Ligation of small RNAs with DNA/RNA chimera linkers at both termini [3' linker oligonucleotide (5'/5Phos/rCrUrGrUAGGCACCATCAATdi-deoxyC-3') and the 5' linker oligonucleotide (5'-ATCGTrArGrGrCrArCrCrUrGrArArA-3')] was followed by reverse transcription and subsequent PCR-amplification of cDNAs using appropriate primers. Then, more than 20 cDNAs were concatenated via BanI restriction sites in the linkers (New England Biolabs, Ipswich, MA, USA) using a DNA ligation kit version 2.1 (Takara Bio, Shiga, Japan). The concatemers of cDNAs were purified with a Geneclean III kit (Qbiogene, Irvine, CA, USA). They were then subjected to TA cloning using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced (864 plasmids per cell line).

Bioinformatic Analysis of Sequence Data

We performed a homology search for all cloned small RNAs and a secondary structural analysis for

all novel miRNA candidates. The sequences of small RNA clones were analyzed for homology with known RNAs, including miRNA (human and non-human), piwi-interacting RNAs (piRNAs), rRNAs, tRNAs, small nucleolar RNAs (snoRNAs), mRNAs, and human genomic DNA. The databases used were as follows: microRNAs (mature and pre), Sanger Data Base (miRBase) v9.2 (http://microrna.sanger.ac.uk/sequences/index.shtml); rRNAs, European ribosomal RNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/); tRNAs, Genomic tRNA database (http://lowelab.ucsc.edu/GtRNAdb/); sn/snoRNAs, RNAdb (http://research.imb.uq.edu.au/randb/); and NONCODE (http://www.noncode.org/). All searches were performed on July 10, 2007. In this study, miRNAs that were the unregistered opposite-strand miRNAs of the known 'unpaired' miRNAs in the miRBase version 9.2 were designated as 'miRNA (opposite)', e.g., *miR-22* (*opposite*).

Clones with 100% homology to human genomic DNA but not matchin known RNAs were termed novel miRNA candidates. The 2-dimensional pre-miRNA configurations of our novel miRNA candidates were predicted as per Mineno *et al.*[23]. Configurations with the least free energy that met the following criteria were assigned to novel miRNA candidates: (i) contains a stem-loop configuration, (ii) cloned mature miRNA sequence portion consists of more than 16 nt in its double-stranded region, (iii) the loop is less than 20 nt long, (iv) the internal loop is less than 10 nt long, and (v) the bulge is less than 5 nt long. Furthermore, novel sequences with overlapping positions in the genome were grouped together.

PCR Analysis of Novel miRNAs

After performing bioinformatic analysis of the sequence data, we further validated novel miRNAs by using a PCR-based method for small RNA detection and quantification by Ro *et al.*[24]. Total RNA from 4 human gastrointestinal tissues (i.e., stomach, colon, liver, and pancreas) was purchased from Stratagene (La Jolla, CA, USA). Small RNAs were isolated from the total RNA samples. Preparation of the cDNAs of small RNAs and semiquantitative PCR analyses of novel miRNAs were performed as reported previously[24]. Primers used for this experiment are listed in **Table 3**. The annealing temperature and the number of PCR cycles were 50℃ and 40 cycles, respectively. As a positive control, the expression of *let-7c* was also analyzed in the same way. The expected cDNA sizes for mature miRNAs are approximately 120 base pairs (bp). The expression levels of the novel miRNAs in the gastrointestinal tissues examined were scored as 4 for high, 3 for medium, 2 for low, 1 for little to none, and 0 for none.

Real-time PCR

The miRNA identification with real-time PCR was performed with an ABI 7300 Fast Real-Time PCR System and a TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers used for *has-miR-22*, *-125a*, *-192*, *-199a*, *-199a**, *-200c*, *-214*, *-376a*, *-424*, and *RNU6B* (*U6*) were purchased from Applied Biosystems. *U6* was used as an endogenous control.

Results

Small RNA Library Sequencing of HIBEpiC and ICC Cell Lines

We collected a total of 2,592 cloned plasmids for sequencing analysis and obtained 35,759 small RNA clones (11,855, 11,506, and 12,398 clones from HIBEpiC, HuCCT1, and MEC, respectively). The average number of small RNA clones in a single sequencing reaction was 13.80 (35,759 clones/2,592 sequences). The small RNA clones were classified as follows (**Fig. 1A**): for HIBEpiC, human miRNA: 10,088 clones (176 genes); piRNA: 84; rRNA: 34; tRNA: 65; snoRNA: 3; mRNA: 44; for HuCCT1, human miRNA: 9,688 clones (123 genes); piRNA: 148; rRNA: 53; tRNA: 86; snoRNA: 0; mRNA: 53; and for MEC, human miRNA:10,767 clones (108 genes); piRNA: 113; rRNA: 28; tRNA: 14; snoRNA: 12; mRNA: 93. We achieved a high level of miRNA cloning efficiency; i.e., more than 80% of the small RNA clones in each line were miRNAs (HIBEpiC, 85.09%; HuCCT1, 84.20%; and MEC, 86.84%) (**Fig. 1B**). The size distribution of the cloned miRNAs in this study is shown in **Figure 1C**. The most

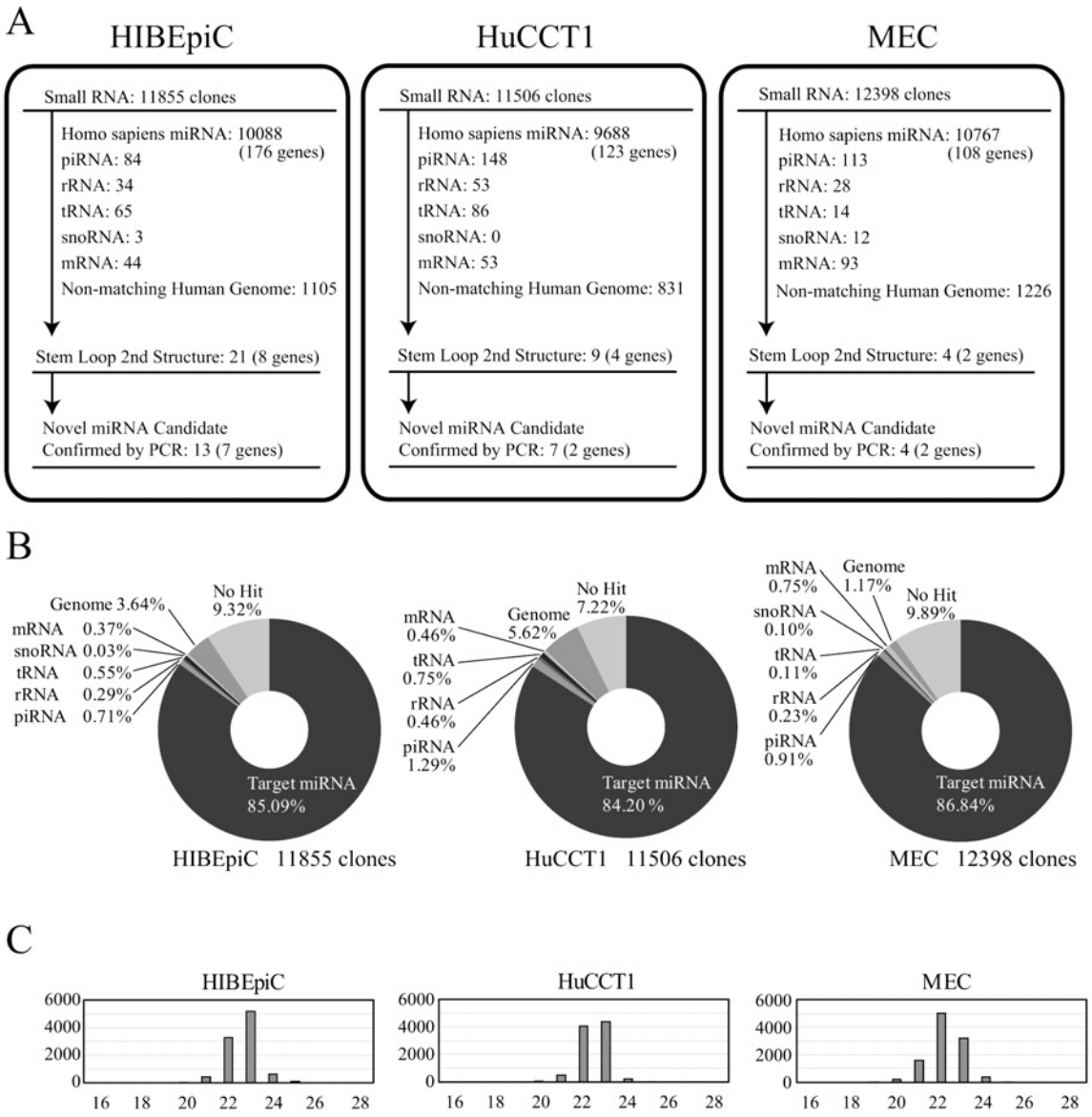

Fig. 1 Bioinformatic analysis of small RNA clones derived from a human normal intrahepatic biliary epithelial cell line (HIBEpiC) and 2 intrahepatic cholangiocarcinoma cell lines (HuCCT1 and MEC). (A) The types of RNAs identified in this study. Sequences composed of 16 to 30 nucleotides (nt) were extracted as valid small RNAs and compared with various RNA databases. (B) Classification of cloned miRNAs. Most (>80%) of the cloned small RNAs were miRNAs. (C) Size distribution of the cloned miRNAs. The nt length of each cloned miRNA is shown on the *x*-axis; the number of miRNA of each length is shown on the *y*-axis. Abbreviations: piwi-interacting RNA (piRNA); ribosomal RNA (rRNA); transfer RNA (tRNA); small nucleolar RNA (snoRNA); messenger RNA (mRNA).

abundant miRNAs in HIBEpiC and HuCCT1 were 23 nt in length, whereas those in MEC were 22 nt in length. This difference is largely due to the differential expression of *miR-21*, which is 23 nt in length.

Cloning Profiles of Known miRNAs in HIBEpiC and ICC Cell Lines

Human *miR-21* (*hsa-miR-21*) was the most highly cloned miRNA in each line (55.04% in HIBEpiC, 53.54% in HuCCT1, and 35.53% in MEC). The other highly cloned miRNAs (greater than 1% of the total miRNA clone population in each cell line) are presented in **Table 1**. Next, our sequencing analysis revealed 10 HIBEpiC-exclusive miRNAs (>0.1% in HIBEpiC and none in HuCCT1 and MEC): *miR-22* (*opposite*) (registered as *miR-22** in miRBase ver. 12.0), *-125a*, *-127*, *-146a*, *-146b*, *-199a* (*-199a-5p* in miRBase

Table 1 Cloning profiles of microRNAs (miRNAs) from the normal intrahepatic biliary cell line (HIBEpiC) and the intrahepatic cholangiocarcinoma (ICC) cell lines (HuCCT1 and MEC) by small RNA library sequencing

HIBEpiC				HuCCT1				MEC			
Mature miRNA	%[b]	Chr	Cluster[c]	Mature miRNA	%[b]	Chr	Cluster[c]	Mature miRNA	%[b]	Chr	Cluster[c]
Highly Cloned miRNAs[a]											
21	55.04	17		*21*	53.54	17		*21*	35.53	17	
221	9.15	X	C1	*221*	11.72	X	C1	*192*	20.75	11	C4
125b	4.05	11, 21		*200c*	2.26	12		*221*	9.41	X	C1
23a	1.91	19		*29b*	2.17	1, 7	C2	*29b*	4.47	1, 7	C2
29b	1.83	1, 7	C2	*31*	2.11	9		*20a*	2.28	13	
222	1.67	X	C1	*125b*	1.97	11, 21		*93*	2.22	7	C5
130a	1.65	11		*93*	1.92	7		*31*	2.17	9	
29a	1.49	7	C2	*29a*	1.78	7	C2	*194*	1.99	1, 11	C4
34a	1.48	1		*100*	1.51	11		*222*	1.91	X	C1
20a	1.33	13		*27a*	1.49	19	C3	*25*	1.33	7	C5
31	1.29	9		*222*	1.46	X	C1	*16*	1.26	3, 13	
*199a**	1.17	1, 19		*200b*	1.25	1		*26a*	1.25	3, 12	
				20a	1.23	13		*29a*	1.23	7	C2
				15b	1.15	3					
				23a	1.04	19	C3				
Predominant miRNAs[a]											
34a	1.48	1		*200b*	1.25	1		*192*	20.75	11	C4
*199a**	1.17	1, 19		*21* (*opposite*)	0.26	17		*194*	1.99	1, 11	C4
181b	0.21	1, 9						*25*	1.33	7	
								26a	1.25	3, 12	
								10b	0.13	2	
Exclusive miRNAs[a]											
199a	0.49	1, 19	C6	*200c*	2.26	12	C8	*196a*	0.79	12, 17	
368	0.46	14	C7	*200a*	0.82	1		*196b*	0.18	7	
214	0.28	1	C6	*7* (*opposite*)	0.27	9		*200a*	0.11	1	
22 (*opposite*)	0.21	17		*141*	0.14	12	C8				
376a	0.19	14	C7								
125a	0.17	19									
424	0.17	X									
127	0.15	14									
146a	0.12	5									
146b	0.12	10									

[a] Highly cloned miRNAs (greater than 1% of the total miRNA clone population in each cell line), predominant miRNAs (more than 5-fold difference between normal and malignant cell lines in cloning frequency, and greater than 0.1% in either cell line), and exclusive miRNAs (greater than 0.1% in each cell line and none in other cell lines).

[b] Percentage of miRNA genes in the total miRNA clone population in each cell line.

[c] Cluster (C1−8) indicates miRNA gene clusters.

Abbreviations: Chromosome (Chr).

ver. 12.0), *-214*, *-368* (registered as *miR-376c* in miRBase version 12.0), *-376a*, and *-424*, and 3 HIBEpiC-predominant miRNAs (HIBEpiC/HuCCT1 and HIBEpiC/MEC ratio of cloning frequency, each >5, and >0.1% in HIBEpiC): *miR-199a**, *-34a*, and *-181b* (**Table 1**). As for *miR-199a**, it may be better to be designated a HIBEpiC-exclusive miRNA rather than a HIBEpiC-predominant miRNA, since 118, 0, and 1 clone of *miR-199a** were obtained from HIBEpiC, HuCCT1, and MEC, respectively. Four HuCCT1-exclusive miRNAs were identified (>0.1% in HuCCT1 and none in HIBEpiC): *miR-7* (*opposite*) (registered as *miR-7-1** in miRBase version 12.0), *-141*, *-200a*, and *-200c*, in addition to 2 HuCCT1-predominant miRNA (HuCCT1/HIBEpiC ratio of cloning frequency >5, and >0.1% in HuCCT1): *miR-21* (*opposite*) (registered as *miR-21** in miRBase ver. 12.0), *miR-200b* (**Table 1**). Finally, there were 3 MEC-

Table 2 Paired-miRNAs Cloned in HIBEpiC, HuCCT1, and MEC

HIBEpiC					HuCCT1					MEC				
Pre-miRNA	Chr	5'-str	3'-str	Ratio of 5'/3'-str	Pre-miRNA	Chr	5'-str	3'-str	Ratio of 5'/3'-str	Pre-miRNA	Chr	5'-str	3'-str	Ratio of 5'/3'-str
21	17	5,552	5	1,110.400	*21*	17	5,187	25	207.480	*21*	17	3,826	7	546.571
125b-1	11	409	1	409.000	*125b-1*	11	191	1	191.000	*let-7b*	22	40	1	40.000
34a	1	149	2	74.500	*93*	7	186	1	186.000	*17*	13	70	2	35.000
17	13	51	1	51.000	*26a-1*	3	18	1	18.000	*let-7a-1, 2*	9, 11	54	2	27.000
15b	3	41	1	41.000	*106b*	7	22	2	11.000	*16-2*	3	136	6	22.667
181-2	9	67	2	33.500	*31*	9	204	31	6.581	*31*	9	234	17	13.765
let-7a-1, 2	9, 11	22	1	22.000	*151*	8	22	7	3.143	*let-7f-1*	9	9	1	9.000
let-7g	3	10	1	10.000	*181-2*	9	3	1	3.000	*106b*	7	30	7	4.286
99b	19	37	4	9.250	*15a*	13	5	3	1.667	*339*	7	4	1	4.000
151	8	22	3	7.333	*193a*	17	4	3	1.333	*151*	8	11	4	2.750
let-7d	9	7	1	7.000	*30d*	8	1	1	1.000	*425*	3	1	2	0.500
30a	6	10	2	5.000	*512-1, 2*	19	1	1	1.000	*574*	4	1	4	0.250
106b	7	10	3	3.333	*574*	4	1	2	0.500	*222*	X	4	206	0.019
let-7f-1	9	6	2	3.000	*324*	17	1	3	0.333	*29a*	7	2	132	0.015
22	17	21	7	3.000	*29c*	1	1	5	0.200					
31	9	130	44	2.955	*141*	12	2	14	0.143					
28	3	2	1	2.000	*7-1*	9	2	26	0.077					
154	14	5	3	1.667	*222*	X	2	141	0.014					
132	17	4	4	1.000	*23a*	19	1	101	0.010					
181c	19	1	1	1.000										
193a	17	5	5	1.000										
377	14	1	1	1.000										
379	14	1	1	1.000										
381	14	1	1	1.000										
574	4	5	5	1.000										
454	17	1	2	0.500										
199a-1, 2	1, 19	49	118	0.415										
455	9	1	5	0.200										
532	X	1	5	0.200										
127	14	1	15	0.067										
29a	7	1	150	0.007										
222	X	1	168	0.006										

Abbreviations: Chromosome (Chr); Clone count of 5'-strand miRNAs (5'-str); clone count of 3'-strand miRNAs (3'-str); Ratio of 5'/3'-strand miRNA clones (Ratio of 5'/3'-str).

exclusive miRNAs (>0.1% in MEC and none in HIBEpiC): *miR-196a*, *-196b*, and *-200a*, and 5 MEC-predominant miRNAs (MEC/HIBEpiC ratio of cloning frequency >5, and >0.1% in MEC): *miR-10b*, *-25*, *-26a*, *-192*, and *-194* (**Table 1**). **Table 1** summarizes miRNA signatures in each cell line. There was chromosome bias in the distribution of the cloned miRNA genes between the HIBEpiC and ICC cell lines. In chromosome 14, one cluster (*miR-368*, *-376a*, *-381*, *-382*, *-495*, and *-655*) was exclusively cloned in HIBEpiC (**Table 1**; percentage of *miR-368*, *-376a*, *-381*, *-382*, *-495*, and *-655* in the total miRNA clone population in HIBEpiC was 0.46, 0.19, 0.01, 0.04, 0.02, and 0.01, respectively). We also examined paired miRNAs that are the 5'- and 3'-strand miRNAs derived from the same pre-miRNAs. In this study, 49 pre-miRNA genes (stem-loop sequence) were detected (**Table 2**). As for some paired miRNA genes (i.e., *pre-miR-21*, *-31*, *-151*, *-222*, and *-574*), the cloning frequencies of the 5'- and 3'-strand mature miRNAs differed between the HIBEpiC and the ICC cell lines (**Table 2**). For example, in HIBEpiC, the ratio of the 5'/3'-strand mature miRNA clone count from *pre-miR-21* was 2 or more times as much as those in HuCCT1 and MEC, suggesting that expression of the 3'-strand mature miRNA (i.e., *miR-*

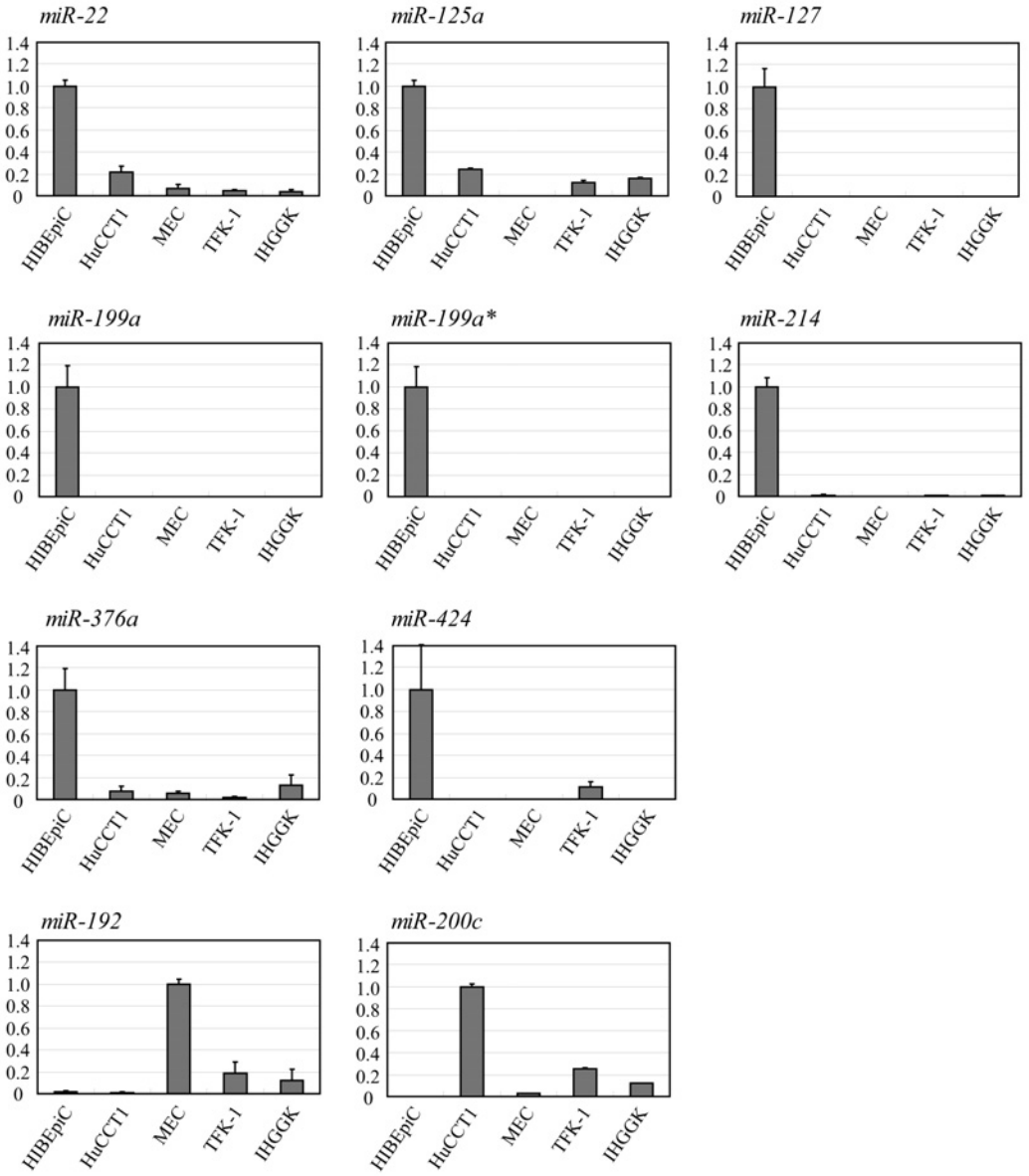

Fig. 2 Real-time PCR analysis of the expression of the 10 miRNAs (*miR-22*, *-125a*, *-127*, *-192*, *-199a*, *-199a**, *-200c*, *-214*, *-376a*, and *-424*) that showed exclusive or predominant expression in each cell line by cloning. Note that the expression levels of *miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424* in the human normal intrahepatic biliary cell line (HIBEpiC) were approximately 5 times higher than those in the cholangiocarcinoma cell lines (HuCCT1, MEC, TFK-1, and IHGGK). *RNU6B* was used as an internal control.

*21**) was upregulated in the ICC cell lines.

Validation of Biliary Epithelial Cell-Characteristic miRNA Signature by Real-time PCR

We next focused on the 10 miRNAs (*miR-22*, *-125a*, *-127*, *-192*, *-199a*, *-199a**, *-200c*, *-214*, *-376a*, and *-424*) that were the exclusively or predominantly expressed miRNAs shown in **Table 1**. Eight of the 10 miRNAs (*miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424*) were relatively abundant in HIBEpiC-exclusive miRNAs; the other 2 miRNAs (*miR-192* and *-200c*) were most abundant in MEC-predominant miRNAs and in HuCCT1-exclusive miRNAs, respectively (**Table 1**). We performed real-time PCR to confirm the differential expression of each between HIBEpiC and the 2 malignant ICC cell lines, HuCCT1 and MEC. We also included 2 other CC cell lines, TFK-1, an extrahepatic bile duct carcinoma cell line[20], and IHGGK, an ICC cell line, in

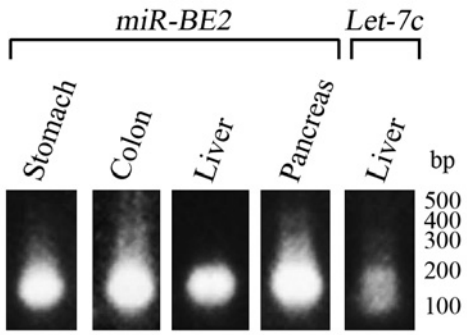

Fig. 3 Analysis of novel miRNAs by semiquantitative PCR. The expression level of *miR-BE2* in gastrointestinal tissues examined in this study is shown as a representative gel picture. The sizes of DNA marker fragments are indicted (bp). *Let-7c* in the liver is shown as a positive control.

our analysis. As shown in **Figure 2**, *miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424* were expressed predominantly in HIBEpiC (their expression levels in HIBEpiC were approximately 5 times higher than those in the 4 malignant cell lines). These results correlate well with our cloning results. *MiR-192* and *-200c* were also strongly detected in MEC and HuCCT1, respectively (**Fig. 2**).

Novel miRNAs

We obtained a total of 1,224 novel small RNA candidates (432, 647, and 145 clones from HIBEpiC, HuCCT1, and MEC, respectively) that matched the human genome but did not match any existing gene databases (miRNA, piRNA, rRNA, tRNA, snoRNA, and mRNA) (**Fig. 1A**). Secondary structural analysis showed that there were 10 genes of novel miRNA candidates that satisfied the stem-loop requirement for pre-miRNAs: 8 genes (21 clones) in HIBEpiC, 4 genes (9 clones) in HuCCT1, and 2 genes (4 clones) MEC (**Fig. 1A**). We further attempted to validate the presence of the 10 novel miRNA candidates with a PCR-based method[24]. Eight of the 10 candidates were detected in gastroenterological organs examined. **Figure 3** shows the expression of *miR-BE2* in gastroenterological organs examined in this study as a representative case. Specific bands for the other 2 candidates were undetectable (data not shown). We assigned tentative miRNA names (*mirR-BE1～8*) to the 8 novel miRNA genes in this study. The novel miRNAs were almost evenly expressed in most, if not all, gastrointesintal tissues examined (**Table 3**). Novel miRNA information, including sequence, location in the genome, and minimum free energy (ΔG), is shown in **Table 3**. The Sanger Data Base (miRBASE) was recently updated to version 12.0. After performing additional analysis of the 10 novel RNAs using miRBASE version 12.0, we found that 2 of the miRNAs (*miR-BE2* and *-BE6*)were newly registered miRNAs as *miR-320* and *-1260*, respectively (see **Table 3**). The novel miRNAs identified in this study were present in a very small percentage (≈ 0.1%) of all cloned miRNAs.

Discussion

The miRNAs are small non-coding RNAs involved in biological processes such as development, differentiation, apoptosis, and proliferation [2]. Moreover, there is increasing evidence of a correlation between miRNA expression and cancer[7]. In this report, we revealed miRNA profiles of human ICC based on small RNA library sequencing from 2 ICC cell lines and 1 normal intrahepatic epithelial cell line. In this study we obtained a number of miRNA clones (approximately 10,000 clones) from each cell line, because the frequency of cloning for individual miRNAs correlates well with their actual expression levels[22,25]. To our knowledge, this is the first report of miRNA profiling of the hepatobiliary system based on a cloning methodology. We identified 27 miRNAs that were expressed exclusively or predominantly in either the normal intrahepatic biliary epithelial cell line (i.e., HIBEpiC) or the ICC cell lines (see **Table 1**). Of these miRNAs, 8 miRNAs were specific for HIBEpiC (*miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424*). Furthermore, we identified several candidate novel miRNAs, most of which were demonstrated to be expressed in representative gastrointestinal tissues (see **Table 3**). For the identification of novel miRNAs, miRNA cloning has a great advantage over other methods, such as microarray analysis. Some of the novel miRNAs we identified may be useful biomarkers for the diagnosis of certain pathological

Table 3 The novel miRNAs identified in this study[a)]

miR[b)]	Sequence	Chr	Start	End	HiB-Epic	HuC-CT1	MEC	ΔG	Expression Level[c)]			
									S	C	L	P
BE 1	ATAGGACTCATATAGTGCCAG	1	66866756	66866776	1	0	0	−38.9	2	4	3	4
BE 2[d)]	AAAAGCTGGGTTGAGAGGGCAAA	1	222511373	222511395	0	0	1	−42.2	4	4	3	4
BE 3	ATCCCCAGATACAATGGACAAT	2	207683011	207683032	1	0	0	−36.4	0	0	1	1
BE 4	AAGAGATTGGCATGCTG	3	180434616	180434632	2	0	0	−30.7	1	1	2	1
BE 5	ACCCCACTCCTGGTACCA	8	10561908	10561925	1	2	3	−31.1	3	3	3	3
BE 6[d)]	ATCCCACCACTGCCACCA	11	95714259	95714276	6	5	0	−55.4	2	3	3	3
BE 7	CAGGAAGGAGGGCGGCGGAG	15	38520410	38520429	1	0	0	−68.8	3	4	3	4
BE 8	AGGCAGAAGTGGGGCTGACAGG	16	2096720 or 15135473	2096741 15135494	1	0	0	−59.9	1	3	3	2

[a)] Cloned cDNA sequence, the number of cloned cDNAs, mature miRNA sequences, the miRNA locus in the human genome, the minimum free energy (ΔG) of the miRNA/miRNA binding site duplex, and the expression levels of the miRNAs in gastroenterological organs.

[b)] We assigned tentative miRNA names (*mirR-BE1−8*) to the 8 genes in this study.

[c)] The expression levels of the miRNAs were classified as follows: 4 for high, 3 for medium, 2 for low, 1 for little to none, and 0 for none.

[d)] *MiR-BE2* and *-BE6* are newly registered as *miR-320* and *-1260*, respectively in miRBase ver. 12.0.

Abbreviations: Chromosome (Chr); stomach (S); colon (C); liver (L); pancreas (P).

states.

The most frequently cloned miRNA in all 3 cell lines was *miR-21*; *miR-221* was the second most highly cloned in HIBEpiC and HuCCT1, and *miR-125b* was the third most highly cloned in HIBEpiC. Our results are somewhat inconsistent with those of a recent study of a human cholangiocyte H69 cell line, in which the increased levels of *miR-31*, *-95*, and *-125a* in cholangiocytes were demonstrated[19]. The change in expression of *miR-125a* is in good agreement between both studies. The expression of *miR-31* was also upregulated in our study; however, we excluded this miRNA from the list of normal biliary epithelial cell-predominant miRNAs because the ratio of the *miR-31* cloning frequency (HuCCT1/HIBEpiC and MEC/HIBEpiC) was less than 5. On the other hand, *miR-95* was not detected specifically or predominantly in HIBEpiC in our study. It appears that the discrepancy between the reports may stem from subtle differences in the methods and samples used. Although the previous study was performed with an miRNA microarray technique [19], distinguishing highly homologous miRNAs (*e.g. hsa-let-7* families) by means of relatively short probes is technically difficult. Furthermore, the previous study was performed with cell lines derived from a metastatic gallbladder carcinoma, a common bile duct carcinoma, and an intrahepatic mixed cholangiocellular carcinoma[19]. In contrast, we used a nonmalignant human intrahepatic biliary epithelial cell line and ICC cell lines.

The thermodynamic stability of the 5'-strand and the 3'-strand in the stem-loop structure of a pre-miRNA have been considered important for preferential selection of the less stable one (designated as the miRNA or guide strand) and elimination of the other one (designated as the miRNA* or passenger strand). However, the strand selection in pre-miRNAs occurs in a tissue-dependent manner[22,26]. In the present study, we observed differential strand selection of some paired miRNA genes between normal and tumor cell lines (see **Table 2**). The different steady-state levels of each strand of a miRNA pair may be independently regulated in different cells, as Ro *et al.* have suggested[26]. Therefore, it is likely that our data reflect differences in miRNA-based posttranscriptional regulation between normal cells and cancer cells.

In conclusion, we have determined miRNA profiles of ICC *in vitro* using a normal intrahepatic epithelial cell line and 2 ICC cell lines. By small RNA library sequencing and subsequent real-time PCR analysis, we found 8 biliary epithelial cell-specific miRNAs

(*miR-22*, *-125a*, *-127*, *-199a*, *-199a**, *-214*, *-376a*, and *-424*) that were downregulated in the ICC cell lines. As the downregulation of hepatocyte-specific *miR-122* is associated with the development of hepatocellular carcinoma[27], the biliary epithelial cell-specific miRNAs identified in this study may serve as potential biomarkers for ICC. Further functional and pathological studies are needed to address the possibilities of miRNA-mediated posttranscriptional regulation and carcinogenesis in the hepatobiliary system.

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