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miR-124 and *miR-203* are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that, in general, negatively regulate gene expression. They have been identified in various tumor types, showing that different sets of miRNAs are usually deregulated in different cancers. Some miRNA genes harboring CpG islands undergo methylation-mediated silencing, a characteristic of many tumor suppressor genes. To identify such miRNAs in hepatocellular carcinoma (HCC), we first examined the methylation status of 43 loci containing CpG islands around 39 mature miRNA genes in a panel of HCC cell lines and non-cancerous liver tissues as controls. Among 11 miRNA genes frequently methylated in HCC cell lines but not in non-cancerous liver tissues, three miRNA genes, i.e. *miR-124*, *miR-203* and *miR-375*, were selected as silenced miRNAs through CpG-island methylation by comparing methylation and expression status and evaluating restored expression after treatment with 5-aza-2′-deoxycytidine. In primary tumors of HCC with paired non-tumorous liver tissues, only *miR-124* and *miR-203* showed frequent tumor-specific methylation, and their expression status was inversely correlated with methylation status. Ectopic expression of *miR-124* or *miR-203* in HCC cells lacking their expression inhibited cell growth, with direct downregulation of possible targets, cyclin-dependent kinase 6 (*CDK6*), vimentin (*VIM*), SET and MYND domain containing 3 (*SMYD3*) and IQ motif containing GTPase activating protein 1 (*IQGAP1*) or ATP-binding cassette, subfamily E, member 1 (*ABCE1*), respectively. Our results suggest that *miR-124* and *miR-203* are novel tumor-suppressive miRNAs for HCC epigenetically silenced and activating multiple targets during hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide (1). It shows a wide geographical variation with high incidence areas in Africa and Asia. In endemic areas, HCC is strongly associated with chronic viral infections of hepatitis types B and C and liver cirrhosis (2). Despite the growing wealth of knowledge on the biology of HCC, little is known about the transcriptional/post-transcriptional regulation of candidate oncogenes and/or tumor suppressor genes (TSGs). Like many other tumors, HCC seems to develop via a multistep process with an accumulation of genetic and epigenetic alterations in regulatory genes or mechanisms, leading to the activation of oncogenes and inactivation of TSGs. An understanding of the molecular mechanisms behind the progression of HCC is crucial to developing effective treatments for this disease.

Abbreviations: COBRA, combined bisulfite restriction analysis; dsRNA, double-stranded RNA; HCC, hepatocellular carcinoma; miRNA, microRNA; PCR, polymerase chain reaction; TSG, tumor suppressor gene; UTR, untranslated region.

MicroRNAs (miRNAs) are an abundant class of endogenous, small, non-coding RNAs, the products of which are small single-stranded RNAs of 19–22 nucleotides with a primary role in post-transcriptional silencing generally through binding to the 3′-untranslated region (UTR) of protein-coding transcripts, in turn triggering messenger RNA degradation or translational repression (3). The recent demonstration of the differential expression of miRNAs and their target messenger RNAs in cancer and the function of some miRNAs as oncogenes or TSGs has spurred considerable interest in elucidating their role in tumorigenesis (4–8). In human cancers, the expression of miRNAs is generally downregulated in malignant tissues compared with the corresponding non-malignant tissues (5,9), suggesting that at least some of these miRNAs act as TSGs in specific tumors, although the expression of selected miRNAs can be increased (OncoMir) (6).

Among various cancer-related gene-silencing mechanisms in the epigenetic pathways, DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many TSGs (10) and several tumor-suppressive miRNAs (11). Recently, in fact, DNA methylation-mediated downregulation of miRNAs by proximal CpG islands has been described by a number of groups, including ours (12–15), although only subsets of miRNA genes contain CpG islands upstream or around them. The altered expressions of several miRNAs and their contribution to tumor growth and/or spread have been described in HCC (16,17). However, tumor-suppressive miRNAs in HCC with DNA methylation-associated silencing remain largely unknown (17,18).

In the study presented here, we focused on miRNA genes containing CpG islands upstream or around them and screened miRNAs with tumor-specific methylation and silencing in cell lines and primary samples of HCC to identify epigenetically inactivated miRNAs leading to HCC. Through this approach, two possible HCC-associated tumor-suppressive miRNAs, *miR-124* and *miR-203*, and novel candidates for their putative targets were identified.

Materials and methods

Cell lines and primary tumor samples

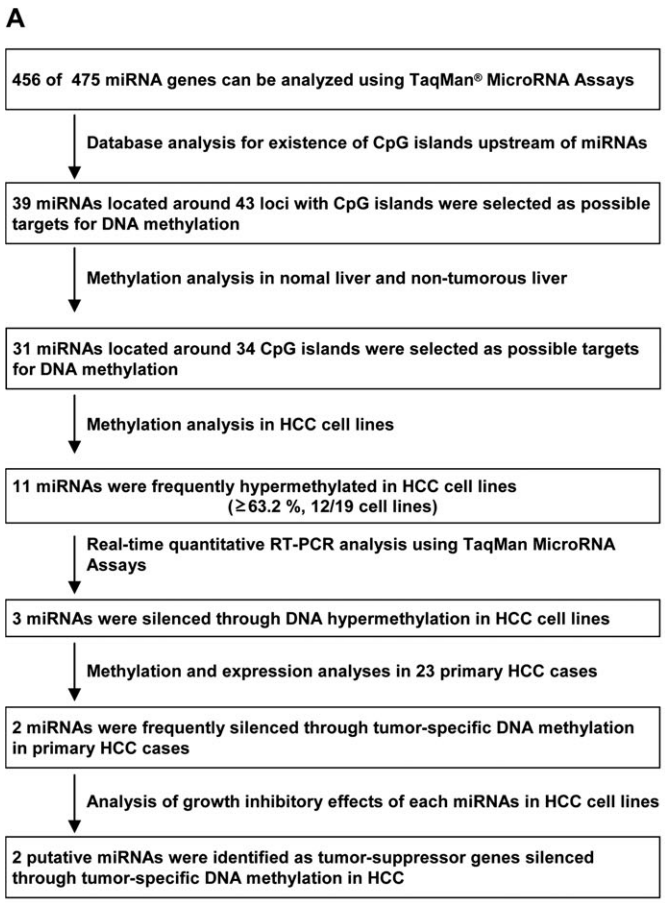
A total of 19 liver cancer cell lines (cHc4, Hep 3B, Hep G2, Hep-KANO, Hep-TABATA, HLE, HLF, huH-1, HUH-6, Huh7, JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, JHH-7, Li7, PLC/PRF/5 and sK-Hep-1) were examined in this study. All cell lines were maintained in appropriate media supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), 2 mM glutamine and 2–10% fetal bovine serum. To analyze the restoration of genes of interest, cells were cultured with or without 10 μM of 5-aza-2′-deoxycytidine for 5 days.

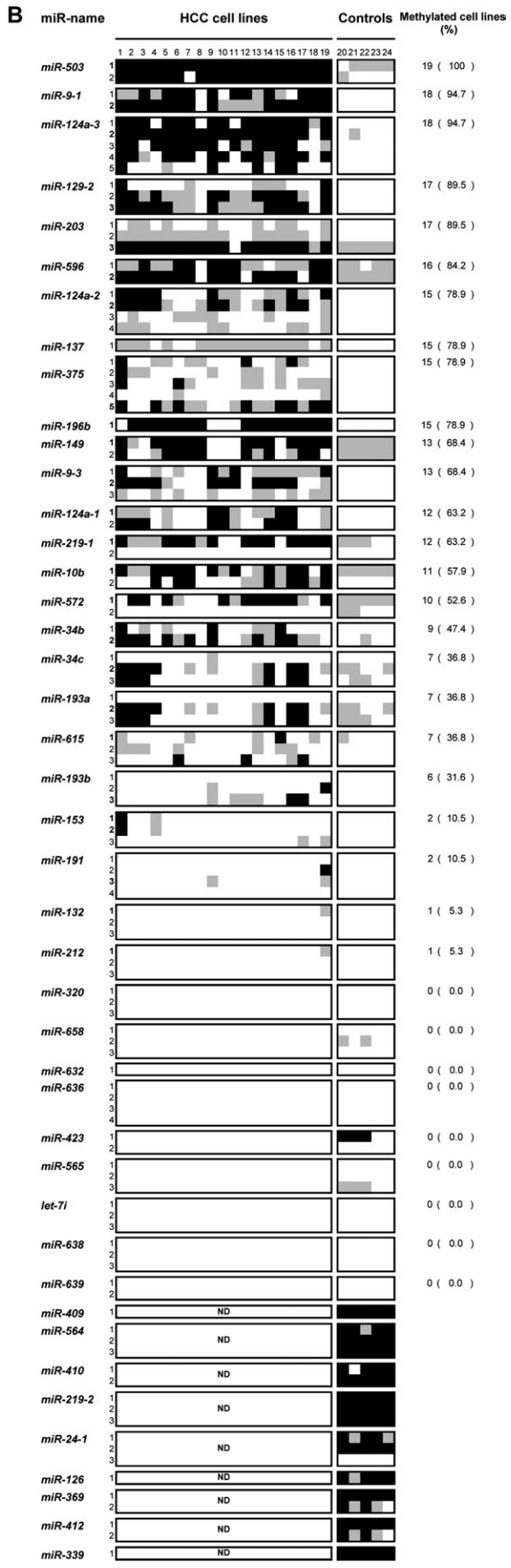
A total of 41 frozen primary tumor samples and corresponding non-tumorous tissue were obtained from HCC patients (stage II, 13 cases; stage III, 16 cases; stage IVA, 9 cases and stage IVB, 3 cases), and two frozen normal liver tissues were obtained from patients with hepatectomy due to metastatic liver tumor treated at Tokyo Medical and Dental University with written consent from each patient in the formal style and after approval by the local ethics committee (supplementary Table S1 is available at *Carcinogenesis* Online). The tumor-nodes-metastasis classification of Union International Contre le Cancer was used.

Genomic DNA and total RNA were extracted from the cell lines and the frozen tissues using a Genomic DNA Purification kit (Gentra, Minneapolis, MN) and Isogen (Nippon Gene, Toyama, Japan), respectively, according to the manufacturer's instructions.

Methylation analysis

Genomic DNA was treated with sodium bisulfite and subjected to a polymerase chain reaction (PCR) using primer sets designed to amplify regions of interest (supplementary Table S2 is available at *Carcinogenesis* Online). For the combined bisulfite restriction analysis (COBRA), PCR products were digested with BstUI or TaqI and electrophoresed (19). After the gels were stained with ethidium bromide, the intensities of methylated alleles (as a percentage) were calculated by densitometry using MultiGauge 2.0 (Fuji Film, Tokyo, Japan).





A methylation density cutoff point of 20% was considered significant as described elsewhere (20). For the bisulfite sequencing analysis, the PCR products were subcloned and then sequenced.

Real-time reverse transcription–PCR

Real-time reverse transcription–PCR was performed using an ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA), TaqMan®

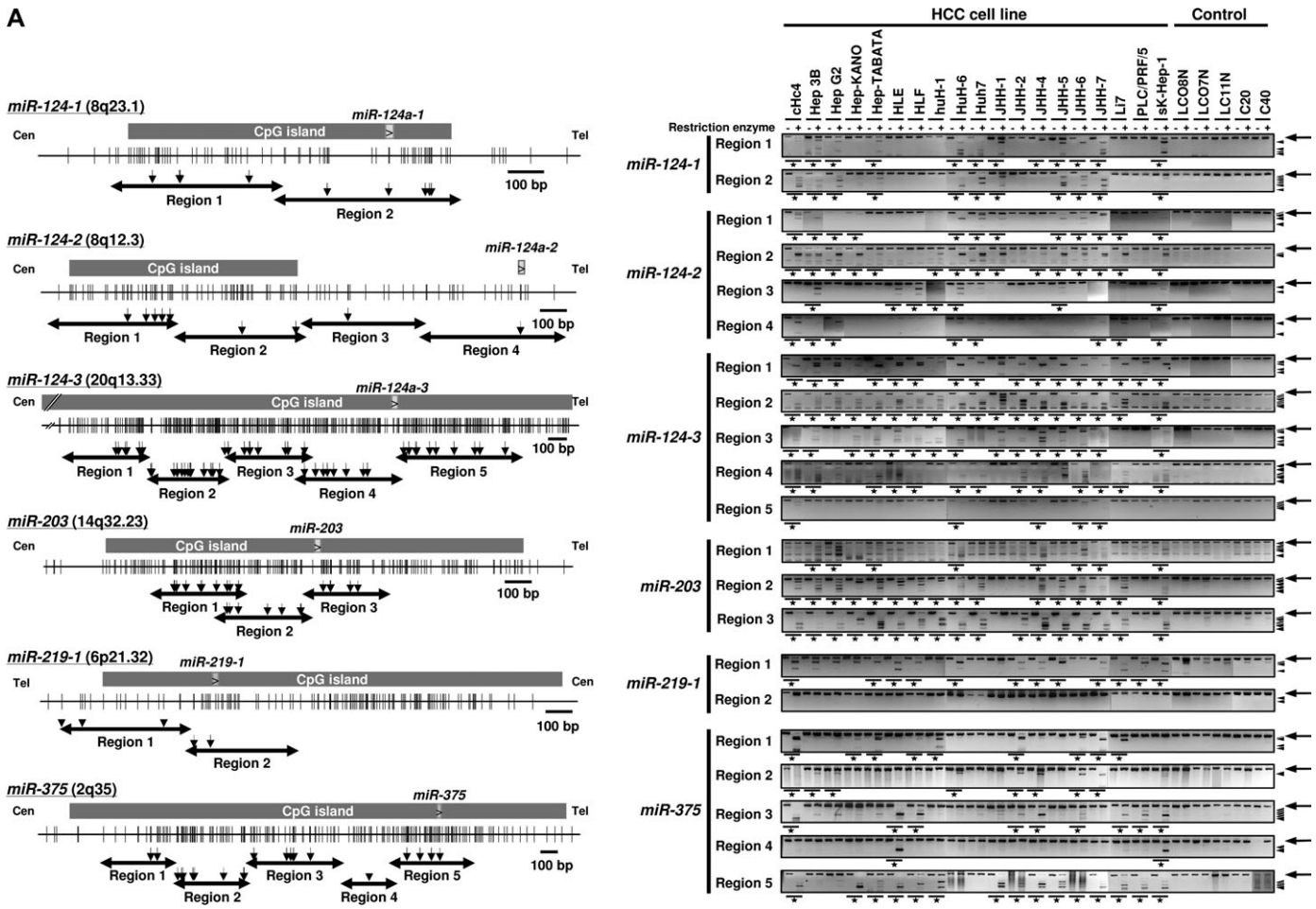


Fig. 2. Analysis for the correlation between methylation and expression status of four candidate miRNAs in HCC cell lines. (**A**) Left: maps of miRNAs, CpG islands, CpG sites and PCR products used for COBRA and bisulfite sequencing. Dark gray boxes, CpG islands; light gray boxes, miRNAs; vertical tick marks, CpG sites; closed arrows, PCR products (primers; supplementary Table S2, available at *Carcinogenesis* Online). For COBRA, PCR products were restricted by BstUI (vertical downward arrows) or TaqI (vertical downward arrowheads). The size of each PCR product is shown under closed arrow. Right: the results of COBRA in HCC cell lines and control non-tumorous liver tissues. Arrows, unmethylated alleles; arrowheads, methylated alleles; stars, samples with significant restricted fragments from methylated alleles (see supplementary Table S5, available at *Carcinogenesis* Online). See legend of Figure 1B for interpretation. (**B**) Expression levels of candidate miRNAs in 19 HCC cell lines and non-tumorous liver tissue. Methylation status determined by COBRA is shown under the expression status of each miRNA. Asterisks indicate frequencies of HCC cell lines, in which DNA methylation accorded with a remarkable downregulation of each candidate miRNA expression (<0.5-fold expression compared with C20 control): the denominator in parentheses is the number of HCC cell lines, in which restricted fragments from methylated alleles were detected, and the numerator is the number of HCC cell lines, in which both DNA methylation around miRNAs and downregulation of expression were detected. (**C**) Effect of treatment with 10 μM 5-aza-2′-deoxycytidine (5-aza-dCyd) on expression of candidate miRNAs in HCC cell lines. Relative expression levels of candidate miRNAs in HCC cell lines are normalized with their expression in non-tumorous liver tissue (C20), and effects of 5-aza-2′-deoxycytidine on expression of miRNAs in each cell line are shown by fold increase relative to their expression without 5-aza-2′-deoxycytidine treatment. Methylation status determined by COBRA is shown under the expression status of each miRNA. Asterisks indicate frequencies of HCC cell lines, in which DNA methylation accorded with restoration of each candidate miRNA expression: the denominator in parentheses is the number of HCC cell lines, in which restricted fragments from methylated alleles were detected, and the numerator is the number of HCC cell lines, in which both DNA methylation around miRNAs and restoration of expression by 5-aza-2′-deoxycytidine treatment were detected.

Fig. 1. Strategy used in this study and results of a methylation-based analysis of miRNAs in a panel of HCC cell lines and control liver tissues. (**A**) Overview of the screening approach used. A combination of experiments *in silico*, molecular analyses in human HCC cell lines and the analysis of primary human HCC specimens with corresponding non-cancerous liver tissues and normal liver was employed for the identification of tumor-suppressive miRNAs silenced by differential methylation in human HCC. (**B**) Summary of DNA methylation status of CpG islands around 39 mature forms of miRNAs located at 43 loci (within 500 bp upstream of each miRNA, http://genome.ucsc.edu/; supplementary Table S4, available at *Carcinogenesis* Online) in 19 HCC cell lines (1–19) and control non-tumorous liver tissues (20–24) determined by COBRA (Figure 2A and data not shown). 1: cHc4; 2: Hep 3B; 3: Hep G2; 4: Hep-KANO; 5: Hep-TABATA; 6: HLE; 7: HLF; 8: huH-1; 9: HuH-6; 10: Huh7; 11: JHH-1; 12: JHH-2; 13: JHH-4; 14: JHH-5; 15: JHH-6; 16: JHH-7; 17: Li7; 18: PLC/PRF/5; 19: sk-Hep-1; 20: LC08N; 21: LC07N; 22: LC11N; 23: C20; 24: C40. For COBRA, PCR products were digested with BstUI or TaqI and electrophoresed (19). After the gels were stained with ethidium bromide, the intensities of methylated alleles as a percentage were calculated by densitometry. A methylation density cutoff point of 20% was considered significant (20). Black, gray and white boxes indicate complete, partial and no digestion, respectively, by restriction enzymes. Since nine of 43 loci were hypermethylated in non-cancerous liver tissues, we excluded them from further analysis in the HCC cell lines (ND, not determined). Notably, 14 loci for 11 mature forms of miRNA were frequently (>60%) hypermethylated in HCC cell lines compared with control non-cancerous liver tissue.

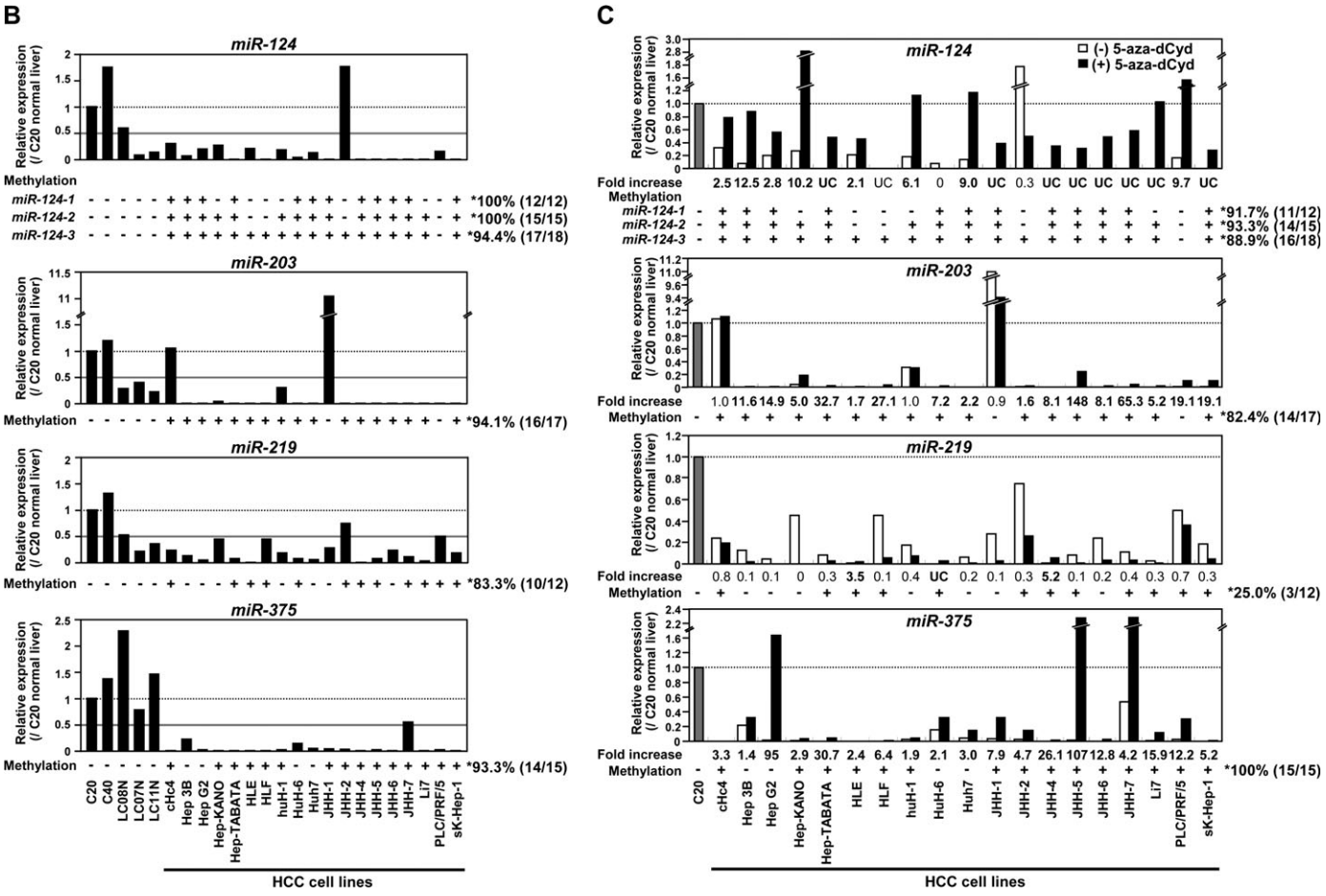


Fig. 2. *continued.*

Universal PCR Master Mix (Applied Biosystems), TaqMan® Reverse Transcription Kit (Applied Biosystems) and TaqMan® MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. Expression levels of miRNA genes were based on the amount of the target message relative to that of the *RNU6B* transcript as a control, to normalize the initial input of total RNA (15).

Transfection with synthetic miRNAs

In total, 10 nM of Pre-miR™ miRNA Precursor Molecule (Ambion, Austin, TX) mimicking *miR-124* or *miR-203* or control non-specific miRNA (Pre-miR™ Negative Control #1, NC#1; Ambion) was transfected into HCC cell lines using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The numbers of viable cells 24–96 h after transfection were assessed by the colorimetric water-soluble tetrazolium salt assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). The cell cycle was evaluated 48 h after transfection by a fluorescence-activated cell sorting as described elsewhere (15). Apoptosis was detected 48 h after transfection by enzymatic labeling of DNA strand breaks using a TdT-mediated dUTP-biotin nick-end labeling staining (MEBSTAIN Apoptosis Kit Direct; Medical Biological Laboratories, Japan) (15). High transfection efficiency of synthetic double-stranded RNA (dsRNA) using Lipofectamine™ RNAiMAX (Invitrogen) in each cell line had been confirmed by (i) a preliminary study using non-specific control small interfering RNA labeled with a fluorescent dye (siGLO RISC-Free small interfering RNA; Dharmacon, Lafayette, CO; data not shown) and (ii) the elevated expression level of mature *miR-124* or *miR-203* after transfection of each dsRNA (data not shown).

miRNA target predictions, western blotting and luciferase activity assay

Predicted targets of *miR-124* or *miR-203* and their target sites were analyzed using miRanda (http://microrna.sanger.ac.uk/sequences/index.shtml), TargetScan (http://www.targetscan.org/), PicTar (http://pictar.bio.nyu.edu/) and microRNA.org (http://www.microrna.org/).

Cells transfected with 10 nM of Pre-miR™ miRNA Precursor Molecule (Ambion) mimicking *miR-124* or *miR-203* or control Pre-miR™ NC #1 (Ambion) using Lipofectamine™ RNAiMAX (Invitrogen) were lysed at an appropriate time after transfection, and cell lysates were analyzed by western blotting using anti-E2F transcriptional factor 6 (Abcam, Cambridge, UK), anti-ATP-binding cassette, subfamily E, member 1 (ABCE1; Abcam), anti-cyclin-dependent kinase 6 (CDK6; Cell Signaling Technology, Beverly, MA), anti-vimentin (VIM; Santa Cruz Biotechnology, Santa Cruz, CA), anti-SET and MYND domain containing 3 (SMYD3; Proteintech Group, Chicago, IL), anti-IQ motif containing GTPase activating protein 1 (IQGAP1; BD Biosciences, San Jose, CA) and anti-β-actin (Sigma, St Louis, MO) antibodies as described elsewhere (15).

Luciferase constructs were made by ligating fragments containing the 3′-UTR target sites of genes of interest prepared by genomic PCR (supplementary Table S3 is available at *Carcinogenesis* Online) downstream of the *luciferase* gene in pMIR-REPORT luciferase vector (Ambion). Then, 0.4 μg of each construct was cotransfected with 0.02 μg of pRL-CMV vector (Promega, Madison, WI) containing *Renilla* luciferase and 10 nM of Pre-miR™ miRNA Precursor Molecule (Ambion) mimicking *miR-124* or *miR-203* or control non-specific miRNA (Pre-miR™ NC #1; Ambion) into HCC cell lines using Lipofectamine™ 2000 (Invitrogen). Twenty-four hours after transfection, firefly and *Renilla* luciferase activity were measured using the Dual-Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate.

Statistical analysis

Differences between subgroups were tested with the Mann–Whitney *U*-test or Student's *t*-test.

Results

Methylation analysis of miRNAs in HCC cell lines and control non-tumorous liver tissues

The present study was designed to identify tumor-suppressive miRNAs silenced by tumor-specific CpG-island methylation in HCC. Strategy

Table I. Methylation and expression status of 14 miRNAs in 19 HCC cell lines

No.	miRNA[a]	CpG-island methylation		Expression	
		Region[a]	Methylated (% total)	Downregulated (% methylated)[b]	Restoration by 5-aza-2′-deoxycytidine treatment (% methylated)[c]
1	*miR-503*	1	19 (100.0)	9/19 (47.4)	ND
2	*miR-9-1*	2	18 (94.7)	6/18 (33.3)	ND
3	*miR-124-3*	2	18 (94.7)	17/18 (94.4)	16/18 (88.9)
4	*miR-129-2*	3	17 (89.5)	6/17 (35.3)	ND
5	*miR-203*	3	17 (89.5)	16/17 (94.1)	14/17 (82.4)
6	*miR-596*	2	16 (84.2)	12/16 (75.0)	ND
7	*miR-124-2*	2	15 (78.9)	>15/15 (100.0)	14/15 (93.3)
8	*miR-137*	1	15 (78.9)	4/15 (26.7)	ND
9	*miR-375*	5	15 (78.9)	14/15 (93.3)	15/15 (100.0)
10	*miR-196b*	1	15 (78.9)	0/15 (0.0)	ND
11	*miR-149*	1	13 (68.4)	1/13 (7.7)	ND
12	*miR-9-3*	2	13 (68.4)	6/13 (46.2)	ND
13	*miR-124-1*	1	12 (63.2)	12/12 (100.0)	11/12 (91.7)
14	*miR-219-1*	1	12 (63.2)	10/12 (83.3)	3/12 (25.0)

ND, not determined. Grey background indicates miRNAs that was frequently downregulated in cell lines with CpG-island methylation (>80% in methylation-positive cell lines).
[a]Most frequently methylated regions around CpG-island of each miRNA detected by COBRA in 19 HCC cell lines.
[b]Numbers of cell lines that showed both DNA methylation of CpG island and downregulation of expression (≤0.5-fold decrease compared with C20 control non-tumorous liver tissue) in each miRNA.
[c]Numbers of cell lines that showed both DNA methylation and restoration of expression after treatment with 5-aza-2′-deoxycytidine (>1.5-fold increase compared with non-treatment control) in each miRNA.

used in this study and results of methylation-based analyses of miRNAs are shown in Figure 1A and B. A search of miRBase (http://microrna.sanger.ac.uk/) and the human genome database browser (University of California Santa Cruz Genome Bioinformatics, http://genome.ucsc.edu/) revealed that, as of October 2007, 456 and 475 miRNAs were listed in the human genome, respectively. Among them, as listed in supplementary Table S4 (available at *Carcinogenesis* Online), 39 mature forms of miRNAs located at 43 loci contained CpG islands around them (within 500 bp upstream). Since multiple copies of some mature miRNAs are transcribed from different genomic loci, the number of mature forms of miRNAs is smaller than the number of genomic loci.

To explore possible target miRNAs for tumor-specific methylation in HCC, we first determined the methylation status around the 43 miRNA CpG islands in normal liver tissue (C20 and C40) and non-tumorous liver tissue from HCC patients (LC07N, LC08N and LC11N) using the COBRA (Figures 1B and 2A; supplementary Table S5 is available at *Carcinogenesis* Online; data not shown). Since nine miRNA CpG islands, i.e. *miR-409*, *-564*, *-410*, *-219-2*, *-24-1*, *-126*, *-369*, *-412* and *-339*, were highly methylated in normal and/or non-tumorous liver tissue, we excluded them from further analyses (Figure 1B and Table I).

We then determined the methylation status of 34 miRNA CpG islands in a panel of 19 HCC cell lines and identified the frequent (>60%) methylation of 14 miRNA CpG islands, for 11 mature miRNAs (Figure 1B). Notably, *miR-124* is represented in three different genomic loci, and all the corresponding CpG islands in three forms [*miR-124-1* (8p23.1), *miR-124-2* (8q12.3) and *miR-124-3* (20q13.33)] showed relatively frequent methylation in HCC cell lines (12/19, 63.2%; 15/19, 78.9% and 18/19, 94.7%, respectively) compared with control non-cancerous liver tissues (Figures 1B and 2A; supplementary Table S5 is available at *Carcinogenesis* Online).

Methylation and expression of candidate miRNAs in 19 HCC cell lines relative to normal liver tissues

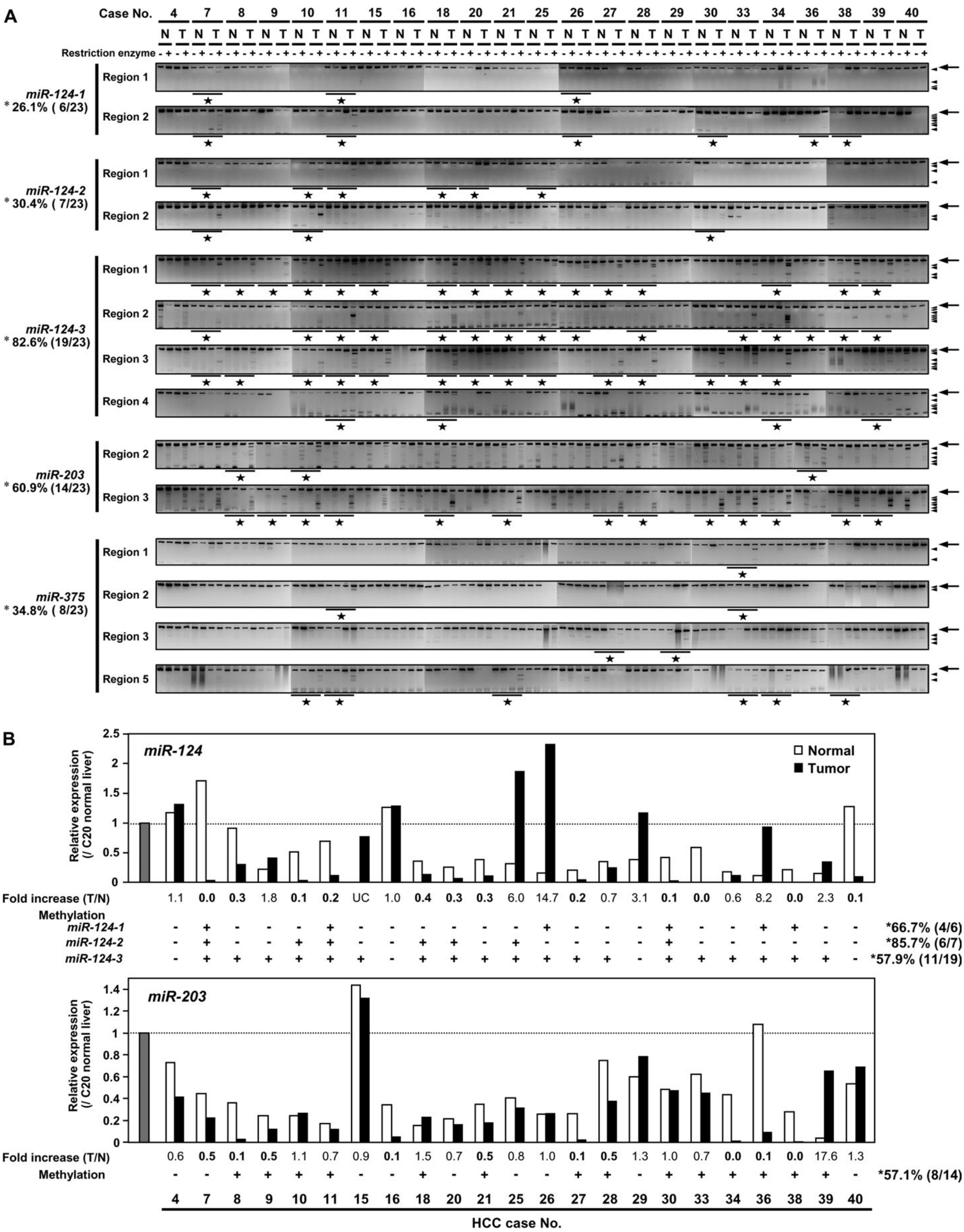
In order to assess the significance of the methylation of CpG islands to miRNA expression, we next determined the correlation between the expression levels of 11 mature miRNAs and the methylation status of their CpG islands in 19 HCC cell lines and control non-cancerous liver tissues (Figure 2B; supplementary Figure S1 is available at *Carcinogenesis* Online). Among 11 miRNAs, *miR-124*, *miR-203*, *miR-219* and *miR-375* were likely to show methylation-dependent downregulation of their expression in HCC cell lines compared with normal liver tissue (Table I): >50% decrease in expression of each miRNA was observed in >80% of CpG-island-methylated cell lines compared with normal liver tissue (C20). Since all methylation density values of CpG islands of those four miRNAs determined by COBRA were lower than a cutoff point of 20%, which we used as described elsewhere (20), in control non-cancerous liver tissues, we adopted this cutoff point for further analysis in primary tumors.

On demethylation of the HCC cell lines with 5-aza-2′-deoxycytidine treatment, the expression of *miR-124*, *miR-203* and *miR-375* was restored at a high frequency, whereas that of *miR-219* was not restored (Figure 2C), suggesting the expression of *miR-124*, *miR-203* and *miR-375* to mainly be suppressed by the aberrant methylation of their CpG islands in HCC cell lines (Table I).

Consistent with the results of COBRA, aberrant DNA methylation of *miR-124-1*, *miR-124-2*, *miR-124-3*, *miR-203* and *miR-375* CpG islands was also shown by bisulfite sequencing in the HCC cell lines lacking the expression of these three miRNAs, but not in normal liver tissue and cell lines expressing these miRNAs (supplementary Figure S2 is available at *Carcinogenesis* Online).

Methylation and expression analyses of miR-124, miR-203 and miR-375 in primary HCC cases

To determine whether the methylation of *miR-124*, *miR-203* and *miR-375* also occurs in primary HCCs in a tumor-specific manner, the methylation status of regions within five CpG islands, which showed frequent hypermethylation in the HCC cell lines, for these three mature forms of miRNA was determined by COBRA initially in 23 primary HCCs (stage II, eight cases; stage III, six cases; stage IVA, six cases and stage IVB, three cases) and corresponding non-cancerous liver tissues (the first set; supplementary Table S5 is available at *Carcinogenesis* Online). We considered the specimens in which significant restricted fragments from methylated alleles (more than a cutoff point of 20%) were detected with higher density by densitometry than in paired normal tissues to be tumor-specific hypermethylation-positive, although there were a few cases in which restricted fragments from methylated alleles were observed in normal tissue. Tumor-specific methylation of *miR-124-1*, *miR-124-2* and *miR-124-3* was detected in 26.1% (6 of 23), 30.4% (7 of 23)



and 82.6% (19 of 23) of primary HCCs, respectively (Figure 3A and Table II), indicating that at least one of the genomic loci for *miR-124* was methylated in 82.6% of primary cases. Since frequent tumor-specific methylation in primary HCCs was also observed in *miR-203* (60.9%, 14 of 23) but not in *miR-375* (34.8%, 8 of 23; Figure 3A) by COBRA, *miR-124* and *miR-203* became our prime candidates for tumor-specific hypermethylation in HCC. The hypermethylation of these CpG islands was also confirmed by bisulfite sequencing of selected positive cases (supplementary Figure S3 is available at *Carcinogenesis* Online).

Expression levels of *miR-124* and *miR-203* in tumors compared with paired non-tumorous liver tissue were reduced (>50% decrease) in 57.9% (11 of 19) and 57.1% (8 of 14) of primary HCC cases, which showed tumor-specific methylation, respectively (Figure 3C and Table II). On the other hand, the expression of *miR-124* and *miR-203* in unmethylated cases was infrequently decreased in tumors compared with corresponding non-tumorous tissue (25%, one of four in *miR-124* and 22.2%, two of nine in *miR-203*), suggesting that those miRNAs also show an inverse correlation between their expression and CpG-island methylation status in primary HCCs.

To further determine an association between CpG-island methylation status of *miR-124* and *miR-203* and clinicopathologic characteristics of HCC in primary cases, we performed a further series of methylation analysis using additional 18 primary HCCs with corresponding non-tumorous tissues (the second set; supplementary Tables S1 and S5 are available at *Carcinogenesis* Online). Although a relatively small numbers of cases (total of 41 cases) were available for analysis, the tumor-specific methylation of *miR-124-2* CpG island in HCC were significantly associated with younger age of patients and multiple tumor nodules and tended to be associated with cancer recurrence. No clinicopathologic difference was observed between tumor-specific methylation-positive and -negative cases in *miR-203* CpG island (supplementary Table S1 is available at *Carcinogenesis* Online) and in *miR-124-1* and *-3* CpG islands (data not shown).

Table II. Methylation and expression status of miRNAs in 23 HCC cases

miRNA[a,b]	HCC cases	
	Methylated (% total)[c]	Downregulated (% methylated)[a,b]
miR-124-1	6 (26.1)	4 (**66.7**)
miR-124-2	7 (30.4)	6 (**85.7**)
miR-124-3	19 (82.6)	11 (**57.9**)
miR-203	14 (60.9)	8 (**57.1**)
miR-375	8 (34.8)	ND

ND, not determined.
[a]miRNAs highly downregulated (>50%) in HCC cases compared with their paired non-tumorous liver tissues with CpG-island methylation are in boldface.
[b]Expression levels of mature forms for *miR-124*, and *miR-203* were evaluated in TaqMan® MicroRNA Assays (Applied Biosystems) as described in Materials and Methods.
[c]Numbers of HCC cases that showed hypermethylation compared with corresponding non-tumorous tissues in at least one region analyzed by COBRA.

Suppressive effects of ectopic miR-124 and miR-203 expression on the growth of HCC cell lines

Since our expression and methylation analyses identified *miR-124* and *miR-203* as candidate miRNAs silenced through tumor-specific hypermethylation in HCC, we examined proliferation-inhibitory effects of *miR-124* and *miR-203* by means of the transient transfection of dsRNA mimicking either *miR-124* or *miR-203* into HCC cell lines lacking the expression of these two miRNAs (JHH-4, sK-Hep-1 and JHH-7 for *miR-124* and Huh7 and Hep 3B for *miR-203*). The expression of mature *miR-124* or *miR-203* was elevated in cells transfected with each dsRNA (data not shown). Restoration of *miR-124* or *miR-203* expression significantly reduced cell proliferation in all the HCC cell lines tested (Figure 4A), suggesting that both *miR-124* and *miR-203* have a proliferation-suppressive function in hepatic cells. Since similar effects were observed in more than two cell lines transfected with those miRNAs, the possibility that their proliferation-inhibitory property is restricted to specific cell lines could be ruled out. In a fluorescence-activated cell sorting analysis using HCC cell lines 48 h after the transfection of dsRNA, *miR-124* caused a decrease in S and G_2–M phase cells and an increase in G_0–G_1 phase cells, whereas *miR-203* caused a decrease in G_0–G_1 and S and G_2–M phase cells and an increase in sub-G_1 phase cells (Figure 4A). These results suggest that *miR-124* exerts its cell-growth-suppressive effect predominantly through the induction of cell cycle arrest at the G_1–S checkpoint, whereas *miR-203* does so mainly through the induction of apoptosis in HCC cells. Apoptotic change in Huh7 and Hep 3B after transfection of *miR-203* was confirmed by the TdT-mediated dUTP-biotin nick-end labeling assay (Figure 4B).

Identification of targets for miR-124 and miR-203 in HCC cells

Next, we explored the underlying molecular mechanism of the growth-inhibitory property of *miR-124* and *miR-203* in HCC cells. Since miRNAs primarily achieve their biological functions in mammalian cells by impeding protein expression, we searched for their potential targets, which may be involved in tumorigenesis, through an approach involving different target prediction algorithms, such as miRanda, TargetScan, Pictar and microRNA.org. Possible oncogenes were sorted out as potential targets from all listed genes by their known or predicted functions *in silico*. We first analyzed protein expression levels for predicted reported putative targets by western blotting 48–72 h after transfection of dsRNA mimicking either *miR-124* or *miR-203* into HCC lines lacking the expression of these miRNAs. Since *CDK6* was reported as a possible target for *miR-124* in various cancer (14,21), we used this protein as a positive control for the analysis. Protein levels of CDK6, SMYD3, VIM and IQGAP1 were reduced in *miR-124* transfectants compared with their control counterparts (Figure 4C). In *miR-203* transfectants, the protein level of ABCE1 and CDK6 was decreased compared with that in the control transfectants (Figure 4D).

To validate whether miRNAs directly alter the expression of those candidate targets, we cloned regions of the 3′-UTR of each target messenger RNA, containing putative binding sites (Figure 4E and F), into downstream of the *luciferase* gene in the reporter vector and used them for reporter assays (15). We used the reporter construct containing the reported *miR-124*-binding site within the 3′-UTR of *CDK6* (14) as a positive control for this assay (Figure 4E). A significant reduction in luciferase activity was observed in HCC cells co-transfected with *miR-124* and a reporter vector containing the *VIM*,

Fig. 3. Methylation and expression analyses in primary HCC cases. (**A**) COBRA for candidate miRNA genes in 23 surgically resected primary HCC tumors (T) and corresponding non-tumorous liver tissue (N). Presence of restriction enzyme processing is indicated with a plus or minus above the results of COBRA. See legend of Figure 1B for interpretation. Stars indicates cases in which tumor-specific methylation was detected (see supplementary Table S5, available at *Carcinogenesis* Online). Asterisks indicate frequencies of cases in which aberrant methylation of candidate miRNA was detected by COBRA. (**B**) Quantitative real-time reverse transcription–PCR analysis for expression levels of candidate miRNAs in primary HCC tumors (closed boxes) compared with paired non-tumorous liver tissue (open boxes). Stars indicate cases in which tumor-specific methylation was detected by COBRA (see supplementary Table S5, available at *Carcinogenesis* Online). Asterisks indicate frequencies of cases, in which a remarkable downregulation of candidate miRNA expression (<0.5-fold expression) was observed compared with paired non-tumorous liver tissue, among cases found to have tumor-specific methylation by COBRA.

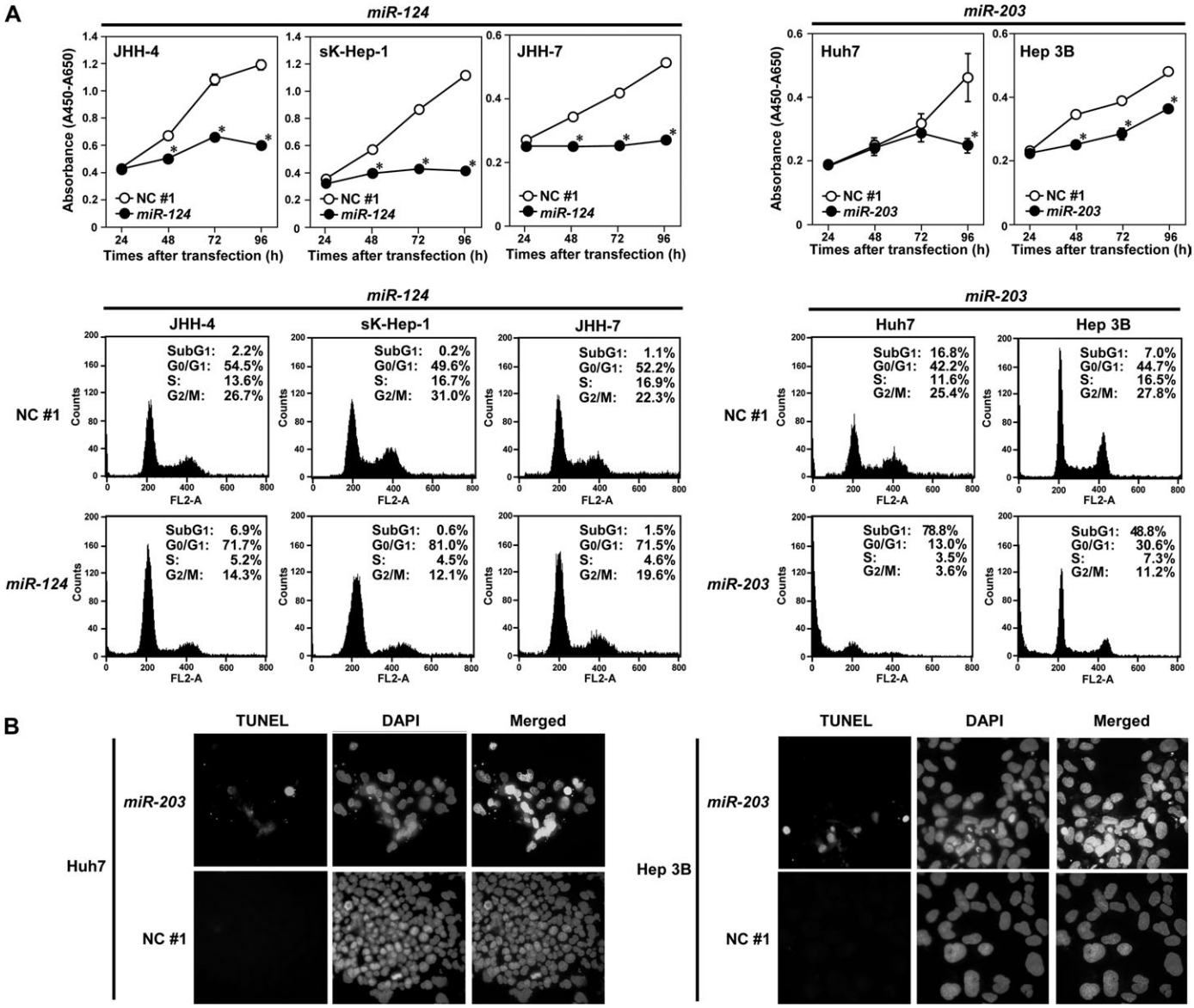


Fig. 4. Growth-suppressive effects of *miR-124* and *miR-203* on HCC cell lines lacking their expression and identification of their predicted targets. (**A**) Upper: growth curves of HCC cell lines in which 10 nM of Pre-miR miRNA Precursor Molecule mimicking *miR-124* (left) or *miR-203* (right) or control non-specific dsRNA (Pre-miR NC #1) was transfected using Lipofectamine™ RNAiMAX. The number of viable cells 24 to 96 h after transfection was assessed by water-soluble tetrazolium salt assay. Points, mean of triplicate determinations in these experiments; bars, standard deviation. $^{*}P < 0.05$ versus Pre-miR NC #1-transfected cells, in a statistical analysis with the Mann–Whitney *U*-test. Lower: representative results of the population in each phase of the cell cycle assessed by fluorescence-activated cell sorting using HCC cell lines 48 h after transfection of Pre-miR miRNA Precursor Molecule mimicking *miR-124* (left) or *miR-203* (right) or Pre-miR NC #1. (**B**) Representative result of TdT-mediated dUTP-biotin nick-end labeling staining in Hep 3B and Huh7 cells at 48 h after transfection of Pre-miR miRNA Precursor Molecule mimicking *miR-203* or control non-specific dsRNA (Pre-miR NC #1, Ambion) under the fluorescence microscope. (**C**) Representative results of western blotting of predicted targets, CDK6, VIM, SMYD3 and IQGAP1, for *miR-124* in HCC cell lines lacking expression of *miR-124* 48 h after transfection of Pre-miR miRNA Precursor Molecule mimicking *miR-124* or control non-specific dsRNA (Pre-miR NC #1). (**D**) Representative results of western blotting of a predicted target, ABCE1 and CDK6, for *miR-203* 72 h after transfection of Pre-miR miRNA Precursor Molecule mimicking *miR-203* or control non-specific dsRNA (Pre-miR NC #1). (**E**) Luciferase reporter assays of a *miR-124*-non-expressing cell line, JHH7, 48 h after cotransfection of pMIR-REPORT luciferase vectors containing 3′-UTR target sites (upper) of *CDK6*, *VIM*, *SMYD3* or *IQGAP1*, Pre-miR miRNA Precursor Molecule mimicking *miR-124* or Pre-miR NC #1, and pRL-hTK internal control vector (lower) using Lipofectamine™ 2000 (Invitrogen). Horizontal open bar, gray boxes and black bars in upper panel indicate 3′-UTR, possible target sites and regions examined in reporter assay, respectively, for each gene. $^{*}P < 0.05$ versus Pre-miR NC #1-treated cells, in a statistical analysis with the Mann–Whitney *U*-test. (**F**) Luciferase reporter assay of a *miR-124*-non-expressing cell line, Hep 3B, 48 h after cotransfection of pMIR-REPORT luciferase vectors containing 3′-UTR target sites (upper) of *ABCE1* or *CDK6*, Pre-miR miRNA Precursor Molecule mimicking *miR-203* or Pre-miR NC #1, and pRL-hTK internal control vector (lower). See legend of Figure 4E for interpretation.

SMYD3 and *IQGAP1* 3′-UTR target sites (Figure 4E) and in those cotransfected with *miR-203* and a vector containing one of the *ABCE1* 3′-UTR target sites (3′-UTR-F1; Figure 4F) compared with control transfectants. On the other hand, no reduction was observed in cells cotransfected with *miR-203* and a vector containing the *CDK6* 3′-UTR target site. These results, together with the results of western blotting, suggest that at least *VIM*, *SMYD3*, and *IQGAP1*, and *ABCE1* are novel possible targets for *miR-124*-mediated and *miR-203*-mediated translational downregulation in hepatocytes, respectively, and that activation of these molecules through methylation-mediated silencing of those two miRNAs may contribute to the pathogenesis of HCC.

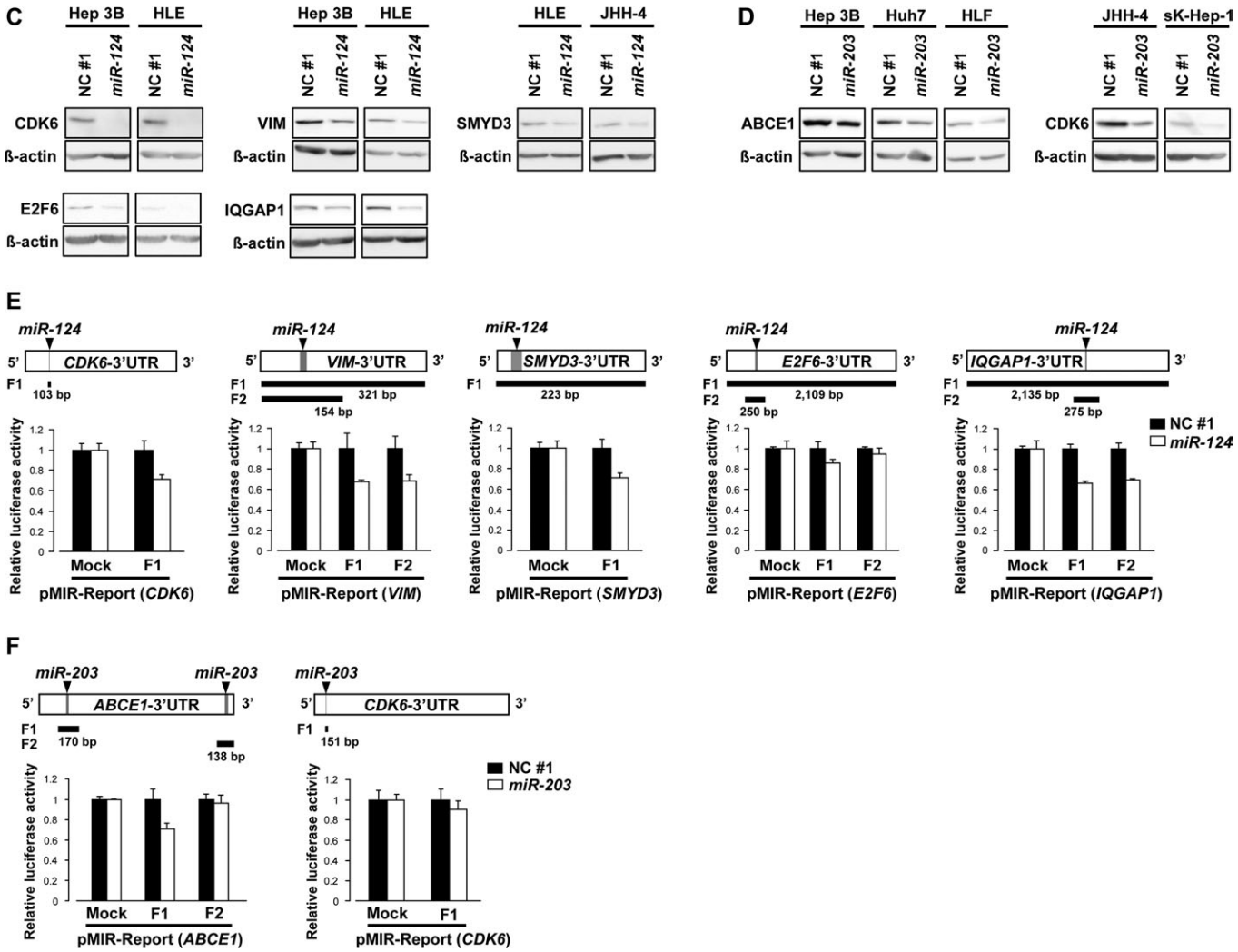


Fig. 4. *continued.*

Discussion

As shown in previous reports, miRNA levels seem to be lower in tumors than normal tissue in cancers, including HCC (9,15,22–24). Similar to various growth-suppressive protein-encoding TSGs, some miRNAs contain CpG islands, which are susceptible to methylation-mediated silencing (11,12,15). Therefore, hypermethylation-mediated silencing of tumor-suppressive miRNAs is likely to be an important mechanism of tumorigenesis through the activation of target oncogenic pathways (25). In the present study, we tried to efficiently identify tumor-suppressive miRNAs contributing to hepatocarcinogenesis in a methylation-dependent manner and having cell proliferation-inhibitory activity by altering the expression of several putative targets using a methylation analysis-based screening approach with further analyses as shown in Figure 1A.

In the course of a program to screen a panel of 19 HCC cell lines for tumor-suppressive miRNAs silenced by tumor-specific aberrant DNA methylation, we first selected *miR-124*, *miR-203* and *miR-375* as possible candidate genes because of (i) consistency in the inverse correlation between DNA methylation status around these miRNAs and their expression patterns in a panel of HCC cell lines; (ii) their higher expression and lower methylation pattern in non-tumorous liver tissues, including inflammatory or cirrhotic livers and (iii) restoration of their expression on treatment with 5-aza-2′-deoxycytidine in cells lacking the expression. Among three miRNAs, however, relatively frequent methylation and an inverse correlation between DNA methylation and expression status in primary tumors of HCC compared with paired non-tumorous liver tissue were observed in *miR-124* and *miR-203* compared with *miR-375*, suggesting the significance of methylation-dependent silencing of *miR-124* and *miR-203* in the tumorigenesis of primary HCC. Recently, *miR-375* was reported to be underexpressed in HCCs, especially β-catenin-mutated tumors, compared with non-tumorous liver tissues, and a direct link between β-catenin activation and repression of *miR-375* was speculated (26), although possible targets for *miR-375* and its biological function involved in hepatocarcinogenesis remain unclear. It will be interesting to clarify the mechanisms and functional significance of *miR-375* repression in HCC. Since we focused on only 39 miRNAs located at 43 loci contained CpG islands around them (within 500 bp upstream) in this study but some miRNAs is known to have their promoter sequences more upstream than 500 bp (27), future studies to delineate miRNA promoters and their relationship with CpG islands may yeild additional information of the epigenetically silenced miRNAs in HCC.

Several recent studies indicated the abnormal expression and pathological significance of *miR-124* and *miR-203* in human cancers (14,21,28), although their precise functions in carcinogenesis, including hepatomagenesis, remain uncharacterized. Using cancer cells genetically deficient in DNA methyltransferase enzymes in combination with miRNA expression profiling, Lujambio *et al.* (14) demonstrated the silencing of *miR-124* through the methylation of CpG islands in several types of human cancer, such as colon, breast and lung cancers, and *CDK6* as a possible target for this miRNA. Decreases in *miR-124* expression were described in anaplastic astrocytomas and glioblastoma multiforme, and growth inhibition in glioblastoma cell lines on

transfection of *miR-124* induced G_1 cell cycle arrest through repression of retinoblastoma 1 and CDK6 (28). Decreased expressions of *miR-124* and *miR-124* restoration-induced non-apoptotic cell cycle arrest with increased CDK6 expression were also reported in medulloblastoma cells (21). Although the mechanisms that silence *miR-124* expression remain unknown in gliomas and medulloblastoma, those results are consistent with our findings showing tumor-specific silencing of *miR-124* at least in part through CpG methylation in HCC and cell cycle arrest at the G_1–S checkpoint on the restoration of *miR-124* expression in non-expressing HCC cells.

One important achievement of this study is that we successfully identified possible direct targets for *miR-124* other than *CDK6* (14,21,28), such as *VIM*, *SMYD3* and *IQGAP1*. Overexpression of the growth-promoting histone methyltransferase SMYD3 in HCC (29) and an association between overexpression of VIM and HCC metastasis were reported previously (30). Although the clinicopathological significance of IQGAP1 in hepatomagenesis remains unknown, the silencing of *miR-124* may contribute to the pathogenesis of HCC by increasing the protein expression of at least some of those possible targets. Indeed, amplification and overexpression of IQGAP1 was reported to be associated with some malignancies, including gastric cancer (31).

The expression pattern of *miR-203* seems to differ among human malignancies: downregulation of *miR-203* expression was described in several types of cancers, such as central nervous system tumors (32), esophageal cancers (33), T-cell lymphomas, chronic myelogenous leukemia and B-cell type acute lymphoblastic leukemia (34), whereas its upregulation was also shown in breast cancer (35), colorectal cancer (36) and ovarian cancer (37). In hepatocellular tumor, significantly decreased expression of *miR-203* was reported in benign tumors when compared with non-tumorous liver tissue and HCC (26). Our previous study excluded *miR-203* as a possible target for methylation-mediated silencing in oral squamous cell carcinoma due to its infrequent tumor-specific methylation in primary cases (15). Recent reports of (i) epigenetic events that silence *miR-203* expression in some hematopoietic malignancies (34); (ii) increased cell proliferation by inhibiting *miR-203* expression in cancer cell line (38) and (iii) induction of apoptosis by restoration of *miR-203* expression in some hematopoietic malignant cell (34) and head and neck tumor cells (39) in an ABL1-dependent and a ΔNp63-dependent manner, respectively, suggest that *miR-203* may work as a tumor-suppressive miRNA in a tissue- or cell-lineage-specific manner. Since, consistent with other malignant cells (34,39), restoration of *miR-203* in HCC cell lines lacking expression of this miRNA induced apoptosis instead of cell cycle arrest, specific regulation of the expression status of *miR-203* may be an alternative approach to molecular targeting therapy for some malignancies, including HCC.

We identified *ABCE1* as a possible novel target for *miR-203*, although expression levels of ABL1 and ΔNp63 were not altered after transfection of *miR-203* in our preliminary experiments (data not shown). ABCE1 encoded by an ATP-binding cassette transporter gene is known to bind with eukaryotic initiation factors, eIF2α and eIF5, and play a role in vertebrate translation initiation (40). ABCE1 inhibitors were shown to efficiently suppress the growth of human tumor cells, suggesting that cancer cells are sensitive to inhibition of protein translation through ABCE1 (40). Among various ATP-binding cassette transporters, ABCE1 was overexpressed in some malignant cells, including melanoma cells (41), and amplification of the *ABCE1* gene was detected in some drug-resistant cancer cell lines (42). Although the clinicopathological significance of ABCE1 to HCC remains unknown, the silencing of *miR-203* may contribute to the pathogenesis through activation of this protein.

In conclusion, the study presented here showed that (i) tumor-specific hypermethylation-mediated silencing of *miR-124* and *miR-203* was a relatively frequent molecular event in primary HCCs and (ii) *miR-124* and *miR-203* exert cell growth-inhibitory effect with the downregulation of their potential targets, resulting in cell cycle arrest at the G_1–S checkpoint and apoptosis, respectively, suggesting that the epigenetic silencing of these miRNA may contribute to hepatic carcinogenesis, although further experiments *in vivo* will be needed to confirm that these miRNAs work as tumor-suppressive miRNAs in this disease.

Supplementary material

Supplementary Figures S1–S3 and Tables S1–S5 can be found at http://carcin.oxfordjournals.org/

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