MicroRNA profiles in various hepatocellular carcinoma cell lines

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-associated mortality worldwide. Although surgery is considered the most effective treatment for patients with HCC, its indication is restricted by limited criteria and a high relapse rate following surgery; therefore, systemic chemotherapy is required for patients with advanced-stage HCC to prolong their survival. MicroRNAs (miRNAs) are endogenous non-coding RNAs of 18-22 nucleotides in length. It has been reported that aberrant expression of miRNAs is a feature shared by various types of human cancer. Previous studies have indicated that the modulation of non-coding RNAs, particularly miRNAs, may be a valuable therapeutic target for HCC. The aim of the present study was to elucidate the miRNA profiles associated with differentiation and hepatitis B virus (HBV) infection observed in HCC cell lines. The human Alex, Hep3B, HepG2, HuH1, HuH7, JHH1, JHH2, JHH5, JHH6, HLE, HLF and Li-7 HCC cell lines were used for an miRNA array. Replicate data were analyzed following their classification into: i) Poorly- and well-differentiated human HCC cells and ii) HBV-positive and -negative human HCC cells. Out of the 1,719 miRNAs, 4 were found to be significantly upregulated and 52 significantly downregulated in the poorly-differentiated cells, as compared with the well-differentiated cells. Conversely, in the HBV-positive cells 125 miRNAs were found to be upregulated and 2 downregulated, as compared with the HBV-negative cells. Unsupervised hierarchical clustering analysis with Pearson's correlation revealed that the miRNA expression levels were clustered both together and separately in each group. In conclusion, miRNA profile characterization based on various parameters may be a novel approach to determine the etiology of HCC.

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Abbreviations: miR/miRNA, microRNA; HBV, hepatitis virus B; HCC, hepatacellular carcinoma

Key words: hepatocellular carcinoma, microRNA profiles, microRNA array, hepatitis B virus-infected hepatocellular carcinoma, differentiation

Introduction

Liver cancer is the third most common cause of cancer-associated mortality worldwide, accounting for an estimated 9.2% of total cancer-associated mortalities in 2008 (1). Surgery is considered the most effective treatment for patients with hepatocellular carcinoma (HCC) (2); however, the indications for surgery are restricted by the size and total number of tumors (2,3). Although the 5-year survival rate of patients with HCC has improved by >30% over the past decade, the recurrence rate following surgery is estimated to be nearly 50% (4); therefore, systemic chemotherapy is required for patients with advanced stages of HCC, in order to prolong their survival.

MicroRNAs (miRNAs) are endogenous non-coding RNAs of 18-22 nucleotides in length (3,5). The effect of miRNAs on the regulation of the expression of various genes is so broad that one miRNA controls >200 genes (6). Aberrant expression of miRNAs is a common feature among various types of human cancer, and has been reportedly associated with patient survival (7-10). Regarding the correlation between miRNAs and HCC, several studies have detected the aberrant expression of specific miRNAs in HCC tissues when compared with normal tissues (11-14). These studies indicated that the modulation of non-coding RNAs, particularly miRNAs, may be a valuable therapeutic target in HCC.

The aim of the present study was to elucidate the miRNA profiles that are associated with differentiation and hepatitis B virus (HBV) infection observed in HCC cell lines. The characterization of miRNA expression patterns using various parameters may be a novel approach for the treatment of patients with HCC.

Materials and methods

Cell lines and culture. The Alex, Hep3B, HepG2, HuH1, HuH7, JHH1, JHH2, JHH5, JHH6, HLE, HLF and Li-7 HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and transported to our laboratory. The cell lines were authenticated by the cell bank using short tandem repeat polymerase chain reaction. The cells were grown in minimal essential medium (Gibco;

Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (catalog no., 533-69545; Wako Pure Chemical Industries, Tokyo, Japan) and penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

Analysis of microRNA array. Total RNA was extracted from the cancer cell lines using a miRNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA samples typically showed $A_{260}/_{280}$ ratios between 1.9 and 2.1 on an Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Following the measurement of the RNA using an RNA 6,000 Nano kit (Agilent Technologies, Tokyo, Japan), the samples were labeled using a miRCURY Hy3/Hy5 Power Labeling kit (Takara Bio Inc., Tokyo, Japan) and hybridized onto a human miRNA Oligo chip (version 19.0; Toray Industries, Inc., Tokyo, Japan). Scanning was conducted with the 3D-Gene Scanner 3,000 (Toray Industries, Inc., Kusatsu, Japan). 3D-Gene extraction software version 1.2 (Toray Industries, Inc.) was used to read the raw intensity of the image. To determine the change in miRNA expression between poorly- and well-differentiated HCC cell lines or HBV-positive and HBV-negative HCC cell lines, the raw data were analyzed using GeneSpringGX version 10.0 (Agilent Technologies). Samples were first normalized to the 28S RNA and the baseline was then corrected to the median of all samples.

Replicate data were analyzed following their classification into: i) Poorly- and well-differentiated human HCC cells, and ii) HBV-positive and -negative human HCC cells, which were organized by the hierarchical clustering in the GeneSpring software. For the $\log_2$ ratios of the miRNA expression intensity between two groups, hierarchical clustering was performed using the furthest neighbor method with the absolute Pearson's correlation coefficient as a metric. The $\log_2$ ratios were median-centered across each miRNA in a color-coding of the heat map. The P-value cutoff was set to 0.05. Only changes of >50% in at least one of the time points for each sample were considered significant. All of the analyzed data were scaled by global normalization.

Statistical analysis. All analyses were conducted using the JMP 8.0 software (SAS Institute, Inc., Cary, NC, USA). A paired analysis between the groups was conducted using a Student's *t* test. $P<0.05$ was used to indicate statistically significant differences between the groups.

Results

Differences in miRNA expression between poorly- and well-differentiated human HCC cell lines. Using a custom microarray platform, the expression levels of 1,719 miRNAs were analyzed in various human HCC cell lines. As shown in Fig. 1 and Tables I and II, of the 1,719 miRNAs, 4 were found to be significantly upregulated and 52 were significantly downregulated in the poorly-differentiated cells, as compared with the well-differentiated cells. Unsupervised hierarchical clustering analysis with Pearson's correlation showed that the poorly-differentiated HCC cell lines clustered both together and separately from the well-differentiated HCC cells (Fig. 1).

Table I. Upregulated expression of miRNA in poorly-differentiated HCC cells, as compared with well-differentiated HCC cells.

Upregulated miRNAs	P-values	PD/WD
hsa-miR-4498	0.026931863	2.867818244
hsa-miR-6842-5p	0.025697328	2.461117562
hsa-miR-6800-5p	0.012943201	1.941309698
hsa-miR-4476	0.030699533	1.759074121

HCC, hepatocellular carcinoma; PD, poorly-differentiated; WD, well-differentiated; miR/miRNA, microRNA.

Differences in miRNA expression between HBV-positive and HBV-negative HCC lines. To examine the effect of HBV infection on alterations in miRNAs, miRNA profiles were analyzed in HBV-positive and -negative human HCC cell lines. As shown in Fig. 2 and Tables III and IV, of the 1,719 miRNAs, 125 miRNAs were found to be significantly upregulated and 2 were significantly downregulated in the HBV-positive HCC cells, as compared with the HBV-negative HCC cells. Unsupervised hierarchical clustering analysis with Pearson's correlation showed that the HBV-positive HCC cell lines clustered both together and separately from the HBV-negative HCC cells (Fig. 2).

Discussion

The aim of the present study was to elucidate the targetable miRNAs associated with the etiology, diagnosis and treatment of HCC. Certain miRNAs, such as miR-26b and miR-132, were found to be downregulated in poorly-differentiated HCC. It has recently been reported that dedifferentiation is involved in the epithelial-mesenchymal transition (EMT), and particularly in the EMT of cancer (15). In order to invade and metastasize to different organs, cancer cells shed their differentiated epithelial phenotype through EMT (15), which suggests that miR-26b or miR-132 may be associated with cancer invasion and metastasis via EMT. In addition, miR-26b has been shown to directly suppress the expression of CDK6 and cyclin E1, resulting in reduced retinoblastoma-associated protein phosphorylation and inhibited cell proliferation (16). miR-132 also inhibits tumor cell proliferation, invasion and migration by targeting Sox5 (17). These studies also indicated that miR-26b and miR-132 may directly inhibit cancer invasion and metastasis.

In the present study, miR-4476 was upregulated in poorly-differentiated carcinoma. Recently, it has been demonstrated that miR-4476 is one of the top 10 validated miRNA markers differentiating pancreatobiliary cancer from other clinical conditions, including other types of cancer and healthy controls (18). Therefore, this result suggests that advanced stages of HCC, which includes poorly-differentiated cells, induce cholestasis in a similar fashion to pancreatobiliary cancers and may increase the miR-4476 upregulation.

Regarding the effect of HBV, miR-99b was found to be upregulated in HBV-infected HCC cells in the present study. It has been reported that the expression of miR-99b is

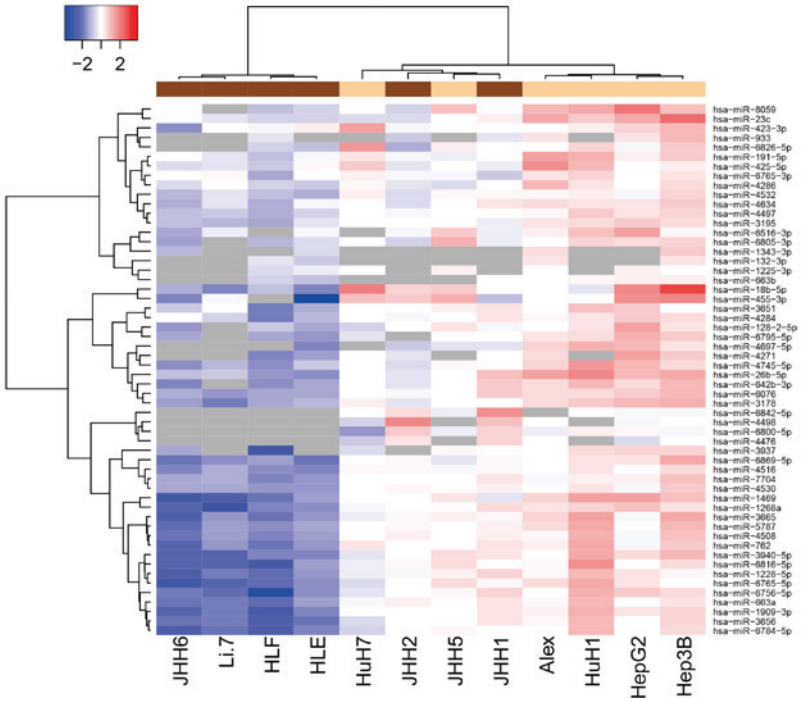

Figure 1. Hierarchical clustering of miRNAs in poorly- and well-differentiated human HCC cell lines. Clustering was performed according to the expression profiles of 56 differentially-expressed miRNAs in poorly- and well-differentiated human HCC cell lines. The columns represent the analyzed samples, while the rows represent the miRNAs. The miRNA clustering tree is shown on the left and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of the miRNAs, with red indicating a high expression level and blue indicating a low expression level. miR/miRNA, microRNA; HCC, hepatocellular carcinoma.

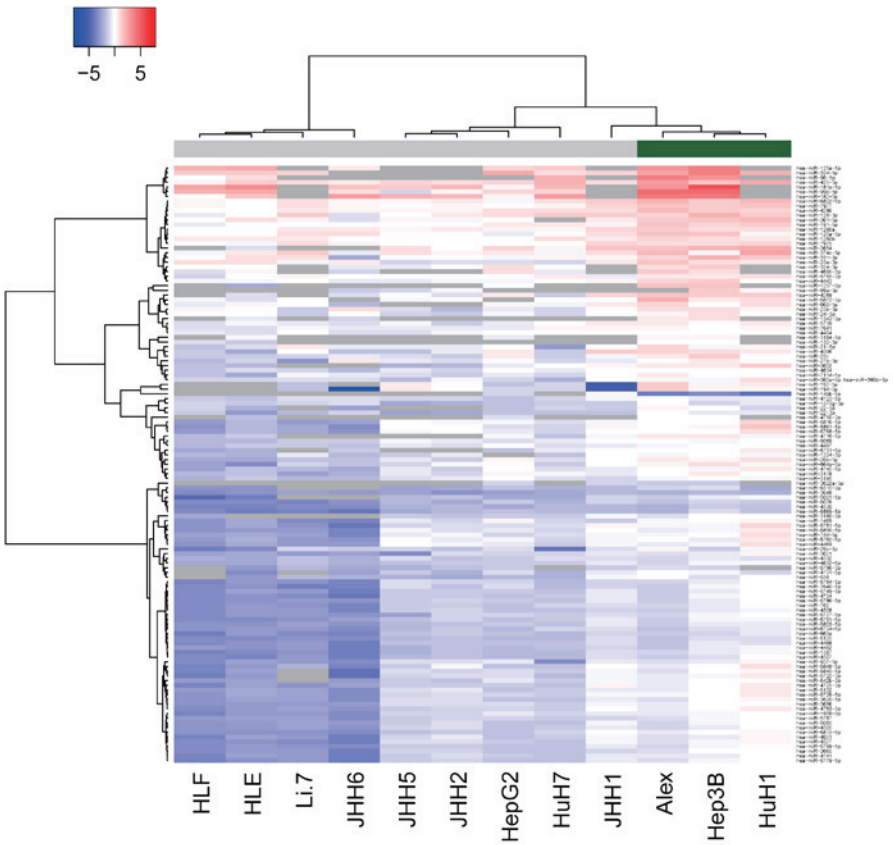

Figure 2. Hierarchical clustering of miRNAs in normal and tumor tissues. Liver tissue clustering was performed according to the expression profiles of 127 differentially-expressed miRNAs in HBV-positive and -negative human HCC cell lines. The columns represent the analyzed samples, while the rows represent the miRNAs. The miRNA clustering tree is shown on the left and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of the miRNAs, with red indicating a high expression level and blue indicating a low expression level. miR/miRNA, microRNA; HCC, hepatocellular carcinoma.

Table II. miRNA downregulation in poorly-differentiated HCC cells as compared with well-differentiated HCC cells.

Downregulated miRNAs	P-value	PD/WD
hsa-miR-3178	0.000974376	0.348146401
hsa-miR-1469	0.001067111	0.256078680
hsa-miR-6805-3p	0.001867476	0.415970562
hsa-miR-3195	0.001905223	0.508164983
hsa-miR-4497	0.002391438	0.540368285
hsa-miR-4532	0.003797834	0.570642150
hsa-miR-4745-5p	0.005612978	0.355402751
hsa-miR-6516-3p	0.008665895	0.446770518
hsa-miR-4634	0.009685127	0.572465267
hsa-miR-8059	0.010182045	0.344828448
hsa-miR-3940-5p	0.010700356	0.359070586
hsa-miR-1909-3p	0.011248665	0.401354333
hsa-miR-6795-5p	0.013039021	0.381096482
hsa-miR-132-3p*	0.014002236	0.500476019
hsa-miR-26b-5p*	0.014113042	0.357904962
hsa-miR-6765-5p	0.014456214	0.375871481
hsa-miR-4516	0.015208421	0.529129777
hsa-miR-1268a	0.015252062	0.430113531
hsa-miR-1225-3p	0.015675303	0.690060509
hsa-miR-191-5p	0.017084147	0.504203776
hsa-miR-5787	0.019066417	0.426932073
hsa-miR-3665	0.019620817	0.372159995
hsa-miR-6784-5p	0.021959677	0.423492063
hsa-miR-762	0.022064969	0.428794025
hsa-miR-425-5p	0.022167838	0.469352798
hsa-miR-6076	0.02338926	0.489615399
hsa-miR-4284	0.025395945	0.607050985
hsa-miR-6816-5p	0.025658929	0.342345756
hsa-miR-6756-5p	0.026680935	0.418869876
hsa-miR-6765-3p	0.028318318	0.681942211
hsa-miR-1343-3p	0.029279009	0.369035375
hsa-miR-4697-5p	0.029348892	0.356689006
hsa-miR-4286	0.029548705	0.606212249
hsa-miR-3656	0.029629729	0.441205506
hsa-miR-6869-5p	0.030683568	0.450864492
hsa-miR-455-3p	0.031351866	0.340391294
hsa-miR-933	0.032763996	0.446918418
hsa-miR-3937	0.032949412	0.428511322
hsa-miR-663b	0.033189114	0.645740828
hsa-miR-1228-5p	0.034737307	0.468155726
hsa-miR-4508	0.035873986	0.465234100
hsa-miR-23c	0.038312747	0.398936550
hsa-miR-642b-3p	0.03853801	0.474336719
hsa-miR-4530	0.03858166	0.564198369
hsa-miR-4271	0.039079038	0.375946014
hsa-miR-18b-5p	0.046265132	0.239201813
hsa-miR-663a	0.046711854	0.497938251
hsa-miR-7704	0.046961909	0.562374269
hsa-miR-6826-5p	0.048141645	0.450205273
hsa-miR-3651	0.048451237	0.591375188
hsa-miR-423-3p	0.04891406	0.585477071
hsa-miR-128-2-5p	0.049142734	0.487264349

HCC, hepatocellular carcinoma; miR/miRNA, microRNA; PD, poorly-differentiated; WD, well-differentiated.

Table III. miRNA upregulation in HBV-positive HCC cells as compared with HBV-negative HCC cells.

Upregulated miRNAs	P-value	HBV(+)/HBV(-)
hsa-miR-99b-5p	0.000000648	9.941304892
hsa-miR-181a-5p	0.033034307	6.386379599
hsa-miR-96-5p	0.021545231	4.758785302
hsa-miR-1237-3p	0.037257957	4.456964302
hsa-miR-182-5p	0.000177429	4.362818593
hsa-miR-125a-5p	0.005613398	3.719262548
hsa-miR-99a-3p	0.045954557	3.494856992
hsa-miR-6861-5p	0.008595301	3.308580171
hsa-miR-6726-5p	0.002146572	3.247738683
hsa-miR-4763-3p	0.003813145	3.075732768
hsa-miR-192-5p	0.018546977	3.068893554
hsa-miR-194-5p	0.027213074	3.058433608
hsa-miR-324-5p	0.047875781	3.025508821
hsa-miR-3665	0.000663551	3.021045838
hsa-miR-6848-5p	0.013784248	3.003340126
hsa-miR-658	0.002645683	2.977949153
hsa-miR-3652	0.006332146	2.969527506
hsa-miR-744-5p	0.00486586	2.967774559
hsa-miR-6132	0.00428827	2.930151490
hsa-miR-26b-5p	0.001262254	2.924882610
hsa-miR-6872-3p	0.013248885	2.880715544
hsa-miR-6813-5p	0.008803711	2.879469305
hsa-miR-4289	0.001809926	2.859805967
hsa-miR-3620-5p	0.004247363	2.837639155
hsa-miR-6779-5p	0.007205138	2.789908382
hsa-miR-6799-5p	0.006060554	2.738799039
hsa-miR-1587	0.005536711	2.737246496
hsa-miR-23c	0.007181466	2.716255574
hsa-miR-4725-3p	0.026222737	2.713962628
hsa-miR-1343-3p	0.029279009	2.709767325
hsa-miR-937-5p	0.016562749	2.704056512
hsa-miR-22-5p	0.012966623	2.690740441
hsa-miR-6816-5p	0.01716898	2.690475807
hsa-miR-6781-5p	0.043635552	2.662786094
hsa-miR-6768-5p	0.009273849	2.658821540
hsa-miR-331-3p	0.001849624	2.656822543
hsa-miR-4327	0.021811096	2.620193911
hsa-miR-6727-5p	0.006674229	2.597922456
hsa-miR-6722-3p	0.024746989	2.593107400
hsa-miR-5787	0.001467763	2.579963522
hsa-miR-29c-3p	0.016910541	2.559268566
hsa-miR-22-3p	0.000110838	2.545731351
hsa-miR-3654	0.007328507	2.536585596
hsa-miR-4507	0.013990723	2.509463513
hsa-miR-4492	0.005952616	2.502548594
hsa-miR-4741	0.009337902	2.486124356
hsa-miR-3621	0.015640016	2.485247656
hsa-miR-4734	0.018933717	2.483317331
hsa-miR-6085	0.014443652	2.479844683
hsa-miR-361-5p	0.000442472	2.474136455
hsa-miR-5001-5p	0.017579881	2.466921307
hsa-miR-6845-5p	0.018960866	2.444791599
hsa-miR-4651	0.031451007	2.433852833
hsa-miR-664a-3p	0.022326221	2.417433070
hsa-miR-6850-5p	0.043000995	2.410122248

Table III. Continued.

Upregulated miRNAs	P-value	HBV(+)/HBV(-)
hsa-miR-3940-5p	0.016650066	2.406661676
hsa-miR-4750-3p	0.034041218	2.405803427
hsa-miR-4716-5p	0.026554669	2.368757616
hsa-miR-365a, b-3p	0.002715735	2.366494444
hsa-miR-4508	0.007102476	2.352557384
hsa-miR-191-5p	0.000120655	2.346171925
hsa-miR-6731-5p	0.024175847	2.333212883
hsa-miR-6822-5p	0.001204907	2.331567699
hsa-miR-4745-5p	0.015938518	2.330093060
hsa-miR-1469	0.039665847	2.329351564
hsa-miR-762	0.010944843	2.327308371
hsa-miR-4505	0.021004934	2.322786379
hsa-miR-3656	0.012652065	2.316840605
hsa-miR-374c-5p	0.046201574	2.309229277
hsa-miR-4306	0.048384309	2.306213437
hsa-miR-4463	0.043512115	2.274070423
hsa-miR-6749-5p	0.034695635	2.258437039
hsa-miR-425-5p	0.005354369	2.258230321
hsa-miR-1909-3p	0.015268122	2.256696389
hsa-miR-4443	0.002035551	2.247501172
hsa-miR-6784-5p	0.020580101	2.233472310
hsa-miR-6791-5p	0.025022751	2.227203891
hsa-miR-6765-5p	0.03907315	2.196934984
hsa-miR-4695-3p	0.049035558	2.196882473
hsa-miR-4731-5p	0.026948571	2.153748402
hsa-miR-324-3p	0.008210293	2.148721766
hsa-miR-7977	0.000154283	2.145089801
hsa-miR-3178	0.022352	2.127295883
hsa-miR-642b-3p	0.020856928	2.086744300
hsa-miR-6786-5p	0.044762899	2.078505858
hsa-miR-6869-5p	0.038412235	2.071725430
hsa-miR-663a	0.030021104	2.045834444
hsa-miR-4488	0.028003788	2.038574665
hsa-miR-7114-5p	0.007743122	2.030825064
hsa-miR-3180-3p	0.025916357	2.023796991
hsa-miR-6125	0.032086556	2.008344893
hsa-miR-21-5p	0.049645958	2.003677073
hsa-miR-125a-3p	0.011495522	1.999755015
hsa-miR-4632-5p	0.009562923	1.999301680
hsa-miR-132-3p	0.014002236	1.998097736
hsa-miR-23b-3p	0.022475719	1.997567414
hsa-miR-27b-3p	0.042591151	1.976679667
hsa-miR-6515-3p	0.043906577	1.956257303
hsa-miR-6803-5p	0.046392317	1.928556399
hsa-miR-6724-5p	0.044214734	1.926601244
hsa-miR-4286	0.000877998	1.922172274
hsa-miR-660-5p	0.029655234	1.920831210
hsa-miR-23a-3p	0.039960215	1.913813878
hsa-miR-1273g-3p	0.008489419	1.887689346
hsa-miR-6076	0.046682307	1.869956757
hsa-miR-3184-5p	0.002114973	1.859834906
hsa-miR-24-3p	0.014737629	1.853159621
hsa-miR-1224-3p	0.024802116	1.852687602
hsa-miR-128-3p	0.034442661	1.852193951
hsa-miR-5739	0.015007524	1.813507960
hsa-miR-6766-3p	0.015357929	1.810550072

Table III. Continued.

Upregulated miRNAs	P-value	HBV(+)/HBV(-)
hsa-miR-6089	0.01949948	1.777100276
hsa-miR-4530	0.045923322	1.741259423
hsa-miR-4497	0.010048462	1.733250168
hsa-miR-4634	0.011175767	1.733040523
hsa-miR-3195	0.048689395	1.633334528
hsa-miR-4532	0.036380915	1.573156813
hsa-miR-6765-3p	0.008380175	1.570194232
hsa-miR-3648	0.018858947	1.557642234
hsa-miR-4723-5p	0.013307402	1.556739349
hsa-miR-1260a	0.009160577	1.550976854
hsa-miR-7641	0.040903303	1.528537769
hsa-miR-7975	0.033849532	1.527016010
hsa-miR-1260b	0.037598062	1.399160141
hsa-miR-4454	0.044853364	1.361601255

HCC, hepatocellular carcinoma; miR/miRNA, microRNA; HBV, hepatitis B virus.

Table IV. miRNA downregulation in HBV-positive HCC cells as compared with HBV-negative HCC cells.

Downregulated miRNAs	P-value	HBV(+)/HBV(-)
hsa-miR-146b-5p	0.012478369	0.125399188
hsa-miR-3622a-5p	0.025065220	0.861532186

miR/miRNA, microRNA; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

associated with the presence of lymph node metastasis (19). In addition, certain miRNAs are associated with the oncogenic processes of HBV-related HCC (3). This data indicates that miRNAs play an important role in the etiology of HBV-related HCC.

In addition, Wang *et al* (20) demonstrated that 10 upregulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 11 downregulated miRNAs (miR-138, miR-214, miR-214, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-5p, miR-483-3p, miRNA-708 and miRNA-1275) were identified in HBV-associated HCC tissues. In the present study, the same microRNAs were not detected in HBV-positive HCC cells; therefore, adjacent normal tissues may be included in the human HCC tissues. These results indicate that the microRNA expression patterns are different from cancer cell lines and cancer tissues. Cell-cell interaction may affect microRNA expression in the microenvironment of cancer tissues.

In conclusion, changes in the regulation of key miRNAs due to differentiation and HBV infection were observed in human HCC cell lines. The present findings suggested that differences in miRNA expression may serve as a novel marker that can aid in elucidating the etiology of human HCC and assist in designing treatments.

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