Table 1 Upregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
miR-17-5p	Upregulated	p38 pathway	Cultured cells, human tissues	[52]
miR-18a	Upregulated	ER1a	Human tissues, cultured cells	[53]
miR-21	Upregulated	C/EBPb	Mouse CDAA model	[54]
	Upregulated	PTEN	Human tissues, cultured cells	[35]
miR-22	Upregulated	ERa, IL-1a	Human tissues, cultured cells, DEN model	[55]
miR-23a	Upregulated	PGC-1a,G6PC	Human tissues, cultured cells	[56]
miR-26a	Upregulated	Lin28B, Zcchc11	Human tissues, xenograft model	[57]
	Upregulated	NF-κB, IL-6 pathways	Human tissues	[58]
miR-30d	Upregulated	GNAI2	Human tissues, cultured cells	[59]
miR-100	Upregulated		Human tissues	[60]
miR-106b	Upregulated	APC	Human tissues, cultured cells	[61]
miR-122	Upregulated		Human tissues	[60]
miR-130b	Upregulated	TP53INP1	Human tissues, xenograft model	[62]
miR-135a	Upregulated	FOXM1, MTSS1	Human tissues, cultured cells, xenograft	[63]
miR-143	Upregulated	FNDC3B	Human tissues, HBX transgenic mouse	[64]
miR-146a	Upregulated in endothelial cells	BRCA, PDGFRA	Cultured cells	[65]
miR-151	Upregulated	FAK	Human tissues, cultured cells	[66]
	Upregulated	FAK, RhoGDIA	Human tissues, cultured cells	[67]
miR-155	Upregulated	SOCS1	Orthotropic transplant model	[68]
	Upregulated	DKK1, APC	Human tissues, cultured cells	[69]
	Upregulated	PTEN	Mouse CDAA model	[54]
miR-181	Upregulated	TIMP3	Mouse CDAA model	[70]
	Upregulated	CDX2, GATA6, NLK	Cultured cells	[71]
miR-183	Upregulated	AKAP12	Human tissues	[72]
miR-186	Upregulated	AKAP12	Human tissues	[72]
miR-200	Upregulated	NRF2 pathway	Rat HCC model,	[73]
miR-210	Upregulated	VMP1	Human tissues, cultured cells	[74]
miR-216a	Upregulated	TSLC1	Human tissues, cultured cells	[75]
miR-216a/217	Upregulated	PTEN, SMAD7	Cultured cells, Human tissues	[76]
miR-221	Upregulated	CDK inhibitors	Transgenic mouse	[77]
	Upregulated	p27, p57, Arnt	Primary hepatocytes	[78]
	Upregulated	Bmf	Cultured cells, human tissues	[79]
	Upregulated	p27, p57	Cultured cells, human tissues	[80]
miR-221/222	Upregulated	p27, DDIT4	Human tissues, mouse model	[81]
miR-224	Upregulated		Human tissues	[82]
	Upregulated	Atg5, Smad4, autophagy	Human tissues, HBV X transgenic mice	[83]
	Upregulated	API-5	Cultured cells, human tissues	[84]
	Upregulated		Human tissues	[85]
	Upregulated	API-5	Human tissues	[86]
miR-423	Upregulated	p21/waf1	Human tissues, cultured cells	[87]
miR-485-3p	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-490-3p	Upregulated	ERCIC3	Human tissues, cultured cells	[89]
miR-494	Upregulated	MCC	Human tissue, mouse liver cancer model	[90]
miR-495	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-517a	Upregulated		Human tissues, cultured cells	[91]
miR-657	Upregulated	TLE1, NF-κB	Human tissues, cultured cells	[92]
miR-664	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-1323	Upregulated		Human tissues	[93]

Table 2 Downregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
let-7a	Downregulated	STAT3	Cultured cells	[94]
let-7c	Downregulated		Human tissues, cultured cells	[95]
let-7g	Downregulated	COL12A	Cultured cells, human tissues	[96]
miR-7	Downregulated	PIK3CD	Cultured cells, human tissues	[97]
miR-10a	Downregulated	EphA4	Cultured cells	[98]
miR-10b	Downregulated		Human tissues	[99]
miR-15a/16	Downregulated		Cultured cells	[100]
miR-21	Downregulated		Human tissues	[82]
miR-26a	Downregulated	IL-6	Human tissues, xenograft model	[101]
	Downregulated	CyclinD2, E2	Cultured cells, mouse model	[102]
miR-29	Downregulated	Bcl2, Mcl1	Human tissues, cultured cells	[103]
miR-29b	Downregulated	MMP-2	Human tissues, cultured cell	[104]
miR-29c	Downregulated	SIRT1	Cultured cells	[105]
miR-34a	Downregulated	CCL22	Human tissues, cultured cells	[106]
miR-99a	Downregulated	PLK1	Human tissues, cultured cells	[107]
	Downregulated	IGF-1R	Human tissues, cultured cells	[108]
miR-100	Downregulated	PLK1	Human tissues, cultured cells	[107]
miR-101	Downregulated	EZH2, EED	Human tissues, cultured cells	[109]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	Mcl1	Cultured cells, human tissues	[110]
	Downregulated	Fos	Human tissues, cultured cells	[111]
miR-122	Downregulated	c-Myc	Human tissues, cultured cells	[112]
	Downregulated		Cultured cells	[113]
	Downregulated	MTTP	Knockout mice	[32]
	Downregulated	IL6, TNF	Knockout mice	[31]
	Downregulated	IGF-1R	Human tissues	[114]
	Downregulated	Cyclin G1	Human tissues, cultured cells	[115]
miR-124	Downregulated	ROCK2, EZH2	Human tissues, cultured cells	[116]
	Downregulated	CDK6, VIM, SMYD3, IQGAP1	Human tissues, cultured cells	[117]
miR-125a/125b	Downregulated		Human tissues, cultured cells	[118]
miR-125b	Downregulated	SUV39H	Human tissues, cultured cells	[119]
	Downregulated	Mcl1, Bclw, IL6R	Human tissues, cultured cells	[120]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	PIGF, MMP-2, MMP-9	Human tissues, cultured cells	[121]
	Downregulated	Lin28B	Human tissues, cultured cells	[122]
miR-139	Downregulated	ROCK2	Human tissues, cultured cells	[123]
miR-139-5p	Downregulated		Human tissues, cultured cells	[95]
miR-140-5p	Downregulated	TGFBR1, FGF9	Human tissues, cultured cells	[124]
		DNMT1	Knockout mice	[125]
miR-141	Downregulated	DLC-1	Human tissues	[126]
miR-145	Downregulated		Human tissues	[60]
	Downregulated	IRS1, IRS2, IGF-1R, b-catenin	Human tissues, cultured cells	[127]
	Downregulated		Human tissues	[85]
miR-148a	Downregulated	c-Met	Human tissues, cultured cells	[128]
	Downregulated	HRIP	Mouse xenograft model, cultured cells	[129]
	Downregulated	e-cadherin	Human tissues, cultured cells	[130]
	Downregulated	c-Myc	Cultured cells	[131]
miR-152	Downregulated	DNMT1, GSTP1, CDH1	Human tissues	[132]

Table 2 continued

miRNA	Expression levels	Targets	Main tested samples	References
miR-195	Downregulated	NF-κB pathway	Cultured cells	[133]
	Downregulated	VEGF, VAV2, CDC42	Cultured cells, human tissues	[134]
	Downregulated	Cyclin D1, CDK6, E2F3	Cultured cells, human tissues	[135]
miR-198	Downregulated		Human tissues	[60]
miR-199a/b-3p	Downregulated	PAK4	Human tissues, cultured cells	[30]
miR-199b	Downregulated		Human tissues	[85]
miR-200a	Downregulated	H3 acetylation	Human tissues, cultured cells	[136]
miR-200b	Downregulated		Human tissues, cultured cells	[95]
miR-200c	Downregulated		Human tissues	[82]
miR-200	Downregulated		Human tissues	[82]
miR-203	Downregulated	ABCE1	Human tissues, cultured cells	[117]
miR-214	Downregulated	HDGF	Human tissues, cultured cells	[137]
miR-222	Downregulated		Human tissues	[82]
miR-223	Downregulated	STMN1	Human tissues	[138]
miR-224	Downregulated		Human tissues	[139]
miR-363-3p	Downregulated	c-Myc	Cultured cells	[131]
miR-375	Downregulated	ATG7	Human tissues, cultured cells	[140]
	Downregulated	AEG-1	Human tissues, cultured cells	[141]
miR-429	Downregulated	Rab18	Cultured cells	[142]
miR-449	Downregulated	c-MET	Xenograft, cultured cells	[143]
miR-520e	Downregulated	NIK	Human tissues, cultured cells	[69]
miR-612	Downregulated	AKT2	Cultured cells, human tissues	[144]
miR-637	Downregulated	STAT3 activation	Human tissues, cultured cells	[145]
miR-1271	Downregulated	GLP3	Human tissues, cultured cells	[99]

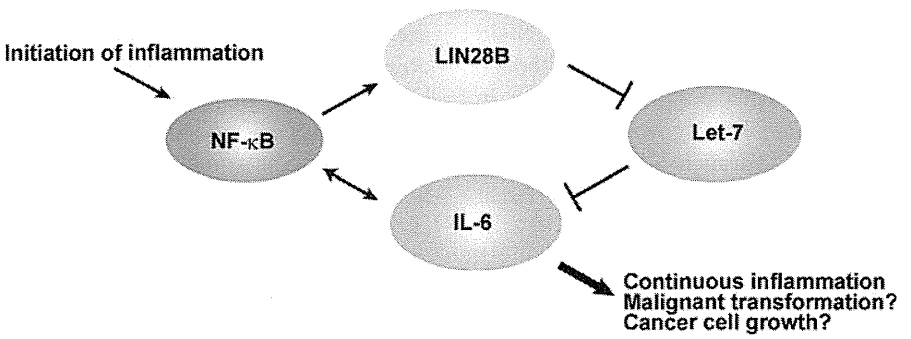

Fig. 2 A model bridging chronic inflammation and transformation by miRNA. Inflammation triggers activation of NF-κB, which leads to transcription of LIN28B. LIN28B inhibits the production of Let-7. Let-7 normally inhibits IL-6 expression, resulting in higher levels of IL-6 than are achieved by NF-κB activation. IL-6 mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop

miRNAs, as a new class of gene expression regulators, may be involved in chronic inflammation-induced carcinogenesis and, in fact, several studies have clarified one such linkage, in which miRNAs may serve as a bridge between continuous inflammation and carcinogenesis.

A flagship report addresses a positive feedback loop of an inflammatory response mediated by NF-κB that activates Lin28B transcription (Fig. 2) [40]. LIN28B, which is an inhibitor of miRNA processing, reduces let-7 levels. Let-7 inhibits IL-6 expression, resulting in higher levels of IL-6 than achieved by NF-κB activation. IL-6-mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop. Although the experiments mainly used MCF10A cells (breast cancer cells), a similar feedback loop was observed in HCC tissues. The authors termed these mechanisms an

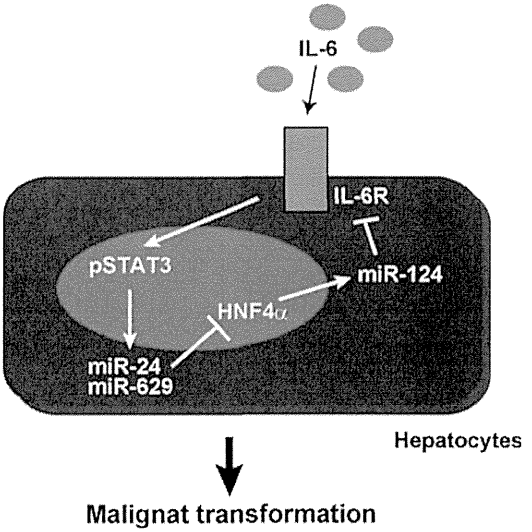

Fig. 3 A model describing a positive feedback loop mediated by miRNAs from transient HNF4α inhibition to transformation. Transient silencing of HNF4α is mediated by miR-24 and miR-629, both of which are induced by STAT3 activation following IL-6 stimulation. miR-124, whose promoter region contains HNF4α-binding sites, targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3, which induces miR-24 and miR-629. This microRNA feedback-inflammatory loop is thought to be crucial in IL-6-mediated liver cancer

"epigenetic switch" because the loop maintains the epigenetic transformed state even in the absence of induction by inflammation (Fig. 2).

Another report addressed hepatocarcinogenesis induced by transient inhibition of HNF4α (Fig. 3) [41]. HNF4α was reported to be involved in liver oncogenesis, although discrepant reports have also been published [42–44]. In that report, transient HNF4α silencing was sufficient to maintain cell transformation. Through a miRNA library screen, miR-24 and miR-629 were identified to target HNF4α. Interestingly, both miRNAs were induced following HNF4α silencing, supporting their involvement in the HNF4α-dependent feedback loop. miR-24 and miR-629 contain the STAT3-binding motif in their promoter region. The authors showed that in response to IL-6, STAT3 binding to their promoters increased, resulting in miRNA expression. They also identified miR-124, whose promoter region contains HNF4α binding sites. miR-124 targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3. The importance of these feedback loops was confirmed in vivo using a mouse HCC model induced by diethylnitrosamine. miR-124 delivery by cationic liposomes prevented tumor development. Thus, these microRNA feedback-inflammatory loops are important and can be a therapeutic target for liver cancer (Fig. 3) [41].

A recent paper reported a similar but distinct observation (Fig. 4). The authors found that when using DEN-induced foci of altered hepatocytes (FAH), LIN28-expressing cells are present in FAH, in which let-7 is down-regulated, resulting in the enhanced expression of IL-6, mediating the progression of malignancies from progenitors. An important difference between the cells in FAH and those in early hepatocarcinogenesis is that IL-6 signaling is autocrine, being mediated by reduced let-7 due to upregulation of LIN28B in FAH cells. This mechanism may contribute to malignant progression from HCC progenitor cells (Fig. 4) [45].

These three reports are from related research groups, and rely on the hypothesis that the IL-6-STAT3 pathway is crucial for hepatocarcinogenesis. Although IL-6 has been implicated as a growth factor in various epithelial cancers [46, 47], its relevance in hepatocarcinogenesis needs to be confirmed to determine the applicability and reproducibility of these findings to the clinical setting.

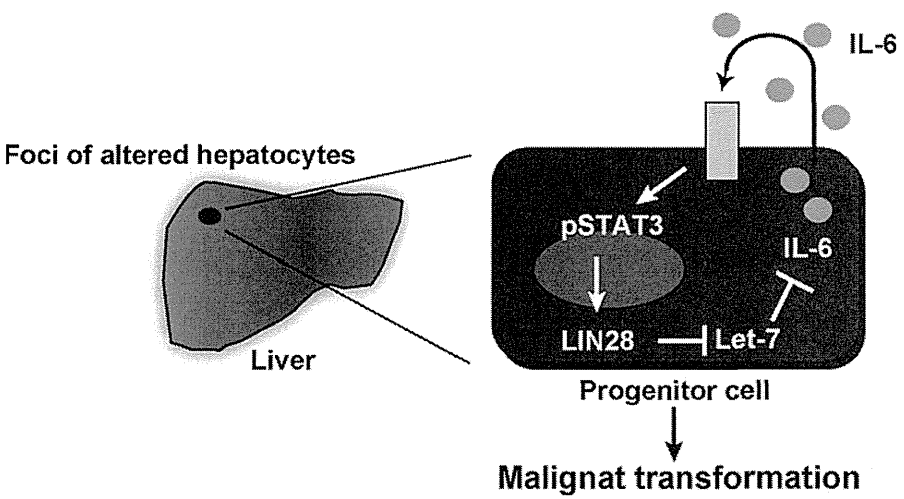

Fig. 4 A model bridging the malignant transformation of precursor cells and autocrine-mediated inflammation by microRNA. LIN28-expressing cells exist in the foci of altered hepatocytes, in which let-7 is downregulated, resulting in enhanced IL-6 expression, which mediates the progression of malignancies from progenitor cells

miRNAs as therapeutic targets in the liver

Recently, miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug for clinical use [48]. It was developed to target HCV, as the stability and propagation of this virus is dependent on a functional interaction between the HCV genome and miR-122 [49, 50]. No harmful events were observed in Phase I studies in healthy volunteers, and Phase II studies proceeded to evaluate the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. The patients were randomly assigned to receive 5 weeks of subcutaneous miravirsen injections at 3, 5 or 7 mg per kg body weight or a placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR-122 binding sites of the HCV genome [48]. The success of miravirsen is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy.

In addition to miravirsen, a clinical trial of MRX34 as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor miR-34 (Mirna Therapeutics, Austin, TX, USA). Further study of MRX34 is being conducted by Mirna Therapeutics, which initiated a Phase I study in May 2013 to examine the effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971). If these oligonucleotide therapies are successful, therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear promising [51].

Issues to be resolved in miRNA involvement in hepatocarcinogenesis

As described above, along with recent discoveries of the diverse effects of miRNAs in hepatocarcinogenesis, miRNA-mediated intervention is promising for the development of new diagnostic, preventive and therapeutic tools. However, the data obtained to date are far from complete. The following are some of the critical issues that we believe need to be resolved.

1. The reason for the non-reproducible results among studies should be determined to utilize the available data more reasonably and efficiently.
2. Identification of crucial driver miRNAs among the diverse deregulated miRNAs is critical to develop useful therapeutics in clinics, although even passive miRNAs may be utilized as markers for diagnosis or prediction of prognosis.
3. Comprehensive target gene analyses using in silico systems biology models should be applied.
4. For effective interventions using miRNA, the delivery method, improved oligonucleotide modification and safety must be further considered. Since miRNAs generally have diverse effects due to targeting multiple mRNAs, undesired outcomes, so called off-target effects, may be encountered, even when a specific miRNA is targeted.

Finding solutions to these issues should be considered as critically important for the near future in order to understand more fully the physiological function of miRNAs in hepatocarcinogenesis and utilize this knowledge in translational research.

Conclusions

The discovery of miRNA has, without doubt, opened up new possibilities for understanding the molecular mechanisms of gene regulation. As numerous findings regarding miRNA, from diverse perspectives, have been reported, the speed of discovery in this field is astonishing. In fact, novel therapeutics targeting miRNAs have already been successfully applied in clinical trials. Some miRNAs may be useful as novel biomarkers. Additionally, the discovery of novel concepts in the pathogenesis of hepatocarcinogenesis frequently involves miRNA. On the other hand, several important issues remain to be resolved in this field. Thus, continuous research in this field is still necessary to develop truly innovative concepts in our understanding of pathogenesis related to miRNA and to transform the obtained knowledge into real clinical applications.

Conflict of interest The authors declare that they have no conflict of interest.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (1): Management of hepatocellular carcinoma

Diagnostic and therapeutic application of noncoding RNAs for hepatocellular carcinoma

Chikako Shibata, Motoyuki Otsuka, Takahiro Kishikawa, Motoko Ohno, Takeshi Yoshikawa, Akemi Takata, Kazuhiko Koike

Chikako Shibata, Motoyuki Otsuka, Takahiro Kishikawa, Motoko Ohno, Takeshi Yoshikawa, Akemi Takata, Kazuhiko Koike, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan
Motoyuki Otsuka, Japan Science and Technology Agency, PRESTO, Kawaguchi, Saitama 332-0012, Japan
Author contributions: Shibata C, Otsuka M and Koike K designed and wrote the manuscript; Kishikawa T, Ohno M, Yoshikawa T and Takata A prepared the tables and references.
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Correspondence to: Motoyuki Otsuka, MD, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 5-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. otsukamo-tky@umin.ac.jp
Telephone: +81-3-38155411
Fax: +81-3-38140021
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Abstract

MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression posttranscriptionally, targeting thousands of messenger RNAs. Long noncoding RNAs (lncRNAs), another class of noncoding RNAs, have been determined to be also involved in transcription regulation and translation of target genes. Since deregulated expression levels or functions of miRNAs and lncRNAs in hepatocellular carcinoma (HCC) are frequently observed, clinical use of noncoding RNAs for novel diagnostic and therapeutic applications in the management of HCCs is highly and emergently expected. Here, we summarize recent findings regarding deregulated miRNAs and lncRNAs for their potential clinical use as diagnostic and prognostic biomarkers of HCC. Specifically, we emphasize the deregulated expression levels of such noncoding RNAs in patients' sera as noninvasive biomarkers, a field that requires urgent improvement in the clinical surveillance of HCC. Since nucleotide-based strategies are being applied to clinical therapeutics, we further summarize clinical and preclinical trials using oligonucleotides involving the use of miRNAs and small interfering RNAs against HCC as novel therapeutics. Finally, we discuss current open questions, which must be clarified in the near future for realistic clinical applications of these new strategies.

Key words: MicroRNA; Long noncoding RNA; Hepatocellular carcinoma; Clinical trials; Biomarker

Core tip: In this review, we summarize the latest findings on deregulated microRNAs (miRNAs) and long noncoding RNAs in hepatocellular carcinomas (HCCs) with a focus on their clinical use as novel diagnostic and prognostic

biomarkers. In addition, we summarize the current status of clinical and preclinical oligonucleotide therapies including miRNAs and small interfering RNAs as novel HCC therapeutics. This review will enable the readers to understand the current status of clinical applications and knowledge of noncoding RNAs in HCC management.

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INTRODUCTION

Noncoding RNAs contain multiple classes of RNAs that are not transcribed into proteins. While most noncoding RNAs studied to date are microRNAs (miRNAs), many noncoding RNAs with various lengths have also been reported.

MiRNAs are short, single-stranded RNAs that are expressed in most organisms[1-3]. Through gene expression regulation at a posttranscriptional level, miRNAs are involved in various physiological and pathological processes[4,5]. Since the discovery of miRNA lin-4 in Caenorhabditis elegans[6,7], as of August 2014, 1881 miRNA precursors and 2588 mature miRNA sequences in humans are deposited in miRBase, a miRNA database by the Sanger Institute[8]. MiRNAs are dysregulated in nearly all types of cancer[9,10], and specific signatures of aberrantly expressed miRNAs in specific cancers may have diagnostic and therapeutic implications[11,12].

Long noncoding RNAs (lncRNAs) also play crucial roles in transcription and translation[13,14]. Similar to miRNAs, their dysregulation is also associated with human cancers[15]. One of the most well-studied lncRNAs is the *HOX* transcript antisense intergenic RNA (HOTAIR). Class I homeobox genes (*HOX* in humans) encode 39 transcriptional factors initially described as master regulators of embryonic development[16] and display a unique gene network organization. HOTAIR, a 2.2-kb-long RNA residing within the *HOXC* locus, was initially described in breast cancer tissues, where it is highly expressed[17]. In addition to HOTAIR, many other lncRNAs are dysregulated in cancer tissues. Thus, lncRNAs may also be candidates for biomarker discovery and therapeutic applications in hepatocellular carcinomas (HCCs)[18].

In contrast to miRNAs and lncRNAs, short interfering RNAs (siRNAs) are double-stranded RNAs that degrade mRNAs through perfect matches with their target sequences. Although human telomerase reverse transcriptase was recently found to function as an RNA-dependent RNA polymerase and contribute to RNA silencing[19], its activities are not dominant in mammals. Additionally, endogenously produced siRNAs may play functional roles under limited circumstances in humans[20]. However, the exogenous application of synthesized siRNAs is an attractive method that could be used to intervene in crucial gene expression under pathological conditions, including cancers[21].

HCC is the third leading cause of cancer-related mortality worldwide[22]. Although advances have been made in early detection and interventional therapies, a continuing need exists to develop novel approaches for the management of advanced HCC[23]. While many reports have described deregulated expression levels or functions of miRNAs and lncRNAs in HCCs, we will focus on the potential clinical use of noncoding RNAs in the very near future for novel diagnostic and therapeutic applications in the management of HCCs.

NONCODING RNAS AS BIOMARKERS FOR HCC

Deregulated expression levels of noncoding RNAs in HCC tissues

Although several published reports have described deregulated expression levels of miRNAs and lncRNAs in HCC tissues[18,24,25], the data thus far vary greatly. The differences may be because of several reasons, including the use of different techniques or samples as controls, normal liver tissues *vs* nonneoplastic tissues around tumors, background livers with various fibrosis staging, inflammation activities, or etiologies, such as hepatitis B, hepatitis C, or steatohepatitis, as well as the age or sex of the tissue-derived patients; any of these factors may cause the differential expression status of miRNAs. Regardless of these limitations, the plenty data about dysregulated miRNAs in HCCs suggests that noncoding RNAs play crucial roles in hepatocarcinogenesis[24].

Deregulated expression of noncoding RNAs in HCC as prognostic/diagnostic markers

Deregulated expression levels of noncoding RNAs in HCC tissues that may be clinically useful as prognostic/diagnostic markers will be described herein. The landmark paper that initially addressed this issue focused on *miR26* expression levels in HCC tissues and was published in the New England Journal of Medicine[26]. In this study, HCC showed frequently reduced levels of *miR26*, and patients exhibited low *miR26* expression with a shorter overall survival but a better response to interferon therapy, indicating that miRNA expression status is associated with survival and response to therapy.

Expression levels of miRNAs have tissue specificities. In the liver, *miR122*, *miR192*, and *miR199a/b-3p* are highly expressed miRNAs of all mRNAs in the liver[27]. The role of *miR122* loss in hepatocarcinogenesis was confirmed in a mouse model[28,29], and its expression is decreased in HCCs, especially non-viral HCCs[27]. Decreased expression of *miR122* is also linked with poor prognosis of HCC[30]. Although *miR192* was not deregulated in HCCs in previous studies, *miR199a/b-3p*

Table 1 Representative noncoding RNAs in sera for Hepatocellular carcinoma diagnosis

MiRNA	Expression levels in HCC	Possible targets	Ref.
MiR21	Upregulated	PTEN, AKT, C/EBPb	[32,39,58]
MiR222	Upregulated	PP2A, p27, DDIT4	[42,43,59]
MiR223	Upregulated	Stathmin	[44]
HULC	Upregulated	IGF2BP1	[45-47]

HCC: Hepatocellular carcinoma; HULC: Highly up-regulated in liver cancer; PTEN: Phosphatase and tensin-like protein; AKT: V-akt murine thymoma viral oncogene homolog; C/EBPb: CCAAT/enhancer-binding protein beta; PP2A: Protein phosphatase 2A; IGF2BP1: Insulin-like growth factor 2 MRNA binding protein 1.

is frequently decreased in HCCs[27]. In contrast, *miR21*, whose expression is increased when rat hepatectomy[31], is upregulated as an onco-miRNA, resulting in the promotion of HCC[32]. *MiR21* expression in HCC tissues confers resistance to the antitumor effect of interferon-α and 5FU combination therapy[33].

Similar to miRNAs, expression levels of lncRNAs are also dysregulated in HCC tissues[18]. Among them, HOTAIR is overexpressed in HCC tissues and may confer chemoresistance[34]. Metastasis-associated lung adenocarcinoma transcript 1, which was initially discovered as an lncRNA associated with metastasis[35], is also upregulated in HCC tissues and may be useful as a biomarker for tumor recurrence. Recently, *HOXA* transcript at the distal tip (HOTTIP) was discovered to be located in physical contiguity with the *HOXA13* gene and upregulated in HCC tissues, and this was also associated with metastasis formation and poor patient survival[36]. These results show the functional importance of lncRNA dysregulation in HCC tissues and indicate their possible use as novel prognostic and diagnostic biomarkers.

Noncoding RNAs in the sera of patients with HCC as diagnostic markers

Although α-fetoprotein (AFP), AFP-L3, and des-gamma-carboxy prothrombin are useful noninvasive biomarkers for HCC surveillance[37], novel and sensitive biomarkers that can detect early HCC are needed. The identification of tumor-specific alterations in circulating nucleic acids of patients with cancer as noninvasive methods of cancer diagnosis is encouraging[38]. Although RNAs are generally considered unstable, they are actually quite stable and readily detected in patient serum and plasma. Microarrays, polymerase chain reaction methods, and next-generation sequencing technologies are generally utilized to detect circulating noncoding RNAs.

Although many reports have described circulating miRNA levels in patients with HCC, only a few tests have been reproducible. For example, data regarding upregulation of circulating *miR21*, *miR222*, and *miR223* in patients with HCC are inconsistent[32,33,38-44]. Highly upregulated in liver cancer, a 1.6-kb lncRNA, is also upregulated in HCC tissues[45-47] and is detected in the plasma of patients with HCC[18,48]. Although these results are encouraging, more work is needed to make the usability of circulating noncoding RNAs as novel biomarkers more reliable (Table 1). Specificity and sensitivity, as well as methods to quantitate small amounts of RNAs in sera with high reproducibility and the universal control to adjust the obtained data from differing times and samples, need to be urgently determined[49].

NONCODING RNAS AS NOVEL THERAPEUTICS AGAINST HCC

Ongoing clinical trials

Mounting evidence suggests that noncoding RNAs are frequently dysregulated in HCCs and possibly involved in oncogenesis and may therefore provide novel molecular targets as a therapeutic intervention. However, due to the complexity associated with pleiotropic miRNA functions and lncRNAs, the number of clinical trials is presently limited[50]. The leading nucleotide-targeting therapy, Miravirsen, an LNA-based *anti-miR122* against hepatitis C virus replication, has been successful in a Phase II a study[51]. In addition, MRX34, a liposome-formulated *miR-34* mimic developed by Mirna Therapeutics, produced complete HCC regression in mouse models[52], and a Phase I study is currently recruiting patients with advanced liver cancer for HCC therapeutic intervention (NCT01829971).

While siRNAs are not endogenous noncoding RNAs, they can be described as noncoding RNAs that have been tried as novel therapeutics against HCC. ALN-VSP (Alnylam Pharmaceuticals), an RNAi therapeutic targeting vascular endothelial growth factor and kinesin spindle protein, has been shown to be well tolerated in Phase I studies (NCT008822180 and NCT01158079) for the treatment of primary and metastatic liver cancer. The results demonstrated disease control lasting more than 6 mo in the majority of patients, including a complete response in a patient with endometrial cancer who had multiple liver metastases. TMK-polo-like kinase 1 (PLK1) (Tekmira Pharmaceuticals), an RNAi targeting PLK1, is also under a Phase I / II trial (NCT01437007). Early results show that TKM-PLK is well tolerated and demonstrates clinical benefits. Although primary results from these potential therapeutics are encouraging, the benefits and unexpected side effects need to be determined, especially under long-term use.

Preclinical trials

Anti-miR21 and *anti-miR221* are under development for clinical use (Regulus Therapeutics). *MiR21* is one of the most validated microRNA targets, with numerous scientific publications suggesting that *miR21* plays an important role in the initiation and progression of cancers, including liver cancer[32,53,54]. Similarly, *miR221* has been identified to be upregulated in multiple cancers including liver cancer[54-56]. *Anti-miR21* and *anti-miR221* prolonged survival time in a preclinical mouse model

Table 2 Representative noncoding RNAs under clinical and preclinical trials for hepatocellular carcinoma therapeutics

Target	Name	Content	Vendor	Current status
MiR34	MRX34	Liposome-formulated miR-34 mimic	Mirna Therapeutics	Phase I
VEGF/KSP	ALN-VSP	RNAi targeting VEGF/KSP	Alnylam Pharmaceuticals	Phase I
PLK1	TMK-PLK1	RNAi targeting PLK1	Tekmira Pharmaceuticals	Phase I / II
MiR21	Anti-miR21	Antisense against miR21	Regulus Therapeutics	Preclinical
MiR221	Anti-miR221	Antisense against miR221	Regulus Therapeutics	Preclinical
MiR7	MiR7 mimic	MiR7 mimic	MiReven	Preclinical

VEGF: Vascular endothelial growth factor; KSP: Kidney-specific cadherin; PLK1: Polo-like kinase 1.

that genetically develops HCC. An *miR7* mimic is also under development (MiReven). Mir7 targets the phosphoinositide 3-kinase (PI3K) pathway and decreases tumor growth both *in vitro* and *in vivo*[57]. These results are summarized in Table 2.

CHALLENGES FOR BETTER CLINICAL TRANSLATION

Several other miRNAs, including lncRNAs, which are dysregulated in HCCs, can be attractive therapeutic targets by RNA mimics, antisense RNA, or siRNA. In fact, many publications have reported their efficacy. However, obstacles remain to be addressed[24]: (1) The more reproducibility of the results should be achieved to make the data more reliable; (2) Identification of driver miRNAs in oncogenesis is important to develop therapeutics targeting such miRNAs, although we may be able to use passive miRNAs as prognostic and diagnostic bio-markers; and (3) The delivery methods of oligonucleotides into specific tissues with improved oligonucleotide modification, and safety need to be seriously considered for utilizing miRNAs in clinical applications. Because miRNAs generally target multiple mRNAs, unexpected outcomes, "off-target effects," may occur, even when targeting a single miRNA.

More research to solve these issues is definitely needed for the improved translational application utilizing the data about miRNAs in HCCs.

CONCLUSION

The discovery of miRNAs and lncRNAs has opened up new possibilities for novel diagnostic and therapeutic tools against HCCs. However, several important issues remain to be resolved. We must conduct continuous research to develop innovative and useful applications of the miRNA data in the clinical management of HCCs.

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Research

DNA methylation at hepatitis B viral integrants is associated with methylation at flanking human genomic sequences

Yoshiyuki Watanabe,[1,2,9] Hiroyuki Yamamoto,[1,9] Ritsuko Oikawa,[1] Minoru Toyota,[3] Masakazu Yamamoto,[4] Norihiro Kokudo,[5] Shinji Tanaka,[6] Shigeki Arii,[6] Hiroshi Yotsuyanagi,[7] Kazuhiko Koike,[8] and Fumio Itoh[1]

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan;* [2]*Internal Medicine, Kawasaki Rinko General Hospital, Kawasaki 210-0806, Japan;* [3]*Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan;* [4]*Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo 162-8666, Japan;* [5]*Hepato-Biliary-Pancreatic Surgery Division, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, University of Tokyo 113-8655, Japan;* [6]*Department of Hepatobiliary Pancreatic Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo 113-0034, Japan;* [7]*Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan;* [8]*Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan*

Integration of DNA viruses into the human genome plays an important role in various types of tumors, including hepatitis B virus (HBV)–related hepatocellular carcinoma. However, the molecular details and clinical impact of HBV integration on either human or HBV epigenomes are unknown. Here, we show that methylation of the integrated HBV DNA is related to the methylation status of the flanking human genome. We developed a next-generation sequencing-based method for structural methylation analysis of integrated viral genomes (denoted G-NaVI). This method is a novel approach that enables enrichment of viral fragments for sequencing using unique baits based on the sequence of the HBV genome. We detected integrated HBV sequences in the genome of the PLC/PRF/5 cell line and found variable levels of methylation within the integrated HBV genomes. Allele-specific methylation analysis revealed that the HBV genome often became significantly methylated when integrated into highly methylated host sites. After integration into unmethylated human genome regions such as promoters, however, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis. The observed dynamic changes in DNA methylation of the host and viral genomes may functionally affect the biological behavior of HBV. These findings may impact public health given that millions of people worldwide are carriers of HBV. We also believe our assay will be a powerful tool to increase our understanding of the various types of DNA virus-associated tumorigenesis.

[Supplemental material is available for this article.]

Hepatitis B virus (HBV) infects more than two billion people worldwide, and 400 million chronically infected individuals are at high risk of developing active hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV carriers with chronic liver disease are at a 100-fold greater risk of developing HCC, which is the third leading cause of cancer-related death worldwide. The HBV genome is integrated into the host genome in 90% of patients with HCC (HBV-HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV-HCCs have been analyzed by comprehensive genome sequencing and high-resolution genome mapping (Kan et al. 2013; Li and Mao 2013; Nakagawa and Shibata 2013). Moreover, the recent deep sequencing of HBV DNA in patients with HCC revealed increased integration events, structural alterations, and sequence variations (Ding et al. 2012; Fujimoto et al. 2012; Jiang et al. 2012; Sung et al. 2012; Toh et al. 2013). A recent study identified a viral-human chimeric fusion transcript, HBx-LINE1, that functions like a long noncoding RNA to promote HCC (Lau et al. 2014). However, the molecular details and clinical impact of HBV integration on the epigenomes of human cells and HBV remain to be defined.

Methylation of exogenous DNA (including viral DNA) that is integrated into the human genome has been studied over the past decade (Doerfler et al. 2001). Within the human genome, cytosine methylation in CpG dinucleotides (CpG sites), which cluster into islands associated with transcriptional promoters, is an important mechanism for regulating gene expression. Additionally, host cells use methylation as a defense mechanism against foreign agents (e.g., viral DNA) (Doerfler 2008; Doerfler et al. 2001). DNA methylation suppresses the expression of viral genes and other deleterious elements incorporated into the host genome over time. Establishment of de novo patterns of DNA methylation is char-

[9]**These authors contributed equally to this work.**
Corresponding author: h-yama@marianna-u.ac.jp.
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acterized by the gradual spread of methylation (Orend et al. 1991). Another attractive possibility is that DNA methylation camouflages the virus from the immune system (Tao and Robertson 2003; Hilleman 2004), resulting in a DNA methylation–related blockade of viral antigen presentation that allows the virus to escape immune control (Fernandez et al. 2009).

The DNA methylome of HBV in human cells may undergo dynamic changes at different stages of disease (Fernandez et al. 2009). For example, DNA methylation at the *HBVgp2* locus, which codes for the S viral proteins, reportedly increases during the progression from asymptomatic lesions to benign lesions, to premalignant disease and malignant tumors. However, because of the significant deletions of the integrated HBV genome detected in this previous study (Fernandez et al. 2009), the DNA methylome of HBV needs to be further characterized. Moreover, the molecular mechanisms involved and the clinical impact of the integration of HBV on the human and HBV epigenomes are unknown. To address these issues, we developed a next-generation sequencing (NGS)–based method for methylation analysis of integrated viral genomes (denoted G-NaVI) and applied this method to the integrative genomic and epigenomic analysis of human hepatoma cell lines and tissues with integrated HBV genomes.

Results

DNA methylation levels in PLC/PRF/5 cells and cancerous tissues obtained from HBV-HCC patients

Methylated CpG island (CGI) amplification (MCA) coupled with microarray (MCAM) analysis (Toyota et al. 1999; Oishi et al. 2012) was performed to detect methylated genes in the human PLC/PRF/5 cell line and in six paired specimens of primary HBV-HCC and adjacent tissues. Compared with the DNA methylation of CGIs in the healthy peripheral blood leukocytes of volunteers or the noncancerous tissues, levels of DNA methylation were not remarkable in the PLC/PRF/5 cells and the cancerous tissues obtained from HBV-HCC patients (Supplemental Fig. 1). These results were confirmed by bisulfite pyrosequencing of candidate tumor-related genes.

DNA methylation of CGIs of *HBx*

We then focused on epigenetic changes in the viral genome. Based on the hidden Markov models for sequence analysis performed on the CpG plugin of bioinformatics software Geneious 5.5.8 (see Methods section), a CpG island was found in only the promoter region of the *HBx* gene in the HBV genome (Fig. 1A; Supplemental Fig. 2; Durbin et al. 1998; Kearse et al. 2012). Host signal transduction pathways and gene expression are disrupted by the expression of *trans*-activating factors derived from the HBV genome, such as the HBx protein and PreS2 activators (Gatza et al. 2005; Lupberger and Hildt 2007). Moreover, transgenic mice expressing high levels of HBx in the liver develop HCC (Kim et al.1991; Koike et al. 1994). The DNA methylation levels of the CGIs of *HBx* were analyzed in 10 HBV-HCC samples and 10 adjacent samples, as well as samples of PLC/PRF/5 cells by bisulfite pyrosequencing (Fig. 1A; Supplemental Fig. 2). We performed advanced methylation quantification in long sequence runs by pyrosequencing on PyroMark Q24 Advanced and PyroMark Q24 instruments. Methylation levels of *HBx* varied across samples (Fig. 1B,C) and were generally lower in HCC tissues than in the adjacent tissues (Fig. 1B). This finding is consistent with a previous report that most HBV genomes, although globally methylated to a greater extent in malignant samples than in premalignant lesions, retain *HBx* in an unmethylated state (Fernandez et al. 2009). Because the pyrosequencing results represent the genome-wide average of DNA methylation levels at the particular CpG site, the results could be affected by the HBV integration site. Therefore, genome-wide methylation analysis of the integrated HBV sequence is necessary in relation to the methylation state of the adjacent human genome. We did not detect an association between *HBx* methylation levels and those of the LINE1 and *Alu*Yb8 repeats (Fig. 1B).

Fluorescence in situ hybridization (FISH) and *Alu* PCR analyses of HBV integration

We developed a FISH technique for detecting HBV DNA in the genome of PLC/PRF/5 cells (Supplemental Figs. 3, 4). Twelve specific primer pairs (FISH probes 1–12) were designed based on the HBV sequences integrated into the genome of PLC/PRF/5 cells; amplification from all primer pairs was confirmed (Supplemental Fig. 4A). These results suggest full-length or partial HBV sequences that are covered by the 12 primer pairs were integrated into the genome of the PLC/PRF/5 cells. The FISH probes were labeled with digoxigenin, and FISH was performed using Carnoy-fixed chromosomal and nuclear specimens. Multiple HBV fluorescent signals (green) were detected in the nuclei (Supplemental Fig. 4B) using probes for *HBx* and its CGI sequences (probes 5 and 6), but not with probes 1–4 or 7–12 (Supplemental Fig. 4C–E). *Alu*-PCR identified one *HBx* integration site in PLC/PRF/5 (Supplemental Fig. 5). The integrated *HBx* sequence was 213 bp and included a promoter region. The *HBx* gene body was located only 13 bases (ATG GCT GCT AGG T) from the transcription start site and was integrated into a noncoding region of the host genome. There were 200 bases of viral DNA sequence upstream of the *HBx* transcription start site. According to the human genome reference sequence (GRCh38) published by the Genome Reference Consortium, this integration site was identified as a noncoding region of host Chromosome 5 1,350,106–1,350,478 that is near the telomerase reverse transcriptase (*TERT*) gene (Supplemental Fig. 5).

NGS analysis of HBV DNA integration site sequences

We developed an NGS analysis technique for sequencing the HBV DNA integration sites (Supplemental Fig. 6A). For efficient genome analysis, we synthesized 12,391 custom baits based on the sequences of the HBV genotypes A to J and on those sequences present in the HBV-transformed PLC/PRF/5 cells that were not related to the human genome sequence (Supplemental Fig. 6B). The average read length was 333.14 bp with a modal length of ~500 bp (Supplemental Fig. 6C). The average read quality was 31.91, corresponding to >99.9% accuracy. We did not detect a common HBV integration site (Fig. 2). The integration sites in the PLC/PRF/5 genome included intergenic (39%), intronic (39%), promoter (8%), and divergent promoter (15%) regions but not exonic (0%) sequences (Fig. 2). HepG2.2.15 cells, which stably express and replicate HBV in a culture system, are derived from the human hepatoblastoma cell line HepG2 (Sells et al. 1987). In the HepG2.2.15 genome, the integration sites included intergenic (29%), intronic (57%), and other (14%) regions but not promoter (0%), divergent promoter (0%), or exonic (0%) sequences (Fig. 2).

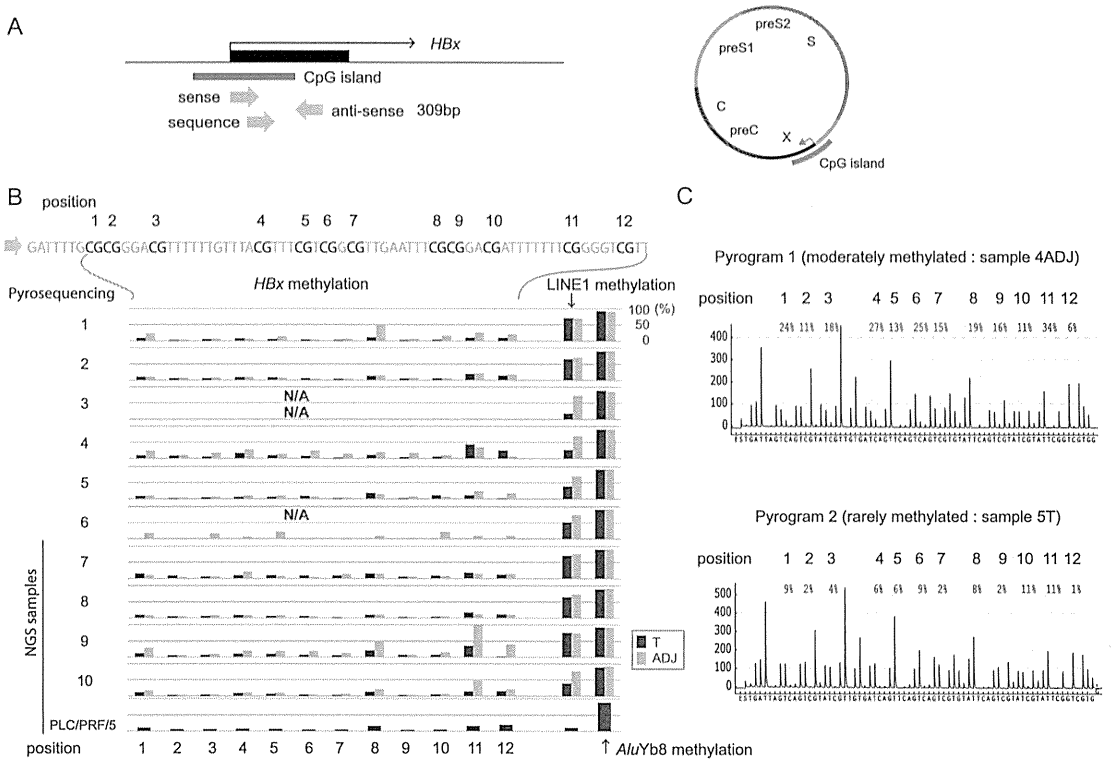

Figure 1. Methylation analysis of the CGI of the *HBx* gene. (*A*) Schema of the CGI of the *HBx* gene. Three arrows show the pyrosequencing primers used for the methylation analysis. (*B*) DNA methylation levels of the CpGs of the *HBx* gene, LINE1, and *AluYb8* in 10 paired HBV-HCC and adjacent nontumor tissue samples and PLC/PRF/5 DNA were analyzed using bisulfite pyrosequencing. Methylation levels of *HBx* varied across samples and were generally lower in HCC tissues than in the adjacent nontumor tissues. An association between *HBx* methylation levels and those of the LINE1 and *AluYb8* repeats was not observed. N/A, could not be analyzed. DNAs from four paired HBV-HCC and adjacent nontumor tissue samples (sample nos. 7–10), PLC/PRF5, and HepG2.2.15 were further analyzed using the NGS (G-NaVI method). (C) Representative pyrograms showing DNA methylation levels of the CpGs of the *HBx* gene. Methylation levels at 12 CpG sites of the *HBx* gene in adjacent nontumor tissue (sample no. 4ADJ) and tumor tissue (sample no. 5T) are shown.

DNA methylation of the integrated HBV genome as well as the adjacent human genome in cell lines

DNA methylation of the integrated HBV genome, as well as the adjacent human genome, was analyzed by bisulfite pyrosequencing. We detected varying levels of methylation of the HBV sequences integrated into the genome of PLC/PRF/5 cells (Fig. 3; Supplemental Fig. 7). Our data suggest DNA methylation in the integrated HBV genome is related to the methylation status of the integration sites within the human genome. We further characterized the methylation status of the HBV genome and human genome by allele-specific DNA methylation analysis (Fig. 3A), which revealed that the HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions such as promoters (Fig. 3B). Integration of the HBV genome did not affect the methylation status of the human genome, including the promoter regions of the *TERT* and *SNX15* genes. Methylation of HBV DNA integrated into HepG2.2.15 cells transformed with HBV DNA (using a head-to-tail dimer) was further analyzed by bisulfite pyrosequencing, which revealed that the HBV genome generally showed significant methylation when integrated into highly methylated regions of the human genome; however, the HBV genome remains largely unmethylated when integrated into unmethylated regions (Fig. 3A).

DNA methylation levels in orthologous loci

We examined methylation levels of orthologous loci in HepG2.2.15 cells and in peripheral blood lymphocytes (PBLs) of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells (Fig. 3B). Similarly, we examined methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were also generally similar to those of HepG2.2.15 cells (Fig. 3B).

DNA methylation of the integrated HBV genome and the adjacent human genome in HCC tissues

To determine whether our results are relevant to human tumors, we used bisulfite pyrosequencing to investigate the methylation status of the HBV and human genomes in surgical specimen pairs of HCC and adjacent nontumor tissues. We detected no common HBV integration site (Fig. 4; Supplemental Fig. 8). Recurrent HBV integration into the *SLC6A13* gene was observed in cancerous tissues. Integration sites were rarely detected in exonic regions of the DNA from HBV-HCC samples (Fig. 4; Supplemental Fig. 8). Similar to the results obtained from the PLC/PRF/5 and HepG2.2.15 cells, our analysis revealed that the HBV genome became significantly

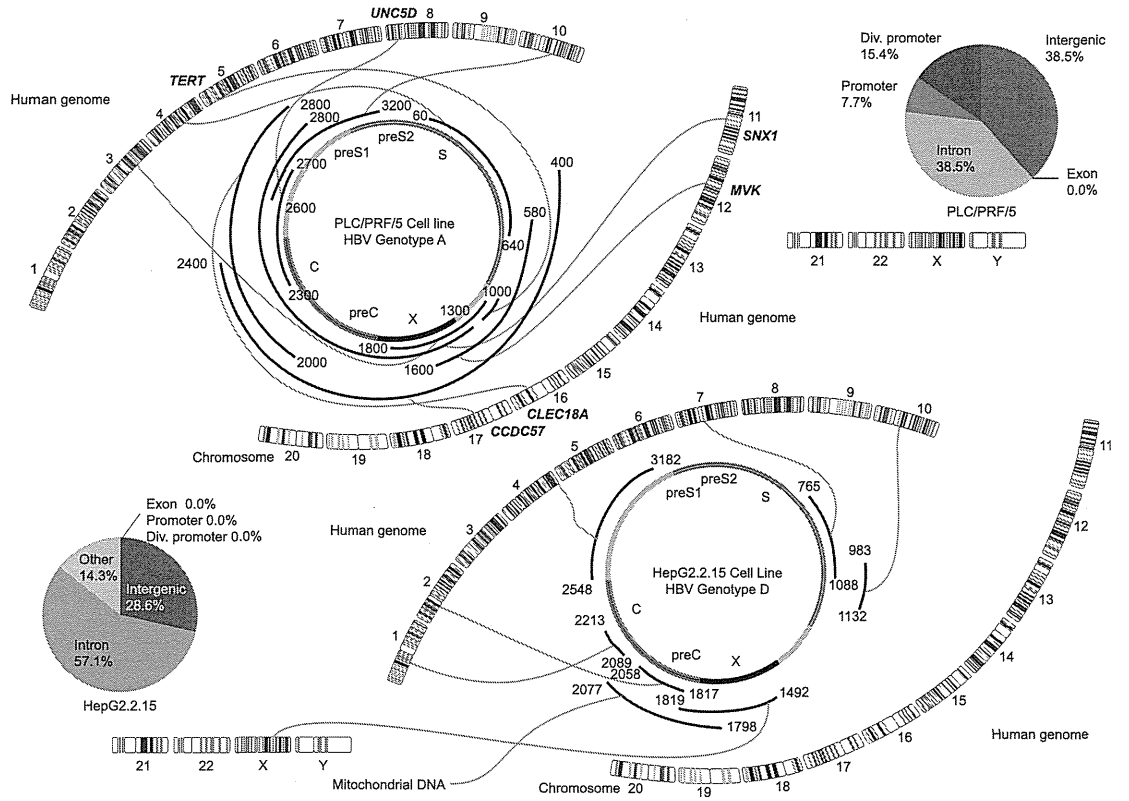

Figure 2. Distribution of the integration sites in the HBV genome and human chromosomes represented by Circos plots of the PLC/PRF/5 genome and the HepG2.2.15 genome. HBV DNA integration was analyzed using the G-NaVI method in the genome of PLC/PRF/5 cells and HepG2.2.15 cells. A common HBV integration site was not detected. Integration sites were not detected in exonic regions of the DNA from cell lines (Venn diagrams). The HBV genes (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and the 24 human chromosomes are shown.

methylated when integrated into highly methylated human genome regions but not when integrated into unmethylated human genome regions (Fig. 4).

Correlation between the methylation pattern of the integrated HBV DNA and the human genome

DNA fragments, including 200 bp of the HBV DNA and 200 bp of the human genome around the boundary, were analyzed for average methylation, GC content, and repetitive sequences. A statistically significant correlation was observed between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples (Fig. 5A–C; Supplemental Table 2). In contrast, average methylation did not correlate with GC content or repetitive sequences in the human and viral genome (Fig. 5D,E; Supplemental Table 2).

Using Bander software, we analyzed the chromatin structure at the integrated HBV site in PLC/PRF/5 and HepG2.2.15. Open chromatin and heterochromatin were observed more frequently at the integrated HBV in PLC/PRF/5 and HepG2.2.15, respectively (Supplemental Table 3). The difference may reflect the fact that PLC/PRF/5 is a naturally derived HBV-positive cell line and HepG2.2.15 is an HBV DNA-transfected cell line.

Discussion

We developed an NGS-based method for structural methylation analysis of integrated viral genomes. This method is a novel approach that enables the enrichment of viral fragments for sequencing using unique baits based only on the sequence of the HBV genome. We detected all regions of the human genome that harbored integrated HBV genomes without conducting unnecessary sequencing of regions where the HBV genome was not integrated. Because this technique only requires sequencing a small region of DNA around the integrated HBV sequences, a sufficient number of sequence reads can be acquired.

Methylation of viral DNA in infected cells may alter the expression patterns of viral genes related to infection and transformation (Burgers et al. 2007; Fernandez et al. 2009) and may clarify why certain infections are either cleared or persist with or without progression to precancer (Mirabello et al. 2012). To the best of our knowledge, we have, for the first time, established that the de novo patterns of DNA methylation in the integrated HBV genome are related to the methylation status of the integration sites within the human genome. A statistically significant correlation between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples has greatly substantiated our findings. It is possible that the HBV genome becomes inactivated by methylation, when it is integrated into highly methylated host sites; therefore, HBV methylation may not contribute to tumor development. However, after integration into unmethylated human genome regions such as promoters, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis (Fig. 6). Because multiple HBV integration sites were present in each of the analyzed samples, there remains the possibility of an asso-

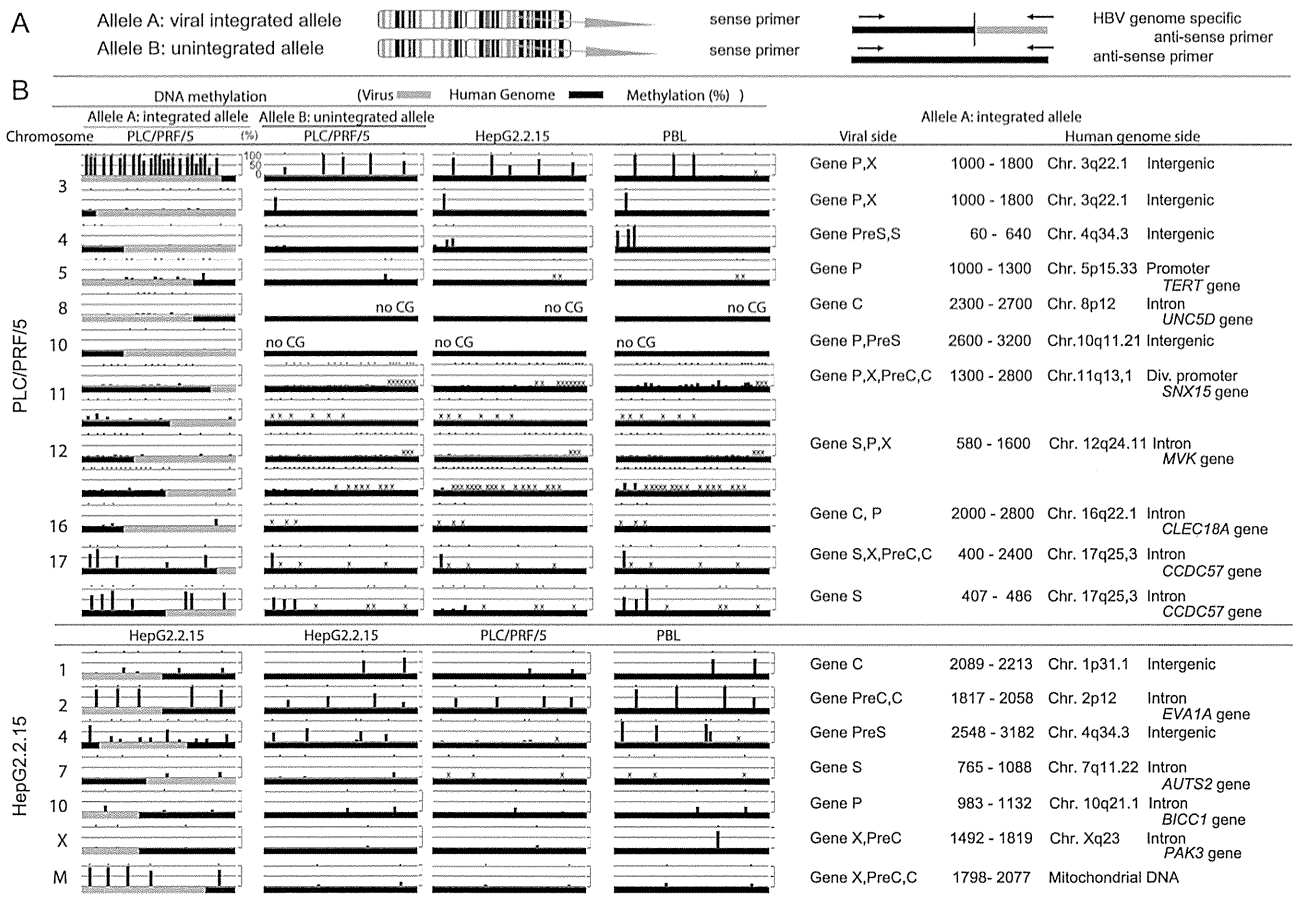

Figure 3. Allele-specific methylation analysis of the PLC/PRF/5 genome and the HepG2.2.15 genome. (*A*) A schema of allele-specific methylation analysis. (*B*) The methylation levels of the HBV and human genomes for the integrated and unintegrated alleles. Detailed results of the HBV integrants (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and flanking host genomes (position, chromosome, location of the genome, and gene names) are shown. DNA methylation of the integrated HBV genome as well as the flanking human genome was examined by allele-specific DNA methylation analysis using bisulfite pyrosequencing. The HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions. Methylation levels of orthologous loci in HepG2.2.15 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells. Similarly, methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were generally similar to those of HepG2.2.15 cells. (×) The desired quantitative methylation levels were not obtained because of technical difficulties with the sequences that were being analyzed.

ciation between methylation and viral transcript levels. The biological impact of methylation on viral transcript levels or viral function, induced by viral insertions, also needs to be further addressed.

Methylation levels of orthologous loci in other samples at the same (empty) target sites of PLC/PRF/5 were generally similar to those of PLC/PRF/5. Similar results were observed in HepG2.2.15. These data suggest that a "before and after" relationship exists between methylation levels at preexisting target sites and those within viral insertions. At the same time, we cannot rule out the possibility that the integration of the virus subsequently affects the methylation established at the flanking target site, perhaps by acting in trans on the empty target site-containing allele. Therefore, this issue needs to be further addressed.

Differences in the integrated viral sequences could have a direct impact on the amount of cytosine methylation observed. In cases where the integration site is a highly active promoter, comparisons of methylation statuses may not be informative. Additional studies, using a large number of samples, are needed to address this issue.

Our results are notable because other studies have detected a statistically significant enrichment of HBV integration into regulatory regions, particularly promoters, in tumors (Sung et al. 2012; Toh et al. 2013); this observation may be explained by the relatively open chromatin structure of promoter regions. Average methylation did not correlate with GC content or repetitive sequences in the human and viral genomes. The relationship between methylation of HBV sequences and chromatin structure remains to be clarified because of the limitation of the Bander software used in this study. Although the mechanism needs clarification, the significant enrichment of HBV integration into regulatory regions would favor integrated HBV nonmethylation and lead to tumorigenesis. Alternatively, while the integration of HBV into the host genome may be random, HBV integration into regulatory regions is positively selected during tumorigenesis (Toh et al. 2013).