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TOPIC HIGHLIGHT

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Diagnostic and therapeutic application of noncoding RNAs for hepatocellular carcinoma

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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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REVIEW

The role of microRNAs in hepatocarcinogenesis: current knowledge and future prospects

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Abstract MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression post-transcriptionally through complementary base pairing with thousands of messenger RNAs. Although the precise biological functions of individual miRNAs are still unknown, miRNAs are speculated to play important roles in diverse biological processes through fine regulation of their target gene expression. A growing body of data indicates the deregulation of miRNAs during hepatocarcinogenesis. In this review, we summarize recent findings regarding deregulated miRNA expression and their possible target genes in hepatocarcinogenesis, with emphasis on inflammation-related hepatocarcinogenesis. Because miRNA-based strategies are being applied to clinical therapeutics, precise knowledge of miRNA functions is crucial both scientifically and clinically. We discuss the current open questions from these points of view, which must be clarified in the near future.

Keywords MicroRNA · Hepatocarcinogenesis · Inflammation

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Introduction

MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs, which are expressed in most organisms, from plants to vertebrates [1]. Since the discovery of the miRNA lin-4 in *Caenorhabditis elegans* [2, 3], 1,872 miRNA precursors and 2,578 mature miRNA sequences in humans have been deposited in miRBase, a public repository hosted by the Sanger Institute, as of November 2013 [4]. Bioinformatic predictions suggest that miRNAs regulate more than 30 % of human protein-coding genes [5–7]. Through the regulation of gene expression, miRNAs are involved in various physiological and pathological processes, including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis and oncogenic suppression [8, 9]. Thus, it is not surprising that deregulation of miRNAs is linked closely to various human pathological conditions. In this review, we will describe the crucial role of miRNAs in liver carcinogenesis, especially inflammation-related hepatocarcinogenesis.

Biogenesis and functions of miRNAs

Transcription is the first step in miRNA expression (Fig. 1). Similar to most protein-coding genes, transcriptional factors, enhancers and silencers are involved in miRNA transcription [10–12]. Epigenetic mechanisms, such as promoter methylation or histone modification, also regulate miRNA transcription, and it was shown that histone deacetylase (HDAC) inhibition results in transcriptional changes in ~40 % of miRNAs [13].

Primary miRNAs, which possess stem-loop structures, are transcribed by RNA polymerase II [8]. These pri-miRNAs are processed by a microprocessor complex

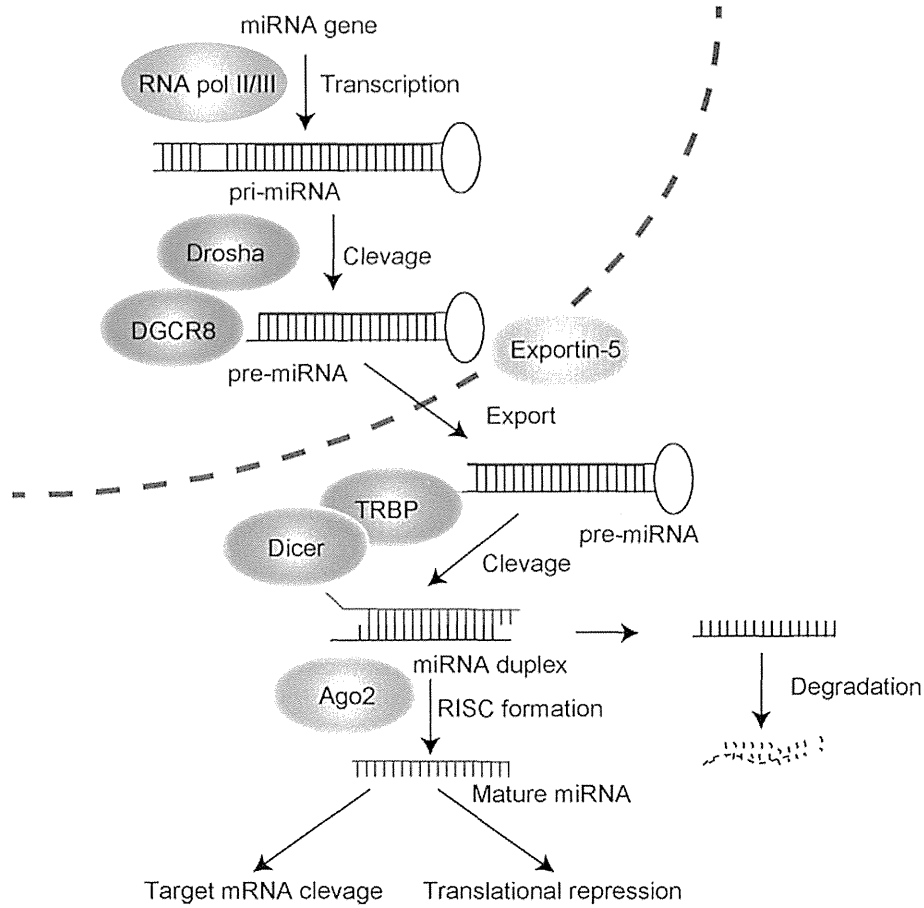

Fig. 1 Biogenesis of miRNAs. The primary miRNA transcript (pri-miRNA) is transcribed from the genome by RNA polymerase II or III. The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA into the precursor hairpin, pre-miRNA in the nucleus. The pre-miRNA is exported from the nucleus by exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein, TRBP, cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage or translational repression. The passenger strand (*black*) is degraded

comprising Drosha (RNAase III) [14] and DGCR8/Pasha [15] in the nucleus [16]. The processed products are approximately 65-nucleotide hairpin-shaped precursors (pre-miRNAs) that are transported to the cytoplasm via exportin-5 [17, 18]. Pre-miRNAs are further cleaved into mature miRNAs by Drosha and Dicer RNA polymerase III. Mature miRNA duplexes are loaded onto an RNA-induced silencing complex (RISC) and are unwound into the single-stranded mature form [19–21]. The resulting co-complex directly targets the 3′-untranslated regions (3′-UTRs) of target mRNAs, depending on the sequence similarities, to negatively regulate their expression by enhancing mRNA cleavage or inhibiting translation (Fig. 1) [8, 22]. Because most miRNAs guide the recognition of imperfect matches of target mRNAs, individual miRNAs have multiple (probably hundreds) of mRNA targets. In addition, multiple miRNAs can cooperate to regulate the expression of the same transcript [6]. Thus, depending upon the identity of the target mRNAs, miRNAs play roles as "fine-tuners of gene expression" in the control of various biological functions.

Identifying functionally important miRNA target genes is crucial for understanding the impact of specific miRNAs on cellular function. However, this is challenging because miRNAs usually have imperfect complementarity with their targets [22]. In mammals, the most consistent requirement for miRNA-target interaction, although not always essential, is a contiguous and perfect pairing of the miRNA (nt 2–8), representing the "seed" sequence [22]. In many cases, the seed sequences determine this recognition, but in other cases, additional determinants are required, such as reasonable complementarity to the miRNA 3′ half to stabilize the interaction. In addition, target pairing to the center of some miRNAs has also been reported [23]. Although public miRNA target prediction algorithms, such as TargetScan [24] and PicTar [25], have facilitated the rapid identification of miRNA target genes [22], candidates should be validated experimentally.

miRNAs and cancer

The involvement of miRNAs in cancer pathogenesis is well established. miRNAs can affect six hallmarks of malignant cells, which are (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) angiogenesis, and (6) invasion and metastasis [26]. miRNAs are frequently

up- or downregulated in malignant tissues and can be considered oncogenes or tumor suppressors, respectively. However, it is essential to test experimentally whether the deregulated miRNAs are actually causative to carcinogenesis, since miRNAs have a very restricted tissue-specific expression and the apparent miRNA modulation in cancer tissues may only reflect the different constituents of a cell population as compared to normal tissues. Extensive analyses have confirmed the causative roles of miRNAs in cancer by using either human cancer cells or genetically engineered animal models, such as transgenic expression of miR-155, miR-21 and miR-15-a/16-1, which are sufficient to initiate lymphomagenesis in mice [27–29]. These results suggest the potential role of miRNAs in the pathogenesis of carcinogenesis and as therapeutic targets.

miRNAs and hepatocarcinogenesis

Numerous reports regarding the deregulated expression of miRNAs in human hepatocellular carcinoma (HCC) are extant. Most studies compared the miRNA expression levels between cancer tissues and background non-tumorous tissues, selected candidate miRNA(s) and revealed their target genes, which may be involved in carcinogenesis. As shown in Tables 1 and 2, many miRNAs have been identified as downregulated or upregulated in recent studies (Tables 1, 2). However, these numerous results are not always superimposable due to the large variances in the results. These significant differences may be due to several reasons, such as the use of different techniques or different samples as controls, normal liver tissues versus peritumoral non-neoplastic tissues. In addition, one may need to take into consideration the fact that HCCs arise in background livers with different etiologies, such as hepatitis B, hepatitis C or steatohepatitis, and also the age or sex of the tissue-derived patients and background liver condition, such as fibrosis staging or inflammation activity, which may result in differences in the expression status of miRNAs. Despite these considerable limitations, the list suggests that diverse miRNAs play crucial roles in hepatocarcinogenesis. We will briefly describe some of them below.

The expression levels of miRNAs have restricted tissue specificities. In the liver, miR-122, miR-192 and miR-199a/b-3p are the three most expressed miRNAs, accounting for 52, 17 and 5 % of all mRNAs in the tissues, respectively [30]. The tumorigenic role of the loss of miR-122 was confirmed in gene-knockout mice [31, 32] and its expression is indeed decreased in half of the HCCs, especially non-viral HCCs [30]. We also reported that decreased expression of miR-122 is linked with poor prognosis of HCC [33]. While miR-192 does not appear to be deregulated in HCC samples in previous studies, miR-199a/b-3p is decreased with high frequency in HCC, which is closely linked to a poor prognosis of HCC [30]. In contrast, miR-21, whose expression is increased following rat hepatectomy [34], is upregulated as a known oncomiRNA and represses PTEN signaling, resulting in promotion of HCC development [35]. Although individual miRNAs may be involved in hepatocarcinogenesis, because miRNAs often function co-operatively, the extent of their involvement remains to be determined.

As described above, miRNAs usually have multiple mRNA targets. Thus, it is not practical to describe only a few genes as being responsible for the phenotypes by deregulation of specific miRNAs, while many studies identify specific genes as targets of specific miRNAs. Nonetheless, the identified targeted genes are generally related to at least one of the hallmarks of cancer, such as cell growth, apoptosis, invasion, and so on. These results suggest that the deregulation of miRNA expression might mediate hepatocarcinogenesis through deregulating the expression of their target genes.

The miRNAs identified as deregulated in hepatocarcinogenesis may be useful as diagnostic and prognostic markers [36], because miRNAs in the circulation are reported to be relatively stable [37]. Also, deregulated miRNAs may be candidate therapeutic and preventive targets against HCC. However, to include the obtained results in clinical interventional applications, it is necessary to confirm if the deregulated miRNAs are truly drivers or are simply passive in hepatocarcinogenesis. To this end, genetically modified mice may provide some information. In addition, to correctly interpret the data, a standard method of normalizing the microRNAome data between studies may also be crucial. Since there are multiple target genes of miRNAs and, conversely, one transcript can be targeted by multiple miRNAs, a more systematic comparison using miRNA data, transcriptome data and proteome data would increase our understanding of the consequences of the deregulation of miRNAs during hepatocarcinogenesis. From this point of view, systematic and comprehensive target gene analyses for in silico systems biology models may be one option to resolve these issues.

miRNAs linked to inflammation-mediated hepatocarcinogenesis

Inflammation is considered to be a major cause of cancer [38, 39]. In the liver, hepatocarcinogenesis frequently occurs in persistently inflamed liver tissues caused by chronic hepatitis viral infection or non-alcoholic steatohepatitis. However, the molecular linkage between chronic inflammation and carcinogenesis is not well characterized.

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]

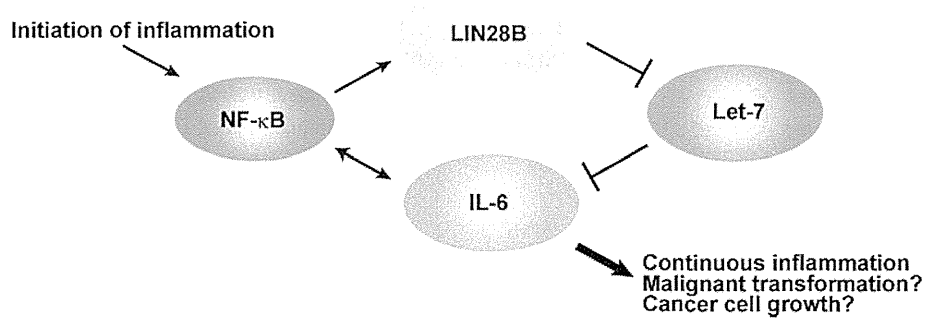

Fig. 2 A model bridging chronic inflammation and transformation by miRNA. Inflammation triggers activation of NF-κB, which leads to transcription of LIN28B. LIN28B inhibits the production of Let-7. Let-7 normally inhibits IL-6 expression, resulting in higher levels of IL-6 than are achieved by NF-κB 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

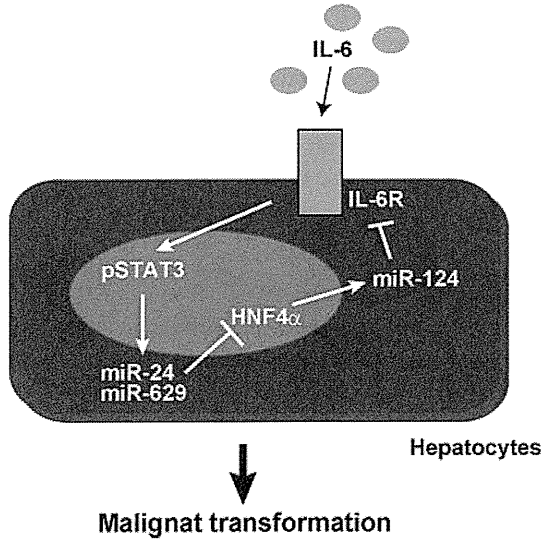

Fig. 3 A model describing a positive feedback loop mediated by miRNAs from transient HNF4α inhibition to transformation. Transient silencing of HNF4α is mediated by miR-24 and miR-629, both of which are induced by STAT3 activation following IL-6 stimulation. miR-124, whose promoter region contains HNF4α-binding 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.

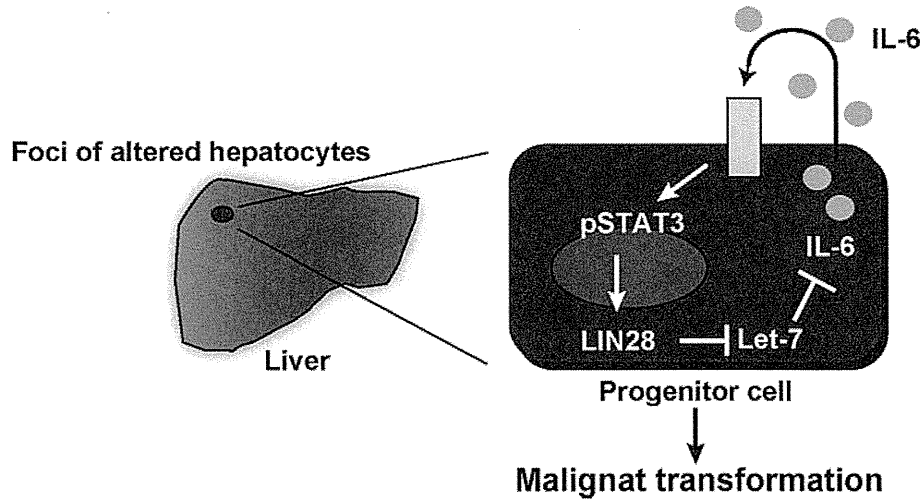

Fig. 4 A model bridging the malignant transformation of precursor cells and autocrine-mediated inflammation by microRNA. LIN28-expressing cells exist in the foci of altered hepatocytes, in which let-7 is downregulated, resulting in enhanced IL-6 expression, which mediates the progression of malignancies 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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Specific delivery of microRNA93 into HBV-replicating hepatocytes downregulates protein expression of liver cancer susceptible gene MICA

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). To date, the lack of efficient in vitro systems supporting HBV infection and replication has been a major limitation of HBV research. Although primary human hepatocytes support the complete HBV life cycle, their limited availability and difficulties with gene transduction remain problematic. Here, we used human primary hepatocytes isolated from humanized chimeric uPA/SCID mice as efficient sources. These hepatocytes supported HBV replication in vitro. Based on analyses of mRNA and microRNA (miRNA) expression levels in HBV-infected hepatocytes, miRNA93 was significantly downregulated during HBV infection. MiRNA93 is critical for regulating the expression levels of MICA protein, which is a determinant for HBV-induced HCC susceptibility. Exogenous addition of miRNA93 in HBV-infected hepatocytes using bionanocapsules consisted of HBV envelope L proteins restored MICA protein expression levels in the supernatant. These results suggest that the rescued suppression of soluble MICA protein levels by miRNA93 targeted to HBV-infected hepatocytes using bionanocapsules may be useful for the prevention of HBV-induced HCC by altering deregulated miRNA93 expression.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, and more than 350 million people globally are chronic carriers of the virus [1]. A significant number of these carriers suffer from either liver failure or hepatocellular carcinoma (HCC) during the late stages of the disease [2]. In fact, chronic infection with HBV is responsible for 60% of HCC cases in Asia and Africa and at least 20% those in Europe, Japan, and the United States [3].

While nucleoside and nucleotide analogs have been applied in the attempts to suppress HBV replication [4, 5], complete elimination of HBV (including cccDNA) remains difficult [6, 7], and an increased understanding of HBV replication and pathogenesis at the molecular level is essential for clinical management of chronic HBV infection. However, the lack of appropriate cell culture systems supporting stable and efficient HBV infection has been a major limitation. Although transient transfection or viral transfer of HBV genes or genomes are used in the study of specific steps of the HBV cell cycle [8-12], they do not accurately reflect the biology of HBV infection and replication. Thus, humanized mice are used for hepatitis virus research [13-18]. Although these mice are useful, immune deficient, chimeric mice are difficult to handle