Analysis of long non-coding RNA (lncRNA) expression in hepatitis B patients

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been implicated in numerous biological processes, including epigenetic regulation, cell-cycle control, and transcriptional/translational regulation of gene expression. Differential expression of lncRNAs and disruption of the regulatory processes are recognized as critical steps in cancer development. The role of lncRNAs in hepatitis B virus (HBV) infection is not well understood. Here we analyzed the expression of 135 lncRNAs in plasma samples of 82 HBV patients (classified as chronic patients, inactive carriers, or resolved patients) at diagnosis and at 12 months of treatment in relation to control group (81 healthy volunteers). We also investigated the effect of small interfering RNA (siRNA)-mediated silencing of *lincRNA-SFMBT2* on HBV-positive human liver cancer cell line. lncRNA expression was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Chemically synthesized siRNAs were transfected into the cell lines using Lipofectamine 2000 Reagent (Thermo Fisher Scientific). HBV DNA and HBsAg and HBeAg were detected in transfected cultures by real-time PCR and ELISA, respectively, using commercial kits. We observed changes in lncRNA expression in all three HBV groups, compared to control group. Most notably, the expression of *anti-NOS2A, lincRNA-SFMBT2*, and *Zfhx2as* was significantly increased and expression of *Y5* lncRNA was decreased in chronic HBV patients. A decreased *Y5* expression and increased *lincRNA-SFMBT2* expression were observed in inactive HBsAg carriers. The expression of *HOTTIP*, *MEG9*, and *PCAT-32* was increased in resolved HBV patients, and no significant change in the expression of *Y5* was observed, compared to control group. siRNA-mediated inhibition of *lincRNA-SFMBT2* decreased the level of HBV DNA in human liver cancer cells. Further research is needed to confirm the prognostic as well as therapeutic role of these lncRNAs in HBV patients.

KEY WORDS: HBV; lncRNA; chronic hepatitis B; inactive HBsAg carrier; resolved hepatitis B
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INTRODUCTION

Hepatitis B virus (HBV) is a double-stranded DNA virus and a member of the Hepadnavirus family. HBV infection can lead to acute and chronic diseases of the liver and represents a global public health problem. Liver diseases associated with HBV are among the most common causes of liver transplantation [1].

Long non-coding RNAs (lncRNAs) are defined as transcribed RNA molecules not translated into proteins and longer than 200 nucleotides. Initially, the length of 200 nucleotides was set as a cut-off in RNA purification protocols to separate long from short ncRNAs, however, this length does not fully explain the functional properties of lncRNAs [2,3]. The majority of the identified lncRNAs are transcribed by RNA polymerase II. Although not necessarily, these transcripts may be polyadenylated, and are localized in the nucleus or cytosol [4]. It is assumed that lncRNAs are more numerous than protein-coding genes in the genome, however, accurate/complete classification and identification schemes for lncRNAs are still not available. Based on their location in relation to protein-coding genes, lncRNAs can be classified as intergenic, sense and antisense (exonic, intronic or overlapping), and bidirectional [5]. According to different functions, lncRNAs may be classified as RNAs that: 1) regulate gene expression, 2) act as microRNA (miRNA) decoys to free target mRNAs, 3) regulate mRNA translation, and 4) regulate protein activities [6].

lncRNAs have been implicated in numerous biological processes, including epigenetic regulation of gene expression

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(e.g., genomic imprinting), apoptosis, cell-cycle control, transcriptional, post-transcriptional, and translational regulation of gene expression, as well as in the development, differentiation and senescence of cells [6-11]. Differential expression of lncRNAs and disruption of such regulatory processes have been recognized as critical steps in cancer development. Moreover, different studies indicated the use of lncRNAs for diagnostic and therapeutic purposes, i.e., as biomarkers for specific cancers, candidates for therapeutic interventions, as well as targets in terms of regulation of lncRNA expression [12].

A popular technique that is used to suppress the expression of specific lncRNAs is RNA interference (RNAi) with small interfering RNAs (siRNAs). siRNAs can selectively target lncRNAs leading to their degradation. In experimental studies, the stability of siRNAs may be improved by different chemical modifications, to achieve prolonged inhibition of targeted lncRNAs.

The role of lncRNAs in hepatitis B infection is not clear. Perz et al. [13] investigated fractions of cirrhosis and hepatocellular carcinoma (HCC) related to chronic HBV or hepatitis C virus (HCV) infection in 11 World Health Organization (WHO)-based regions and reported that, globally, about three quarters of HCCs were attributed to HBV or HCV infection [13]. Worldwide, chronic HBV infection explains around 50% of all cases of HCC including all childhood cases [14]. Chronic HBV infection was estimated to be the main risk factor for HCC in countries with developing economies [15].

The HBV genome is a partially double-stranded DNA molecule containing four overlapping open reading frames (ORFs) encoding the envelope, core, polymerase, and HBx proteins. X ORF encodes the small regulatory protein HBx of 154 amino acids in length [16]. HBx promotes the expression and replication of viral genes through the transactivation of cellular promoters and enhancers important for continuous viral infection [17]. HBx also affects cell survival, proliferation, migration and transformation by interrupting several cell signal transduction pathways [18,19]. Current evidence supports an important role of HBx in the pathogenesis of HBV-mediated HCC [20].

In this study, we analyzed the expression of 135 lncRNAs in three HBV groups (chronic, inactive carriers, and resolved patients) at diagnosis and at 12 months of treatment in relation to control group. We also investigated the effect of siRNA-mediated silencing of *lincRNA-SFMBT2* on HBV-positive human liver cancer cell line.

MATERIALS AND METHODS

Patient and control groups

The patient group included 82 randomized patients with HBV who had been referred to the Tepecik Training and Research Hospital for Infectious Diseases and Clinical Microbiology Clinic. Patients with decompensated cirrhosis, concomitant hepatitis A virus (HAV), HCV, hepatitis D virus (HDV), hepatitis E virus (HEV), human immunodeficiency virus (HIV), and any other viral infection or cause of liver disease were excluded. According to the treatment/follow-up response at 6 months and the American Association for the Study of Liver Diseases (AASLD) guidelines, the patients in HBV group were classified as follows: 27 patients (14 females, 13 males) with chronic HBV infection, 27 (23 F, 4 M) inactive hepatitis B surface antigen (HBsAg) carriers, and 28 (11 F, 17 M) resolved HBV patients. The control group included age-matched 81 healthy volunteers (41 F, 40 M) with no history of infectious diseases (including HBV), psychiatric, neurological, or metabolic disorder. Demographic and clinical features of HBV and control groups are given in Table 1.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study.

lncRNA expression profiling

lncRNA expression profiling included the following steps: collection of plasma samples from HBV patients and controls, total RNA isolation, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Changes in lncRNA expression were analyzed at diagnosis and at 12 months after treatment/follow-up in the three HBV groups, in relation to control group.

Collection of plasma samples

Peripheral blood samples (4 mL) of patients, at diagnosis and 12 months after treatment, and of controls were collected, transferred to microcentrifuge tubes, and centrifuged at 200 × g for 10 minutes at 40°C. The supernatant was then transferred to a new microcentrifuge tube and centrifuged at 12,000 × g for 10 minutes at 4°C to remove all cellular components. The upper plasma layer was collected for total RNA isolation.

lncRNA isolation

Total RNA, including lncRNAs, was isolated from plasma samples using the RNeasy mini kit (Qiagen, Germany). The purity and concentration of RNAs were determined by measuring the absorbance at 260/280 nm and 230/260 nm on a Nanodrop instrument (Thermo Scientific, USA). RNA samples with A260/A280 and A230/A260 absorbance ratios >2.0 were considered "pure" and used in further analysis.

TABLE 1. Demographic and clinical features of patients with hepatitis B virus (HBV) and control group

Parameter		Chronic HBV patients	Inactive HBsAg carriers	Resolved HBV patients	Control	*p*
Gender	Female	14	23	11	41	0.004
	Male	13	4	17	40	
Age	Mean±SD	40.11±14.26	43.19±10.16	46.54±12	45.26±11.52	0.173
	Median (range)	39 (21-65)	43 (25-60)	48 (26-67)	45 (19-79)	
HBsAg	Negative	0	0	0		<0.0001
	Positive	27	27	28		
Anti-HBs	Negative	27	27			>0.05
	Positive	0	0			
	Mean±SD			30.05±63.02		
	Median (range)			12.20 (0.17-290)		
Anti-HBe	Negative	8	0	1		0.001
	Positive	19	27	27		
HBeAg	Negative	19	27	27		0.001
	Positive	8	0	1		
Treatment	Entecavir	8				
	Tenofovir	19				
Albumin	Mean±SD	4.08±0.68				
	Median (range)	4.30 (1.50-4.60)				
AST	Mean±SD	36.52±18.29	23.59±8.95	21.79±6.24		<0.0001
	Median (range)	33.00 (13-83)	22 (13-50)	2.50 (10-35)		
ALT	Mean±SD	43.15±29.51	19.81±8.12	20.71±7.03		<0.0001
	Median (range)	32.00 (15-130)	18 (11-42)	19 (10-39)		
TBil	Mean±SD	0.84±0.67	0.73±0.35	0.74±0.20		0.615
	Median (range)	0.70 (0.10-3.90)	0.70 (0.10-1.70)	0.70 (0.40-1.30)		
DBil	Mean±SD	0.25±0.39	0.14±0.11	0.14±0.13		0.198
	Median (range)	0.14 (0.05-2.02)	1.20 (0.01-0.60)	1.20 (0.60-0.80)		
AFP	Mean±SD	2.73±1.10	3.05±2.09			0.491
	Median (range)	2.33 (1.54-5.77)	2.59 (2.96-11.50)			
Hb	Mean±SD	13.60±1.79	12.69±1.40			0.041
	Median (range)	14.20 (9.3-16.30)	12.70 (9.9-15.70)			
Plt	Mean±SD	220.19±51.78	241.15±62.55			0.186
	Median (range)	225 (122-331)	244 (60-346)			
PT/INR	Mean±SD	1.03±0.21	1.02±0.10			0.778
	Median (range	1.06 (0.08-1.21)	1.03 (0.84-1.20)			

HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibody for HBsAg; HBeAg: Hepatitis B envelope antigen; Anti-HBe: Antibody for HBeAg; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TBil: Total bilirubin; DBil: Direct bilirubin; AFP: Alpha-fetoprotein; Hb: Hemoglobin; Plt: Platelets; PT/INR: Prothrombin Time and International Normalized Ratio

cDNA synthesis

Total RNA was converted into cDNA using the RNA-Quant cDNA Synthesis Kit (System Biosciences, USA) which allows the detection of lncRNAs, following manufacturer's instructions.

qRT-PCR analysis

Changes in lncRNA expression levels in the three HBV groups and control group were analyzed for a panel of 135 lncRNAs. These lncRNAs were selected from a lncRNA database (http://www.lncrnadb.org/), which provides a comprehensive list of lncRNAs that have been associated with biological functions in eukaryotes [21]. lncRNA expression was analyzed by qRT-PCR using the lncRNA Profiler qPCR Array Kit (Human) and Disease-Related lncProfiler 96-well Primer Sets (System Biosciences, USA) and Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA). Amplifications were performed on a LightCycler 480 II (Roche Life Science, Germany). The average values of reference gene expressions (7SL, small conditional [scRNA], 5.8S ribosomal RNA [rRNA], U87 Small Cajal body-specific RNA [scaRNA], U6 non-coding small nuclear RNA (snNA), *ACTB*, *B2M*, *PGK1*, *GAPDH*, *HPRT1*, *RPL1A*, and *RPL13A*) were used for normalization. The relative expression of lncRNAs in HBV groups at diagnosis and 12 months of treatment/follow-up was determined according to the $2^{-\Delta\Delta C}{}_{T}$ method in relation to control group.

The expression profiles of the following 135 lncRNAs were analyzed: *21A, 7SK, 7SL, AAA1, aHIF, Air, AK023948, Alpha 250, Alpha 280, ANCR, ANRIL, anti-NOS2A, antiPeg11, BACE1AS, BC017743, BC043430, BC200, BCMS, BIC, CAR Intergenic 10, CCND1, CMPD, DD3, DGCR5, DHFR upstream transcript, DISC2, Dio3os, DLG2AS, E2F4 antisense, EGO, EGO B, Ego A, Emx2os, Evf1, EVF2, GAS5, GOMAFU, H19, H19 antisense, H19 upstream conserved 1 & 2, H19-AS, HAR1A, HAR1B, HOTAIR, HOTAIRM1, HOTTIP, HOXA11AS, HOXA1AS_AA489505,*

HOXA3as, HOXA3AS_BE873349, HOXA3AS_BI823151, HOXA6as, HOXA6AS_AK092154, HULC, IGF2A, IPW, Jpx, Kcnq1ot1, KRASP1, L1PA16, LIT, lincRNA-p21, lincRNA-RoR, LincRNA-SFMBT2, LincRNA-VLDLR, LOC285194, LUST, MALAT1, mascRNA, MEG3, MEG9, MER11C, NCRMS, ncR-uPAR, NDM29, NEAT1, Nespas, NRON, NTT, p53 mRNA, PANDA, PAR5, PCAT-1, PCAT-14, PCAT-29, PCAT-32, PCAT-43, PCGEM1, PCGEM1, PR antisense transcript, PR-AT2, PRINS, PRINS, PSF repressor RNA, PTENP1, RMRP, RNCR3, ROR, SAF, SCA8, snaR, SNHG1, SNHG3, SNHG4, SNHG5, SNHG6, Sox2OT, SRA, ST7OT, ST7OT1, ST7OT2, ST7OT3, ST7OT4, TEA ncRNAs, Telomeraz_RNA, TMEVPG1, Tmevpg1, TncRNA, Tsix, TU_0017629, TUG1, TUG1, UCA1, UCA1, UM9-5, WT1-AS, Xist, Y RNA-1, Y1, Y3, Y4, Y5, ZEB2NAT, Zeb2NAT, Zfas1, and *Zfhx2as.*

Analysis of lncRNA function and potential mechanism of action

lincRNA-SFMBT2 lncRNA, which showed significant changes in its expression at diagnosis and at 12 months of treatment/follow-up in HBV groups in relation to control group, was analyzed *in vitro* using HBV-positive human liver cancer cell line (Celprogen, USA). The cell line was cultured with Human Liver Cancer Cell Culture Full Growth Medium (Celprogen, USA) on flasks coated with Extracellular Matrix of Human Hepatic Cancer Cell Culture (Celprogen, USA). The cells were incubated at 37°C with 95% humidity and 5% CO_2 until they reached the proliferation stage.

siRNA-mediated suppression of lncRNA

To investigate the effect of siRNA-mediated suppression of lncRNA on HBV-positive human liver cancer cell line, we selected siRNA for *lincRNA-SFMBT2* using the online siDESIGN program (Dharmacon, USA). The designed siRNA sequence is given in Table 2. siRNAs were synthesized on a 10 nmol scale. The chemically synthesized siRNAs were transfected into HBV-positive human liver cancer cell lines using Lipofectamine 2000 Reagent (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. At 48, 96 and 144 hours after the transfection, the level of lncRNA was determined by qPCR. HBV DNA was isolated from the culture supernatant with the QIAamp DNA mini kit (Qiagen, Germany) and quantified with the ready-to-use *artus* HBV PCR Kit (Qiagen, Germany) on a Rotor-Gene cycler (Qiagen, Germany). The detection of HBsAg and hepatitis B envelope antigen (HBeAg) in the culture supernatants was performed with enzyme-linked immunosorbent assay (ELISA) kit (Beijing Wantai Biological Pharmacy, Beijing, China), according to the manufacturer's protocol.

Statistical analysis

Categorical variables were expressed as frequencies and compared with chi-squared test between the groups. Quantitative variables were expressed as mean ± standard deviation (SD) and median (range) and compared with one-way analysis of variance (ANOVA) between the groups. Log2 transformation was applied to the $2^{-\Delta\Delta Ct}$ values of lncRNA expression in control and HBV groups. The expression of 135 lncRNAs in each HBV group was determined at the initial diagnosis and at 12 months of treatment/follow-up in relation to control group and the values between HBV groups and control group, as well as between the two time points in each HBV group, were compared using Student's t-test and false discovery rate (FDR)-corrected *p* values. The change in lncRNA expression of ± 2-fold in relation to control group and FDR-corrected *p* values <0.05 were considered significant. lncRNA expression data analysis was performed using the CLC Main Workbench software (Qiagen Bioinformatics, USA). A *p* value <0.05 was considered significant.

RESULTS

Analysis of lncRNA expression in patients with chronic HBV infection

In the group of 27 patients with chronic HBV infection, the expression of 15 lncRNAs increased by 2 fold from the time of initial diagnosis to the 12th month of treatment/follow-up, in relation to control group ($p < 0.05$). The expression of 7 lncRNAs was decreased more than 2 fold at 12 months of treatment/follow-up compared to the expression values at the initial diagnosis, and in relation to control group ($p < 0.05$). A significantly increased expression of *anti-NOS2A*, *lincRNA-SFMBT2*, and *Zfhx2as* and decreased expression of *Y5* lncRNA in patients with chronic HBV infection compared to control group, indicate their potential use as biomarkers for monitoring the course of disease and response to treatment (Table 3, Figure 1).

TABLE 2. RNA sequence of *lincRNA-SFMBT2*-specific small interfering (siRNA)

5′			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	3′
			G	G	G	C	U	G	A	G	C	U	G	U	U	G	U	U	C	U	A	U	U	
	U	U	C	C	C	G	A	C	U	C	A	A	C	A	A	C	A	A	G	A	U			
3′	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1			5′

TABLE 3. Long non-coding RNAs (lncRNAs) with ±2 fold changes in the expression between two time points (initial diagnosis/12 months of treatment) in hepatitis B virus (HBV) patients compared to control group

lncRNA	Chronic HBV patients		Inactive HBsAg carriers		Resolved HBV patients	
	Initial diagnosis	12th month of treatment/follow-up	Initial diagnosis	12th month of treatment/follow-up	Initial diagnosis	12th month of treatment/follow-up
21A			2.41	–2.35		
7SL			–19.26	–7.53		
AK023948					–45.48	–6.92
Alpha 280	–4.07	5.22				
Anti-NOS2A	2.39	10.72	–13.61	–2.43		
AntiPeg11			–25.39	–3.15	–3.29	4.03
BACE1AS	2.44	5.22	–37.53	–3.8		
Dio3os	2.16	4.58			–24.48	–4.47
DLG2AS	2.9	2.44	–47.44	–7.08	–6.9	–7.51
Evf1 and Evf2			–16.04	–3.35	–9.66	–2.28
GAS5	–7.78	–3.7	–12.37	–5.02	–25.07	–3.58
H19	–15.44	2.75	–19.39	–2.41		
H19-AS	–4.17	–2.69	–2.73	–2.62		
HAR1A					–24.43	–7.29
HAR1B					–22.16	–4.9
HOTAIR					–3.67	–3.9
HOTAIRM1			–9.83	2.29	–2.48	3.59
HOTTIP					2.43	8.7
Hoxa11as	–3.1	2.77	–6.61	–2.61		
HULC	2.5	–3.33	–2.5	–21.23		
L1PA16	5.53	11.38				
LIT					–47.24	–2.95
lincRNA-RoR					2.33	–2.21
lincRNA-SFMBT2	41.84	46.14	2.08	2.46	1.11	–1.87
lincRNA-VLDLR	2.35	3.24			–3.09	–25.28
LOC285194					–28.4	–5.9
Malat1			–18.68	–6		
MEG9			–26.52	–7.42	3.38	7.87
NCRMS					–2.16	–27.49
NTT	–3.81	2.68				
PARS			5.08	–4.15		
PCAT-1					–5.6	–3.22
PCAT-14	2.24	2.99				
PCAT-29			–3.2	–9.33	–24.58	–4.98
PCAT-32	–3.48	–2.5			5.06	9.19
PCAT-43	–2.43	–2.7	–2.67	–5.43		
PR A			–22.27	–16.18	–2.39	–13.12
PRINS	2.88	4.75			–26.56	–3.54
RMRP	–2.21	–2.28				
SCA8			–6.28	2.86		
SNHG4					2.21	–3.88
SRA	2.28	3.51				
ST7OT			–27.43	–7.52		
ST7OT2			–2.01	–2.27		
ST7OT3	6.02	2.08			–12.87	–9.16
Tsix			–12.2	–2.15		
TU_0017629	–2.74	–2.99			–18.56	–7.09
TUG1	2.3	4.62	–22.17	2.56		
WT1-AS	20.45	3.47			–45.48	–6.92
Xist	2.09	5.07				
Y RNA-1			–7.54	2.08	–5.05	0.32
Y5	–4.02	–9.8	–2.83	–5.3		
Zeb2NAT			–6.48	6.75		
Zfh×2as	6.63	30.3				

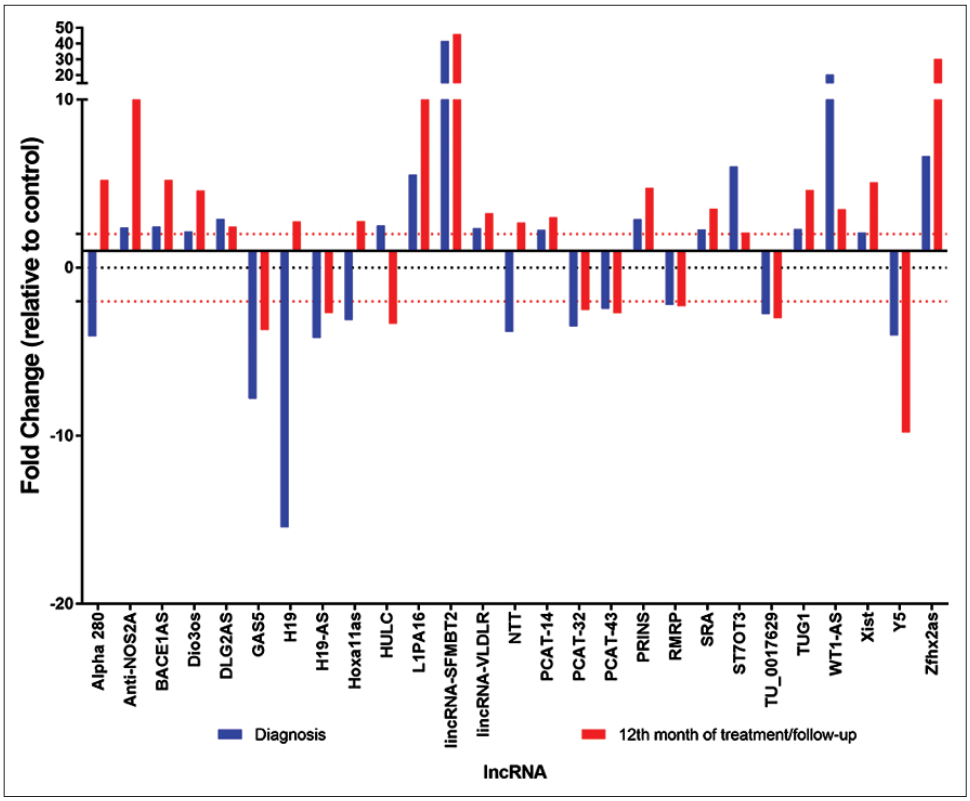

FIGURE 1. In chronic hepatitis B virus (HBV) patients (n = 27), the expression of 15 long non-coding RNAs (lncRNAs) increased by 2 fold from the time of initial diagnosis to the 12th month of treatment/follow-up, compared to control group ($p < 0.05$). The expression of 7 lncRNAs was decreased more than 2 fold at 12 months of treatment/follow-up compared to the expression values at the initial diagnosis, in relation to control group ($p < 0.05$). A significantly increased expression of *anti-NOS2A*, *lincRNA-SFMBT2*, and *Zfhx2as* and decreased expression of *Y5* lncRNA was observed in this group. The change in lncRNA expression of ± 2-fold in relation to control group and false discovery rate (FDR)-corrected *p* values <0.05 were considered significant.

Analysis of lncRNA expression in inactive HBsAg carriers

In 27 inactive HBsAg carriers, the expression of *lincRNA-SFMBT2* lncRNA increased by 2 fold from the initial diagnosis to the 12th month of treatment/follow-up, compared to control group ($p < 0.05$). The expression of 21 lncRNAs was significantly higher at the initial diagnosis and at 12 months of treatment/follow-up, compared to control group ($p < 0.05$). A decreased expression of *Y5* and increased expression of *lincRNA-SFMBT2* lncRNA in inactive HBsAg carriers suggest the role of those lncRNAs as prognostic biomarkers in patients with HBV (Table 3, Figure 2).

Analysis of lncRNA expression in resolved HBV patients

In 28 resolved HBV patients, the expression of *HOTTIP*, *MEG9*, and *PCAT-32* lncRNAs increased more than 2 fold from the initial diagnosis until 12 months of treatment/follow-up, compared to control group ($p < 0.05$). The expression of 20 lncRNAs was decreased more than 2 fold at 12 months of treatment/follow-up compared to the control ($p < 0.05$). No significant change was observed in the expression of *Y5* between resolved HBV patients and control group, confirming the potential use of *Y5* lncRNA as prognostic marker (Table 3, Figure 3).

siRNA-mediated inhibition of lncRNA expression

The expression of *lincRNA-SFMBT2* in HBV-positive human liver cancer cell line transfected with the specific siRNA decreased 5 times until day 4 compared to control cells, and it was decreased 1.59 times at day 6 of transfection (Figure 4). These results indicate that the siRNA-mediated inhibition of *lincRNA-SFMBT2* in the HBV-positive human liver cancer cell line was successfully achieved.

HBsAg and HBeAg levels in siRNA-treated HBV-positive human liver cancer cell line

HBsAg and HBeAg were detected in the culture supernatant of HBV-positive human liver cancer cell line transfected with *lincRNA-SFMBT2*-specific siRNA and with negative control siRNA, at 48, 96 and 144 hours after transfection. These findings indicate that the replication of HBV DNA was continuously present in the cell line.

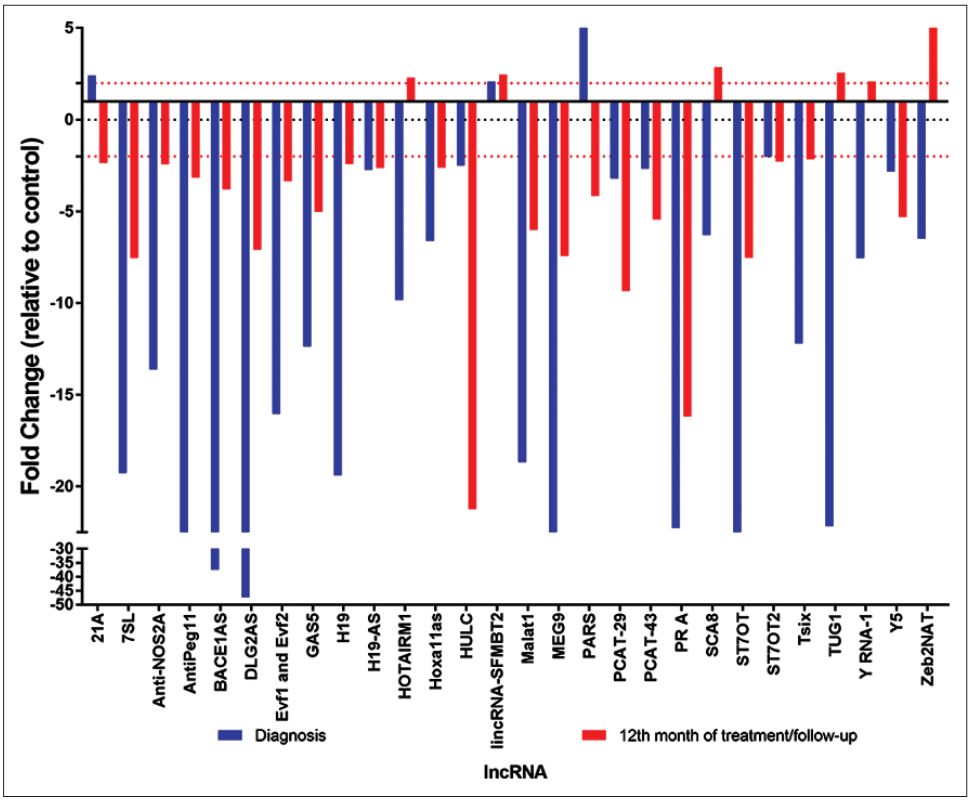

FIGURE 2. In inactive HBsAg carriers (n = 27), the expression of *lincRNA-SFMBT2* increased by 2 fold at the initial diagnosis and the 12th month of treatment/follow-up, compared to control group, while the expression of *Y5* was decreased 2.83 and 5.3 fold at initial diagnosis and at 12 months of treatment/follow-up, respectively ($p < 0.05$). The expression of 21 long non-coding RNAs (*lncRNAs*) was significantly higher at the initial diagnosis and at 12 months of treatment/follow-up, compared to control group ($p < 0.05$). The change in lncRNA expression of ± 2-fold in relation to control group and false discovery rate (FDR)-corrected p values <0.05 were considered significant.

Quantification of HBV DNA in siRNA-treated HBV-positive human liver cancer cell line

The amount of HBV DNA in the culture supernatant of HBV-positive human liver cancer cell line transfected with *lincRNA-SFMBT2*-specific siRNA was decreased 1.55, 1.66 and 1.22 times at 48, 96, and 144 hours respectively, and the difference was significant compared to the cells treated with negative control siRNA (Figure 5). These results suggest that the siRNA-mediated inhibition of *lincRNA-SFMBT2* affected the level of HBV DNA in the liver cancer cells.

DISCUSSION

The gene for *AK023948* lncRNA was mapped to the 8q24 region, which also contains two overlapping protein-coding genes, thyroglobulin (*TG*) and Src-like adaptor (*SLA*). In the same study, the expression of *AK023948* was significantly downregulated in majority of papillary thyroid carcinomas (PTC) [22]. In the group of resolved HBV patients, we observed a 45-fold reduction in the expression of *AK023948* at diagnosis compared to control group, and a 7-fold reduction at 12 months of treatment/follow-up. Despite the increase in the expression of *AK023948* during the 12-month period, *AK023948* expression was still lower in resolved HBV patients compared to control group. Moreover, a significant reduction in the expression of *AK023948* lncRNA was not observed in the other two groups of HBV patients, suggesting a role of *AK023948* in the recovery from HBV.

anti-NOS2A is a ~1.9 kb intronless polyadenylated lncRNA transcribed from a locus located on 17q23.2, which has a high sequence similarity with the *NOS2A* gene that encodes the inducible isoform of nitric oxide synthase. Korneev et al. [23] reported several observations for the *anti-NOS2A* locus: the average sequence identity between the *anti-NOS2A* and the corresponding regions of the *NOS2A* gene was ~80%; this indicates that the *anti-NOS2A* is the result of gene duplication and subsequent internal DNA inversion; *anti-NOS2A* RNA was expressed in brain tumors such as meningiomas and glioblastomas; finally, *anti-NOS2A* likely regulates the neuronal differentiation of human embryonic stem cells by modulating the expression of *NOS2A* gene [23].

NO is a free radical produced by the inducible NO synthase (iNOS) in the liver. It was indicated that NO mediates the antiviral activity of interferon γ (IFN-γ) in HBV transgenic mice heterozygous or homozygous for the iNOS null mutation, and decreases the expression of viral antigens in the cell by inhibiting viral replication. Moreover, the absence of NO increased the severity of liver disease

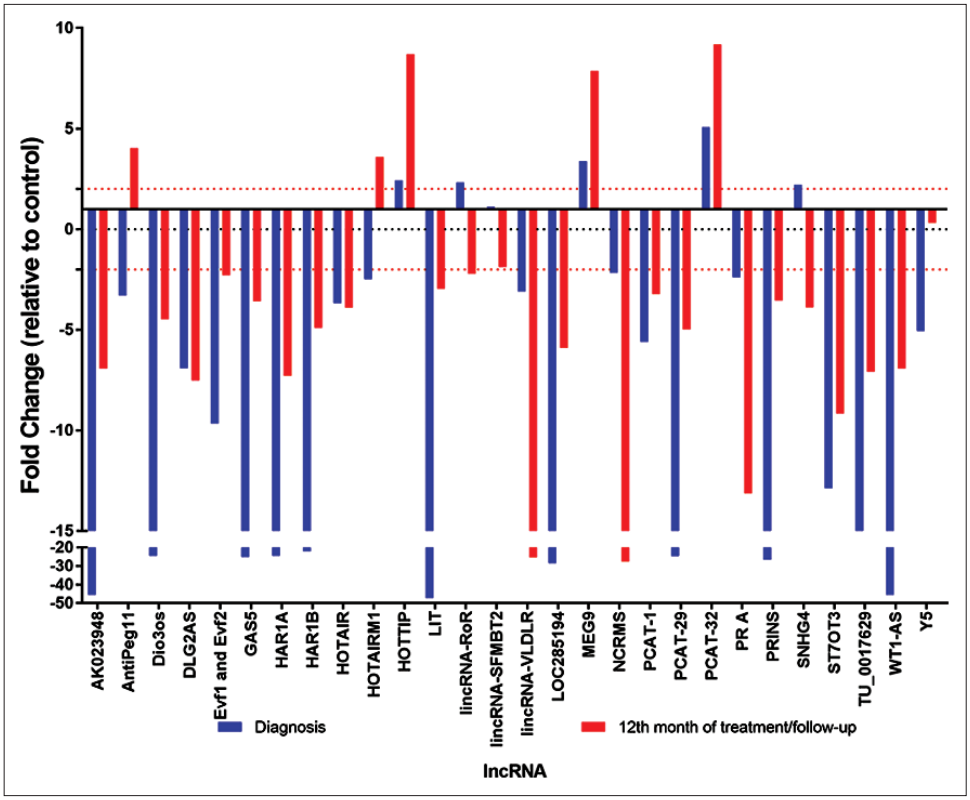

FIGURE 3. In resolved hepatitis B virus (HBV) patients (n = 28), the expression of *HOTTIP*, *MEG9*, and *PCAT-32* increased more than 2 fold from the initial diagnosis until 12 months of treatment/follow-up, compared to control group ($p < 0.05$). The expression of 20 long non-coding RNAs (lncRNAs) was decreased more than 2 folds at 12 months of treatment/follow-up compared to the initial diagnosis, and in relation to control group ($p < 0.05$). No significant change was observed in the expression of *Y5* between resolved HBV patients and control group. The change in lncRNA expression of ± 2-fold in relation to control group and false discovery rate (FDR)-corrected *p* values <0.05 were considered significant.

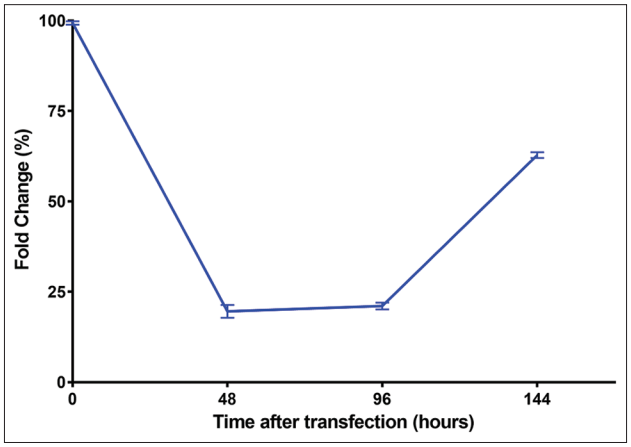

FIGURE 4. The expression of *lincRNA-SFMBT2* in hepatitis B virus (HBV)-positive human liver cancer cell line transfected with the specific small interfering RNA (siRNA) decreased 5 times until day 4 compared to control cells, and it was decreased 1.59 times at day 6 of transfection.

in those animals [24]. The expression of *anti-NOS2A* in our patients with chronic HBV infection was significantly increased both at diagnosis and at 12 months of treatment/follow-up, compared to control group, which may have affected the NO synthesis in these patients. On the other hand, *anti-NOS2A* expression was significantly lower in the inactive HBsAg carriers compared to control group, confirming the potential prognostic and therapeutic role of this lncRNA.

Growth arrest specific 5 (*GAS5*) is lncRNA involved in the regulation of cell cycle progression. The overexpression of several *GAS5* transcripts induced growth arrest and apoptosis in mammalian cell lines, including human breast cancer cells. Moreover, the *GAS5* expression was significantly downregulated in the breast cancer cells compared with adjacent healthy tissue [25]. Chang et al. [26] suggested that low expression of *GAS5* in HCC indicates a poor prognosis. In our study, *GAS5* expression was significantly lower at both time points (initial diagnosis and 12 months of treatment/follow-up) in all three patient groups compared to control group, suggesting the role of *GAS5* in HBV infection.

H19 is an imprinted, maternally expressed gene (i.e., monoallelic expression) that transcribes to lncRNA. *H19* RNA is normally expressed only during embryonic development, but may be re-activated in some processes such as adult tissue regeneration and tumorigenesis [27-29]. Biallelic expression due to loss of imprinting (LOI) of *H19* and the associated paternally expressed insulin-like growth factor 2 gene (*IGF2*) gene, was demonstrated in HCC and several other tumor types, indicating the role of this process in epigenetic mechanism of tumor development [28]. Matouk et al. [29]

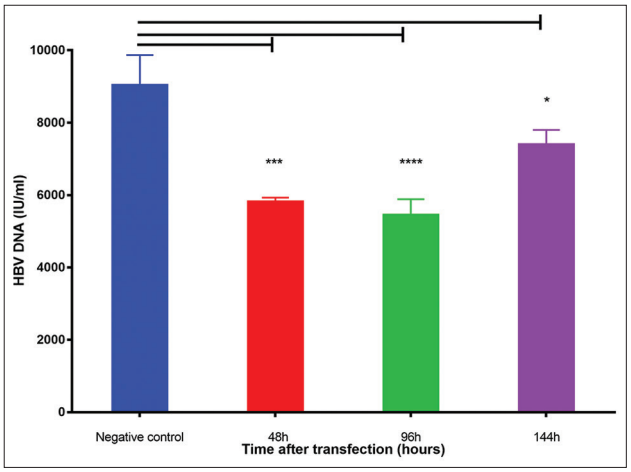

FIGURE 5. The amount of hepatitis B virus (HBV) DNA in the culture supernatant of HBV-positive human liver cancer cell line transfected with *lincRNA-SFMBT2* specific small interfering RNA (siRNA) was decreased 1.55, 1.66 and 1.22 times at 48, 96, and 144 hours respectively, and the difference was significant compared to the cells treated with negative control siRNA.*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

showed that *H19* RNA is upregulated in HCC and bladder carcinoma cell lines in response to hypoxic stress, and suggested that *H19* acts as an oncogene, promotes tumorigenesis and affects tumor growth. Similarly, Iizuka et al. [30] showed that the *H19* and *IGF2* genes, as well as genes involved in signal transduction, transcription and tumor metastasis were upregulated in HBV-associated HCC [30]. Moreover, a 120-kb-long transcript (*91H*) antisense to the *H19* gene, was characterized within the human and mouse *H19/IGF2* locus. *91H* RNA, preferentially expressed from the maternal chromosome, did not affect imprinting of the *H19*/*IGF2* locus in human cells, but it did downregulate *IGF2* expression in trans on the paternal allele [31]. In another study, lncRNA array analysis of 90 well-annotated mouse lncRNAs showed that the level of *91H* (also named *H19-as*) was reduced in cultured vitamin D receptor (VDR)-deleted mouse keratinocytes [32]. The expression of *H19* in our chronic HBV patients and inactive HBsAg carriers at diagnosis was significantly lower compared to control group. At 12 months of treatment/follow-up, we observed a 2-fold decrease in *H19* expression in inactive HBsAg carriers and a 2-fold increase in chronic HBV group, compared to control group. The expression of *H19-as* in chronic HBV patients and inactive HBsAg carriers was lower both at the initial diagnosis and at 12 months of treatment/follow-up compared to control group. Lower *H19* and *H19-as* expressions observed in our patient groups compared to controls may be associated with a suggested tumor-suppressor role of *H19* [33].

Highly Accelerated Region 1A (*HAR1A* or *HAR1F*) is a 2.8 kb-long 2-exon transcript, it overlaps with *HAR1B* (*HAR1R*), and both of these genes contain the Human Accelerated Region (*HAR1*) [34]. Liu et al. [35] analyzed alterations in lncRNA expression in the breast invasive carcinoma dataset of the Cancer Genome Atlas (TCGA), containing ~1,000 cases. According to their results, the upregulation of *HAR1A* (and 8 other lncRNAs) could predict breast cancer recurrence [35]. *HAR1A* and *HAR1B* have also been associated with Alzheimer's disease [36]. Moreover, transcriptional repression of *HAR1* by RE1-Silencing Transcription factor (REST) has been shown in the striatum of patients with Huntington's disease [37]. Zhou et al. [38] indicated the role of the REST corepressor 1 (CoREST)/REST complex in the suppression of Herpes Simplex Virus 1 gene expression, during its productive as well as latent infection in sensory neurons. The expression of *HAR1A* and *HAR1B* lncRNAs was significantly decreased in our resolved HBV patients both at the initial diagnosis and at 12 months following the treatment/follow-up, compared to control group. The inhibition of lncRNA as well HBV gene expression might be associated with the epigenetic mechanisms of the CoREST/REST complex.

HOX Transcript Antisense RNA (*HOTAIR*) is a 2.2 kb lncRNA involved in transcriptional modulation. By recruiting the polycomb repressive complex 2 (PRC2) and the lysine-specific demethylase 1 (LSD1)/coREST/REST complex, *HOTAIR* mediates the trimethylation of histone H3 at lysine 27 and the demethylation of histone H3 dimethyl Lys4 at promoters of target genes, leading to gene silencing. A number of studies reported that the overexpression of *HOTAIR* in HCC was associated with poor prognosis in those patients [39]. In our study, the expression of *HOTAIR* in resolved HBV patients was significantly decreased at the initial diagnosis and 12 months following the treatment/follow-up, compared to control group.

HOX antisense intergenic RNA myeloid 1 (*HOTAIRM1*) was first identified as a 483 nt long transcript located between the human *HOXA1* and *HOXA2* genes, and expressed specifically in the myeloid lineage. Short hairpin (shRNA)-mediated knockdown of *HOTAIRM1* downregulated the expression of 3′ *HOXA* neighboring genes and affected the expression of the alpha-M beta-2 integrin subunits, *CD11B* and CD18 genes, indicating the regulatory role of *HOTAIRM1* in myelopoiesis [40]. Similarly, a tumor suppressor role of *HOTAIRM1* was demonstrated in colorectal cancer (CRC) [41]. *HOTAIRM1* lncRNA was significantly decreased in our inactive HBsAg carrier and resolved HBV patient groups at the initial diagnosis compared to control group, and it showed a 2-fold increase at 12 months of treatment/follow-up. Considering the tumor suppressor function of *HOTAIRM1* in other cancer types, our results suggests that *HOTAIRM1* may be used as a positive prognostic marker in HBV patients.

HOXA transcript at the distal tip (*HOTTIP*) is an antisense lncRNA located at the 5′ end of the *HOXA* cluster. *HOTTIP* lncRNA is involved in the activation of several 5′ *HOXA* genes by binding to WD repeat-containing protein

5 (WDR5) and targeting the WDR5/MLL complex to the *HOXA* locus, which further mediates trimethylation of histone H3 lysine 4 (H3K4me3) and thus maintains the active state of transcription [42]. Deregulation of *HOX* genes was indicated in hepatocarcinogenesis, and the upregulation of *HOTTIP* and *HOXA13*, in snap–frozen needle HCC biopsies prior to any treatment, was associated with increased metastasis and decreased overall survival in HCC patients [43]. In our resolved HBV patients, the expression of *HOTTIP* was significantly higher at both time points (initial diagnosis/12 months of treatment) compared to control group, which might serve as a good prognostic indicator in HBV patients.

Psoriasis susceptibility-related RNA Gene Induced by Stress (*PRINS*) is a 3.6 kb long transcript located on 10p12.1, comprised of two exons and both harbor Alu elements. Increased expression of PRINS was demonstrated under stress conditions, such as ultraviolet-B (UV-B) irradiation, HSV infection, and translational inhibition [44]. In our study group, chronic HBV patients had significantly higher *PRINS* expression at the initial diagnosis/12 months of follow-up compared to control group. In contrast, the expression of *PRINS* was significantly decreased in resolved HBV patients at both time points. These findings suggest, for the first time, that HBV infection may lead to increased *PRINS* expression and *PRINS* may be useful in distinguishing between chronic and resolved HBV patients.

The *RAY1/ST7* locus is located at 7q31 and its complex multi-transcript system has been implicated in disease states, such as autism and cancer. The multigene system of the *RAY1/ST7* locus is comprised of two noncoding sense strand genes (*ST7OT3* and *ST7OT4*) overlapping with many alternative forms of the coding *RAY1/ST7* transcript, and two noncoding antisense strand genes (*ST7OT1* and *ST7OT2*) [45]. Our study is the first to provide information on the expression of *ST7OT1*, *ST7OT2*, *ST7OT3* and *ST7OT4* transcripts in HBV. The expression of *ST7OT3* was significantly higher in chronic HBV compared to control group. Moreover, a significant suppression of *ST7OT3* in resolved HBV group suggests it has an oncogenic function in HBV. Similarly, the expression of *ST7OT1* and *ST7OT2* was significantly decreased in inactive HBsAg carrier group.

WT1 (Wilms' tumor 1) antisense transcript (*WT1-AS*), transcribed from an antisense promoter within *WT1* intron 1 [46], is a 0.4-3 kb transcript present in multiple splicing isoforms [47]. Reduced expression of *WT1-AS* has been associated with increased cell proliferation and invasion in gastric cancer [48]. However, our results indicate that *WT1-AS* has an oncogenic role in HBV, i.e., the expression of *WT1-AS* was increased in chronic HBV group and decreased in resolved HBV patients, compared to control group.

Y RNAs are family of highly conserved small ncRNAs, 83-112 nt long. In humans, the family includes four types of ncRNAs, *Y1*, *Y3*, *Y4* and *Y5*, which are transcribed by RNA polymerase III from individual genes. Y RNAs have a highly conserved stem-loop structure, where the stem (a double stranded region formed by the terminal 5′- and 3′-sequences of Y RNA) contains a sequence-specific binding site for Ro protein, forming together a ribonucleoprotein (RNP). These RNAs have an essential role in the initiation of DNA replication in mammalian cells [49,50]. In carcinomas and adenocarcinomas of the urinary bladder, cervix, colon, kidney, lung and prostate the relative expression levels of the four Y RNAs were significantly higher compared to normal nonmalignant tissues. Also, degradation of *Y1* and *Y3* RNAs by siRNAs in human cell lines led to the inhibition of cell proliferation [51]. It was also demonstrated that multiple small RNA fragments (including those derived from the 5′-ends of specific Y RNA) circulate in the blood. These RNAs may serve as signaling molecules in various cellular processes, however, their targets as well as functions are still not completely understood [52]. The possibility of their use as markers of specific disease states, particularly cancer, has been indicated in breast cancer [53], lung cancer [51], and head and neck squamous cell carcinoma [54]. Overall the expression of *Y5* was low in our HBV groups. Furthermore, *Y5* expression was decreased in chronic HBV patients and inactive HBsAg carriers compared to control, while it was close to normal (control group) in resolved HBV patients.

Together with 7 other lncRNAs, *lincRNA-SFMBT2*, *lincRNA-VLDLR* and *lincRNA-ST8SIA3* (also named *lincRNA-RoR*), were identified to have higher expression in induced pluripotent stem cells (iPSCs) compared to embryonic stem cells (ESCs), indicting their role in the development of iPSCs. Moreover, knock-down or overexpression of *lincRNA-ST8SIA3* directly correlated with the formation of iPSCs colonies. Colocalization of transcription factors OCT4, SOX2 and NANOG near the *lincRNA-ST8SIA3* promoter suggested that its expression is induced by those pluripotency factors. Also, *lincRNA-ST8SIA3* negatively regulated *TP53* expression, thus affecting cell cycle arrest and apoptosis [55,56]. In our study, the expression of *lincRNA-SFMBT2* was significantly higher in chronic HBV patients and inactive HBsAg carriers. In addition, *lincRNA-VLDLR* expression was high in chronic HBV patients. *lincRNA-ST8SIA3* expression was significantly different between the three HBV groups. Expression of *lincRNA-SFMBT2* has been shown to increase in HepG2 cells 9.58 fold compared to malignant human hepatocytes (HepG2 cells) and non-malignant human hepatocytes (HH cells) [57].

Overall, different lncRNAs showed changes in the expression between HBV groups and control group, between initial diagnosis and 12 months of treatment in each HBV group, and between the three HBV groups (chronic HBV patients,

inactive HBsAg carriers and resolved HBV patients). Most notably, *Y5* expression was decreased in chronic HBV patients and inactive HBsAg carriers, and the level was close to normal (control group) in resolved HBV patients, suggesting its use in monitoring the course of disease. *lincRNA-SFMBT2* expression was increased in chronic HBV patients and inactive HBsAg carriers, and siRNA-mediated inhibition of *lincRNA-SFMBT2* decreased the level of HBV DNA in human liver cancer cells. In addition, *AK023948*, *H19*, *H19-As* and *HOTAIRM1*, as potential tumor suppressor lncRNAs, as well as *HOTTIP* may be important in the prognosis of HBV infection, while *ST7OT3* and *WT1-AS* may have an oncogenic role. Similarly, *GAS5* could play an important role in HBV infection. In addition to *lincRNA-SFMBT2*, *WT1-AS* might have an effect on HBV replication. The changes in the expression of *HAR1A*, *HAR1B*, and *HOTAIR* might indicate their involvement in epigenetic suppression of HBV genes via the CoREST/REST complex. Further research is needed to confirm the prognostic as well as therapeutic role of these lncRNAs in HBV patients.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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