

Association of single nucleotide polymorphisms in non-coding RNAs (miRNA-100 and MALAT1) with susceptibility to hepatitis B virus infection

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Abstract. Non-coding RNAs (ncRNAs) play a vital role in the diagnosis and treatment of hepatitis B virus (HBV). ncRNAs include major classes, such as microRNAs (miRNAs/miRs) and long ncRNAs (lncRNAs). The present study focused on miR-100 and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) single nucleotide polymorphisms (SNPs) and their expression levels. In addition to their dual effect on susceptibility to hepatitis B virus (HBV) infection, new molecular biomarkers of HBV infection are suggested. In the present study, 100 patients with HBV infection vs. 100 healthy controls were enrolled. miR-100 SNP (rs1834306T/C) was detected using the polymerase chain reaction sequence-specific primers technique, while MALAT1 SNP (rs619586A/G) was detected

using the restriction fragment length polymorphism-PCR technique. Their expression levels were measured using reverse transcription-quantitative PCR. As per the miR-100 genotyping results, the TC genotype represented the most frequent genotype in all subjects. However, in MALAT1 SNP, only the dominant AA genotype was detected. A significant upregulation of both miR-100 ($P<0.01$) and MALAT1 ($P<0.05$) expression was observed in the patient group compared to the controls. A positive correlation was found between the viral load and an elevation in miR-100 and MALAT1 expression levels ($r=0.508$, $P<0.01$; and $r=0.282$, $P<0.05$, respectively). On the whole, the present study demonstrates that miR-100 and MALAT1 may be considered as potential molecular markers for the prognosis of patients with HBV infection. To the best of our knowledge, this is the first observational prospective case-control study to scrutinize all the possible correlations between miR-100 (rs1834306T/C) and MALAT1 (rs619586A/SNPs) and their expression levels. Further extensive studies with large sample sizes are recommended to confirm the findings obtained herein.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; EDTA, ethylene-diamine-tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; lncRNAs, long non-coding RNAs; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miRNAs/miRs, microRNAs; NEAT2, nuclear-enriched abundant transcript 2; ORF, open reading frame; PCR, polymerase chain reaction; PCR-SSP, PCR-sequence-specific primers; RT-qPCR, reverse transcription-quantitative PCR; RFLP-PCR, restriction fragment length polymorphism-PCR; SNPs, single nucleotide polymorphisms

Key words: HBV, miR-100, MALAT1, polymorphism, viral load

Introduction

Hepatitis B infection is a life-threatening liver disease resulting from the hepatitis B virus (HBV) (1). There is high inter-individual variability in the clinical presentation of HBV infection, ranging from self-limited to acute fulminant hepatitis. This can cause chronic liver inflammation leading to cirrhosis and hepatocellular carcinoma (HCC) (2). Despite the presence of effective antiviral therapies and vaccines (3), the mortality rates have increased from 0.8 to 1.4 million from 1990 to 2013 (4). Therefore, it is crucial to consider the molecular aspects affecting HBV.

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into proteins (5). They regulate diverse cellular functions and processes by controlling gene expression (6). High-throughput DNA sequencing and array-based technologies have revolutionized the classification of ncRNAs (7).

Among several types of ncRNAs, short ncRNAs, including microRNAs (miRNAs/miRs) and long ncRNAs (lncRNAs), have been considered a standpoint (8).

miRNAs are ~19 to 22 nucleotides (nt) in length (9). To date, >2,000 miRNAs have been registered in the 'miRbase' database; however, the functional role of the majority of miRNAs remains unclear; they have emerged as eminent players in human pathophysiological processes (10). miRNAs affect gene expression through various mechanisms, such as de-adenylation, targeting mRNA cleavage and suppression of translation, supporting the evidence that a single miRNA can regulate hundreds of genes; hundreds of miRNAs (11) can also regulate a single gene. An example of miRNAs that have been highly expressed in the liver is miR-100, which is located on chromosome 11 at 11q24.1 (12). It promotes HBV protein production, DNA replication and progeny secretion (13).

lncRNAs, the most recent acknowledged class of ncRNAs, are transcripts with lengths >200 nt without protein-coding capacity (14). lncRNAs are messenger RNA (mRNA)-like transcripts, but without stable open reading frames (ORFs). The majority of lncRNAs can regulate gene expression through chromatin modification, transcription and post-transcriptional processing (15). An increasing number of lncRNAs have been characterized in studies, focusing on their roles in regulating gene expression (16,17).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is one of the most abundant lncRNAs in normal tissues and is highly conserved between humans, also known as nuclear-enriched abundant transcript 2 (NEAT2) (18). It consists of >8,000 nt and is coded by chromosome 11q13. MALAT1 has been reported to regulate gene expression; there is substantial evidence to suggest the vital role of MALAT1 in liver cell proliferation (19,20). Recently, MALAT1 has gained considerable attention due to its association with a number of diseases, also acting as a potential biomarker for the diagnosis, prediction and therapeutic target for numerous types of cancer (21).

Single nucleotide polymorphisms (SNPs) have been suggested to be biological markers for revealing the evolutionary history and common genetic polymorphisms that explain the heritable risk for common diseases (22). SNPs in ncRNAs have been reported to alter their secondary structure or modify expression levels, thereby influencing their regulatory function, contributing to disease development (23). In a previous study, the authors examined the presence of SNP (rs1834306 T/C) in miR-100 in HBV-infected patients and its effect on gene expression (24). In continuation of this, the present study focused on examining SNP rs619586 (A/G) in MALAT1, and its expression level in HBV-infected Egyptian patients compared to other healthy controls. Possible correlations between both miR-100 and MALAT1 in HBV infection were also investigated.

Subjects and methods

Ethics approval. All subjects provided written informed consent for genetic analysis in the present observational prospective case-control study. All methods and analyses were carried out following the guidelines of the Ministry of Health and approved by the Research Ethics Committee for

Experimental and Clinical Studies at the Faculty of Pharmacy, University of Cairo, Egypt [BC (1837)].

Patients and study design. A total of 200 subjects; 100 outpatients (70 males and 30 females; under the medical supervision of the National Liver Institute, Menoufia University, Menoufia, Egypt) with an approved diagnosis of HBV infection by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) were included in parallel to 100 individuals (64 males and 36 females) with normal liver function test results, no history of hepatic diseases and negative for HBV and hepatitis C virus (HCV) serology, which served as controls. As previously described by Motawi *et al* (24), viral assessment in all subjects was performed. Patients with HCV infection or other viral or hepatic disorders were excluded from the study. All biochemical investigations included alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. According to the manufacturer's instructions, total bilirubin, albumin and creatinine levels were measured in blood for all subjects using a Cobas 6000 analyzer (Roche Diagnostics GmbH).

SNP selection. Based on the data from the HapMap (<http://www.hapmap.org>); NCBI dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) and miRNAs (<http://microrna.sanger.ac.uk>) databases, miR100 (rs1834306 T/C) and MALAT1 (rs619586 A/G) SNPs were selected for analyses in the present study.

DNA extraction and SNP genotyping. Genomic DNA was extracted from 5 ml of venous blood samples [collected in ethylene-diamine-tetra-acetic acid (EDTA) sterile vacutainer] from each participant using the GentraPuregene Blood kit (Qiagen GmbH) according to the manufacturer's instructions. A Nanodrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, Inc.) was used to assess the purity and the concentration of the extracted DNA. The extracted DNA was applied to 1% agarose gel electrophoresis to confirm its integrity.

miR-100 rs1834306 T/C was analyzed using PCR-sequence-specific primers (PCR-SSP), as previously described by Motawi *et al* (24). However, MALAT1 polymorphism rs619586 A/G was genotyped by restriction fragment length polymorphism-PCR (RFLP-PCR). The primer sequences of both miR-100 and MALAT1 were designed using Primo SNP 3.4: SNP PCR Primer Design (<https://www.changbioscience.com/primo/primosnp.html>) and secondly checked using primer blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table I). A 25 µl PCR reaction mixture contained MyTaq™ Red Mastermix (2X; Meridian Life Science, Inc.), 10 pmoles of each primer and 150 ng DNA. The PCR reaction conditions were as follows: 95°C for 10 min (one cycle) followed by 35 cycles of 94°C for the 30 sec, 59°C for 30 sec, and 72°C for 1 min, then a final extension step at 72°C for 7 min. All PCR reactions were performed in a 2720 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). A 2% agarose electrophoresis stained with ethidium bromide (10 mg/ml) was used to visualize the PCR product (188 bp) in comparison to the 100 bp DNA ladder (Fermentas; Thermo Fisher Scientific, Inc.) (Fig. 1). The PCR product was digested by the addition of *BvuI* (*Bsp*MI) restriction enzymes (Fermentas; Thermo Fisher Scientific, Inc.). The restriction

Table I. PCR primers sequences used for the amplification of miR-100 and MALAT-1 in patients with HBV and the controls.

SNP	Primers	PCR Product (bp)	Enzyme	Restriction product
miR-100	Forward T: 5'-GTGGAACCAAGGGAAGCACGT-3'	301	-	-
rs1834306	Forward C: 5'-TGGAACCAAGGGAAGCACGC-3'			
T/C	Reverse: 5'-ATAAGCAAAGCCCCAGGTCC-3'			
MALAT-1	Forward: 5'-AAAGTCCGCCATTTTGCCAC-3'	188	<i>Bsp</i> MI	AA:188
rs619586	Reverse: 5'-CACAAAACCCCGGAACCTT-3'			AG:188/121/67
A/G				GG:121/67

MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA; SNP, single nucleotide polymorphism.

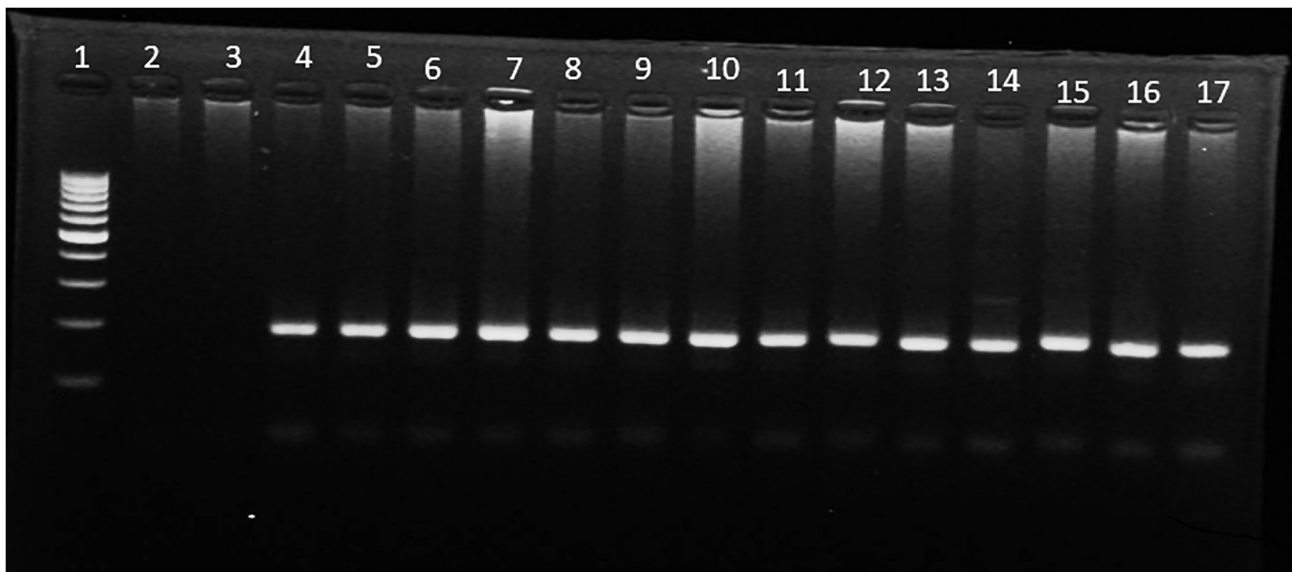


Figure 1. A 2% agarose electrophoresis stained with ethidium bromide (10 mg/ml). Lane 1, 100 bp DNA ladder; lanes 4-17, PCR product of the metastasis-associated lung adenocarcinoma transcript 1 single nucleotide polymorphism (188 bp).

product size was 188bp for the A/A genotype, 121/67 bp for G/G and 188/121/67 bp for A/G. The digestion products were visualized by 3% agarose gel electrophoresis and estimated by comparing with the 50 bp DNA Ladder (Fermentas; Thermo Fisher Scientific, Inc.). In total, 10% of samples were randomly selected to be sequenced to control genotyping quality and validate the results.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA (from 200 μ l plasma samples) was purified using the miRNeasy Mini kit (cat. no. 217004; Qiagen, Inc.) according to the manufacturer's instructions. For the miR-100 expression level, cDNA was prepared using the miScriptII RT kit (cat. no. 218061; Qiagen, Inc.). qPCR was performed using the miScript SYBR-Green PCR kit (cat. no. 218073; Qiagen, Inc.), as previously described in the study by Motawi *et al* (24).

To determine the transcripts of the gene of interest (MALAT1), RNA was reverse-transcribed using the High-Capacity DNA Reverse Transcription kit (cat no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.). The target cDNA was then amplified using the

TaqMan™ Universal Master Mix II (cat no. 4440043; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for quantitative normalization. RT-qPCR amplification began with an initial holding period at 50°C for 2 min, 95°C for 10 min followed by a PCR program consisting of 40 cycles of 95°C for 15 sec and 60°C for 1 min. Differences in the Ct values (Ct) between MALAT1 and GAPDH were calculated using the formula $\Delta\Delta Ct = \Delta Ct$ (tested sample) - ΔCt (control sample) to determine the relative expression levels; the fold change in MALAT1 was calculated using the $2^{-\Delta\Delta Ct}$ method (25).

Statistical analysis. All statistical analyses were performed using the clinical Statistical Package for Social Science (SPSS) version 19 (SPSS, Inc.). Data are presented as the mean $\pm$ standard deviation/error (SD/SE). An independent paired t-test was applied to compare numerical variables between the patients with HBV and controls for quantitative variables. SNP/STAT was performed using the online tool (<http://bioinfo.iconco-logia.net/SNPstats>). The receiver operating characteristic

Table II. ROC curve of miR-100 and MALAT-1.

Parameters	Cut-off	AUC	Sensitivity	Specificity	95% CI	P-value
miR-100	1.017	0.778	76.9%,	63.2%	0.644-0.913	P<0.01
MALAT1	0.830	0.670	66.7%	75%	0.510-0.831	P<0.05
Combined		0.853			0.727-0.978	P<0.001

MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA.

(ROC) curve analyzed the sensitivity versus specificity of the scoring system. Pearson's correlation analysis was used for correlation analysis. All P-values were two-tailed; A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Demographic and biochemical characteristics of patients with HBV vs. the control subjects. All patients with HBV were found positive for hepatitis B surface antigen (HBsAg) and HBV-DNA. The demographic and biochemical characteristics of the patients with HBV vs. the control subjects have been previously described in the study by Motawi *et al* (24)

Genetic variation of MALAT1 rs619586 A/G and miR-100 rs1834306 T/C. The study of the genotypes of MALAT1 rs619586 A/G revealed the presence of only one genotype: The dominant AA genotype, with a complete disappearance of other genotypes in the two studied groups. On the contrary, all genotypes of miR-100 rs1834306 (T/C) were found, although no statistically significant difference in the genotype distribution between patients with HBV and the normal controls was demonstrated. The genotype and allelic frequency of MALAT1 rs619586 (A/G) and miR-100 rs1834306 (T/C) are presented in Fig. 2.

Expression of miR-100 and MALAT1. The analysis of the miR-100 and MALAT1 expression levels revealed a significant upregulation of miR-100 (P<0.001) and MALAT1 (P<0.001) expression in patients with HBV vs. the controls (Fig. 3). The results of the analysis of the ROC curve for both miR-100 and MALAT1 are summarized in Table II. Combined ROC curve analysis resulted in an improvement in the diagnostic potential of both ncRNAs, leading to 75% sensitivity and 92% specificity (Fig. 4).

Correlations between increased miR-100 and MALAT1 expression levels with some biochemical tests and the viral load in patients HBV vs. the controls. No statistically significant correlation was found between MALAT1 and miR-100 concerning their genotyping or expression level in patients with HBV. A positive correlation between the viral load of HBV, and both MALAT1 expression and ($r=0.282$ and $r^2=0.079$; P<0.05) and miR-100 expression ($r=0.489$ and $r^2=0.239$; P<0.001) was found. In view of the biochemical tests, a positive correlation ($r=0.316$ and $r^2=0.099$; P<0.05) between MALAT1 expression and the ALT level was detected (Table III).

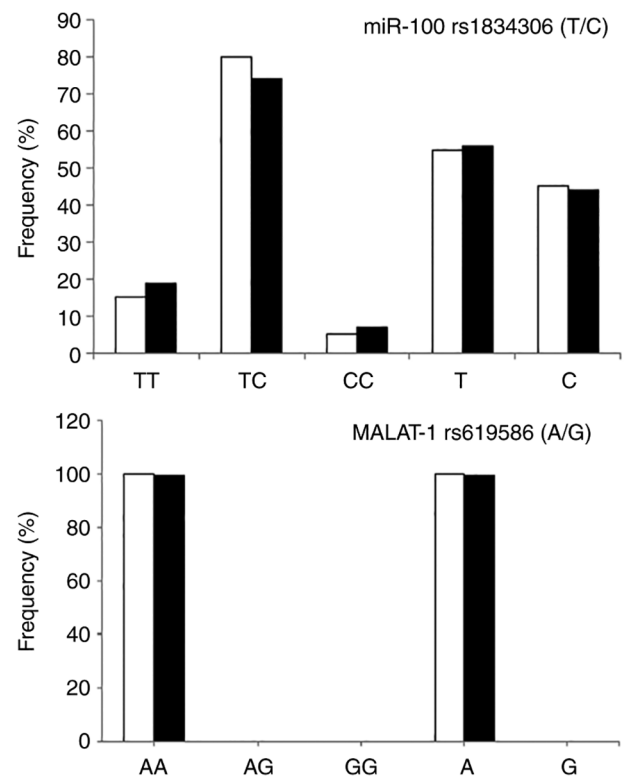


Figure 2. Genotype distribution and allelic frequency of miR-100 rs1834306 (T/C) and MALAT1 rs619586 (A/G) in the controls (white bars) and patients with hepatitis B virus (black bars). MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA.

Discussion

Viral infections are of global public health concern; HBV is one of the leading causes of mortality (26), and the main obstacle to its treatment is the inability to achieve a full cure for HBV (27). Thus, it is urgent to consider the molecular field affecting HBV. Recent research has devoted ample attention to genetic alterations (28). In this respect, diverse classes of ncRNAs, ranging from miRNAs to lncRNAs, play a crucial role in the epigenetic regulation of gene expression; genome stability also acts as a defense against foreign genetic elements (29).

Over the past decade, an increasing number of scientific studies and research have focused on the pivotal biological functions of one of the key lncRNAs, MALAT1, which was originally discovered as a prognostic marker for lung cancer metastasis, and has been linked to several other human tumor

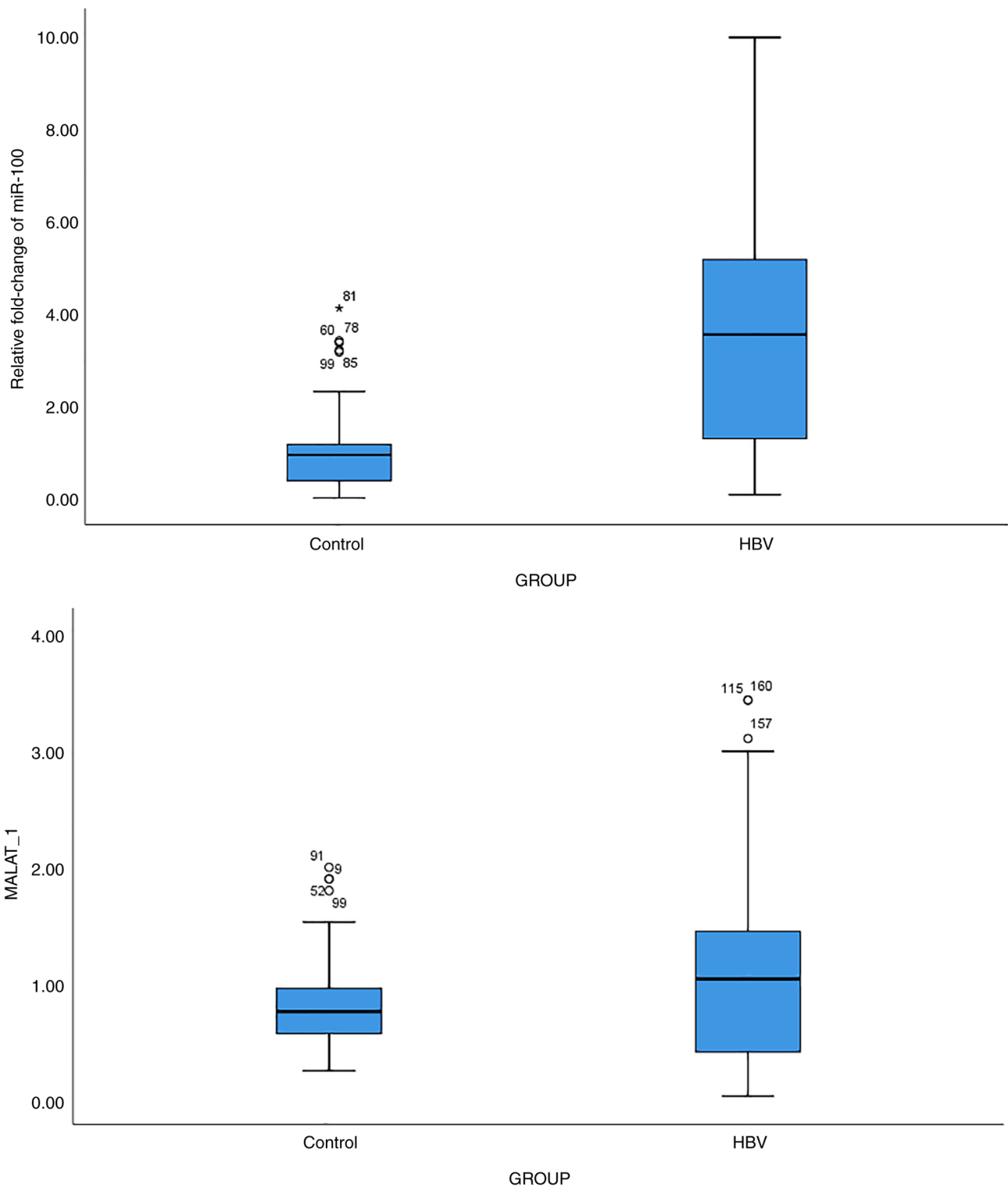


Figure 3. Relative fold change in the expression of miR-100 and MALAT-1 in the controls and patients with HBV. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA; HBV, hepatitis B virus.

entities (30). Previous studies have revealed that MALAT1 gene polymorphisms are associated with disease susceptibility; for example, the MALAT1 rs619586 has been found to be associated with a decreased risk of developing HCC and colorectal cancer (31-32).

With respect to an SNP of MALAT1 rs619586 A/G in HBV-infected patients vs. the control group, the genotyping results of the present study revealed the dominant appearance

of AA genotype in both groups. By contrast, the AG and GG genotypes were lacking. In agreement with these results, Motawi *et al* (33) demonstrated that the AA genotype was more frequent than AG or GG genotypes in HBV-infected Egyptian patients. An earlier study on the Chinese population reported that the AA genotype was the most frequent with a lack of significance, apart from reporting no significant association between MALAT1 rs619586 SNP and HBV clearance (31).

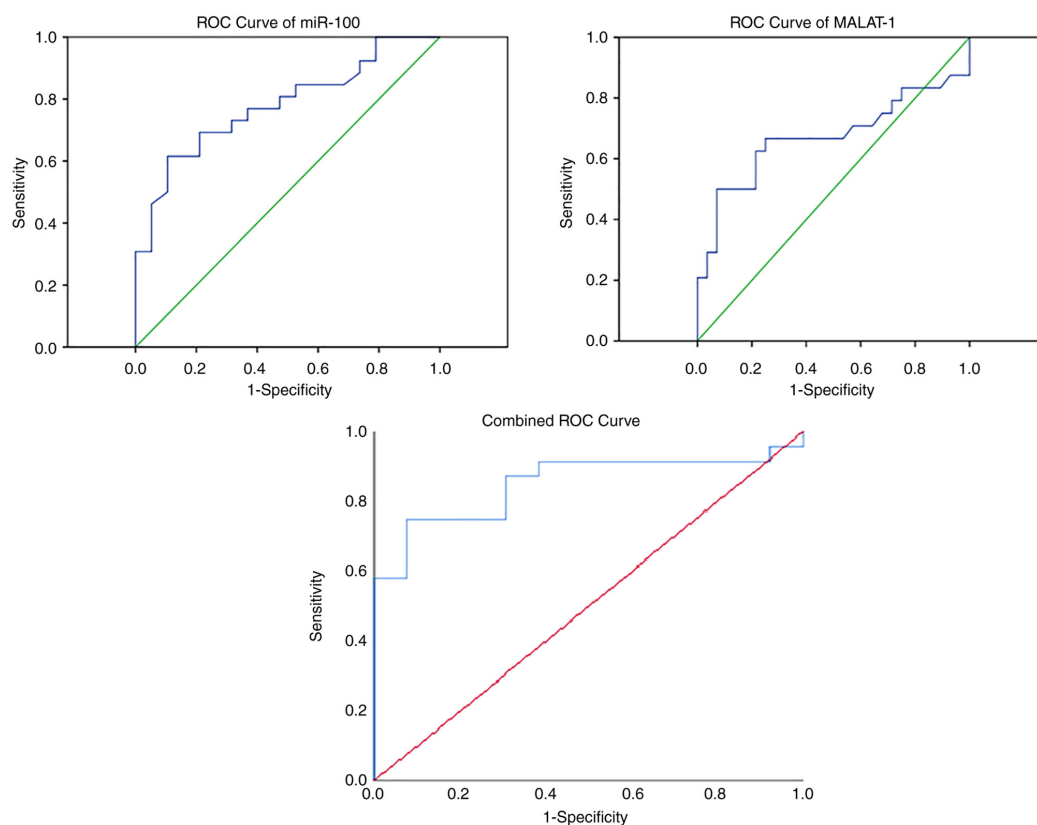


Figure 4. ROC curve of miR-100 and MALAT1. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA.

Table III. Correlation between biochemical tests and the viral load with miR-100 and MALAT1 expression level.

	r/r ² value and P-value	Group	AST	ALT	Albumin	Total bilirubin	PCR	miR-100	MALAT1
Group	r	1							
AST	r	0.365 ^b	1						
	r ²	0.133							
	p	<0.001							
ALT	r	0.368 ^b	0.865 ^b	1					
	r ²	0.135	0.748						
	p	<0.001	<0.001						
Albumin	r	-0.718 ^b	-0.069	-0.078	1				
	r ²	0.515	0.004	0.006					
	p	<0.001	NS	NS					
Total bilirubin	r	0.353 ^b	0.724 ^b	0.781 ^b	-0.051	1			
	r ²	0.124	0.524	0.609					
	p	<0.001	<0.001	<0.001	NS				
PCR	r	0.598 ^b	0.698 ^b	0.722 ^b	-0.280 ^b	0.732 ^b	1		
	r ²	0.357	0.487	0.521	0.078	0.535			
	p	<0.001	<0.001	<0.001	<0.001	<0.001			
miR-100	r	0.478 ^b	0.356 ^a	0.334 ^a	-0.302 ^a	0.401 ^b	0.489 ^b	1	
	r ²	0.228	0.126	0.111	0.091	0.160	0.239		
	p	<0.001	<0.05	<0.05	<0.05	<0.01	<0.001		
MALAT1	r	0.294 ^a	0.236	0.316 ^a	-0.151	0.248	0.282 ^a	0.102	1
	r ²	0.086	0.055	0.099	0.022	0.061	0.079	0.010	
	p	<0.05	NS	<0.05	NS	NS	<0.05	NS	

^aP<0.05 and ^bP<0.01/0.001, indicate statistically significant differences. NS, not significant; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

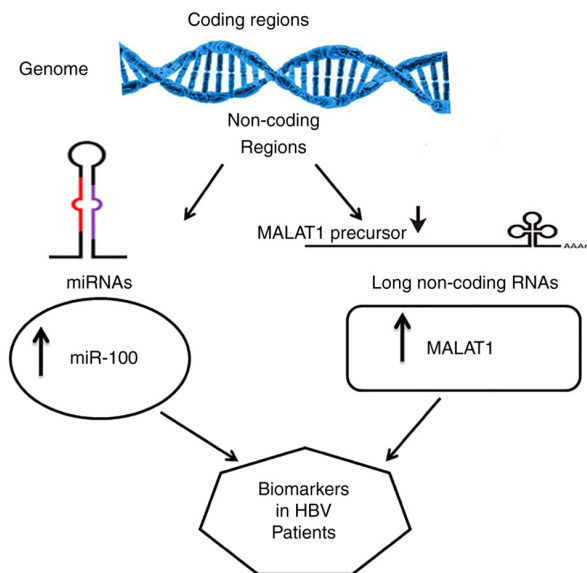


Figure 5. Hypothesized effects of both miR-100 and MALAT1. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, miRNA.

In the Taiwanese population, Yuan *et al* (34) pointed to an insignificant association between MALAT1 rs619586 and the risk of developing HCC in the HBV-positive subgroup, with higher frequency persistence of the AA genotype over the AG and GG genotypes. However, Wang *et al* (35) found that the MALAT1 rs619586 polymorphism decreased the risk of developing HCC under a dominant model, indicating that this SNP has the potential to be a biomarker for HCC risk and prognosis. In general, only a limited number of studies have focused on the genetic variation of MALAT1 rs619586 A/G in HBV infection. In other diseases, the AA genotype is the most dominant genotype, such as in lung cancer (36), congenital heart disease (37), ischemic stroke (38), thyroid carcinoma (39) and recurrent miscarriage (40).

It is not surprising that disease-associated SNPs can alter their gene expression levels (23). Supporting this issue, recent studies have focused (41-46) on the dysregulation of MALAT1 in various diseases. The present study demonstrated a significant upregulation of MALAT1 expression in the plasma of patients with HBV compared to healthy controls. Generally, only a limited number of studies are available to date which quantify MALAT1 expression in patients with HBV. Consistent with the results obtained herein, Konishi *et al* (41) reported that MALAT1 plasma levels were progressively and significantly elevated in both hepatic disease and patients with HCC. Evidence from other recent studies has reported an increase in the MALAT1 level in HCC tissues (42,43). Multiple lines of evidence have reported the prognostic usefulness of MALAT1 across various types of cancer (44-45), such as being a putative non-invasive biomarker in HCV-induced HCC (46).

In addition to MALAT1, miR-100 is another type of non-protein-coding transcript, miRNA. Previously, it was reported by Motawi *et al* (25) that miR-100 was significantly upregulated in patients with HBV, and this elevation is synchronized with the presence of the T allele, suggesting that miR-100 may be considered a potential molecular marker to appraise the prognosis of patients with HBV. As lncRNAs

may interact with miRNAs and modulate each other's expression (47), MALAT1 has been described to regulate several miRNAs (48).

The present study hypothesized the presence of correlations between miR-100 and MALAT1. Although no significant correlation was observed, miR-100 and MALAT1 were significantly upregulated in the HBV-infected patients. A positive correlation between the viral load of HBV and both miR-100 and MALAT1 was detected. Both miR-100 and MALAT1 may be regarded as non-invasive molecular markers in HBV infection in the Egyptian population (Fig. 5). Therefore, the present preliminary study focused on two major classes of ncRNAs; miR-100 and MALAT1. Both ncRNAs were upregulated in patients with HBV, and both were found to be positively correlated with the HBV viral load. Accordingly, they may be considered a molecular biomarker in HBV infection. To confirm these findings, further studies with larger sample sizes with other SNPs in both genes are required to clarify the associations between SNPs and their susceptibility to HBV infection in the Egyptian population.

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Availability of data and materials

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

The present study was carried out in collaboration between all authors. RMT designed the research and contributed new reagents/analytic tools. SZE provided the patient samples and clinical data. AEM and EAEM performed the experiments. RMT and EAEM analyzed and interpreted the data. SMR and TKM participated in the experimental design and in the writing of the manuscript. RMT, AEM and EAEM confirm the authenticity of the raw data. All authors wrote, and have read and approved the final manuscript.

Ethics approval and consent to participate

All subjects provided written informed consent for genetic analysis in the present observational prospective case-control study. All methods and analyses were carried out following the guidelines of the Ministry of Health and approved by the Research Ethics Committee for Experimental and Clinical Studies at the Faculty of Pharmacy, University of Cairo, Egypt [BC (1837)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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