

Article

Association Analysis of Genetic Variants of Sodium Taurocholate Co-Transporting Polypeptide NTCP Gene (*SLC10A1*) and HBV Infection Status in a Cohort of Egyptian Patients

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Abstract: Background: Single nucleotide polymorphisms (SNPs) in the *SLC10A1* gene, coding for a functional receptor of hepatitis B virus (HBV), sodium taurocholate co-transporting polypeptide (NTCP), may influence the susceptibility, the outcome, and disease course of HBV infection in some populations. Aim: to determine the prevalence of SNPs of NTCP gene, rs2296651 and rs943277, and their relationship with chronic HBV infection in a group of Egyptian patients. Methods: 137 patients with HBV and 65 healthy controls were enrolled, and the patients were divided into two groups; group I chronic HBV infection (68 patients with normal ALT and minimal or no liver necroinflammation or fibrosis) and group II chronic hepatitis B (69 patients with elevated ALT and moderate or severe liver necroinflammation). They were subjected to full history taking, clinical examination, laboratory investigations, abdominal ultrasound, and liver stiffness measurement using both Echosens® Fibroscan and acoustic radiation force impulse (ARFI). Real time PCR TaqMan 5' allelic discrimination assay was applied to detect the SNPs in NTCP gene, rs2296651 and rs943277. Results: On studying the rs2296651 variant, all controls and patients had genotype GG without any significant association with HBV infection or disease progression. However, the rs943277 variant in all controls and 98% of patients had genotype GA, except for two chronic HBV infection patients who had genotype AA, but no significant difference between patients and controls was found. The non-invasive methods for liver fibrosis assessment ARFI, AST/platelet's ratio (APRI), and fibrosis-4 score (FIB-4) could predict the stages of fibrosis in agreement with Fibroscan with AUCOR 0.8, 0.79, and 0.76, respectively. Conclusion: These findings may suggest that there is no relation between these SNPs of the NTCP gene and susceptibility or chronicity of HBV infection in the Egyptian population. We also suggest that the use of the non-invasive methods for liver fibrosis assessment, ARFI, FIB-4, and APRI may decrease the need for liver biopsies in prediction of significant hepatic fibrosis in chronic HBV patients.

Keywords: Functional receptor; Hepatitis B virus; Polymorphism; Sodium taurocholate co-transporting polypeptide; hepatic fibrosis; Egypt

1. Introduction

Although effective hepatitis B virus (HBV) vaccines are in use worldwide, infection with HBV is still a serious global health problem with significant morbidity and mortality. In 2017, the World Health Organization (WHO) estimated that approximately 2 billion people have evidence of past or present infection with HBV [1]. A prevalence of 1.4% in Egypt is reported [2]. The number of HBV-related deaths due to complications of chronic HBV infection like liver cirrhosis and/or hepatocellular carcinoma (HCC) increased by 33% between 1990 and 2013 [3].

Factors that can affect persistence or clearance of HBV infection and its variable clinical outcomes include viral, environmental, and host factors [4]. Studying these factors allows an understanding of the viral life cycle, the natural history of the disease, and the development of new therapeutic agents aimed at curing HBV. One of the host factors is the genetic variations of *NTCP* receptor genes. HBV enters the hepatocytes via an entry receptor, sodium taurocholate co-transporting polypeptide (NTCP, also known as solute carrier family 10 member 1), which specifically interacts with the pre-S1 region of HBV [5]. This co-transporter, encoded by the *NTCP* (*SLC10A1*) gene, is highly expressed on the sinusoidal membranes, and plays a crucial role in bile duct enterohepatic circulation and regulating functions of the hepatocytes [6].

The relationship between *NTCP* polymorphisms and HBV infection, liver cirrhosis, or HCC is controversial, with some studies concluding that *NTCP* polymorphisms have been associated with resistance to HBV infection [7,8], while others have demonstrated that it promotes HBV infection [9-11]. Accordingly, it remains to be explored whether *NTCP* polymorphisms influence the susceptibility to HBV infection and the occurrence of liver cirrhosis or HCC.

The rs2296651 SNP is a non-synonymous G/A single nucleotide transition substitution, missense variant in the fourth exon of *SLC10A1* gene, located on Chr.14 [12]. The rs943277 SNP is a G/A single nucleotide transition substitution in the first intron of the *SLC10A1* gene. It has been reported that variants in the intron might also influence gene expression via regulating a transcription enhancer or silencer [13]. The prevalence of rs2296651 or rs943277 SNPs of the *NTCP/ SLC10A1* gene is controversial in different geographic areas. To the best of our knowledge, there are no studies investigating the prevalence of rs2296651 or rs943277 SNPs of the *NTCP* gene in Egypt. Consequently, the present study was conducted in a group of Egyptian patients to determine the prevalence of these SNPs, and to explore if there is a possible relation between them and HBV infection status.

2. Results

The basic clinical and demographic data of the patients and controls are described in **Tables 1**. Age and sex were well-matched between controls and patients, though ages were significantly higher in the group of patients with more advanced liver disease (P 0.002 and 0.01 respectively). Among patients, history of undergoing surgical operations or dental procedures were the main risk factors of acquisition of HBV infection.

Regarding laboratory data, serum hepatic transaminases and total bilirubin were significantly higher in the patients group compared to the control group, and increased with progression of liver disease ($P < 0.0001$), while platelet count and albumin were significantly lower in the patients than in controls, and decreased with progression of liver disease; HBV DNA levels were significantly higher in the CHB patients group compared to the chronic HBV patients' group ($P < 0.0001$) (**Table 2**). From HBV markers, HBeAg was negative in 95% of patients.

Among all studied patient groups, the degree of fibrosis was absent or mild in 49.64% (47 patients F0, 6 patients F0-F1, 15 patients F1), and significant in 50.36% (32 patients F2-3, 17 patients F3, 11 patients F3-F4, 9 patients F4) using Fibroscan (Echosens®).

Table 1. The basic characteristics of the patients.

Variable	Healthy control (HC) n=65	Patients group I HBV infection n=68	Chronic chronic hepatitis B (CHB) n=69	Patients group II P1 value	P2 value
Age (yrs)	mean \pm SD	40 \pm 11	37	43	
	min - max	23 - 75	22 - 62	23 - 68	N.S 0.002
Sex	Male	39 (60%)	41 (60.3%)	55 (79.7%)	N.S 0.01
	Female	26 (40%)	27 (39.7%)	14 (20.3%)	
Residence	Rural	8 (12.3%)	11 (16.2%)	22 (31.9%)	0.01 N.S
	Urban	57 (87.7%)	57 (83.8%)	47 (68.1%)	
Comorbidities	DM	3 (4.6%)	5 (7.4%)	6 (8.7%)	
	HTN	4 (6.2%)	3 (4.4%)	4 (5.8%)	N.S N.S
	No	58 (89.2%)	59 (86.8%)	59 (85.5%)	
Manifestation of CLD	---	0 (0.0 %)	6 (8.7%)	-	0.02
BMI (kg/m ²)	28 \pm 3.5	27 \pm 3.4 *	27 \pm 2.6	0.04	N.S

Table 2. Laboratory data of the patients and controls.

Variable	HC n=65	Patients group I Chronic HBV infection n=68	Group II CHB n=69	P1 value	P2 value
Hb (13-18 g/dl)	13 \pm 1.4	14 \pm 1.5	13 \pm 1.6	0.19 N.S	0.19 N.S
TLC (4-11 $\times 10^3$ /mm ³)	6.2 \pm 1.8	6.5 \pm 1.6	6.1 \pm 1.7	0.3 N.S	0.3 N.S
PLT (150-450 $\times 10^3$ /mm ³)	294 \pm 83	258 \pm 76	186 \pm 85	<0.0001	<0.0001
INR	1 \pm 0.06	1.1 \pm 0.1	1.1 \pm 0.18	<0.0001	<0.0001
ALT (up to 41 IU/L)	27 \pm 6.3	30 (11-78)	39 (13-114)	<0.0001	<0.0001
AST (up to 40 IU/L)	22 \pm 5.6	28 (10-50)	37 (16-152)	<0.0001	<0.0001
T. Bilirubin (0.5-1.2 mg/dl)	0.64 \pm 0.19	0.7 (0.3-1.4)	0.8 (0.2-3.1)	<0.0001	<0.0001
Albumin (3.4-5.4 g/dl)	4.5 \pm 0.58	4.3 (3.5-5.7)	4 (2.9-5.5)	<0.0001	<0.0001
AFP (up to 10 ng/ml)	---	4 (0.4-20)	4.8 (0-59)	0.7 N.S	0.7 N.S
Creat (0.6-1.2 mg/dl)	0.62 \pm 0.2	0.8 (0.3-3.9)	0.8 (0.3-2)	0.0004	N.S
HBV PCR (x10 ³ IU/ml)	---	1.446 (0.198-5298.090)	5.030 (0.074-170000)	<0.0001	<0.0001
HBsAg	+Ve	1 (1.5 %)	5(7.2 %)	-	0.03
	- Ve	67 (98.5%)	64(92.8 %)		

The mean values of non-invasive methods for hepatic fibrosis measurement, including ARFI, APRI, and FIB-4 were significantly higher in CHB patients compared to the chronic HBV infection group and could predict stages of fibrosis in agreement with liver stiffness measured by Fibroscan ($p < 0.0001$). On the other hand, there was no statistically significant difference in AAR between both groups (**Table 3**).

An ROC curve was plotted to discriminate predictive value of ARFI, APRI and FIB4 in prediction of significant fibrosis \geq F2 (**Table 4**).

- ARFI showed the highest AUC of 0.8 ($p < 0.0001$, significant) with best cut off \leq 1.3, at which sensitivity and specificity were 80% and 74%, respectively.
- APRI showed AUC of 0.79 ($p < 0.0001$, significant) with best cut off \leq 0.34, at which sensitivity and specificity were 74% and 71%, respectively.
- FIB-4 showed AUC of 0.76 ($p < 0.001$, significant) with best cut off \leq 0.83, at which sensitivity and specificity were 74% and 60%, respectively.

Table 3. Relationship between Fibroscan as an acceptable tool for fibrosis assessment and the other non-invasive methods for prediction of fibrosis.

	Fibroscan				P value	
	< F2 (N= 68)		≥ F2 (N= 69)			
	No	%	No	%		
ARFI MEDIAN	< F2	61	89.7	33	47.8	
	≥ F2	7	10.3	36	52.2	
APRI	< F2	61	89.7	33	47.8	
	≥ F2	7	10.3	36	52.2	
FIB-4 index	< F2	60	88.2	36	52.2	
	≥ F2	8	11.8	33	47.8	
AAR	< F2	3	4.4	4	5.8	
	≥ F2	65	95.6	65	94.2	

Table 4. Area under curve (AUC), confidence interval and Cut-off values of other noninvasive methods for prediction of fibrosis than Fibroscan with its sensitivity and specificity.

Variable(s)	AUC	p value	95% Confidence Interval		Cutoff value	Sensitivity (%)	Specificity (%)
			Lower Bound	Upper Bound			
ARFI MEDIAN	0.8	<0.0001	0.71	0.88	1.3	80	74
APRI	0.79	<0.0001	0.72	0.87	0.34	74	71
FIB-4 index	0.76	<0.0001	0.69	0.84	0.83	74	60

2.1. NTCP rs2296651 variants among the studied groups

All studied subjects had genotype GG of SNP rs2296651 of NTCP gene. Accordingly, it did not show a statistically significant difference between patients and controls.

2.2. NTCP rs943277 variants among the studied groups

The heterogeneous mutant genotype GA of SNP rs943277 of NTCP gene was the most prevalent genotype among the controls (100%) and patients (98.5%), with only two patients from the chronic HBV infection group having the homogenous mutant genotype AA. None of the genotypes showed a statistically significant difference neither among patients' groups nor between different patient groups and controls (**Table 5 and 6**). Analysis of data of the two patients with homogenous mutant genotype AA of SNP rs943277 of NTCP gene is demonstrated in **Table 7**.

Table 5. NTCP rs943277 variant among the studied groups.

Variable	HC n=65	Group I Chronic HBV infection n=68		Group II CHB n=69	P value
rs943277 vari- ant	GG	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.13
	GA	65 (100%)	66 (97%)	69 (100%)	
	AA	0 (0.0%)	2 (3%)	0 (0.0%)	
	Allele G	65 (50%)	66 (48.52%)	69 (50%)	
	Allele A	65 (50%)	70 (51.47%)	69 (50%)	

Table 6. NTCP rs943277 variant among patient groups versus controls.

rs943277 variant	Group I against HC		Group II against HC	
	P value	OR (95%CI)	P value	OR (95%CI)
GG	REF	REF	REF	REF
GA + AA	0.49	Infin	>0.99	1
Allele G		1.06		1
Allele A	0.9	(0.665-1.72)	>0.99	(0.619-1.61)

Table 7. Analysis of data of the two patients with homogenous mutant genotype AA of SNP rs943277 of NTCP gene.

Variable	Patient 1	Patient 2
Age	38	35
Sex	Male	Male
Risk factors	No	H/O of operation
PLT (150-450 $\times 10^3/\text{mm}^3$)	221	360
INR	1	1.1
ALT (up to 41 IU/L)	19	19
AST (up to 40 IU/L)	18	10
T. Bilirubin (0.5-1.2 mg/dl)	0.7	0.7
Albumin (3.4-5.4 g/dl)	3.8	3.9
HBV PCR ($\times 10^3\text{IU/ml}$)	1739	450
HbeAg	- ve	- ve
HbeAb	+ ve	+ ve
Fibroscan (Kpa)	5.8	4.7
Fibroscan metavir score	F0-F1	F0
ARFI median	1.52	1.12
APRI	0.2	0.07
FIB-4	0.71	0.22
AAR	0.95	0.53
rs2296651	GG	GG

3. Discussion

Single nucleotide polymorphisms (SNPs) in *SLC10A1* gene, coding for sodium taurocholate co-transporting polypeptide (NTCP) functional receptor of HBV, has been associated with natural history of HBV infection in some populations.

Based on genome Aggregation Database (genomAD), the minor allele frequency of rs2269951 SNP is A=0.005 in global population, A=0.095 in East Asian, A=0.002 in African, A=0.002 in American, and A=0.00005 in European populations (SNPs with a minor allele frequency of 0.05 (5%) or greater are considered a common variant in this population) [12]. According to a 1000 genomes phase 3 combined population database, the minor allele frequency of rs943277 SNP is A=0.078 in global population, A=0.003 in East Asians, A=0.03 in South Asians, A=0.007 in Africans, A=0.25 in Americans, and A=0.174 in Europeans [14].

However, the genetic mutants of NTCP and their relation to HBV infection have not yet been studied in the Egyptian population. The fundamental target of the present study was to determine the prevalence of SNPs of NTCP gene, rs2296651 and rs943277, in Egyptians, explore the possible association between these SNPs and HBV susceptibility in Egyptian patients, and correlating their presence, if any, to disease progression.

Surprisingly, we detected only wild type GG genotype in all our studied Egyptian participants, and none of them presented the mutant GA or AA genotypes, with no difference between groups. Our results were similar to those of other populations like Moroccan [15], Spanish Caucasian [16], Polish Caucasian [17], European Americans, and African Americans [18] populations, where the rs2296651 mutant genotypes were not detected in any of their participants.

In contrast to our results, several previous studies in Chinese Han population showed that rs2296651 mutant variants were inversely correlated with HBV susceptibility, where GA and AA genotypes had lower frequencies in patients with CHB compared with healthy controls and were demonstrated to be protective, reducing the risk of liver cirrhosis, hepatocellular carcinoma, and liver failure in CHB patients [8,19-22]. Some studies done in other Asian populations, such as Taiwanese [7,23], Vietnamese [24], Korean [25], and Taiwanese [26] populations, reported that the *SLC10A1* A allele consistently decreased HBV infection risk compared with the G allele. The possible protective role of rs2296651 polymorphism was also demonstrated in a meta-analysis that extracted 8 studies, including 14,591 chronically HBV-infected patients and 12,396 healthy controls from different populations (Chinese, Taiwanese, and Moroccan). It concluded that the mutant variant was inversely associated with the risk of HBV infection (OR = 0.593, P = 0.028) where the A allele and GA genotype frequencies were lower in the CHB group compared with HCs group [27].

However, there were conflicting data in Asian populations that the rs2296651 mutant variant may be associated with increased susceptibility to HBV infection [9] or may not be a risk factor for HBV infection at all [10,11,28]. These rather contradictory results suggest that the rs2296651 variant varies among different ethnic populations and may be specific to Asian populations, and that it might display its advantage in conferring resistance to HBV infection.

On the other hand, our study population presented the heterogeneous mutant GA genotype of rs943277 SNP of *NTCP* gene, except for only two patients from the chronic HBV infection group that had the homogenous mutant AA genotype, with no statistically significant difference found between the studied groups.

SNP rs943277 of *NTCP* gene was studied in a group of Chinese Hans (1232 HC and 2453 CHB patients) by Wang et al. in 2017 [27] and found that the wild GG genotype was the most prevalent among HC (99.3%) and CHB patients (98.4%). While the heterogeneous mutant GA genotype was 0.7% and 1.6% in HC and CHB patients, respectively, with only one CHB patient showing homogenous mutant AA genotype, this difference in GA genotype was statistically significantly, being higher in CHB patients than in HC (p=0.014), and revealed to be a potential hazardous mutant, facilitating susceptibility to HBV infection.

Due to limited baseline research on *NTCP*, especially the rs943277 GA mutant, the function of the rs943277 GA mutant in influencing *NTCP* expression is still poorly understood. Further comprehensive studies using large scale multicenter populations of varying ethnic origins, with different outcomes of infection and therapeutic schedules, are needed to determine the prevalence of *NTCP* polymorphisms and their exact role in the pathogenesis of HBV infection.

With reference to basic data results of our study, the mean age of our patients was 44±13 years that matches a previous Egyptian study conducted by Ismail et al. in 2017, who showed a similar HBV infection risk peak among those aged 35-44 [2]. There was a significant increase in age of patients with higher degree of liver disease progression, which is consistent with Li et al.'s study in 2017 who found that the older the age, the higher the stage of fibrosis [29]. This could be explained by the longer duration of exposure to HBV infection [30]. Male predominance was obvious in our study (70%), matching another Egyptian study by Maklad et al. in 2018 (with a male: female ratio of about 5:1), which may be due to more exposure to risk factors of viral acquisition among males [31].

In our study, the non-invasive serum biomarkers for predicting liver fibrosis APRI and FIB-4 were significantly higher in the CHB patients group compared to the chronic HBV infection group, while AAR did not show a significant difference between both groups. Zhang et al. in 2016 [32] also reported that the mean values of FIB-4 and APRI in patients with HBV infection were significantly higher for each successive fibrosis stage (p <0.05), but there were no differences between successive fibrosis stages for AAR. To discriminate between significant and non-significant fibrosis by serum APRI, we found the

cut-off value of 0.34 to have sensitivity of 74% and specificity of 71%, with AUC 0.79. This is in accordance with the findings of Yue et al. in 2019 [33] who proposed 0.35 as a cut off for significant fibrosis ($F \geq 2$) in patients with CHB with sensitivity 78% and specificity 72%. And for serum FIB-4 we found the cut off value of 0.83 to have sensitivity of 74% and specificity of 60% with AUC 0.76. The findings suggested by Ucar et al. in 2013 [34] were 0.68 AUC of FIB-4 for cut-off value 1.08 with sensitivity 70.7% and specificity 62.5% in CHB patients.

In addition, our study showed that mean values of the LSM by ARFI elastography for predicting liver fibrosis was significantly higher in CHB patients group compared to the chronic HBV infection group. Another study by Tseng and colleagues in 2018 [35] concluded that the value of ARFI measurement increased with the severity of liver fibrosis and found a significant correlation between fibrosis stage and ARFI ($p = 0.0001$), APRI ($p = 0.012$), and FIB-4 ($p = 0.004$). However, LSM should be interpreted with caution among patients with elevated ALT and should not be used in patients with extensive liver necro-inflammation (ALT levels $> 10 \times$ ULN). Transient elastography also has limited use in hepatic congestion, cholestasis, obese patients, and patients with ascites [36].

Furthermore, in our work, ARFI achieved AUC=0.8 with p value < 0.0001 for cut-off value 1.3 m/s in prediction of significant fibrosis with sensitivity 80% and specificity 74%. These results agree with the results of previous studies done by Friedrich-Rust et al. in 2012 [37] who proposed 1.34 m/s as a cut-off for the diagnosis of significant fibrosis, with AUC=0.75 and sensitivity 81.0% and specificity 64.2%.

4. Materials and Methods

4.1. Study subjects

The present study comprised 137 patients with HBV infection and 65 age-matched healthy volunteers as a control group recruited from Cairo Fatemic Hospital, Ministry of Health and Population (MOHP), and Cairo University Center for Hepatic Fibrosis (CUC-HF), Cairo University, between January 2019 and September 2019. All participants were Egyptians.

All patients were HBV treatment naïve with positive HBsAg for more than 6 months, and detectable HBV DNA and negative HCV, HIV, and autoimmune antibodies. Liver enzymes and transient elastography were performed to determine the stage of hepatic fibrosis. The patients were then divided into two clinical subgroups:

Group I: chronic HBV infection (n=68) characterized by normal ALT according to traditional cut-off values [ULN approximately 40 IU/L] and minimal or no liver necro-inflammation or fibrosis [38].

Group II: chronic hepatitis B (n=69) characterized by elevated ALT and moderate or severe liver necro-inflammation and accelerated progression of fibrosis [38].

The concept of the study was clearly explained to all participants who then provided informed consent. The study protocol conformed to the ethical guidelines of the declaration of Helsinki and was approved by National Committee for combating Viral Hepatitis (NCCVH) ethical committee.

4.2. Methods

All patients were subjected to full history taking, proper physical examination, basic laboratory investigations, and hepatitis markers by EIA: HBsAg, HBeAg, anti-HBe, and HCV Antibodies, quantitative HBV DNA by real time PCR (with a lower limit of detection of 16 IU/ml), abdominal ultrasonography, and assessment of liver fibrosis by the following non-invasive methods:

4.2.1. Indirect serum indices

APRI was calculation using the formula (AST/upper limit of normal \times 100)/platelet count [39]. FIB-4 was calculation using the formula (Age [yrs.] \times AST /platelet count \times ALT) [40]. AAR index was calculation using the formula (AST/ALT ratio) [41].

4.2.2. Liver stiffness measurement:

I) Transient elastography (TE) using a FibroScan® device (Echosens, Paris) [42]

II) Acoustic Radiation Force Impulse (ARFI) elastography: using a Siemens ACUSON S3000 Ultrasound System (Siemens AG, Erlangen, Germany) [43]

Both Transient elastography (TE) and ARFI elastography were done at the Cairo University Center for Hepatic Fibrosis (CUC-HF); funded by Science and Technology Development Fund (STDF) (5274 Center of excellence).

Since non-invasive measurements became widely available and, according to EASL guidelines for management of HBV, Transient elastography (TE) is an acceptable tool for fibrosis assessment in the setting of chronic hepatitis B infection, and due to the limitations of using liver biopsy, tissue elastography was considered the standard diagnostic tool for the assessment of liver fibrosis [36].

4.2.3. Determination of the SLC10A1 gene SNPs rs2296651 and rs943277 by real time PCR TaqMan 5'

Allelic discrimination assay in both patients and control subjects were conducted as described in [26]. Two milliliters of blood were put into a vacutainer containing ethylene diamine tetra-acetic acid (EDTA) for DNA extraction and analysis of *SLC10A1* gene polymorphisms. Total genomic DNA of patients and controls was extracted using Qiagen extraction kit (Cat. No.51104, USA). To detect the variants in this gene both SNPs rs2296651 and rs943277 kit of Cat. No: 4362691 and 4351379 (Applied Biosystems, ThermoFisher Scientific, USA) were used for real time PCR assay. The kit contains both forward and reverse primers in addition to probes for both wild and minor type alleles. TaqMan universal PCR master mix contains AmpliTaq Gold® DNA polymerase, dNTPs, optimized buffer component and was purchased from Applied Biosystem.

Genotyping of the SNPs was carried out using the TaqMan genotyping assay on the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, USA). Each Custom TaqMan® SNP Genotyping Assay consists of a single tube containing: two primers for amplifying the polymorphic sequence of interest, and two TaqMan® MGB probes for distinguishing between the two alleles. Each TaqMan MGB probe contains a reporter dye at the 5' end of each probe (VIC® dye is linked to the 5' end of the Allele 1 probe; FAM™ dye is linked to the 5' end of the Allele 2 probe) and a non-fluorescent quencher (NFQ) at the 3' end of each probe.

During PCR, each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. When the oligonucleotide probe is intact, the proximity of the reporter dye to the quencher dye results in quenching of the reporter fluorescence primarily by Förster-type energy transfer [44,45]. AmpliTaq Gold® DNA polymerase extends the primers bound to the template DNA. AmpliTaq Gold DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. The increase in fluorescence signal occurs when probes that have hybridized to the complementary sequence are cleaved. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample. So, a substantial increase in VIC-dye fluorescence only indicates homozygosity for Allele 1, while a substantial increase in FAM-dye fluorescence only indicates homozygosity for Allele 2. An increase in both VIC- and FAM-dye fluorescence indicates Allele 1-Allele 2 heterozygosity. The rs2296651 and rs943277 genotyping assays were read at the PCR end point. GG genotype

was considered as the wild genotype, GA as the heterozygous genotype, and AA as the homogenous minor genotype.

4.3. Statistical analysis:

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23 and GraphPad Prism 7. Data was summarized using median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and MannWhitney tests. For comparing categorical data, a chi-square test was performed. An exact test was used instead when the expected frequency was less than 5. Genotype and allele frequencies were compared between every 2 groups using chi-square tests. The odds ratio (OR) with 95% confidence intervals was calculated. The receiver operating characteristic (ROC) curve was used for prediction of cut-off values. P-values less than 0.05 were considered as statistically significant.

5. Conclusions

Based on the results of the current study we can conclude that the rs2296651 variant within the *NTCP* gene is not prevalent in the studied group of Egyptian population, with subsequent absence of relation with susceptibility to HBV infection or disease progression which could be related to our ethnic group. Genotype GA of the rs943277 variant within the *NTCP* gene was the prevalent genotype among our studied group of Egyptian healthy controls and 98% of patients, but no association was similarly found between this variant and HBV infection susceptibility or disease progression. The non-invasive methods for liver fibrosis assessment ARFI, FIB-4, and APRI displayed significantly higher values among patients with significant fibrosis, and their use will decrease the need for liver biopsies in prediction of significant hepatic fibrosis in chronic HBV patients.

Author Contributions: design of the study, M.E.S.E.R. and N.A.Z.; data acquisition, A.Y., M.H., M.E.S. and W.D.; contribution by analysis tools, A.Y. and R.M.S.H.; writing—original draft preparation, F.E.; writing—review, editing, and finalization M.E.S.E.R., N.A.Z., Y.S.I., H.G.E. and V.N.U.; supervision, M.E.S.E.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Cairo Fatemic Hospital and Faculty of Medicine, Cairo University (protocol code I-170318, date of approval 2018, July).

Informed Consent Statement: Written informed consent has been obtained from all subjects involved in the study to use their clinical, investigational data for research and subsequent publication of research results.

Data Availability Statement: all data we have already presented in our manuscript, the supporting data will be found at data bank such as Medline, PubMed, Scopus, and web of sciences.

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