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Association of MicroRNA-30a rs1358379 single nucleotide polymorphism with susceptibility to hepatitis B virus Infection in Patients with End-Stage Renal Disease

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Abstract

Background: Occult hepatitis B virus (HBV) could be infective through blood transfusion or organ transplantation. MicroRNA-30a rs1358379 polymorphism plays a crucial role in the development of end-stage renal disease (ESRD).

Objectives: We aimed at revealing the association between CC genotype of MicroRNA-30a rs1358379 polymorphism and occult HBV infection in ESRD Egyptian patients.

Methods: We performed real-time PCR for the quantification of HBV-DNA in the serum of 139 ESRD patients and for diagnosis of MicroRNA-30a rs1358379 polymorphism in the serum of patients and 100 healthy controls. **Results:** Out of 139 patients, 125 (89.9%) were HBsAg negative. We observed a high percentage of the CC genotype among patients (106=76.2%), while the CT and TT genotypes were (19=13.7%) and (14=10.1%), respectively. The C allele represented 83.1% in patients whereas the T allele was 16.9%. The CC and CT genotypes in patients had a statistically significant difference in the mean level of PCR. The CT genotype in patients among males and the TT genotype amongst females had the higher statistically significant percentages. The presence of C allele declared a statistically significant difference in the mean levels of AST and PCR. **Conclusion:** We found that the high percentage of C allele or CC genotype of MicroRNA-30a rs1358379 polymorphism in ESRD Egyptian patients might be responsible for the existence of HBV DNA with lack of exhibited hepatitis B surface antigen.

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INTRODUCTION

Occult "or latent" hepatitis B virus "HBV" infection is the presence of HBV DNA in plasma and/or serum of HBV infected patients, while the HBV surface antigen (HBsAg) is absent. This condition could sustain for years without incarnating manifestations of apparent HBV infection(1). Co-infections(2), drug abuse and/or addiction (3) or immuno-suppressive drugs (4) can arouse a boost in HBV DNA level without increasing HBsAg. HBV could be transmitted from patients with occult HBV infection through multiple routes that mainly includes transfusion of blood or organ transplantation (5). It may represent a major risk factor not only for the infected persons, but also for the surrounding community.

HBV infection and end-stage renal disease (ESRD) are prevalent and represent a considerable general health problem all over the world. Both have been accompanied by serious morbidity and mortality (6).

HBV infection in ESRD patients is considered a different clinical problem considering the immunosuppression influence of renal failure, the susceptibility for de novo infection and nosocomial transmission, the long-term consequences on morbidity and mortality, and the alteration in clinical course after kidney transplantation. The natural history of HBV infection in ESRD patients might differ in conformity with the timing of infection, genotype, and location (7).

Genetic determinatives for detection of HBV infection have yet to be recognized. Many host and viral molecular agents are identified to affect susceptibility to HBV infection, capability to get rid of the virus, continuation of a chronic status and proceed to late stages of hepatic diseases (8).

An essential factor influencing HBV infection that is getting attention is microRNAs which are a group of endogenous, small noncoding RNAs that are commonly 20-22 nucleotides in length. They regulate many biologic processes, like inflammatory reactions, cellular growth, differentiation, multiplication and apoptosis (9). MiRNA-30 family members are implicated in pathogenesis of many renal diseases (10). It was revealed that glucocorticoid sustained miR-30 expression was accompanied by decreased Notch1 stimulation and palliated podocytes injury, associated with changed expression of Notch1 and p53, denoting the essential role of miR-30 family in renal diseases (11).

It has been showed that single nucleotide polymorphisms (SNPs) detected in miRNA genes influence miRNA expression and interactions with target mRNAs. Many researches have reported that SNPs in miRNA genes are associated with HBV infection (12)

The prospective aim of the current study was to scout about if there is a coincidence of MicroRNA-30a rs1358379 SNP in ESRD Egyptian patients with the receptivity to HBV infection.

Subjects and Methods:

This study included 139 patients with end-stage renal disease on regular hemodialysis consecutively recruited, in the period from January 2019 to June 2019, from the hemodialysis Units, Fayoum University Hospital, Faculty of Medicine, Fayoum University and 100 healthy age and sex matching volunteers. The study was approved by the research ethical committee (REC) at Faculty of

Medicine, Fayoum University. It was accomplished in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments related to human-beings. Written informed consent that was approved by the REC, was signed by all subjects enrolled in the study, and kept with us for documentation. We performed a real-time PCR for quantification of HBV-DNA and for diagnosis of miRNA-30a rs1358379 SNP. Inclusion criteria included ESRD on regular hemodialysis (for at least 6 months' duration). Exclusion criteria included other causes of liver dysfunction (e.g. primary biliary cirrhosis, autoimmune hepatitis, continued alcohol abuse, autoimmune hepatitis, and HIV infection) and being on treatment with interferon and/or ribavirin or sovaldi. A complete medical history, including previous infection with hepatitis B or HBV vaccination history, duration of hemodialysis, etiology of ESRD, past history of blood transfusion, schistosomiasis, other risk factors for HBV as surgical or dental operations and thorough physical examination were performed for all patients.

Sample Collection:

5 ml venous blood was obtained by venipuncture from all subjects into plain tube and incubated at 37°C for 15 minutes, centrifuged at 3000g for 10 minutes to separate serum and stored in aliquots at −80°C.

Serological markers:

HBsAg HBcAg and Anti-HIV were tested by commercial enzyme linked immunosorbent assay ELISA (Dia Sorin, USA).

DNA Extraction:

Serum hepatitis B viral DNA was isolated with QIAamp Blood Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

Quantification of HBV viral DNA in serum by real-time PCR:

Extracted DNA was amplified by using primers specific for the pre-S1/pre-S2 region of HBV (nucleotides 3025 to 80 from the theoretical *Eco*RI site of the 3,221-nucleotide HBV sequence). The sense primer was preS1F (5`-AGGTRGGAGYGGGAGCATTCGG-3`), and the antisense primer was preS1R 5`-CCTGAACTGGAGCCACCAGCAGG-3` (R is A and G and Y is C and T). Thermal cycling parameters involved 40 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s. If the 277-bp product was not initially detected, the extracted DNA was amplified by nested PCR with outer primers that produce a 479-bp product (sense primer, 5`-TCACCATATTCTTGGGAACAAGA-3`; antisense primer, 5`-TTCCTGAACTGGAGCCACCA-3`)[13], followed by second-stage amplification with the pre-S primers described above. Reaction tubes for PCR contained 5 µl of DNA extract or the first-stage PCR product, AmpliTaq Gold reaction buffer (Applied Biosystems, Foster City, Calif.), 0.2 mM deoxynucleoside triphosphates (Invitrogen Life Technologies, Burlington, Ontario, Canada), 2.5 mM $MgCl_2$, 25 pmol of each primer, and 2.5 U of AmpliTaq Gold polymerase.

PCR for diagnosis of miRNA-30a rs1358379 polymorphism:

Template DNA was then amplified by using a pair of sense oligonucleotide primer of 5`-CTGGAGACCACTCCCATCCTTTCT-3` and

antisense primer 5`-GATGGTGGC CATCACATTCGTCAGAT-3`, 10 pmol of each primer. The PCR mixture contained 20 ng of genomic DNA, 3 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 5% dimethyl-sulphoxide (DMSO), each of 0,5 mM deoxyribonucleoside triphosphate (dNTPs) and 1 unit of Taq polymerase (Pharmacia, Uppsala, Sweden) in a final volume of 50 µL. Amplification was performed by denaturation at 94ºC for 1 min, annealing (nucleotide primer attachment) at 58ºC for 1 min, and extension (DNA elongation) at 72ºC for 2 min for 30 cycles followed by a final extension at 72ºC for 4 min. Results of PCR were separated by electrophoresis on a 2% agarosa gel, which had been enriched with ethidium bromide (0.1%), then visualized by ultraviolet light and were documented by using the gel doc (Biometra, Germany). The PCR product was 244 bp fragment in the presence of T allele and 159 bp fragment in the presence of C allele.

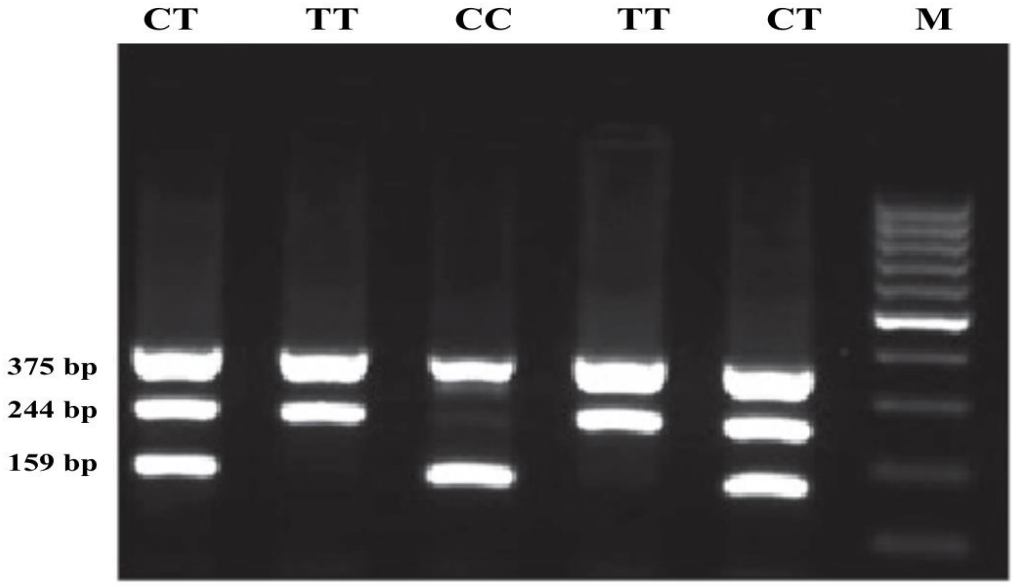

Figure 1: M, DNA marker; lanes 1 and 5, miRNA-30a rs1358379 CT; lanes 2 and 4, TT; lane 3, CC.

Statistical Analysis:

Data were assembled and coded to enable their handling with double-entry into Microsoft Access. Their analysis was uttered using SPSS software version 25 in windows 7 (IBM, Chicago, IL, USA). Simple descriptive analysis in the form of numbers and percentages for qualitative data, and arithmetic means as central tendency measurement, standard deviations as measure of dispersion for quantitative parametric data, and inferential statistic test; For quantitative parametric data, One way ANOVA test in comparing more than two independent groups of quantitative data with benferroni post-hoc to test significance between each two groups. For quantitative non parametric data, kruskal wallis test used in comparing more than two independent groups. For qualitative data, Chi square test to compare two of more than two qualitative groups. The P-value $\leq$ 0.05 was considered the cut-off value for significance.

Results:

This study was conducted on 139 patients with end-stage renal disease on regular hemodialysis, they were 67 males (48.2%) and 72 females (51.8%) with mean age = 40.19 ± 0.11, and 100 healthy age and sex matching controls, they were 50 males (50%) and 50 females (50%) with mean age = 42.89 ± 0.53.

Table (1): Description of demographic and laboratory data among the hemodialysis group.

Variables			
Sex	**No**	**%**	
Male	67	48.2%	
Female	72	51.8%	
	Mean	**SD**	**SE**
ALT	89.4	22.1	1.8
AST	78.5	17.5	1.5
Urea	178	57.4	4.9
Creatinine	2.8	0.85	0.07
qRT-PCR for HBV (IU/ml)	150677.7	394934.2	45006.9
	No	**%**	
Positive HBsAg	14	10.1%	
Positive HBcAg	77	55.4%	
Positive HIV	0	0%	

Concerning the liver enzymes; the mean levels of ALT and AST among the patients were (89.4±22.1) and (78.5±17.59), respectively. As regards the kidney function; the mean urea and creatinine levels were (178±57.4) and (2.8±0.85), respectively. Finally for quantitative real time-PCR for HBV, the mean level was (150677.7± 394934.2). We observed that only 14 patients (10.1%) were positive for HBsAg, whereas 77 patients (55.4%) showed a positive HBcAg. All patients were HIV-free. **(table 1).**

Table (2): Comparison of MiRNA-30a rs1358379 SNP genotypes in patients and control groups.

Variable		**Patients (N=139)** (%)	**Controls (N=100)** (%)	**P-value**
rs2273773 C>T	**CC**	106 (76.2%)	8 (8%)	**< 0.001***
	CT	19 (13.7%)	22 (22%)	
	TT	14 (10.1%)	70(70%)	
	C allele	231 (83.1%)	38 (19%)	**< 0.001***
	T allele	47 (16.9%)	162(81%)	

*Significant at p-value ≤ 0.05

The allocation of **miRNA-30a gene rs1358379** polymorphism among patients elucidated a high proportion of the CC genotype (106=76.2%). Meanwhile, the CT and TT genotypes were (19=13.7%) and (14=10.1%), respectively. While in control group, the CC genotype was 8 = 8% and the CT and TT genotypes were 22 = 22% and 70 = 70%, respectively.

Concerning the frequency of alleles among patients, the C allele represented 83.1% of alleles whereas the portion of T allele was 16.9%, while in control group the C allele represented 19% of alleles whereas the portion of T allele was 81% (table 2).

Table (3): Comparisons of laboratory investigations in different miRNA-30a rs1358379 Polymorphism genotypes among hemodialysis group.

Variables	miRNA-30a rs1358379 Polymorphism			p-value
	CC	CT	TT	
	Mean± SE	Mean± SE	Mean± SE	
ALT	88.8±2.2	100.3±2.9	79±5.6	0.1[a], 0.3[b] **0.01[c]***
AST	78.5±1.7	84.7±2.5	69.6±4.7	0.5[a], 0.2[b] **0.04[c]***
Urea	173.3±5.7	188.3±11.6	199.8±14.9	0.8[a], 032[b] 0.9[c]
Creatinine	3.82±0.08	3.75±0.16	3.8±0.29	0.9[a], 0.9[b] 0.9[c]
qRT-PCR for HBV (IU/ml)	**434666**.3± 214966	109961.4± 40787	47589.9± 20926	**0.03[a]*.** 0.9[b], 01[c]

a: statistical significance between mir-30a rs1358379 Polymorphism CC and CT
b: statistical significance between mir-30a rs1358379 Polymorphism CC and TT
c: statistical significance between mir-30a rs1358379 Polymorphism CT and TT

Although the laboratory investigations among the patients revealed high levels, only the mean levels of ALT and AST were statistically significantly higher in the CT genotype when compared to the TT genotype. Meanwhile, the CC genotype didn't show any statistically significant difference when compared to the CT and TT genotypes. As regards the mean level of PCR, the only statistically significant difference was displayed between CC and CT genotypes. Meanwhile, the other genotypes didn't show any statistically significant difference. On the other hand, the mean levels of urea and creatinine didn't show any statistically significant difference between the different genotypes (p-value >0.05) (table 3).

Table (4): Comparisons of sex, HBsAg and HBcAg in different miRNA-30a rs1358379 Polymorphism genotypes among hemodialysis group.

Variables		miRNA-30a rs1358379 Polymorphism genotypes			p-value
		CC	CT	TT	
		No. (%)	No. (%)	No. (%)	
Sex	Male	49(46.2%)	**14(73.7%)**	4(28.6%)	**0.03***
	Female	57(53.8%)	5(26.3%)	**10(71.4%)**	
HBsAg	-ve	94(88.7%)	17(89.5%)	14(100%)	0.4
	+ve	12(11.3%)	2(10.5%)	0(0%)	
HBcAg	-ve	47(44.3%)	8(42.1%)	7(50%)	0.9
	+ve	59(55.7%)	11(57.9%)	7(50%)	

*: statistical significance of the highlighted group of Polymorphism

Concerning sex, there was a statistically significant difference between different genotypes, with higher percentage of CT type among males versus TT type among females. Meanwhile, there was no statistical significant distinction between different genotypes as regards hepatitis B surface and core antigens (table 4) .

Table (5): Comparisons of variables in different study alleles of miRNA-30a rs1358379 Polymorphism among hemodialysis group.

Variables	C allele (N=231)			T allele (N=47)		
	No	Yes	p-value	No	Yes	p-value
	Mean ±SE	Mean ±SE		Mean ±SE	Mean ±SE	
ALT	79±5.6	90.6±1.9	0.06	88.8±2.2	91.3±3.4	0.6
AST	69.6±4.8	**79.5±1.5**	**0.04***	78.5±1.7	78.3±2.7	0.9
Urea	199.8±14.9	175.6±5.1	0.1	173.3±5.6	193.2±9.1	0.08
Creatinine	3.8±0.28	3.8±0.07	0.9	3.8±0.08	3.7±0.15	0.8
qRT-PCR (IU/ml)	47589.9± 20926.7	160986.5±49 33.4	**0.03***	109961.4± 40787.9	284136.6± 136990.2	0.1
Sex						
Male	4(6%)	63(94%)	0.2	49(73.1%)	18(26.9%)	0.4
Female	10(13.9%)	62(86.1%)		57(79.2%)	15(20.8%)	
HBsAg						
-ve	14(11.2%)	111(88.8%)	0.4	94(75.2%)	31(24.8%)	0.5
+ve	0(0%)	14(100%)		12(85.7%)	2(14.3%)	
HBcAg						
-ve	7(11.3%)	55(88.7%)	0.8	47(75.8%)	15(24.2%)	0.9
+ve	7(9.1%)	70(90.9%)		59(76.6%)	18(23.4%)	

There was a statistically significant difference in AST and PCR mean levels in different C alleles of miRNA-30a rs1358379 Polymorphism; as patients who had C-allele show high mean levels of AST and PCR. There was also no statistically significant difference as regards ALT, urea, creatinin, sex, HBsAg, and HBcAg. Meanwile, there was no statistical significant distinction in various investigations and sex as regards the presence of T allele (table 5).

Discussion:

The latency of occult yet manifest HBV-infection may extend to long periods of mild necro-inflammation that participate in developing hepatic cirrhosis and cancer. On the other hand, occult HBV may be oncogenic through integrating itself into the host-genome and maintaining active transcription that synthesize proteins with prospective proto-oncogenic features. Therefore, persons with negative HBsAg should be tested for HBV DNA to identify those at high risk for evolving hepatic cancer(14). HBV carriers may face a promoting hazard of deteriorating to cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC)(15,16). The poor understanding of occult HBV patterns may be related to unrecognizable mutated surface-protein, deleted pre-S1 region, reduced expression of HBsAg by mutated HBV regulatory region or mutated posttranslational proteins(17). Notable studies were accomplished to localize the tangible gene that predisposes to ESRD(18). To establish the desired gene, multiple studies have been conducted in assorted populations with paradoxical findings (19,20).

MiRNA-30 family members are implicated in pathogenesis of many renal diseases (10). A study was carried out in Saudi Arabia involving 1,352 HBV-infected patients and 600 healthy controls displayed that SNPs in various microRNA genes,

involving miRNA- 30a rs1358379, have been related to HBV infection(21).

Thence, this study aimed to elucidate if polymorphic variants in miRNA-30a gene is predominant in ESRD patients and whether polymorphisms of this gene is correlated with the receptivity to HBV infection.

Our study was conducted on 139 ESRD patients on regular hemodialysis, and 100 healthy age and sex matching controls. Only 14 patients were positive for HBsAg, whereas 125 were negative. Meantime, the mean level of quantitative real time-PCR (qRT-PCR) for HBV was (150677.7± 394934.2). Though the negativity of HBsAg, the concealed high levels of qRT-PCR, ALT and AST implied the serious lethal activity of cryptic HBV. Thus, a routine HBV-PCR for patients on hemodialysis should be a must.

Our results elucidated a higher proportion of the CC genotype of miRNA-30a rs1358379 SNP among patients (106=76.2%). While in control group, the CC genotype was 8 = 8%. Concerning the frequency of alleles among patients, the C allele represented 83.1% of alleles whereas the portion of T allele was 16.9%, while in control group the C allele represented 19% of alleles whereas the portion of T allele was 81%. Relying on the frequency of C allele and the percentage of CC genotype among patients and in comparison with the control group, we might consider the C allele to play a crucial role in the pathogenesis of ESRD.

Many previous studies detected that miR-30a rs1358379 SNP is associated with various cancers (22,23,24). But, there is deficiency of data to correlate rs1358379 SNP alleles or genotypes with susceptibility to HBV infection. Al-Qahtani et al. revealed that the C allele of miR-30a rs1358379 SNP was associated with susceptibility to HBV infection and the T allele was associated with an high ability of HBV-infected patients to clear the virus. On trial to reveal the influence of miR-30a rs1358379 on disease prognosis, it was reported that the rs1358379 C allele was related to increased risk of liver cirrhosis and hepatocellular carcinoma compared to T allele(21).

In the present study, the existence of the C allele was associated with increased mean levels of ALT and AST in both CC and CT genotypes in patients, while the latter aroused a statistically significant difference when compared to the TT genotype. Meanwhile, the CC genotype displayed a statistically significant higher mean level of the qRT-PCR for HBV when compared to the CT genotype in patients.

Concerning sex, the higher percentages that had statistically significant differences were revealed by the CT genotype among males versus the TT genotype amongst females in patients. Meantime, the mean levels of urea, creatinine and hepatitis B surface and core antigens didn't show any statistically significant difference between the different genotypes.

To our knowledge, there were no prior researches based on miR-30a rs1358379 SNP polymorphism that connect ESRD subjects with the susceptibility to HBV; this was the first comprehensive report.

In our study, the involvement of C allele declared a statistically significant difference in the mean levels of both AST and qRT-PCR for HBV in patients. On the contrary, the T allele was not accompanied by any significant difference. This could explain the ambiguous role of the C allele of miR-30a rs1358379 SNP in ESRD Egyptian

patients that might be responsible for the existence of HBV DNA with lack of exhibited hepatitis B surface antigen

MiR-30a rs1358379 polymorphism seems to be responsible for HBV-induced hepatic cirrhosis. The genotypic CC may have a crucial role in developing and progressing of hepatic fibrosis. These observations might be helpful in therapeutic collaboration.

Conclusion:

We found that the high percentage of C allele or CC genotype of miR-30a rs1358379 SNP in ESRD Egyptian patients might be responsible for the existence of HBV DNA with lack of exhibited hepatitis B surface antigen. We hope for further researches that could aid in establishing a direct genetic linkage in the future.

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Conflicts of Interest:

All authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in our study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standard.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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