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Heparanase gene rs12331678single nucleotide polymorphism increases risk of hepatocellular carcinoma in patients with HCV related liver cirrhosis

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ABSTRACT

Worldwide, HCC represents the sixth most common cancer and the third cause of cancer-related death. Heparanase activity is involved in cancer growth and development in humans and single nucleotide polymorphisms (SNPs) in the heparanase gene (HPSE) are associated with tumors. The studyaims to detect the association of SNPs rs12331678 in HPSE with the risk of HCC in patients with HCV-related cirrhosis. We found no significant association regarding HPSE rs12331678between the studied cirrhotic patients and controls. CA, AA genotypes, and A allele showed significant association with HCC when compared to the controls, with a higher risk to develop HCC. Also, CA, AA genotypes and A allele showed significant association with HCC when compared to cirrhotic patients, with a higher risk to develop HCC. HPSE SNP rs12331678 is more frequent in HCC patients than in cirrhotic patients or controls. This significant association is not related to the number, size of focal lesions, BCLC, or MELD score. In addition, the marked difference of HPSE SNP rs12331678 between HCCs and liver cirrhosis tissues suggests that its levels may function as a marker for the differential diagnosis of these disorders.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a universal problem and its epidemiological data showed variation from place to place. HCC represents the sixth most common cancer and the third most common cause of cancer-related death [1]. Egypt ranks third in Africa and 15th most populous country worldwide. In Egypt, the number of HCC patients increased two fold over a decade [1].

Hepatitis C virus (HCV) infection is a major cause of advanced hepatic fibrosis and cirrhosis, with a significantly increased risk for the development of HCC [2].

Despite well-established risk factors, the specific underlining pathogenesis of HCC remains unclear. Genetic and genomic alterations are common and may be associated with the development and progression of cancer including HCC [3].

HPSE is a kind of multifunctional extracellular hydrolase, which is up-regulated in almost all malignant tumors. HPSE promotes tumor growth, metastasis, and angiogenesis by degrading HSPGs and releasing vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) from the extracellular cytokines [4].

HPSE gene (HPSE-1) contains 14 exons and 13 introns. It is located on chromosome 4q21.3 and expressed as two mRNA [5]. Single nucleotide polymorphisms (SNPs) are the most common genetic variation in the human genome. [6].

Previous reports suggested that SNPs in HPSE are associated with various types of cancers, including ovarian carcinoma, hematological malignancies, and gastric cancer [7- 10].

The allele loss and reduced HPSE expression are closely correlated with tumor progression and poor prognosis in HCC patients. HPSE is a tumor suppressor gene based on the fact that tumor suppressor genes usually cause loss of heterozygosity (LOH) in carcinogenesis [9]. The role of HPSE in HCC is currently controversial.

1.1 Aim of the study

The study aims to detect the association of SNPs rs12331678 in HPSE with risk of HCC in patients with HCV-related cirrhosis.

2. Methods

Study Design: This is a case-control study that was conducted at Outpatient clinics and admission units at internal medicine department of Specialized Medical Hospital at Mansoura University and Shebin al-Kom Teaching Hospital.

Study Period: 2 years.

Study Population: This study included 50 Egyptian patients, from 18 to 60 years of both gender with post hepatitis C cirrhotic patients, 50 patients with HCC related to HCV, and 100 healthy individuals as a control group. Non-compliant patients, patients with a prior liver transplant, malignancies other than HCC, presence of severe co-morbidity such as (Hypertension, Diabetes, chronic kidney disease, and Ischemic heart diseases), Other causes of cirrhosis as (Chronic Hepatitis B virus(HBV) and human immunodeficiency virus(HIV), Alcoholic cirrhosis, Cholestatic liver cirrhosis, autoimmune liver cirrhosis, Pediatric congenital liver diseases, and Metabolic liver diseases), Budd-Chiari syndrome, other vascular diseases, Drug-induced liver failure, and Acute fulminant liver failure were excluded from the study.

Sampling Method: Simple random sampling

Sample Size: 200 subjects.

Study Procedures: The EDTA blood sample of patients and controls were subjected to genomic DNA extraction using gene get purification kits (Thermo scientific). The Nanodrop 2000 was used for estimation

of the concentration and purity of DNA. The genomic extracted DNA was amplified by PCR then the amplified products were digested with the appropriate restriction enzyme (RsaI, Thermo scientific, USA) for HPSE polymorphism by the technique of PCR-RFLP, the digestion products were subjected to gel electrophoresis and visualized under UV transilluminator for detection of different genotypes of HPSE gene polymorphism. The sequence of bases in rs12331678:

Primer forward:5-CTATAGTATTTCCTACATTATAGAGTTTGGA-3
Primerreverse: 5-TGGATTAGGCAATGGTCATCA-3

Picture of gel electrophoresis for detection of different polymorphisms in our SNP: two photos added
AA□→ 240bp(PCR)
CC□→ 209 + 31 bp
AC□→ 240 + 209 + 31 bp

Study Interventions: All selected individuals have been subjected to the following: Full medical history taking. Full clinical examination, complete blood count, serum creatinine, serum bilirubin, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum albumin and International Normalized Ratio (INR), viral marker as; HCV Ab by ELISA technique, hepatitis B exclusion by (HBsAg) and (HBcIgG), serum alpha fetoprotein (AFP), Abdominal and pelvic ultrasound, Triphasic computed topography scan of the abdomen. Laboratory data were collected from the patient sheet. All patients were classified according to Child-Pugh score(CTP), Barcelona clinic liver cancer staging(BCLC),and model for end stage liver disease(MELD).

Specific Laboratory tests:
heparinase gene SNPs(rs12331678)2 ml of blood was delivered into EDTA tubes and stored frozen at -20ºC till work for DNA extraction and rs12331678 single nucleotide gene polymorphism

Statistics
The collected data was revised, coded, tabulated using Statistical Package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. Student T-Test was used to assess the statistical significance of the difference between two study group means. Mann Whitney Test (U test) was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. The Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Hardy–Weinberg equilibrium (HWE): Deviations from Hardy–Weinberg equilibrium expectations were determined using the chi-squared test. Polymorphisms and genotype frequencies were evaluated by gene counts. The data were tested for the goodness of fit between the observed and expected genotype frequencies. HWE indicates that the selected groups of study are reasonable for performing genetic analysis of this SNP. Regression analysis: Logistic and ordinal regression analyses were used for the prediction of risk factors, using generalized linear models.

An odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure.

The 95 % confidence interval (CI) is used to estimate the precision of the OR. A large CI indicates a low level

of precision of the OR, whereas a small CI indicates a higher precision of the OR.

Probability of results: A *p*-value is considered significant if <0.05 at confidence interval 95%.

3. Results

Table (1): Demographic data of the studied groups.

		Control	**LC**	**HCC**	**P1**	**P2**	**P3**
		N=100	N=50	N=50			
Age (years)	mean±SD	58.8±1.2	58.5±1.3	58.9±1.2	0.210	0.745	0.172
Males	N, %	(46)46%	(26)52%	(25)50%	0.488	0.644	0.841
Females	N, %	(54)54%	(24)48%	(25)50%			

SD, standard deviation; P1, comparison between control and LC; P2, comparison between control and HCC; P3, comparison between LC and HCC.

The mean age of the HCC group was 58.9 years and 58.5 years for the LC group. The study included 100 healthy control subjects of matched age and gender (p>0.05 for each).

Table (2): Comparison of laboratory data of the studied groups.

		LC	**HCC**	**P**
		N=50	N=50	
Bilirubin (mg/dL)	Median, range	2.1(1-2.7)	2.2(1.2-5.4)	0.008
Creatinine (mg/dL)	mean±SD	1.1±0.3	1.3±0.2	0.102
AFP (ng/mL)	Median, range	46(10-95)	136(25-298)	<0.001
ALT (U/L)	mean±SD	50.7±14.7	61.7 ±15.7	0.001
AST (U/L)	mean±SD	49.8 ±15.2	62.2 ±15.7	<0.001
Albumin (g/dL)	mean±SD	3.3 ±0.4	2.9 ±0.2	<0.001
INR	mean±SD	1.4 ±0.2	1.8 ±0.3	<0.001
Hemoglobin (g/dL)	mean±SD	12.1 ±1.1	10 ±0.9	<0.001
Platelets (X109 /L)	mean±SD	225.1 ±57.0	341.9 ±108.3	<0.001
TLC (X109 /L)	mean±SD	7 ±1.6	9 ±2.9	<0.001

ALT: Alanine aminotransferaseAST: Aspartate aminotransferaseTLC: Total leukocytic court INR: International Normalized Ratio

The HCC group showed significantly higher bilirubin, AFP, ALT, AST, INR, platelets, significantly lower albumin and hemoglobin when compared to liver cirrhosis (LC) cases. Creatinine did not differ significantly between both groups.

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Regarding HCC cases, 74% had PVT, 26% had metastasis, 16% had BCLC grade B, 26% had BCLC grade C and 58% had BCLC grade D. Mean number of HFL was 3.9, mean size of HFL was 5 cm.

Table (3): Distribution of HPSE (genotypes and alleles) in the cirrhotic patients and healthy control subjects.

HPSE		**Control N=100**		**LC N=50**		***P***	**OR**	**95% CI**	
		N	**%**	**N**	**%**				
Genotypes	***CC***	77	77	36	72	-	**1**	**Reference**	
	CA	22	22	11	22	0.873	1.042	0.630	1.724
	AA	1	1	3	6	0.098	3.146	0.810	12.219
	CA+AA	23	23	14	28	0.505	1.176	0.730	1.893
Alleles	***C***	176	88	83	83	-	**1**	**Reference**	
	A	24	12	17	17	0.240	1.285	0.846	1.952

OR, odds ratio; CI, confidence interval.

In table 3, we found no significant association was found regarding HPSE (genotypes and alleles) between the studied LC patients and healthy controls.

Table (4): Distribution of HPSE (genotypes and alleles) with HCC patients and healthy control subjects.

HPSE		**Control N=100**		**HCC N=50**		***P***	**OR**	**95% CI**	
		N	**%**	**N**	**%**				
Genotypes	***CC***	77	77	17	34	-	**1**	**Reference**	
	CA	22	22	21	42	**0.008**	1.452	1.103	1.910
	AA	1	1	12	24	**<0.001**	2.090	1.450	3.013
	CA+AA	23	23	33	66	**<0.001**	1.600	1.249	2.050
Alleles	***C***	176	88	55	55	-	**1**	**Reference**	
	A	24	12	45	45	**0.009**	1.334	1.074	1.658

OR, odds ratio; CI, confidence interval.

In contrast, when we compared the genotype distribution between HCC patients and healthy controls, the CA, AA, CA+AA genotypes and A allele showed significantly higher association with HCC with a higher risk to develop HCC.

Table (5): Distribution of HPSE (genotypes and alleles) in HCC and cirrhotic patients.

HPSE		**LC N=50**		**HCC N=50**		***P***	**OR**	**95% CI**	
		N	**%**	**N**	**%**				
Genotypes	***CC***	36	72	17	34	-	**1**	**Reference**	
	CA	11	22	21	42	**0.003**	2.382	1.349	4.206
	AA	3	6	12	24	**0.001**	3.696	1.654	8.256

	CA+AA	14	28	33	66	**<0.001**	2.708	1.617	4.533
Alleles	***C***	83	83	55	55	**-**	**1**	**Reference**	
	A	17	17	45	45	**<0.001**	2.357	1.588	3.498

OR, odds ratio; CI, confidence interval.

Table 5 shows the Distribution of HPSE (genotypes and alleles) in HCC and cirrhotic patients where the A allele is highly expressed in the HCC group compared to the cirrhotic group.

Table (6). Comparison between cirrhotic group and HCC acording to genotype and allel in different child, MELD score and BCLC staging

			LC				**HCC**			
			CC **N=36**	CA **N=11**	AA **N=3**	*P*	CC **N=17**	CA **N=21**	AA **N=12**	*p*
CTP	**A**	**N** **%**	6 16.7%	1 9.1%	0 0%	0.631	-	-	-	
	B	**N** **%**	30 83.3%	10 90.9%	3 100%		9 52.9%	10 47.6%	3 25%	0.298
	C	**N** **%**	-	-	-	-	8 47.1%	11 52.4%	9 75%	
MELD		**Mean ±SD**	13.6 ±2.3	14.1 ±2.5	13 ±2.6	0.745	18.2 ±2.9	17.9 ±3.2	19.2 ±3.5	0.569
BCLC	**B**	**N** **%**					2 11.8%	5 23.8%	1 8.3%	0.222
	C	**N** **%**					7 41.2%	5 23.8%	1 8.3%	
	D	**N** **%**					8 47.1%	11 52.4%	10 83.3%	

CTP: Child-Pugh score MELD:Model for end stage liver diseaseBCLC: Barcelona clinic liver cancer staging

No significant difference were found regarding HBSE genotype and alleles with child classes and MELD score in HCC and LC groups. Also, BCLC staging in HCC group.

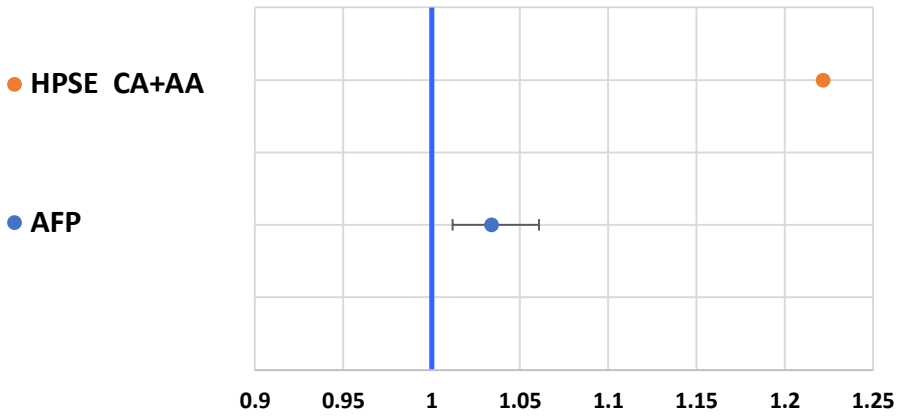

Figure (1): Forest plot of multivariable analysis for prediction of HCC susceptibility

Multivariable analysis for prediction of HCC susceptibility was shown in figure 2 where Higher AFP, presence of HPSE CA or AA genotypes were suggested being independent predictors of HCC risk in multivariate analyses.

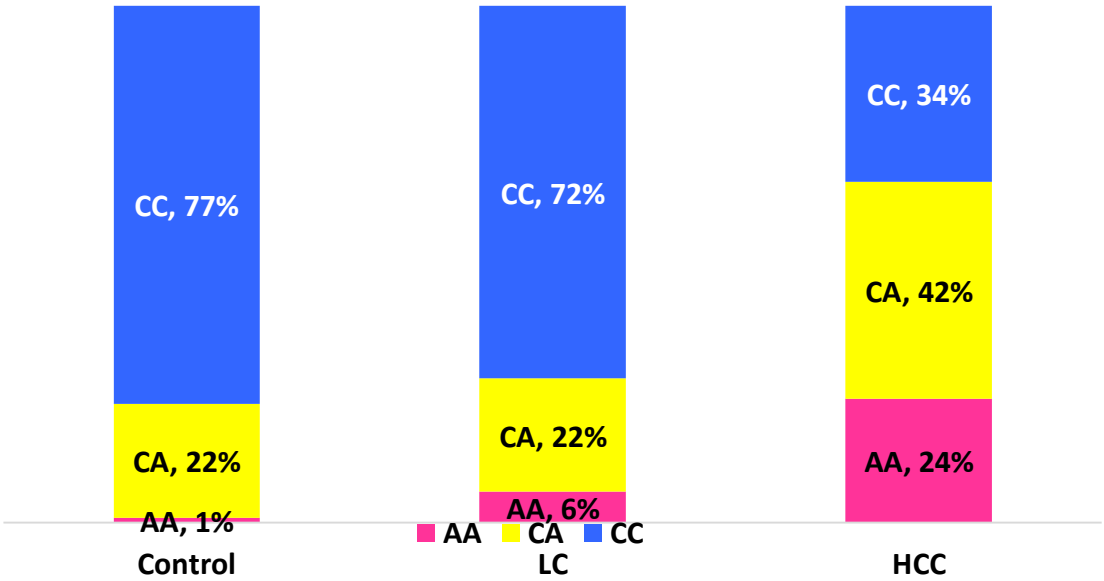

Figure (2): HPSE genotypes in studied LC, HCC patients and healthy control subjects.

Considering CC genotype as a reference CA, AA, CA+AA genotypes and A allel showed signicantly higher frequency associated with HCC when compared to control subject and LC group, with risk to develop HCC.

4. Discussion

The present study was conducted on 50 liver cirrhosis and 50 HCC cases. The mean age of the HCC group was 58.9 years compared to 58.5 years in the cirrhotic group. The mean age of patients in our study was comparable to a study conducted by [11] who found that the cases suffering from proved HCC and cirrhosis with a mean age of 57.1±6.8 years old and the cases suffering from cirrhosis only with a mean age of 54.8±7.6 years old. This mean age is lower than the mean age in the United States, it has been found that the mean age at diagnosis among women is 65-69 years, whereas the mean age among men is between 60 and 64 years

[12]. Also, [13] detected that HCC was common in persons of an average age of nearly 64 years.

It has been reported that the occurrence of HCC at a younger age among the Egyptians can be due to early risk factors exposure and the HCV infection endemicity. Furthermore, in Egypt, other environmental factors may have a significant role in the injury of the liver including the aflatoxin presence in numerous stuff of food, bilharziasis that presented in approximately 40% of patients and contamination of food with insecticides [14].

In the present study, we found no significant differences regarding age, gender, and family history between the studied groups.

In agreement with our results, [15] analyzed forty-five patients, with HCV infection. Patients were divided into 2 groups, the first group consisted of 21 patients with histologically proven HCC and the second one consisted of 24 patients with liver cirrhosis without HCC. There was no statistically significant difference in the structure of the compared groups of patients as regards ageand sex. Also, [16] conducted a study included 480 healthy controls and 400 incident cases suffering from HCC, they found that The 2 groups had no significant difference according to drinking status, age, and pack-years of smoking.

Also, [17] found no significant differences between control and the cases suffering from HCC in terms of age and gender.

As regard laboratory data in our study, we found that the HCC group showed significantly higher bilirubin, AFP, ALT, AST, INR, platelets, significantly lower albumin and hemoglobin when compared to LC cases. This was parallel to [18], who detected that cirrhosis has been found in 80–90% of cases suffering from HCC. The accumulated risk at 5 years for HCC development in the patients suffering from cirrhosis ranges from 5 to 30% based on the cirrhosis etiology, ethnic group, stage, and the region of cirrhosis. The HCC risk of development augments in the cases suffering from not controlled LC [19].

Significant correlation between HCC with high AST compared with the group having LC shows a vital significance that HCC is commonly related to high fibrosis degree because AST is included in most of the scoring systems of fibrosis like FIB-4 index, AST-platelet ratio index, and Forns index and therefore, logically, high AST levels lead to increasing these scores reflecting high fibrosis degree.

In line with our results [11] found that AFP had a significant difference between the two groups with a median value greater in the group suffering from HCC (25 ng/ml in the control group vs 305 ng/ml than in the group suffering from HCC; $p < 0.001$). Also, [20] found a statistically significant association of HCC with high AST, hypoalbuminemia, increased bilirubin, and prolonged prothrombin time.

Also, [21] found that in patients suffering from HCC, the levels of AFP was higher than in cirrhotic patients, total bilirubin, and INR in HCC patients were higher ($p < 0.0001$) than in patients suffering from LC while albumin and platelets counts in HCC patients were lower than in patients suffering from LC.

In the present study, considering the CC genotype as a reference guide by [16] we found no significant association regarding HPSE (genotypes and alleles) between studied LC patients and healthy control subjects [8]. Also, it is essential to declare that CA, AA, CA+AA genotypes, and A allele showed significantly higher association in patients with HCC when compared to the control subjects, with ahigher risk to develop HCC that suggesting that the heparanase gene has a significant role in the late carcinogenesis steps.

In the present study, we found that CA, AA, CA+AA genotypes, and A allele showed significantly higher association in patients with HCC when compared to LC, with a higher risk to develop HCC. Additionally, it is important to mention that these findings confirmed the preliminary hypothesis that the HPSE gene may participate in the HCC pathogenesis.

In agreement with our results, [16] found an increased A allele frequency for rs12331678 in subjects suffering from HCC [11]. The frequency of the A allele was significantly greater in the group including the cases suffering from HCC than in the group including the controls (0.11 vs. 0.074, p = 0.006). In addition, the rs12503843-T allele could be a risk factor for HCC development. [22] reported that HPSE was associated with metastasis and poor prognosis of patients suffering from HCC.

Contrarily, [17] found that the rs12331678 genotype distributions were 1.4% AA, 31.4% CA, and 67.1% CC. The C and A alleles showed no significant difference between HCC and control [11].

In the present study, we found no significant differences regarding HPSE genotypes with FH in CTP classes and MELD score and laboratory data in the LC group and HCC group. In agreement with our results, [17] found that no significant correlation has been detected between the rs12331678 and rs12503843 of HPSE and all clinic-pathologic status and laboratory markers (AST,AFP) [11].

We found no significant association between HPSE genotypes and PVT, metastasis, BCLC grades, number, or size of focal hepatic lesions (FHL).

Logistic regression analysis was conducted for HCC prediction susceptibility, using FH, AFP, and HPSE genotypes as confounders. Higher AFP, presence of HPSE CA or AA genotypes was suggested to be independent predictors of HCC risk in uni and multivariable analyses. [9] suggested that HPSE protein was an independent predictor of overall survival in HCC patients. ALSO, [23] found that AFP elevation is a risk factor for developing HCC and is used to help define the at-risk populations [24] found that levels of AFP, platelets, and ALT, along with age, which increased the predictive value for identifying patients with HCV-associated cirrhosis likely to develop HCC within 6 months.

The disparity between our results and the previous finding may be explained by differences in the genetic effect among ethnic groups, e.g., population differences in the LD pattern, or allele frequencies of HPSE. For example, there may be a small, population-specific effect of HPSE rs4693608 on the development of HCC. Indeed, the allelic and genotypic frequencies of HPSE rs4693608 vary with ethnicity.

5. Conclusion

HPSE SNP rs12331678 is more frequent in HCC patients more than patients with liver cirrhosis or controls. However, this significant association is not related to the number, size of focal lesions, BCLC or MELD score. We hypothesize that heparinase SNP rs12331678 is involved in the pathogenesis of HCC. In addition, the marked difference of HPSE SNP rs12331678 patients with HCCs and those with liver cirrhosis suggests that its levels may function as a marker for the differential diagnosis of these disorders.

List of abbreviations

Single nucleotide polymorphisms (SNPs)Heparanase gene (HPSE) Hepatocellular carcinoma (HCC)Hepatitis C virus (HCV)Vascular endothelial growth factor (VEGF)basic fibroblast growth factor(bFGF) loss of heterozygosity (LOH)Hepatitis B virus(HBV)Human immunodeficiency virus(HIV)

Aspartate aminotransferase (AST)
Alanine aminotransferase (ALT)
International Normalized Ratio (INR)Alpha fetoprotein (AFP)Child-Pugh score(CTP)Barcelona clinic liver cancer staging(BCLC)Model for end stage liver disease(MELD)Mansoura medical ethics Committee (MMEC) Hardy–Weinberg equilibrium (HWE)Odds ratio (OR) Liver cirrhosis (LC) Focal hepatic lesions (FHL)

Declarations
Ethics
Written consents from patient sand controls who participated in the study or from their families were obtained and approved by the Mansoura medical ethics Committee (MMEC) of the faculty of medicine.

Availability of data and material: All data generated or analyzed during this study are included in this published article [and its supplementary information files] and readily available for share.

Conflict of interest: The authors declare that they have no conflict of interest.

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Authors' contributions
The protocol of the study, study design, methodology, follow up the patients, collecting data, data analysis, and writing - original draft preparation:,AMS,MEAK and HH; Lab investigation: HAT; Writing - review and editing: AS. Supervision: AO and AS.

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