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ORIGINAL ARTICLE

Role of Interleukin-28 B Gene Polymorphism and Cytomegalovirus Coinfection in Hepatocellular Carcinoma Patients with Chronic Hepatitis C Virus

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ABSTRACT

Background: *Liver diseases including HCC are life-threatening morbidities. Mechanisms of HCC are still unclear. Cytomegalovirus (CMV) is DNA virus characterized by latency and cellular transformation. Genetic polymorphisms especially SNPs are the most common genetic variation. It plays a critical role in regulation of cellular interaction and cytokine production. CMV infection and its role in HCC is under investigation.* ***Objectives:*** *to profile IL28B single-nucleotide polymorphism in development of HCC in chronic HCV, and identify the relationship between human cytomegalovirus infection, IL28B SNPs and HCC.* ***Methodology:*** *120 blood samples were obtained from HCV patients; divided according to clinical, radiological and laboratory data into three equal groups, forty patients each: HCV, HCC, LC groups, and 40 normal persons as controls. SNPs of interleukin-28 (IL28B rs12979860) by RFLP and CMV viral DNA by real-time polymerase chain reaction were investigated.* ***Results:*** *IL28B /CC genotype was increased significantly in controls and TT genotype in HCC group. A positive correlation among HCV viral load, CMV with the genotype IL28B rs12979860/TT especially in HCC group.* ***Conclusions:*** *IL28B rs12979860/TT is expressed in HCC patients making its role in development of HCC in CMV positive HCV patients cannot be denied.*

INTRODUCTION

Hepatic malignancy; namely HCC is the primary hepatic malignancy with the second major number of deaths all over the world[1]. HCV and human cytomegalovirus (CMV) are prominent viruses that establish a persistent latent infection with alterations in Natural Killer cells[2]. CMV is *Beta herpes virinae* subfamily member that causes opportunistic infection among immunocompromised patients with increased morbidity and mortality[3]. CMV coinfection in the occurrence of HCC, and in incidence of hepatic fibrosis is not clear. Impairment of the cytokine system is an effective viral method to evade the immune surveillance[4].

IFNL3 encodes IFN lambda-3 (IFN-λ3), also known as interleukin 28B, which belongs to the type III IFN-λ family consisting of IL29/IFNL1, IL28A/IFNL2, IL28B/IFNL3, and newly discovered IFNL4[5]. IL28B is secreted by immune cells and macrophages have potent antiviral role by stimulating the formation of interferon-stimulated genes (ISGs) and ISGF3 complex (IRF9, STAT1, and STAT2) with efficient antiviral response [6].

IL28B gene SNPs may change sequence of action of HCV as it have been associated with natural and treatment-induced clearance of HCV[7], fibrosis progression in chronic HCV infection and severity of HCV recurrence after liver transplantation [8]. The association between *IL28B* rs12979860 polymorphism and the expansion of HCC has been investigated with inconclusive and inconsistent results[9]. A link between IL28B rs12979860 SNP and the initiation of HCV-related HCC is suggested[10]. The aim of our work is to explore the role of IL28B rs12979860 polymorphism in HCV induced HCC and CMV infection effect in HCC development.

METHODOLOGY

Ethical considerations

The study procedures were approved by the Institutional Ethical Committee of National Liver Institute, Menoufia University, as mentioned in Declaration of Helsinki as a statement of ethical principles. This study was performed in the National Liver Institute, Menoufia University from May 2018 to December 2019 on liver disease patients. They were grouped into 3 groups each contains 40 patients: **Group 1**: Chronic hepatitis C group **Group 2**: Liver cirrhosis group and **Group 3**: Hepatocellular carcinoma group.

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In addition to a fourth group of apparently healthy, age and sex matched as Control group.

All patients were subjected to: History taking, full Clinical examination. Triphasic abdominal Computed Tomography (CT) and Laboratory Investigations were done.

***Blood sampling*:**

The blood sample was divided into vacutainer tubes and EDITA containing tube for CBC. Serum was separated by centrifugation (**Kaida centrifuge, China)** at 3000 rpm for 10 minutes [11] and used for: Liver, kidney profiles: liver enzymes, serum albumin, total bilirubin, urea, creatinine and random blood sugar. HCV RNA and HBV DNA were detected by Taqman, Real-Time PCR System (ROCHE, USA). Also, Anti HCV, HBV serological markers (HBsAg and anti HBc) were done using electro chemiluminescence immunoassay "ELIZA" using **Cobas 6000 (ROCHE, Germany).**

Detection of cytomegalovirus DNA: It was done by PCR QIAGEN Real-Time PCR system **(QIAGEN, GmbH);** Genomic CMV-DNA was obtained according to manufacturer's instruction**.** The thermal cycling protocol was as follows: 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles using Biometra T1 thermal cycler (Biometra, Germany), then the nested PCR product was analyzed on agarose-gel electrophoresis. Results were positively documented with a visible 100-bp product [12].

- CMV Primers:
  CMV1:
  5'GAGGACAACG AAATCCTGTTGGGCA3';
  CMV2:
  5'GTCGACGGTGGAGATACTGCTGAGG3';
  CMV3:
  5'ACCACCGCACTGAGGAATGTCAG3';
  CMV4: 5TCAATCATGCGTTTGAAGAGGTA3'.

Detection of IL28B single-nucleotide polymorphism by PCR:

By polymerase chain reaction-based restriction fragment length polymorphism assay (PCR-RFLP) assay[13] in the following steps:

- ***DNA extraction*:** Genomic DNA was extracted from whole blood samples by means of the Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, USA) according to manufacturer's instruction.
- ***PCR* by** QIAGEN REAL-Time PCR system (QIAGEN, GmbH) Using A 139 base pair (bp) product with:
  5`- CCAGGGCCCCTAACCTCT-GCA-3` and
  5`-GGGAGCGCGGAGT-GCAATTCA - 3`,
  by the NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
- ***Restriction fragment length polymorphism (RFLP) analysis for IL28B genotypes:***
  Ten ul of the amplicons were digested with BstU-I restriction endonuclease. Electrophoresis on 4% agarose gel was performed. A band of 139 bp illustrates the TT genotype on the gel, 109 bp + 30 bp that seen on the gel indicates the CC genotype, and the invisible (139 bp + 109 bp+ 30 bp) revealed the CT genotype[13].

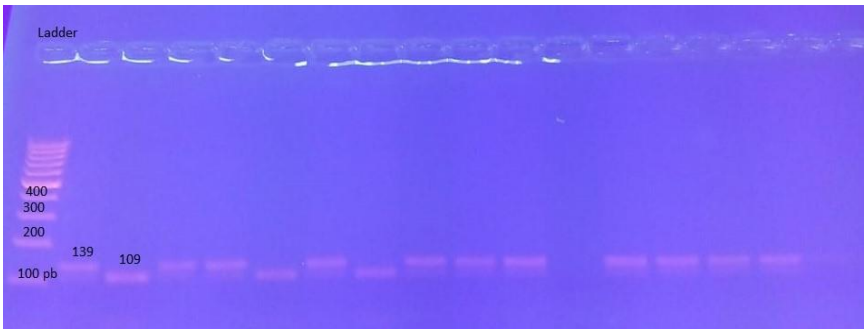

Fig. 1: RFLP of IL28B rs12979860 illusterated as TT genotype in HCC patients at 139 bp and CC genotype in control group at 109bp

Statistical analysis:

Data were collected, tabulated, and analyzed by SPSS (statistical package for social science) version 20 on IBM compatible computer

RESULTS

This was a case- control study done in the National Liver Institute, Menoufia University. It involved 120 chronic HCV cases and 40 healthy volunteers (control group). The patient groups were 120 patients (79 males and 41 females) with mean age of 53.3±6.44 years which was not significant rural distribution (P ≤0.05) (table 1).

Table 1: Demographic data among the studied groups

	The studied groups				Test	P value
	Group A (CHC) N = 40	**Group B (LC) N =40**	**Group C (HCC) N = 40**	**Group D (Control) N = 40**		
Age (year)					F	
X ±SD	56.2±6.02	55.65±5.24	53.65±7.03	52.93±6.35	2.56	0.06
Range	45 – 63	40 – 65	42 – 68	40 – 64		
Sex						
Male	25 (62.5)	23 (57.5)	31 (77.5)	23 (57.5)	4.65	0.20
Female	15 (37.5)	17 (42.5)	9 (22.5)	17 (42.5)		
Residence						
Rural	27 (67.5)	34 (85.0)	32 (80.0)	24 (60.0)	7.98	0.046*
Urban	13 (32.5)	6 (15.0)	8 (20.0)	16 (40.0)		
Smoking						
Yes	10 (25.0)	13 (32.5)	14 (35.0)	8 (20.0)	2.81	0.42
No	30 (75.0)	27 (67.5)	26 (65.0)	32 (80.0)		

F = F of ANOVA, qualitative data were compared by Chi square test

Clinical characters of the studied patients:

CT/TT genotypes of IL28B was significantly increased in hepatic cancer patients with Child C classification with moderate ascites with hematemesis and hypertensive and on Child B classification. Non-HCC patients showed high level of CC genotype of IL 28 in those with no comorbid conditions, Child A, fibrosis stage 1-2, no hematemesis, no encephalopathy, no ascites.

IL28B rs12979860 polymorphism distribution among the studied groups:

Table 2 shows that; IL28B allele C with (CC) genotype was highly significant (P<0.005) predominance in the CHC and control groups. In HCC group, TT genotype of IL28B and T allele is highly significantly prevalent (22 (55.0%)) in comparison to control, CHC group and LC group. CHC group shows the highest significant expression of (CT) genotype (20 (50.0%)) in relation to other studied groups. The LC group showed non-significant increase in both CT /TT genotypes (16 (40.0%)) with predominant T allele.

Table 2: Distribution of IL28B genotypes and alleles among the studied groups:

	The studied groups				Test	P value
	Group A (CHC) N = 40	**Group B (LC) N =40**	**Group C (HCC) N = 40**	**Group D (Control) N = 40**		
IL28B genotypes						
CC	14 (35.0)	9 (22.5)	4 (10.0)	16 (40.0)	20.53	0.002*
CT	20 (50.0)	15 (37.5)	14 (35.0)	14 (35.0)		
TT	6 (15.0)	16 (40.0)	22 (55.0)	10 (25.0)		
IL28B alleles						
C	48 (60.0)	33 (41.2)	22 (27.5)	46 (57.5)	22.24	<0.001*
T	32 (40.0)	47 (58.8)	58 (72.5)	34 (42.5)		

CMV infection among the studied population:

Cytomegalovirus infection was significantly higher (P<0.05) (37.5%) in HCC group compared with control (0%) and CHC group (15%) while no significant increase in CMV infection in HCC group in relation to liver cirrhosis group as shown in figure 2.

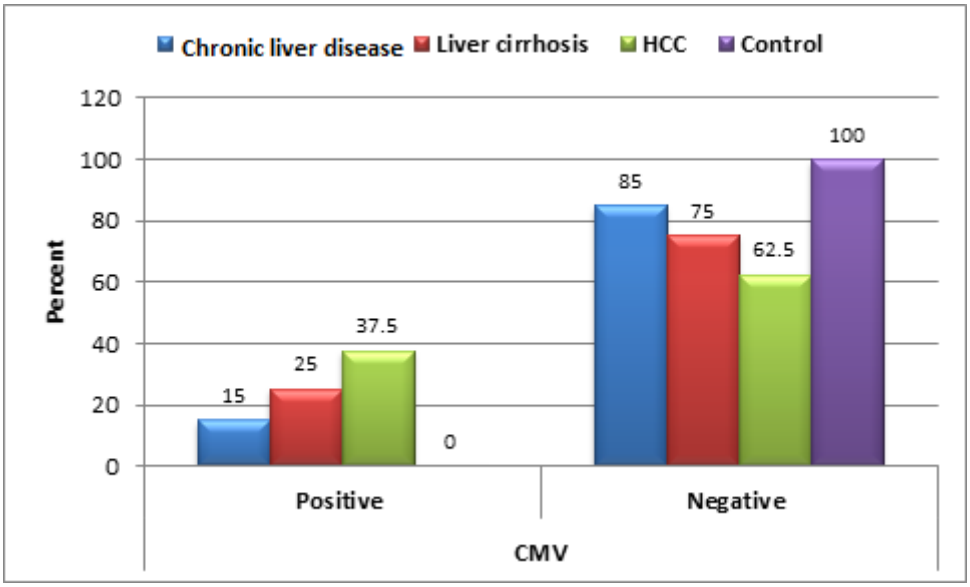

Fig. 2: CMV infection among the studied population.

IL28B genotypes and alleles among CMV infected HCC patients:

In Table 3**;** In HCC group, CMV positive cases shows non-significant elevated expression of homozygous TT genotype (71.4%). An increased level of CT/TT genotypes is seen in CMV negative HCC cases. CMV positive HCC shows non- significant increase in TT genotype distribution than non -HCC while CMV negative HCC shows equal distribution of both TT and CT IL28B genotypes with predominant T allele. CMV negative non-HCC (LC &CHC) shows IL28B CT /C allele genotypic predominance which was non-significant.

Table 3: Interleukin 28B genotypes and alleles in relation to CMV infection among the HCC and non- HCC cases

	HCC group		Test P value	Non -HCC group (CHC +LC)		Test P value
	CMV positive N = 14	CMV negative N =26		CMV positive N = 16	CMV negative N =64	
IL28B genotypes			X^2			X^2
CC	2 (14.3%)	2 (7.7%)	4.09	2 (12.5%	22 (34.4%	3.97
CT	2 (14.3%)	12 (46.2%)	0.13	4 (25.0%	24 (37.5%	0.14
TT	10 (71.4%)	12 (46.2%)		10 (62.5%	18 (28.1%	
IL28B alleles			X^2			X^2
C	6 (21.4%)	16 (30.8%)	0.25	14 (43.8%	68 (53.1%	0.90
T	22 (78.6%)	36 (69.2%)	0.61	18 (56.2%	60 (46.9%	0.34

X^2 = Chi square test

HCV viraemia in HCC:

Sever HCV viraemia-HCC patients showed significant increase in IL28B TT genotype than those with moderate and low viraemia, also in Non -HCC while CT genotype prevails among HCC and Non -HCC patients with moderate viraemia as in table 4.

Table 4: Relation between HCV viral load and IL28B genotypes among the HCC and non- HCC cases:

IL28B		viral load			X^2	P. value
		low	moderate	severe		
HCC	CC	10(83.3%)	2(10.0%)	0(0.0%)	12.48	0.014*
	CT	2 (16.7%)	10(50.0%)	2(25.0%)		
	TT	0(0.0%)	8(40.0%)	6(75.0%)		
Non-HCC	CC	2 (33.3%)	10(22.7%)	6(20.0%)	1.998	0.736
	CT	2(33.3%)	20(45.5%)	10(33.3%)		
	TT	2(33.3%)	14(31.8%)	14(46.7%)		

IL28B rs12979860 effect on Hepatocarcinogenesis:

Odds ratios (ORs) and 95% confidence intervals (CIs) of IL28B rs12979860 polymorphism was presented in table 5. In healthy control CC/CT genotypes and C allele is on significantly elevated than in HCC patients (OR = 4.0, CI = 0.71 – 22.51, P> 0.05). In HCC group, TT genotype and T allele is highly significantly elevated than in healthy controls (OR = 8.8, CI = 1.61 – 48.23, P <0.05).

Table 5: Multivariable Logistic regression of IL28B rs12979860 polymorphism in cases (HCC on top of chronic HCV-infected patients) versus controls:

	The studied groups		Test	P value	Odds ratio	95% CI
	Group D (Control)* N = 40	Group C (HCC) N = 40				
IL28B genotypes						
CC	16 (40.0%)	4 (10.0%)	NA	NA	(1)	
CT	14 (35.0%)	14 (35.0%)	2.70	0.14	4.0	0.71 -22.51
TT	10 (25.0%)	22 (55.0%)	7.43	**0.008***	8.8	1.61 –48.23
IL28B alleles						
C	23 (57.5%)	11 (27.5%)	NA	NA	(1)	
T	17 (42.5%)	29 (72.5%)	7.37	**0.01***	3.57	1.4 – 9.09

Odds ratio were constructed considering rs12979860-CC genotypes as reference.
OR: Odds ratio; Ref = reference category, * = reference group, CI = Confidence interval

IL28B rs12979860 relation to early detection of HCC:

Multivariable Logistic regression of IL28B rs12979860 polymorphism in cases of HCC versus CHC cases was demonstrated in table 6; that CT genotype is non-significantly elevated in CHC than HCC group (OR = 2.45, CI = 0.44 – 13.59, P >0.05). TT genotype and T allele is significantly elevated in HCC cases in contrast to CHC cases (OR = 12.83, CI = 2.15 – 76.45, P <0.05).

Table 6: Comparison of IL28B genotypes and allele between patients with CHC and HCC

	The studied groups		Test	P value	Odds ratio	95% CI
	Group A (CHC)* N = 40	Group C(HCC) N = 40				
IL28B genotypes						
CC	14 (35.0)	4 (10.0)	---	---	Ref (1)	
CT	20 (50.0)	14 (35.0)	1.09	0.44	2.45	0.44 – 13.59
TT	6 (15.0)	22 (55.0)	9.41	0.004*	12.83	2.15 – 76.45
IL28B alleles						
C	48 (60.0)	22 (27.5)	----	----	Ref (1)	
T	32 (40.0)	58 (72.5)	11.27	0.001*	3.95	1.73 – 9.03

DISCUSSION

HCV infection is a prevalent health problem throughout the world[14]. Chronic HCV infection may lead to LC which progress to HCC[9]. our goal was to characterize IL28B genetic polymorphism in HCV induced liver cancer cases and if co infection with cytomegalovirus share in carcinogenesis process.

In this study, 31 (77.5%) from forty patients with HCC were males with mean age of 53.3±6.44 years which is not significantly different from other groups. Thirty-Two (80.0%) of HCC patients were significantly rural resident with comorbid diabetes mellitus. They developed HCC on top of chronic HCV liver disease. Those results was in agreement with other studies [15].

Current study illustrated that, IL28B rs12979860 allele C with (CC) genotype (CC 40.0%), CT (35.0%) and (25.0%) TT showed highly significant (P<0.005) predominance in Control and CHC groups. This finding is with agree with some investigators[16,17].

Our study revealed that, HCC group, TT genotype and T allele is highly significantly prevalent (TT 22 of 40 HCC cases (55.0%)) in comparison to control, CHC group and Liver cirrhosis group. Some investigators agreed with our results as he informed that rs12979860 polymorphism to be significantly associated with HCC risk in Caucasians[18]. Other studies emphasize these results[9],[19]. Some authors found that IL28B rs12979860 CC and rs8099917 TT genotypes were predominant in patients with chronic HCV infection in Tianjin, China[20]. Such results indicate that the presence of the T/T genotype may be associated with HCV persistence. Since the IL-28B gene encodes IFN-λ3, which participates in antiviral responses and the inflammatory process and since allelic variants of the IL-28B polymorphism may affect the efficiency of the inflammatory process, chronic hepatitis patients with the T/T genotype of IL-28B polymorphism may suffer more injury to liver cells, which could lead to cirrhosis or HCC [21].

In this study, CHC group shows the highest significant expression of IL28B rs12979860 (CT 50.0%) genotype in relation to other studied groups followed by liver cirrhosis group (CT 37.5%). *Others* reported similar results [22],[23], [17].

In the current study, Cytomegalovirus infection was significantly higher (P<0.05) in HCC group (37.5%) compared with control (0%) and CHC group (15%). These result is in agreement with some studies [24],[25] The coexistence of CMV and HCV infection impacts on the progression of liver diseases with higher incidence of CMV among HCV genotype 4 infected patients with less response to IFN therapy [12] and treatment naïve patients having HCC [26],[27].

The current study showed that IL28B genotypes (TT) (73.3%) and alleles (T) (80.0%) is non-significantly prevalent among CMV infected HCC patients in comparison to other groups. Another study showed that Genotype frequencies in the group (33.3% carriers of CC and 66.7% carriers of CT or TT genotypes) did not differ from the genotype frequencies detected in the group without CMV disease (38.6% carriers of CC and 61.4% carriers of CT or TT, P = 0.32) [3]

In the current study, IL28B rs12979860 association with HCC development was presented as odds ratio and 95% confidence intervals. HCC group compared with healthy controls, this could reveal the IL28B rs12979860 genotype TT is highly significantly (OR = 8.8, 95% CI = 1.61; 48.23, P< 0.05) associated with increased susceptibility to HCC progression. *IFNL3* rs12979860 polymorphism with T allele may be a factor which increases the risk of chronic HCV-related HCC in the Chinese patients as mentioned in other studies [9].[5],[28].

According to our study, the risk for HCC development was presented as odds ratios (ORs) and 95% confidence intervals (CIs), In healthy control IL28B CC/CT genotypes and C allele is on significantly elevated than in HCC patients (OR = 4.0, 95% CI = 0.71 – 22.51, P> 0.05), So, CC genotype and C allele seems to be protective against HCC progression. Some investigators suggested a role of protection of the C allele in HCC initiation [29],[13],[16].

The allelic variants of the *Il28B genetic* polymorphism may disturb the proficiency of the immune modulatory process, which could lead to HCC[30]. It strengthens the seriousness of LC, and HCC. Carrying the T allele was associated with advanced HCC stages and the major C allele has a protective role against HCC development [31].

CONCLUSION

Our results concluded that CMV infection occurs in patients with HCC significantly higher than in patients without HCC and is clearly linked with Il28B rs12979860 genetic polymorphism TT in cirrhotic patients. IL28B polymorphism seems to be involved in the development of HCV-induced HCC and significantly correlated with HCV viral load. T allele may be regarded as a genetic risk factor for HCV-related hepatic carcinogenesis. If CMV infection plays a significant act in HCC, CMV infection eradication via the development and administration of treatments or vaccines may reduce HCC mortality rates. That in turn reduce the incidence of HCC related to CMV infection .

Limitation of the study:

The number of patients to be increased. Further studies including large-scale samples should be done to investigate the role of CMV in HCC.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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