RESEARCH ARTICLE

DETECTION OF IL-28 rs 12979860 GENE POLYMORPHISM BY REAL TIME PCR IN HEPATOCELLULAR CARCINOMA PATIENTS.

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Manuscript Info   *Abstract*

Background:- Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. It commonly develops on cirrhotic livers, and surveillance programs have therefore been suggested to identify early HCC, at a stage when it remains suitable for surgical therapy and has a better clinical outcome.

Aim:- This study aimed to investigate the association between rs12979860 SNP of IL-28 gene and prediction of HCC in Egyptian patients.

Methods:- This study was conducted on a total number of 110 participants admitted to Hepatology and Gastroenterology Department in NHTMRI (National Hepatology& Tropical Medicine Research Institute). The participants of this study were divided into three groups as following; Group I: 30 cases CLD, Group II 30 patients LC group and Group III as HCC individuals, in addition to 20 healthy subjects (as controls).Liver enzymes activities and serum levels of total proteins, albumin, and alpha fetoprotein were measured. Also, Genotyping of IL-28B rs12979860 C/T allele Polymorphism was carried out using Real Time PCR.

Results:- Our results showed in chronic hepatitis C group, the genotype CC represents 50%, while CT genotype was 36.4% and TT genotype represents only 13.6%. In cirrhosis group, the genotypes frequencies were as follow: CC (43.4%), CT (40%) and TT (16.6%), whereas, in HCC group, the TT genotype frequency increases up to 23.6% while the CT (40%) and CC genotype was only 36.4 %. The genotype TT was more frequent in HCC group in comparison to chronic hepatitis group (P= 0.083).

Conclusion:-This study demonstrated the beneficial role of SNPs rs12979860 allele of IL28B gene for predicting the Hepatocellular carcinoma related to HCV patients.

Introduction:-
Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and a leading cause of death in Africa and Asia. The rate of HCC is increasing in Egypt where the major risk factors are chronic infections with hepatitis B and C viruses (HBV & HCV), other risk factors involves aflatoxin B1 exposure, pesticides, alcohol consumption, and genetic defects (But et al., 2008).In Europe and North America, it commonly develops on cirrhotic livers, and surveillance programs have therefore been suggested to identify early HCC, at a stage when it

remains suitable for surgical therapy and has a better clinical outcome. Ramsey and Wu-Gy (1995) stated that alpha-fetoprotein (AFP) level and abdominal ultrasonography remain the cornerstones of screening for HCC. Recently American Association for the Study of Liver Diseases (AASLD) practice guideline (2005) recommended that surveillance for HCC should be performed using ultrasonography and AFP for screening at 6 to12 month intervals. Viral and host factors influence the development of liver fibrosis, cirrhosis and HCC in patients with HCV (Missiha et. al., 2008). In HCV infection, secretion of inappropriate amounts of cytokines may be associated with chronicity or resistance to interferon (IFN) treatment (Thio, 2008). Multiple genome wide association studies have identified single nucleotide polymorphisms (SNPs) near the IL-28B gene (encoding IFN λ3) to be strongly associated with spontaneous clearance of HCV infections (Rauch et. al., 2010).One of these SNPs, rs12979860 was pivotal in predicting the resolution of HCV infections (Lagging et. al., 2011). The SNP rs12979860 is found ~ 3 kb upstream from the IL-28B gene, and encodes the type III interferonλ3 (IFN λ3). Little is known about the IFNλ3family, but evidence is mounting to support a role for them in the immune response to viral infections (Pineda et. al., 2011). There are few and controversial data available on the association between IL-28B SNPs and severity of liver fibrosis, presence of cirrhosis or developing HCC (Di Marco et. al., 2011). Further studies are needed to assess the role and impact of the rs12979860 single nucleotide polymorphism (SNP) on hepatitis C outcomes in different populations and its impact on the outcome of HCV genotypes non-1 such as HCV genotype 4 (Sharafi and Alavian, 2011). So, we aimed in thisstudy to investigate the association between rs12979860 SNP of IL-28B gene and prediction of HCC in Egyptian patients.

Subject and Methods:-

This was a cross section prospective study, an informed written consent was obtained from all patients. The ethical consideration was informed consent and would been taken from every patients. This study was conducted for one year on 90 patients with liver disease and 20 healthy subjects as control admitted to Hepatology and Gastroenterology Department, National Hepatology and Tropical Medicine Research Institute (NHTMRI). The age of these patients ranges from 30-75 years. These patients were selected for this study according to the following inclusion and exclusion criteria. Exclusion criteria, ,we excluded in the current study most of the factors that may contribute to disease progression or considered as risk factors for the development of HCC such as abnormal renal function, chronic alcohol intake, diabetes mellitus, high BMI and other metabolic disorders or co-infections (e.g. schistosomiasis, chronic HBV or even occult Hepatitis B infection). The inclusions criteria were adult patients with liver disease were classified as in table 4.The patients under this study will be classified into three groups;twenty healthy subjects (control) group, Group I include 30 patients with chronic liver disease (CLD), Group II include 30 patients with liver cirrhosis (LC) and the Group III include 30 patients with hepatocellular carcinoma (HCC). All patients were subjected to the following category; firstly, thorough history taking with particular attention to manifestations of liver disease especially abdominal ultrasonography and abdominal CT scanning to patients with hepatic focal lesion (table 2) and/or elevated AFP.Secondary the full general and local examination looking for signs of liver disease will be recoded. Finally full investigations of renal function tests (serum creatinine and blood urea) and liver function tests, transaminases (ALT& AST), serum total, serum albumin and Prothrombin time (PT) were done for all participants in addition to serum alpha fetoprotein detected by Eliza procedures.

DNA Extraction:-

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leucocytes using QIA amp DNA Blood Mini Kit supplied by Qiagen GmbH (Hiden, Germany) (Schur et al., 2001). The DNAconcentration was determined from absorbance at 260 nm. All samples had a 260/280 nm absorbance ratiobetween 1.59 and 1.77 indicating purity of DNA. The integrity of theDNA was checked by electrophoresis on 0.8 % agarose gel stained with ethidium bromide.

Detection of IL28 polymorphism:-

DNA from patients was extracted from Venus blood using standard methods. Genotyping of the rs12979860 was performed using a TaqMan 5′ allelic discrimination assay (Applied Biosystems). The probes were labelled with the fluorescent dyes VIC and FAM respectively. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the Taq polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCRthermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Genotyping of each sample was automatically attributed for allelic discrimination.

Statistical analysis:-

Data was analyzed using SPSSwin statistical package version 15 (SPSS Inc., Chicago, IL). Chi-square test (Fisher's exact test) was used to examine relationships between qualitative variables. For quantitative data, comparison between two groups was undertaken using Mann-Whitney test and comparison between 3 groups with ANOVA test or Kruskal-Wallis test followed post- Hoc "Schefe test". Spearman-rho method was used to test correlation between numerical variables. Multivariate analysis was performed using multiple linear regression model using forward method for the significant factors affecting fibrosis on univariate analysis. Multivariate analysis (logistic regression) was performed to find the predictors for HCC development All tests are two-tailed; a P-value<0.05 was considered significant.

Results:-

This study was conducted on four groups of patients without non significant difference of mean age between subjected cases (table 1). Transaminases, total bilirubin and prothrombin time observation indicate significant elevation levels in CLD, LC and HCC cases comparing with control healthy individuals (table 1). As usual albumin, platelets, hemoglobin, and hematocrit recorded significant decreasing levels in chronic hepatitis C group (CLD) (table 1).As regards AFP induce a significant difference provoke between HCC group and healthy control (P=0.03), as well as CLD group and liver cirrhosis group (P=0.01), while there was a highly significant difference increasing between HCC group comparing with CLD cases (P=0.001) as in table 2.

Table 1:-Biochemical findings in different groups (mean± SD).

Test		Control group (N= 20)	CLD group (I) (N=30)	LC group (II) (N=30)	HCC group (III) (N=30)	Test (U)	P value
Age (years)	mean	51.50 ± 22.2	55.05 ± 20.3	55.60 ± 22.4	60.15 ± 34.9	0.023^a	0.083
	range	$33 – 77$	$33 – 71$	$32 – 78$	$35 - 76$	$<0.0001^b$ $<0.0001^c$	
AIT (U/L)	mean	17.35 ± 9.6	92.90 ± 33.5	75.25 ± 28.4	64.65 ± 33.9	$<0.0001^a$	0.0001
	range	$11 – 31$	$32 – 176$	$12 – 255$	$34 – 103$	0.007^b 0.85^c	
AST (U/L)	mean	22.05 ± 4.1	69.80 ± 30	96.45 ± 33.5	133.35 ± 77.4	$<0.0001^a$	0.0001
	range	$13 – 34$	$30 – 108$	$31 – 402$	$80 - 300$	0.003^b 0.02^c	
Albumin (gram %)	mean	4.01 ± 1.5	2.76 ± 1.0	2.53 ± 1.1	2.79 ± 0.69	0.04^a	0.0001
	range	$2.80 – 5.10$	$1.90 – 3.60$	$1.30 – 3.90$	$1.70 – 3.40$	0.005^b 0.99^c	
T. bilirubin (mg %)	mean	0.70 ± 0.14	2.65 ± 1.09	5.74 ± 2.08	3.72 ± 1.9	0.001^a	0.003
	range	$0.40 – 0.90$	$1.00 – 5.40$	$1.10 – 13.40$	$1.50 – 6.01$	0.001^b 0.001^c	
P T (sec.)	mean	14.05 ± 1.0	16.20 ± 2.0	18.95 ± 3.1	15.80 ± 3	$<0.0001^a$	0.124
	range	$13.0 – 16.0$	$14.0 – 19.0$	$11.7 – 16.7$	$14.2 -19.6$	$<0.0001^b$ $<0.0001^c$	
TLC (x10³/cmm)	mean	6.97 ± 3.4	8.40 ± 3.0	10.37 ± 3.2	10.37 ± 5.9	0.002^a	0.168
	range	$4.0 – 10.2$	$3.1 – 16.2$	$3.0 - 16.2$	$3.0 – 16.1$	0.008^b $<0.0001^c$	
Hb (g/dl)	mean	13.72 ± 2.1	9.90 ± 2.0	10.09 ± 2.2	11.80 ± 3.1	0.008^a	0.0001
	range	$12.1 – 15.7$	$6.1 – 13.0$	$4.1 – 16.0$	$9.0 – 15.0$	0.004^b $<0.0001^c$	
Hct (%)	mean	42.0 ± 11.3	31.0 ± 12	28.97 ± 9.8	28.97 ± 8.8	0.006^a	0.0001
	range	$40 – 50$	$20 – 40$	$13 – 44.4$	$13.3 – 44.4$	0.001^b 0.001^c	
Plt (x10³cmm)	mean	262.10 ± 80	233.95 ± 76	161.60 ± 67	123.85 ± 34	0.034^a	0.0001
	range	$150 – 450$	$73 – 431$	$48 – 331$	$60 - 160$	0.005^b 0.09^c	
U (Mann-Whitney test), SD (standard deviation), T (total) PT (prothrombin time), TLC (total leucocytes count), Hb (hemoglobin), Hct (hematocrit) and Plt (platelets count). [a]=Comparing control & HCC group (III).							

b=Comparing control & CLD group (I).
c=Comparing CLD group (I) & HCC group (III).
(a, b, C) P-value < 0.05 was significant.

Table 2:- Radiological examination of studied groups (Control, CLD, LC and HCC patients) Sonar and Computed tomography (CT).

Parameters	Control N (%)	CLD N (%)	LC N (%)	HCC N (%)	P=value
Liver:					P<0.001*
-Normal liver	20(100%)	10(40%)	0(0%)	0(0%)	
-Bright liver	0(0%)	15(60%)	0(0%)	0(0%)	
-Coarse liver	0(0%)	0(0%)	30(100%)	30(100%)	
Focal lesion	0(0%)	0(0%)	0(0%)	30(100%)	
Ascitis:					P=0.008*
No	20(100%)	25(100%)	18(60%)	24(80%)	
Mild	0(0%)	0(0%)	7(23.3%)	2(6.7%)	
Mod	0(0%)	0(0%)	3(10%)	3(10%)	
Severe	0(0%)	0(0%)	2(6.7%)	1(3.3%)	
PVT:					P=0.19
Yes	0(0%)	0(0%)	3(10%)	3(10%)	
No	20(100%)	25(100%)	37(90%)	27(90%)	
Splenomegaly:					P=0.01*
Yes	0(0%)	0(0%)	3(10%)	7(23.3%)	
No	20(100%)	25(100%)	27(90%)	23(76.7%)	
Hepatomegaly:	0(0%)				P=0.12
Yes	20(100%)	0(0%)	4(13.3%)	3(10%)	
No		25(100%)	26(86.7%)	27(90%)	
Hypertension:					P=0.052
Yes	0(0%)	0(0%)	3(10%)	0(0%)	
No	20(100%)	25(100%)	27(90%)	30(100%)	
*P-value < 0.05 significant, PVT (portal vein thrombosis).					

Table 3:- Prevalence of IL28 gene polymorphism among the studied groups

SNP	Control group(N=20)	CLD group (I) (N=30)	LC group (II) (N=30)	HCC group (III) (N=30)	P value
CC	17(85%)	15(50%)	13(43.4%)	11(36.4)	0.083
CT	2(10%)	11(36.4%)	12(40%)	12(40%)	
TT	1(5%)	4(13.6%)	5(16.6)	7(23.6%)	

The genotypes and alleles frequencies in different clinical groups are shown in table 3.

In controls Fifty (85%) participants were CC homozygous genotype, whereas, the other remaining were either homozygous TT (1 participant, 5%) or heterozygous CT (2 participants, 10%).In chronic hepatitis group, the genotype CC represents 50%, while CT genotype was 36.4% and TT genotype represents only 13.6%. In cirrhosis group, the genotypesfrequencies were as follow: CC (43.4%), CT (40%) and TT (16.6%),whereas, in HCC group, the TT genotype frequency increases up to 23.6% while the CT (40%) and CC genotype was only 36.4 %.The genotype TT was more frequent in HCC group in comparison tochronic hepatitis group (P= 0.083).

Table 4:- Roc curve of AFP for prediction of HCC.

Test	Cut-off value	AUC	sensitivity	Specificity
AFP	> 20 ng/ml	0.67	70%	77%

ROC=receiver operating curve,AFP= alpha-fetoprotein, AUC=area under curve

The above table showed the receiver operating curve where the area under curve (AUC) for AFP was 0.67 when we use 20 ng/mL as a cutoff point which gives the optimum balance between sensitivity, specificity the sensitivity was 70% and a specificity of 77%.

Discussion:-

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy of adults. It is the sixth most common cancer worldwide and the third most common cause of cancer death. In Egypt, liver cancer forms 11.75% of the malignancies of all digestive organs and 1.68% of the total malignancies (Lavanchy D 2009). HCC constitutes 70.48% of all liver tumors among Egyptians (El-Raziky et al., 2007). HCC represents the main complication of cirrhosis, and shows a growing incidence in Egypt, which may be the result of a shift in the relative importance of hepatitis B virus (HBV) and HCV as primary risk factors (Ray et al.,2001) and improvements in screening programs and diagnostic tools(Lyra et al., 2004). HCC represents an important public health problem in Egypt. In many Egyptian regional registries, liver cancer is the first most common cancer in men and the second in women (Ge et al., 2009).

Since the progression of HCV related liver disease could be related to many factors, other than the genetic background of the patients (Missiha et al., 2008), we excluded in the current study most of the factors that may contribute to disease progression or considered as risk factors for the development of HCC such as abnormal renal function, chronic alcohol intake, diabetes mellitus, high BMI and other metabolic disorders or co-infections (e.g. schistosomiasis, chronic HBV or even occult Hepatitis B infection).

The present study was conducted for one year on 90 patients with liver disease and 20 healthy subjects as control admitted to Hepatology and Gastroenterology Department, National Hepatology and Tropical Medicine Research Institute (NHTMRI) wherethe median age of the studied HCC patients was 55.5 years. This agreed with Baghdady et al. 2014, who reported that the age of the HCC patients ranged between 42 and 70 years (mean 58.70 ± 5.76 years) and also with Shaker et al., who found that the most frequent age category affected by HCC in Egypt was between 51 and 60 years.

AFP, the golden marker of HCC, has frequently normal levels in patients with small HCC and moderate levels in a significant proportion of patients with early stage, potentially curable HCC. Therefore there is an increased need for new tumor markers that may be more sensitive and specific for HCC (Baghdady et al., 2014).

The current study shows significantly higher levels of AFP in patients with HCC than other patients and controls ,this finding came in agreement with the work of many authors(El- Lyra et al.,2004) and (Raziky et al. 2007). Based on this significant higher level, an attempt was done to evaluate AFP in diagnosis of HCC using ROC curve analysis (Ciesla et al., 2012). According to the previous authors, the area under the ROC curve (AUC) is used as an indicator for the acceptance or rejection of a marker in diagnosis. The minimal accepted AUC is 0.7 (Ciesla et al., 2012) while inthe present study AFP recorded an AUC of 0.67 indicating its validity as a diagnostic marker of HCC in a cirrhotic population.

In our study, AFP sensitivity was 70% and specificity was 77% at cut-off value more than 20 ng/ml (table 3), while in a previous recent study the best cutoff was 10 ng/ ml with sensitivity 66.3% and specificity 80.6% when used as a screening test (Biselli et al., 2015). Usually patients with a higher AFP level were associated with more severe cirrhosis, more frequent vascular invasion, higher tumor burden and poorer performance status. Patients with AFP less than 20 ng/mL had significantly better long-term survival than patients with AFP more than 20 ng/mL and patients with AFP less than 400 ng/mL had significantly better overall outcome than patients with AFP more than 400 ng/mL (Hsu et al., 2015).This finding came not in agreement with El-Houseini et al.

The natural history of hepatitis C virus (HCV) infection varies greatly. However, it is still unclear to what extent do genetic variations at the IL28B locus contribute to the severity and time to progression in HCV-associated liver disease (Marabita et al., 2011), especially in the Egyptian population, which has the highest prevalence of HCV infection worldwide.

Many studies showed that the IL-28rs 12979860 gene genotype is the commonest genotype in hepatitis C virus patients. Suggesting its possible role for this genotype in the transition of a normal hepatocyte into an abnormal one.

Gene polymorphism and its importance in chronic HCV Egyptian patients with genotype 4 showed that CC genotype is associated with favorable response, while absence of C allele is associated with failure of Interferonresponse in hepatitis C virus (HCV) infection (McCarthy, et al.,2010).

In our study, the prevalence of IL 28 gene polymorphism among the studied groupsshowed the genotypes and alleles frequencies in different clinical groups are shown in table 3.

In controls Fifty (85%) participants were CC homozygous genotype, whereas, the other remaining were either homozygous TT (1 participant, 5%) or heterozygous CT (2 participants, 10%).In chronic hepatitis group, the genotype CC represents 50%, while CT genotype was 36.4% and TT genotype represents only 13.6%. In cirrhosis group, the genotypes frequencies were as follow: CC (43.4%), CT (40%) and TT (16.6%), whereas, in HCC group, the TT genotype frequency increases up to 23.6% while the CT (40%) and CC genotype was only 36.4 %.The genotype TT was more frequent in HCC group in comparison to chronic hepatitis group (P= 0.083). Moreover, in patients with chronic HCV infection, carriage of the T/T genotype occurred more frequently in those patients affected by end-stage liver disease (ESLD) than in those with mild chronic hepatitis. Our findings confirm the results of Fabris et al., (2011).

Other results regarding the frequency of the protective C/C genotype (about 30% of the Egyptian healthy control) and the slightly higher frequency of the C than the T allele give the Egyptian population a near modest frequency of the protective (C) allele compared to its high frequencies in East Asian populations and its low frequencies in the sub-Saharan Africa (Thomas et al., 2009). This highlights the role of ethnic variation in relation to the genetic environment, which should be extensively explored in large, population based studies including larger numbers of patients and control subjects in order to determine the exact frequency and contribution of SNPs in different ethnic groups at different disease stages. Also, Eurich et al., (2012), in a study of patients who received a liver graft due to severe HCV-induced liver disease, found that the IL-28B rs12979860 T/T genotype was more frequent in the 61 patients with HCC on the explanted liver than in the 106 patients without HCC, suggesting that the major C allele plays a protective role against the development of HCC and this confirmed our results.

Regarding the etiology of Hepatocellular Carcinoma in the current study revealed that all of the HCC cases were positive for hepatitis C viral infections (HCV). This was in agreement with Goldman et al. who reported that up to 90% of the HCC cases in Egypt were attributable to HCV infection.

This study suggests that SNPs at rs12979860 allele of IL28B gene may be useful for predicting the Hepatocellular carcinoma related to HCV patients.This conclusion is supported by El-Houseini et al.,who studied Hepatocellular carcinoma related to HCV patients is related to SNPs inIL28B gene.

Competing Interests:-
Authors have declared that no competing interests exist. The authors alone are responsible for the content and writing of the paper. The authors did not receive any funds from any source

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