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ORIGINAL ARTICLE

S Association of CYP24A1 promoter methylation with hepatocellular carcinoma on top of HCV infection.

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ABSTRACT

Background (purpose): Since HCV, infection may lead to hepatocellular carcinoma (HCC) .we investigated if CYP24A1 gene hyper methylation involved in HCC and the relationship between CYP24A1 gene and HCC

Methods:-Fifty patients were selected from Tropical Medicine department and outpatient's clinic of Zagazig University Hospitals. The patients were classified into: 15 patients with chronic HCV patients with cirrhosis, 15 patients with chronic HCV without cirrhosis and 20 patients have HCC on top of HCV .All patients were subjected to proper history taking, full physical examination, laboratory investigations, abdominal U.S and triphasic helical CT as well as real time PCR for detection of hyper methylated CYP24A 1

Results:-The three groups were matched together as regards age and sex. There are a high statistically difference among the three studied groups regarding level of CYP24A1 ,which was higher among HCC group 14.62±7.74 and the lowest level was among HCV group without cirrhosis 5.3±3.46 (P value =0.000)

CON CLUSION: CYP24A1 promoter methylation increase in HCC patient on top of HCV infection

Keywords: viral hepatitis, hepatocellular carcinoma, CYP24A1, vitamin D, epigenetics.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most widely recognized primary liver tumour [1] **.** It is the fifth most common form of cancer worldwide and its mortality rate is the second highest in the world as well as the therapeutic options are limited [2] .Hepatitis C virus (HCV) infection is a major cause of HCC worldwide[3] . HCC always develops in the setting of chronic hepatitis or cirrhosis in which there are continuous inflammation and regeneration hepatocytes [4] . HCC often occurs in the back ground of cirrhotic liver with few exception [5].Egypt has the highest HCV prevalence in the world [6]. In which HCV gene type 4 represented the commonest genotype of HCV in Egypt as reported formerly by[7]**.** Interfamilial transmission was thought to be a major underlying factor to the high prevalence of HCV infection in Egypt [8]. chronic hepatitis C reported declining the strict preventive rules on blood transfusion and surgical techniques ,as well as the development of the newly direct acting antiviral therapy ,however the rates of cirrhosis and hepatocellular carcinoma are expected to declined [9] **.**

The WHO targeted for eliminating HCV by 2030 have been deemed ambitious by many Cytochrome P450 24A1 (CYP24A1) is a gene belongs to the phase I/ II xenobiotic metabolizing enzyme family and these enzymes play an important role in protecting cells from cytotoxic and carcinogenic agent DNA methylation level of CPG islands around promoters inversely regulate gene expression and closely involved in carcinogenesis. The major role of CYP24A1 is to maintain 1.25 di hydroxyl vitamin D3 homeostasis and have role also in catalysis the hydroxylation of 20 hydroxy vitamin D3 producing di hydroxyl derivatives that show very effective antitumorgenic activities **[11]** DNA methylation might plays an important role in HCV related HCC by silencing cancer related pathway inhibitors [12] who said also that, hyper methylation of CYP 24A1 was reported in HCC, they explained this as disruption of detoxification enzyme might cause excessive reactive oxygen species and result in initial HCC.

on HCC aberrant methylation of promoters sequences occurs not only in advanced tumor, it has been also observed in premalignant condition just as chronic viral hepatitis B or C and cirrhotic liver [13]

Recently DNA methylation level of CPG islands around promoters inversely regulate gene expression and closely involved in carcinogenesis. So, this study was done to evaluate the CYP24A1 promoter methylation in HCV patients and its relation in development of HCC [14]

PATIENTS AND METHODS

The study was based on case control study, it involved 50 subjects were provided from Tropical medicine department and its out patients clinic of Zagazig University Hospitals over a period of May 2016 to April 2018 after approved from IRP .ZU had been obtained. The patients were classified as,20 of them HCC patients with age mean ± SD of 56 ± 8.15 (13 male and 7 female) they were selected that they have HCC on top of HCV infection ,15 of them were patients with HCV infection associated with cirrhosis with age mean ± SD of 54.9 ± 7.81 (10 male and 5 female) and patients with HCV infection without cirrhosis with age mean ± SD of 41.1 ± 4.89 serves as control group

Inclusion criteria

Patients age is more than 20 years old, patients infected with HCV without cirrhosis ,patients infected with HCV with cirrhosis ,patients with HCC on top of HCV and patients have informed consent before study

Exclusion criteria

Patients with other types of tumors, cholangiocarcinoma or liver metastasis as proved by spiral CT , Patients with HBV infection as proved to be free of HBV infection by HBSAg detection ,Patients refuse the informed consent and Patients age < 20 years

Ethical considerations

Written informed consent was obtained from all participants and the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Methods

All patients were submitted to the following: An informed written consent was taken from all patients. Detailed history taking: age and sex .Clinical examination .Radiological investigation which include US and spiral CT for detection of hepatic focal lesion , liver metastasis , hepatosplenomegaly and presence of ascites. Laboratory investigation include the following : Complete blood count(CBC) was done on automated cell counter ,model XN 2000(Sysmex,Japan) together with examination of Leishman stained peripheral blood smears for differential Leucocytic count prothrombin time (PT) were done on automated blood coagulation analyzer ,model CS 2100 (Sysmex,Japan) . Liver function test(LFT) and kidney function test(KFT) were done on cobas c 311/501 analyzers while hepatitis marker and alpha feto protein(AFP)were done by using electrochemiluminescence immunoassay cobas e411 immunoassay analyzers (Roche diagnostics Germany) , Real time PCR for confirmation of HCV patients done by Cobas®TaqMan® (Roche diagnostics Germany) and special investigation include detection of hypermethyation CYP 24 A1 concentration by real time PCR

A) ten ml of blood was collected from each patient under strict sterile condition was taken , 3 ml of venous blood were collected on a sterile gel separator tube (Greiner –bio)for DNA extraction from serum .Another ml of blood was ruled in plain tube for other investigations

B) Analysis of hyper methylated CYP24A1 concentration :DNA extraction was done using QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany),Bisulfite conversion was done using Epi Tect Bisulfite kit (Qiagen GmbH, Hilden, Germany) .The Epi Tect bisulfite procedure comprises a few simple steps: bisulfite –mediated conversion of un methylated cytosine ,binding of the converted single –strand DNA to the membrane of an Epi Tect spin column washing , desulfonation of membrane –bound DNA ,washing of membrane –bound DNA to remove desulfonation agent ,and elution of the pure converted DNA from the spin column and Taqman methylation specific real time PCR which include the following :- 2 primers one for CYP24A1 gene (gene of interest) one for Actin Beta gene (ACTB) internal controle gene **[15]** primer designed using primer express software (Applied biosystem) sequence of primer for CYP24A1 :-

F: GCGATAGGTTTTTTACGGTTTGA, R: TCGCCATACCTACTAAAAACGCTATA

sequence for primer for ACTB gene F: TGGTGATGGAGGAGGTTTAGTAAGT,

R: AACCAATAAAACCTACTCCTCCCTTAA , Applied Biosystem INC 850 lincoln centre drive foster city,CA94404.USA, 2 probe one for CYP24A1 Gene one for ACTB Gene

Probe designed using primer Express software (applied Biosystems) , Master Mix -EpiTect methyl light PCR kit (Qiagen) and Epi Tect® control DNA (human) ,methylated and bisulfite converted (100% methylated control)(Qiagen)

Equipment

Applied Biosystems step one TM Real –Time PCR system

Procedure:

The Epi Tect methylight mastermix,primer and probe solution ,RNase –free water and converted DNA were thawed ,individual solution s were mixed –Real time cycler was programmed according to initial PCR activator 95o C for 5 min then 2- step cycling denaturation for 15 s at 95 ° C annealing /Extension for 60 s at 60 o C in 45 cycle

Interpretation Of Results

Was done by using the PMR (percent methylation reference) PMR value was used as a universal measure for the percentage of fully methylated alleles of a DNA sample It utilize absolute method of quantitation for real –time PCR,which is based on mean values derived from a standard curve of defined initial template quantities ,by comparing to a control reaction and to a reference sample ,this absolute method was turned into a relative method [16].the formula to calculate PMR value represents the quotients of two ratios (x100) ,thus the formula is (gene /reference) $_{\text{sample}}$ /(gene /reference)$_{100\%\ \text{methylate control}}$ × 100 once the real-time PCR program is finished the CT value were converted to mean value using the standard curve , PMR value of 10 and above is considered as evidence for specific gene methylation

Statistical Analysis

Data collected and analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and stander deviation or median and range . qualitative data were expressed as frequency and percentage chi square test or fisher's (x^2) was used to examine the relation between qualitative variables . for not normally distributed quantative data ,comparison between to groups was done using Mann-Whitney test (non-parametric test) ,comparison between three groups was done using Kruskal-Wallis test (non-parametric ANOVA) .The Receiver operating characteristic (ROC) curve was used for prediction of cut off values .A P value <0.05 was considered significant and P value < 0.01) was considered highly significant .

RESULTS

Result of this study was statistically calculated and tabulated

Table (1) shows that both groups were matched regarding sex and there was no statistical significant difference, while age of HCC group was higher than the other groups with a high statistical significant difference

Table(2) shows no statistical significant difference among HCC patients and HCV patients with cirrhosis regarding CHILD score, while most of HCC patients (60%) were among score C and 60% of HCV patients were among score B.

Table (3) shows statistical significant difference on comparing the liver function test including ALT, AST, Albumin, TB.L,DB.L(direct bilirubin and INR between total bilirubin .the three groups (P value =0.000, 0.003, 0.000, 0.000, 0.000 and 0.000 respectively) . all the blood parameter (HB, TLC and Platelets) in complete blood picture showed statistically significant difference on comparing the three groups (P value =0.001, 0.000 and 0.006)

Table (4) and figure (1) showed the level of hyper methylated CYP24A1 among three studied group. There were statistical significant difference between group with highest among HCC group (17.62 ± 7.74) and lowest in HCV without cirrhosis (5.3 ± 3.46) (p < 0.000)

Table (5) and figure (2) showed AFP among 3 studied group there were statistical significant difference between groups with highest among HCC group (76.2 ± 191) and lowest in HCV without cirrhosis group (3.25 ± 0.93) (p= 0.02)

Table (6) showed the level of hypermethylated CYP 24A1 among two studied group there were highly statistical significant difference between groups Being highest among patients with ascites (13.58 ± 7.5) and lowest in none ascetic group (5.3 ± 3.66) (p= 0.00)

Table(7) shows that sensitivity of CYP 24A1 in differentiation of HCC patients from non-HCC patients (HCV patients with and without cirrhosis) was 95% and could exclude 77% of negative cases ,while sensitivity of AFP was 73.3% and could exclude 55% of negative cases

Figure |(3) shows that the curve has an AUC of 0.05 and P value 0.004 the point of best cutoff for AFP was 22.7 with sensitivity 73.3% and specificity of 50%.As well as the CYP24A1 at a cut off value of 10.5 differentiat HCC patients from non-HCC patients with a sensitivity of 95% and specificity of 77%.

Table 1: General data of the three studied groups.

	HCC (n=20)		HCV with cirrhosis (n=15)		HCV without cirrhosis (n=15)		X^2	p-value
	N	**%**	**N**	**%**	**N**	**%**		
Sex Male	**13**	**65**	**10**	**66.7**	**11**	**73.3**		
Female	**7**	**35**	**5**	**33.3**	**4**	**26.7**	0.291	0.865

	HCC (n=20)		HCV with cirrhosis (n=15)		HCV without cirrhosis (n=15)		X^2	p-value
	N	**%**	**N**	**%**	**N**	**%**		
Age Mean ±SD	**56.6±8.15**		**54.9 ± 7.81**		**41.1 ± 4.89**		**22.1**	**0.000****

Fisher's exact test (X^2) **P-value < 0.001 is high significant

Table 2: Comparison of CHILD score between group 1 (HCC) & group 2 (HCV with cirrhosis)

CHILD score	Group				X^2	P
	HCC (n=20)		**HCV with cirrhosis (n=15)**			
	N	**%**	**N**	**%**		
A	**0**	**0.0**	**1**	**6.7**	3.29	0.193
B	**8**	**40**	**9**	**60**		
C	**12**	**60**	**5**	**33.3**		

Fisher's exact test (X^2) **P-value > 0.05 is non-significant

Table3: Comparison between the three studied groups regarding laboratory data.

	HCC (n=20)	HCV with cirrhosis (n=15)	HCV without cirrhosis (n=15)	F test\ KW	p-value
AST (IU/L)	159.3± 82.3 55 -325	77 ±26.4 24 - 105	45.2 ±20.1 25 -100	**20.1***	**0.000****
ALT (IU/L)	82.1 ±31.01 29 -149	50.87± 17.1 19 -70	59.4 ±26.7 31 -150	**6.71***	**0.003****
Albumin (gm/dl)	2.65 ±0.38 2.1 -3.1	2.85 ±0.34 2.1 -3.3	4.19 ±0.27 3.9 – 4.7	**99.03**	**0.000****
TB.L Range	3.7± 1.79 2.4-10.7	2.53± 0.56 1.8-3.2	0.57 ±0.12 0.4- 0.8	**30.2**	**0.000****
DB.L Range	2.13 ±1.28 1.3 -7.3	1.17 ±0.32 0.7 -1.7	0.195 ±0.08 0.1- 0.3	**23.02***	**0.000****
INR Range	1.73± 0.41 1.27-2.5	1.54± 0.304 1.16-2.1	1.08 ±0.07 1-1.16	**19.3**	**0.000****
Hb (gm/dl)	10.9 ±1.59 8 - 13	10.9± 1.96 8 - 14	13.1 ±1.73 10.3 – 16.4	**8.3**	**0.001****
PLT (1000/cmm)	94200± 50405.3 50000-250000	83667±32210.6 44000-140000	169270±40675.5 100000-250000	**18.4***	**0.000****
TLC (1000/cmm)	5495 ±1926.1 3200-10700	4513.3 ±491.2 3500-5300	6646.7± 2215.8 3400-10200	**5.6**	**0.006****

*Kruskal-Wallis test of non-parametric data **P-value <0.001 is a highly significant
*P-value <0.05 is significant

Table4: Comparison between the three studied groups regarding levels of hypermethylated CYP24A1.

	HCC (n=20)	HCV with cirrhosis (n=15)	HCV without cirrhosis (n=15)	KW*	p-value
CYP24A1 Mean ±SD	17.62 ±7.74	8.2 ±1.08	5.3 ±3.46	**36.67**	**0.000****
Range	10 – 36	7 – 10	0.87 – 13.14		
median	16	9	5.4		

*Kruskal-Wallis test of non-parametric data **P-value <0.001 is a highly significant

Table 5: Comparison between the three studied groups regarding AFP.

	HCC (n=20)	HCV with cirrhosis (n=15)	HCV without cirrhosis (n=15)	F test	p-value
AFP ng/ml Mean ±SD	**76.2± 191.1**	**16.4 ±5.85**	**3.25 ±0.93**	**4.03**	**0.02***
Range	**1.8 - 600**	**2.7 - 25**	**1. 8 – 5.2**		
median	**81**	**18**	**3.1**		

F-test P-value < 0.05 is significant

Table 6: Comparison of CYP24A1 in patients with and without ascites.

	Patients with ascites	Patients without ascites	MW	P –value
CYP24A1 Mean ±SD	13.58 ± 7.5	5.3 ± 3.66	**4.55**	**0.000**

*Mann-Whitney test of non-parametric data **P-value <0.001 is a highly significant

Table 7: Reliability data of AFP and hyper-methylated CYP24A1 in HCC VS non HCC (HCV with and without cirrhosis).

	Cutoff	**AUC**	**Sensitivity**	**Specificity**	**P**
CYP24A1	10.5	0.987	95%	77%	0.000**
AFP	22.7	0.05	73.3%	50%	0.004*

Figure 1: Levels of hypermethylated CYP24A1 among the three studied groups.

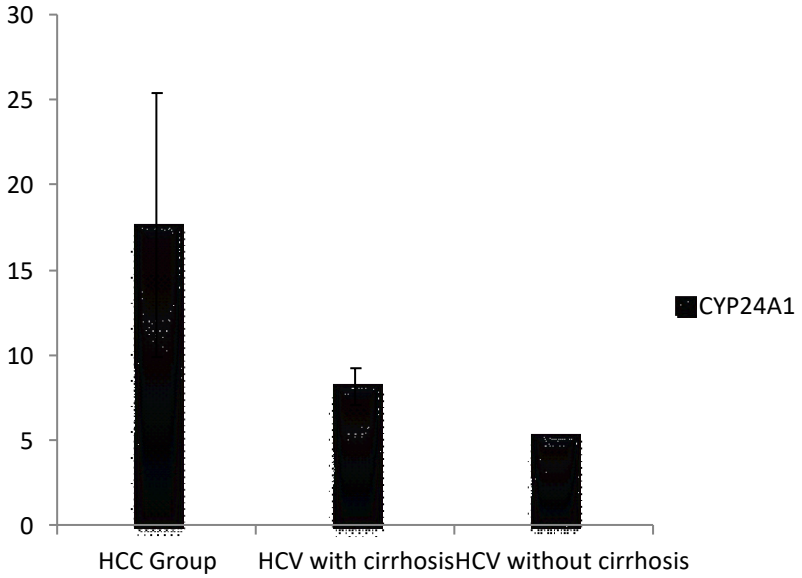


Figure 2: Comparison between the three studied groups regarding AFP

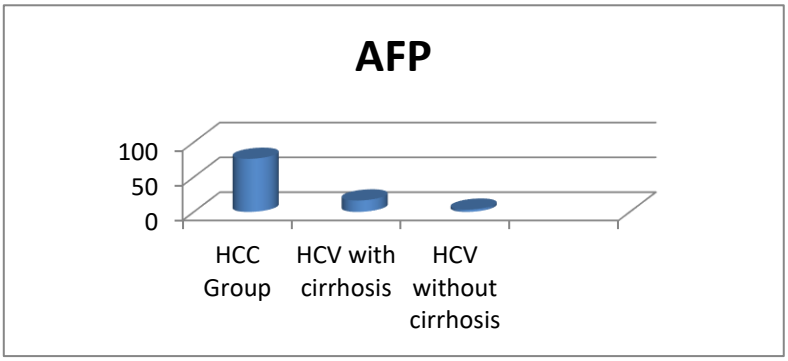
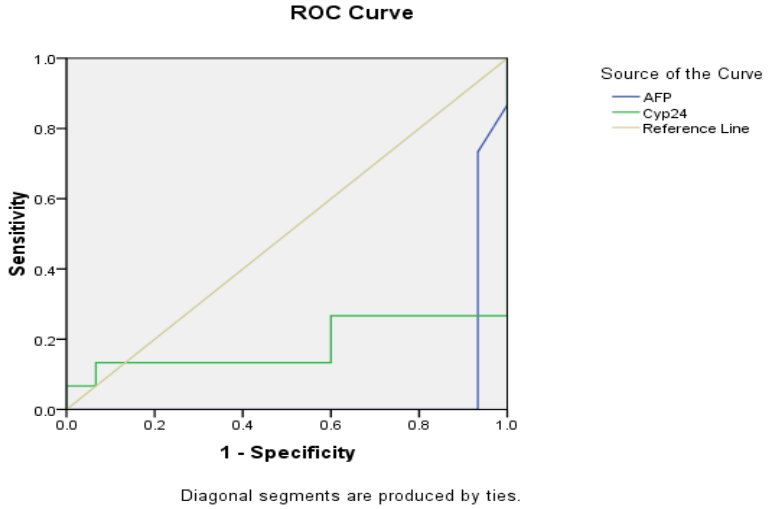


Figure 3: ROC curve of AFP and hyper methylated CYP 24A1 in prediction of HCC Vs. Non-HCC (HCV with and without cirrhosis)



DISCUSSION

Hepatocellular carcinoma being the most common type of primary malignant liver tumor with a typical poor prognosis [1]**.**Major risk factors for hepatocellular carcinoma include infection with HBV or HCV ,alcoholic liver disease, and most probably nonalcoholic fatty liver disease (NASH). Less common causes include hereditary hemochromatosis, alpha1-anti trypsin deficiency, autoimmune hepatitis, some porphyria and Wilson's disease. Risk factor among patients with hepatocellular carcinoma is highly variable, depending on geographical region and ethnic group [**17]** . Most of these risk factors leads to the formation and progression of cirrhosis .The five years cumulative risk for development of hepatocellular carcinoma in patients with cirrhosis ranges between 5% and 30% depending on the cause with the highest risk among those infected with HCV [18].Egypt has highest HCV prevalence in the world [6].CYP24A1 is a gene belongs to phase I / II xenobiotic metabolizing enzyme family and these enzymes play an important role in protecting cells from cytotoxic and carcinogenic agents , hypermethylation of CYP24A1 was reported in HCC . Disruption of the detoxification enzyme might cause excessive reactive oxygen species and result in initiation of HCC [12] .theoretically, elevated level of CYP24A1 should stimulate degradation of 1,25 dihydroxy vitamin D3 with attenuation of it is antitumorgenic action enabling tumor growth or progression [19].in our study we found no statistical significant difference between group as regard sex in contrast to [**20]** found HCC is more prevalent among male,. Also, we found among studied group being higher in HCC group than other groups .these finding are in agreement with [**21]** who demonstrated that HCC increase with aging .our study showed that statistical significant increase in ALT,AST and AFP among studied group being higher in HCC group and lower in HCV without cirrhosis group (P value 0.003 , 0.00 , 0.02) this result was in agreement with that of [22]**.** Our study ,investigated the presence of hypermethylated CYP24A1 in sera of HCC patients in comparison to cirrhotic HCV chronic patients and non cirrhotic HCV patients, Hypermethylated CYP24A1 was detected in sera of HCC patients, non cirrhotic HCV patients and cirrhotic HCV chronic patients

group but there was a highly statistical significant difference in hypermethylated CYP24A1 among studied group being higher among HCC group and lower .HCV group without cirrhosis (P value = 0.000) this agree with **[12]** who found that CYP24A1 was significantly methylated in HCV – related HCC compared with HBV-related HCC (P value < 0.05) [23] reported that a positive score for methylation was given if PMR for a given gene was > 10% in agree to our result using the Roc Curve . We found that the PMR cut off value of 10.5 (with sensitivity and specificity 95%, 77% respectively) was powerful in discrimination between HCC and non HCC group in comparison to AFP at cut off 22.7 (with sensitivity and specificity 73.3%, 50 % respectively) . CYP24A1 promoter methylation might play an important role in HCV-related HCC by silencing cancer-related pathway inhibitors such as epigenic inactivation of tumor suppressor genes and disruption of detoxification enzymes that might cause excessive reactive oxygen species these finding are in agreement with [12,13]

CONCLUSION

CYP24A1 promotor methylation was found in HCC group, HCV with cirrhosis group and HCV without cirrhosis group but it higher in HCC group so CYP24A1 promoter methylation might use for early predication of HCC on top of HCV infection

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