Egyptian Journal of Medical Research (EJMR)

Study of Epidermal Growth Factor Gene Polymorphism in Hepatitis C Virus Induced Liver Cirrhosis with or without Treatment

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Abstract:

Background: Epidermal growth factor (EGF) is a mitogen for hepatocytes, and plays a critical role in liver tissue regeneration. Many signaling pathways especially pathways that regulate the physiological processes such as tumor cells growth, differentiation, migration, apoptosis, and angiogenesis had been studied in the era of HCC development such as the EGF signal pathway. During the past few years, there have been enormous efforts to understand the structure and life cycle of hepatitis C virus (HCV) as well as to develop specific medications targeting different viral proteins such as NS3/4A protease, NS5B polymerase, and NS5A replication complex. The discovery of direct acting antiviral agents (DAAs) represented a revolution in the management of chronic hepatitis C virus infection **Objectives:** to investigate polymorphism of the gene encoding EGF in HCV-induced liver cirrhosis whether receiving antiviral treatment or not. **Methods:** Sixty Egyptian patients were enrolled in the study. Twenty patients pre-diagnosed as HCV-induced liver cirrhosis and received antiviral treatment in the form of Daklatasvir, Sofosbuvir and Ribavirin for 3 months and sustained virological response (SVR) was obtained, 20 patients diagnosed as HCV-induced liver cirrhosis and not receiving treatment yet, and 20 healthy person of matched age and sex as control group). Genotyping for EGF gene was performed by real time PCR. **Results:** With regards to polymorphism of EGF gene there are three genotypes; A/A, A/G and G/G that were detected in the three studied groups. The frequency of EGF gene polymorphism in HCV patients who received treatment was (AA 5%, AG 60%, GG 35%), while was (AA10%, AG 45%, GG 45%) in HCV patients who did not receive treatment. In

control group, the frequency was (AA 65 %, AG 15%, GG 20%). Upon examining the allelic discrimination, A allele was present in 35% of HCV patient who received treatment versus 32.5% of HCV patient who did not receive treatment and 72.5% of control group. On the other hand, G allele was present in 65% of HCV patients who received treatment versus 67.5% of HCV patients who did not receive treatment and 17.5% of control group. **Conclusion:** No significant effect of antiviral treatment on EGF gene.

Keywords: HCV infection; Liver cirrhosis; Epidermal growth factor gene; Gene polymorphism; Egypt

1. Introduction:

Hepatitis C virus (HCV) infection is one of the most serious global health problems. The incidence of HCV infection is increasing, with over 185 million people infected worldwide. Moreover approximately 700,000 HCV-infected individuals die of liver-related causes each year.[1] HCV-related liver disease can progress in an insidious manner over several decades. The advanced forms of the disease are liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Approximately 20-30% of subjects chronically infected with HCV are estimated to develop liver cirrhosis 15-25 years later .[2]

In 2013, a systematic review reported that for HCV-infected patients with compensated liver cirrhosis, 2.8-11.7% develop hepatic decompensation, 1.8-8.3% develop hepatocellular carcinoma, and 2.7-6.7% die or undergo liver transplantation each year.[3]

In the absence of antiviral therapy, 67-91% of patients with HCV-related liver cirrhosis die due to liver-related causes including HCC or hepatic failure .[4] Hence, liver cirrhosis is a critical stage in HCV-related chronic liver disease. Liver cirrhosis can also be attributed to other causes including hepatitis B virus (HBV), alcohol abuse and nonalcoholic steatohepatitis (NASH).[5]

Epidermal growth factor (EGF) stimulate cell growth and differentiation by binding to its receptor. Human Epidermal Growth Factor is a 6-kDa protein with 53 amino acid residues and three intra molecular disulfide bonds.[6]

The present study aim to evaluate EGF gene polymorphism in HCV-induced liver cirrhosis with or without treatment and if

there is change in gene polymorphism after receiving treatment.

2. Materials and methods:

2.1. Study group

This case control study was conducted on 60 patients, 40 of them suffer from HCV-induced liver cirrhosis plus 20 healthy persons of matched age and sex. The patients were recruited from Beni-Suef University hospital in Egypt during the period from April to October 2019.

The study was approved by the local Research Ethical Committee at Beni-Suef University's hospital. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Faculty of Medicine, Beni-Suef University) and with Helsinki Declaration of 1975, as revised in 1983. All participants provided informed consent to participate in this study.

Inclusion of the patients was based on diagnosis of HCV-induced liver cirrhosis. Patients with cryptogenic liver cirrhosis, liver cirrhosis secondary to autoimmune hepatitis, HBV infection, advanced liver cell failure and patients with hepatocellular carcinoma were excluded from the study.

Patients were further subclassified equally to 2 groups based on receiving antiviral treatment of HCV or not. Twenty patients were receiving (Sofosbuvir, Daclatasvir and Ribavirin for 3 months), while the other 20 patients did not receive any antiviral treatment yet. This is in addition to 20 healthy persons of matched age and sex as a control group.

All patients were subjected to thorough history taking and proper clinical examination. Biochemical analysis was done for the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, CBC, urea, creatinine, total and direct bilirubin, α-fetoprotein (AFP), and pelvic-abdominal ultrasonography to ensure liver cirrhosis and exclude hepatic focal lesions.

2.2. EGF Genotyping

Genotyping of epidermal growth factor gene polymorphism was determined using real-time PCR, allelic discrimination assays using Taq-Man single nucleotide polymorphism (SNP) genotyping assays (Applied Bio systems Inc.). Reactions were carried out according to the manufacture's protocol. The assay was done as follows: DNA extraction, amplification and post PCR plate read and analysis

2.2.1. DNA extraction

DNA extraction was done from EDTA-anticoagulated whole blood using QIAamp DNA Mini Kit.

2.2.2. Amplification of the target genes by (RT-PCR)

The successive cycles of DNA synthesis result in an exponential amplification of the target DNA sequence leading to a 10^5 to10^6 folds increase in the amount of DNA sequence of interest.[7]

The PCR reaction volume was 20 µL containing: 40 ng/µl of gDNA, 10 µL 2X universal TaqMan master mix II , 0.5 µL 20X SNP assay mix and adjusted to a final volume of 20 µL using nuclease free water.

The PCR was performed by StepOne™ Real Time PCR (Applied Biosystem) under the following conditions: initial enzyme activation at 95°C for 10 min, followed by 40 cycles of amplification as follows: denaturation at 95°C for 15 secs, annealing/extension for 1 min at 60°C. Florescence data collection at annealing/extension step for 6 FAM and VIC dye.

Interpretation of data was done using allelic discrimination plot. Life Technologies Real-Time instrument software was used to generate the plots of the results of the allelic discrimination data as a scatter plot of Allele 1(VIC® dye) Versus Allele 2 (FAM day).

2.3. Statistical analysis

Analysis of the data was performed using SPSS v. 25 (Statistical Package for Social science) for Windows. Description of variables was presented as follows: description of quantitative variables was in the form of mean, standard deviation (SD), median and range (min-max). Description of qualitative variables was in the form of numbers (No.) and percentage (%). Chi-Square was used to detect the difference between both groups regarding categorical variables. One-way ANOVA was used to detect the difference between groups regarding the scale variables. The significance of the results was assessed in the form of P-value that was differentiated into: Non-significant when P-value > 0.05, Significant when P-value ≤ 0.05 and Highly significant when P-value ≤ 0.001.

3. Results:

3.1. General characteristics of study groups

The mean age of the different groups was as follows: control group (**47.4 ±7.1**), HCV-infected patients with treatment (**50.2 ±7.702**), and HCV-infected patients without treatment (**49.3 ±6.664**). The age and sex of the 3 studied groups were matched with statistically non-significant difference (**P-value >0.05**).

Different clinical and laboratory characteristics of both groups are shown in Table 1. There is no significant difference between control group and HCV with treatment group regarding the ALT, AST, total and direct bilirubin levels (P-value >0.05) but there was a significantly higher level of ALT, AST, total and direct bilirubin levels in HCV without treatment than HCV with treatment groups and control group (P-value <0.05). The INR level didn't differ significantly among the three studied groups (P-value >0.05).

Table 1: Characteristics of the different study groups

Characteristics	Groups			P-value
	Control group (No=20)	HCV with ttt (No=20)	HCV without ttt (No=20)	
		Urea		
Mean ±SD	52.1 ±6.5	51.6 ±11.9	57.3 ±12.1	
Range (Min-Max)	44 - 63	40 - 94	45-87	0.173
Median	50	49.5	53.5	
		Creatinine		
Mean ±SD	0.91 ±0.19	0.93 ±0.19	1.1 ±0.3	
Range (Min-Max)	0.6 - 1.3	0.5 - 1.3	0.5 - 1.9	0.097
Median	0.9	0.9	1.1	
		ALT		
Mean±SD	27.4 ±3.9	25.5 ±4.9	60.2 ±15.1	P1 = 0.802
Range(Min-Max)	20 - 33	18 - 34	30 - 94	**P2 <0.001****
Median	28	24.5	59	**P3 <0.001****
		AST		
Mean±SD	33.6±6.8	37.5±5.9	77.8±13.9	P1 = 0.411

Range(Min-Max)	22-54	28-48	59-98	**P2 <0.001****
Median	32	38	78	**P3 <0.001****
		Albumin		
Mean ±SD	4.1 ±0.4	3 ±3.5	3.04±0.3	**P1 <0.001****
Range (Min-Max)	3.5-4.9	2-3.2	2.4-3.4	**P2 <0.001****
Median	4	2.6	3.1	**P3 <0.001****
		Total bilirubin		
Mean ±SD	1 ±0.18	1.15 ±0.3	1.9 ±0.68	P1 = 0.684
Range (Min-Max)	0.7-1.3	0.8-1.8	0.8-3.20	**P2 <0.001****
Median	1	1.1	1.9	**P3 <0.001****
		Direct bilirubin		
Mean ±SD	0.18 ±0.05	0.28 ±0.09	0.5 ±0.2	P1 = 0.053
Range (Min-Max)	0.1 - 0.3	0.2 - 0.5	0.2 - 0.9	**P2 <0.001****
Median	0.2	0.2	0.5	**P3 <0.001****
		INR		
Mean ±SD	1.05 ±0.08	1.03 ±0.05	1.1 ±0.12	
Range (Min-Max)	1 - 1.30	1 - 1.2	1 - 1.4	0.105
Median	1.02	1	1.03	
Scale data was presented as mean ±SD				
P-value is insignificant>0.05				
Scale data was presented as mean±SD				

3.2. Distribution of EGF genotype in the study groups:

Table 2 compares the EGF genotype between the three groups. It shows that there was a statistically significant difference between the three groups regarding the distribution of different genotype of the EGF genotype (P-value<0.001). Regarding the AA genotype, it was prevalent in 65% of the control group, in 5% of the HCV infection with treatment, and in 10% of the HCV infection without treatment. The distribution of AA was significantly higher in the control group than in the HCV with treatment and without treatment, but there was no significant difference of AA

distribution between the HCV with treatment and without treatment.

Regarding the AG genotype, it was prevalent in 15% of the control group, in

60% of the HCV infection with treatment, and in 45% of the HCV infection without treatment. The AG distribution was significantly lower in the control group than the HCV with treatment and without treatment, also there was a significant increase of AG distribution among the HCV with treatment than those without treatment.

In spite of non-statistically significant difference of GG distribution between the three studied groups, there was clinically significant difference between the 3 groups regarding GG expression, as the highest expression of GG was found in HCV group (45%) followed by the treatment group (35%) and finally the control group (20%).

Table 2. Comparison between the three groups regarding the EGF genotype:

EGF	Groups			P-value	X^2
	Control group (No=20)	**HCV with ttt (No=20)**	**HCV without ttt (No=20)**		
AA	13(65%)	1(5%)	2(10%)	**<0.001**	23.8
AG	3(15%)	12(60%)	9(45%)		
GG	4(20%)	7(35%)	9(45%)		

Categorical data was presented as number (%) P-value is highly significant<0.001 (different letters denotes statistical significance).

3.3. Distribution of EGF allelotype in the study groups:

Table 3 and Figure 1 revealed that there was a statistically significant difference between the three groups regarding the distribution of different allelotype of the EGF genotype (P-value <0.001). Regarding the A allele, it was prevalent in 72.5% of the control group, in 35% of the HCV infection with treatment and in 32.5% of the HCV infection. The distribution of A allele was significantly higher in the control group than in the HCV with treatment and without treatment, but there was no significant difference of AA distribution between the

HCV with treatment and without treatment.

The G allele was prevalent in 17.5% of the control group, in 65% of the HCV infection with treatment and in 67.5% of the HCV infection without treatment. The distribution of G allele was significantly lower in the control group than in the HCV with treatment and without treatment, but there was no significant difference of AA distribution between the HCV with treatment and without treatment

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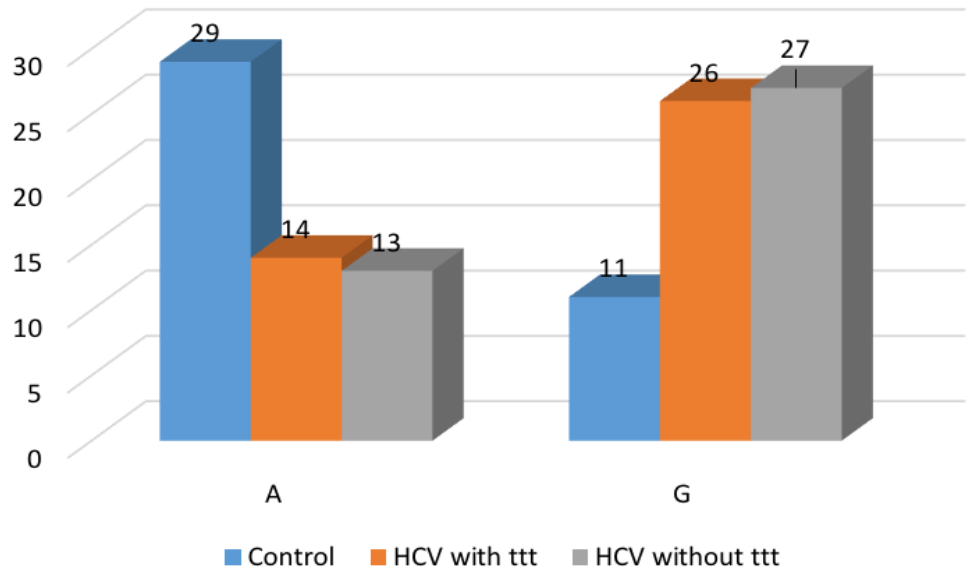
Table (3): Comparison between the three groups regarding the EGF allelotype:

EGF	Groups			P-value	X^2
	Control group (No=40)	**HCV with ttt (No=40)**	**HCV without ttt (No=40)**		
A	29(72.5%)	14(35%)	13(32.5%)	**<0.001**	23.8
G	11(17.5%)	26(65%)	27(67.5%)		

Categorical data was presented as number (%) P-value is highly significant<0.001 (different letters denotes statistical significance)

Figure 1. Distribution of EGF allelotype in the three groups



4. Discussion:

Hepatitis C virus (HCV) infection in Egypt represents a massive challenge facing the country with high prevalence rates within the Egyptian population of different age.[8] everal recent studies discussed the epidemiology of HCV infection worldwide. HCV genotype 4 infection is common in the Middle East, Northern Africa, and Sub-Saharan Africa. In Egypt, 15% of an estimated populatioof 80 million were HCV positive, of which 93% were infected with genotype 4.[9]

During the past few years, there have been enormous efforts to understand the structure and life cycle of hepatitis C virus (HCV) and to develop specific medications targeting different viral proteins such as NS3/4A protease, NS5B polymerase, and NS5A replication complex. The discovery of direct acting antiviral agents (DAAs) represented a revolution in the management of chronic hepatitis C virus infection .[10]

Many signaling pathways especially pathways that regulate the physiological processes such as tumor cells growth, differentiation, migration, apoptosis, and angiogenesis had been studied in the era of HCC development such as the epidermal growth factor (EGF) signal pathway.[11] EGF gene polymorphism and overexpression of human EGF has been associated with multiple human malignancies including HCC, lung, breast, colon, and bladder malignancies. [12]

Genetic polymorphisms may be the result of chance processes or may have been induced by external agents such as viruses or radiation. If a difference in DNA sequence among |individuals has been shown to be associated with disease, it will usually be called a genetic mutation. Changes in DNA sequence that have been confirmed to be caused by external agents are also generally called mutations rather than polymorphisms. SNPs are the most common type of genetic variations in humans.[13]

Epidermal growth factor (EGF) is a mitogen for hepatocytes and plays a critical role in liver tissue regeneration. Mounting evidence supports a role for EGF in malignant transformation, tumor growth and progression. Over-expression of a secreted human EGF fusion protein enhances the transformation of fibroblasts to fibrosarcomas and induces the development of HCC in transgenic mice.[14]

Several mechanistic studies support relation between HCV and EGF. HCV cellular entry is facilitated by a mechanism mediated by (EGFR) Epidermal Growth Factor receptor .[3]

The present study was performed on a group of sixty Egyptian patients , The

first group consists of twenty patients diagnosed as hepatitis c virus induced liver cirrhosis and exclusion of patients with other causes of liver cirrhosis with confirmation of hepatitis c virus infection thorough specific labs and received treatment of hepatitis c virus in the form of sofosbuvir, daclatasvir and ribavirin for 3 months with sustained virological response. The second group consist of twenty patients diagnosed as the above group (liver cirrhosis due to HCV infection with exclusion of other causes of liver cirrhosis) but not received treatment yet. The third group consist of twenty healthy persons of matched age and sex as a control group.

As regard polymorphism of Epidermal Growth Factor gene there were three genotypes; A/A, A/G and G/G that were detected in the three studied groups this study showed that there was a statistically significant difference between the three groups regarding the distribution of different genotype of the EGF genotype (P-value<0.001). The distribution of AA was significantly higher in the control group than in the HCV with treatment and without treatment, but there was no significant difference of AA distribution between the HCV with treatment and without treatment.

This goes in agreement with the Egyptian study that include 150 patients divided as (group 1 (50) patients with HCV related cirrhosis, and Group II which included 50 patients with newly diagnosed HCC on top of HCV related cirrhosis, and (50) persons of matched age and sex as control group. As found that A/A, A/G and G/G that were detected in the three studied groups, The genotype A/A was more dominant in the control group, The A/G genotype was more dominant in the cirrhotic group, while the G/G genotype was more dominant in the HCC group. Comparison between the studied groups in relation to number and percentage of EGF genotypes was statistically significant (P-value < 0.0001).[15]

Other studies reported that the single-nucleotide polymorphism (SNP) A to G mutation of the EGF gene is associated with an increased risk of various malignant tumors.[16] Looking into the association between EGF polymorphism and the risk of developing HCC that was initially reported by .[16] in two case-control studies. The EGF 61*G allele in patients with alcoholic and HCV-associated cirrhosis, was highly associated with the increased risk of HCC compared with the A allele, where, cirrhotic patients with G/G and A/G genotype had a 4 and 2.4 fold for developing HCC, respectively when compared with A/A genotype patients. [16]

Recent studies reported a protective value in having A allele gene from developing HCC. As results indicate that the G allele may have a key role in hepatocarcinogensis, while A/A genotype may have a protective role.[17]

In comparison to **Suenaga et al., 2013** study that included (208) patients with liver cirrhosis induced HCV infection who received treatment , and (290) patients with liver cirrhosis but without HCV infection treatment reported the ratios of EGF polymorphism as follows (A/A 5.3%, A/G 42.8%, and G/G 51.9%) in the first group, and (A/A 8.6%, A/G 35.9%, and G/G 55.5%) in the second group with insignificant difference. By continuing studying to assess the incidence of HCC related to the three genotypes it reported that G allele (A/G and G/G) had higher risk for developing HCC especially in HCV patients when compared with A/A patients. But they could not explain why HCC was higher in patients with A/G genotype when compared with the G/G genotype; however, this has been observed occasionally, in other studies**.** This was in agreement with the study of **(Yuan et al., 2013)** who observed that among non-Asians in Los Angeles, patients with G allele had higher risk of HCC when compared with the A/A genotype even after adjustment for multiple risk factors for HCC.[18]

On the other hand, **(Qi et al., 2009)** certificated that there was no association of EGF polymorphism and HCC in Chinese patients with chronic HCV infection. The researches perceive that the association between EGF polymorphism GG genotype and the risk of HCC is still controversial and ambiguous.[19]

Prospective trials will be needed to address questions of the durability of SVR after DAA treatment, the incidence of HCC after achievement of DAA-mediated SVR, and the benefit of continued screening in SVR patient.

5. Conclusion:

The present study showed that even if DAAs has an evoluting effect in the treatment of HCV infection, they may lack ability to protect against HCC. Despite an improved understanding of direct and indirect mechanisms leading to HCV-induced HCC and despite the development of highly potent DAAs for HCV therapy, HCV-related HCC will remain a major health challenge in the coming decades. Some limitations are found in our study. Firstly, the number of collected samples from control groups and patients with chronic hepatitis C with or without receiving treatment was small, so significant association between EGF gene polymorphism and chronic hepatitis c

treatment may become statistically remarkable if larger samples were collected. Secondly, lack of studies of association between effect of Direct anti-viral agents and epidermal growth factor gene polymorphism. Finally, lack of comparison in this study between EGF gene polymorphism in HCC patients and HCV patients who received DAAs treatment as HCC patients were excluded from this study.

6. Rferences:

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