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The Link between an Epidermal Growth Factor Gene Functional Polymorphism and Hepatocellular Carcinoma in a Cohort of Hepatitis C Egyptian Patients

Bothaina A. Madkour[1], Ola M. Mahmoud[1], Ola B Abo El Nil[1*], Mohamed A. Aboul-Ezz[2], Shereif A. Morsy[2], Ahmed R. Mashaal[2], Omar M. Sabry[1]

[1]Hematology Department, [2]Hepato-gastroenterology Department, Theodor Bilharz Research Institute, Giza, Egypt.

***Corresponding author:** Ola B Abo El Nil, **Mobile:** (00201005071442), **E-mail:** olabadr81@gmail.com

ABSTRACT

Background: Chronic hepatitis C (HCV) infection is implicated in hepatocarcinogenesis. Hepatocellular carcinoma (HCC) progression is influenced by a number of genetic and epigenetic mechanisms. One of the most critical variables in early hepatocarcinogenesis is the dysregulation of the epidermal growth factor receptor (EGFR) or the epidermal growth factor (EGF) signaling pathway. This study aimed to assess the association between the incidence of HCC and the EGF 61A/G polymorphism in chronic HCV Egyptian cases.

Patients and methods: A comparative study was carried out with a total of 165 single ethnic Egyptian. They were classified into 3 groups: Chronic HCV (n = 55), HCC (n = 60), and healthy cases (n = 50). Genotyping was performed for all participants using RT-Multiplex PCR.

Results: The frequencies of genotypes were in HCV G/A 32.7%, G/G 60.0%, and A/A 7.3%, and in HCC G/A 51.7%, G/G 30.0%, and A/A 18.3%. The control group was G/A 52.0%, G/G 20.0%, and A/A 28.0%. The alleles frequencies in HCV were G 76.4%, and A 23.6%, in HCC were G 55.8% and A 44.2%. In the control group were G 46.0% and A 54.0%. The difference regarding the genotype and allele frequency among studied groups was highly significant. The G/G genotype (P-value 0.006) were protective against HCC development. However, HCV patients with the A/G genotype (P-value 0.04) might be at a higher risk to develop HCC.

Conclusion: The indications for EGF 61A/G gene polymorphism's correlation with susceptible threat for HCC development in HCV patients in Egypt.

Keywords: EGF 61A/G gene polymorphism; HCC; HCV; Comparative study; Theodor Bilharz Research Institute.

INTRODUCTION

HCC is cancer affects the liver and considers primary malignant tumor in liver tissue mainly affects people have cirrhosis and chronic liver disease (CLD) [(1)]. It's Egypt's fourth most prevalent cancer [(2)].

During chronic inflammation, inflammatory process continues for extended length of time, and constant apoptosis/regeneration occurs as a result of the process known to increase possibility of malignancy, particularly HCC [(3, 4)].

The EGF gene, 110 kb, has 24 exons and 23 introns, is localized at chromosome 4q25-27. The EGF 61A/G polymorphism (rs4444903) is single nucleotide polymorphism (SNP) that affects the expression of EGF identified in the EGF gene's 5′-untranslated region (5′-UTR) [(5,6)].

The aim of the present study is to assess the association between the incidence of HCC and the EGF 61A/G polymorphism in chronic HCV Egyptian cases.

PATIENTS AND METHODS

Study Design: Cohort Study
Study Setting: Theodor Bilharz Research Institute
Study Period: One year.

Data collection:

165 subjects categorized into 3 groups group (A) comprised 55 patients with established diagnosis of chronic HCV infection, group (B) comprised 60 patients diagnosed with HCC on top of chronic HCV infection, and group (C) who constituted 50 healthy individuals to serve as the control group.

Group A included 27(49.1%) males besides 28(50.9%) females with their age ranged from 34 to 67 years (mean ± SD = 44.3 ± 13.9). While in group B comprised 42(70%) males as opposed to 18(30%) females with age ranged from 48 to 60 years (Mean ± SD = 46.8±15.9) were enrolled. Finally, group C comprised 36 (72.0%) males and 14 (28.0%) females with their age ranged from 32 to 57 years (Mean ± SD= 46.7±13.3).

Sample size calculation:

A Cohort (Longitudinal) study of subjects in which we will regress their values of the patients against control. Prior data indicate that the standard deviation of control is 0.8 and the standard deviation of the regression errors will be 1.9. If the true slope of the line obtained by regressing patients against control is 1.0, we will need to study 50 subjects for each group to be able to reject the null hypothesis that this slope equals zero with probability (power) 90%. The Type I error probability associated with this test of this null hypothesis is 0.05.

Study population and demographic information:

The study was executed at Theodor Bilharz Research institute (TBRI) where 165 subjects were involved and categorized into 3 groups; group (A) comprised 55 patients with established diagnosis of chronic HCV infection, group (B) comprised 60 patients diagnosed with HCC on top of chronic HCV infection

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and classified according to Barcelona clinic liver cancer (BCLC) system and Triphasic CT scan for HCC staging. Finally, group (C) who constituted 50 healthy individuals to serve as the control group. Exclusion criteria included those with HBV comorbidity, schistosomiasis, alcohol consumption as well as patients receiving antiviral therapy. A written informed consent was attained from the participants. All the procedures used were approved by TBRI ethics committee according to Helsinki Declaration.

Group A included 27(49.1%) males besides 28(50.9%) females with their age ranged from 34 to 67 years (mean ± SD = 44.3±13.9). While in group B comprised 42(70%) males as opposed to 18(30%) females with age ranged from 48 to 60 years (Mean± SD = 46.8±15.9) were enrolled. Finally, group C comprised 36 (72.0%) males and 14 (28.0%) females with their age ranged from 32 to 57 years (Mean ± SD= 46.7±13.3). The study work setting was held through 2019, began on Jan15.

Those with HBV co-morbidity, Schistosoma infection, autoimmune liver disease, alcohol drinking habits, or antiviral medication were all not included in the study.

Genomic DNA extraction:

The QIAamp DNA Mini Kit was used to acquire genomic DNA (Qiagen; catalog No.: 51104). 5mL intravenous peripheral entire blood was withdrawn in a vacuum tube that is sterilized supplemented with EDTA attached to the bottom of the tube for genomic DNA extraction using a conventional process involving proteinase K. Three times, using lysis buffer, red blood cells were lysed. After that, the residual white cells were treated with 10% sodium dodecyl sulphate (SDS) supplemented with 10 µl proteinase K and droplets of guanidine HCL, then incubate the sample for 10 min at 56^{o}C to inhibit all nucleases such as DNAs.

After intracellular nucleic acids were precipitated, they were attached to specific pre-packed glass fiber in a highly elevated purification filtered tube, and a number of washing and spinning processes by adding 500 µl Buffer solution 1 and 500 µl Buffer solution 2 were performed to remove contaminants. Lastly, 200 l elution buffer. Buffer AE was administered to samples, and the nucleic acid was released from the glass fiber after 1 minute of incubation at 15-25^{o}C. The concentration of DNA product was determined by spectrophotometer at 260 nm.

EGF Genotyping:

A polymorphism in the EGF 61A/G gene (rs4444903) was observed using the Taq Man SNP genotyping assay. 2.5 µl of diluted DNA with a concentration of 5 ng/l, 12.5 µl of 2× TaqMan Universal PCR Master Mix, 1.25 µl of 20× TaqMan SNP Genotyping Assay Mix, and 8.75 µl of Distilled water (d.H_2o) make up each PCR reaction tube. In an ABI 7500 thermal cycler, A denaturation stage of 95°C for 10 min was proceeded by 40 cycles of 95°C for 25 sec and 1 min at the annealing temperature in the PCR process Finally, using the ABI Prism that acts as a genetic analyzer a threshold of 0.1 is established for analysis. This includes identifying the size of the amplified PCR output of fragments by trying to compare them to PCR output in a size standard, as well as quantifying the amplified PCR output or fragments.

Ethical Approval:

The study was approved by the Ethical Committee of Theodor Bilharz Research Institute, which was fully aligned with the code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans (Ethical approved No. FWA 00010609). All the participants signed a written informed consent form.

Statistical analysis

Microsoft Excel 2010 and the statistical program for social science (SPSS version 22.0) for Windows (SPSS IBM., Chicago, IL) were used to analyze the data. Normally distributed data were expressed as mean associated with a 95% confidence interval, whereas categorical and non-parametric variables were represented as alleles and genotype frequencies and percentages; a p-value of less than 0.05 was reportedly statistically significant. The Student's t-test has been used to make a comparison of the average means of normally distributed data between groups. The prevalence of continuous variables between groups was determined using ANOVA accompanied by Tukey-Kramer as a post-hoc check in multi groups, χ^2 test, or Fisher's exact test. A logistic regression analysis model has been used to perform a comparison among the groups from where the odds ratio analysis of HCC, including many factors as sex and age as well as genotype.

RESULTS

In the midst of the current study, 55 patients associated with chronic HCV infection and 60 patients associated with HCC on top of chronic HCV infection were included. RT-Multiplex PCR was utilized to validate the presence and the quantification of the EGF 61A/G gene polymorphism (rs4444903). In the HCV group, the genotype ratios were *A/A*=7.3%, *A/G*= 32.7% and *G/G*= 60%, whereas in the HCC group, the genotype ratios were *A/A*= 18.3%, *A/G*= 51.7% and *G/G*= 30% (Table 1).

Table 1: Analysis of EGF genotypes among the studied groups:

EGF polymorphism		Studied Groups			Total	*P*. value		
		Control N=50	HCV N=55	HCC N=60		Control & HCV	Control & HCC	HCV & HCC
EGF Alleles	Wild *A/A*	14(28.0%)	4(7.3%)	11(18.3%)	29	0.001**	0.04*	0.03*
	Hetero *A/G*	26(52.0%)	18(32.7%)	31(51.7%)	75	0.001**	0.2	0.01*
	Homo *G/G*	10(20.0%)	33(60.0%)	18(30.0%)	61	0.001**	0.05*	0.001**
Total	*A* allele	54(54.0%)	26(23.6%)	53(44.2%)	133	0.001**	0.01*	0.001**
	G allele	46(46.0%)	84(76.4%)	67(55.8%)	197			

*P-value ≤ 0.05 demonstrates the significance of data analyzed, **P-value ≤ 0.001 demonstrates the high significance of data analyzed.

The adjusted odds ratio (AOR) for the HCC development incidence was assessed by statistical analysis tools using the A/A genotype acted as a genetic reference in the study of HCC susceptibility (Table 2). The AOR of inducing HCC in A/G individuals was 1.6 (95% confidence interval [CI]: 0.44–5.76, P=0.04) with significant results, while in G/G patients, it was 0.32 (95% confidence interval [CI]: 0.14–0.72, P=0.006) with highly significant results.

Table 2: Comparison of the HCV and HCC groups associated with the risk of HCC according to EGF Genotype:

	HCV N=55	HCC N=60	AOR**(95%CI)	*P*-value
Wild *A/A*	**4(7.3%)**	**11(18.3%)**	1(reference)	
Hetero *A/G*	**18(32.7%)**	**31(51.7%)**	1.6 (0.44– 5.76)	0.04*
Homo *G/G*	**33(60.0%)**	**18(30.0%)**	0.32 (0.14– 0.72)	0.006*

AOR: Adjusted Odds Ratio; CI: Confidence Interval; *P-value ≤ 0.05 significant.

DISCUSSION

Chronic HCV infection is thought to be strongly correlated to hepatocarcinogenesis. HCC will evolve in a certain percentage of HCV patients who have been infected for a long time. In this context, various genomic and epigenomic elements can influence HCC Development and progression [7]. The deregulation of the EGF/EGFR signaling pathway is regarded to become a major contributor in primary hepatocarcinogenesis among these genetic changes [8].

Due to chronic infection, the EGF/EGFR signaling pathway has been reported to be a key modulator of hepatocyte proliferation potential and liver regeneration. This regenerative liver action mediator has been hypothesized to change EGF levels instead of a modification in EGF receptor known expression. These results indicate that the correlation between the EGF 61A/G functional polymorphism and HCC is due to incompetence to down regulate the EGF pathway once cirrhosis has progressed, rather than a more severe liver fibrosis phase. As a result of these findings, early hepatocarcinogenesis and unregulated development of early HCC occur [9].

In this cohort of HCV Egyptian patients, the allelic frequencies of the G and A alleles were 0.76 and 0.24, sequentially, which are remarkably comparable to what other researchers have found. This is striking compared to Caucasians, who had allelic frequencies of 0.40 for the G and 0.60 for the A. The prevalence of the EGF 61A/G1 polymorphism varies by race, according to many research studies [9-12].

The current study demonstrated that the G allele frequency among studied HCC and HCV Egyptian patients was (76.4% and 55.8% consecutively) when

making a comparison to normal individuals (46.0 percent). This is in accordance with Yang et al. [13] and ***El Sergany et al.*** [14], who found the 61G allele to be a potential risk for HCC and the 61A allele to be protective.

The results of the current experimental study are in agreement with ***Liu et al.*** [15], who demonstrated that EGF expression was substantially higher in HCCs compared to normal tissues, demonstrating that EGF is prevalently expressed in the microenvironment of HCC. Moreover, there was a considerable variance in serum EGF levels across the three groups evaluated. In the HCC group, EGF levels were considerably greater than in the HCV and control groups (unpublished data). Again, these results were agreed with the studies performed by ***El Sergany et al.*** [14] ***and Liu et al.*** [15] who discovered that a higher EGF level in the blood was linked to a higher tumor grade, implying that EGF may promote the growth of HCCs**.**

Similarly, several study reports showed that the *A* allele was mainly prevalent in the control group 62%. While, *G* allele was dominant significantly in HCC patients 63% compared to 42% in cirrhotic patients. The *G* allele showed a significantly high risk for HCC compared to cirrhosis (95% CI) 2.35 (*P* =0.015). These findings showed that the *G* allele might carry the risk of hepatocarcinogenesis, while the *A* allele might be protective [16,17,18].

Furthermore, the A/G genotype was shown to be the most common genotype in HCC (51.7%), accompanied by the G/G genotype (30%). However, in HCV Egyptian patients, the G/G genotype (60%) was the most common genotype, accompanied by the A/G genotype (32.7%). These findings are consistent with those of ***Suenaga et al.*** [19], who found that in HCC Japanese patients, The A/A, A/G, and G/G genotype ratios in HCV patients were 5.3%, 42.8%, and 51.9%, consecutively, whereas the ratios in HCV patients were 8.6%, 35.9%, and 55.5%, with no significant difference was observed.

In contrast to the current investigation findings, ***El Sergany et al.*** [14] discovered that in HCC Egyptian patients, the G/G genotype was the most prevalent genotype (84%) accompanied by the A/G genotype in only 10%. In cirrhotic patients (70%), the A/G genotype was the most common, while in the control group, the A/A genotype was the most common (84%). The discrepancy in genotype frequency might be attributed to the difference in used technique, small sample size.

HCV Egyptian individuals with the A/G genotype (AOR 1.6) who had the EGF 61A/G gene polymorphism (rs4444903) had an increased chance of inducing HCC. On the other hand, the *G/G* genotype (AOR 0.32) might be protective against HCC development. Similarly, when making a comparison to A/A patients, ***Suenaga et al.*** [19] ***and Yuan et al.*** [20] found that the G allele had a greater chance of developing HCC, especially in HCV patients. They were baffled as to why the A/G genotype had a greater HCC risk than the G/G genotype. These data demonstrated that EGF *61A/G* gene polymorphism (rs4444903) is an additive potential risk for HCC development in HCV-infected Egyptian patients.

CONCLUSION

Egypt has a high prevalence of HCC in chronic HCV patients. In cirrhotic HCV Egyptian patients, the EGF 61A/G gene polymorphism is linked to a high potential risk of HCC development. More well-designed large studies are needed to validate the relevance between the EGF 61A/G polymorphism and the incidence of HCC. Targeted therapy with EGFR inhibitors may provide an alternative therapy for advanced HCC patients with EGF *61A/G* gene polymorphism, and patients carrying the risk alleles should be closely followed up for early diagnosis and better treatment outcome.

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Conflicts of interest disclosed:

None

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