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RESEARCH ARTICLE

Epidermal Growth Factor rs4444903 A>G Gene Polymorphism Association with Chronic Hepatitis B Liver Disease Progression among Egyptian Children: A Multicenter Study

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Abstract:

Background:

Polymorphisms of genes encoding the pro-inflammatory and anti-inflammatory cytokines can affect the clinical presentation of the infection. We aimed to assess the role of EGF gene single-nucleotide polymorphism in the outcome of chronic hepatitis B virus (HBV) infection in children.

Methods:

One hundred HBV-infected children and 75 healthy matched controls were enrolled in this prospective study. Patients included 18 chronic inactive and 82 chronic active carriers. EGF rs4444903 A>G genotypes were determined using allele-specific amplification.

Results:

Significant differences regarding EGF genotypic frequency ($p=0.001$) in patients compared to controls ($p=0.001$). Eighteen percent were inactive, and 82% were active carriers. AA, AG and GG genotypic frequency were 66.7%, 33.3%, 0% and were 3.7%, 37.8% and 58.5% in the inactive and active carriers, respectively, with significant differences regarding AA, AG, GG genotypic frequency ($p=0.001$ for all). EGF AA, AG, GG genotypes frequency were 1.9%, 33.3%, and 64.8%, respectively, with significant differences between cirrhotic and non-cirrhotic patients regarding AA, AG, GG genotypic frequency ($p=0.001$ for all).

Conclusion:

Increased G allele frequency in EGF rs4444903 A > G polymorphism in HBV- Egyptian children is associated with worse outcomes.

Keywords: HBV, Carriers, Cirrhosis, EGF, Polymorphism, Egyptian children.

Article History

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1. INTRODUCTION

Hepatitis B is a potentially life-threatening liver infection, and it is a major global health problem. It can cause chronic infection with subsequent cirrhosis and hepatocellular carcinoma. An estimated 257 million people are living with hepatitis B virus infection worldwide [1]. Liver disease due to the hepatitis B virus (HBV) affects about 240 million people worldwide, with nearly 600,000 deaths per year. HBV is a foremost cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in developing countries [2, 3]. The risk of a chronic infection depends on the age at which a person becomes infected; therefore, children infected with hepatitis B and aged less than 6 years are at greater danger of developing chronic infections [3].

Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer, which may develop in patients with chronic liver diseases, mainly hepatitis B virus (HBV) [4]. Zhang *et al.* reported that hypomethylation around HBV integration sites aids low-pass cfDNA WGBS to serve as a non-invasive approach for early hepatocellular carcinoma (HCC) detection [5].

Other researchers found that highly dynamic cfDNA methylation profiles in support of HBV-related HCC development. They identified a panel of DMGs that are predictive for the early, middle, and late stages of HCC development. These are potential markers for the early detection of HCC as well as the screening of high-risk populations [6].

Chen *et al.* in 2020, achieved the efficient enrichment of plasma cell-free chimeric DNA from the integration site and demonstrated that chimeric DNA profiling in plasma is a promising non-invasive approach to monitor HBV integration in liver cancer development and to determine the ability of integrated sequences to express viral proteins that can be targeted by immunotherapies [7].

Gene polymorphisms such as Single Nucleotide Polymorphism (SNP) in a gene's promoter region may cause increased or decreased output of the protein concerned. The presence of such types of inherited gene polymorphisms may affect susceptibility to certain diseases [8].

Epidermal Growth Factor (EGF) was first isolated in 1962 [9]. It promotes epidermal and epithelial tissue proliferation, differentiation, and tumorigenesis by binding to its receptor (EGFR) and thus activating several signal pathways [10 - 13].

EGF level can be modulated by a functional polymorphism in EGF at position 61 (A > G) (SNP rs4444903) identified recently with the G/G genotype with higher gene expression than the A/A genotype. This genotype has a great chance to share in progression to HCC compared to cirrhotic patients with the A/A genotype [14].

In this study, we aimed to identify the EGF gene polymorphism at different stages of chronic HBV infection in Egyptian children and its association with the disease progression.

2. MATERIALS AND METHODS

2.1. Patients

The study included a total of 100 children with HBV attending the hepatology outpatient clinics of the 3 hospitals: El Sahel teaching hospital, South Valley University Hospital, Minia Children, and Maternity University hospital. Cases were selected consecutively during the period from July 2017 to February 2020. All patients were clinically assessed and investigated for; liver enzymes, {alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT)}, serological markers as {HBV surface antigen (HBsAg), HBV e antigen (HBeAg), anti-HBeAg, anti-HB core antigen (IgM and IgG against HBcAg), and anti-hepatitis C antibodies (anti-HCV)}, virologic (HBV-DNA viral load) tests and abdominal ultrasound were also performed. The patients' initial clinical and laboratory results were attained from patients' clinical records to be included in the study. Studied patients were classified as active (DNA level > 2000 IU/ml) and inactive (DNA level < 2000 IU/ml) carriers according to their serological and virologic markers. Diagnosis of cirrhosis was based on; elevated AST and ALT, with AST > ALT, slightly elevated Alkaline phosphatase but less than 2-3 times the upper limit of normal. Elevated GGT, bilirubin, and reduced Albumin levels in addition to radiological diagnosis by ultrasonography [15, 16].

2.2. Eligibility

Persons between 2-18 years of both sexes with HBsAg for more than 6 months, positive HBV DNA, and patients with or without high ALT, AST, and GGT values.

2.3. Exclusion Criteria

All individuals who did not meet the above-mentioned criteria, were co-infected with hepatitis C (HCV) and D (HDV) viruses, patients with human immunodeficiency virus type 1 (HIV-1), also, patients who had used or were treated with specific anti-HBV therapies were excluded.

2.4. Control Subjects

Matched (age and sex) seventy-five outwardly healthy children were collected from the outpatient's clinics of the same hospitals included in the study, during their routine follow-up after resolution of an upper respiratory tract infection, and they were excluded from having any liver disease by history, clinical examination and full hepatitis B and C serologic investigations. For inactive chronic HBV carriers, eighteen age and sex-matched healthy children were selected from the total 75 enrolled control children for statistically balanced comparison.

Both patients and controls were subjected to detailed history taking, including HBV vaccination status, and complete clinical examination, including anthropometric measurements

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(weight, height, BMI) that were plotted on growth charts optimal for their age, sex, chest, heart, abdominal and neurological examination.

The study was designed respecting the expected ethical aspects according to the Declaration of Helsinki 1975, as revised in 2008, and was approved by the Research Ethics Committee of El-Minia University and South Valley University hospitals. All guardians of included children agreed to contribute to the study by signed informed consent forms.

2.5. Sampling

Vacuum tubes containing ethylene-diamine-tetra-acetic acid (EDTA) as an anticoagulant were used to obtain blood samples from patients and controls. After centrifugation, peripheral blood cells and plasma were collected and processed at -20°C before the study.

All collected serum samples were tested for the presence of hepatitis B surface antigen (HBsAg) using commercialized kits (AUSRIA, Abbott Laboratories, North Chicago, IL). Negative samples were further tested for hepatitis B core antigen (anti-HBc) using standard testing kits (Corab, Abbott Laboratories, North Chicago, IL) and hepatitis B envelope antigen (HBeAg) and antibodies (anti-HBe) serum using commercialized kits (AUSRIA, Abbott Laboratories, North Chicago, IL). All samples were tested using ELISA (Chiron, Emeryville, CA) for the presence of antibodies to the hepatitis C (anti-HCV) and hepatitis D (anti-HDV) virus in the serum according to manufacture instructions.

2.6. EGF Genotyping

2.6.1. EGF Genotyping was Done According to Sergany et al. 2017 Using

In vitro blood kit for DNA purification (PureLink® Genomic DNA Kits). Genotyping assay was performed for EGF polymorphism at position 61 (A > G) (SNP rs4444903) using qPCR based on TaqMan probes (TaqMan® Universal Master Mix II) based on manufacturer-designed allele-specific probes. Primer sequences used in PCR amplification, which was performed in a thermal cycler (Applied Biosystems) was done according to Sergany et al., 2017 [17].

2.7. Statistical Analysis

Data were computed and analyzed by SPSS (Statistical Package for the Social Sciences) program version 19.0. Non-numerical data were presented in terms of percentages, while numerical data were presented as mean \pm SD. Data that were not normally distributed were expressed as a median. Chi-square was used to compare between non-parametric tests. The significance level was set if p -value ≤ 0.05 .

3. RESULTS

In this study, 100 chronic HBV patients were enrolled (43 were males and 57 were females), their median age was 12 years, while 75 healthy children with a median age of 11 years were enrolled as controls (37 were males and 38 were females). Significant differences between patients and controls were found regarding AST, ALT, ALP, GGT, and total bilirubin ($p < 0.001$ for all), as shown in Table 1.

Regarding EGF, the percentage of AA and AG genotypes was significantly lower in HBV infected children than controls but, the GG genotype was significantly higher in HBV infected children than the control group ($p < 0.001$). Also, total G alleles were significantly higher than total A alleles in HBV ($p < 0.001$), as shown in Table 2.

The inactive carrier group involved 18 patients (18%) while active carriers were 82 (82%), 89% were in phase IV, and 11% were in phase I of chronic HBV infection status. In the inactive carriers, the percentage of AA genotypic expression was higher than AG expression, and no GG expression was found in this group. An allele was 100% expressed, while total G allele expression was 33.3% among the HBV-inactive group. GG was expressed in more than half of patients with active HBV. Moreover, total G allele presentation was 96.3% of active HBV carriers ($p < 0.001$ for all), as shown in Table 3.

In the disease group, cirrhotic changes were found in 54 (54%) children, while 46 (46%) of them were non-cirrhotic with higher GG expression in 64.8% of cirrhotic patients. A predominance of G alleles was presented in 98.1% of them ($p < 0.001$ for all), as shown in Table 4.

Table 1. Some demographic and laboratory parameters of studied children.

Parameter	Active carrier N= 82	Control N=75	p-value	Inactive carrier N= 18	Control N= 18	p-value
Median age (years)	11	13	0.7	12	11	0.66
Sex (M/F)	33/49	36/39	0.54	8/10	9/9	0.81
Median age at the appearance of 1 st symptoms (month)	21.2	-----	-----	25.1	-----	-----
Weight for age centile (th)	53	58	0.49	51	58	0.61
Height for age centile (th)	61	69	0.68	66	69	0.71
BMI centile (th)	77	72	0.7	75	72	0.76
Median TB (mg/ml)	1.9	0.8	0.001*	1.3	0.8	0.04*
Median DB (mg/ml)	0.5	0.2	0.001*	0.3	0.2	0.7
Median AST (IU/L)	71	25	0.001*	60	25	0.001*
Median ALT (IU/L)	60	28	0.001*	48	28	0.01*
Median GGT (IU/L)	52	30	0.001*	39	30	0.04*

(Table 1) contd....

Median ALP (IU/L)	230	190	0.001*	178	171	0.65
HBe Ag positivity (%)	95%	-----	-----	55%	-----	-----
Anti-HBe positivity (%)	3%	-----	-----	21%	-----	-----
Anti-HBc positivity (%)	100%	-----	-----	85%	-----	-----
Median Serum albumin (mg/ml)	3.6	3.9	0.001*	3.2	3.9	0.03*
Median Glucose (mg/dl)	88	70	0.001*	90	73	0.01*
Median WBC	8.8	5.9	0.001*	8.8	5.9	0.04*

th= percentile, TB= total bilirubin, DB=Direct bilirubin, ALT=alanine aminotransferase, AST=aspartate aminotransferase, GGT=gamaglutamyl aminotransferase, ALP= alkaline phosphatase, WBC= white blood cell count. *= Significant ($p<0.05$).

Table 2. Comparison between patients and controls regarding EGF genotypes.

Parameter	Active carrier N=82	Control N=79	p-value	In active carrier N=18	Control N=18	p-value
AA	3 (3.75%)	29 (38.7%)	0.001*	12(66.7%)	16	0.04*
AG	31 (37.8%)	44(58.7%)		6 (33.3%)	2	0.03*
GG	48 (58.5%)	2 (2.7%)		0 (0%)	0	-----
A allele	34 (41.5%)	73 (97.3%)		18 (100%)	18	-----
G allele	79 (96.3%)	20 (26.7%)		6 (33.3%)	2	0.04*

*= Significant ($p<0.05$)

Table 3. Relation between the outcome of HBV in children with EGF genotyping and some demographic data.

Parameter	Outcome of chronic HBV infection		
	Inactive carrier N=18	Active carrier N= 82	p-value
Age (years)	12	11	0.88
Sex (M/F)	8/10	33/49	0.79
HBe Ag positivity (%)	95%	55%	0.01*
Anti-HBe positivity (%)	3%	21%	0.001*
Anti-HBc positivity (%)	100%	85%	0.001*
EGF genotyping			
AA (n %)	12(66.7%)	3 (3.75)	0.001*
AG (n %)	6 (33.3%)	31 (37.8%)	
GG (n %)	0 (0%)	48 (58.5%)	
A allele (n %)	18 (100%)	34 (41.5%)	0.001*
G allele (n %)	6 (33.3%)	79 (96.3%)	0.001*

M= male, F=female. *= Significant ($p<0.05$)

Table 4. Association between the presence of cirrhosis with EGF genotyping and some demographic data.

Parameter	Liver cirrhosis		
	Non-Cirrhotic changes N= 46	Cirrhotic changes N= 54	p-value
Age (years)	13	12	0.79
Sex (M/F)	21/25	20/34	0.42
EGF genotyping			
AA (N/%)	14 (30.4%)	1(1.9%)	0.001*
AG (N/%)	19 (41.3%)	18 (33.3%)	
GG (N/%)	13 (28.3%)	35 (64.8%)	
A allele(N/%)	33(71.7%)	19 (35.2%)	
G allele(N/%)	32 (69.6%)	53 (98.1%)	

M= male, F=female. *= Significant ($p<0.05$).

4. DISCUSSION

EGF rs4444903 G allele was associated with the severity

of the chronic HBV disease progression in adults' research. In addition, end-stage liver disease patients and those who are

eligible for liver transplantation were found to have a maximal G allele representation and are less common among active and inactive chronic HBV carriers [14].

As the EGF rs4444903, A > G is a functional polymorphism, the longer half-life of EGF messenger RNA in the G genotype augments excessive EGF production and secretion. The allelic and genotypic distributions of this polymorphism show ethnic differences in HBV-infected patients [14].

To the best of our knowledge, this study is the first to search about the association between the outcome of chronic HBV infection in children and EGF rs4444903 A > G gene polymorphism.

No significant differences were noted between our patients with HBV and controls regarding age, sex distribution, or anthropometric indices. Significant differences between them regarding their laboratory investigations, including their liver functions that were significantly elevated in HBV infected children. Also, a higher mean fasting blood glucose and total leucocytic count were detected.

This study revealed that the most prevalent EGF gene polymorphism in HBV infected children was GG genotype. In the control group, AG genotype was found with dominant G allele in patients, whereas A allele was dominant in controls. Different studies documented early expression of the G allele early in the disease course with accelerated disease progression [16, 18 - 20].

The “inactive carrier” state indicates stoppage of the active viral replication when HBeAg becomes negative, hepatitis B e antibody (anti-HBe) appears (sero conversion), and transaminase levels normalize with persistent lower HBV DNA (phase III chronic HBV). On the other hand, chronic active hepatitis is the other sequelae of chronic HBV infection, a condition that may progress to chronic hepatic insufficiency, hepatic decompensation, cirrhosis, and end-stage liver disease (phase IV chronic HBV infection) [21].

Among studied HBV infected children, 82% were active carriers, and (12%) were inactive carriers. The G allele, as well as the G/G genotype of the EGF rs4444903 A > G polymorphism, were often detected in chronic active HBV carriers and in liver cirrhotic patients carrying more adverse disease outcome. More cirrhosis incidence was associated with the carriage of at least one G allele with stabilization of other factors. In chronic hepatitis C virus patients (with similar hepatic pathology to HBV infection), the G allele expression showed faster disease morbidity progression [19].

In an Egyptian study on EGF Gene Polymorphism among HCC patients with hepatitis C G/G genotype was dominated by 84%, whereas genotype A/G was found in only 10%. Among cirrhotic patients, A / G was detected in 70% of cirrhotic patients. On the other hand, A / A was dominating in the control group by 84%. This is also supported by the study of Jiang *et al.* 2015 [22]. These findings suggest that the G allele could have a role in hepatocarcinogenesis, whereas the A/A genotype could have a protecting role [23].

A previous study showed high expression among end-stage

HBV patients who were eligible for liver transplantation, which is less frequent between chronic HBV active and inactive carriers and G alleles expression among cirrhotic compared to non-cirrhotic children [18]. This expression is followed by EGF/EGFR signaling pathway, which mediates hepatocyte proliferation and liver regeneration in response to chronic injury [24 - 26].

Opposite to our results was the study included a cohort of clinically stable chronic inactive HBV patients compared to a group of cirrhotic HBV patients and healthy controls. No significant differences were found in EGF rs4444903 polymorphism allelic and genotypic expression among inactive HBV patients and those with or without cirrhosis or HCC [20]. Also, a study conducted by Li *et al.* showed no difference in the allelic and genotypic presentation of EGF in cirrhotic patients when compared to healthy ones [27]. The previous two studies contradict the probability of the EGF rs4444903 A > G polymorphism role in an unfavorable disease outcome of chronic HBV infection. The difference between our results and previous studies may be due to genotype ethnic differences between Egyptian children and their study population, which makes this comparison of limited value.

CONCLUSION

EGF rs4444903 A > G polymorphism with dominant G allele is associated with chronic active HBV and a higher chance of cirrhosis development, predicting worse disease outcome in chronic HBV infected children. In contrast, the A/A genotype may play a protective role.

LIMITATIONS OF THE STUDY

The limitations of the current study are mainly the limited number of patients as the study was not funded.; There was a lack of information concerning maternal HBsAg/HBeAg status. Most children acquiring HBV-positive in the post-vaccination era had received vaccinations appropriate for their ages. The small number of enrolled patients, lack of adequate financial resources to make the HBV genotypic assay, and lack of hepatic biopsy confirmation for the presence of cirrhosis which was difficult to perform for ethical considerations, were the limitation factors of our study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Research Ethics Committee of El-Minia University and South Valley University Hospitals.

HUMAN AND ANIMAL RIGHTS

No animals were used as the basis of this study. All the human research procedures were conducted according to the Declaration of Helsinki 1975, as revised in 2008.

CONSENT FOR PUBLICATION

Written consents were obtained from patients' caregivers.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

Dr. Sherief Abd-Elsalam is the Associate Editorial Board Member of The Open Biomarkers Journal.

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