# Epidermal growth factor polymorphism (rs4444903) predicts hepatocellular carcinoma in Egyptian population

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## ABSTRACT

*Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy in the world, with an increasing worldwide prevalence. Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide. Epidermal growth factor (EGF) and its receptor play critical roles in carcinogenesis. A functional polymorphism in the EGF gene has been linked to increased cancer susceptibility. This study was aimed to investigate the association between both the EGF +61A/G polymorphism & its serum level and the risk for hepatocellular carcinoma (HCC) in Egyptian patients.* ***Methods:*** *We analyzed 140 (HCC) & 100 chronic hepatitis patients without any focal lesion. In addition to 110 subjects as a control group. All were subjected to PCR and genotyping of EGF +61A/G polymorphism was assessed by PCR-restriction fragment length polymorphism.* ***Results:*** *We found a significant difference between patients with HCC and those with chronic hepatitis versus controls in terms of the G allele.* ***Conclusion****: EGF +61GG genotype might be correlated with development of HCC as a risk factor in Egyptian patients with chronic liver disease.*

***Key words:*** *Carcinoma, Hepatocellular, HCC, Polymorphisms, rs4444903.*

## 1. INTRODUCTION

Hepatocellular carcinoma is one of the five most common cancers worldwide, with a particularly high prevalence in Asian countries due to endemic hepatitis B virus infection ***(Parkin et al., 2001).*** The incidence of HCC is also rising in Western countries as a result of increasing hepatitis C virus infection ***(El-Serag et al., 2003)***. More than 80% of patients with HCC have associated cirrhosis and impaired liver function, making treatment of HCC more difficult than many other cancers.

Hepatocellular carcinoma (HCC) is highly endemic in Africa (***Patel et la., 2015).*** There is a general anecdotal consensus that persons with HCC induced by viral hepatitis or other causes in Africa present at younger ages than in other regions of the World. This has resulted in recommended guidelines that surveillance of African born persons at risk for HCC should begin at the age of 20 years ***(Boespflug et al., 2015)***

EGF was discovered by Dr. Stanley Cohen more than half a century ago ***(Cohen***

special issue for the second international conference on genetic engineering & biotechnology "biotechnology and artificial intelligence towards sustainable development" (baisd-2019), 25-26th december, 2019, alexandria, Egypt.

***1962)***. EGF was isolated, purified, and characterized. It is a single-chain polypeptide consisting of 53 amino acids that is derived from the cleavage of a large precursor, prepro-EGF. Urogastrone, an inhibitor of gastric acid secretion, was independently isolated from human urine and was subsequently found to be structurally and functionally identical to mouse EGF and was proven to be human EGF ***(Gregory1975)***. EGF is now known as the prototype of the group I EGF family that also includes transforming growth factor- α (TGF- α), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen ***(Schneider & Wolf 2009)***.

EGF expression EGF has been detected in a variety of body fluids, such as milk ***(Nojiri et al., 2012)***, saliva (***Carpenter & Cohen 1979***), urine ***(Fisher et al., 1989)***, plasma (***Carpenter & Cohen 1979***), intestinal fluid ***(Nair et al., 2008)*** , amniotic fluid ***(Hofmann et al., 1990)***, and others ***(Fisher et al., 1990)***, which is locally produced and secreted by the lactating breast, sub maxillary gland, kidney, Brunner's glands of the duodenum, and placenta, respectively. Sub-maxillary gland is the major EGF producing site in mice, where it is synthesized, processed and stored in granules of the tubular duct cells. Consequently, EGF concentrations are high in mouse saliva ***(Carpenter & Cohen 1979).***

The epidermal growth factor (EGF) rs4444903 A > G polymorphism, involving an A > G transition at position 61 of the 50 untranslated region of the EGF gene, is associated with several types of cancer ***(Zhang et al., 2010)***, including malignant melanoma ***(Randerson-Moor et al., 2004)***, esophageal cancer ***(Lanuti et al., 2008)***, gastric cancer ***(Hamai et al., 2005)***, breast cancer ***(Araujo et al ., 2009)*** and colorectal cancer ***(Wu et al., 2009).*** Carriers of the G allele, especially those with the G/G genotype, may have an increased risk of developing hepatocellular carcinoma (HCC), as shown in a series of European ***(Tanabe et al., 2008)***, American ***(Abu Dayyeh et al., 2011)*** and Asian studies ***(Chen etal., 2011)***. The association with HCC, however, was not confirmed by all studies ***(Qi et al., 2009)***. So. The aim of this study is to investigate the association of the EGF +61A/G polymorphism & its risk for hepatocellular carcinoma (HCC) in Egyptian patients.

## 2. MATERIALS AND METHODS

### 2.1 Patients

The study included 140 consecutive Egyptian patients with HCC. The majority of the patients had been referred to National Liver Institute Hospital, Menoufia University, Egypt for the diagnosis and treatment of HCC. Approximately two thirds of patients had chronic hepatitis. The diagnosis was confirmed in all cases by the histological evaluation of a liver biopsy specimen and laboratory findings such as hypo-albuminemia, INR increase and low platelets count. The control group consisted of 110 healthy Egyptians, Control subjects did not have any clinical and/or laboratory evidence of liver disease or of any other major pathological condition. The median age was 56 years for HCC group, 57 for Chronic Hepatitis group and 55 for healthy group.

### 2.2 Biochemical parameters

Liver function tests were done for all patients including ALT, AST, Albumin, Bilirubin Total and Direct. Creatinine, AFP, Prothrombin time with INR. In addition to hematological parameters including Hb%, platelets and total leucocytes count.

### 2.3 Molecular biology

Genotyping of the EGF rs4444903 A > G polymorphism was performed using a polymerase chain reaction-based restriction fragment length polymorphism assay. Genomic DNA was extracted from whole blood samples using ABIOpure ™ Total

DNA (Version 2.0) Blood / Cell / Tissue Extraction Alliance Bio. According to the manufacturer's instructions. And with elution in 200 µl with elution buffer DNA abtained then was ready to use. A 242 base pair (bp) product was obtained by polymerase chain reaction technique by using specific primer as follow: 5´- TGT CAC TAA AGG AAA GGA GGT-3, (forward) 5´- TTCACAGAGTTTAACAGCCC-3 (reverse), which were designed using NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

PCR amplification was carried out in a total volume of 25 µL. Samples containing 10 ng of genomic DNA were subjected to pre denaturation at 95ºC for 10 min followed by 35 cycles of denaturation (at 95 ºC for 30 s), annealing (at 58 ºC for 45 s) and elongation (at 72 ºC for 60 s). In a total volume of 30 µL, 10 µL of the amplicons were digested with 1 unit of the Alu-I fast digest restriction enzyme (#FD0014-Thermo Fisher Scientific Inc.) at 37 ˚C for one hour. The digested fragments were 102, 94, 34 and 15 bp for the A allele and 193, 34 and 15 bp for the G allele variant. The fragments were resolved by electrophoresis on 2.5% agarose gels after staining with ethidium bromide dye.

## 2.4 Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

### 2.5 The used tests were

1 - Chi-square test
For categorical variables, to compare between different groups

2 - F-test (ANOVA)
For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons.

3 – Kruskal Wallis test
For abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons.

4– Hardy-Weinberg
The population of the studied sample was explored to find its equilibrium with Hardy-Weinberg equation.

## 3. Results

There were no statistically significant differences (P>0.05) between all groups in terms of age and sex distributions (table1).

### 3.1 Biochemical parameters results

The serum levels of AFP, albumin, Prothrombin, INR, platelets, bilirubin total &direct, alanine aminotransferase and aspartate aminotransferase were significantly higher in HCC and CH compared to control groups ($P < 0.001$) (Table). Whereas Serum creatinine levels were significantly higher in HCC compared to the controls ($P < 0.001$) than CH patients compared to the controls ($P < 0.006$) (table2).

### 3.2 EGF genotyping of patients

RFLP was used to determine the EGF SNP genotype in 140 patients with HCC and 100 patients with either hepatitis or liver cirrhosis. The ratios of A/A, A/G, and G/G genotypes were 12.1%, 35.7%, and 52.1%, respectively, in the 140 patients with HCC, whereas in the 100 patients with either hepatitis or liver cirrhosis were 17.0 % , 38.0% , 45.0% respectively. The frequencies of the EGF polymorphism in

this study population were consistent with Hardy–Weinberg equilibrium (P=0.077)(table4).

## 4. Discussion

Hepatocarcinogenesis is thought to be deeply associated with chronic HBV or HCV infection, and in fact, a certain proportion of chronically infected HBV and HCV individuals will develop HCC. Thus, multiple genetic and epigenetic factors may affect HCC development in this background ***(Liovet et al., 2008)***. Among the genetic alteration, the dysregulation of EGF/EGFR signaling pathway is thought to be one of the most important factors in early Hepatocarcinogenesis ***(Komuves et al., 2000).***

More over some patients without known risk factors eventually develop HCC (***El-Serag & Mason 2000)***. Therefore genetic predisposition may contribute to the process of hepatocarcinogenesis**.** Some studies have reported an association between EGF 61*A/g and HCC risk ***(Abu Dayyeh et al ., 2011)***. The EFGR signaling pathway is thought to be an important mediator of hepatocyte proliferative capacity and liver regeneration as a result of chronic liver injury ***(Natarajan et al., 2007)***. Dysregulation of the EGF receptor signaling pathway plays an important role in early hepatocarcinogenesis and another tumorigenesis ***(Ettinger 2006)***. Modulating EGF levels by which the EGF gene polymorphism may lead to increased risk of HCC. Other studies of the EGF 61*A/G polymorphism and HCC risk failed to find an association (Table 3).

The most likely reason for the inconsistencies among these studies is that most are single case control studies with small sample sizes. To help resolve these conflicting results using a larger sample size. Our results for the total population suggest an increased HCC risk for subjects carrying the EGF 61*G/G genotype, and a protective effect for the A/A genotype. The 61*G allele was highly associated with increased risk of HCC based on allelic contrast, homozygote comparison and the recessive genetic model.

Our findings are in line with those of a recently published meta-analysis showing that the EGF 61*G/G genotype in Caucasians is associated with increased risk of glioma ***(Tan et al., 2010)***, and recurrence of liver metastases ***(Kovar et al., 2009)***.

EGF up regulations are a characteristic of cirrhotic liver disease ***(Feren et al., 2000).*** EGF is a mitogen for adult and fetal hepatocytes grown in culture and its expression is up regulated during liver regeneration ***(Mullhaupt et al., 1994)***. Mounting evidence supports a role for EGF in malignant transformation and tumor progression ***(Stoscheck & King 1986)***.

The mechanism by which increased EGF expression is associated with the polymorphism of the 5-untranslated region of the EGF gene is currently unknown. One plausible explanation is the proximity of the – 61G locus to a region involved in EGF gene regulation ***(Bhowmick et al., 2004).*** To date, the role of EGF genetic variants in HCC susceptibility of Egyptian patients with chronic liver diseases has not been reported. Therefore, the association between the functional EGF 61A/G polymorphism in the promoter region and the risk of HCC in patients with chronic liver diseases was investigated in this study.

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**Table (1): Comparison between the three studied groups according to demographic data**

| | Group I (n = 140) | | Group II (n = 100) | | Group III (n = 110) | | Test of Sig. | p |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | | |
| **Sex** | | | | | | | | |
| **Male** | **78** | **55.7** | **57** | **57.0** | **53** | **48.2** | $\chi^{2=}$ | 0.365 |
| **Female** | **62** | **44.3** | **43** | **43.0** | **57** | **51.8** | 2.014 | |
| **Age (years)** Mean ± SD. | 55.62 ± 6.78 | | 56.69 ± 8.78 | | 54.85 ± 6.04 | | F= 1.727 | 0.179 |

$\chi^2$: **Chi square test** **F**: **F for ANOVA test**
p: p value for comparison between the three group
**Group I**: HCC patients **Group II**: Chronic Hepatitis **Group III**: Control

**Table (2) Biochemical data of all studied group (mean ±SD)**

| parameters | Group I Control (n=110) | Group II Chronic hepatitis (n=100) | Group III HCC (n=140) | P |
|---|---|---|---|---|
| | Mean ±SD | Mean ±SD | Mean ±SD | |
| TLC | 5.82±0.55 | 5.97±1.66 | 5.82±2.13 | 0.162 |
| Hemoglobin | 12.81±0.89 | 12.44±1.53 | 12.67±1.64 | 0.168 |
| Platelets | 193.5±49.53 | 140.0±58.66 | 131.8±57.81 | <0.001* |
| Alpha-fetoprotein | 1.19±1.57 | 5.48±5.25 | 690.33±125.30 | <0.001* |
| Total bilirubin | 0.82±0.19 | 1.16±0.55 | 1.26±0.63 | <0.001* |
| Direct bilirubin | 0.17±0.03 | 0.58±0.94 | 0.55±0.63 | <0.001* |
| Albumin | 4.57±0.49 | 3.56±0.74 | 3.40±0.63 | <0.001* |
| Prothrombin (%) | 98.29±2.29 | 67.64±12.91 | 75.36±15.47 | <0.001* |
| ALT | 24.06±6.29 | 49.78±37.78 | 44.4±32.47 | <0.001* |
| AST | 28.45±5.24 | 57.74±35.88 | 58.77±46.8 | <0.001* |
| Creatinine | 0.891±0.19 | 0.89±0.23 | 0.92±0.23 | <0.001* |

P:P value for comparison between the three groups
*: statistically significant at p≤0.05

**Table (3):Comparison between the three studied groups according to RFLP**

| | Group I (n = 140) | | Group II (n = 100) | | Group III (n = 110) | | $\chi^2$ | p | Post hoc test | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | | | I vs. II | I vs. III | II vs. III |
| **RFLP** | | | | | | | | | | | |
| AA | 17 | 12.1 | 17 | 17.0 | 39 | 35.5 | 31.285* | <0.001* | 0.436 | <0.001* | 0.001* |
| AG | 50 | 35.7 | 38 | 38.0 | 46 | 41.8 | | | | | |
| GG | 73 | 52.1 | 45 | 45.0 | 25 | 22.7 | | | | | |
| **Allele** | | | | | | | | | | | |
| A | 84 | 30.0 | 72 | 36.0 | 124 | 56.4 | 37.545* | <0.001* | 0.166 | <0.001* | <0.001* |
| G | 196 | 70.0 | 128 | 64.0 | 96 | 43.6 | | | | | |

$\chi^2$: **Chi square test** p: p value for comparison between the three group
**Group I**: HCC patients **Group II**: Chronic Hepatitis **Group III**: Control

**Table (4): A simple calculator to determine whether observed genotype frequencies are consistent with Hardy-Weinberg**

| RFLP | Observed | Expected | $\chi$2 | P |
|---|---|---|---|---|
| HCC patients (n=140) | | | | |
| AA | 17 | 12.6 | | |
| AG | 50 | 58.8 | 3.136 | 0.077 |
| GG | 73 | 68.6 | | |
| Chronic Hepatitis (n=100) | | | | |
| AA | 17 | 13.0 | | |
| AG | 38 | 46.1 | 3.075 | 0.079 |
| GG | 45 | 41.0 | | |
| Control (n=110) | | | | |
| AA | 39 | 34.9 | | |
| AG | 46 | 54.1 | 2.471 | 0.116 |
| GG | 25 | 20.9 | | |

If P < 0.05 - not consistent with HWE.
Not accurate if <5 individuals in any genotype group.

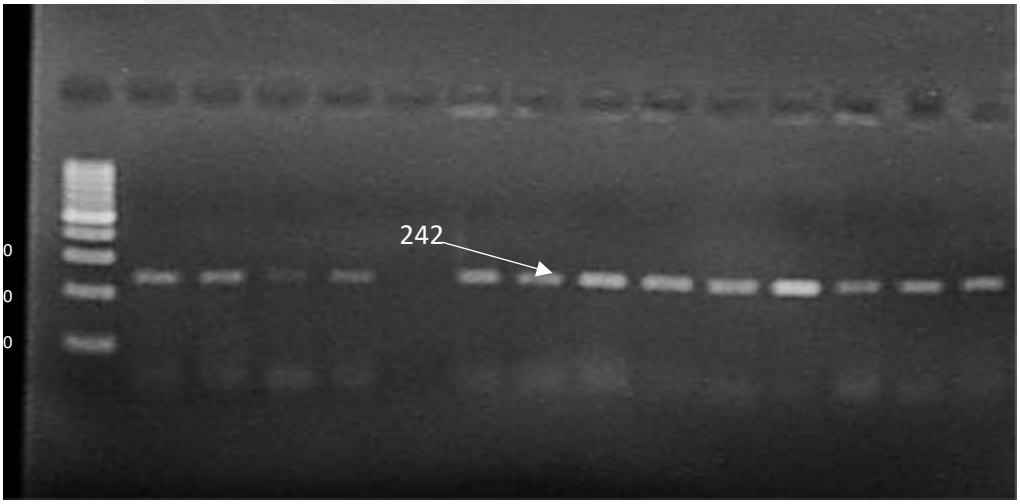


**Fig 1: Electrophoresis on 2 % agarose Gel for EGF gene showing band in 242bp.**

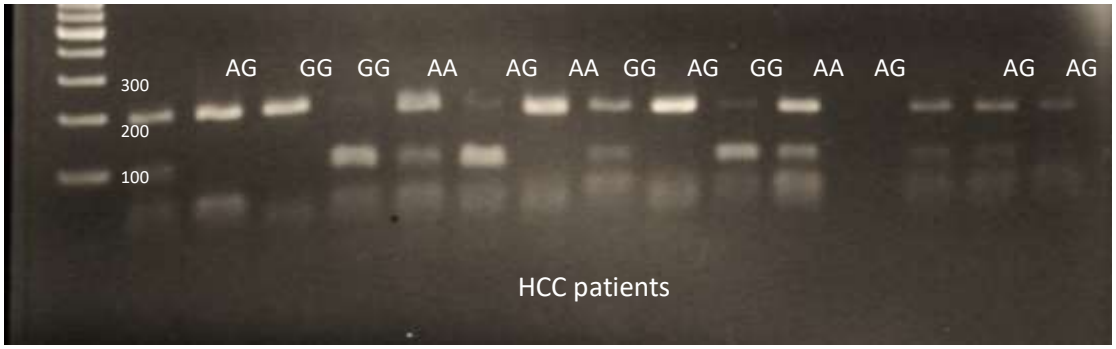


**Fig 2: 242 pb PCR /RFLP product electrophoresis on 2.5% agarose gel For HCC patients show that bands for G allele were 193.34and 15 and for A allele were 102 ,91,34 and 15.**

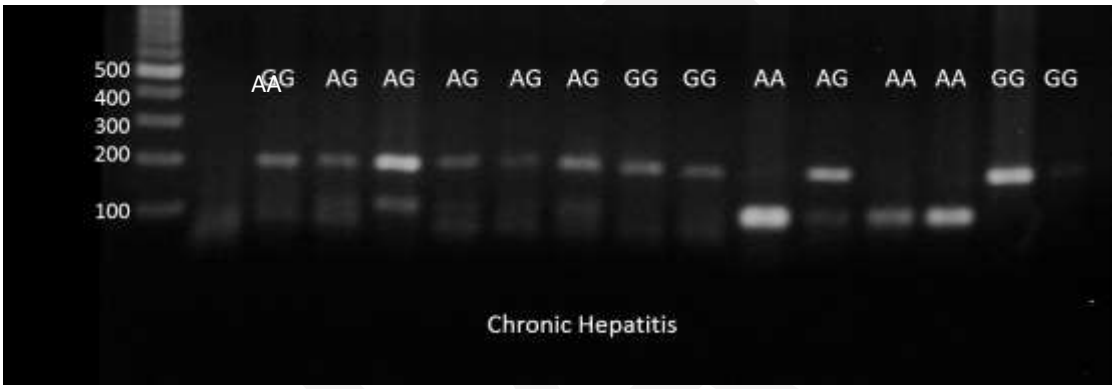


Fig 3: 242 pb PCR /RFLP product electrophoresis on 2.5% agarose gel For Chronic Hepatitis patients show that bands for G allele were 193.34and 15 and for A allele were 102 ,91,34 and 15.

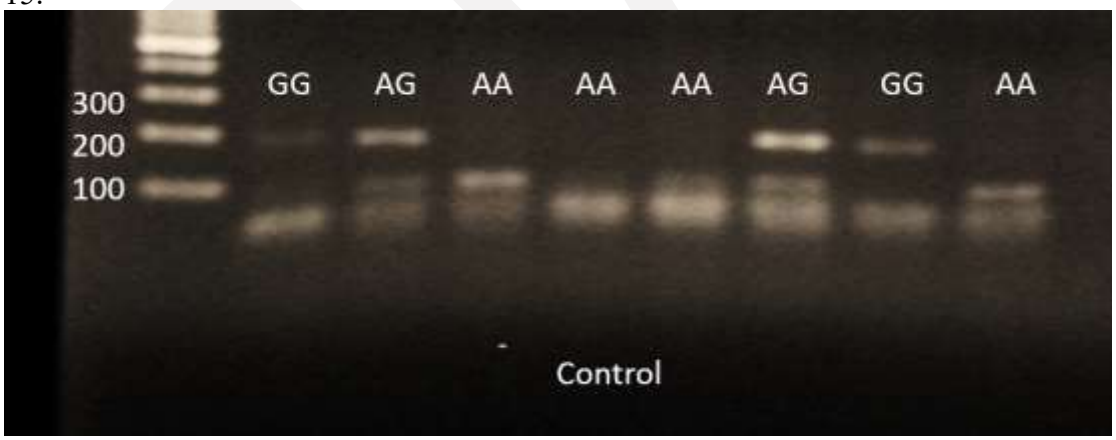


**Fig 4: 242 pb PCR /RFLP product electrophoresis on 2.5% agarose gel For healthy control show that bands for G allele were 193.34and 15 and for A allele were 102 ,91,34 and 15..**