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RESEARCH ARTICLE

EPIDERMAL GROWTH FACTOR GENETIC POLYMORPHISM AND ITS CIRCULATING SERUM LEVEL PREDICT THE RISK OF HEPATOCELLULAR CARCINOMA IN EGYPTIAN PATIENTS WITH HCV (Genotype-4)-RELATED CIRRHOSIS.

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Abstract

Background & Aim of the work: 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 HCV (G4) - related cirrhotic Egyptian patients**. Methods**: We analyzed 133 (HCC) (group I) & 105 HCV (genotype-4)-related cirrhotic patients without any focal lesion (group II). In addition to 100 subjects as a control group (group III). All were subjected to PCR HCV, HCV genotyping & ELISA assay of serum level of EGF. EGF +61A/G polymorphism was assessed by PCR-restriction fragment length polymorphism. **Results**: The carriage for allele G of EGF +61A/G (SNP) was significantly associated with development of HCC compared with long term cirrhotic group (OR = 4.0404, 95 % CI 2.3275. 7.014, p= 0.0001). A significantly elevated EGF serum level in HCC group was found when compared with both cirrhotic and normal control groups (P<0.001). It is found that the value of 375 ng/ml is a cutoff point level of developing HCC among cirrhotic patients at 85.6% sensitivity and 87.6% specificity. **Conclusion**: Our data suggest an increased risk to develop HCC in Egyptian patients with HCV (G4) carrying the G allele of EGF +61A/G SNP and with serum level of >375 ng/ml.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the third most common cause of cancer mortality (El-Serag, 2004 and Williams, 2006). This tumour, which arises from hepatocytes, is often associated with liver cirrhosis resulting from chronic liver diseases. Among the environmental risk factors, the prevalence of chronic hepatitis B and C (HCV) virus infections is directly linked to the incidence of HCC (Dragani, 2010).

In Egypt, the incidence of HCC has been nearly doubled over the last decade (Freedman,2006) and Egypt has simultaneously been plagued with the highest prevalence of HCV in the world, ranging from 6% to 28% (Khattab et al., 2011). The prevalence of serological markers of HCV infection in patients with HCC is nearly 80% (Lehman and Wilson, 2009)**.** The incidence and mortality rates for HCC are virtually identical, reflecting the overall poor survival of patients with this tumor.

The definite molecular mechanism of hepatocarcinogenesis is far from clear. The pathogenesis of HCC is quite diverse and influenced by a variety of environmental and genetic factors of the host. Functional polymorphisms that influence an individual's susceptibility to liver cancer include gene products involved in activation of cell proliferation. Gene polymorphisms of this candidate gene (s) play key roles in individual susceptibility to liver cancer. Thus genetic polymorphisms may explain why individuals with shared environmental exposures do not always share cancer morbid (Aravalli et al 2008 and John and Colerangle 2010) .

Despite recent improvements in surveillance programs and diagnostic tools, only 30-40% of HCC patients are eligible for liver resection or transplantation which is the only curative treatment options to date (Llovet &Bruix, 2003). For this reason, identification of molecular markers associated with increased risk of HCC would better define high-risk populations of HCC, helping to improve prevention and treatment strategies.

Epidermal growth factor (EGF) gene is mapped to long arm of chromosome 4 (chromosome 4q25). It is encoded by a 4.8-kilobase (kb) mRNA transcripted from a 110-kb gene containing 24 exons (Salomonet al., 1995). EGF and EGF receptor (EGFR) interaction plays a pivotal role in cell proliferation, differentiation, and tumorigenesis of epithelial tissues. In recent years, numerous studies have been associated a single-nucleotide polymorphism involving an A-to-G mutation at position 61 of the 5' untranslated region of the EGF gene (61 A/G, rs4444903) with the risk of tumorigenesis in multiple human cancers (Shahbazi et al., 2002, Lanutiet al., 2008 and Wang et al., 2009) EGF is one of the candidate genes for HCC.

The aim of the current study was to evaluate genetic factors related to individual susceptibility to HCC on top of HCV (G4). Specifically, individual genetic differences in EGF +61G/A (rs4444903) polymorphism in addition to its serum level were both investigated in HCC cases and long term HCV-related cirrhotic patients.

Study population:

This case-control study enrolled 238 patients, all of which had successful DNA genotyping (133 patients with HCC and 105 patients with HCV (genotype-4)-related liver cirrhosis). In addition to 100 normal healthy blood donor subject served as a control group. Patients were recruited at Tropical Medicine, Mansoura University Hospital between 2008 and 2011. All 238 patients were positive for HCV& had a history of chronic liver diseases for a duration ranging from 8-10 years (when they were first diagnosed). Data abstracted from the medical records of the decompensated patients revealed that signs of liver decompensation appeared 3-5 years ago. All patients and control were subjected to through clinical examination routine liver function tests, abdominal ultrasound; Triphasic computed tomography, α-feto protein (AFP), polymerase chain reaction (PCR) , genotyping for HCV and ELISA assay of serum level of EGF. The diagnosis of HCC was verified histologically, or based on the finding of typical radiological features in two different liver imaging studies showed a mass lesion with characteristics of HCC (vascular enhancement, wash out), or by a single positive imaging technique associated with AFP>400ng/ml. All The exclusion criteria were as follows: autoimmune liver disease, alcohol abuse, hepatitis B virus or HIV coinfection, haemochromatosis , diabetes mellitus and patients with a history of chronic liver disease less than 8-10 years duration.

All participants provided informed written consent. The research protocol was reviewed and approved by Medical Mansoura University Ethics Committee for Human Subject Research. Approval for this study was granted by the research boards at the Mansoura University.

EGF ELISA:

Aliquot of stored plasma (at -80 °C) of our study group was used for assay of plasma EGF using RayBio^{R} Human EGF ELISA Kit (RayBiotech, Inc.). The procedures were done according to manufacturer's instructions as follow:

Each sample was diluted 10 fold with provided diluents. Each sample and standard was analyzed in duplicate. 100 µl of each standard and sample into appropriate wells were incubated over night at 4°C followed by 4 times washing and addition of 100 µL of biotinylated antibody for 1 h and 100 µL of TMB substrate solution for 30 min. Finally, 50 µL of Stop Solution was added to each well. The plate was read immediately in a plate reader (Sunrise TECHAN) with an excitation wavelength of 450 nm. The standard curve was generated for every set of analysis. Samples assayed using the standards provided in the kit and the results were expressed in ng/ml.

DNA extraction and genotyping:

DNA genotyping Laboratory Testing:
Blood specimens were processed immediately after collection and DNA were stored at −70°C until subsequent testing. Genotyping analysis was done on genomic DNA isolated from lymphocytes using QIAamp DNA Blood Mini Kits (Qiagen, Germany).

The EGF rs4444903 A>G gene polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay as previously described by (Guo-yang., et al., 2009).

PCR primers were designed to amplify products of exactly 248 pb using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/) software to generate more appropriate allele-discriminating DNA fragments. A DNA fragment of 248 bp containing the A/G polymorphism of EGF rs4444903 gene polymorphisms was amplified using specific primers: forward primer 5'-AGCAAAGCTGAGTCATTCCACc-3', and reverse primer 5'-TGTTTCTTTGGAAGCCAGTAAGA-3'. PCR was conducted on the ABI 2400 (Applied Biosystems, Foster City,CA,USA) in a system with total volume of 25 µl containing 1 ul genomic DNA, 2.5 µl 10×PCR Buffer, 2.5 mM MgCl2, 0.3 µM each primer, 0.4 mM dNTP, AND 0.40 µl Taq DNA polymerase (Qiagen Germany) (figure 1). The cycling parameters were: initial preheating 94°C for 10 min; 35 cycles at 94°C for 1 min, 56°C for 1 min s, 72°C for 1 min s; and a final extension step at 72°C for 10 min. For each PCR product, 5 µl the PCR reaction was then electrophorsed using 1.5 agrose gel stained with ethidium bromide to check a PCR reaction. Ten microliter of remaining PCR products were digested using 10x NE buffer and 2.0 U of appropriate restriction enzymes (AluI). 10 % of our samples were selected randomly to regenotype at different set as quality control group. The results of the genotyping showed 100% concordance of our study group and control groups.

HCV Genotyping:

HCV genotyping was determined by VERSANT HCV Genotype Assay (LiPA), (Bayer Corporation, Tarrytown, NY, USA). The Amplicor HCV kit and the LiPA were performed according to manufactures′ instructions.

Liver biopsy:
Liver biopsies were obtained with an 18-gauge or larger needle with a minimum of five portal tracts and were routinely stained with hematoxylin-eosin stain. Biopsies were interpreted according to the scoring schema developed by the METAVIR group. Needle liver biopsy specimens were examined by a pathologist unaware of the laboratory results. METAVIR score was used to stage fibrosis (F0–F4). Fibrosis was scored on a 5-point scale: F0, no fibrosis; F1, portal fibrosis alone; F2, portal fibrosis with rare septae; F3, portal fibrosis with many septae; F4, cirrhosis (Poynard et al1997).

Statistical analysis:
Data were computed and statistically analyzed using SPSS soft ware program version 16. For comparison of qualitative variables (presented as frequencies and percentages) Chi square of Fisher Exact test were used. *Hardy*-Weinberg equilibrium (HWE) was assessed in each group separately using χ^2 tests. Logistic regression was used to determine the most predicting variable for HCC. The difference was considered significant if $P \leq 0.05$.

RESULTS:

Our study was included 133 HCC cases and 105 patients with long term HCV (genotype-4)-related liver cirrhosis without any radiological finding for focal lesion. In addition to 100 healthy control subjects were included in this study. All patients were recruited from the Tropical Medicine Department, Mansoura University Hospital. The clinical & laboratory characteristics of the studied groups are summarized in Table (1) where a significant association was found between higher EGF serum level and development of HCC compared with either cirrhotic or

normal healthy control groups. A significant elevation of EGF serum level was found in both HCC& cirrhotic groups (527.4±130.6, 278.2±85.5 respectively) when compared with normal control group (142.1±33) (P<0.001). Furthermore, a significantly elevated EGF serum level in HCC group was found when compared with cirrhotic group (P<0.001).

The distribution of the different EGF polymorphism among different groups is presented in table (2). The allele carriages of EGF SNP in all groups were in Hardy-Weinberg equilibrium (all P > 0.05).

The distribution of EGF polymorphism was 42.9% G/G, 32.3% G/A, and 24.8% A/A in patients with HCC, while in cirrhotic control group was 8.6 % G/G, 34.3 % G/A, and 57.1 % A/A and in normal control group the distribution of genotyping was 50% G/G 36% G/A and 14% A/A.

Also our finding showed that subjects carriage allele G of EGF +61A/G SNP was significantly associated with the development of HCC compared with both long term cirrhotic group (OR = 4.0404, 95 % CI 2.3275 - 7.014, p= 0.0001) and normal control group (OR = 3.0, 95 %CI :1.7209- 5.2298 - P< 0.0001) (Table 3).

Multivariate logistic regression analysis in table (4) showed that the most predictable variables for occurrence of HCC were: level of total Bilirubin (odds ratio = 10.6 (95%CI=(1.6-6.89) , EFG(OR=1.05- 95%CI= 1.01-1.1) and albumin (odd ratio = 37.3(95%CI=1.8-6.98) according to the model in the table (P< 0.001). HCC prediction can be calculated as follow:

HCC prediction= constant + ß1x albumin level + ß2 x EGF level + ß3 x Total bilirubin level. When the result is near 1 the prediction of HCC occurrence increased.

By applying roc curve to determine the reliable cut off point of developing HCC among cirrhotic patients(figure 2) , it is found that the value 375 ng/ml is a cutoff point level of developing HCC among cirrhotic patients at 85.6% sensitivity and 87.6% specificity.

Table (1): Clinical & laboratory Characteristics of studied groups.

	HCC (133)	Liver cirrhosis (105)	Normal control(100)	Significance test		
Sex: M F	118(89.4) 14(10.6)	90(85.7) 15(14.3)	87(87%) 13(13%)	χ^2=0.763, P=0.68		
	Mean±SD	Mean±SD	Mean±SD	P1	P2	P3
Age	56.1±6.2(57.0)	56.5±6.2(57.0)	54.3±7.3(55.0)	P=0.7	P=0.4	P=0.38
Albumin	3.4±0.5(3.4)	2.9±0.5(2.9)	4.39±0.44(4.45)	P≤0.001	P≤0.00	P≤0.00
Bilirubin. Total	8.3±4.0(7.2)	3.3±0.9(3.3)	1.33±0.22(0.8)	t =12.4, P≤0.001	P=0.00	P=0.00
Bilirubin. Direct	3.0±0.8(2.5)	0.8±0.3(0.8)	0.13±0.09(0.12)	t =9.4, P≤0.001	P=0.00	P+0.00
SGPT	50.6±22.8(48)	44.1±8.2(38)	27.14±5.4(27.0)	t =2.4, P=0.018	P=0.00	P=0.00
SGOT	44.6±12.7(43.0)	43.2±12.4(41)	23.35±4.2(22.5)	t =0.8, P=0.4	P=0.00	P=0.00
Alkaline Phosphate	10.9±4.6(10.0)	25.5±1.9(8)	4.17±1.2(4)	t =6.6, P≤0.001	P=0.00	P=0.00
AFP	1172.5±1746.1(592)	25.5±51.8(9)	7.32±1,95(7)	Z*=12., P≤0.001	P=0.00	P=0.00
EGF.serum	527.4±130.6(523.0)	278.2±85.5(295.0)	142.1±33.(141.0)	t =16.9, P≤0.001	P=0.00	P=0.00

P1 when comparing HCC with Cirrhosis, P2 when comparing HCC with normal,
P3 when comparing Cirrhosis with normal. Statistical tests used are χ^2 , Student t test and Mann-Whitney test.

Table (2): Distributions of the EGF +61A/G polymorphism among different groups.

	HCC (133) N (%)	Liver cirrhosis (LC)(105) N (%)	Normal control (NC)100 N(%)	Significance
EGF genotype				
A/A	33 (24.8 %)	60 (57.1%)	50 (50%)	χ^2=30.3
A/G	43 (32.3%)	36 (34.3%)	36 (36%)	P≤0.001
G/G	57 (42.9%)	9 (8.6%)	14(14%)	
Allele frequencies				
A	123 (46.6)	155 (73.8)	68 (68)	χ^2=35.7,
G	141 (53.4)	55(26.2)	32(32)	P≤0.001

Table (3): Association between EGF +61 A/G SNP and development of HCC.

	OR+	95 % CI		Chi-square	*P*
		lower	high		
Overall G HCC vs‡ LC	4.0404	2.3275	7.014	24.04	<.0001
G (%) HCC vs LC	2.8082	1.535	5.1374	10.57	0.001149
Overall G HCC vs NC	3.0	1.7209	5.2298	3.874	0.0001
G (%) HCC vs NC	2.4541	1.234	4.1265	3.44	0.0025

\+ *OR= Odds ratio.*

‡ *Vs= versus*

Table (4): Multivariate logistic regression analysis of independent predictors of HCC.

	β	P	OR(95%CI)
Albumin (continuous)	-3.6	0.019	37.3(1.8-69.8)
EGF (continuous)	0.1	0.018	1.05(1.01-1.1)
Bilirubin total (continuous)	2.4	0.013	10.6(1.6-68.9)
Constant	-53.5		
Model χ^2	312.7,	≤0.001	
% correctly predicted	99.2		

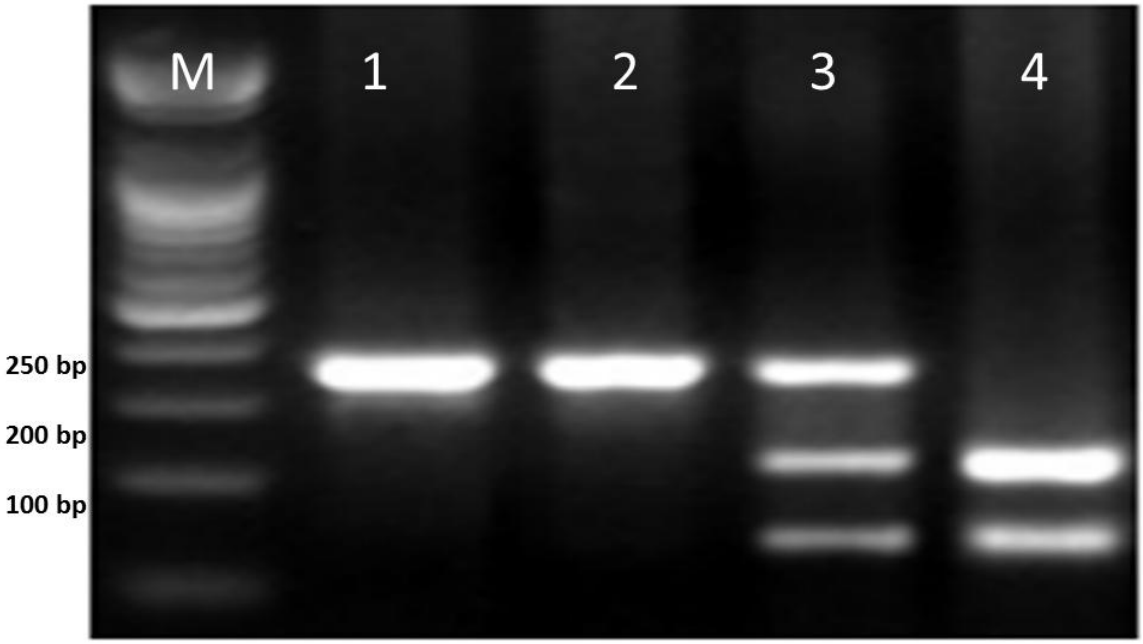
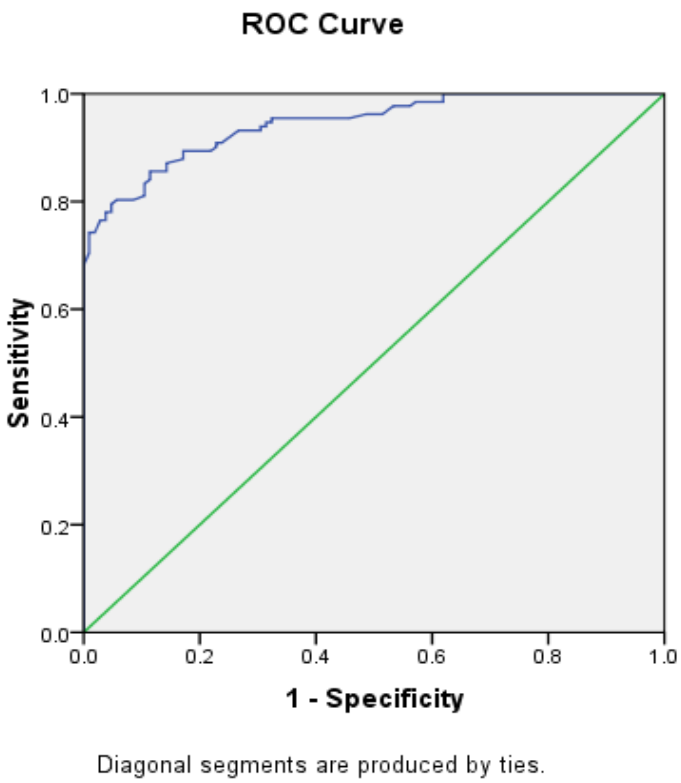


Figure 1 *AluI* RFLP genotyping of *EGF +61A/G*n polymorphism. *AluI* RFLP products were electrophoresed on a 1.5% agarose gel. An example of each genotype is shown and the fragment sizes obtained are indicated in base pairs (bp). The 248 bp of undigested PCR product in lane (1). Lane 2; GG genotype 241 bp. The lane 3; GA genotype with fragment length 241, 150. and 91 bp. The genotype AA is shown in lane 4 with two fragments 150 and 91 bp. . An 7 bp fragment present in all *AluI* genotypes is not visible.

Figure(2):



DISCUSSION:

HCC is a complex, heterogeneous malignancy, the pathogenesis of which involves multiple genetic and epigenetic alterations and modulation of molecular signaling pathways implicated in malignant transformation of hepatocytes and tumor progression (Llovet &Bruix, 2008).

Cirrhosis associated with HBV and/or HCV infection and alcohol are the most well established environmental risk factors for HCC around the world. In fact, cirrhosis is considered a precancerous stage to some extent, although only a fraction of cirrhotic patients and HCV-infected individuals develop HCC later in life (Bowen and Walker 2005). Moreover, some patients without known risk factors eventually develop HCC (El-Serag and Mason, 2000). Therefore, genetic predisposition may contribute to the process of hepatocarcinogenesis.

Dysregulation of the EGF/EGFR signaling pathway is thought to be important in early hepatocarcinogenesis (Komuves et al., 2000). A functional polymorphism in the 5' untranslated region of the EGF gene (61*G) that modulates tissue-specific EGF gene expression has been associated with multiple human malignancies including HCC (Lanutiet et al., 2008 and Xu et al., 2010**]**. This study aimed to evaluate the association between the individual genetic differences in EGF +61G/A (rs4444903) polymorphism and the risk for HCC development in HCV patients.

Previous studies which evaluate the association of the EGF +61A/G polymorphism and the development of HCC have showed conflict results. Our findings seem to suggest that the role of genetic factor in predisposition and/or pathogenesis of HCC. The results confirmed the possible key role for EGF in the mechanism of progression of HCV-cirrhotic liver into HCC stage. Our study found around a 4-fold increased HCC risk with the G allele of + 61 SNP of EGF. As for +61A/G polymorphism of EGF, our results revealed that EGF +61 A allele could be a protective allele against HCC on the top of HCV in Egyptians after adjusting for confounders and other clearly defined HCC risk factors. Also a significant association was found between higher EGF serum level and development of HCC compared with either cirrhotic or normal healthy control groups.

The results of this study were in agreement with some studies. Tanabe et al., 2008, found a significant association of G allele of EGF +61A/G SNP and development of HCC with odd ratio around 4.0 [21]. Also Abu Dayyeh et al., 2011

reported a significant association of EGF genotype G/G with increased risk for HCC in American Caucasian as well as Afro-Americans.

On the other hand, Qi et al. 2009 failed to find a significant association between EGF +61A/G SNP and risk of HCC in Chinese patients with chronic hepatitis B virus infection. This may be due to using healthy individual as control group or may be due to anther loci is linked to HCC on the top of HBV.

EGF promotes cell survival, growth, cell proliferation, differentiation and tumorigenesis via the activation of several integrated signaling pathways (Limaye et al 2008). Over expression of EGF is also associated with growth and invasion of some malignant tumors via autocrine and paracrine pathways (Stoscheck and King 1986). Results from clinical trials indicate that therapies directed against EGFR are promising in the treatment of a variety of cancers, including esophageal cancer (Karamouzis et al 2007).

One mechanism by which the EGF gene polymorphism may lead to increased risk of HCC is by modulating EGF levels. Tanabe et al. (2008) found that EGF secretion was 2.3-fold higher in 61G/G hepatocellular carcinoma cell lines compared to A/A cell lines, and that mRNA transcripts with the G allele showed a longer half-life and increased stability. Tanabe et al. (2008) concluded that the EGF polymorphism rs4444903 is associated with risk for development of hepatocellular carcinoma in liver cirrhosis through modulation of EGF levels.

The EGF/EGFR signaling pathway has been shown to be an important mediator of hepatocyte proliferative capacity and liver regeneration in response to chronic injury (Natarajan et al., 2007). Modulation of EGF levels rather than alteration in EGF receptor expression has been suggested to be the mediator of this regenerative liver response (Komuves et al., 2000). These studies lend biological plausibility to the observation of lower liver fibrosis progression rates among subjects with the 61*G functional polymorphism in the 5' untranslated region of the EGF gene, which predicts increased EGF mRNA expression in hepatocytes and stability in serum. They argue that the observed association between the EGF 61*G functional polymorphism and HCC is not mediated by a more aggressive liver fibrosis course, but perhaps more likely by the inability to down regulate the EGF pathway once cirrhosis has developed, leading to early hepatocarcinogenesis and uncontrolled progression of early HCC.

CONCLUSION

Our findings suggest that increased risk to develop HCC in patients with HCV (genotype-4) - related cirrhosis carrying the G allele of EGF +61A/G single nucleotide polymorphism. Also our study recommend that the EGF serum level with cutoff value 375 ng/ml can be used for clinical prediction of patients with HCV related-cirrhosis who have relative HCC risk for early detection and management of HCC.

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