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Epidermal growth factor gene polymorphism rs4444903 in hepatocellular carcinoma in an Egyptian population: Association with the Circulating Tumor Cells.

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ABSTRACT

The association of hepatocellular carcinoma (HCC) and epidermal growth factor (EGF) gene +61A/G polymorphism (rs4444903) has been studied in several populations. However, the results are contradicted. Circulating tumour cells (CTCs) have long been considered a monitor of tumor cell behavior and aggressiveness. The aim of this study was to address the association of+61A/G EGF rs4444903 polymorphism and association with Circulating Tumour Cells (CTCs) in Egyptian patients with HCC. A total of 140 HCC patients, 100 chronic hepatitis patients and 110 healthy individuals were enrolled in this study. Genomic DNA was extracted from peripheral whole blood of all patients and control. Genotyping of the rs4444903 (A/G) polymorphism was done by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. CTCs was detected by using flow cytometry. Genotyping for HCC patients shows that 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. Mean values of CTCs were significantly higher in HCC patients with EGF genetic variants. CTCs show significantly high correlation with AFP ($p<0.001$) and tumor size ($p<0.008$). Our findings proposed that the gene of EGF (rs4444903) +61A/G polymorphism could be a risk factor for HCC susceptibility in Egyptian population. CTCs counts correlates with tumor size and AFP, suggesting that it may indicates disease progression in HCC patients. Our findings may recommend the assessment of CTCs in patients with HCC especially that carry G-allele of the EGF 61* polymorphism (rs4444903) to improve the clinical outcomes in patients at risk of HCC.

Keywords: Epidermal growth factor, EGF +61A>G polymorphism, Hepatocellular carcinoma. Circulating tumor cell, HCC risk.

Abbreviation's list
Epidermal growth factor (EGF); Hepatocellular carcinoma (HCC)
Circulating tumor cell (CTC); Single Nucleotide Polymorphisms (SNPs)
Mitogen-activated protein kinase (MAPK); Phosphatidylinositol-3' kinase (PI_3K)
Alpha-fetoprotein (AFP); Insulin-dependent diabetes mellitus (IDDM)

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INTRODUCTION

Hepatocellular carcinoma consider one of the major leading cause of cancer-related deaths and it is considered the fifth common malignancy worldwide (**Torre et al., 2015**). HCC vary in the incidence and distribution among different nations with the highest incidence estimated in African and Asian populations **(Choo et al., 2016; Bosch et al., 2004)**. These data suggest a role of genetic factors in the HCC development **(Jain et al., 2010)**.

At the biological level, distinctive genetic signatures could be determined by detecting single nucleotide polymorphisms (SNPs) in human genes. Several SNPs located in human genes sequence have been accompanied with the hepatocarcinogenesis process **(Liao et al., 2018; Jin et al., 2011)**. The epidermal growth factor gene encodes EGF which plays a vital role in tumor progression, invasion, migration, and ultimately metastasis in a variety of malignancies **(Ma et al., 2012; Dong et al., 1991; Zheng et al., 2013).** EGF exerts these biological effects upon binding to its receptor EGFR that results in activation of tyrosine kinase and transduction of the proliferation and survival signals primarily mediated by both mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3′ kinase (PI_3K) **(Zhou *et al*., 2007; Defoe and Grindstaff, 2004)**. Therefore, it is not unusual to find that EGF and EGFR have been involved in malignant transformation of many different cell types **(Kurachi et al., 1985; Bade et al., 2011)**. A SNP at position 61 (rs4444903 -61A>G) of 5′- untranslated region (5′-UTR) of the EGF gene was found to be a functional mutation that results in increased EGF expression and risk of skin malignant melanoma **(Shahbazi et al., 2002)**. Subsequently, several studies have confirmed the relation between EGF A61G and predisposition of various malignancies including HCC **(Oliveira et al., 2010; Lanuti et al., 2008; Jiang et al., 2015)**. Other studies found EGF +61 A>G genotype associated with tumor progression and poor prognosis and shorter overall survival **(Pinto et al., 2009; Yang et al., 2014)**.

Alpha-fetoprotein (AFP) in conjunction with ultrasound every 6 to 12 months are currently the gold standard biomarkers for patients at risk for HCC **(Trinchet et al., 2011; Sherman, 2010)**. However, some studies have indicated that these procedures are far from perfect and have significant limitations as screening tests **(Abdel-Aziz et al., 2016; Singal et al., 2009)**. Moreover, AFP is a less sensitive and specific indicator in predicting the prognosis of patients with HCC, especially for small HCC **(Giannini et al., 2012)**. With the recent advances of cell isolation technologies and in molecular diagnostic techniques, circulating tumor cells (CTC) enumeration and characterization have proved indispensible for clinicians that provide critical information about cancer staging and prognosis **(Vona et al., 2004).** Conclusions of recent meta-analysis studies indicated that CTC positivity is correlated with poor prognosis in patients with HCC **(Fan et al., 2015; Sun et al., 2017)**.

CTC are tumor cells disseminated from both primary and metastatic lesion through the blood circulation that is expected to occur during early stages of tumor development as a result of the activation of epithelial-to-mesenchymal transition (EMT) process **(Mendonca et al., 2019; Yu et al., 2013)**. CTCs can be isolated by non-invasive procedure from the blood or the lymphatic vessels of cancer patients **(Yu et al., 2011; Balic et al., 2005)**. It can thus be used as a ‘‘liquid biopsy’’ to monitor patient’s condition over time **(Alix-Panabieres and Pantel, 2012)**. Several studies reported that the elevated CTCs, despite of ongoing treatment, is an indication of succeeding rapid disease development and worse overall survival in many malignancies such as HCC **(Wang et al., 2019; Wang et al., 2019; Josefsson et al., 2017; Chen et al., 2019)**.

We aimed to determine the feasibility of correlating +61A/G polymorphism of EGF gene with the poor-prognosis indicator CTC as well as with other clinical and biochemical markers used in diagnosis of HCC in Egyptian population.

MATERIAL AND METHODS

Study Subjects

This case-control study included a total of 350 Egyptian subjects grouped into two groups: HCC group (140 patients), and healthy control group (110 individuals). The study was conducted over a period of five months from October 2017 to February 2018. The HCC patients were recruited from the Hospital of National Liver Institute, Menoufia University, Egypt. HCC patients were diagnosed by expert physicians based on pathologic and histopathological criteria **(ICGHN , 2014)**. Patients were excluded for viral or bacterial infection, renal damage, insulin-dependent diabetes mellitus and other malignancy within 5 years. Patients being too old, having received

prior treatment for HCC, having inadequate blood counts, or having another chronic disease were also excluded. The control group was matched with HCC patients for age, sex and ethnical origin. The study was approved by the Ethics Committee and performed in commitment of Good Clinical Practice Guidelines and the Declaration of Helsinki. The study protocol was approved by the Local Ethics Committee of the National Liver Institute (NLI-001.09.2017/1) and all patients and healthy individuals were given a written informed consent. Demographic and clinical data were collected from medical records of patients.

Blood sampling and Laboratory investigation

Fifteen ml of peripheral venous blood were collected from patients and controls and divided into three parts. The first part of blood consists of 7.5 ml collected in ethylene diamine tetra-acetic acid (EDTA) was processed for CTCs enumeration using flow cytometry. The second part consists of 2.5 ml blood in EDTA that was divided into 1 ml for CBC and 1.5 ml for genomic DNA extraction and molecular testing. The third part consists of 1.8 ml of blood that was collected on 0.2 ml sodium citrate (3.8%) for prothrombin time (PT) assay and 3 ml of blood that were placed in a plain tube, left to clot, centrifuged and serum was separated to determine routine liver function tests and Alfa-fetoprotein (AFP).

The number of erythrocytes count, hemoglobin concentration, hematocrit, blood indices, total number of leucocytes, and platelets were determined by cell blood counter Labomed, Inc. SK9000 Sino thinker and the result confirmed by sysmex kx- 21N automated hematology analyzer. All biochemical parameters were measured by standard automated laboratory methods using HumaStar 200 analyser (Human, Wiesbaden, Germany) for the liver function tests (ALT, AST, GGT, albumin, and total bilirubin) and using Tosoh AIA-360 immunoassay analyzer (Tosoh Bioscience, Tokyo, Japan) for the serum AFP according to recommendations provided by manufacturers.

DNA extraction and EGF Genotyping

Genomic DNA was purified from peripheral whole blood using ABIOpure ™ Total DNA (Alliance Bio, USA). Concentration of DNA and purity were checked with kit Nanodrop (Thermo scientific, USA).

The EGF +61A>G (rs4444903) polymorphism was further genotyped using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (PC-RFLP) method, as previously described **(Shahbazi et al., 2002)**. In brief, genomic DNA was subjected to amplification using PCR under the following conditions: 95° C for 7 min followed by 35 cycles of 95° C for 30 s, 58° C for 30 s, 72° C for 1 m and a final step of extension at 72° C for 10 minutes. Sequences of the primers used were as follows: 5´- TGT CAC TAA AGG AAA GGA GGT-3´ (Forward), 5´- TTC ACA GAG TTT AAC AGC CC-3´(Reverse). Subsequently, 1µg of the amplified PCR product was digested with 5 units of AluI fast digest restriction enzyme (#FD0014-Thermo Fisher Scientific Inc. USA) for two hours at 37 °C. Then, RFLP products were separated on a 3% agarose gel electrophoresis and visualized by Gel-Doc Imaging System (E-Box VILBER, France). The 242-bp PCR products containing the +61 A allele were digested by *Alu1*restriction enzyme the into15-, 34- , 91- , and 102-bp fragments whereas the +61 G allele produced 15-, 34- , and193- bp fragments.

Detection of Circulating Tumor Cells (CTCs) by flow Cytometry

Mononuclear cells from whole peripheral blood were obtained by gradient density centrifugation by busing biological grade of Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). Then the obtained cells were fixed for 1 hour by using 1% paraformaldehyde at room temperature followed by permeabilization with 0.01% Triton X-100 at room temperature for 1 hour. Then the fixed cells were incubated with mouse serum (cat no. M5905-Sigma-Aldrich, St. Louis, MO, USA) for 30 min, at room temperature, followed by addition of fluorescent-labeled antibodies: CK19-FITC and CD45-PE, or FITC-mouse IgG1 isotype antibody (MACS; Milteny Biotec, Gladbach, Germany). After incubation for 30 minutes in the dark, cells were washed twice with permeabilization buffer and then were run in Accuri C6 plus flow cytometer. The number of CD45-/CK19+ cells was assessed as the CTCs number. The results were documented for each sample and the mean was obtained and represented as the CTCs number in 7.5 mL of blood. A cut-off of 5 CTCs/7.5 mL was taken to determine the test as positive **(Polzer et al., 2014)**.

Statistical analysis of the data

Quantitative data were presented as mean ±SD. For comparison of the two groups' mean, the Student's t-test was used, while for the comparison of the three groups' mean, one-way analysis of variance (ANOVA) was used followed by Post Hoc test. If the SD was >50% of the mean and/or if the 2 SDs below or above the mean were outside the range, we considered the data to be nonparametric. Non-parametric quantitative data were expressed as median (range), Kruskall -wallis and Mann-whitney tests were used for comparison of means. Qualitative data was expressed as frequencies and percentages. Association between qualitative data was done using Chi- square test. Hardy-Weinberg equilibrium (HWE) was used for genotype distribution in the studied groups. Non-parametric Pearson correlation analysis was used to evaluate the associations between CTCs numbers and other tested parameters. All statistical analyses were done by SPSS (version 20.0, Armonk, NY: IBM Corp), and two-sided significance tests at the 5% level were used throughout.

RESULTS

Population characteristics

Our study comprised of 140 patients with HCC and 110 healthy controls as shown in (Table 1). The healthy control group consists of 53 males anf 57 females with ages ranged between 30-66 years and with mean of 54.85 ± 6.04. As regards the HCC patients group, it consists of 78 males and 62 females, their ages ranged from 38-74 with a mean of 55.7±6.78 years. There wasn't statistically significant difference among the two groups as regards age and sex (P=0.250, P=0.237 respectively).

Clinical and laboratory data

Comparing the two groups regarding the laboratory data revealed a significant difference between them as regards all laboratory data (table 1).

EGF genotyping of patients

RFLP was used to determine the EGF SNP genotypes in 140 patients with HCC and 100 patients with either hepatitis or liver cirrhosis and 110 subjects as a healthy control (able 2). The frequencies of the EGF polymorphism in this study population were consistent with Hardy–Weinberg equilibrium (P<0.077). We studied the risk estimation in HCC, chronic hepatitis groups in relation to the control group; and we found that there weren't statistically significant differences among the chronic hepatitis group and the control group in AG/AA (P = 0.079), G/A allele (P = 0.001), and GG/AA (P = 0.001). However, there were statistically significant differences among HCC and controls in GG/AA (P = 0.001), GA/ AA (P = 0.010), and G/A Alleles (P = 0.001).

Associations of EGF genotyping and CTCs with Laboratory data

There wasn't significant relation between EGF genotyping and age, sex, Child score nor tumor focal lesions (Table 3). As regard association with laboratory findings, significant statistical relations were found between HCC patients owning the normal or the mutant genotypes of EGF (rs4444903) as regards ALT, AST, AFP, and CTCs (Table 4). CTCs numbers were found significantly different between HCC patients as regard with Child scores and total size of focal lesions (table 5). Spearman correlation analysis (Table 6) showed that there were positive significant correlations of CTCs with Tumor size (P<0.001*) and with AFP (P<0.008*).

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Table 1: Patient characteristics with studied groups.

variables	HCC	Control	P-value*
	(No.= 140)	(No.= 110)	
AST (U/L)	58.77 ± 68.85	28.45 ± 5.24	<0.001
ALT (U/L)	44.46 ± 32.47	24.06 ± 6.29	<0.001
T. Bill (mg/dl)	1.26 ± 0.63	0.82 ± 0.19	<0.001
D. Bill (mg/dl)	0.55 ± 0.63	0.17 ± 0.03	<0.001
Alb (gm/dl)	3.40 ± 0.63	4.57 ± 0.49	<0.001
Creat (mg/dl)	0.92 ± 0.23	0.81 ± 0.19	<0.001
PT (seconds)	1.24 ± 0.21	1.04 ± 0.06	<0.001
INR	75.36 ± 15.47	98.29 ± 2.29	<0.001
Platelets (/mm)	131.8 ± 57.81	193.5 ± 49.53	<0.001
AFP (ng/ml)	690.33±1730.30	1.19 ± 1.57	<0.001

*: p value for comparison between groups, <0.001 as highly statistically significant

Table 2: Frequencies of EGF genotypes in patients and controls

	HCC (n = 140)		Controls (n = 110)		OR	p	95 % C.I	
	No.	%	No.	%			LL	UL
RFLP								
AA®	17	12.1	39	35.5	1.000			
AG	50	35.7	46	41.8	2.494*	0.010*	1.24	5.0
GG	73	52.1	25	22.7	6.699*	<0.001*	3.23	13.89
Allele								
A®	84	30.0	124	56.4	1.000			
G	196	70.0	96	43.6	3.014*	<0.001*	2.08	4.36

®: Reference
OR: Odd`s ratio between group I and Group III
L.L: Lower limit
U.L: Upper limit
C.I: Confidence interval
*: Statistically significant at p ≤ 0.05

Table 3 Relations between EGF genotyping and clinical data.

Variables		RFLP			Test of Sig.	p-value
		AA (n = 17)	AG (n = 50)	GG (n = 73)		
Sex	Male	9 (52.9%)	26 (52%)	43 (59%)	χ2= 0.634	0.728
	Female	8 (47.1%)	24 (48%)	30 (41%)		
Age	Min – Max	43.0 – 63.0	37.0 – 68.0	44.0 – 73.0	F= 0.307	0.736
	Mean ± SD.	54.41 ± 5.83	55.84 ± 7.57	55.75 ± 6.46		
	Median	56.0	57.50	56.0		
CHILD	A	8 (47.1%)	18 (36.0%)	25 (34.2%)	χ2= 1.482	0.830
	B.	6 (35.3%)	20 (40.0%)	27 (37.0%)		
	C	3 (17.6%)	12 (24.0%)	21 (28.8%)		
Focal Lesions	Min – Max	2.70 – 43.0	1.10 – 139.0	2.13 – 247.0	H=3.177	0.204
	Mean ± SD.	14.03 ± 2.85	31.54± 3.74	29.66 ± 3.04		
	Median	9.0	16.05	18.60		

H: H for Kruskal Wallis test, p: p value for association between Child and CTCs in HCC patients
p1: p value for comparison between A and B, p2: p value for comparison between A and C, p3: p value for comparison between B and C, p-value <0.001 considered highly statistically significant

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Table 4 Relations between EGF genotyping and laboratory data.

Variables		RFLP			Test of Sig.	p-value
		AA (n = 17)	AG (n = 50)	GG (n = 73)		
ALT					H= 2.575*	0.002*
	Mean ± SD.	29.71 ± 16.84	51.68 ± 36.62	42.96 ± 31.13		
	p-value between groups	p1<0.001*, p2=0.018*, p3=0.060				
AST					H= 8.850*	0.012*
	Mean ± SD.	39.06 ± 12.57	60.66 ± 35.74	62.06 ± 90.24		
	p-value between groups	p1=0.003*, p2=0.033*, p3=0.159				
ALB					F= 0.262	0.770
	Mean ± SD.	3.41 ± 0.75	3.34 ± 0.59	3.43 ± 0.63		
Creatinine					H= 1.055	0.590
	Mean ± SD.	0.93 ± 0.19	0.92 ± 0.27	0.92 ± 0.21		
T. Bilirubin					H= 1.391	0.499
	Mean ± SD.	1.16 ± 0.70	1.21 ± 0.55	1.32 ± 0.67		
D. Bilirubin					H= 1.169	0.557
	Mean ± SD.	0.43 ± 0.33	0.66 ± 0.91	0.50 ± 0.40		
PT					H= 3.860	0.145
	Mean ± SD.	1.15 ± 0.11	1.25 ± 0.19	1.26 ± 0.23		
Hemoglobin					F= 0.728	0.485
	Mean ± SD.	12.52 ± 1.63	12.49 ± 1.58	12.83 ± 1.68		
TLC					H= 0.519	0.771
	Mean ± SD.	5.79 ± 1.26	5.64 ± 1.79	5.96 ± 2.49		
Platelets					H= 0.120	0.942
	Mean ± SD.	132.4 ± 57.04	134.3 ± 58.99	129.9 ± 57.90		
AFP					H= 9.411*	0.009*
	Mean ± SD.	413.1 ± 692.9	1407.3 ± 3631.2	2162.1 ± 10917.2		
	p-value between groups	p1=0.016*, p2=0.495, p3=0.007*				
Focal Lesions					H=3.177	0.204
	Mean ± SD.	14.03 ± 2.85	31.54± 3.74	29.66 ± 3.04		
CTCs					H=12.125*	0.001*
	Mean ± SD.	20.12 ± 456.20	45.96 ± 22.03	54.36 ± 23.94		
	p-value between groups	p1=0.005*, p2=0.001, p3=0.048*				

H: H for Kruskal Wallis test, p: p value for association between Child and CTCs in HCC patients.
p1: p value for comparison between A and B, p2: p value for comparison between A and C, p3: p value for comparison between B and C, p-value <0.001 considered highly statistically significant

Table 5 Relation between CTCs, Child and Focal region in HCC patients group

		CTCs			H	p-value
		Min – Max	Mean ± SD.	Median		
Child	A (n = 19)	7.0 – 44.0	19.67 ± 8.05	19	H=93.064*	<0.001
	B (n = 53)	11.0 – 86.0	41.91 ± 16.45	42		
	C (n = 36)	15.0 – 205.0	90.19 ± 39.51	87.5		
	p-value between groups	p1<0.001, p2<0.001, p3<0.001				
Tumor Size	< 5cm	13.00-57.00		34.5	χ2= 7.534*	<0.001
	>5cm	8.00-117.00	47.97±24.54	43.5		

H: H for Kruskal Wallis test, p: p value for association between Child and CTCs in HCC patients.
p1: p value for comparison between A and B, p2: p value for comparison between A and C, p3: p value for comparison between B and C, p-value <0.001 considered highly statistically significant

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Table 6 Correlation between Cytokeratin 19 and different parameters

Variables	CTCs	
	rs	p
Tumor Size	0.487	<0.001*
AFP	0.212	0.012*
ALT	0.113	0.184
AST	0.129	0.129
ALB	-0.203	0.043*
Creatinine	-0.008	0.923
Total Bilirubin	0.097	0.255
Direct bilirubin	0.054	0.528
PT	0.064	0.454
INR	-0.103	0.224
HB	-0.088	0.303
TLC	0.115	0.176
PLT	0.126	0.139

r_s: Spearman coefficient

*: Statistically significant at $p \leq 0.05$

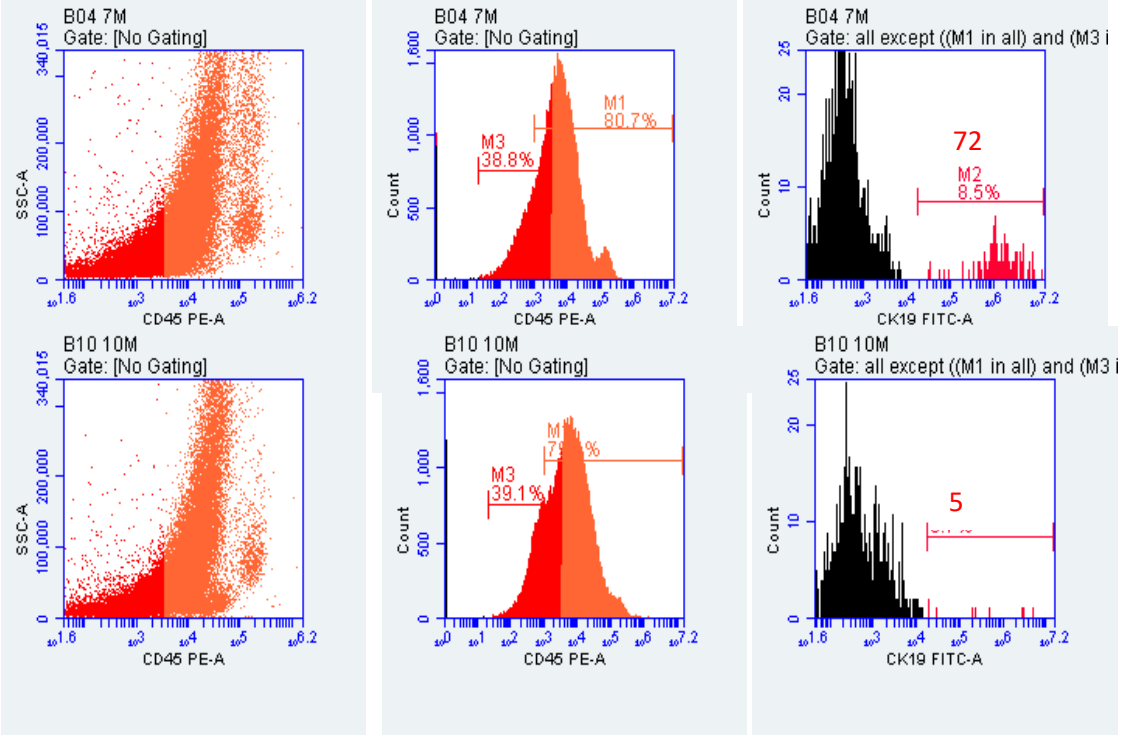

Figure 1: Flow cytometry results for a case of hepatocellular carcinoma. Percentage of CTCs cells positive for Cytokeratin-19 (FITC) and negative CD45 (PE).

DISCUSSION

HCC remains the most public health problem worldwide. In Egypt, HCC is consider the second major common type of cancer in men and also the sixth major common cancer in women **(Ziada et al., 2016)**. Development of HCC; like any other cancer; is affected by multiple epigenetic and genetic factors **(Herceg and Paliwal, 2011; Yam et al., 2010)**. Moreover, certain genes have been found implicated in HCC progression, invasion, migration, and ultimately metastasis **(Ni et al., 2019)**. EGF is among the genes that not only affects HCC development **(Fuchs et al., 2014; Tanabe et al., 2008)**, but also affects tumor progression and metastasis **(Liu et**

al., 2017). Moreover, genetic polymorphism of EGF was found associated with a higher risk of HCC recurrence after therapeutic hepatectomy **(Yoshiya et al., 2014)**. Though, few studies have concerned about the association of DNA variations in EGF with HCC prognosis.

In this study, we investigated the association between EGF+61A/G polymorphism and susceptibility of HCC. A total of 140 cases with HCC, 100 cases with Chronic Hepatitis diseases and 110 cases healthy control were finally involved in this study. Our data showed that the frequency of the EGF +61G allele in HCC patients was significantly higher than the healthy controls, suggesting that EGF+61G allele is risk allele for HCC patients. Additionally, it proposes that the polymorphism of EGF+61A/G was significantly correlated with an elevated HCC risk in all genetic models.

The results of our study were in accordance with those of Suenaga et al., **(Suenaga et al., 2013)**, who found that the odds ratio of developing HCC was higher in patients with the EGF+61 G allele in a Japanese population. However, the authors found that there were no statistically significant differences between the EGF rs4444903 genotype and disease free or overall survival, suggesting that the EGF rs4444903 could be associated with a risk for the progression of HCC in Japanese cohort but not with the prognosis. Furthermore, the EGF rs4444903 GG genotype was found to be associated with higher susceptibility to HCV-related LC and HCC in the Chinese Han population **(Zhang et al., 2017)**. Correlation with the clinical data revealed no significant difference of the clinical parameters between HCC patients with the different genetic variants of EGF. However, there were significant differences between patients with the different EGF genetic variants as regard liver enzymes ALT and AST, AFP levels and number of CTCs.

Recent studies suggested that the hostile behavior of HCC could be partially assigned to its ability to disseminate cancer cells into blood in the early stage and survive without clinical representation **(Ye et al., 2019)**. These malignant liver cells may form micrometastases, an important initial step leading to recurrence and the formation of distant metastases **(Qi et al., 2018)**. Earlier studies have revealed that the existence of CTCs in patient's blood is an early marker of tumor recurrence occurring before clinical symptoms present **(Chen et al., 2017)**. Therefore, monitoring CTCs could lead to improved prognostication and selection of appropriate adjuvant treatments.

The concept of determination of CTCs has always been a point in the modern medicine, however as these cells are few in the circulation makes it difficult to isolate **(Weng et al., 2018)**. When compared with other diagnostic methods, detection of CTCs is a rapid, easy, and frequently noninvasive and relatively have lower cost. These advantages stimulated its use to nullify metastases mechanisms, monitor treatment outcome, help clinicians to do perfect decisions and recognize new drug targets **(Esposito et al., 2017)**. Therefore, many research groups have developed several CTC assays that are vary considerably in the methods and markers used for CTC isolation and detection **(Cheng et al., 2019; Kalinich et al., 2017;Park et al., 2017)**. However, given the significant heterogeneity of CTCs, it is not clear whether these methods detect all CTCs or even the same subpopulations of CTCs, since epithelial-to-mesenchymal transition, a common feature of CTCs, may prevent CTC identification by the use of epithelial markers **(Yu et al., 2013)**.

Earlier studies have found that cytokeratin 19 (CK19) is a promising tumor marker that may significantly improve the detectability of CTCs **(Morales et al., 2018; Chen et al., 2013)**. In our study, we used flow cytometry for detection of CTCs as a fast and mostly sensitive technique for the monitoring of HCC patients at different stages of disease. This was achieved by detecting circulating CK19-positive cell which is found in the cytoplasm after non-epithelial (CD45) cells negative selection. Our data revealed that flow cytometry has the ability to determine a significantly higher values of CTCs (CD45-/CK19+) in the HCC Patients blood. In our study CTCs were significantly associated with tumor size ($p<0.001$) and AFP ($p<0.008$). In addition, CTCs showed a highly significant association with Child-Pough score and Tumor size > 5cm. On the other hand, EGF gene polymorphism did not show significant association with these clinical parameters. Suggesting that both genetic variants of EGF and CTCs are independent parameters in HCC patients.

In conclusion, the present study suggests that the G-allele of the EGF 61* polymorphism (rs4444903) is corelated with increased risk of HCC, while the A-allele contributes to decreased susceptibility to HCC, especially in the Egypt cohort. Also, these findings proposed that the functional polymorphism (rs4444903) of EGF gene was associated with the risk of HCC development. These results supported the hypothesis that genetic variants of EGF polymorphisms In addition, results showed that CTCs significantly positive correlated with size of tumor

and AFP and were also positively associated with Child-Pough score suggesting it might become a biological biomarker for the prediction of HCC carcinogenesis progression. However, no association was detected between CTCs and genetic variants of EGF, suggesting that both CTCs and EGF 61* polymorphism are independent variables in HCC, and thus their determination can complement each other in the HCC risk assessment and disease progression. Finally, our study may recommend the assessment of CTCs in patients with HCC especially that carry G-allele of the EGF 61* polymorphism (rs4444903) to improve the clinical outcomes in patients at risk of HCC.

Authors' contributions

RF, MS and ME participated in collection of samples and lab experiments. RF, MS, ME, and SM participated in the data analyses, and manuscript writing. All authors share and approved the final manuscript.

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The authors thank all the individuals participated in this study, and the cooperating clinicians for their contribution.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The authors declare that there is no conflict of interest

Research involving Human Participants

The study protocol was approved by the Local Ethics Committee of the National Liver Institute hospital, Menoufia University, Egypt (NLI-001.09.2017/1)

Informed consent

All patients and healthy individuals were given a written informed consent.

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