Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and accounts for 7% of all cancers worldwide. It is considered the third cause of cancer related deaths**.** In Egypt, there is a growing incidence of HCC (10–120/100,000). It has nearly doubled over the last decade from 4.0% in 1993 to 7.2% in 2003 among patients with chronic liver diseases ***(El-Zayadi et al., 2010 and Elbaz et al., 2013).***

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) are considered major risk factors for developing HCC. Other risk factors include exposure to aflatoxin B, oral contraceptives, androgens intake and cigarette smoking ***(Baghdadi et al., 2014).***

Ultrasound (US) represents the primary radiologic tool for HCC surveillance of at-risk populations. However, US has limited sensitivity (<60%) to detect small HCC especially in obese patients and those with underlying cirrhosis. The gold standard test for diagnosis of HCC is spiral computerized tomography (C.T.) which has advanced sensitivity and specificity over US ***(Elbaz et al., 2013).*** Unfortunately, its use in HCC surveillances is limited, as the diagnostic efficacy of CT is diminished in small tumors (less than 2 cm) owing to the hypo-vascularization of small-sized tumors, also it requires administration of contrasting materials to patients that may be potentially harmful and it is not available in many medical centers. This has driven the search for an early, more reliable,

more sensitive and non-invasive tumor marker for early diagnosis of HCC ***(Sun et al., 2010 and Kim, 2011).***

Alpha fetoprotein (AFP) is still the non-invasive widely used marker, though its sensitivity is not satisfactory. Moreover, its specificity is relatively low because moderately raised levels also found in some patients with uncomplicated liver disease. This highlights the need for new more reliable biomarker with better sensitivity and specificity ***(Hu et al., 2014).***

Cytochrome P450 (CYP) is a super family of mono-oxygyenases responsible for metabolizing many endogenous and exogenous substances. This is through their major role in Phase I enzymes reaction in the liver ***(Booven et al., 2010).***

Cytochrome P450 genes have been studied in which certain alleles are likely to be associated with HCC development and the polymorphism associated with altered enzymes activities have been shown to be related to hepatic carcinogenesis**.** Among the CYP 450 family, CYP-2D6; encoded by CYP-2D6 gene located on chromosome 22q13.2; is perhaps the most widely studied polymorphically expressed drug metabolizing enzyme in human and its polymorphism shows high clinical importance ***(Sayed and Imam, 2012).***

Mostly, allele four (CYP-2D6*4), the major variant allele, has been shown to be associated with various carcinogenic processes, such as cancer of the lung or larynx and with hepatocellular carcinoma (HCC) ***(Zimmermann et al., 2011).***

Aim of the Work

The aim of the present study was to study CYP2D6*4 genotype by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) in peripheral blood of patients with hepatocellular carcinoma to evaluate its clinical utility as a diagnostic marker. Moreover, the expression of CYP2D6*4 will be compared to serum Alfa feto protein(AFP).This may offer earlier diagnosis with higher sensitivity, leading to intervention at an early stage and may have its impact on the therapeutic regimens.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC), the most common type of primary liver tumor, arises directly from hepatocytes and accounts for about 90 % of all primary hepatic malignancies. It is the fifith most common cancer worldwide, with 600,000 new cases each year. The highest rates of HCC are in areas with high rates of chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections ***(Saleh et al., 2015).***

In Egypt, HCC forms 12% of the malignancies of all digestive organs and 1.7% of the total malignancies ***(El Azab et al., 2016).***

A- Risk Factors:

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) are considered major risk factors for developing HCC. Other risk factors include exposure to aflatoxin B, oral contraceptives, androgens intake and cigarette smoking. In addition, hemochromatosis, alcoholism, and non-alcoholic fatty liver disease (NAFLD cirrhosis) are considered risk factors for developing HCC (Figure 1) ***(Baghdadi et al., 2014).***

1-Hepatitis B Virus (HBV):

Hepatitis B virus is the most common cause for HCC worldwide, accountable for an estimated 54% of all liver cancers. Chronic infection with HBV increases the relative risk for developing HCC 15 to 20 folds with a mortality rate of

approximately 30% to 50% among all cases of chronic HBV infection ***(Levrero and Zucman, 2016).***

Hepatitis B virus contributes to hepatocellular carcinoma development through direct and indirect mechanisms. HBV DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces both genomic instability and direct insertional mutagenesis of diverse cancer-related genes ***(Kew et al., 2008)***.

2- Hepatitis C Virus (HCV):

Hepatitis C virus is the second most common risk factor for HCC, with an estimated 10%–25% of all cases around the world. Chronic HCV infection is associated with a 20 to 30-fold increased risk of developing HCC as compared to uninfected individuals ***(Miao et al., 2010)***.

Elimination of HCV infection rate has been reduced globally through implementation of a combination of laboratory measures such as screening of blood and blood products, public health initiatives such as identification and counseling, and treatment of infected and high-risk individuals ***(Baghdadi et al., 2014*)**.

3- Aflatoxins:

Aflatoxin is a fungal product of Aspergillus flavus and related species. It is a potential contaminant of many farm products (the common food staples, grain, and peanuts) that are

stored under warm and humid conditions for some time. The risk of developing HCC due to aflatoxin is exposure dose and duration dependent and is heightened in those with HBV ***(Magnussen and Parsi, 2013).***

4-Alcohol and Tobacco:

Alcohol abuse has been associated with HCC development that occurs on a background of hepatitis and cirrhosis. Alcohol abuse can potentiate HCV and HBV to increase the incidence of HCC by at least two folds ***(Mckillop and Schrum, 2009)***.

Smoking contributes to the induction and progression of HCC in a dose dependent and synergistic manner ***(Koh et al., 2011)***.

5- Hereditary Hemochromatosis:

Hereditary hemochromatosis is an autosomal recessive genetic disorder of iron metabolism that causes excessive intestinal absorption of dietary iron and deposition of iron in organs including the liver. The latter leads to development of fibrosis and cirrhosis. ***(Aranda et al., 2010)***. The incidence of HCC in hereditary hemochromatosis is increased over 100 times relative to a comparative control population ***(Nahon et al., 2010)***.

6-Non-Alcoholic Steatohepatitis (NASH):

With the increasing prevalence of obesity and type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD) has become a public health problem particularly in developed countries ***(Akiyama et al., 2011)***. NASH is the most serious form of NAFLD. Once steatosis has developed, cellular adaptations may occur to allow the cell to survive in the new stressful environment and enhance vulnerability to a second hit, leading to necro-inflammatory changes of NASH where different mediators are involved in such pathogenesis .Several studies revealed that NASH leads to fibrosis of the liver, cirrhosis and eventually HCC ***(Kawai et al., 2011)***.

7- Genetic Factor:

Some Studies showed that individuals with a first-degree family history of HCC were roughly four times more likely to develop liver cancer than individuals without such a family history. Thus, monitoring individuals with family history of HCC, particularly those with positive viral hepatitis markers, could help to identify HCC at an earlier stage, and hence potentially reduce mortality from HCC ***(Brito et al., 2016).***

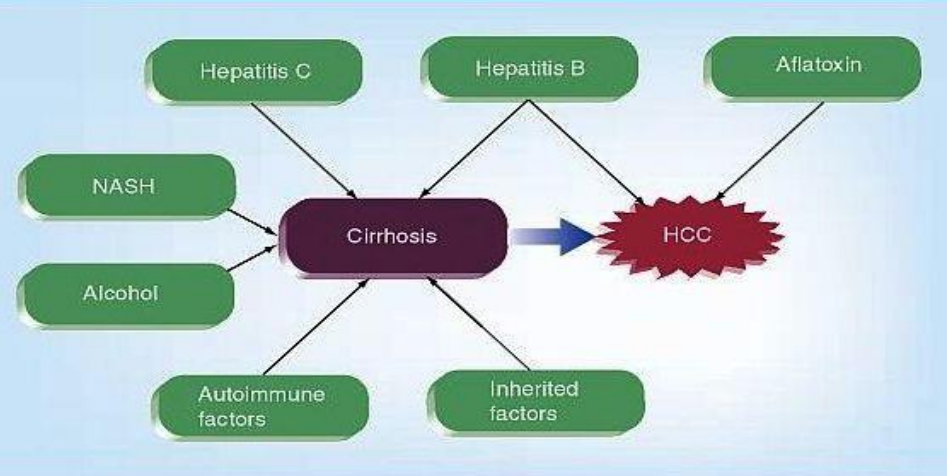

Figure (1): Risk factors for hepatocellular carcinoma development ***(Baghdadi et al., 2014).***

HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

B-Pathogenesis of HCC:

The pathophysiology of HCC is clearly a multi-factorial event. The disease processes, which result in malignant transformation, include a variety of pathways, many of which may be modified by external and environmental factors and eventually lead to genetic changes that delay apoptosis and increase cellular proliferation. Hepatocellular carcinoma pathogenesis includes genetic alteration pathways, tumor suppressor genes, cell cycle regulators, oncogenes and their receptors, apoptosis, angiogenesis, adenomatous hyperplasia and liver cell dysplasia ***(Blum and Spangenberg, 2007).*** Moreover, dysregulation of several signaling pathways have been implicated in HCC pathogenesis. These include WNT-β-catenin, PI3K/AKT/MTOR, RAS/MAPK, IGF, HGF/MET, VEGF, EGFR and PDGF ***(Meguro et al., 2011).***

C- *Staging Systems of Hepatocellular Carcinoma*

1-TNM Staging

The TNM staging system is the conventional system certified by the American Joint Committee on Cancer (AJCC). It contains variables related to tumor stage table (1) ***(Kudo et al., 2003).***

Table (1): TNM Staging System

T	**Primary tumor**
T1:	Solitary mass, without vascular invasion.
T2:	Solitary mass with vascular invasion, or multiple tumors, non invasive >5 cm.
T3:	Multiple masses >5 cm or tumor involving a major branch of the portal or hepatic vein(s).
T4:	Tumor with direct invasion of adjacent organs, other than the gallbladder or with perforation of the visceral peritoneum.
N	**Regional lymph nodes**
N0:	Negative regional lymph nodes.
N1:	Positive regional lymph nodes.
M	**Distant metastases**
M0	No distant metastases.
M1	Distant metastases present.
Stages	**Nomenclature**
Stage I	T1 N0 M0.
Stage II	T2 N0 M0.
Stage IIIA	T3 N0 M0.
Stage IIIB	T4 N0 M0.
Stage IIIC	Any T any N M0.
Stage IV	Any T any N M1.

(Kudo et al., 2003)

2-Child-Pugh Staging System:

Child-Pugh score is the best established method to predict the risk of postoperative liver cell failure occurring in patients of HCC undergoing surgical treatment. The classification determines the severity of the liver disease according to the degree of ascites, the plasma concentrations of bilirubin and albumin, the prothrombin time, and the degree of encephalopathy Table (2) ***(Pugh et al., 1973).***

Table (2): Child-Pugh Scoring System

Parameter	Points assigned		
	1	**2**	**3**
Ascites	Absent	Slight	Moderate
Bilirubin, mg/dL	≤ 2.0	2.0-3.0	>3.0
Albumin, g/dL	>3.5	2.8-3.5	<2.8
Prothrombin time *Seconds over control * INR	 1-3 <1.8	 4-6 1.8-2.3	 >6 >2.3
Encephalopathy	None	Grade 1-2	Grade 3-4

(Pugh et al., 1973)

Interpretation of Child –Pugh score: A total score of 5-6 is considered Child class A (well-compensated disease); 7-9 is Child class B (significant functional compromise); and 10-15 is Child class C (decompensated disease).

3- Barcelona Clinical Liver Cancer (BCLC) Staging System for HCC:

HCC is classified into different stages and different treatment strategies for each stage as shown in figure 2.

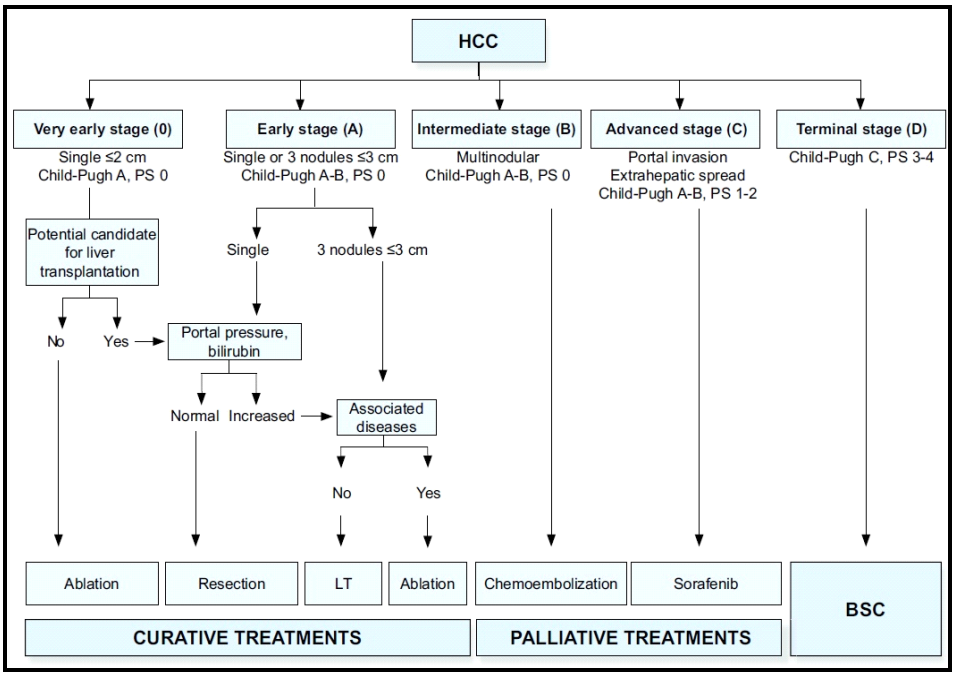

Figure (2): BCLC staging and treatment strategy
(Parikh and Hyman, 2007).

BSC: best supportive care; **LT**: liver transplantation; **PS**: performance status.

D- Diagnosis of Hepatocellular Carcinoma:

1- Clinical Picture of Hepatocellular Carcinoma:

Most patients have non-specific upper abdominal pain, malaise, fatigue, weight loss, and sometimes awareness of an abdominal mass. Jaundice, fever and gastrointestinal or esophageal variceal bleeding are inconstant findings. Clinical

features of metastases such as lymph node, bone, brain and pulmonary metastases (dyspnea) are late symptoms due to large size tumor ***(Sherolk and Dooly, 2011).***

2- Radiological investigations:

HCC can be diagnosed radiologically, without the need for biopsy if the typical imaging features are present ***(Sun et al., 2010 and Kim et al., 2011).***

Ultrasound (US) represents the primary radiologic tool for HCC surveillance of at-risk populations. However, US has limited sensitivity (<60%) to detect small HCC especially in obese patients and those with underlying cirrhosis ***(Bargellini et al., 2014)***.

The gold standard test for diagnosis of HCC is spiral computerized tomography (C.T.) which has advanced sensitivity and specificity over US ***(Bargellini et al., 2014)***. By dynamic imaging through the contrast enhanced study (Dynamic CT scan) or MRI, the sensitivity of four phase CT in detecting HCC is up to 100% for tumors greater than 2 cm in size, 93% for tumors 1-2 cm in size, and for tumors less than 1 cm in size, it is 60% ***(Sun et al., 2010 and Kim et al., 2011).***

3- Laboratory Investigations:

a- Laboratory Investigations Using Blood Samples:

Anemia, thrombocytopenia, prolonged PT, elevated liver enzymes (AST and ALT), increased serum bilirubin,

hyponatremia and hypoglycemia are considered conventional laboratory findings found in HCC patients. Moreover, multiple tumor markers that may aid in diagnosis of HCC are seen in table (3) ***(Gomaa et al., 2009).***

i- Alfa-fetoprotein (α-fetoprotein, AFP):

Alfa-fetoprotein (α-fetoprotein, AFP) is a fetal-specific glycoprotein synthesized primarily by the embryonic liver and by fetal intestinal tract in the first trimester of pregnancy. It has a half-life of 5–7 days. The serum concentration of AFP declines rapidly after birth and its expression is repressed in adults ***(Toyoda et al., 2015).***

AFP is elevated in HCC, embryonic carcinomas, and in gastric and lung cancer. AFP is synthesized by approximately half of HCC, and is used in differential diagnosis and follow-up of HCC patients. However, serum AFP is associated with two main problems. The first problem is the transient rise in the serum level of AFP in CLD patients especially during exacerbation of hepatitis to more than 100 ng/mL. Moreover, slight increases in serum AFP is usual in acute hepatitis, chronic hepatitis and cirrhosis; thus it has low specificity. The second problem is that among all patients diagnosed with HCC, AFP levels may be normal in up to 40% of patients, particularly during the early stages (low sensitivity) ***(Ajlan, 2016).***

ii- Carcinoembryonic antigen (CEA):

CEA is a glycoprotein that belongs to the oncofetal antigen family.

It is expressed in normal mucosal cells. It has a normal value of less than 5 ng/mL in smokers and up to 2.5 ng/mL in non smokers. Serum carcinoembryonic antigen is a broad spectrum biomarker of multiple types of tumors including colorectal cancer, breast cancer and lung cancer and has important significance for monitoring prognosis of patients with these tumors. Regarding HCC, CEA is less helpful in the diagnosis of the primary tumors, but may be used to differentiate primary HCC from hepatic metastasis of gastrointestinal cancer ***(Liu et al., 2016).***

iii- Protein induced by vitamin K absence or antagonist-II (PIVKA-II):

Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II), also known as Des-γ-carboxy-prothrombin (DCP), is another marker specific for HCC. Many studies have shown that PIVKA-II is applicable for HCC follow up and prognosis with remarkably good results ***(Wu et al., 2010 and Yu et al., 2016).***

iv- AFP mRNA:

The detection and analysis of circulating tumor cells (CTCs) is helpful for both predicting recurrence after surgery and for selecting an appropriate surgical strategy. Thus, as a tool for

detecting CTCs is the presence of alpha-fetoprotein (AFP) mRNA-expressing cells in patients with HCC. Sensitivity and specificity are 25% and 88% respectively ***(Okajima et al., 2017).***

v- Alpha-L-fucosidase (AFU):

It is a glycosidase primarily found in lysosome. The alterations of AFU catalytic activity in human cells, tissues and body fluids have a diagnostic value for human tumors including ovarian cancer ***(Abdel-Aleem et al., 1996)***, colorectal cancer ***(Ayude et al., 2000)*** and HCC ***(Lin et al., 2006)***. Moreover, persistently elevated AFU level in sera of patients with liver cirrhosis was claimed to be an early detector of HCC ***(Zhou et al., 2006).***

vi- Squamous cell carcinoma antigen (SCCA):

It is a member of the serpin family (serine protease inhibitor), and is physiologically expressed in the skin and other squamous epithelial cells. High levels have been reported in tissues of head and neck cancer and other epithelial cancers. It has also been reported to be over expressed in tissues and blood of patients with HCC as well as in patients with malignant dysplastic nodules ***(Guido et al., 2008)***.

vii- Glypican-3 (GPC3):

Glypican-3 is a cell-surface glycoprotein. It is a member of the glypican family of glycosyl-phosphatidyl inositol anchored cell surface heparin sulfate proteoglycans ***(Wright et al., 2007)***. It is expressed in 72% of HCC tissues, while it is