Detection of Aberrant P16 "Tumour Suppressor Gene" Methylation in Sera of Egyptian Patients with Liver Cirrhosis and Hepatocellular Carcinoma

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List of Abbreviations

AASL	**American association of studying liver disease**
AFP	**Alpha-fetoprotein**
AFP-L3	**L.culinaris-reactive AFP**
ALT	**Alanine aminotransferase**
APC	**Adenoma polyposis coli gene**
APC	**Anaphase promoting complex**
AR	**Androgen receptors**
AST	**Aspartate aminotransferase**
bp	**Base pair**
C	**Cytosine**
Ca	**Calcium**
CDK	**Cyclin dependent kinase**
DCP	**Des-gamma-carboxy prothrombin**
DNA	**Deoxy ribonucleic acid**
DNMT	**DNA methyl transeferase**
DR	**Direct repeat**
Dsh	**Dishevelled**
EASL	**European association society of liver disease**
ELISA	**Enzyme linked immunosorbant assay**
FNA	**Fine needle aspiration**
G	**Guanine**
GP73	**Golgi protein73**
GPC-3	**Glypican-3**
HBV	**Hepatitis B virus**
HBX	**Hepatitis B X antigen**
HCC	**Hepatocellular carcinoma**
Hcr	**Hepatocarcinogenesis resistance**
Hcs	**Hepatocarcinogenesis susceptibility**
HCV	**Hepatitis C virus**
HGDN	**High grade dysplastic nodule**
HGF	**Hepatocyte growth factor**
IGF	**Insulin like growth factor**

IL	**Interleukin**
iNOS	**Inducible nitric oxide synthase**
kb	**kilobasepair**
KDa	**kilodalton**
LC	**liver cirrhosis**
LCF	**liver cell failure**
LOH	**loss of heterozygosity**
miRNA	**Micro RNA**
MSP	**Methylation specific polymerase chain reaction**
MTS1	**Multiple tumor suppressor**
ng	**Nanogram**
PC	**Prothrombin concentration**
PCR	**Polymerase chain reaction**
pRb	**Protein product of the gene**
RB	**Retinoblastoma gene**
RCT	**Randomized controlled trial**
RNA	**Ribonuecliec acid**
ROS	**Reactive oxygen species**
RT	**Reverse transcriptase**
SFRP1	**Secreted frizzled related protein 1**
SV40	**Siamian virus of the polyamavirus family**
T	**Thymine**
TAA	**Tumour derived auto antibodies**
TGF	**Tumour growth factor**
TNF	**Tumour necrosis factor**
US	**Ultrasound**
Wnt	**Wingless gene family**

Abstract

Background: In Egypt, HCC is the third common cancer in males and the sixth among females. HCCs major known etiologic factors are HBV and or HCV infection. The *p16INK4A* tumor suppressor gene frequently displays genetic alteration in HCC tissues. **Aim of work:** The present study was performed to examine the incidence of methylated p16 in the sera of liver cirrhosis and HCC patients and its relation to HCV and HBV infection, and to evaluate its role as a tumor marker of HCC. **Patients and methods:** The sera of 20 LC patients and 25 HCC patients were examined in this study. The methylation status of p16 was evaluated by methylation-specific PCR of serum samples, AFP level were assessed by RIA and HBsAg and HCV-Ab were assessed by ELIZA. **Results:** Methylated p16 was detected in 20% (4/20) of LC patients and in 80% (20/25) of HCC patients with a sensitivity of 80%, a specificity of 80%, and a positive predictive value of 83.3% and a negative predictive value of 76.2%. in sera of HCC patients, methylated p16 was detected in all HBV positive patients and in 19 (79.2%) of HCV positive patients and in all co-infected patients. No association was demonstrated between p16 methylation and serum AFP level.

Conclusions: Our observations suggest that $p16^{INK4A}$ hypermethylation may contribute to hepatocarcinogenesis and that viral infections may be associated with $p16^{INK4A}$ hypermethylation in hepatocarcino-genesis, and methylated P16 DNA may play an important role as a tumor marker in detection of HCC.

Key Words *:*

P16INK4A Methylation; PCR, Methylation-Specific; Carcinoma, Hepatocellular; Liver Cirrhosis; ELIZA; RIA; AFP; HBV; HCV.

Introduction:

Hepatocellular carcinoma (HCC) is a major cause of cancer morbidity and mortality in many parts of the world**.** The major known etiologic factors associated with development of HCC are infection with hepatitis B (HBV) and/or hepatitis C (HCV) virus and lifetime exposure to high levels of aflatoxin B_1 in diet**.** ***(*Block TM et al**., **2003*)***

Detailed knowledge of the etiology of HCC has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease, and this knowledge is beginning to be translated to preventive interventions in high-risk populations. HBV is a significant risk factor for HCC in the developing world where there are >400 million viral carriers**.** ***(*Arbuthnot p et al., 2001*)***

The contribution of HBV to the pathogenesis of liver cancer is multifactorial and is complicated by the identification of mutant variants of HBV that modulate the carcinogenesis process. The HBV genome encodes its essential genes with overlapping open reading frames; therefore, a mutation in the HBV genome can alter the expression of multiple proteins**.** ***(*Arbuthnot p et al 2001*)***

The onset of these mutations has also been associated with increasing severity of the HBV infection and cirrhosis. Thus, the tracking of this mutation with disease outcomes makes it a candidate biomarker for the early detection of HCC risk in individuals. Finally, the emergence of HCV infection as an etiologic factor in HCC raises the potential for further viral-viral and viral-chemical interactions in the pathogenesis of this disease **(Poynard T et al 2003).**

Several studies have now shown that DNA isolated from serum and plasma of patients with cancer contains the same genetic aberrations as DNA isolated from an individual's tumor**.** The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis, or other processes**. *(*shuang-yuan kuang et al 2005*).***

Inactivation of tumor suppressor genes is important in the development of cancers, and leads to abnormal proliferation, transformation, invasion, and metastasis. Inactivation of tumor suppressor genes can occur due to silencing as a result of methylation of tumor suppressor gene promoters, as well as genetic mutation, loss of heterozygosity, or deletion of homozygosity**. *(*Hyung Junchu et al 2004*)***.

The tumor suppressor gene p16 is located on chromosome 9p21and encodes the p16 protein, which binds selectively to CDK4 to inhibit activation of the CDK4/cyclinD complex in G1phase. Recent studies have indicated the occurrence of structural changes in p16 in HCC ***(*Saito Y et al 2001*).***

Inactivation of this gene, which normally inhibits progression to G1 phase of the cell cycle, is involved in the initiation of tumors. The methylation of p16 is known to silence transcription of the gene**.** ***(*Weihrauch M et al 2001*).***

Aim of work:

i. Examine the frequency of methylated p16 in the sera of liver cirrhosis and HCC patients.

ii. Evaluate its role as a tumour marker.

iii. Frequency of p16 hypermethylation in relation to presence of HCV and/or HBV infection.

Chapter One

Hepatocellular Carcinoma

HCC can be considered as a common complication of chronic liver diseases. The age-adjusted worldwide annual incidence is between 5.5 and 14.9 people per 100,000 populations, resulting in approximately 600,000 to 1,000,000 deaths annually **(Bosch et al., 2005).** Males are more commonly affected than females in the ratio of 3:1 to 4:1**(Carr et al., 2004)**

The mean age of presentation of hepatocellular cancer in Europe and the United States is approximately 60 years. This is in contrast with patients in Asia and Africa, where it is between 20 and 50 years. **(Tsukuma et al., 1993)**

Its frequency is higher in some geographic areas, such as Eastern and South-Eastern Asia and sub-Saharan Africa (>15 per 100,000 population), than in United States, Canada and Western Europe **(Seeff and Hoofnagle, 2006).** However, the incidence of HCC is increasing also in Europe and in the United States **(El-Serag and Mason, 1999).**

In Egypt, a high incidence of HCC has been reported; HCC is the third common cancer in males and the sixth among females **(Al-Attar et al., 2002)**. The incidence of HCC is expected to increase significantly in the next decade because of the high prevalence rate of HCV in the general population which accounts for most of the cirrhosis and HCC cases **(Hassan et al., 2001).** HCC is now the second in males and fourth in females**. (www.NCI.com).**

Though there are solid cancers that are more common, HCC is at this moment the third or fourth cause of death for cancer worldwide. Epidemiologic studies have identified several risk factors for HCC and it is well known that its development is strongly dependent on the presence of liver cirrhosis; more than 90% of HCC in the Western World develop in patients with liver cirrhosis **(Mu¨ ller, 2006).**

The molecular pathogenesis of HCC seems to involve multiple genetic aberrations in the molecular control of hepatocytes proliferation, differentiation, and death and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes, and other genes. Epigenetic alterations are also involved in cancer development and progression (**Feinberg et al., 2001)**

Methylation of promoter CpG islands is known to inhibit transcriptional initiation and cause permanent silencing of downstream genes. Hypermethylation of p16, a cyclin dependent kinase inhibitor gene that regulates the cell cycle, has been detected frequently in human cancers **(Esteller et al., 2001).**

Detection of methylated DNA has been suggested as a potential biomarker for early detection of cancer **(Baylin et al., 2006)**

Among the main *risk factors* for HCC, HBV and HCV are the most important; accounting for more than 70% of cases worldwide **(Wurmbach et al., 2007).**

Viral hepatitis and hepatocarcinogenesis

The etiological agents leading to HCC have been largely established ; In Japan, Europe, and America about 60% of the patients

with HCC are attributed to chronic hepatitis C (HCV) infection, whereas 20% are attributed to chronic hepatitis B (HBV) infection and about 20% between cryptogenic and alcoholic liver disease **(Bosch et al.,2004)**

In Egypt, Prevalence for HBV and HCV were 6.7% and 13.9% among healthy populations, and 25.9% and 78.5% among HCC cases**. (Lehman et al., 2008)**

1. HBV infection:

i. Prevalence of HBV infection in hepatocellular carcinoma

Chronic HBV infection is the main causal factor for HCC in the world, and the geographical distribution of HBV infection closely reflects that of HCC (**Bosch et al., 2005).**

In high prevalence areas such as Eastern Asia, China and Africa, about 8% of the population is chronically infected as a result of vertical (mother-to-child) or horizontal (child-to-child) transmission. The pattern of transmission is different in areas with lower prevalence of HBV such as North America, Western Europe, and Australia where infection mostly occurs in adulthood through sexual and parenteral routes. The higher prevalence of chronic HBV, as well as the longer period of exposure to infection largely explains the higher HBV related HCC risk in endemic areas**. (Carlos et al., 2007)**

In areas of high HBV endemicity (i.e., Asian countries), persons with cirrhosis have an approximately 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis and a 16-fold higher risk of HCC than the inactive carrier; the corresponding numbers to a low