p53 MUTATIONS AS A SOURCE OF ABERRANT β-CATENIN ACCUMULATION IN CANCER CELLS

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By

TOLGA ÇAGATAY

September, 2002

To My Family;
ÇAGATAYs

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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Prof. Dr. Mehmet Öztürk

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

p53 MUTATIONS AS A SOURCE OF ABERRANT β-CATENIN ACCUMULATION IN CANCER CELLS

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β-catenin is involved in both cell-cell interactions and wnt pathway-dependent cell fate determination through its interactions with E-cadherin and TCF/LEF transcription factors, respectively. Cytoplasmic/nuclear levels of β-catenin are important in regulated transcriptional activation of TCF/LEF target genes. Normally, these levels are kept low by proteosomal degradation of â-catenin through Axin1- and APC-dependent phosphorylation by CKI and GSK-3β. Deregulation of β-catenin degradation results in its aberrant accumulation, often leading to cancer. Accordingly, aberrant accumulation of β-catenin is onberved at high frequency in many cancers. This accumulation correlates with either mutational activation of CTNNB1 (β-catenin) or mutational inactivation of APC and Axin1 genes in some tumors. However, there are many tumors that display β-catenin accumulation in the absence of a mutation in these genes. Thus, there must be additional sources for aberrant β-catenin accumulation in cancer cells. Here, we provide experimental evidence that wild-type β-catenin accumulates in hepatocellular carcinoma (HCC) cells in association with mutational inactivation of p53 gene. We also show that worldwide p53 and β-catenin mutation rates are inversely correlated in HCC. These data suggest that inactivation of p53 is an important cause of aberrant accumulation of β−catenin in cancer cells.

ÖZET

KANSER HÜCRELERiNDEK SIRADISI β-KATENiN BiRiKiMiNiNE
p53 MUTASYOUNUNUN KATKISI

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β-katenin, E-kaderin ve TCF/LEF ifade faktörleri ile etkileserek hücre-hücre etkilesim ve wnt-bagimli hücre kaderi belirlenmesinde rol alir. β -kateninin sitoplazmik/çekirdek seviyeleri TCF/LEF hedefi olan genlerin kontrollu ifadesi için önemlidir. Nomalde bu seviyeler β-kateninin Aksin-1'e ve APC'ye bagli olarak CK-I ve GSK-3β tarafindan fosforlanmasini takiben poteozomal darak parçalanmasi ile düsük seviyelerde tutulur. β-katenin parçalanmasindaki bozukluklar, sIklIkla kansere neden olnan siradisi β -katenin birikimi ile sonuçlanir. Ayni dogrultuda birçok kanserde siradisi β-katenin birikimi yüksek bir sIklIkla gözlenmektedir. Bazi tümörlerde bu birikim ya CTNNB1 (β-katenin)'in mutasyonal olarak aktivasyonu ya da APC veya Aksin1'in mutasyonal olarak inaktive edilmesi ile iliskilidir.Birçok tümörde ise bahsi geçen genlerde herhangi bir mutasyon olmadigi halde β -katenin birikimi gözlenmektedir. Bundan dolayi, kanser hücrelerindeki siradisi β-katenin bitirikimine katkisi olan baska ek nedenler olmalidir. Bu çalismada normal β-katenin tasiyan hepatoselüler kanser hücrelerindeki siradisi β-katenin biriminin p53 genin mutasyonel olarak inaktive edilmesi ile iliskili oldugu deneysel olarak gösteriyoruz. Ayrica, dünya çapindaki p53 ve β -katenin mutayonlarinin ters oratili gidisatini göstermekteyiz. Bu veriler p53 inaktivasyonunun kanser hücrelerinde gözlenen siradisi β-katenin birikimine önemli bir katkisi olduðunu düsündürmektedir.

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"The best part of a scientific research is that it has a joyful, game-like nature in itself. We were saying "we're gonna have fun", we had actually and we have been continuously to have it " - J. Dausset, 1983-

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ATP	adenine triphosphate
â-catenin	beta-catenin
bp	base pair
cDNA	complementary DNA
CKI	casein kinase I
CKII	casein kinase II
cm	centimeter
dATP	adenosine deoxyribonucleoside triphosphate
dCTP	cytosine deoxyribonucleoside triphosphate
dGTP	guanosine deoxyribonucleoside triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dTTP	thymine deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetra-acetic acid

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GSK-3â	glycogen synthase kinase 3 beta
xg	gravity
g	gram
h	hour
H33258	the fluorochrome dye H33258
kb	kilobase
kV	kilovolt
LB	Luria-Bertani medium
M	molar
mM	millimolar
ml	milliliter
min	minute
mRNA	messenger RNA
ms	millisecond
OD600	optical density at 600 nm
PBS	phosphate buffered saline
rpm	revolution per minute
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
sec	second
TAE	tris/acetic acid/EDTA buffer
TGF-â	transfORming growth factor beta

Tris	2-amino-2-(hydroxymethyl)- 1,3 propandiol
U	unit
V	volt
v/v	volume for volume
w/v	weight for volume
w/w	weight for weight

CHAPTER 1. INTRODUCTION

1.1 Liver Cancers

The liver is one of the largest organs in the body, filling the upper right side of the abdomen and protected by the rib cage. The liver has many functions. It plays an important role in changing food into energy and also filters and stores blood. Primary liver cancer is different from cancer that has spread from other places in the body to the liver (liver metastases). Roughly, there are two different types of primary liver cancer (Durr and Caselmann, 2000):

- The widespread kinds are called hepatoma, or hepatocellular carcinoma, and arise from the main cells of the liver (the hepatocytes). This type is usually confined to the liver, although occasionally it spreads to other organs.
- The other type of primary liver cancer is called cholangiocellular carcinoma or bile duct cancer, because it starts in the cells lining the bile ducts

Childhood liver cancer, also called hepatoma, is rare. Hepatoblastoma is more common in young children before age 3. Children infected with hepatitis B or C (viral infections of the liver) is more likely to develop hepatocellular cancer than other children. Immunization to prevent hepatitis B may decrease the chance of developing hepatocellular cancer (Aguayo and Patt, 2001).

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer. A hepatocellular carcinoma cell resembles, to some extent, the morphological features of a hepatocyte. Although it is classified in several types (trabecular, clear cell, etc.), these morphological distinctions have no reflection in biological behavior (Engstrom et al., 1981); the exception being fibrollamelar carcinoma that appears in younger individuals, often arises in non-cirrhotic livers, and has a more benign course. People who have hepatitis B or C (viral infections of the liver) or a disease of the liver called cirrhosis are more likely to develop adult primary liver cancer than other people (Caselmann, 1996; Caselmann and Alt, 1996; Craig, 1997).

1.2 General Mechanism of Hepatocellular Carcinogenesis

Hepatocellular carcinoma (HCC) belongs to the group of epithelial tumors; it is the most common liver tumor and is one of the most frequent tumor forms worldwide (Parkin, 2001). There is a striking difference in tumor incidence with reported incidence rates based on tumor registries all over the world ranging between 0.2/100,000 in Guyana to 54292/100,000 in China (Ince and Wands, 1999; Parkin, 2001).

Based on the accumulated experimental data with chemically induced carcinogenesis in various models, the progress of HCC, as within the other organs, takes place in three stages: initiation, promotion, and progression. A mutational change caused by a carcinogen can lead to the *initiation* of malignant transformation in an irreversible manner. *Promotion* can be depicted as the clonal expansion of the abnormal cells and formation of foci. Growth of tumor from the dysplastic lesions refers to *progression.* The whole cascade is generally supported by an imbalance between oncogene activation and tumor suppressor gene inactivation. Also, during tumor progression gross molecular changes, such as chromosomal aberrations can occur.

Table 1 Risk factors that contribute to the development of HCC

Infectious agents	Hepatitis B virus[a], hepatitis C virus[a]
Pathology	Chronic liver disease (macronodular cirrhosis)[a], neonatal hepatitis[b]
Dietary	Long-term aflatoxin consumption[a], dietary iron overload[b], low vegetable intake[b], chronic alcoholism[b]
Hormone related	Oral contraceptives (female)[b], anabolic steroids[b], elevated serum levels of testosterone[b]
Other environmental	Vinyl chloride[a], cigarette smoking[b], inorganic arsenic ingestion[b], radioactive thorium dioxide (thorotrast) exposure[b], pesticides (?)
Genetic associations[c]	Hereditary tyrosinemia[a], á$_1$-antitrypsin deficiency[a], idiopathic hemochromatosis[a], porphyria (HCV)[b],Wilson's disease[b], genetic polymorphisms of cytochrome P450 2E1 and 2D6 and arylamine N-acetyltransferase 2(involved in detoxification)[b] and L-*myc*[b], mutations in GST[d], familial aggregation[e]
Other	Elevated TGFá[a], age (45)[a], gender (male)[b], elevated expression of *neu* oncoprotein[b], low serum retinol (?)

[a]Strong association with the development of HCC. [b]Weak or inconsistent association with the development of HCC. [c]Cirrhosis, and not the underlying genetic mutation(s), appears to be the major risk factor associated with the development of HCC. [d]A dose-response relationship between AFB exposure and HCC has been documented in HBV carriers with a GST null genotype. [e]Familial aggregation of HCC may result from environmental agents such as HBV or HCV infections, although a genetic basis for HCC susceptibility in such families has not been excluded.
(Adapted from (Feitelson et al., 2002)).

Under normal physiological conditions, adult hepatocytes are non-dividing cells. Indeed, only a minor fraction of hepatocyes undergo cell division in response to cell loss due to ageing or apoptosis (programmed cell death) (Ozturk and Cetin-Atalay, In press). HCC is most frequently associated with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, with chronic exposure to the mycotoxin, aflatoxin B_1 (AFB_1), and also is a complication of alcoholic cirrhosis. Considering the other risk factors given in Table 1, there is a slight increase in HCC incidence in recent decades (Abel and Becker, 1987; Taylor-Robinson et al., 1997; Deuffic et al., 1998; El-Serag and Mason, 1999).

1.3 Pathogenesis of Hepatocellular Carcinoma

Within the multifactorial pathogenesis of HCC, environmental, infectious, nutritional, metabolic, and endocrine factors contribute directly or indirectly to hepatocarcinogenesis. There is considerable variability in contribution of these factors depending on environmental and socioeconomic influences.

1.3.1 Significance of Viral Hepatitis in Hepatocellular Carcinoma

The epidemiologic association of chronic HBV or HCV infection with HCC has been well established. The availability of cloned HBV and HCV genomes made it possible to detect hepatitis viruses in hepatocellular carcinomas, and their involvement in hepatocarcinogenesis. Oncogenic mechanism of HBV and HCV infection may be simply defined as releasing the growth control of hepatocytes by coding for a factor like the X protein of HBV (HbX) that activates otherwise dormant genes or activates proto-oncogenes or silences anti-oncogenes; by inserting its DNA sequences that can activate and influence the transcription of cellular genes; by causing chronic inflammation with cell death and hepatocyte regeneration and with fibrosis; and by activation of the immune system liberating cytokines at the wrong time at the wrong place.

Regarding the contribution of HBV to hepatocarcinogenesis, the role of integration of HBV DNA into host chromosomes and the subsequent chromosomal instabilities is an interesting issue. Integration of HBV DNA into HepG2 cells or into transgenic mouse chromosomes has resulted in chromosomal instability that may lead to loss of heterozygosity (LOH) in many loci during chronic infection (Hino et al., 1991; Livezey and Simon, 1997). It has also been shown that HBV chronic carriers display a higher incidence of chromosomal instabilities thant he corresponding the uninfected population (Simon et al., 1991; Laurent-Puig et al., 2001).

Transcriptional activation of a wide range of viral, as well as cellular genes such as *c-fos ,c-myc, insulin-like growth factor 2* (IGF2), *insulin-like growth factor I receptor* (IGF_{R1}) and *b-interferon* , were shown to be induced by HBV encoded X antigen (HBxAg) (Twu and Schloemer, 1987; Colgrove et al., 1989; D'Arville et al., 1991; Caselmann, 1996; Kim et al., 1996). In chronic HBV infection, it has been shown that HBxAg binds and functionally inactivates the tumor suppressor p53 (Ueda et al., 1995; Huo et al., 2001) and the negative growth regulator $p55^{sen}$ (Ueda et al., 1995; Feitelson, 1999), both of which are involved in senescence related pathways. Inactivation of the retinoblastoma (Rb) tumor suppressor by hyperphosphorylation resulting in the activation of E2F1 and the trigger of the cell cycle has been reported in HbxAg-positive HCC cells (Sirma et al., 1999). It has also been shown that HBxAg can down regulate the expression of translational factor sui1, and cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1/SDI1}$(Feitelson et al., 1999; Sirma et al., 1999). As with HbxAg, carboxyterminal truncated middle hepatitis B surface protein ($MHBS^{t}$) can activate various viral and cellular gene promoters (Caselmann et al., 1990; Kekule et al., 1990). Recent data suggests that HbxAg contributes to HCC development also by mechanisms other than transactivation. HBxAg binds to the X-associated protein 1 and possibly disturbs its function in nucleotide excision repair mechanism (Becker et al., 1998). It has also been shown that HBxAg-stimulated cell growth is associated with constitutive activation of the ras/raf/MAPK and NKκ-B signal transduction pathways (Lucito and Schneider, 1992; Benn and Schneider, 1994; Shirota et al., 2001).

Studies have recently begun to clarify the molecular mechanisms of HCV-induced carcinogenesis. Studies with HCV proteins showed that viral proteins interact with various cellular proteins, including 14-3-3 protein, apolipoprotein AII, tumor necrosis factor (TNF) receptor, lymphotoxin-ß receptor, DEAD domain of RNA helicase, nuclear ribonucleoprotein, double-stranded RNA protein kinase (PKR), p53 and SNARE-like protein (Ghosh et al., 1999; Shimotohno, 2000; Ray and Ray, 2001).

In chronic HCV infection, inactivation of p53 can be achieved either by HVC core protein which transcriptionally represses the *p53* promoter (Ray et al., 1997; Pontisso et

al., 1998), or by nonstructural protein 3 (NS3) and NS5A which bind and most likely inactivates p53 (Ishido and Hotta, 1998; Majumder et al., 2001). Recently, stable expression of HCV core protein in HepG2 cells was shown to result in constitutive activation of the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Hayashi et al., 2000).

1.3.2 Alcohol and Cirrhosis

Beside for the HBV and mostly HCV-associated cirrhosis, non-viral induced cirrhosis is also coupled with elevated risk of HCC (Furuya et al., 1988; Floreani et al., 1999). Following liver cell necrosis, inflammation, regeneration, and fibrosis, quiescent hepatocytes start to proliferate. During this physiological process, irregular regeneration of hepatocytes through clonal expansion may lead to loss of control over growth and development of HCC (Ueno et al., 2001) .

It has been shown that alcoholic cirrhosis is associated with both HCC and cholangiocarcinoma. In the absence of cirrhosis, however, alcohol has not been shown to be carcinogenic in animal studies. It appears that, in large part, the relationship of alcoholic cirrhosis to liver cancer may be due to concomitant infection with HCV, although the possibility that alcohol exerts a promoting effect in the absence of HCV remains open (Farber, 1996).

1.3.3 Hepatotoxic Chemicals

Aflatoxin B1 (AFB_1), benzo(a)pyrene and vinly chloride were shown to have a well-defined genotoxic effect in hepatocarcinogenesis. Consumption of food contaminated with aflatoxins, toxic metabolites of some species of Aspergillus fungi, has been allied with both human and animal HCCs. It has been shown to induce a specific mutation in codon 249 of the *p53* gene leading to G:C to T:A transversion (Bressac et al., 1991; Hsu et al.,

1991). Similarly, genotoxic effect of benzo(a)pyrene and vinly chloride on *p53* has been supported by human and animal data (Puisieux et al., 1991; Barbin et al., 1997).

1.3.4 Hemochromatosis and Iron

Iron overload has been associated with a high risk of HCC in patients with untreated hemochromatosis and dietary iron intake in patients without hereditary iron overload (Hann et al., 1989; Fargion et al., 1992). One possible mechanism that involves iron overload as a cofactor for hepatocarcinogenesis is the iron deposition which occurs in hepatocytes, Kupffer cells and bile ducts. It results in increased production of oxygen-free radicals, peroxidation of membrane lipids, and other reactive oxygen species (ROS)-mediated damages (Kicic et al., 2001; Rocken and Carl-McGrath, 2001).

1.4 Molecular Pathogenesis of Hepatocellular Carcinoma

Cancer is not a single disease; it can be defined as great variety of malignant and benign tumors that arise from the same critical process of uncontrolled cell proliferation. Cancer proceeds through accumulation of mutations in genes that govern the cell proliferation and death, and it is believed that hepatocarcinogenesis also shares this common molecular pathogenesis as well as common biological features.

The absence of an evidently inherited predisposition to liver cancer has obstructed the identification of specific critical genes in hepatocarcinogenesis. However, HCCs present various genomic alterations, including DNA rearrangements, loss of heterozygosity (LOH), chromosomal amplification, loss of imprinting and mutations (Buendia, 2000; Pineau and Buendia, 2000).

Table 2 lists the genes that have been implicated in the pathogenesis of HCC, and may be divided into four functional groups: (1) genes regulating DNA damage response,

(2) genes involved in cell cycle control, (3) genes involved in growth inhibition and apoptosis, and (4) genes responsible for cell-cell interaction and signal transduction (Ozturk, 1999).

1.4.1 Oncogenes in HCC

Beta-catenin (b-catenin) gene mutations that lead to oncogenic activation have been recently described as one of the most frequent mutations in both HCCs and hepatoblastomas. Reported incidence of β-catenin mutation in HCC varies between 8% and 24% depending on the geographical origin of the tumors (de La Coste et al., 1998; Miyoshi et al., 1998a; Huang et al., 1999; Kondo et al., 1999; Terris et al., 1999; Hsu et al., 2000; Satoh et al., 2000; Devereux et al., 2001; Fujie et al., 2001; Laurent-Puig et al., 2001; Wong et al., 2001). However, childhood hepatoblastomas represent much higher rate of *b-catenin* mutations that varies between 48% and 89% (Blaker et al., 1999; Koch et al., 1999; Jeng et al., 2000; Wei et al., 2000; Park et al., 2001; Takayasu et al., 2001; Udatsu et al., 2001).

The amplification and overexpression of the *cyclin D1* gene that results in the rapid growth and aggressiveness of tumor was observed in 11-13 % of HCCs (Zhang et al., 1993; Nishida et al., 1994; Albrecht et al., 1995; Kim et al., 2000; Joo et al., 2001). Cyclin A is an S- and G2-M-phase regulatory protein, and its abnormal expression has been implicated in 39% of HCCs (Chao et al., 1998).

The constitutive activation of c-myc expression is key to the genesis of many cancers (Dang et al., 1999; Fearon and Dang, 1999). The overexpression of *c-myc* mRNA and gene product without amplification may be a characteristic of HCC. The reported frequencies of *c-myc* gene overexpression diverge between 0% and 87%. (Hansen et al., 1993; Saegusa et al., 1993; Wu et al., 1996).

Table 2 Gene alterations in hepatocellular carcinomas

Mutated gene	Alteration (%)*	Additional references
c-myc	0–87	
N-myc	0	
p53	28	
p73	0–13	(Mihara et al., 1999; Peng et al., 2000)
p51	0	(Hamada et al., 2000)
IGF II	23-27	(Cariani et al., 1988; Uchida et al., 1997; Eriksson et al., 2001)
M6P/IGF2R	0–33	
TGFRb2	0	
Smad2	0–2	
Smad4	0–6	
Smad6	0	(Kawate et al., 2001)
Smad7	0	(Kawate et al., 2001)
RB1	15	
$p15^{INK4B}$	0	
$p16^{INK4A}$	0–55	
$p21^{CIP1}$	5	
Cyclin D	11–13	
Cyclin A	19	
E-Cadherin	p.u.	
b-Catenin (CTNNB1)	8–24	
AXIN1	7-9	(Satoh et al., 2000; Taniguchi et al., 2002)
MET protooncogene	11	(Park et al., 1999b)
hMSH1	p.u.	
hMSH2	<30	(Yano et al., 1999)
K-ras	0–17	
N-ras	0–16	
H-ras	0–10	
BRCA2	5	
BCL10	57	(Jihua et al., 2000; Cheng et al., 2001)
PTEN/MMAC1	3	(Yao et al., 1999; Fujiwara et al., 2000)
TRa_1	65	(Lin et al., 1999)
TRb_1	76	(Lin et al., 1999)

M6P/IGF2R: Mannose 6-phosphate/insulin-like growth factor 2 receptor gene, RB1: Retinoblastoma gene,hMSH1: Human Mut L homolog-1, hMSH2 Human Mut S homolog-2, BRCA2: Breast cancer susceptibility gene 2, p.u. = Prevalence unknown, ***:** mutation, amplification and *de nova* methylation.
(adapted form Ozturk, 1999)

Aberrant proto-oncogene expression of the "*ras*" family of genes has been implicated in hepatic cell proliferation, transformation and carcinogenesis using rat models (Sills et al., 1999; Boivin-Angele et al., 2000), but there is little evidence of activation in human tumors. The mutation rate in *K-ras* and *N-ras* genes have been reported to be between 0% and 17%, and *N-ras* mutation seems to be less frequent (Tsuda et al., 1989; Challen et al., 1992; Bjersing et al., 1996; Luo et al., 1998; Boivin-Angele et al., 2000; Boix-Ferrero et al., 2000; Weihrauch et al., 2001).

The *c-met* proto-oncogene encodes the tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), a potent mitogen and motogen for epithelial cells. There is only one report about the c-met mutations in HCC by Park *et. al*. who described three missense mutations in 10 childhood HCCs, whereas there is no mutation identified in adult HCCs, cholangiocellular carcinomas, or hepatoblastomas (Park et al., 1999b). However, c-met over expression has been reported in some HCC tumors (Ljubimova et al., 1997; Ueki et al., 1997; Okano et al., 1999; Tavian et al., 2000).

The insulin-like growth factor II (IGF II) gene expression is monoallelic in all the normal tissues other than the liver because of the imprinting of the maternal allele. However, interestingly, most of the cancers arising in these tissues are reported to show biallelic expression (relaxation) of the *IGF II* gene (Suzuki et al., 1994; Quinn et al., 1996; Steller et al., 1996). Allelic imbalance (re-imprinting) of *IGF II* gene expression is often seen in HCC as well as hepatoblastomas (Li et al., 1995; Sohda et al., 1996; Takeda et al., 1996; Kim and Lee, 1997; Li et al., 1997; Uchida et al., 1997; Li et al., 1998; Sohda et al., 1998; Eriksson et al., 2001).

1.4.2 Tumor Suppressor Genes in HCC

p53 gene is the best characterized and known tumor suppressor in terms of its implications in human carcinogenesis (Levine et al., 1991). In human HCC, LOH at chromosome 17p13 has been observed in 26-60 % of tumors in different studies, and the

global prevalence of *p53* can be estimated to be around 28% (reviewed in (Ozturk, 1999; Buendia, 2000).

Reduced *p21(WAF1/CIP1)* expression, which is mainly associated with *p53* gene mutation in HCCs, contributes to hepatocarcinogenesis, but a p53-independent pathway also plays a role in the regulation of *p21/WAF1* expression (Hui et al., 1998; Qin et al., 1998; Bhardwaj et al., 1999). It has been reported that reduced *p21(WAF1/CIP1)* expression with wild-type *p53* participate in hepatocarcinogenesis with only 5% occurrence in studied HCCs (Furutani et al., 1997; Hui et al., 1997).

Tumor suppressor function of the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) was first proposed by De Souza *et al*. by the identification of 6q26-27 chromosomal deletion in HCC samples and its role in activation of transforming growth factor-beta 1 (TGF-β1) and promoting the degradation of IGFII (De Souza et al., 1995). Mutations in the *M6P/IGFIIR* have been detected in 0 to 33% of HCCs (Piao et al., 1997; Wada et al., 1999; Saeki et al., 2000; Kishimoto et al., 2001; Oka et al., 2002).

Inactivation of the *retinoblastoma (Rb)* gene is considered to play a fundamental role in the genesis and progression of several human cancers. In human HCCs, it has been shown that inactivation of *Rb* gene occurs either via strongly down-regulated pRb protein in 30-50% of tumors or via LOH at chromosome 13q where the Rb locus is found (25-48%); (Murakami et al., 1991a; Fujimoto et al., 1994; Hsia et al., 1994; Zhang et al., 1994; Hada et al., 1996). Although no mutation has been reported in *Rb* gene itself so far, a recent publication from Higashitsuji *et al* (2000) proposes a novel pathway that leads to inactivation of Rb via a new oncogene called gankyrin (an ankyrin-repeat protein homologoues to the p28 subunit of 26S proteosome) (Higashitsuji et al., 2000).

Regional *de nova* methylation of CpG islands in transcriptional regulatory region represents an alternative mechanism for loss of function of a number of tumor suppressor genes in human cancer. Notably, hypermethylation of normally under-methylated CpG islands in transcriptional regulatory regions of *CDKN2A*, a gene located on 9q21, has been

observed, and shown to be the main mechanism for inactivation of *p16^{INK4}* in variety in HCC. *CDKN2A* encodes p16^{INK4}, an inhibitor of cyclin-dependent kinase -4 and -6, and p14^{ARF} which target p53 via inhibition of mdm-2. (Lin et al., 1998; Liew et al., 1999a; Wong et al., 1999; Iwata et al., 2000; Jin et al., 2000; Kanai et al., 2000; Azechi et al., 2001; Weihrauch et al., 2001; Shen et al., 2002). LOH at 9q occurs with 20% and homologous deletion of 9q21 has been detected only in four HCC cases (Liew et al., 1999b). In HCC, germline and somatic mutations of *CDKN2A* seems to be quite rare (<10% of studied cases) (Chaubert et al., 1997; Liew et al., 1999a).

The *adenomatous polyposis coli (APC)* gene mutations were suggested to occur in HCCs (Koch et al., 1999; Wei et al., 2000). *APC* mutations result in up-regulated wild-type β-catenin levels and constitutive Wnt signaling. The finding of rare LOH at 5q where the *APC* gene is located and the absence of truncation mutations in the HCC samples analyzed indicate that APC can not be considered as a major contributor to hepatocarcinogenesis (Ding et al., 1993; Devereux et al., 1999; Su et al., 2001).

Another tumor suppressor gene referred as *Human Mut S homolog-1 (hMSH1)* is located on chromosome 5q, but its role and contribution to HCC, if any, remains to be identified. Mutations in another mismatch repair gene know as *Human Mut S homolog-2 (hMSH2)* has been reported at about 30% of HCCs examined (Yano et al., 1999), but this data has not been confirmed yet.

LOH of 16q may also be involved in the HCC transformation via loss of the *Axin* gene which normally promotes ubiqitine-dependent and -regulated degradation of β-catenin (Laurent-Puig et al., 2001). Recently, input of *Axin 1* mutations to HCC development was reported to be around 7-9 % (Satoh et al., 2000; Taniguchi et al., 2002) and that of *Axin2* mutations was reported as ~3% in studied HCCs but, not in HBs (Taniguchi et al., 2002). LOH of 16q also correlates with metastatis in HCC and therefore can be a link to another candidate tumor suppressor gene, namely *E-cadherin* which is located at 16q22 (Niketeghad et al., 2001). Its role as a receptor in adherence junctions is essential both for the maintenance of tissue structure and regulation of free cytoplasmic β-

catenin levels. In addition to LOH (Slagle et al., 1993), hypermethylation of *E-cadherin* promoter has been proposed as an alternative mechanism for WNT activation in HCC (Kanai et al., 1997).

PTEN/MMAC1 tumor suppressor gene has recently been shown to block growth-stimulatory and survival signals mediated by PI3 kinase and converging on the activation of protein kinase B/Akt (Downward, 1998; Stambolic et al., 1998). Somatic mutations of *PTEN* gene were found in 4% of HCCs (Kawamura et al., 1999; Yao et al., 1999; Fujiwara et al., 2000), and 27% of HCCs display LOH in the same gene (Kawamura et al., 1999; Yeh et al., 2000).

Low frequency of mutation in the *Smad2, Smad4,* and *TGF-**b** RII* genes has been reported by two independent studies implicating a disruption of TGF-β pathway in less than 10 % of HCCs (Kawate et al., 1999; Yakicier et al., 1999). It has been also shown that mutations of the *Smad6* and *Smad7* genes are not the main cause of the TGF-beta resistance in human HCC (Kawate et al., 2001).

The recently described *bcl10* gene has been suggested to be a major target gene for inactivation in a variety of human cancers and the wild type acts as a tumor suppressor, while mutant type behaves like an oncogene. (Bose et al., 1999; Dyer, 1999; Gill et al., 1999; Lambers et al., 1999; Lee et al., 1999b; Stone et al., 1999). It has been shown that BCL10 promotes apoptosis, pro-caspase-9 maturation and activation of NF-kappaB (NF-κB) via NF-κB-inducing kinase (NIK) and IkB kinase (IKK) (Koseki et al., 1999; Willis et al., 1999; Ruland et al., 2001). Recently, the *Bcl 10* gene was reported to have high mutation frequency in liver cancer (57 %); (Jihua et al., 2000; Cheng et al., 2001).

BRCA2 and *p73* mutations have been rarely detected in HCC (Katagiri et al., 1996; Mihara et al., 1999; Peng et al., 2000).

Thyroid hormone nuclear receptor (TR) mediates the diverse biological effects of thyroid hormone through interaction with a large network of regulatory proteins. High

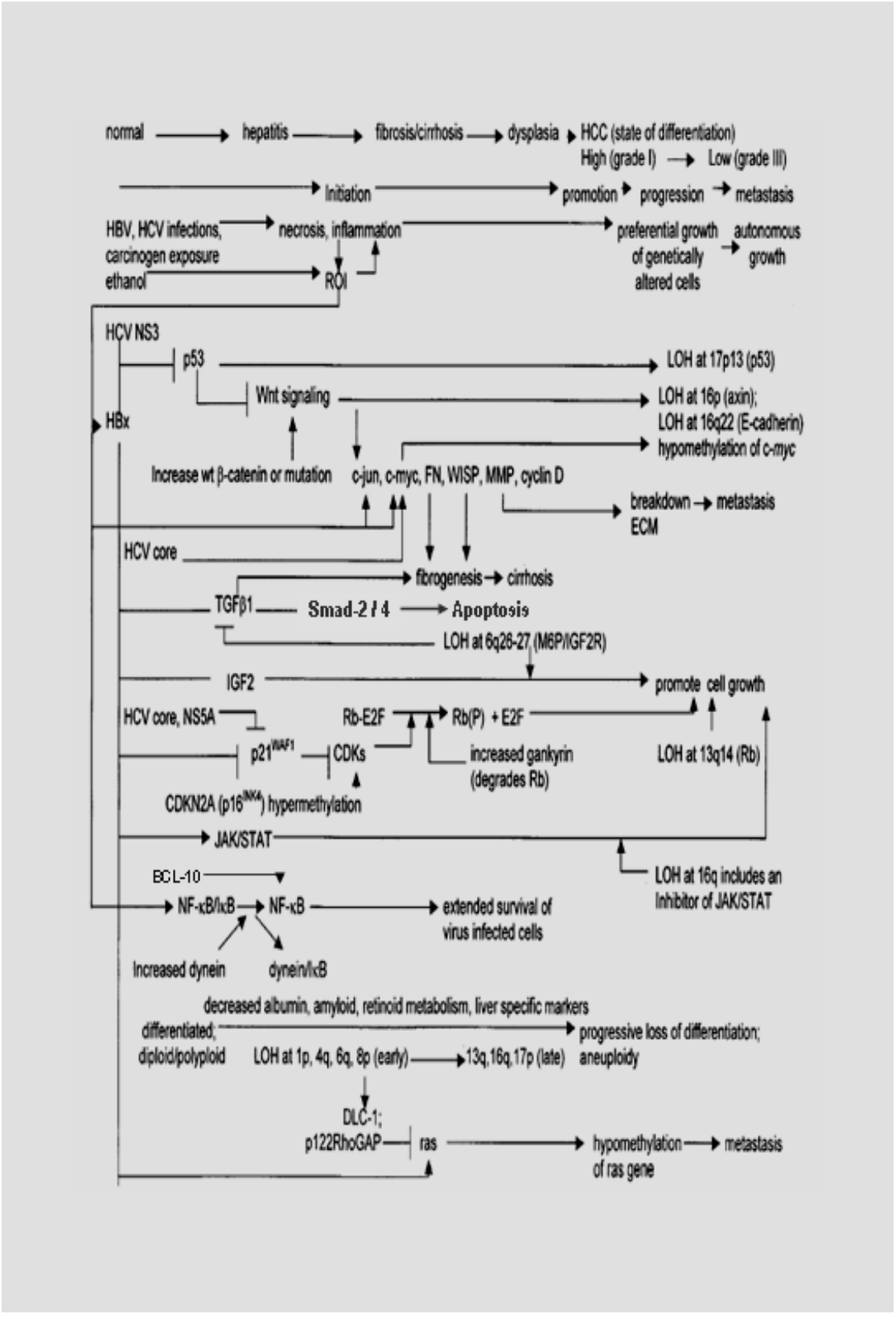
frequencies of mutations of *TR-alpha (TRα)* and/or *TR-beta (TRβ)* genes have been reported in human hepatocellular, renal clear-cell, and papillary thyroid carcinomas (Lin et al., 1995; Lin et al., 1996; Kamiya et al., 2002; Puzianowska-Kuznicka et al., 2002). Lin *et al.* reported that point mutations in $TR\alpha_1$ and $TR\beta_1$ are highly frequent (65%-76%) in HCC (Lin et al., 1995; Lin et al., 1999), however the role of TR in this cancer needs further investigations.

1.4.3 Genetic Mechanisms of Hepatocellular Carcinoma

Hepatocarcinogenesis is accompanied with a variety of genetic alterations such as loss of LOH (*RB1, M6P/IGF2R, E-cadherin, BRCA2*), somatic mutation (*p53, RB1, $p16^{INK4A}$, β-catenin, APC, ERs*), *de-novo* methylation (*$p16^{INK4A}$, E-cadherin*) and/or functional inactivation (*p53*). Variation in number and types of these molecular changes depend on genetic and geographical background of the host, and the etiology of the tumor which can be viral, chemical or both (Wong et al., 2000).

Overall, the consequences of these changes suggest that the pathogenesis of HCC is accompanied by a progressive loss of differentiation, normal cell adhesion, and extracellular matrix, as well as constitutive activation of selected proliferative and survival signal transduction pathways. Figure 1 presents an overview of the common changes that contributes to the pathogenesis of HCC.

Figure 1. Proposed model of multistep hepatocarcinogenesis by Feitelson et al (2002).



1.5 The p53 Tumor Suppressor

p53 is a tumor suppressor mutated in more than 50% of all human tumors, and its germ line mutations in Li-Fraumeni syndrome patients are known to pre-dispose to a variety of cancers (Hansen and Oren, 1997; Levine, 1997). The *p53* gene can be classified as either a "gatekeeper" or a "caretaker" tumor suppressor gene. As an inducer of apoptosis, p53 appears to act as a gatekeeper, however the critical role of p53 in maintaining genomic integrity has earned it the nickname "guardian of the genome" (Lane, 1992) .

1.5.1 Activation of p53

Excessive wild-type p53 activity results in several cellular responses, most prominently cell cycle arrest and apoptosis. Such severe outcomes become very undesirable and harmful if occurring in normal, resting cells. Therefore p53 activation should be kept under tight control.

Under normal physiological conditions, p53 is likely to be in its latent form and does not interfere with significant cellular processes; since p53 knock-out mice in most cases undergo appropriate development and maturation (Donehower et al., 1992). On the other hand, a variety of conditions cause a rapid transition of latent p53 form into a physiologically active form. The common characteristic of these environmental insults are that they create a cellular stress condition, like deoxyribonucleic acid (DNA) damage (Kastan et al., 1991), microtubule disruption (Cross et al., 1995), matrix detachment (Wu and Schonthal, 1997; Park et al., 1999a), hypoxia (Graeber et al., 1996), ribonucleotide depletion (Wahl et al., 1997), oxidative stress (Forrester et al., 1996) or oncogene activation (Lane, 1992; Hermeking and Eick, 1994).

Activation of the p53 in response to stress conditions occurs primarily through alteration in p53 protein. Change in the rate of p53 expression plays a minor role, if any, in such induction. Activation of p53 was first thought to be achieved at transcriptional level

either by a transcriptional repressor sequence present at the 3'-untranslated region of p53 mRNA (Fu et al., 1996) or by p53 itself which binds to its own mRNA (Mosner et al., 1995; Fontoura et al., 1997), however it has been well-documented that post-translational modifications are the major mechanism that cause accumulation of active p53 in response to stress. The p53 is usually a very labile protein and its turnover rate is as short as a few minutes (Rogel et al., 1985). Therefore significant increase in p53 levels requires stabilization of p53 protein.

A key component in the regulation of p53 is the Mdm2 protein. Mdm2 is a product of an oncogene, whose oncogenic activation was observed in several human cancers (Lozano and Montes de Oca Luna, 1998; Freedman et al., 1999). Mdm2 inhibits p53 activity either by binding to transactivation domain of p53 (Lu and Levine, 1995), or by interacting with p53 protein to act as an active transcription repressor (Thut et al., 1995). However, it is now clear that Mdm2 is a p53-specific E3 ubiquitine-protein ligase, which covalently attaches ubiquitine groups to p53 for its complete elimination by proteolytic degradation. On the other hand p53 binds to the *mdm2* gene and activates its transcription. The p53-Mdm2 negative feedback/ autoregulatory loop serves as a tightly controlled shift mechanism for activation and inactivation of p53 according to the presence or absence of triggering stress factors (Wu et al., 1993; Honda and Yasuda, 1999). It has also been reported that c-jun N-terminal kinase (JNK) has a possible role in p53 ubiquitination and degradation (Fuchs et al., 1998).

Covalent modifications of p53 – particularly phosphorylation- also play an important role in activation of p53 protein upon stress. p53 becomes phosphorylated at serine residues in both amino- and carboxy-terminal regions *in vivo* in response to various types of stress, and many stress-activated kinases such as casein kinase I and II (CKI / II) can phosphorylate p53 *in vitro* (Milne et al., 1992; Hall et al., 1996). Double-stranded DNA-activated protein kinases (Lees-Miller et al., 1990; Shieh et al., 1997), ATM and ATR (Banin et al., 1998; Canman et al., 1998), cyclin-dependent kinase(cdk) activating kinase (CAK) (Ko et al., 1997), cdk2 and cdc2 (Bischoff et al., 1990; Price et al., 1995) and protein kinase C (PKC) also phosphorylate p53 protein (Baudier et al., 1992).

p53 acts as a transcriptional activator which relies on sequence- specific DNA binding activity of p53 (Weintraub et al., 1991; Donehower et al., 1992; Crook et al., 1994; Levine, 1997). It has been reported that p53 acetylation and gylcosylation activate the latent sequence-specific DNA-binding activity of p53 (Hupp et al., 1992; Shaw et al., 1996; Sakaguchi et al., 1998) .

1.5.2 Biochemical and Physiological Functions of p53

p53 is a sequence-specific transcriptional factor, and most of p53 functions are carried out by its target genes (Amundson et al., 1998; el-Deiry, 1998). The p53 protein is capable of binding to specific DNA sequences with its central core domain (Figure 2). The common feature of these downstream genes is that they contain one or more p53 consensus binding sites in their regulatory regions. The p53 consensus binding site contains two or more copies of a 10 bp half-site 5'-PuPuPuC(A/T)(T/A)GpyPyPy-3' (el-Deiry et al., 1992). The amino-terminal region of p53 functions as a transcriptional activation domain, and the carboxy-terminal region appears to be required for p53 to form dimers and tetramers with itself (Cho et al., 1994; Ko and Prives, 1996).

Genotoxic and other stress signals may trigger the increase of p53 protein, which has three major functions: growth arrest, DNA repair and apoptosis (cell death). The growth arrest stops the progression of cell cycle, preventing replication of abnormal DNA. In the mean time, p53 may activate the transcription of genes involved in DNA repair. On the other hand, p53 could also induce apoptosis, which is the "last resort" to avoid proliferation of abnormal cells.

p53 has been shown to activate transcription of anumber of genes with roles in the control of the cell cycle, including $p21^{WAF1/CIP1}$ which encodes a regulator of Cdk activity (el-Deiry et al., 1993), *gadd45;* a growth arrest DNA damage-inducible gene (Kastan et al., 1992), *mdm2*, as note above, encodes a protein that is a known negative regulator of p53 (Juven-Gershon and Oren, 1999) and 14-3-3σ; (a regulator of G2/M progression) (Hermeking et al., 1997).

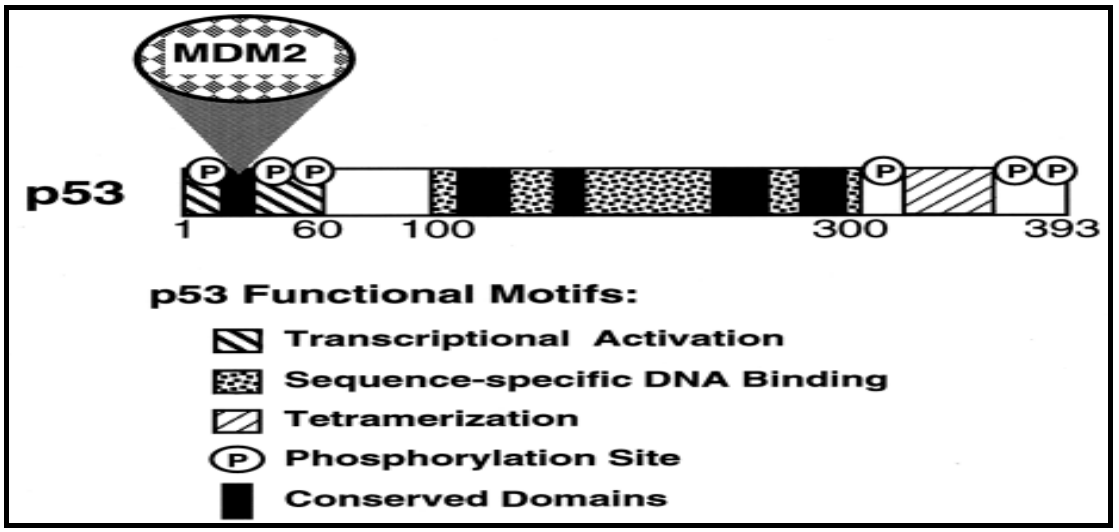


Figure 2 p53 functional motifs.
Domains of p53 involved in transcriptional activation, sequence-specific DNA binding, tetramerization, and binding by the MDM2 protein are indicated. The five distinct regions of p53 that are highly conserved between p53 proteins of diverse species are indicated. In addition, the locations of several sites in the protein that are phosphorylated (P) and that regulate p53 function are indicated

p53 can also induce apoptosis by activating some apoptotic genes including *Bax* (a pro-apoptotic Bcl-2 related protein) (McCurrach et al., 1997), *Apaf-1* (apoptotic protease activating factor 1) (Kannan et al., 2001), *Puma* (p53 upregulated modulator of apoptosis) (Nakano and Vousden, 2001), *NoxA* (a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins) (Oda et al., 2000). Involvement of p53 in DNA repair processes has been proposed in several reports (Kastan et al., 1992; Lakin and Jackson, 1999; Lozano and Elledge, 2000; Khanna and Jackson, 2001; MacLachlan et al., 2002) and a solid evidence came from a report showing that p53 induces transcription of *p53R2* (p53 induced ribonucleotide reductase small subunit-like protein) in response to ultraviolet and gamma-irradiation and adriamycin treatments (Tanaka et al., 2000).

While the specific mechanisms of p53 repression are not well understood, several candidate targets of p53 repression have been suggested, including the gene for the microtubule-associated protein MAP4 (Murphy et al., 1996; Murphy et al., 1999), the multi drug resistance associated protein 1 (MRP1) (Wang and Beck, 1998) and the gene for FKBP25, an FK506/rapamycin-binding protein (Ahn et al., 1999).

1.5.3 p53 and Oncogenic Stress

Deregulated expression of oncoproteins (like adenovirus E1A, Ras, Myc and β-catenin) cause p53 accumulation and activation, often in an $p14^{ARF}$-dependent manner (de Stanchina et al., 1998; Stott et al., 1998; Damalas et al., 2001; Palmero et al., 2002) . At the biochemical level, excess activity of oncoproteins leads to enhanced transcription of *$p14^{ARF}$* gene, at least some of which is mediated by the E2F transcriptional factor (Bates et al., 1998) . The induced $p14^{ARF}$ protein binds to the Mdm2 protein and this binding sequesters Mdm2 in the nucleolus, where it can not target p53 for ubiquitinylation and $p14^{ARF}$ directly inhibits the E3 activity of Mdm2 (Honda and Yasuda, 1999; Tao and Levine, 1999; Weber et al., 1999; Lohrum et al., 2000; Weber et al., 2000). Therefore oncogenic stress activated $p14^{ARF}$ inhibits ubiquitin-dependent p53 degradation, and as a consequence p53 accumulation with subsequent stimulation of p53 mediated cellular responses take place. This is believed to serve as a failsafe mechanism that defends cells against the tumorogenic outcomes of oncogene activation (Sherr, 1998).

1.5.4 The p53 Tumor Suppressor in HCC

p53 is functionally inactivated by structural mutations, viral proteins, and endogenous cellular mechanisms in greater part of human cancers (Hollstein et al., 1991; Greenblatt et al., 1994). The majority of base substitutions target DNA sequences that encode the highly conserved central domain of the protein that mediates sequence-specific DNA binding and transcriptional activation (Murakami et al., 1991b; Hollstein et al., 1994). Loss of p53 function also occurs through allelic deletions at chromosome 17p13 where the gene is located. A detailed analysis of *p53* gene mutations indicates that the sites and features of DNA base changes differ among the variety of human tumor types (Hollstein et al., 1991; Hollstein et al., 1994).

In the case of HCC, LOH at chromosome 17p13 has been detected in 24-65% of tumors, and approximately 28% of HCC cases harbor p53 mutations (Ozturk, 1999; Buendia, 2000). Although in most HCC tumors Knudson's "two hit" model is observed in

p53 inactivation (besides 17p LOH, mutations can occur in the remaining allele) (Oda et al., 1992; Nishida et al., 1993), a considerable percentage of HCC have either a single wild-type *p53* allele or a heterozgous *p53* mutation (Rashid et al., 1999). Noteworthy studies have shown that there is a significant correlation between *p53* mutation and 13q LOH where *Rb* gene is located at late tumor-stage (Murakami et al., 1991a; Unsal et al., 1994) and between *p53* mutation and LOH of chromosome 4p (Rashid et al., 1999), suggesting that the corresponding tumor suppressor genes might be combined forces in HCC transformation.

Mutational screening studies in sporadic HCCs have shown that *p53* gene mutations display a heterogenic pattern regarding the tumor's geographical origins (Ozturk, 1991; Buetow et al., 1992; Unsal et al., 1994). In this regard, a significant worldwide study by Ozturk and colleagues put forward a close correlation between presence of G:C to T:A transversion leading to an Arg to Ser mutation at codon 249 of p53 and high risk of AFB_1 intake (Ozturk, 1991). Now it is principally confirmed and well established that codon 249 *p53* mutations in HCC is mostly seen in some regions of Africa and Asia where the AFB_1 content of diets is high and chronic HBV infection is highly epidemic (Bressac et al., 1991; Hsu et al., 1991; Coursaget et al., 1993; Hosono et al., 1993; Li et al., 1993; Diamantis et al., 1994; Unsal et al., 1994) .

The p53 protein is now well established as a crucial player in the regulation of cell cycle arrest, apoptosis, differentiation, angiogenesis, and senescence. Therefore inactivation of this key regulator molecule presumably facilitates malignant progression by promoting extended hepatocyte survival and acquisition of mutations through genomic instability. For that reason, frequent involvement of p53 inactivation either via viral protein inactivation early in carcinogenesis, or via mutation or/and LOH much later during HCC tumor progression would not be surprising.

1.6 The Canonical WNT/Wg (Wingless) Signaling

The canonical Wnt/Wingless signaling pathway is involved in a large variety of developmental processes, including segment polarity in *Drosophila*, axis specification in *Xenopus*, mesoderm induction in *Caenorhabditis elegant* development of vertebrate limbs and urogenital system (Wodarz and Nusse, 1998). Recently, inappropriate reactivation of the Wnt pathway in adult tissues has shown to play a central role in the etiology of human cancers (Kondoh et al., 2001; Smalley and Dale, 2001; Taipale and Beachy, 2001; Moon et al., 2002).

In most instances, the Wnt pathway is initiated by secreted Wnt protein (paracrine factors; at least 15 members of this family in vertebrates), which binds to a class of seven-pass transmembrane protein encoded by the *frizzled* (Fz) gene (Yang-Snyder et al., 1996; He et al., 1997). By the activation of Fz, the cytoplasmic protein Dishevelled (Dsh) is recruited to the membrane (Axelrod et al., 1998; Boutros et al., 2000) and becomes phosphorylated (Lee et al., 1999a). Phoshorylated Dsh associates with Axin1 and causes the disintegration of multiprotein complex containing Axin 1, glycogen synthase kinase 3β (GSK-3β) the APC tumor suppressor and β-catenin, and prevents glycogen GSK-3β from phosphorylating critical targets (Itoh et al., 1998; Kishida et al., 1999; Smalley et al., 1999). Although, the exact mechanism of Dsh-mediated inactivation of this complex is not understood yet, it has been shown that GBP/Frat-1 inhibit GSK-3β- Axin 1 binding (Yost et al., 1998; Farr et al., 2000; Salic et al., 2000). The GSK-3β substrates include the Axi1, APC and β-catenin (Rubinfeld et al., 1996; Yost et al., 1996; Yamamoto et al., 1999).

Disintegration of multiprotein complex in response to Wnt signal results in cytoplasmic accumulation of hypo-phosphorylated free cytoplasmic-β-catenin, which then translocates into the cell nucleus. Nuclear β-catenin interacts with T cell factor/lymphocyte enhancer binding factor (TCF/LEF) family of transcriptional factors and stimulate transcription of a variety of target genes (Behrens et al., 1996; Molenaar et al., 1996)

which modulate cell fate, proliferation, and apoptosis (Mann et al., 1999; Chen et al., 2001; Kawakami et al., 2001; Kofron et al., 2001; Olson, 2001; van Gijn et al., 2001).

In the absence of Wnt signal, β-catenin is destabilized by the multiprotein complex (mentioned above). Axin serves as a scaffolding component for binding of APC, GSK-3β and β-catenin. Interaction between Axin and GSK-3β facilitates phosphorylation of N-terminal located serine/theronine residues of β-catenin by GSK-3β/CK-I dual kinase system (Polakis, 2000; Amit et al., 2002; Liu et al., 2002) leading to degradation initiated by ubiquitin-mediated proteolysis of β-catenin via β-TRCP (an E2 ubiquitin ligase) (Aberle et al., 1997; Hart et al., 1999; Latres et al., 1999; Liu et al., 1999; Sadot et al., 2002).

Cleary, the stability of free cytoplamic β-catenin is the heart of the canonical Wnt pathway and most of the effects of Wnt signaling are mediated by nuclear β-catenin and TCF.

The canonical Wnt pathway is highly conserved between *Drosophila* and vertebrates, and the Figure 3 showing the core elements of the canonical WNT pathway is undoubtedly an oversimplification, because different cells use this pathway in different ways.

1.7. Canonical WNT Signaling in Cancer

1.7.1 Ligand and Receptor

The Wnt ligands are secreted glycoproteins that can be categorized according to their ability to promote neoplastic transformation (Wodarz and Nusse, 1998). Oncogenic potential of Wnt was first described by Nusse and Varmus almost 20 years ago inciting intense investigation into the role of *Wnt* genes in human cancer (Nusse and Varmus, 1982). Subsequent studies covered the way for assembling a signaling pathway and consequently identification of candidate cancer-causing genes within the same pathway.

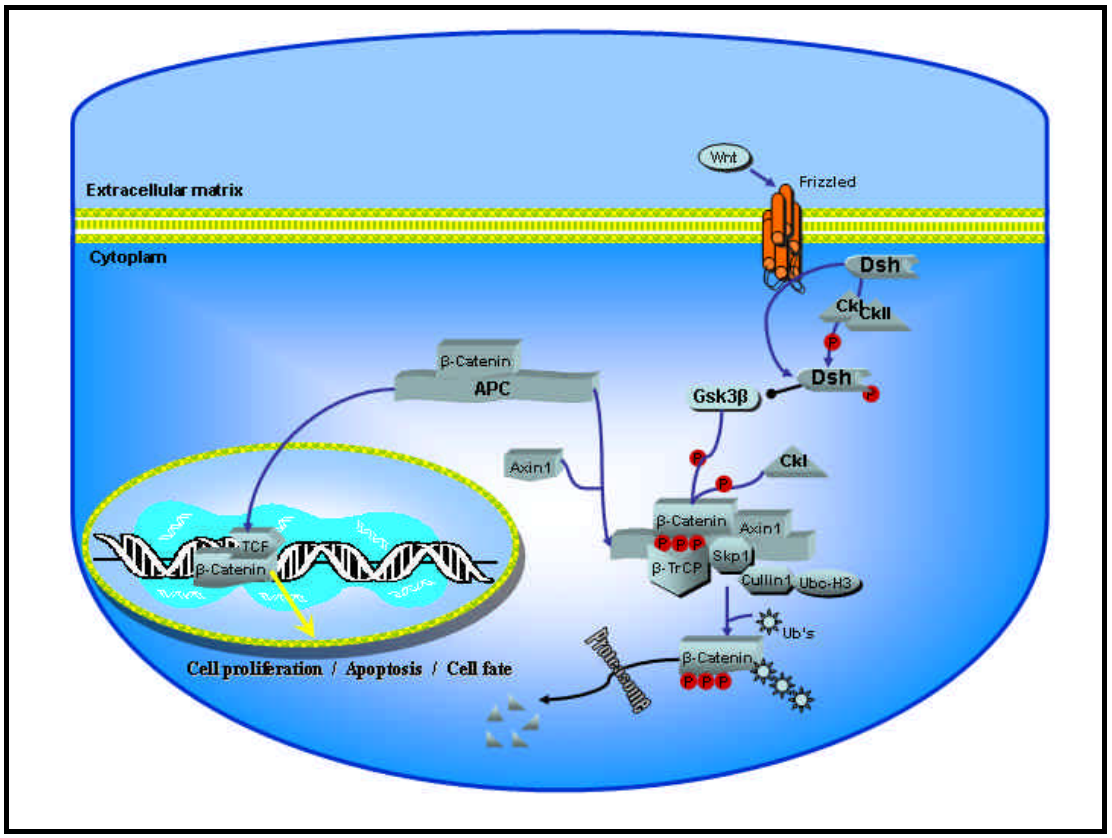


Figure 3 Canonical WNT pathway.

Core elements of WNT/β-catenin pathway are shown, illustrating how activation of Frizzled receptor by the Wnt ligand leads to activation of β-catenin. This activates gene expression leading to diverse cellular responses in both embryonic development and in adults.

Regarding the ligand-receptor interaction, at least 11 vertebrate *frizzled* genes have been identified. However except for few published data (Tanaka et al., 1998; Gazit et al., 1999; Sheldahl et al., 1999), there is no direct evidence for involvement of Frizzled in neoplastic transformation.

1.7.2 Dishevelled (Dsh)

Dishevelled is a phosphoprotein that becomes hyperphosporylated on serine /threonine residues when wnt signaling is activated. It is located downstream of the receptor (e.g. frizzled) and upstream of β-catenin (Noordermeer et al., 1994). Under normal physiological conditions, Dsh is localized in the cytoplasm, by the activation of Wnt signaling it is recruited to the membrane (Yang-Snyder et al., 1996). Overexpression of

Dsh in the absence of wnt activation leads to accumulation of β-catenin (Yanagawa et al., 1995). However, its involvement in human cancer has not been reported. One possible connection to Dsh in human cancer can be via its interaction and phosphorylation, thereby activation by casein kinase II (CK II). It has been reported that in *CKII* transgenic mice develop lymphomas (Seldin and Leder, 1995; Willert et al., 1997; Song et al., 2000).

1.7.3 Glycogen Synthase Kinase 3b (GSK-3b)

The serine/threonine kinase GSK-3β is an integral component and negative regulator of Wnt signaling in vertebrates (Dominguez et al., 1995; He et al., 1995). GSK-3β carries out its regulatory effect by phosphorylating several substrates in the Wnt pathway (Rubinfeld et al., 1996; Ikeda et al., 1998; Itoh et al., 1998; Li et al., 1999; Yamamoto et al., 1999). The down-regulatory nature of GSK-3β could be qualified as putative tumor suppressor in neoplastic transformation. However GSK-3β displays no mutation in colorectal cancers, which are known to activate Wnt pathway (Bienz and Clevers, 2000). The participation of GSK-3β in human cancers can be linked to its inactivation by Frat-1, a GSK-3β binding protein antagonistic to Wnt signaling in mammalian cells (Li et al., 1999; Thomas et al., 1999). Recently, Frat-1 was found to be involved in to cancer progression in transgenic mice model and human cancer-associated upregulation of Frat-1 has been reported (Jonkers et al., 1999; Saitoh and Katoh, 2001; Saitoh et al., 2001).

1.7.4 Axin 1

Axin was identified as an inhibitor of Wnt signaling was positioned downstream of Dsh and GSK-3β, and upstream of β-catenin and shown that the Axin protein can bind to GSK-3, APC, β-catenin and Dsh (Peifer and Polakis, 2000). The apparent lack of enzymatic function and its direct interaction with other critical component of the Wnt pathway suggest that Axin1 functions as a scaffold to assemble critical Wnt pathway component in close proximity. In this regard, Axin 1 facilitates the phosphorylation of β-

catenin by GSK-3β and CKII (Polakis, 2000; Amit et al., 2002; Liu et al., 2002). The tumor suppressor role, thereby biallelic inactivation of *Axin1* has been verified by Satoh *et al.* in human hepatocellular cancers and cell lines (Satoh et al., 2000). Tumor suppressor nature of Axin was also shown by other studies (Dahmen et al., 2001; Lustig et al., 2002; Taniguchi et al., 2002).

1.7.5 The Adenomatous Polyposis Coli (APC)

The adenomatous polyposis coli protein can bind to β-catenin and promotes its degradation in vertebrates (Su et al., 1993; Munemitsu et al., 1995; Rubinfeld et al., 1995). Loss of *APC* is observed in the majority of human colon cancers and leads to elevated β-catenin levels (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). This and many other observations suggest that the main function of APC as a tumor suppressor is to control β-catenin levels. Despite the massive amount of data implicating APC in a β-catenin regulation, its exact role in Wnt pathway is not firmly established. As negative regulator of β-catenin, APC phosphorylation by GSK-3β is needed for its association with β-Catenin (Cavallo et al., 1997; Willert and Nusse, 1998). Recently, it has been proposed that APC is a nuclear-cytoplasmic shuttling protein, and can act as a β-catenin chaperone, providing a specific transport route for nuclear β-catenin to the cytoplasm (Henderson, 2000; Henderson et al., 2002).

1.7.6 Beta Catenin (b-catenin)

β-catenin is a 92 kDa multifunctional protein and initially considered to be a cell-cell adhesion protein. It binds to the intracellular domain of cadherins, transmembrane proteins that mediate calcium-dependent cell-cell adhesion. β-catenin links cadherins to the actin cytoskeleton (DeMarais and Moon, 1992). β-catenin also behaves as an essential component of the Wnt/Wingless signaling pathway. Another line of evidence strongly suggesting a role for β-catenin in signal transduction stems from the observation that β-

catenin interacts directly with the epidermal growth factor receptor (EGFR) and that EGF induces an immediate tyrosine phosphorylation of β-catenin (Hoschuetzky et al., 1994; Shibamoto et al., 1994). Similarly, tyrosine phosphorylation of β-catenin has been observed after stimulation of cells with hepatocyte growth factor (HGF) (Shibamoto et al., 1994). More recently, c-met receptor of HGF was shown to associate with β-catenin, and upon binding of HGF ligand, β-catenin dissociates and translocates to nucleus in a Wnt-independent manner. (Monga et al., 2002)

The cellular level of the β-catenin protein, as well as its subcellular localization are very tightly regulated (Figure 4). When not associated with cell-cell junctions, β-catenin forms a large complex with the protein encoded by APC, GSK-3β, and Axin 1 (Behrens et al., 1998). In the absence of Wnt signal, constitutive phosphorylation of β-catenin by CKI/GSK-3β dual-kinase system lower its levels via ubiquitin-mediated proteolysis (Aberle et al., 1997; Kitagawa et al., 1999; Liu et al., 2002). Activation of the Wnt pathway upon binding of the Wnt ligand leads to inactivation GSK-3β that give rise to stabilization of and accumulation of hypo-phosphorylated free-β-catenin, which then translocates into the cell nucleus. Nuclear β-catenin interacts with TCF/LEF family of transcriptional factors and stimulate transcription of a variety of target genes (Hulsken et al., 1994; Molenaar et al., 1996). The nature of the target genes of the β-catenin/LEF complex remain mostly unknown, except for the recently documented *c-myc , c-jun, fra-1* and *cyclin D1* genes in colorectal cancers (He et al., 1998; Mann et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999).

β-catenin seems to be an essential component of intercellular junctions as well as wnt growth factor signaling pathways. The free cytosolic β-catenin is considered to represent the signaling pool of β-catenin (Sadot et al., 2001; Simcha et al., 2001). In addition, modulation of APC/-catenin association has been suggested to play a role in the regulation of both cadherin-based cell-cell adhesion and cell motility(Hermiston and Gordon, 1995b; Hermiston and Gordon, 1995a; Adams et al., 1996; Nathke et al., 1996)

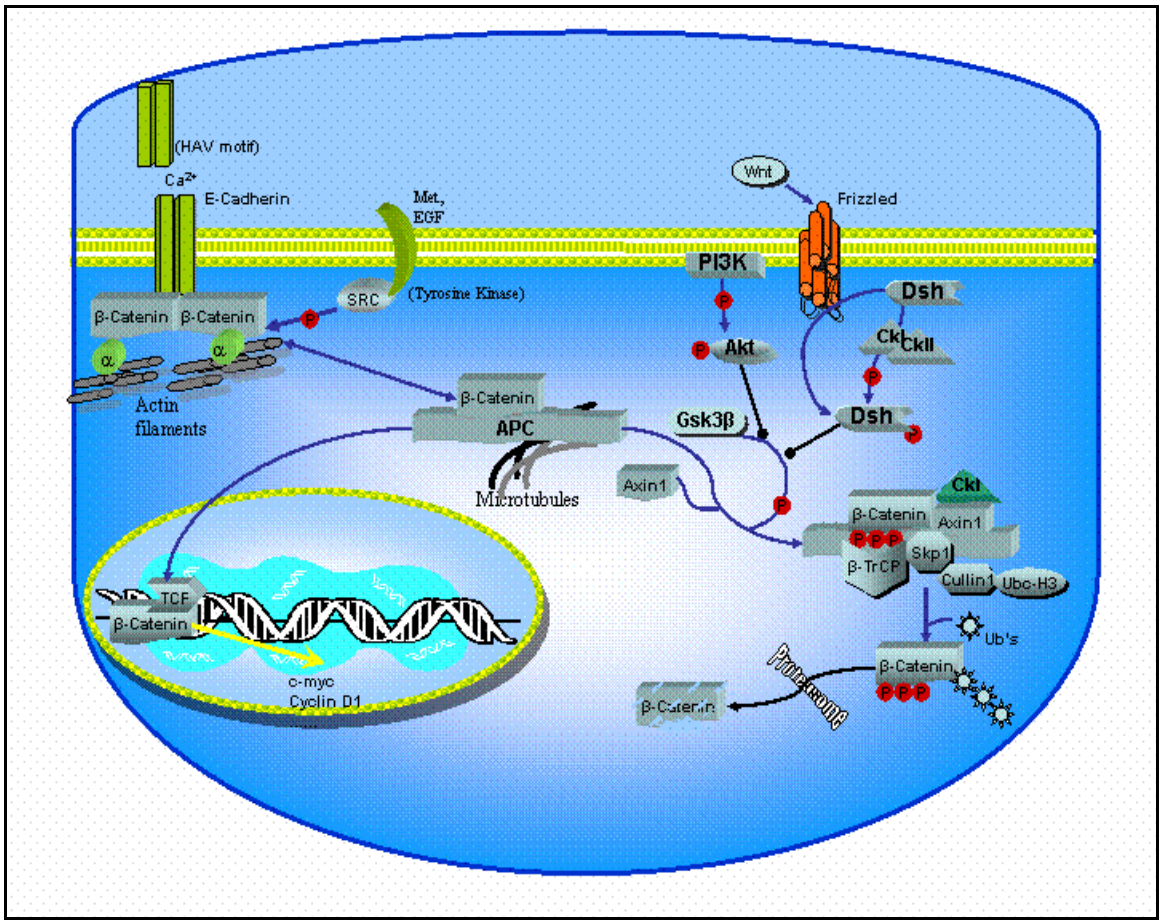


Figure 4 b-catenin is a key molecule in E-cadherin-mediated cell-cell adhesion and in the WNT signaling pathway.

The primary structure of the β-catenin protein reveals that it contains a 130 amino acid amino-terminal domain, an armodillo domain composed of 12 imperfect repeats of 42 amino acids (arm repeats), and a carboxy-terminal domain of 100 amino acids (Figure 5). Several proteins bind to β-catenin through the central region (arm repeats) and this positively-charged groove may serve as a binding motif including TCF/LEF, APC and adherent junction protein E-cadherin. (Rubinfeld et al., 1995; Yost et al., 1996; Huber et al., 1997).

The carboxyl terminal domain functions as a transcriptional activator when fused to the GAL4 DNA-binding domain (van de Wetering et al., 1997), whereas the amino terminus of β-catenin is known to be important for regulating the stability of β-catenin. This region of β-catenin, also called destruction box, contains a series of serine and threonine

residues, which may be phosphorylated. Phosphorylation of serine/threonine residues at positions 29, 33, 37, 41 and 45 by GSK-3β in complex with Axin and APC appears to be a prerequisite for ubiquitination (Aberle et al., 1997; Orford et al., 1997). Recently, another Axin-associated kinase, Casein kinase I (CKI) has been shown to be both necessary and sufficient to initiate the β-catenin phosphorylation-degradation cascade (Liu et al., 2002). Complex of Axin and CKI induces β-catenin phosphorylation at a single site: serine 45, and creates a priming site for subsequent GSK-3β phosphorylation cascade finally hitting the S33/S37 of β-catenin (Amit et al., 2002; Ding and Dale, 2002; Gao et al., 2002; Liu et al., 2002). FWD1 (mouse homologoue of Slimb/βTrCP), an F-box/WD40-repeat protein, specifically forms a multi-molecular complex with β-catenin, Axin, GSK-3β and APC and facilitate ubiquitination and degradation of β-catenin. FWD1 also serves as an intracellular receptor for phosphorylated β-catenin (Kitagawa et al., 1999).

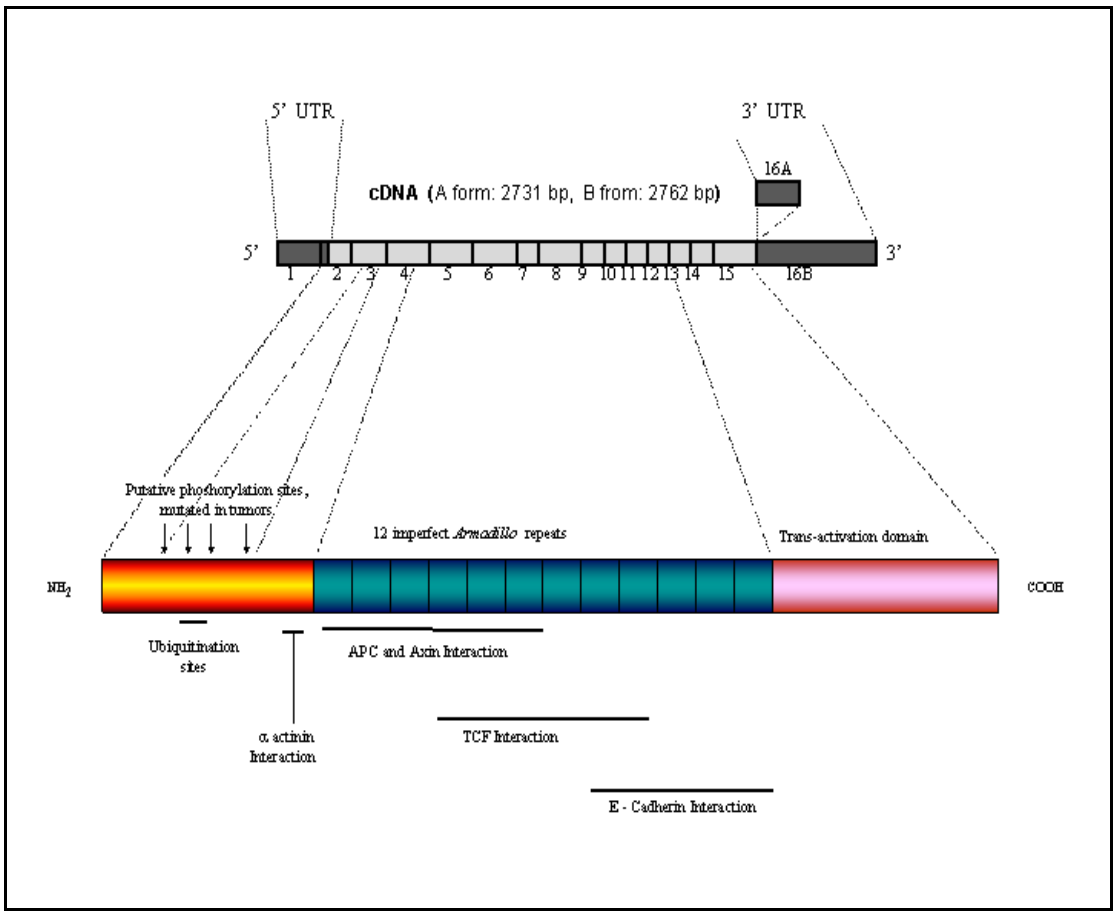


Figure 5 cDNA and protein structure of human b-Catenin Gene (*CTNNB1*)

Presently, several colon, endometrial, anaplastic thyroid, hepatocellular carcinomas, pilomatrixomas, small colorectal adenomas and childhood hepatomas contain elevated levels of β-catenin which can be attributed to point mutations that change either the critical serine and threonine residues and/or their neighbourhood amino acid residues within the destruction box, thereby blocking its phosphorylation and subsequent degradation (Fukuchi et al., 1998; Miyoshi et al., 1998b; Muller et al., 1998; Palacios and Gamallo, 1998; Voeller et al., 1998; Zurawel et al., 1998; Iwao et al., 1999; Koch et al., 1999; Samowitz et al., 1999; Wright et al., 1999; Ueda et al., 2001a).

As shown in Table 3, regulatory sequences in destruction box of β-catenin is mutated in a wide range of human cancers as well as in chemically induced animal tumors. Importantly, ***b-catenin*** mutations in tumors are exclusive to those that inactivate *APC*. This is particularly evident in colorectal cancer where the huge majority of these tumors contain *APC* mutation and display very low ***b-catenin*** mutation frequency (Kitaeva et al., 1997; Sparks et al., 1998; Samowitz et al., 1999). This inverse correlation between *APC* and ***b-catenin*** mutations were also shown in colorectal cancers without *APC* mutations (Iwao et al., 1998; Sparks et al., 1998). Not surprisingly, the exclusivity of *CTNNB1* and *APC* mutations were also found in over half of 42 desmoids analyzed, whereas *APC* mutations were detected only in nine tumors (Shitoh et al., 1999; Tejpar et al., 1999). Relatively high incidence of ***b-catenin*** mutations in sporadic hepatoblastomas and gastric cancers which are also less likely to have biallelic inactivating mutation in *APC*, indicates components in the Wnt pathway other than *APC*, such as ***b-catenin***, might make easier targets for oncogenic mutations (Koch et al., 1999; Park et al., 1999c; Jeng et al., 2000; Wei et al., 2000).

Notably, HCCs and hepatoblastomas have become one of most common tumors harboring mutations in the Wnt pathway. Several independent studies have revealed that overall ***b-catenin*** mutation rate in HCCs is around 24%, and ~60% of hepatoblastomas displays ***b-catenin*** mutation (de La Coste et al., 1998; Miyoshi et al., 1998a; Huang et al., 1999; Legoix et al., 1999; Nhieu et al., 1999) (Table 3).

Table 3 Beta-catenin mutations in human cancers (modified fromPolakis, 2000)

Amino acids affected by mutations in CTNNB1 gene are indicated for the sequence located between serine-29 (S29) and lysine-49 (K49). The overall frequency (freq.) of mutations in each tumor type is represented as the number of tumors with mutations/total number of tumors analyzed. The amino acid affected by the mutation and the number of mutations at each position are listed across columns. Interstitial deletions affecting one or more amino acids in the sequence are indicated as Δ.

Tissue	Freq.	S29	Y30	I31	D32	S33	G34	I35	H36	S37	G38	A39	T40	T41	T42	A43	P44	S45	Δ
Colorectal	11/294						1							4				6	
Colorectal-w/o APC mut.	20/85					2	1							3				5	8
Colorectal HNPCC	12/28				2		2			1				2				5	
Colorectal w MSI	13/53																	6	
Colorectal w/o MSI	0/27																		
Desmoid, sporadic	23/43													11				12	
Endometrial w/ MSI	3/9				2	1													
Endometrial w/o MSI	10/20				3	1	2			3				1					
Gastric	7/26	2			5														
Hepatocellular	62/277				14	6	6	1	1	6			1	8				17	7
Hepatoblastoma	47/79				4		6	1		2				6				2	27
Kidney, Wilm's tumor	6/40													1				2	
Medulloblastoma	3/47					2				1									
Melanoma	1/65																	1	
Ovarian, endometriod	20/87				3	3	2			10				2					
Pilomatricoma	12/16				2	4	3			2				1					
Prostate	5/104				1	2								1				1	
Thyroid anaplsatic	19/31					1				3	1		8	2	1	1	4	2	
Uterine endometrium	10/76					1				2				4				3	

1.7.7 T-Cell Factor/Lymphocyte Enhancer Binding Factor (TCF/LEF) Family of Transcriptional Factors

Wnt signal-activated and uncomplexed β-catenin is stabilized in cytoplam, which can then transcolate into the nucleus to combine with T cell factor/lymphocyte enhancer binding factor (TCF/LEF) family of transcriptional factors. Although LEF/TCFs bind directly to DNA through their HMG-domains, they are incapable of activating gene transcription (Roose and Clevers, 1999). HMG-domain proteins appear to have intrinsic ability to either activate or repress transcription of target gene, a decision that may be regulated by interaction with β-catenin (Brannon et al., 1997; Rocheleau et al., 1997; Thorpe et al., 1997). The founding members of the TCF/LEF family of transcription factors, TCF-1 and LEF-1, were identified in screens for T cell-specific transcription factors (Travis et al., 1991; van de Wetering et al., 1991). In more recent years, two additional family members were identified in mammals: TCF-3 and TCF-4 (Korinek et al., 1998). All members of the TCF family can bind β-catenin through a conserved N-terminal stretch of 55 amino acids. β-catenin thus functions as a classical coactivator of transcription (Molenaar et al., 1996; Korinek et al., 1998; Duval et al., 2000). Recent studies have indicated *TCF/LEF* mRNAs undergo extensive alternative splicing. (Duval et al., 2000). While the functional relevance of the alternative splice products in *TCF/LEF* genes remains unclear at present, alternative promoter usage in the *TCF*-and *LEF-1* gene generates protein isoforms that either carry or lack the N-terminal β-catenin interaction domain.

The *TCF*s mutations do not directly contribute to cancer progression but can act in additive manner. Regarding this suggestion, it has been shown that *TCF-4* was mutated in a subset of colorectal tumors (Brannon et al., 1997; Rocheleau et al., 1997; Thorpe et al., 1997), and also transgenic mice homozygous for mutations in *TCF-1* developed adenomatous intestinal polyps(Roose et al., 1999).

1.8 The p53 –WNT Cross Talk

Defects leading to activation of the Wnt pathway could occur in signaling systems that are apparently disparate to Wnt signaling. One potential mode of cross talk includes the p53 tumor suppressor. As activation of wnt/β-catenin signaling is thought to contribute to tumorogenesis of certain tumors, including liver cancers, the repression of this signal by tumor suppressor p53 may be one mechanism of tumor suppression by p53.

Forced overexpression of β-catenin is believed to mimic the oncogenic activation of this protein. Recently, it was reported that such overexpression results in the accumulation of wild-type p53, apparently through interference with its proteolytic degradation (Damalas et al., 1999). Suppression p53 activity via competitive binding of β-catenin to CBP/p300 was also reported. The CBP/p300 is a transcriptional coactivator, central regulator of gene expression and important in cell differentiation, cell cycle, and anti-oncogenesis (Miyagishi et al., 2000). Another study displaying the negative regulator nature of p53 in wnt pathway was the demonstration that Dickkopf-1 (Dkk-1) which is an inhibitor of Wtn signaling (Fedi et al., 1999), is induced by p53 (Wang et al., 2000).

Recently, additional evidence for the presence of negative feedback control between β-catenin and p53 was provided (Damalas et al., 2001; Liu et al., 2001; Matsuzawa and Reed, 2001; Sadot et al., 2001). It has been shown that oncogenic activation of β-catenin induces $p14^{INK4A}$ expression and augments transcription from the of $p14^{INK4A}$ promoter in colorectal carcinoma(CRC)-derived cell as well as fibroblasts, thereby preventing p53 degradation and allowing accumulation of functional wild-type p53. The $p14^{INK4A}$ upregulation is significantly eliminated by the introduction of dominant-negative TCF, and also is partially mediated by E2F1 manner (Damalas et al., 2001). Subsequent β-catenin degradation mechanism after induction of functional active p53, was shown to be linked to a p53-induced gene called *Siah-1* (Amson et al., 1996; Nemani et al., 1996), which binds to ubiquitin-conjugating enzymes via an N-terminal RING domain and targets other proteins to degradation (Hu et al., 1997a; Hu et al., 1997b), and requires APC/β-catenin protein complex (Liu et al., 2001; Matsuzawa and Reed, 2001).

However, there are two conflicting observations for active degradation of β-catenin in a p53-dependent manner. Sadot *et al.* has shown that the inhibitory effect of p53 on β-catenin is apparently carried out by β-TrCP ubiquitination system and requires an active GSK-3β (Sadot et al., 2001). On the other hand, a novel degradation mechanism was implied by Matsuzawa and Reed (Figure 6). A network of protein interaction in which Siah-1, SIP (a novel Siah-interacting protein, which provides a physical link between Siah-1 and Ebi), Skp1 (central component of E3 (Koepp et al., 1999)) and Ebi (an F-box protein that binds to β-catenin independently of the phosphorylation site recognized by GSK-3β) collaborate in a pathway controlling the β-catenin levels in response to p53 activation (Matsuzawa and Reed, 2001).

1.9 WNT/b-catenin Signaling in Liver Cancer

Studies in recent years clearly established that the deregulation of Wnt signaling pathway via oncogenic activation of β-catenin plays a key role in hepatocellular carcinomas, both in man and rodents. Activating mutations in *b-catenin* are quite prevalent in human hepatocellular cancers. The preliminary reports from de La Coste *et al.* and Miyoshi *et al.*, have revealed that *CTNNB1* (gene encoding β-catenin) mutations are relatively frequent in human and mouse HCCs (de La Coste et al., 1998; Miyoshi et al., 1998a). Succeeding studies have demonstrated that prevalence of aberrant accumulation of β-catenin is between 33-69%, while only 8-13% of studied HCCs display *b-catenin* mutations (Hsu et al., 2000; Devereux et al., 2001; Wong et al., 2001). The second most likely component of Wnt pathway for oncogenic β-catenin activation is *Axin 1*, a putative tumor suppressor gene. The overall mutational and/or LOH screening studies in HCC samples have shown that only a small fraction of screened samples (7-9%) were subject to *Axin 1* mutations (Satoh et al., 2000; Laurent-Puig et al., 2001; Taniguchi et al., 2002).

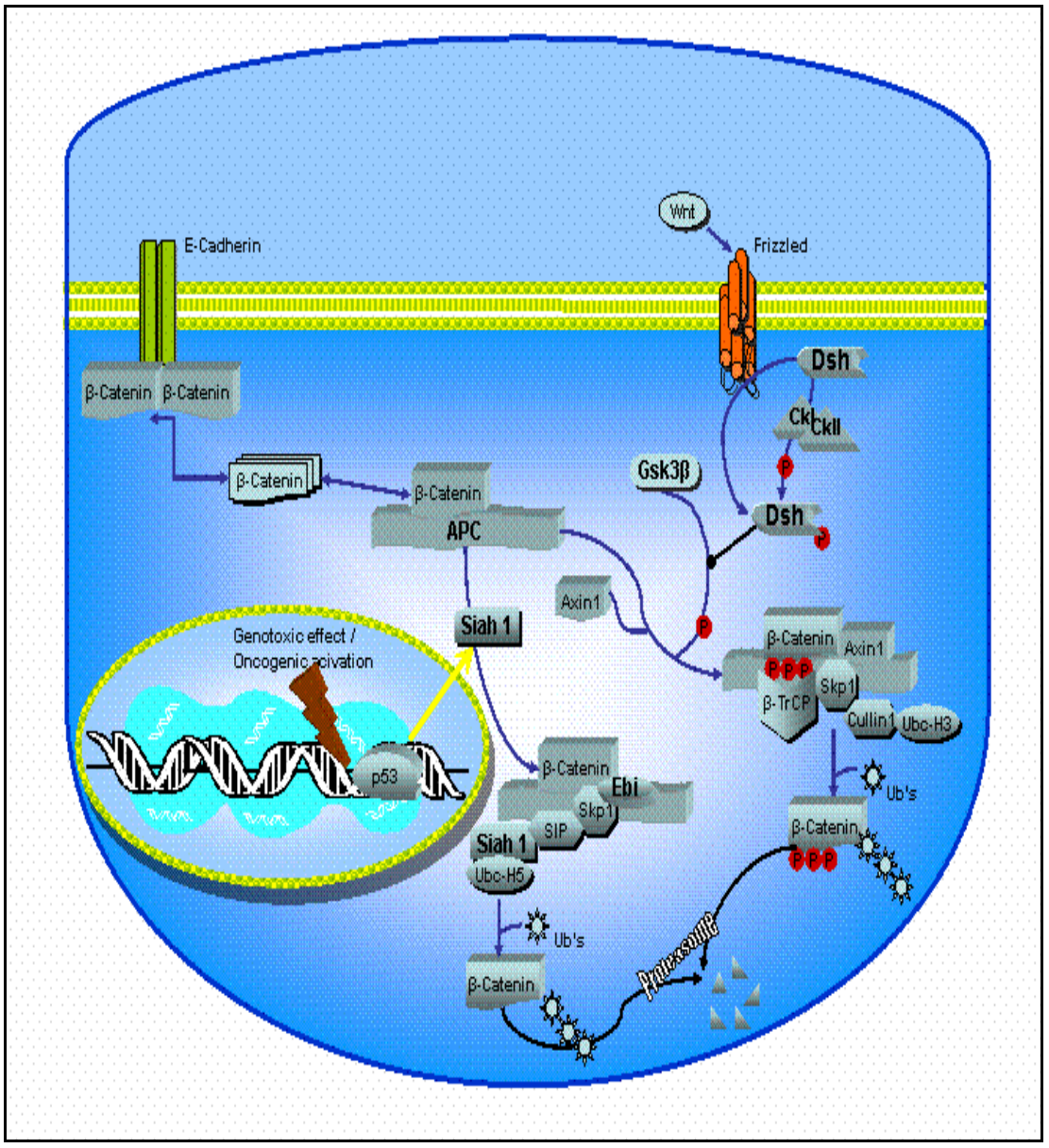


Figure 6 b-catenin degradation pathways (Adapted form Matsuzawa and Reed, 2001)

Two alternative pathways for regulation of -catenin levels are presented, involving different F box proteins (Ebi versus -TrCP). One pathway (left) is initiated by increases in the expression of Siah-family proteins, which can be induced, for example, by p53 in response to DNA damage, and involves sequential protein interactions with SIP, Skp1, and Ebi. Ebi binds -catenin, thus recruiting it to the Siah-1–SIP–Skp1 complex for polyubiquitination and subsequent proteosome-mediated degradation. Siah-1 binds the E2 UbcH5. The other pathway (right) is regulated by Wnt signals(Dsh)
and possibly PI3K/Akt. This pathway is phosphorylation dependent and involves GSK3-induced phosphorylation of Ser-33 and Ser-37 on -catenin, allowing -TrCP binding, resulting in recruitment of β-catenin to Skp1–Cullin-1–-TrCP complexes (SCF). Cullin-1, in collaboration with other proteins not shown, supplies this SCF complex with E2s, such as UbcH3. APC is required for both pathways as a scaffold protein, binding -catenin via one domain and also binding Siah-1 (left) and GSK3 (right) (Liuet al., 2001)

The molecular etiology for accumulation of β-catenin in HCC is not completely understood. Studies in transgenic mice and chemical carcinogenesis have shown that both the pattern and frequency of *b-catenin* mutations are heterogenous (Tsujiuchi et al., 1999; Calvisi et al., 2001; Devereux et al., 2001). Almost half of the *b-catenin* aberrations in human hepatobastoma are interstitial deletions covering the third exons of the gene (Koch et al., 1999; Wei et al., 2000). However, the role of β-catenin in cancer formation and/or progression in cholangiocarcinoma has not been well-studied. There are only two reports which showed that *b-catenin* mutations were present in 8 of 107 (7.5%) studied biliary tract cancer form China, and only 15% of cholangiocarcinoma displayed aberrant nuclear accumulation of β-catenin without genetic mutation (Rashid et al., 2001; Sugimachi et al., 2001).

The finding of mutated *b-catenin* in early stages of human HCC (de La Coste et al., 1998; Miyoshi et al., 1998a; Hsu et al., 2000),in adenomas in c-myc transgenic mice, and rapid development of hepatomegaly in mutated β-catenin expressing transgenic mice suggest (Cadoret et al., 2001; Calvisi et al., 2001) that aberrations in wnt pathway can be considered as an early event in hepatocarcinogenesis.

An unexpected feature of HCC is the high frequency of β-catenin accumulation despite the presence of rather low levels of *b–catenin* and *Axin1* mutations. This suggests that β-catenin accumulation can be caused by additional mechanisms.

CHAPTER 2. OBJECTIVES AND RATIONALE

Cytoplasmic and/or nuclear accumulation of β-catenin is a highly frequent event affecting various cancers including colorectal, lung, breast, cervix cancers, melanoma, HCC and hepatoblastoma (Candidus et al., 1996; Rimm et al., 1999; Chung, 2000; Lin et al., 2000; Park et al., 2001; Shinohara et al., 2001; Ueda et al., 2001a; Hommura et al., 2002; Inagawa et al., 2002). In colorectal cancers, β-catenin accumulation is often associated with mutational inactivation of *APC* gene, which affects almost 80% of these tumors (Kinzler and Volgelstein, 1998). About 50% of colorectal cancers with wild-type *APC* gene display oncogenic mutations affecting ***b-catenin*** directly (Sparks et al., 1998). In hepatoblastomas, *APC* mutations are rare, but these tumors display ***b-catenin*** mutations at very high frequency (Koch et al., 1999; Wei et al., 2000; Park et al., 2001). In other tumors, aberrant accumulation of β-catenin protein is not always associated with a mutation affecting these genes. For example, it has been recently reported that 8-13% of HCCs are affected by a ***b-catenin*** mutation, whereas 33-69% of these tumors display aberrant β-catenin protein accumulation (Hsu et al., 2000; Devereux et al., 2001; Wong et al., 2001). Among other candidate genes, *Axin1* was found to be mutated in a small fraction (7-9 %) of HCCs (Satoh et al., 2000; Laurent-Puig et al., 2001), whereas *APC* mutations were exceptional, if not absent (Huang et al., 1999). Thus, it is estimated that in between 35 and 80% of HCCs, the aberrant β-catenin accumulation is not associated with a mutation affecting ***b-catenin,*** *Axin1* or *APC* gene. Aberrant accumulation of β-catenin protein without associated mutations on ***b-catenin,*** *APC* or *Axin1* gene is not limited to HCC, since mutations affecting these genes are absent or exceptional in several other common

malignancies such as cancers of the breast, lung and cervix, and melanomas (Candidus et al., 1996; Rimm et al., 1999; Lin et al., 2000; Shinohara et al., 2001; Ueda et al., 2001a; Hommura et al., 2002). Thus, in many tumors, aberrant accumulation of β-catenin cannot be linked to a known mutation affecting *wnt* pathway, raising the possibility that mutations affecting the genes involved in other pathways may also contribute to aberrant accumulation of β-catenin in cancer cells.

In this study, we investigated the contribution of β-catenin to the development of HCC in Southern Africa and China sample populations, and scrutinized the aberrant accumulation of wild-type β-catenin protein in association with *p53* gene mutation in HCC cells.

CHAPTER 3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO, U.S.A), Farmitalia Carlo Erba (Milano, Italy) and Merck (Schucdarf, Germany) with the following exceptions: Ethanol was from Delta Kim Sanayi ve Ticaret A.S (Turkey). Midi-prep kit and Qiaex kit (for recovery and extraction of DNA from agarose gel) were from Qiagen (Chatsworth, CA, U.S.A). Agar, tryptone and yeast extract were obtained from Gibco, BRL Life Technology Inc. (Gaithersburgs, MD, U.S.A).

3.1.2 Bacterial Strain

The bacterial strain used in this work was: *E. coli*: DH5α and JM109

3.1.3 Enzymes

Restriction endonuclease Hinf I and Taq DNA polymerase form *Thermus aquaticus* BM were purchased from Roche Diagnostics GmbH (Mannheim, Germany). DNase free RNase was from Promega (Madison, WI, U.S.A)

3.1.4 Nucleic Acids

DNA molecular weight standard and ultrapure deoxyribonucleotides were supplied by MBI Fermentas GmbH (Germany). Eukaryotic cloning and expression vector pEGFP-N2 (C-terminal protein fusion vector) was purchased from CLONTECH Laboratories, Inc. (CA, U.S.A). *p53* complementary DNA cloned into a eukaryotic expression vectors, namely p53-V143A, p53R248W and p53-R249S, were gift from T. Frebourg, (INSERM EPI9906, Faculté de Médecine et de Pharmacie, Rouen Cedex, France).

3.1.5 Oligonucleotides

The sequencing-primer used for cycle sequencing reactions and oligonucleotides used in polymerase chain reaction (PCR) was synthesized by ÝONTEK (Bursa, Turkey). Sequence of the primers used throughout this syudy is given in Table 4.

3.1.6 Electrophoresis and Photography

Electrophoresis grade agarose was supplied from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO, U.S.A). Horizontal electrophoresis apparatuses were from Stratagene (Heidelberg, Germany) and E-C Apparatus Corporation (Florida, U.S.A). The power supply Power-PAC300 was from Bio Rad Laboratories (CA, U.S.A). Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software used in agarose gel profile visualizing were from BioRad Laboratories (CA, U.S.A).

3.1.7 Tissue Culture Reagents and Cell Lines

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum was obtained from BIOCHROM AG Seromed (Berlin, Germany). L-glutamine, gentamycin, calcium and magnesium-free phosphate buffered saline (PBS), Penicillin / Streptomycin mixture was from Biological Industries (Haemel, Israel). Tissue culture flasks, petri dishes, 15 ml polycarbonate centrifuge tubes with lids and cryotubes were purchased from Costar Corp. (Cambridge, England). Geneticin (G418 sulfate) was from GibcoBRL, Life tech. (U.S.A)

3.1.8 Tumor Specimens

In a previous study, 80 hepatocellular carcinomas from three different continents have been investigated for *p53* gene mutations and HBV sequences (Unsal et al., 1994). In the present study, we investigated 33 of these tumors obtained from patients lived in Mozambique (n= 10), South Africa (n=12) and China (n=11).

3.1.9 Radioisotopes

á-^{32}P-dCTP (3000Ci/mmol) were from Amersham Pharmacia Biotech Ltd. , (Buckinghamshire, U.K)

3.1.10 Antibodies and Chemiluminesence

For western blot studies; mouse anti-human β-catenin monoclonal antibody (clone E5) supplied form Santa Cruz Biotech. (U.S.A) and mouse anti-human GSK-3β monoclonal antibody (clone 7) from Transduction Labs (U.S.A) were used. The mouse anti-human p53 monoclonal antibody (clone 6B10) was produced in the laboratory (Yolcu et al., 2001)

For immunofluorescence studies; mouse anti-human β-catenin monoclonal antibody (clone 6F9) was purchased from Abcam Ltd (Cambridge, U.K) and FITC conjugated secondary antibody, goat anti-mouse immunoglobulins-F(ab')2 was from DAKO Corporation (CA, U.S.A) (Cat. No. F0749).

Detection of proteins immobilized on membranes was performed by using the ECL Western Blotting kit Amersham Pharmacia Biotech Ltd. (Buckinghamshire, U.K) according to the manufacturer's instructions.

3.2 SOLUTIONS AND MEDIA

3.2.1 General Solutions

50X Tris-acetic acid-EDTA (TAE):	2 M Tris-acetate, 50 mM EDTA pH 8.5 Diluted to 1X for working solution.
Ethidium bromide:	10 mg/ml in water (stock solution), 30 ng/ml (working solution)
1X Gel loading buffer:	0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 1mM EDTA

Solutions for plasmid DNA isolation :

Solution I	50 mM Glucose, 25 mM Tris.Cl, pH 8.0, 10 M EDTA. Sterilize in autoclave.
Solution II	0.2 N NaOH, 1% (wt/vol) SDS
Solution III	3 M Potassium acetate, pH 4.8

Bradford Stock Solution	4.75 ml of 95 % ethanol, 10 ml of 85% phosphoric acid , 17.5 mg Coomassie Brilliant Blue. Stored in dark at 4°C.
Bradford Working Solution	21.25 ml double distilled water, 0.75 ml of 95% ethanol, 1.5 ml of 85% phosphoric acid, 1.5 ml of Bradford stock solution. Filtered through Whatman No.1 paper and prepared freshly.

3.2.2 Microbiological Media and Antibiotics

Luria-Bertani medium (LB)	*Per liter:* 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl. For LB agar plates, add 15 g/L bacto agar.
Glycerol stock solution	65% glycerol, 0.1 M $MgSO_4$, 0.025 M Tris.Cl, pH 8.0
Ampicillin	100 mg/ml solution in double-distilled water, sterilized by filtration and stored at -20°C (stock solution).100 µg/ml (working solution)
Kanamycin	300 mg/ml solution in double-distilled water sterilized by filtration and stored at -20°C (stock solution). Working solution was 30 µg/ml.

3.2.3 Tissue Culture Solutions

DMEM	*For 500 ml* DMEM*:* 2mM Fetal calf serum, 100 U/ml Penicillin, 0.1 mg/ml streptomycin. Stored at -4 °C.
10X Phosphate-buffered saline (PBS)	*Per liter:* 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, pH 7.4
BES Transfection solutions:	
2.5 M $CaCl_2$	3.675 g $CaCl_2$ in 10 ml double-distilled water. Sterilized by filtration through 0,2ìm filter. Stored at -20°C
100 mM BES, pH 6.95	0.2132 g BES (N,N-bis(2-hydroxyethly) in double-distilled water. pH was adjusted to 6.95 with NaOH at room temperature. Stored at -20°C
2X BBS (BES buffer saline)	50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4 in double-distilled water. Sterilized by filtration through 0,2ìm filter. Stored at -20°C.
Geneticin (G418 Sulfate)	500 mg/ml solution in double-distilled water, sterilized by filtration and stored at -20°C (stock solution). 500 ìg/ml (working solution for stable cell line selection) and 250 ìg/ml (working solution for maintenance of stable cell line).

3.2.4 Single Strand-Conformational Polymorphism (SSCP) Solutions

(40%) (75:1) Acrylamide solution	39.5 g acrylamide, 0.53 g bisacrylamide the volume was adjusted to 100 ml by adding ddH_2O. Filtered and stored at 4°C (stock solution).
(40%) (99:1) Acrylamide solution	98.6 g acrylamide, 0.4 g bisacrylamide the volume was adjusted to 100 ml by adding ddH_2O. Filtered and stored at 4°C (stock solution).
SSCP loading buffer	95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol
10X Tris-boric acid-EDTA (TBE)	*Per liter:* 108 g Tris HCl, 55 g boric acid,20 ml 0.5 M EDTA, Filtered and autoclave sterilized. Stored at 4°C.

3.2.5 SDS (Sodium Deodecyl Sulfate)-PAGE (Polyacrylamide Gel Electrophoresis) Solutions

30% Acrylamide mix (1:29)	*Per 100 ml*: 29 g acrylamide, 1 g bis-acrylamide in double-distilled water, filtered and stored at 4°C (stock solution).
5X SDS gel-loading buffer	3.8 ml double-distilled water, 1 ml of 0.5 M Tris-HCl, 0.8 ml glycerol, 1.6 ml of 10% SDS, o.4 ml of 0.05% bromophenol-blue, 400 ìl 2-β-Mercaptanol. (Prepared freshly). Diluted to 1X for working solution

5X SDS-electrophoresis buffer	*Per liter:* 15.1 g Tris base, 72 g Glycine, 5 g SDS. Diluted to 1 X for working solution. Stored up to 1 month at 4°C.
10% Ammonium persulfate (APS)	0.1 g/ml solution in double distilled water (Prepared freshly)
1.5 M Tris-HCl, pH 8.8	54.45 g Tris base (18.15 g/100 ml) ~150 ml distilled water Adjust to pH 8.8 with 1 N HCl. Make to 300 ml with distilled water and store at 4° C.
0.5 M Tris-HCl, pH 6.8	6 g Tris base ~ 60 ml distilled water Adjust to pH 6.8 with 1 N HCl. Make to 100 ml with distilled water and store at 4° C.

3.2.6 Immunoblotting Solutions

Semi-dry transfer buffer	*Per liter*: 48 mM Tris, 39 mM glycine, 0.037% SDS, 20% ethanol pH 9.2. Do not adjust the pH.
10X Tris-buffer saline (TBS)	*Per liter:* 100 mM Tris-base, 1.5 M NaCl, pH 7.5 in double distilled water.
TBS-Tween (TBS-T)	0.5% Tween-20 solution in TBS (Prepared freshly)
Blocking solution	3% (w/v) non-fat milk, 0.5% Tween-20 in TBS. (Prepared freshly).

3.2.7 RNA Study Solutions

DEPC-treated water	0.1% Diethylpyrocarbonate (DEPC) (v/v) in double-distilled water was stirred in loosely plugged bottle. Then autoclaved and stored at room temperature.
5X Formaldehyde gel running buffer	*Per liter:* 20 ml of 2M Sodium Acetate, 20.6 gr MOPS, 780 ml of DEPC treated distilled water. pH was adjusted to 7.0 with 5M NaOH. Then 10 ml of 0.5 M EDTA pH 8.0 and volume was completed to 1 liter with DEPC-treated water. Stored at room temperature.
RNA loading buffer	50% formamide, 20% Formaldehyde, 15% 5X running buffer, 15% glycerol-dye. Stored at -20 °C.

3.2.7 Immunofluorescence Solutions

H33258 fluorochromo dye	1 mg/ml solution in double-distilled water and stored at -20 °C. Working solution was 300 µg/ml.
3% paraformaldehyde	3 g paraformaldehyde, 5 mM NaOH in PBS, pH 7.4. Stable at 4°C for a week.
PBS-TritonX-100 (PBS-T)	0.1 TritonX-100 in PBS.

3.3 METHODS

3.3.1 General Methods

3.3.1.1 Transformation of *E.coli*

Transformation of plasmid DNA into *E.coli* was achieved by using calcium chloride method. The following procedure is based on Ausubel *et al.* (Ausubel et al., 1991).

Preparation of competent cell

500 µl of appropriate *E. coli* strain glycerol stock solution was inoculated into 5 ml of LB medium containing selective agent and cells were grown at 37°C, shaking at 200 rpm to an optical density at 590 nm (OD_{590}) of 0.4 (approximately for 3 h). 1.5 ml of growing cells were centrifuged at 13,000 rpm for 1 min at 4°C and gently resuspended in 500 µl ice-cold 50 mM $CaCl_2$. After preparation, competent cells were used within 24 h or stored at -80°C for future use.

Transformation

Competent cells were suspended with 500 µl ice-cold 50 mM $CaCl_2$ and centrifuged at 13,000 rpm for 1 min at -4°C. The pellet was resuspended gently in 100 µl of ice-cold 50 mM $CaCl_2$. 1 µl plasmid DNA (1ng/µl) was mixed with the competent cells and incubated on ice for 30 min. The competent cells were heat shocked at 42° for 90 seconds and the cells were then incubated on ice for 2 min. 1 ml of LB medium was added onto competent cells and incubated at 37°C, 200 rpm for 1 h to allow the expression of antibiotic resistance gene before plating. After the incubation, 200 µl of transformation mixture was plated onto LB agar plates containing 100 µg/ml ampicillin or 30 µg/ml kanamycin to provide a selection for positive colonies carrying the newly introduced antibiotic-resistance gene via transformed plasmid and incubated at 37°C overnight for the selection of antibiotic-resistant tranformants.

3.3.1.2 Growth and Storage of Bacterial Strains

A single bacterial colony picked from either an agar plate or a loopfull of bacterial glycerol stock was inoculated into 5 ml LB broth in 15 ml screw capped tubes. The tubes were incubated at 200 rpm at 37°C for overnight in a rotator-incubator.

Bacterial strains were stored at -70°C in LB medium containing 50% bacterial glycerol stock solution for long term storage. Recombinant clones were stored under the same condition in media containing the appropriate antibiotic. Strains were maintained as isolated colonies on LB agar plates at 4°C for short term storage. Bacterial strains used in this study are defined in section 2.1.2.

Purification of plasmid DNA using the Qiagen Kit

The Qiagen 100 kit was used for large scale isolation of pure plasmid DNA. The method is based on the "midi-prep" instructions supplied with the QIAGEN Plasmid Midi Kit (Cat. No. 12145) by Qiagen (Germany). This procedure yields approximately 60-150 µg of plasmid DNA for 100 ml initial LB culture.

3.3.1.3 Preparation of Genomic from Cultured Cells

Cultured cells were grown in 15mm tissue culture Petri dish to 70-80% confluency. Adherent cells were trypsinized from 150 cm^2 flasks and washed with PBS by centrifugation at 13.000 x g for 2 min. The pellet was resuspended in 0.5 ml freshly prepared digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5 mM SDS and 0.1 gm/ml Proteinase K). After incubation at 65 °C overnight, DNA was extracted as explained in the following "phenol extraction" and "Ethanol precipitation" sections.

Phenol extraction

The DNA solution was mixed with an equal volume of 25:24:1 phenol/chloroform/isoamylalcohol and vortexed vigorously. The aqueous and organic phases were separated by centrifugation at 13,000 rpm for 2 min. The top (aqueous) phase was transferred to a new tube. In order to improve the recovery of DNA (especially in cases where starting specimen was too small) the organic phase was re-extracted with 100 µl double-distilled H_2O and the second extract was pooled with the first extract.

Ethanol precipitation

DNA solution or the aqueous phase collected from phenol extraction was mixed with $^1/_2$ volume of 7.5 M ammonium acetate, pH 5.2 and mixed by vortexing briefly. After addition of 2 volume of ice-cold absolute ethanol, the sample was left at -20°C for an hour. The pellet was recovered by centrifugation at 13,000 rpm for 20 min and washed by 1 ml of 70% ethanol. The pellet was air-dried and resuspended in 150-300 µl sterile distilled water.

3.3.1.4 Quantification and Qualification of Nucleic Acids

Concentration and purity of the double stranded nucleic acids (plasmid and genomic DNAs,), oligonucleotides and total RNAs were determined by using the Beckman Instruments Du Series 600 Spectrophotometer software programs (ds DNA ,Oligo DNA Short and RNA methods) on the Beckman Spectrophotometer Du640 (Beckman Instruments Inc. CA. U.S.A).

3.3.1.5 Restriction Enzyme Digestion of DNA

Restriction enzyme digestions were routinely performed in 20 µl reaction volumes and typically 2-10 µg DNA was used. Reactions were carried out with the appropriate reaction buffer and conditions according to the manufacturer's recommendations.

Digestion of DNA with two different restriction enzymes was performed in the same reaction buffer to provide the optimal condition for both restriction enzymes. If no single reaction buffer could be found to satisfy the buffer requirements of both enzymes, the reactions were carried outsequentially.

3.3.1.6 Gel Electrophoresis of Nucleic Acids

Horizontal agarose gels of DNA samples

DNA fragments were fractionated by horizontal electrophoresis by using standard buffers and solutions. DNA fragments less than 1 kb were generally separated on 1.0 % agarose gel, those greater than 1 kb (up to 11 kb) were separated on 0.8 % agarose gels.

Agarose gels were completely dissolved in 1x TAE electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30 μg/ml. The DNA samples were mixed with one volume loading buffer and loaded onto gels. The gel was run in 1x TAE at different voltage and time depending on the size of the fragments at room temperature.

Vertical native-polyacrylamide gels of DNA samples

The glass plates were assembled according to the manufacturer's instructions (BioRad). In all of the experiments 12% acrylamide gel was used. The polymerization reaction of the gel mixture (*for 50 ml*: 20 ml 30% Acrylamide solution, 1 ml 50X TAE, 29 ml double-distilled water, 0.25 ml of 10 APS) was catalyzed by addition of appropriate amount of TEMED. After 32 hr. run at constant 1 watt in cold room, gel was stained in 30ug/ml ethidium bromide solution for 1 min, and destained for 3 min in double-distilled water.

Gel electrophoresis of total RNA

RNA was fractionated through 1% (w/v) agarose gels containing formaldehyde which disrupts hydrogen bonds. 0.5 g agarose was melted in 1X formaldehyde gel running buffer, allowed to cool, and 10 ml of 37% formaldehyde was added. The gel was

immediately poured in a laminar hood. 5ìl of RNA sample was mixed with 15 ìl of RNA loading buffer and heated at 70 °C for 5 minutes. Samples were quenched on ice and loaded onto gel. Electrophoresis was performed at a constant voltage (85 V) for 4 hr at 4 °C in 1X formaldehyde gel running buffer. Following electrophoresis, gel was soaked for 5 min in 5 volumes water to remove formaldehyde. This step was repeated for 3 times. The gel was stained in 30ug/ml ethidium bromide solution for 5 min, and destained overnight in double-distilled water.

Nucleic acids were visualized under ultraviolet light (long wave, 340 nm) and GeneRuler (MBI Fermentas) DNA size markers was used to estimate the fragment sizes.1 kb DNA ladder for horizontal agarose gels and 100 bp ladder for vertical agarose gels.

3.3.2 Computer Analysis of DNA Sequences

Restriction endonuclease maps of the plasmid DNAs and the *b-catenin* cDNA were analyzed by using the WebCutter program (Max Heiman, 1995, maxwell@minerva.cis.yale.edu) available for free and public use at http://www.medkem.gu.se/cutter and http://firstmarket.com/firstmarket/cutter.

The annealing temperatures for primers were calculated using the Tm determination program provided by The Alces WWW Server, Virtual Genome Center (VCG) at http://alces.med.umn.edu/rawtm.html (stew@lenti.med.umn.edu).

3.3.3 Tissue Culture Techniques

3.3.3.1 Cell Lines

14 HCC derived cell lines (Huh7, FOCUS, Mahlavu, Hep40, Hep3B, HepG2, PLC/PRF/5, SK-Hep1, SNU 182, SNU 387, SNU 398, SNU 423, SNU 449 and SNU

475) were used in this study. The characteristics of these cell lines were obtained from http://www.attc.org web site (Appendix A).

HepG2 cell line clones ectopically expressing mutant forms (p53-V143A, p53-R248W, p53-R249S) of p53 were generated by stable transfection with corresponding plasmids (Frebourg et al., 1992), as described previously (Ponchel et al., 1994).

3.3.3.2 Thawing Cell Lines

One vial of the frozen cell line from the liquid nitrogen tank was taken and immediately put into ice. The vial was left 1 minute on the bench to allow excess nitrogen to evaporate and then placed into 37°C water bath until the external part of the cell solution was thawed (takes approximately 1-2 minutes). The cells were resuspended gently using a pipette and transferred immediately into a 15 ml. sterile tube containing 10 ml cold fresh medium. The cells were centrifuged at 1500 rpm at 4°C for 5 minutes. Supernatant was discarded and the pellet was resuspended in 10 ml 37°C culture medium to be plated into 100 mm dish. After overnight incubation in a humidified incubator at 37°supplied with 5% CO_2, culture mediums were replenished.

3.3.3.3 Growth Conditions

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1 mM glutamine and penicillin and streptomycin (50 mg/ml) was used to culture the HCC cell lines. The cells were incubated in at 37°C in an incubator with an atmosphere of 5% CO_2 in air.

The cells were passaged before reaching confluence. The growth medium was aspirated and the cells were washed once with calcium and phosphate-free PBS. Trypsin was added to the flask to remove the monolayer cells from the surface. The fresh medium

was added and the suspension was pipetted gently to disperse the cells. The cells were transferred to either fresh petri dishes or fresh flasks using different dilutions (from 1:2 to 1:10) depending on requirements.

All media and solutions used for culture were kept at 4°C (except stock solutions) and warmed to 37°C before use.

3.3.3.4 Cryopreservation of Cell Lines

Exponentially growing cells were harvested by trypsinisation and neutralized with growth medium. The cells were counted and precipitated at 1500 rpm for 5 min. The pellet was suspended in a freezing solution containing 10% DMSO, 20% FCS and 70% DMEM at a concentration of $4x10^6$ cells/ml. 1 ml of this solution was placed into 1 ml screw cap cryotubes. The tubes were left at -70°C overnight. The next day, the tubes were transferred into the liquid nitrogen storage tank.

3.3.3.5 Transient Transfection of Eukaryotic Cells Using BES Method

$5x10^5$ cells were plated into a 10 cm tissue culture dish the day before the transfection to obtain 50-60% confluency on the day of transfection. 20 ug supercoiled eukaryotic expression vector in which mutant p53 cDNAs were cloned; namely p53-V143A, p53R248W and p53-R249S constructs, pCDNA3.1.A and pEGFP-N2 plasmids were ethanol precipitated and washed with 70% ethanol. The samples were dried in the sterile hood and dissolved in 20 µl sterilized double-distilled water. All transfections were performed as co-transfection with pEGFP-N2 reporter plasmid for determination of transfection efficiency.

1 hr before transfection, growth medium supplemented with antibiotics (penicillin/streptomycin) was aspirated and 9 ml growth medium without antibiotics was added. Then 15 ìl (15 ug) resuspended plasmid of interest and 5 ìl (5 ug) pEGFP-N2 was mixed with 430 ìl double-distilled water. 50 ìl 2.5 mM $CaCl_2$ was added in dropwise

manner while votexing the DNA solution. After incubation at room temperature for 10 min, 500 ìl of 2X BBS was added to DNA-$CaCl_2$ solution in dropwise fashion, and incubated at room temperature for 40 min till calcium phosphate-DNA precipitates. Calcium phosphate-DNA solution (1ml) was added dropwise to the plate of cells, and the mixture was stirred gently and incubated for 18 hr at 37°C in an incubator with an atmosphere of 5% CO_2 in air. The medium was aspirated and rinsed twice with fresh growth medium, refed and placed back to the incubator for a further 24 hr.

3.3.3.6 Generation of Stable Cell Lines

SKHep-1 cells were transfected with appropriate plasmids expressing various mutant p53 proteins by using BES tranfection method as described in the previous section. The pCDNA3.1.A was used to generate negative control clones.

After the 24 hr incubation at 37°C in an incubator, selection was initiated by addition of Geneticin (G418-sulfate) to a final concentration of 800ìg/ml .Cells were allowed to die in the presence of selective growth medium (complete DMEM with 800 ug/ml G418-sulfate) until the colony formation was observed. Plates containing visible colonies were washed with PBS (after a month period of selection), then all fluid was removed. Sterile 5-10 mm diameter cloning rings were dipped in sterile adhesive (vasaline) and lightly dabbed against a dry surface to spread the adhesive evenly around the outer edges. The rinds were applied around the colonies to be harvested. 60 ìl of trypsin was added into each ring with sterile micropipette tips. After 30 seconds, the colony was broken up by pipetting through cotton plugged pasteur pipette 2-3 times. The cells were transferred to a 24-multiwell plate containing 500 ìl growth medium per well.

After 4 hours, the plate was examined under the microscope to determine if the cells were attached. The medium was replaced with fresh selective medium to remove any remaining tyrosine. Once the small cultures were confluent they were passaged to large culture vessels (usually 6-multiwell plates first).

After colonies were picked up, plates were fixed in 50% methanol and 50% acetone at room temperature for 10 min. After washing the cells 3 times with fresh PBS, cell were stained with Giemsa solution (Sigma) for 5 min, and rinsed with double-distilled water, air dried and counted to compare the colony formation efficiency of each introduced p53-mutant plasmid in SKHep-1 cells.

3.3.4 Amplification of DNA by Polymerase Chain Reaction (PCR)

Cold-PCR

Polymerase chain reactions were performed using the GeneAmp System 9600 (Perkin-Elmer, USA) thermal cycler. PCR was carried out in 50µl of reaction mixture containing 20-100 ng of genomic DNA. Standard conditions were 5pmol/ul each primer, 200 ìM deoxyribonucleotide triphosphates dNTPs, 1X PCR buffer containing 1.5 $MgCl_2$, 1% DMSO and 0.25 U *Taq* DNA polymerase. In order to amplify the desired region of β-catenin and Axin1 genes sets of different primer pairs were used. The primer sequences, expected fragment sizes, optimal $MgCl_2$ concentration, and PCR conditions are summarized in Table 4.

Radioactive PCR

PCR amplifications for single strand-conformational polymorphism (SSCP) studies were carried out as described above with the following modifications. Final PCR reaction volume was lowered to 10 ìl to diminish the amount of radioactive liquid-waste. 0.2 µCi of (^{32}P)á-dCTP (3000 Ci/mol, Amersham) was added into each 10 ìl reaction and the concentration of non-radioactive dCTP was reduced to 0,2 mM to produce a radioactive product. The cycle number was decreased to 30 cycles to reduce possible PCR/Taq-based mutation.

Table 4 List of primers and PCR conditions

All PCR started with 5 min 95°C initial denaturing step and finished with final 10 min extension at 72°C. Number of cycles was 35 for all PCR, unless indicated. (R: reverse, F: forward primers, REA. Restriction enzyme analysis, SSCP: SSCP hot PCR, SP: Sequencing primer, DA: Deletion Analysis, S-PCR Semi- quantitative PCR, gDNA: Genonic DNA)

Primer	Sequence (5'□3')	Product(bp)	Tm value (°C)	Used in/as	Target Gene	Reference
BCAT-1 / F BCAT-2 / R	GATTTGATGGAGTTGGA TGTTCTTGAGTGAAGGACTGAG	215	64	REA,SSCP and SP	*b-catenin*	(Koch et al., 1999)
BCAT-3 / F BCAT-4 / R	AAATCCAGCGTGGACAATGG TGTGGCAAGTTCTGCATCATC	1115 form gDNA 466 from cDNA	62	DA and SP	*b-catenin*	(Koch et al., 1999)
AxcDNA / F AxcDNA / R	CAACGACAGCGAGCAGCAGCAGA GAGGCAGCTTGTGACACGGC	480	62	DA and SP	*Axin-1*	-
Siah-1 / F Siah-1 / R	TTGTAGACGGAGCGTGTTG CTTTCTCCATAGCCAAGTTG	327	60	S-PCR	*Siah-1*	-
Cyc D1 / F Cyc D1 / R	GAGAAGCTGTGCATCTACACCGAC CACATCTGTGGCACAAGAGGCAAC	215	62	S-PCR	*Cyclin D1*	-
c-myc / F c-myc / R	GGAACTATGACCTCGACTACGACTC GCGGCGGCCGAGAAGCCGCTCCACAT	396	62	S-PCR	*c-myc*	-
BCAT-1 / F BCAT-4 / R	GATTTGATGGAGTTGGA TGTGGCAAGTTCTGCATCATC	668 from gDNA 434 from cDNA	62	S-PCR	*b-catenin*	(Koch et al., 1999)
GAPDH / F GAPDH / R	GGCTGAGAACGGGAAGCTTGTCAT CAGCCTTCTCCATGGTGGTGAAGA	250 from gDNA 151 from cDNA	62	S-PCR	*GAPDH*	-

3.3.5 Extaction of Total RNA from Tissue Culture Cells and First Strand cDNA Synthesis

Extraction of total RNA

The isolation of RNA requires pure reagents and care in preparation due to the sensitivity of RNA to chemical breakdown and cleavage by nucleases. Therefore, all the solutions (except Tris) were made either with DEPC-treated water or treated with 0.1% DEPC overnight at 37 °C. The centrifuge tubes were soaked overnight in a solution of 0.1% DEPC prior to autoclaving and all glassware were baked at 200°C overnight.

Exponentially growing monolayer cultures were washed twice with ice-cold PBS, scraped with scraper, pelleted and snap frozen in liquid nitrogen, and stored at -80°C until needed for RNA preparation. Frozen cells were quickly thawed and lysed with Tripure. Alternatively, cells were lysed directly in 150 mm tissue culture dishes using Tripure reagent. The Tripure (Roche) isolation reagent was used for isolation of total RNA from cultured cells. The Tripure is a monophasic solution of phenol and guanidine thiocyanate, which allows the isolation of total RNA, DNA and protein from the same sample in single-step liquid separation. The procedure is an improvement of the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Tripure reagent RNA isolation protocol was followed as suggested by manufacturer. Total RNA pellets were dissolved in 50 ìl of DEPC-treated water. This procedure yields approximately 5-10 ìg RNA /10^6 cells. Integrity and quality of total RNAs were checked with gel electrophoresis (as described in section 2.3.1.7) and quantification was performed spectrophotometrically as described in section 2.3.1.5.

First strand cDNA sythesis

First strand cDNA synthesis from total RNA was performed using RevertAid First Strand cDNA synthesis kit (MBI Fermantas, Germany). The RevertAid kit relies on genetically engineered version of Moloney Murine Leukemia Virus reverse transcriptase (RevertAid M-MuLV RT) with low RNase H activity. This allows the synthesis of full-length cDNA from long templates. The first strand reactions were primed with oligo(dT)18 primer

to specifically amplified mRNA population with 3'-poly(A) tails. As the reaction conditions and components of this kit and those of conventional PCR are compatible, first strand synthesized with this system can be used as a template for PCR.

3.5 ìg total RNA was used to synthesize the first stand cDNA following the manufacturer's instruction. After 1:1 dilution of total reaction products in DEPC-treated water, 2 ìl of diluted first strand cDNA was used for PCR.

3.3.5.1 Primer Design for Expression Analysis by Semi-Quantitative PCR

The primer pairs that have been used in expression profile analyses were designed carefully. Forward and reverse primer were positioned on different exons of the gene of interest, so that the primer pair was either be able to produce a longer amplicon from genomic DNA or not be able to amplify from the covered genomic DNA regioin in a given PCR condition (critical parameter was extension time). Therefore the amplicon, which was amplified from cDNA, was not be longer than 1500 bp. Primers used for expression analysis have been designed strictly considering these criteria, and listed in Table 4.

3.3.5.2 Fidelity and DNA Contamination Control in First Strand cDNAs

The fidelity and genomic DNA contamination of first strand cDNAs were checked before performing expression analyses. 2ìl of diluted first strand cDNA was used for cold-PCR amplification (see section 3.3.5) of the *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* transcript. *GAPDH* primer pair for this analysis was designed to produce a 151 bp fragment from cDNA and 250 bp fragment from genomic DNA (Table 4).

3.3.6 Expression Analysis of a Gene by Semi-Quantitative PCR

Determination of optimal cycle of a gene for semi-quantitative PCR

Using equal amount of templates for PCR amplifications of a gene of interest give comparable results at a certain number of PCR cycles. The number of optimal PCR cycle was determined by an initial study for each gene by performing 35-cycle PCR during which PCR amplicon samples were collected by 2-cycle intervals. Agarose gel analysis of samples from 20^{th}, 23^{rd}, 26^{th}, 29^{th}, 32^{nd}, and 35^{th} cycles of PCR with an equal load defined the minimum number of cycle to visualize the product on agarose gel and the saturation cycle. Agarose gels were analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software (BioRad). The determined cycle number was used for amplification of the gene of interest.

***GAPDH* Normalization**

Equal volume (2ìl) of all first strand cDNA samples was used for cold-PCR amplification of *GAPDH* transcript using the pre-determined optimal cycle number for GAPDH. Then an equal volume of each sample was loaded onto agarose gel and intensity of each band was analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software. After intensities were determined, intensity of sample with the highest densitometric reading and 2 ìl loading volume were used as reference points for normalization of input loading volume of other samples for expression analysis of both *GAPDH* and gene of interest by cold PCR amplification. Amplification products were analyzed in computer.

3.3.7 Mutation Screening Methods

3.3.7.1 SSCP

Two different SSCP gel recipes were employed: 10% 75:1 acrylamide: bisacrylamide and 10% 99:1 acrylamide: bisacrylamide. Gels were also prepared with or

without glycerol, a neutral compound that alters the strand separation and increases the resolution. The acrylamide gel formulations are given below:

10% acrylamide (100 ml)	10% acrylamide(100 ml) without gylcerol
25 ml acrylamide 75:1 or 99:1	25 ml acrylamide 75:1 or 99:1
59 ml ddH_20	69 ml ddH_20
6 ml 10X TBE	6 ml 10X TBE
10 ml glycerol	-
400 µl 10% APS	400 µl 10% APS
40 µl TEMED	40 µl TEMED

All the gel components except APS and TEMED were combined and filtered using a Nalgene apparatus with 0.2 µm cellulose acetate filters (Corning Costar Corp., Cambridge, MA, England) and a Hetomaster jet water pump (Heto-Holten A/S, Allerød, Denmark). Vacuum was applied for 5 minutes in order to degas the acrylamide solution. Immediately before pouring the gel 10% APS and TEMED were added. EC 160 (E-C Apparatus Corp., Holbrook, NY, USA) vertical gel apparatus was set up according to the manufacturer's instructions. The gel was allowed to polymerize for at least 2 hours. The standard running buffer 0.6X TBE was used.

The SSCP reaction was performed by mixing 2 µl radioactive PCR product with 18 µl SSCP loading buffer, heating at 95°C for 2 minutes and putting the tubes on ice for 5 minutes. The samples were then immediately loaded onto the gel. Electrophoresis was performed at constant power (5 W) either at room temperature (25°C) or at 4°C. After electrophoresis, the gel was removed from glass plates using Whatman 3MM paper and dried on the SGD 2000 slab gel dryer (Savant, Holbrook, NY, USA) at 80°C for 2 hours. Radioactive bands were detected by autoradiography.

For autoradiography, the dried gel was placed into a cassette with X-ray film (Kodak, Rochester, NY, USA) and an intensifying screen. The cassette was placed at -80°C for exposure. After the exposure, the film was developed using the Hyperprocessor developer (Amersham, Buckinghamshire, England).

3.3.7.2 Direct PCR Sequencing

PCR products were purified using spin columns (QIAquick PCR purification kit; Qiagen, IC.) and 90 ng of purified DNA was subjected to direct sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) following the manufacturer's instructions.

Denaturing PAGE is used for DNA sequencing to get resolution of 1 bp difference. 4% polyacyrlamide gel (5 ml of 20% 19:1 acrylamide solution, 5 ml of 10X TBE, 18 g urea) was prepared and degassed as described in 3.3.6.2. After casting the glasses according to the manufacturer's instructions, gel was immediately poured and allowed to polymerize for at lest 2 hr. 1X TBE was used as running buffer system.

Sequencing reactions were run on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA), and the data was collected by ABI Data Collection 1.0.4 software and analyzed using ABI Prism Sequencing Analysis 3.3 software (Applied Biosystems). Sequencing was performed in both directions using the primer pair used in the SSCP amplification.

3.3.8 Protein Extraction from Tissue Culture Cells

Crude total protein extraction

Adherent monolayer cells (both stable and parental cells) were grown to 70% confluency in growth medium lacking selective antibiotic. After removal of growth medium, cells were washed twice with ice-cold PBS to remove any serum residue. 300 ìl of lysis-buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 % NP-40 and 1X Complete Protein Inhibitor mix (Roche) was added into 10 cm tissue culture petri dish, and cells were scraped with rubber scrapper. Complete lysis was achieved by pipetting of crude cell lysates several times and by incubating the lysates on ice for 30 min, and then centrifuged at 15.000xg. Total cell protein was collected as supernatant.

Extraction of NP-40-soluble (cytosolic) and –insoluble (non-cytosolic) proteins

Cells were grown as described above and washed twice with ice-cold PBS. To obtain the soluble cytosolic fraction; 1 ml NP-40 buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5 % NP-40 and 1X Complete Protein Inhibitor mix) was added into 10 cm tissue culture petri dish and evenly distributed over the cells. After 3 min incubation at room temperature, dishes were tilted at ~45° to surface to drain the buffer and the NP-40-soluble fraction was colledted. Collected fraction was centrifuged at 10.000xg for 10 min at 4°C. Supernatants were used in SDS-PAGE analysis.

To acquire the insoluble non-cytosolic fraction; 600 ìl of ice-cold RIPA buffer (1% NP-40, 0.5 Sodium deoxycholate, 0.1 SDS and 1X Complete Protein Inhibitor mix in 1X PBS) was added onto the remain cells on dish. Cells were scraped and transferred into 1.5 ml eppendorf tube using a 2.5 gaughe syringe. Plates were washed once more with 400 ìl ice-cold RIPA buffer and buffer was transferred onto previously obtained cell lyaste. Collected cell lysate combination was passed through 2.5 gaughe syringe 3 times. Collected fraction was centrifuged at 10.000xg for 10 min at 4°C. Supernatants were used in SDS-PAGE analysis.

Extraction of cytosolic and non-cytosolic proteins fraction by using Sucrose-Lysis buffer system

The previously method described by Iwai *et al*. (Iwai et al., 1995), was used with following modifications.

Cells were grown as described above and washed twice with ice-cold PBS. Then cells were scraped in 2 ml ice-cold PBS, transferred into 15 ml falcon tubes and pelleted at 2500 rpm for 15 min at 4°C. Supernatant was discarded and pellets were snap frozen in liquid nitrogen and thawed quickly. 2 ml of Sucrose-Lysis buffer (5 mM Hepes, 320 mM Sucrose, 5 mM β-mercaptoetanol, 3 mM EGTA, 0.5 mM $MgSO_4$) was added onto pellet. Using glass-glass (tight) homogenizer, cells were burst with 20 strokes. Homogenized cell lysates were transferred into 4.9 ml OPTISeal ultra-centrifuge tubes (Beckman) and volume

was completed to 5 ml with mineral oil. After centrifugation at 100.000xg for 1 hr (Beckman XL-90 Ultracentifuge with NVT-90 rotor), mineral oil was removed carefully without disturbing the pellet and withdrawing the supernatant. Supernatant was collected as cytosolic fraction and pellet resuspended in equal volume of fresh Sucrose-Lysis buffer to be used as non-cytosolic fraction in western blot analysis.

3.3.9 Western Blotting

The conventional Bradford protein assay was employed to quantity the protein in the lysates obtained from either Crude total protein extraction or NP-40 fractionation. Equal volume of Sucrose-Lysis fractions were used for western blot analysis. After protein quantification, protein lysates were aliquoted into fresh tubes and, stored at –70°C.

10% resolving gel and 5% stacking gel was used in SDS-PAGE analysis of protein lysates. The acrylamide gel formulations are given below:

10% resolving gel (50 ml)	5% stacking gel (10 ml)
10.0 ml 30% Acyrlamide mix	1.7 ml 30% Acyrlamide mix
26.6 ml ddH_20	6.8 ml ddH_20
12.5 ml 1.5 M Tris, pH 8.8	1.25 ml 1.0 M Tris, pH 8.8
0.5 ml 10% SDS	0.1 ml 10% SDS
0.5 ml 10% APS	0.1 ml 10% APS
0.04 ml TEMED	0.01 ml TEMED

EC-120 (E-C Apparatus Corp., Holbrook, NY, USA) and ProteanII-xi (BioRad) vertical gel system was set up according to manufacturer's instructions. The gel was allowed to polymerize for at least 1 hr. The standard SDS-electrophoresis buffer system was used.

Equal amounts of cell lysates were solubilized in 1X SDS gel-loading buffer, denatured at 100°C for 10 min and incubated on ice for 2 min. After a quick spin, samples were loaded onto 10% SDS-polyacrylamide gel. After electrophoresis at 8 mA for 2 hr

following by 16 mA for 10 hr, proteins were transferred onto PVDF western blotting membrane (Roche) by using Transblot-Semi Dry (BioRad) eletroblotting apparatus according to the manufacturer's instructions at 12V for 70 min for ProteanII-xi gel or at 12V for 45 min for EC-120 gel.

Membrane was immediately treated for an hour in blocking solution at room temperature and probed with primary antibody either for an hour at room temperature or overnight at 4°C. After washing 3 times for 5 min in TBS-T solution at room temperature, the membrane was incubated with appropriate HRP-conjugated secondary antibody for 1 hr. The membrane was washed 3 times for 5 min in TBS-T solution at room temperature. After final wash, the blot was exposed to ECL western blot detection kit (Amersham) according to manufacturer's instructions. The chemiluminescence emitted was captured on X-ray film within 30 sec. to 1 min. exposure times.

3.3.10 Immunofluorescence Staining

Autoclaved-sterilized coverslips were placed into the well of 6-multiwell plates. 6x 10^4 cells were seeded onto each coverslip and grown overnight in 1 ml growth medium. Cells were washed with PBS and fixed in 1 ml of 3% formaldehyde for 3 min. After fixation cells were permeabilized with 0.5% TritonX-100 in PBS for 3 min and blocked in 1 ml blocking solution (3% bovine serum albumin-FractionV, 0.1 TritonX-100 in 1X PBS) for 30 min at room temperature. Coverslips were probed with primary antibody in appropriate dilution for 1 hr at room temperature. After 3 times washing for 5 min aech with PBS-T, appropriate FITC-conjugated secondary antibody was applied for 45 min at room temperature. Cells were washed 3 times for 5 min with PBS-T and DNA counter staining was performed by with Hoechst 33258 for 3 min. Hoechst 33258 was aspirated and destaining was done in double-distilled water for 15 min. Immediately after coverslips were taken out from the well and excess water removed by tissue paper, coverslips were mounted onto slides containing 10 ìl 80% glycerol. All steps after the addition of FITC-conjugated secondary antibody were performed in the dark.

Stained cells were examined under fluorescence microscope (ZEISS) and pictures were captured in a digital Kodak Camera (DC290, Eastman Kodak Co., Rochester, NY), using Adobe Photo Deluxe (Adobe Systems Inc.) software. The pictures were edited using Adobe Photoshop 5.0 (Adobe Systems Inc.) software. Digital images were magnified when needed during picture editing.

CHAPTER 4. RESULTS

4.1 Mutational Screening of *CTNNB1 (b-catenin)* Gene

Most of the published mutational screening studies in HCC have shown that structural aberrations in *CTNNB1* gene causing an oncogenic activity of β-catenin are harbored in the coding region spanning from exon 2 to exon 5. These structural aberrations attribute to either point mutations that change either the critical serine and threonine residues and/or their neighborhood amino acid residues in destruction box or interstitial deletion that result in complete loss of exon 3 resulting in the deletion of this destruction box (Figure 7). Therefore we scanned this region for all possible aberrations (interstitial deletions, point mutations) by indirect screening methods (fragment length analysis, restriction enzyme digestion and SSCP) as well as nucleic acid sequencing.

For cell lines, both genomic DNA and RNA materials were used. For tumors, we used only genomic DNA for technical reasons. Genomic DNA was isolated from 14 HCC cell lines using phenol/chloroform extraction method. Isolated DNA samples were evaluated quantitatively and qualitatively by UV spectrophometry and agarose gel electrophoresis. All DNA samples had a 260/280 optical ratio of 1.7-1.8, and displayed intact, high molecular weight band on agarose (Figure 8.a). The concentration of DNA samples were between 140-280 ìg/ml

Genomic DNAs of 33 tumor samples obtained from patients who lived in Mozambique (n= 10), South Africa (n=12) and China (n=11), have been isolated years ago for *p53* mutation studies (Ozturk, 1991).

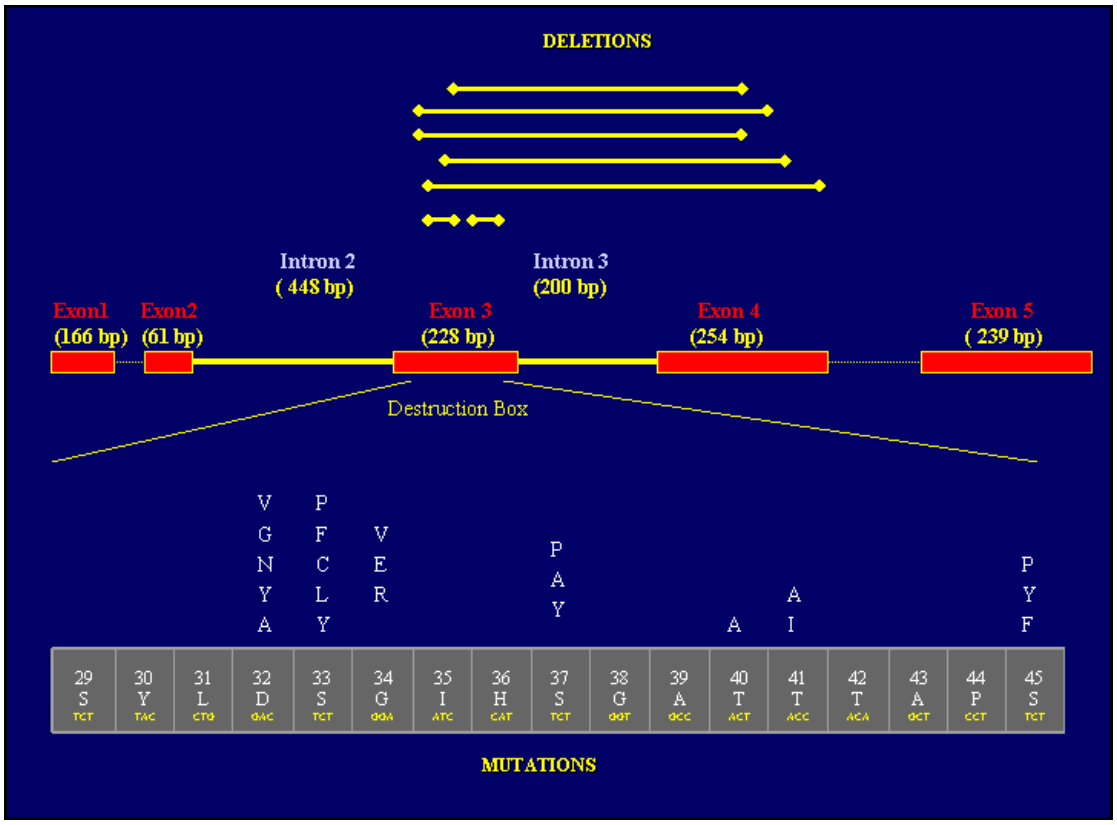


Figure 7 A summary of genetic alteration of *b-catenin* gene in HCC

The data have been complied from seven independent reports (de La Coste et al., 1998; Miyoshi et al., 1998a; Huang et al., 1999; Kondo et al., 1999; Legoix et al., 1999; Nhieu et al., 1999; Terris et al., 1999). Mutations at other positions were reported only occasionally. The interstitial deletions involved invariably the destruction box.

Total RNA content of 14 HCC cell line were extracted by TriPure (Roche) extraction method. Extracted total RNAs were run on RNA gel agarose for quality check (Figure 8.b) and quantified by spectrophotometry. First strand cDNA was prepared from 3.5 ug total RNA by oligo-dT primering following the instructions of the cDNA preparation kit, as described in Materials and Methods section.

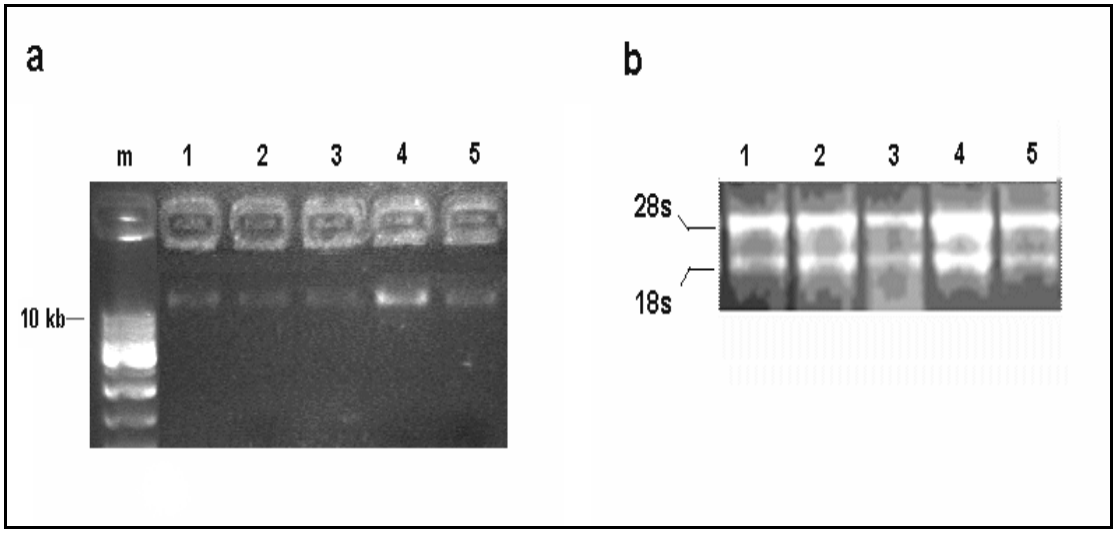


Figure 8 Quality control of genomic DNA and RNA samples
a. Total genomic DNA samples were electrophorezed in a 0.8% agarose gel.
b. Total RNA extracts were electrophorezed in a 1% formaldehyde agarose gel
m: marker, **1.** Skhep1, **2.** Hep3B, **3.** Focus, **4.** Mahlavu, and **5**. SNU 182 cell lines.

4.1.1 Interstitial Deletions of *b-catenin* Gene are Rare in Primary HCC Tumors and Cell Lines

Interstitial deletions were screened using the cold-PCR products in both 14 HCC cell lines and 33 primary HCC tumor samples. PCR amplification of *CTNNB1 (b-catenin)* gene was performed with BCAT-3/BCAT-4 primer pair which produces an 1115-bp genomic DNA fragment that covers the region between exon 2 though exon 4. No alterations in size of the fragments were detected in any of the 13 HCC cell lines and 33 primary HCC tumor samples (Figure 9.a and .b). As reported previously, HepG2 cell line displayed an interstitial deletion leading to a truncated protein product lacking amino acids 24 to 141 (de La Coste et al., 1998) (Figure 9c).

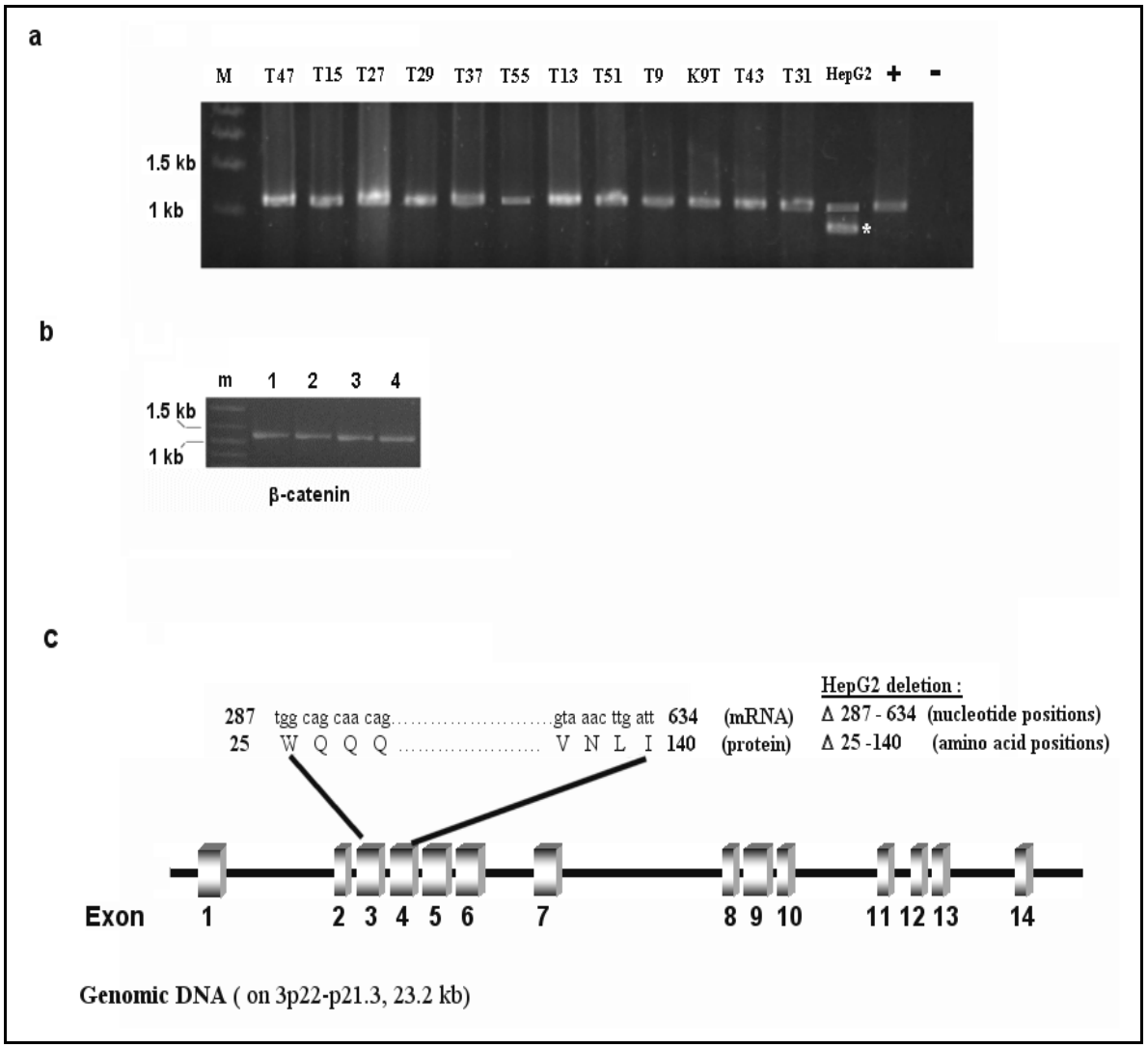


Figure 9 Large deletion analysis in *b-catenin* gene by PCR of genomic DNA
(a) Absence of β-catenin mutations in HCCs from southern Africa and China. Picture shows that a genomic DNA region of β-catenin gene (exons 2-4) with an expected sized PCR product (1115 bp) in all tumor samples (coded by **T**); Genomic DNA from HepG2 cell line was used as a positive control for deletion (*). **M**: marker,. + and – show positive (normal DNA) and negative (no DNA) controls for PCR reaction. **(b)** Analysis of same genomic DNA region in HCC cell lines display expected size PCR product; **1.**Mahlavu **2.** Huh7 **3**. SkHep-1 **4**. Hep3B (representative sample for HCC cell lines).**(c)** The diagram shows the deletion of exon 3-4 of β-catenin observed in HepG2 cell line. Nucleotide and amino acid positions are given according to the GenBank mRNA sequence of *CTNNB1* gene (X87838)

4.1.2 Missense *b-catenin* Mutations are Rare in Primary HCC Tumors and Cell Lines

For detection of missense mutations affecting exon 3 of *b-catenin*, several methods were used. As a rapid test, we first used *Hinf I* restriction enzyme digestion method to detect most mutations effecting codons 32, 33, 34 and 35. As shown in Table 5,

codon codon 32, 33, 34 and 35 are frequently mutated in both human and mouse HCCs. The nucleotide sequence encoding these exons habours 2 *Hinf I* recognition motif (GANTC) as shown in figure 10. In the absence of mutation, *Hinf I* digestion of 215 bp fragment encompassing exon 3 yields 3 sub-fragments of 132 bp, 70 bp and 7 bp, respectively. Any mutation affecting four nucleotides of the motif GANTC would result in a different digestion pattern.

Table 5 Frequently observed point mutations in codons 32, 33, 34 and 35 in HCCs

Tumor Type	Organism	Codon	Mutation { Number of case}
HCC	Human	32	GAC (Asp) to AAC (Asn) {3}
HCC	Human	32	GAC (Asp) to TAC (Tyr) {4}
HCC	Human	32	GAC (Asp) to GGC (Gly) {2}
HCC	Human	32	GAC (Asp) to GCC (Ala)
HCC	Human	32	GAC (Asp) to GTC (Val)
HCC	Human	33	TCT (Ser) to CCT (Pro)
HCC	Human	33	TCT (Ser) to TGT (Cys) {2}
HCC	Human	33	TCT (Ser) to TTT (Phe) {3}
HCC	Human	33	TCT (Ser) to CTT (Leu)
HCC	Human	33	TCT (Ser) to TCC (Ser)
HCC	Human	34	GGA (Gly) to GTA (Val) {2}
HCC	Human	34	GGA (Gly) to GAA (Glu)
HCC	Human	34	GGA (Gly) to AGA (Arg) {2}
HCC	Human	35	ATC (Ile) to AGC (Ser) {2}
HCC	HuH6	34	GGA (Gly) to GTA (Val)
HCC	Mouse	32	GAC (Asp) to CTC (Val)
HCC	Mouse	32	GAC (Asp) to TAC (Tyr)
HCC	Mouse	34	GGA (Asp) to GAA(Glu)
Hepatoblastoma	Human	32	GAC (Asp) to TAC (Try) {2}
Hepatoblastoma	Human	32	GAC (Asp) to AAC (Asn)
Hepatoblastoma	Human	32	GAC (Asp) to GGC (Gly)
Hepatoblastoma	Human	34	GGA (Gly) to GTA (Val) {3}
Hepatoblastoma	Human	34	GGA (Gly) to GAA (Glu)

The data have been comp iled from seven independent reports (de La Coste et al., 1998; Miyoshi et al., 1998a; Huang et al., 1999; Kondo et al., 1999; Legoix et al., 1999; Nhieu et al., 1999; Terris et al., 1999)

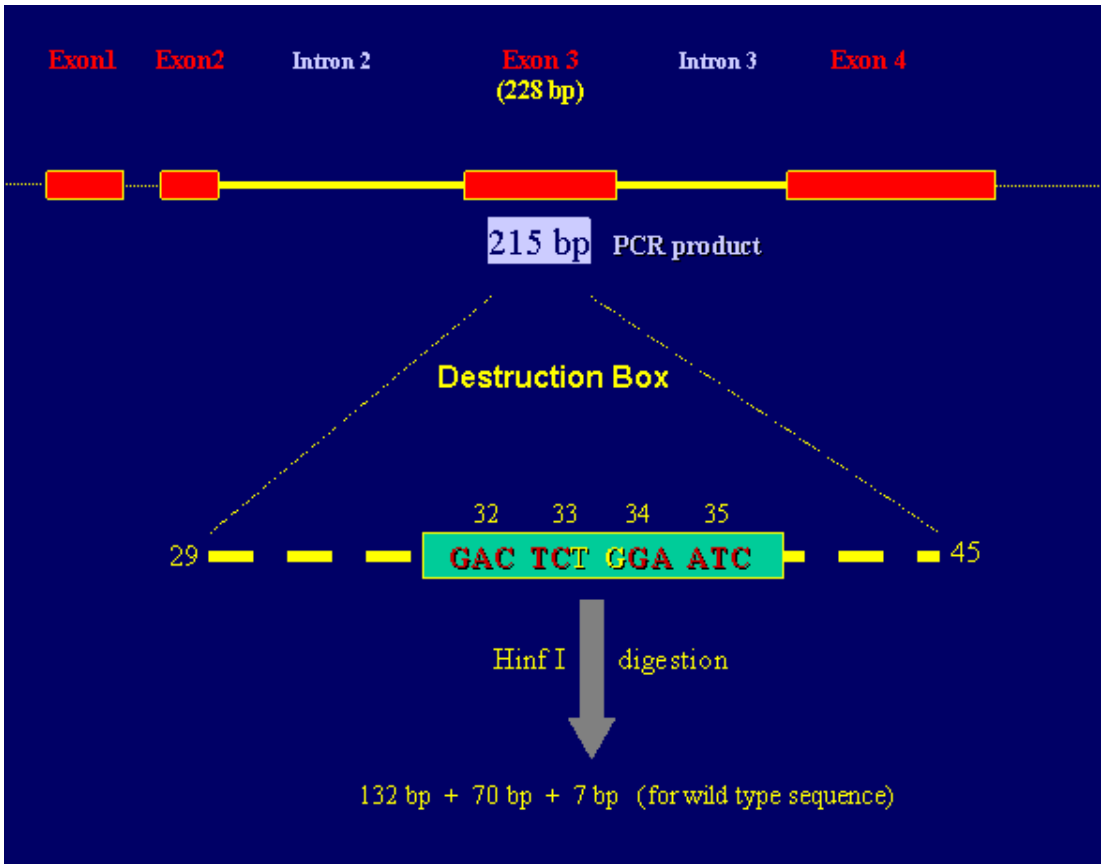


Figure 10 Schemetic representation of *Hinf* I analysis of exon 3 of b-catenin

215 bp fragment of Exon 3 was amplified by cold-PCR using primer pair BCAT-1 /BCAT-2 for all HCC cell line and primary tumor DNA samples. Then, PCR products were subjected to *HinfI* digestion and analyzed by acrylamide gel electrophoresis. All samples tested produced two bands of 132 bp and 70 bp, indicating that there was no mutation affecting G, A, T or C nucleotide of Hinf *I* recognition sites. The expected 7 bp band was not seen under these conditions because of its tiny size (Figure 11).

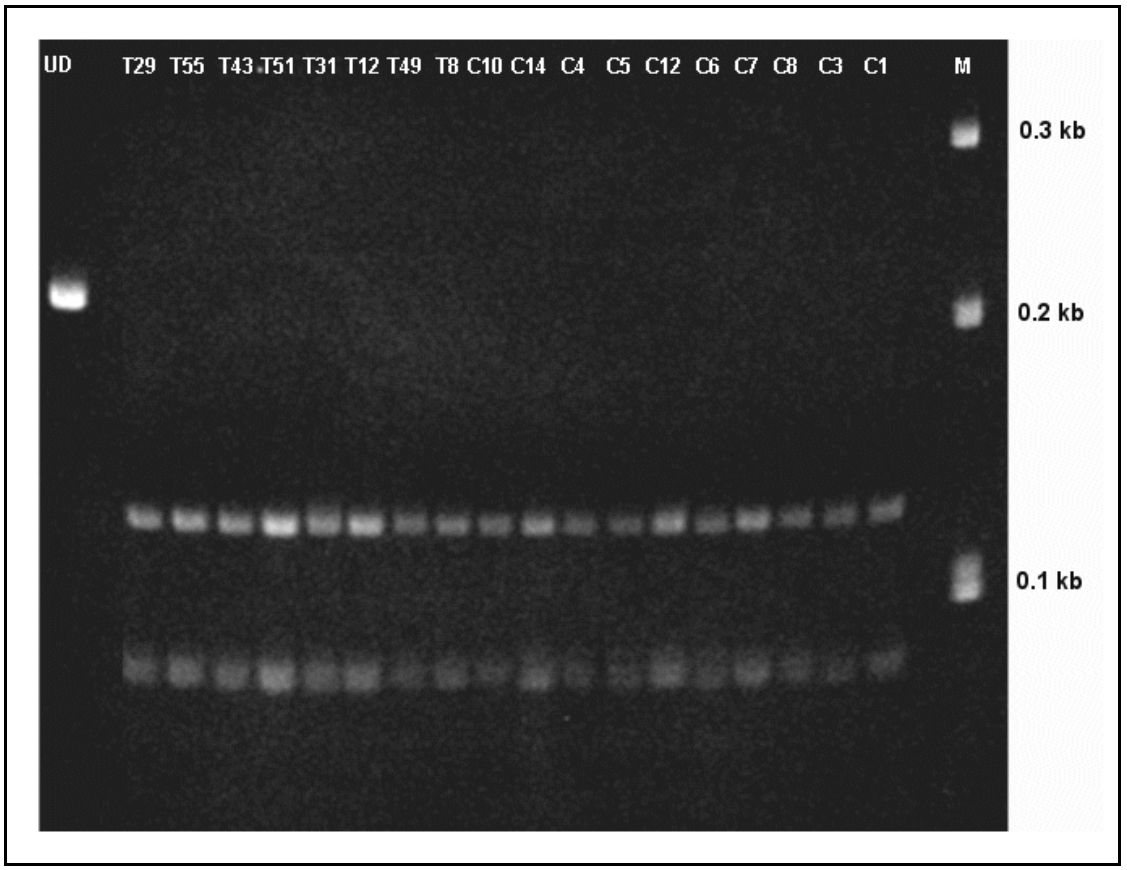


Figure 11 *Hinf I* digestion profiles of primary HCC tumor samples

Absence of ***b-catenin*** mutations at codons 32, 33, 34 and 35 in primary HCCs from southern Africa and China. All samples display wild type digestion profile; only 132 bp and 70 bp fragments are seen here, since 7 bp fragment is too small. **M:** marker, Codes above the gel image (e.g. **T8**, **C15**) stand for the name of the tumor sample analyzed. **UD:** Undigested PCR reaction product.

Next, we screened all samples for mutation using SSCP (single strand-DNA conformational polymorphism) assay on a fragment covering the exon 3 of ***b-catenin***. .

Genomic DNAs from HCC cell lines and tumor samples were amplified using BCAT1/BCAT2 primer pair by radioactive PCR. Radioactive-PCR products were run onto SSCP gel with different recipes at different condition as given below:

Condition	10% 99:1 Acrylamide	10% 75:1 Acrylamide	10% Glycerol	Temp(°C)
I	✓		✓	25
II	✓			25
III	✓		✓	4
IV	✓			4
VI		✓	✓	25
VII		✓		25
VIII		✓	✓	4
IX		✓		4

A previously described ***b-catenin*** mutation S33Y was used as a control (Fukuchi et al., 1998). As shown in the Figure 12, using the experimental condition III, a band shift was visible. But, no band shift was observed within any of the tested HCC cell lines and tumor samples. This mutation was not visible with any of the other conditions.

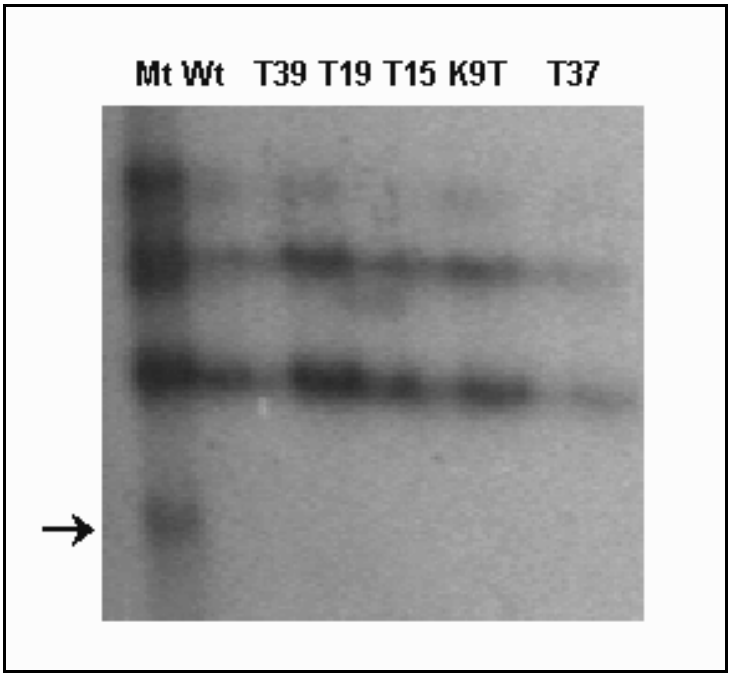


Figure 12 SCCP autoradiogram of exon 3 of *b-catenin* gene

Arrow indicates aberrantly migrating bands. **Mt:** pCI-S33Y, previously described point mutation control in β-catenin sequence (amplified from pCI-S33Y eukaryotic expression plasmid). **Wt:** pCI-wt-β-catenin, wild type β-catenin sequence (amplified from pCI-β-catenin$_{XL}$ eukaryotic expression plasmid). Codes above the gel image (e.g. **T37**, **K9T**) stand for the name of the tumor sample analyzed.

All these indirect techniques suggested us that primary HCCs from southern Africa and China, as well as most of the HCC cell lines, lacked *b-catenin* mutations. To cover the whole exon3 for possible mutations DNA sequencing was performed.

For 33 primary HCC tumor samples, the cold-PCR amplification products from section 4.1 were used as templates used for DNA sequencing analysis of 33 tumor samples. Complete coding sequence of exon3 was sequenced by BigDye Terminator Cycle Sequencing using the BCAT1 and BCAT2 primers.

For HCC cell lines, by the availability total RNA samples, unlike the 33 primary HCC tumor samples for which we had only the DNA extracts, the 466 bp coding region covering the exon 2 to exon 4 of *b-catenin* gene was completely sequenced. The 466 bp fragment was amplified from first strand cDNAs of 14 cell lines by cold-PCR by using BCAT-3/BCAT4 primer pairs (Figure 13.a). Then 466 bp PCR products were purified using spin columns (QIAquick PCR purification kit; Qiagen, IC.) and were subjected to direct sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) with same primer pair.

We found no genetic alteration of *CTNNB1* in any of the 33 HCC primary tumor samples. For the 14 HCC cell lines, except for the previously reported interstitial deletion in HepG2 (de La Coste et al., 1998), SNU398 cells displayed a heterozygous TCT to T(C/G)T mutation leading to a serine to cystein substitution (S37C) of a GSK-3β phosphorylation site in β-catenin protein (Figure 13.b). On the other hand, in our hands, it was SNU398, but not SNU449, which displayed a missense *b-catenin* mutation, in apparent opposition to previously, published data (Satoh et al., 2000). No mutation was observed in the remaining 12 cell lines.

In summary, 2/14 HCC cell lines, but none of 33 primary tumors displayed *b-catenin* mutation. Thus, we deciced to continue our mutation search, on *Axin 1* gene, which was reported to be mutated in HCC (Satoh et al., 2000; Taniguchi et al., 2002)

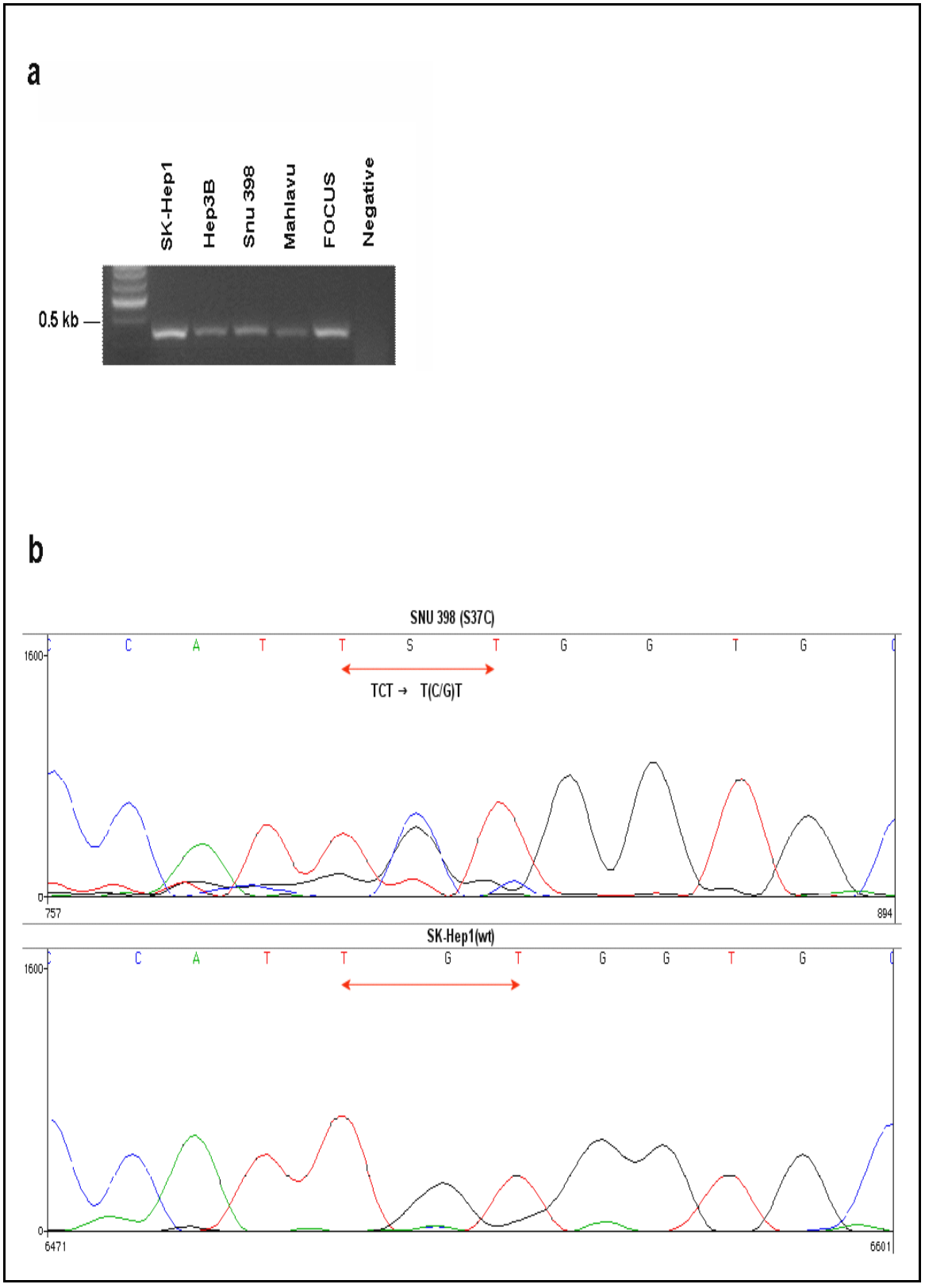


Figure 13 ***b-catenin*** **mutation statuses of hepatocellular carcinoma cell lines**

(a) Cold-PCR product of β-catenin gene from first strand cDNA, **(b)** Direct sequencing of exons 2-4 of genomic DNAs from 13 cell lines showed a point mutation in SNU398 (top), but not in other cell lines (SK-Hep1 data shown as an example).

4.2 Mutational Analysis of *Axin1* Gene in HCC Cell Lines

As shown in Figure 14, *Axin 1* gene is composed of 10 exons spaced by very large introns (e.g. intron 1: 4475 bp ,intron 2: 5531 bp) and most of the exons have relatively high GC contents (e.g. exon1: 67% GC , exon6: 66% GC). Therefore, we preferred to use cDNAs as a starting material for efficient and effective mutational screening methodology. Consequently, mutational screening for *Axin1* gene was performed for 14 HCC tumor cell lines. We could not include the 33 primary HCCs since we did not have fresh tumor samples.

First strand cDNAs of 14 HCC cell line were amplified by cold-PCR for *Axin1* gene with AxcDNA-F/AxcDNA-R primer pair. The 480 bp PCR product covers the region of *Axin1* gene spanning exon 2 to exon 4 (Figure 14). This region encodes GSK-3β-binding site of Axin1 protein. Cold-PCR products were purified with Qiagen PCR purification columns and the expected sizes of the PCR products were confirmed by comparison of observed DNA bands with the 1 kb DNA ladder.

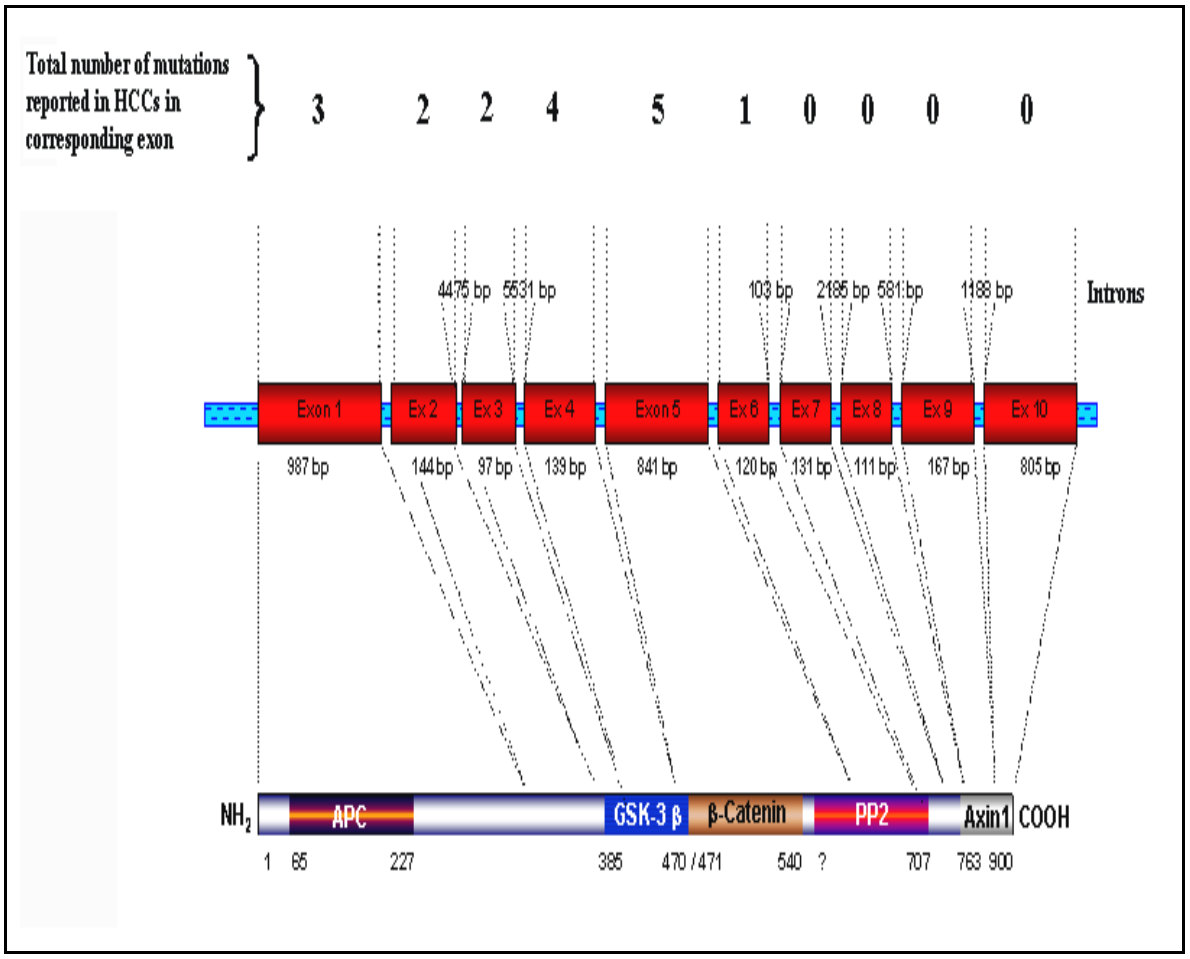


Figure 14 Genomic and protein structure of *Axin 1*

Colored region on protein illustration indicate corresponding to each protein-binding sites, APC, GSK-3β, β-catenin, PP2Ac (protein phosphotase 2Ac). Dashed lines indicate the regions encoded by responsible exons. The overall mutations in each exon is represented as bold number at the top of the figure (Satoh et al., 2000; Taniguchi et al., 2002).

4.2.1 Analysis of *Axin1* Transcript by RT-PCR

Wild-type PCR product that spans the exon2 to exon4 coding region of *Axin1* mRNA can be observed on agarose as 480 bp fragment. For scanning a possible size difference and/or presence of the *Axin1* transcript, cold-PCR products (obtained in 4.2) were visualized in Nusieve-agarose (Figure 15).

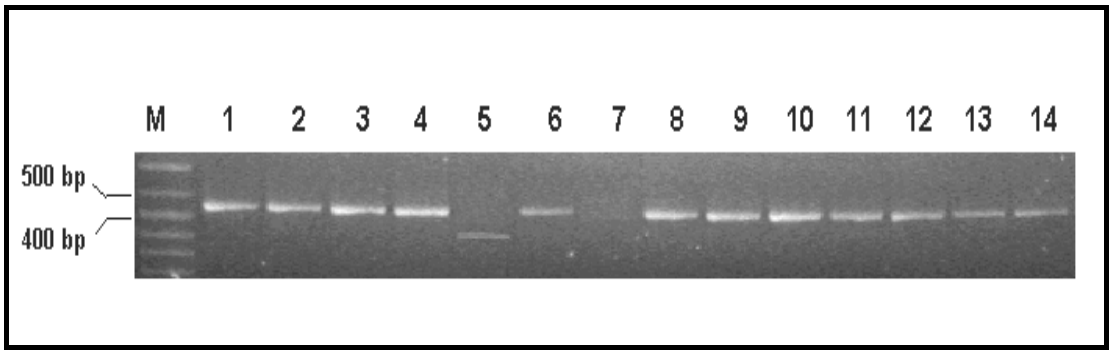


Figure 15 Interstitial deletion analysis of 14 HCC cell lines

M: 100 bp marker, **1.**Huh7, **2.**Hep3B, **3.**SK-Hep1, **4.**FOCUS, **5.**PLC/PRF/5**, 6.**Mahlavu**, 7.**Snu475, **8.**Hep40, **9.**HepG2, **10.**Snu182, **11.**Snu387, **12.**Snu398, **13.** Snu423 and **14.**Snu449.

As shown in Figure 15, previously described loss of exon 4 in *Axin 1* transcript in PLC/PRF/5 (Lane 5) was confirmed. There was no amplification in Snu475 cold-PCR (Lane 7) due to deletion of forward primer(AxcDNAF) position in *Axin1* gene (Satoh et al., 2000). Transcripts from the remaining 12 cell lines were of normal size.

4.2.2 Analysis of Point Mutations and Small Deletion in *Axin1* Transcript

Except for the Snu475, all 13 HCC cell lines were subjected to direct sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit. The coding region of *Axin1* from exon2 to exon 4 was sequenced and no mutations were observed in Huh7, Hep3B, SK-Hep1, FOCUS, Mahlavu, HepG2, Snu182, Snu387, Snu398 and Snu449 cell lines. We confirmed the homozygous exon 4 deletion in PLC/PRF/5 (Figure 16.a) and 13 bp lost in Snu423 cell lines (Figure 16.b) reported by Satoh *et al.* (Satoh et al., 2000).

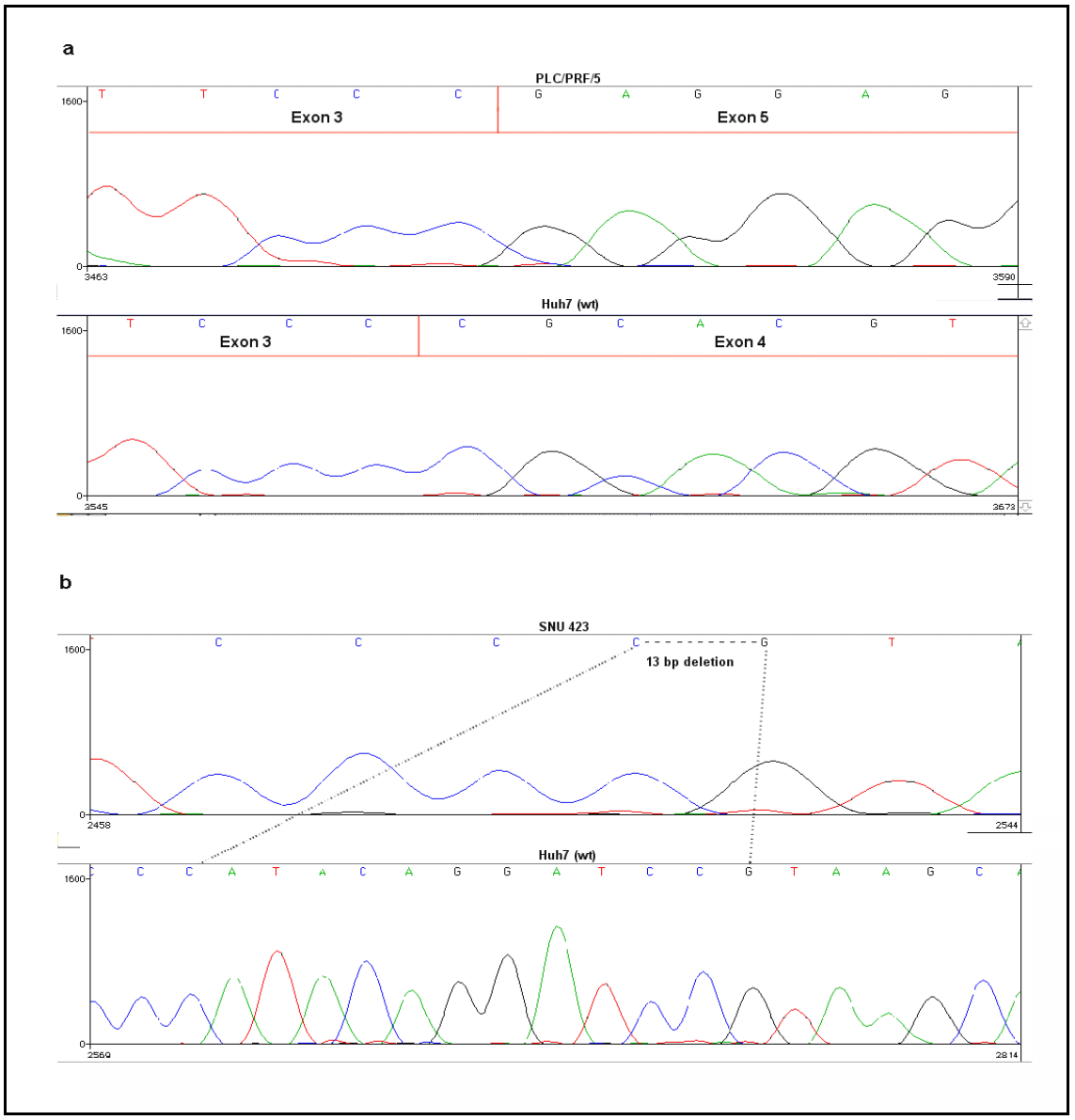


Figure 16 Sequence analysis of *Axin1* cDNA of PLC/PRF/5 and Snu423

(a) The deletion of Exon4 in PLC/PRF/5 cells and **(b)** Homozygous 13 bp deletion observed in Snu423 cell line. cDNA sequence data of the Huh7 cell line was used as wild-type reference sequence.

In addition, we detected an Arg→His substitution (CGC → CAC) at codon 454 in exon 4 of *Axin1* in the Hep40 cell line. Mutation was confirmed by sequencing in both directions. This mutation was homologous since no wild type sequence was observed on the eletropherogram (Figure 17.a). The histidine amino acid residue at positon 454 was conserved throughout Human, Mouse, Rat, Chicken, Zebra-fish, and Xenopus Axin1 protein sequences, as shown by a protein multi-alignment program, BioEdit version 5.0.6 (Indiana University at ftp://iubio.bio.indiana.edu/molbio/seqpup, 2001); (Figure 17.b). It

was also noteworthy, that no other cell line displayed such mutation, nor this mutation was reported previously (Satoh et al., 2000; Taniguchi et al., 2002).

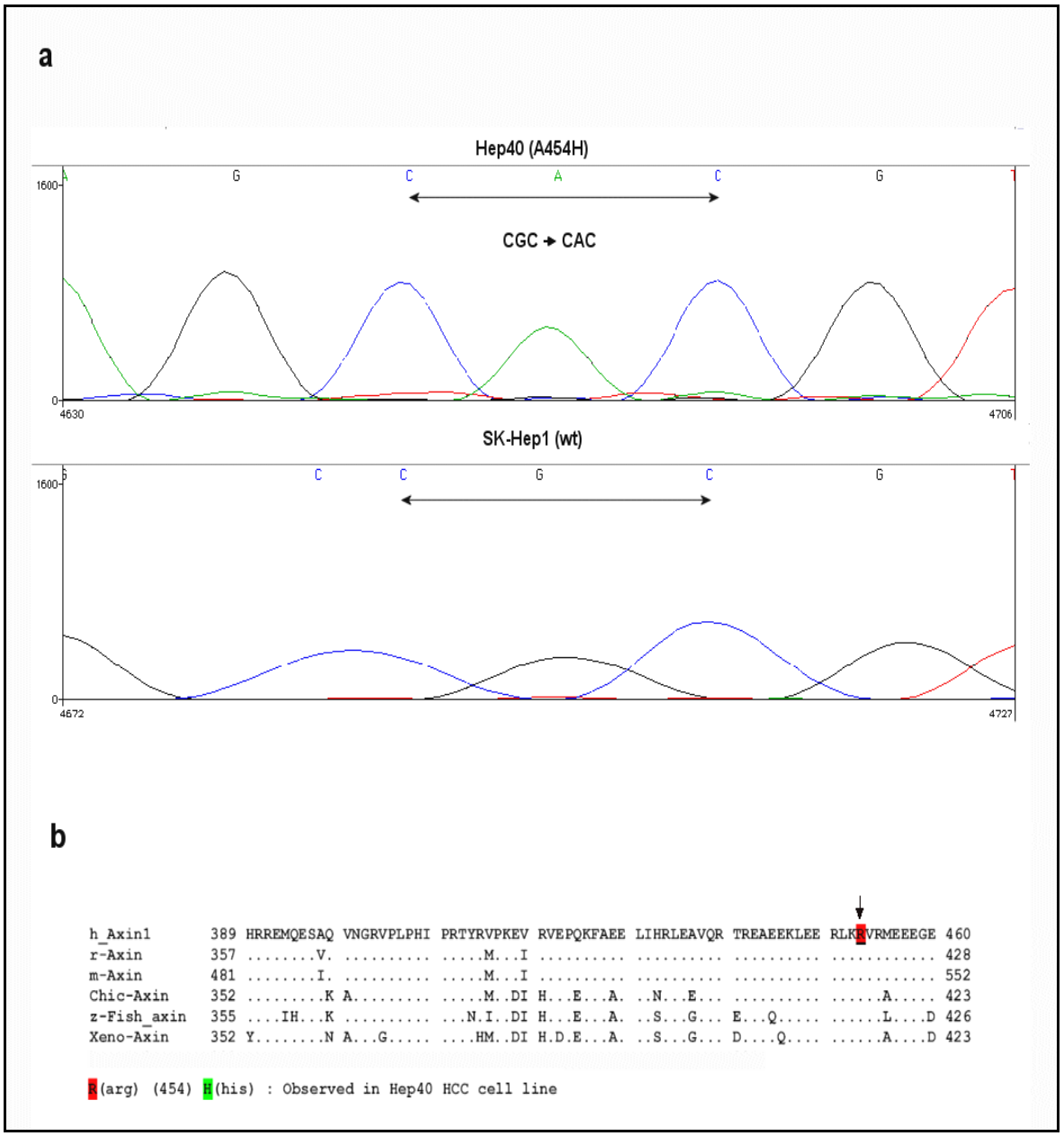


Figure 17 The Arg(454)His substitution (CGC → CAC) in exon 4 of *Axin1* in the Hep40 cell line

(a) Sequence analysis showing the Arg(454)His substitution (CGC → CAC) in Hep40 cell line.
(b) Amino acid alignment of Axin1 protein sequences spanning the region 70 amino acids of exon 4; **h-Axin1:** human Axin1 protein sequence, **r-Axin:** rat Axin1 protein sequence, **m-Axin:** mouse Axin1 protein sequence, **Chic-Axin:** chicken Axin1 protein sequence, **z-fish-axin:** zebra-fish Axin1 protein sequence, **Xeno-Axin:** xenopus Axin1 protein sequence. Arrow and red highlight indicated highly conserved Arginine residue at codon 454 of Axin1 protein sequence, Green highlight indicates Histidine residue.

4.3 Summary of Mutational Screening Analysis

4.3.1 Mutational Screening of the *CTNNB1* and *Axin1* Gene in 14 HCC Cell Lines

Table 6 summarizes the mutational screening data for the 14 HCC cell lines we have analyzed. 2 (HepG2 and Snu398) of the studied HCC cell lines displayed β-catenin mutation, while 4 (Snu475, Snu423,PLC/PRF/5,and Hep40) cell lines harbored *Axin1* genetic alterations. Except for Hep40 cell line which was shown to carry an Arg(454)His substitution in *Axin1* gene, no new sequence alterations were found in any of the screened genes in studied HCC cell lines. It is important to indicate that only exons 2-4 of *Axin 1* gene were analyzed. Since this gene displays mutation in other exons (1 and 5), it is possible that additional cell lines also have mutation. With regard to Hep40 mutation, we notice that it is a missense mutation. Therefore, it is not clear whether this is a polymorphism or a true mutation.

Table 6 Results of mutational screening of *CTNNB1* and *Axin1* genes in HCC 14 cell lines

Cell Line	*b- catenin Status*	*Axin1 Status*	*p53 Status*
Hep G2	Δ Exon 3✸	w.t.+	w.t.
Snu 398	Ser(37)Cyc TCT(S)→TGT(C)+✓	w.t.+	mut.
Snu 475	w.t.	Δ Exon 1-2+	mut.
Snu 423	w.t.	Δ Exon 4+	mut.
PLC/PRF/5	w.t.	Δ Exon 4 (13bp)+	mut.
Hep 40	w.t.	Exon 4 ; Arg(454)His) CGC → CAC	mut.
Hep 3B	w.t.	w.t.+	Δ
Huh 7	w.t.	w.t.+	mut.
Sk Hep1	w.t.	w.t.+	w.t.
Mahlavu	w.t.	w.t.+	mut.
Focus	w.t.	w.t.+	Δ
Snu 182	w.t.	w.t.+	mut.
Snu 387	w.t.	w.t.+	mut.
Snu 449	w.t.✓	w.t.+	mut.

w.t., wild type; **D**, deletion ; **mut,** mutant.+ mutation status was reported by de La Coste et al., 1998. ✸ mutation status was reported by Satoh et al., 2000. ✓ It was SNU398, but not SNU449, which displayed a missense β-catenin mutation, in apparent opposition to previously, published data (Satoh et al., 2000). (p53 status were compiled from Sayan et al. 2001a and Olivier et al., 2002), + denotes wild type partial *Axin1* sequences between exon2 and exon 4.

4.3.2 Mutational Screening of the *CTNNB1* in 33 Primary HCC Tumor Samples from Southern Africa and China

Mutational screening data for *b-catenin* gene for the 33 primary HCC tumor samples is summarized in Table 7. No mutations were found in any of the tumor sample. Table 7 also shows the data of *p53* and HBV status of the tumors that is compiled from previous work by Unsal *et al.* (Unsal et al., 1994).

Table 7 Lack of b-catenin mutation in hepatocellular carcinomas from Africa and China

Sample	Origin	b–catenin ✹	HBV	*p53*
T47	Mozambique	wt	+	V157F
T15	Mozambique	wt	+	R249S
T27	Mozambique	wt	+	R249S
T29	Mozambique	wt	-	R249S
T37	Mozambique	wt	+	R249S
T55	Mozambique	wt	+	R249S
T13	Mozambique	wt	+	wt
T51	Mozambique	wt	+	wt
T9	Mozambique	wt	+	wt
K9T	Mozambique	wt	+	wt *
T43	South Africa	wt	+	286 (8bp del. ; Frameshift)
T31	South Africa	wt	+	wt
T33	South Africa	wt	+	wt
T23	South Africa	wt	+	wt
T12	South Africa	wt	-	wt
T35	South Africa	wt	+	wt *
T19	South Africa	wt	-	wt
T49	South Africa	wt	+	wt
T41	South Africa	wt	+	wt
T8	South Africa	wt	+	Wt
T39	South Africa	wt	+	wt
C10	China	wt	+	R245S
C4	China	wt	+	251 (ATC del. ; 1 aa del.)
C14	China	wt	+	D281E
C5	China	wt	+	wt
C12	China	wt	+	wt
C6	China	wt	+	wt

Table 7 (cont'd)

Sample	Origin	b–catenin	HBV	*p53*
C7	China	wt	+	wt
C8	China	wt	+	wt
C3	China	wt	+	wt
C1	China	wt	NT	wt*
C15	China	wt	+	wt*
	TOTAL	(0/33)	28/32	10/33
	(%)	(0.0)	(87.5)	(30.3)

β-catenin mutations were studied by SSCP, Direct sequence analysis of exon 3 and by interstitial deletions of genomic region of exon2 to exon 5. p53 and HBV status were obtained from Unsal *et al.* **wt,** wild-type, **NT**, Not tested or no data available. **+,** samples with detectable HBV DNA sequences, -, absence of detectable HBV DNA sequences. * (asterisk) denotes wild type partial *p53* sequences either between exon5 and exon 7 or only exon 7. ❁, wild type for exon 3.

4.4 b-catenin Protein Levels in HCC Cell Lines

4.4.1 Aberrant Accumulation of Wild-Type b-Catenin in Mutant p53 Cell Lines

Mutations *of b-catenin, Axin 1* or *APC* genes result in abberant accumulation of β-catenin protein in cancer cells including HCC. It is also known that β-catenin accumulation in some cancers is not associated with a mutation in these genes (Candidus et al., 1996; Rimm et al., 1999; Lin et al., 2000; Shinohara et al., 2001; Ueda et al., 2001a; Hommura et al., 2002).

Table 6 compares the status of *b-catenin* and *p53* genes in 14 HCC-cell lines. The great majority of HCC cell lines (11/14; 79%) displayed a *p53* mutation, in contrast to only 2/14(17%) cell lines displaying a *b-catenin* mutation. Either *p53* or *b*-catenin was mutated in these cell lines, except SNU398 cells, which displayed both p53 and β-catenin mutations, and SK-Hep1 was wild type for both *p53* and *b-catenin*. We have selected 12 HCC cell lines, namely SK-Hep1, Huh7, Snu449, Snu398, Mahlavu, Snu182, FOCUS,

Hep3B, PLC/PRF/5, Snu475, Snu 387 and HepG2 for possible accumulation of β-catenin by western blot.

After isolation of total crude protein contents, equal amounts of 15 ìg of crude protein extract were load onto SDS-PAGE gel. Proteins were immobilized on PVDF membrane and then probed by 0.8 ìg/ml monoclonal β-catenin antibody. After incubation with 1:1500 diluted HRP-conjugated secondary antibody, proteins were detected by non-enhanced chemiluminesence kit; ECL Western Blotting kit (Amersham).

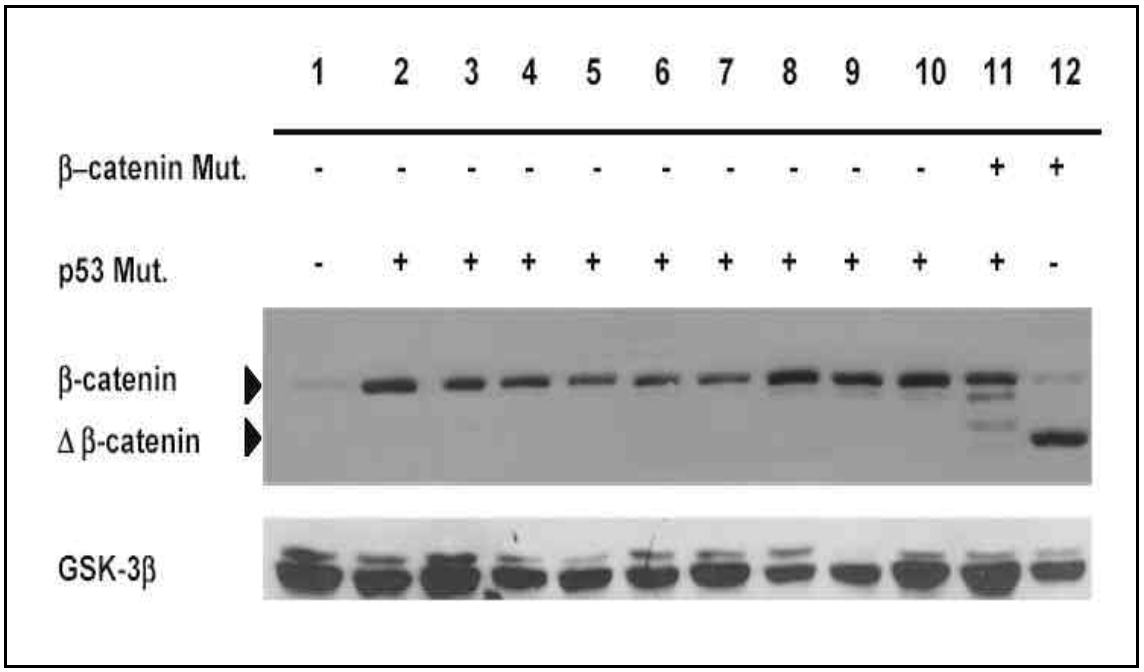


Figure 18 Increased b-catenin protein levels in HCC cell lines displaying a p53 mutation

1.SK-Hep1, **2.**Huh7, **3.**SNU449, **4.**SNU387, **5.**Mahlavu, **6.**SNU182, **7.**FOCUS, **8.**Hep3B, **9.**PLC/PRF/5, **10.**SNU475, **11.**Snu398 and **12**.HepG2. GSK-3β serves as a protein loading control. **+,** presence of mutation, **-,** absence of mutation. **D b-catenin:** Truncated mutant β-catenin protein , **b-catenin:** Full-length wild-type β-catenin protein.

As shown in Figure 18, both HepG2 and SNU398 displayed a strong increase in the levels of mutant β-catenin proteins. Selective accumulation of the mutant, but not wild-type β-catenin was most evident in HepG2 cell line due to faster migration of truncated β-catenin (Δ β-catenin) protein (Figure 18). As expected, SK-Hep1 cells expressed wild-type β-catenin at low levels. On the other hand, all remaining cell lines (n=9) displayed increased levels of wild-type β-catenin protein (Figure 18). This unexpected accumulation

of wild-type β-catenin was as important as the accumulation of mutant β-catenins (compare lanes 2-8 with lanes 11 and 12 in Figure 18) and/or as the accumulation of wild-type β-catenin protein in Axin1-mutant cell lines, namely PLC/PRF/5 and Snu 475 (compare lanes 2-8 with lanes 9 and 10 in Figure 18).

When we analyzed the β-catenin protein levels considering the p53 mutational status of cell lines, wild-type p53-expressing cell lines SK-Hep1 (lane 1) and HepG2 (lane 12, high molecular weight band) express low levels of wild-type β-catenin protein. The Δ β-catenin lacking region amino acids between codon 25-140 observed in HepG2 (lane 12) and non-truncated mutant β-catenin in SNU398 (lane 11) serve as examples of stabilized mutant β-catenin proteins. In contrast, all mutant p53 cell lines (Table 8) shown in lanes 2 (Huh7), 3 (SNU449), 4 (SNU387), 5 (Mahlavu), 6 (SNU182), 7 (FOCUS), 8 (Hep3B), 9 (PLC/PRF/5), and 10 (SNU475) display increased levels of wild-type β-catenin protein.

Table 8 Comparative analysis of p53 and b-catenin status of HCC cell lines

Cell Lines	P53 gene	b-catenin gene	Axin 1 gene	b-catenin protein levels
SK-Hep1	w.t.	w.t.	w.t.	Low
HepG2	w.t.	w.t. Δ25-140	w.t.	Low Increased
Huh7	Y220C	w.t.	w.t.	Increased
Snu449	K139R A161T	w.t.	w.t.	Increased
Snu387	L164X	w.t.	w.t.	Increased
Mahlavu	R249S	w.t.	w.t.	Increased
Snu182	S215I	w.t.	w.t.	Increased
FOCUS	–/–	w.t.	w.t.	Increased
Hep3B	–/–	w.t.	w.t.	Increased
PLC/PRF/5	R249S	w.t.	Δ Exon 4 (13 bp)	Increased
Snu475	N239D N288S C275R	w.t.	Δ Exon 1-2	Increased
Snu398	–/–	w.t./S37C	w.t.	Increased

w.t., wild type; **-/-,** gene deletion and/or protein loss. Data on the status of p53 gene in SK-Hep1, HepG2, Hep 3B, PLC/PRF/5 and Huh7 has been previously reported (see ref. Sayan et al., 2001a), data for SNU475, SNU449, SNU387 and SNU182 was obtained from the IARC p53 mutation database version R6 (http://www.iarc.fr/p53/Index.html; Olivier et al,2002).

4.4.2 Accumulated b-catenin Level Evident in Both Cytosolic and Non-Cytosolic Protein Fractions

Cytosolic and non-cytosolic fractions of selected 12 HCC cell lines were prepared by two different methods.

Cells were grown to 70-80% confluency and NP-40-soluble (cytosolic) and –insoluble (non-cytosolic) protein fractions were extracted. 15 ìg of extracted fractions were run on SDS-PAGE gel. Probing and chemiluminesense assay were performed as described in section 4.4.1 (Figure 19).

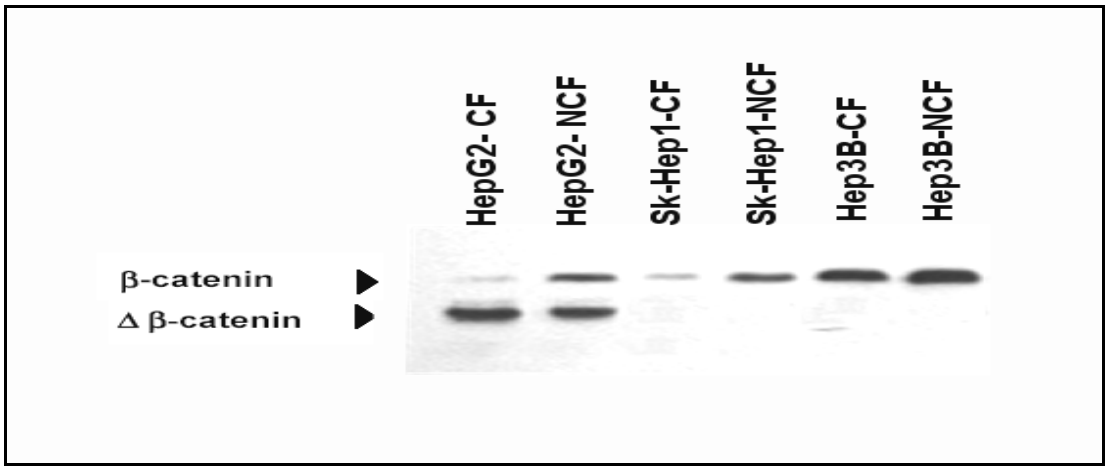


Figure 19 Anlaysis of b-catenin in NP-40-soluble (cytosolic) and –insoluble (non-cytosolic) protein fractions

CF suffix denotes ctyosolic fraction, **NCF** suffix stands for non-ctosolic fraction.

Extraction of cytosolic and non-cytosolic proteins fraction by using Sucrose-Lysis buffer system was used as a secondary methodology. Cells were seed to a 15cm petri plates day before the extraction. Cell at 70-80% confluency were fractioned to cytosolic and non-cytosolic fractions and equal volumes of the fractions were analyzed as performed in NP-40 fractionation (Figure 20).

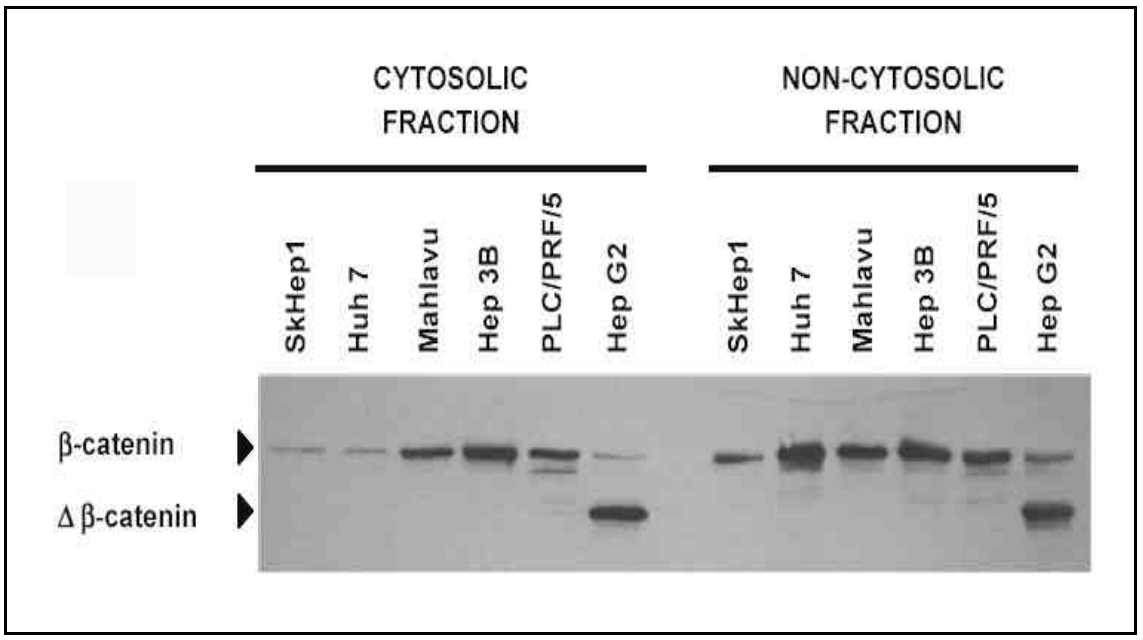


Figure 20 Analysis of b-catenin in cytosolic and non-cytosolic fractions extracted by Sucrose-Lysis buffer system

Fractions were load in equal volumes. **D b-catenin:** Truncated mutant β-catenin protein, **b-catenin:** Full-length wild-type β-catenin protein.

The accumulation was evident in both cytosolic and non-cytosolic forms of wild-type β-catenin in mutant p53 cell lines tested (Mahlavu, Hep3B and PLC/PRF/5), similarly to mutant forms, with the exception of Huh7, which displays an accumulation in non-cytosolic fraction only in both fractionation methodology. Since both techniques has almost the same efficiency and quality, regarding the simplicity of the techniques we decided to use NP-40 fractionation for the following proteins studies.

4.5 Analysis of Ectopic Expression of Mutant p53 Effect on Wild-Type b-Catenin Protein

When we analyzed β-catenin protein levels in 12 HCC cell lines there was an unexpected accumulation of wild-type β-catenin protein that was as important as the accumulation of mutant β-catenin protein (Figure 18). Detailed scrutiny of this comparative study in HCC cell lines in line with their p53 mutation status (Table 8), led us to hypothesize that p53 mutations may lead to aberrant accumulation of wild-type β-catenin protein in cancer cells.

4.5.1 Generation of Isogenic Cell Lines Ectopically Expressing Wild-Type and Mutant p53

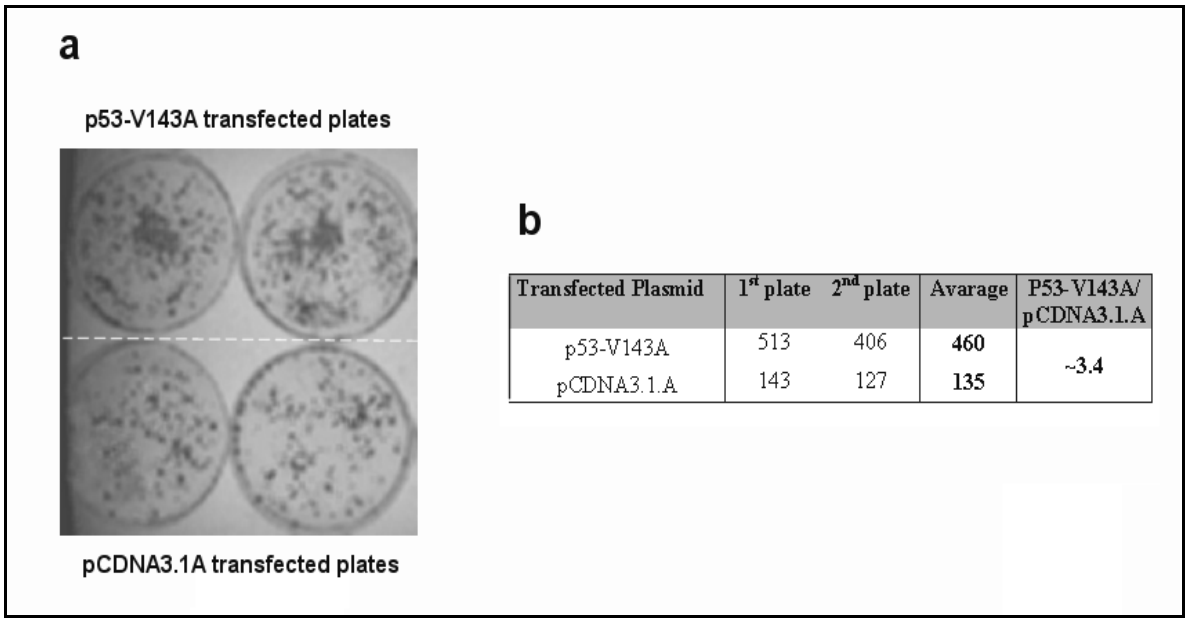
To test whether p53 mutation is a direct cause of wild-type β-catenin accumulation in HCC cells, we employed several stable clones obtained by transfection of wild-type p53-expressing HepG2 cell line with mutant p53 expression plasmids. HepG2-143, HepG2-248 and HepG2-249 clones expressing mutant p53-V143A, p53-R248W and p53-R249S proteins respectively have been generated previously, as described in Ponchel *et al.* (Ponchel et al., 1994).

We also generated stable SK-Hep1 cell lines ectopically expressing mutant p53 (V143A) .Wild-type p53-expressing SK-Hep1 parental cells were transfected with p-V143A eukaryotic expression construct (Frebourg et al., 1992) and with pCDNA3.1.A to

be used as negative control clones. The p53-V143A construct contains a Neomycine^R (G418^R) gene allowing the selection of stable clones. After selection in 800 ìg/ml G418 containing growth medium for 5 weeks, single colonies were picked and grown and maintained in growth medium containing 400 ìg/ml G418. Around 50 colonies were picked randomly from each tissue culture petri plate. Remaining colonies were stained with Giemsa and counted to determine the colony formation efficiency (Figure 21.a). The number of total colonies in each plate was shown in Figure 21.b. Introduction of mutant *p53* to parental Sk-Hep1 cells having both wild-type *b-catenin* and *p53* genes provided a growth advantage compared to the parental SK-Hep1 tranfected with pCDNA3.1.A. The average colony number was about 3.4 fold higher in mutant p53-transfected SK-Hep1 cells.

Picked-up colonies were grown in 6-multiwell plates as duplicates and one set was used for expression analysis of introduced mutant p53 protein. As shown in Figure 22, all clones displayed increased expression of p53 protein (Lanes, 3- 10) as compared to parental SK-Hep1 cell line and negative tranfection control clone (Lane 1 and 2).

Three mutant p53 expressing clones were selected for future studies: SK-Hep1-143-2, SK-Hep1-143-3, and SK-Hep1-143-8.



Transfected Plasmid	1st plate	2nd plate	Avarage	P53-V143A/ pCDNA3.1.A
p53-V143A	513	406	460	~3.4
pCDNA3.1.A	143	127	135	

Figure 21 Giemsa staining and colony formation efficiency of SK-Hep1 stable tranfection

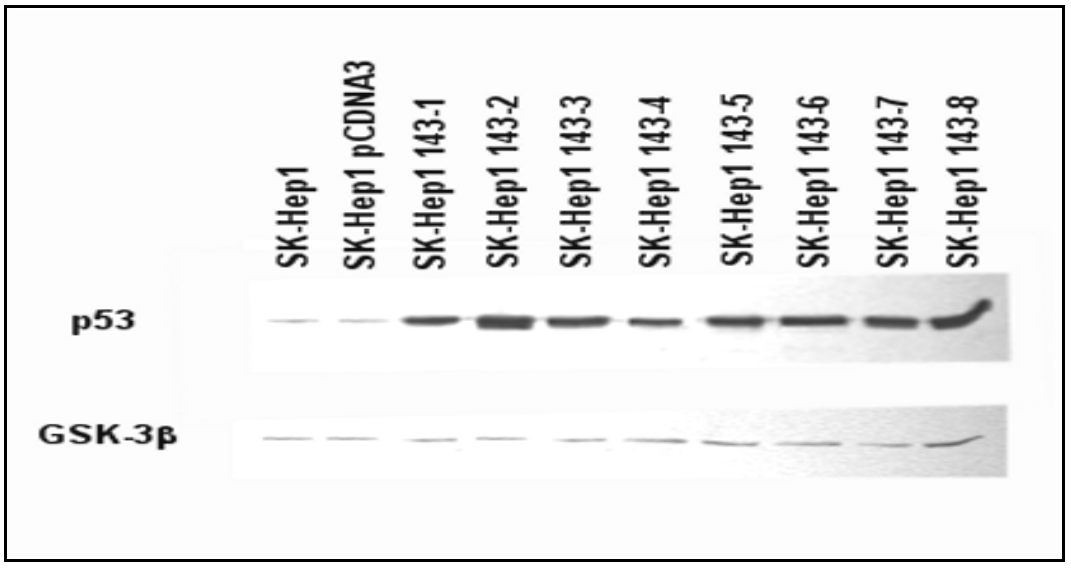


Figure 22 Selection of SK-hep1 clones expressing the mutant p53-V143A

G418-resistant clones were selected and tested for p53 protein by western blotting. Parental Sk-Hep1 and Sk-Hep1 pCDNA.3 (negative transfection clone) were used as reference for endogenous p53 protein level.

4.5.2 Ectopic Expression of Mutant p53 Causes the Accumulation of Wild-Type b-catenin Protein

We have analyzed effect of the ectopic expression of mutant p53 on wild-type β-catenin protein in HepG2 mutant p53 clones and in Sk-Hep1 mutant p53 clones. Six stable cell lines and their transfection negative controls were grown in 10cm tissue culture plates to 70-80% confulency in the absence of G418 in the growth medium. NP-40 soluble fractions were isolated and protein levels of p53 and β-catenin were analyzed by western blotting.

As shown in figure 23(a), HepG2 clones expressing mutant p53 displayed increased wild-type β-catenin protein levels as compared to parental HepG2, while no change was observed in mutant truncated β-catenin protein levels in HepG2 clones (Δ−β-catenin).

We have also confirmed this accumulation in HepG2 stable p53 mutant clones in a time- and temperature-dependent manner. Cells were left for 18h after plating at 37°C, and the incubation temperature was then shifted to 32°C in one set of plates, and β-catenin was tested at times 0, 8 and 12h by western blotting. Regardless of the temperature thereby conformational change of p53-V143A (Friedlander et al., 1996) and time, HepG2-A143V clone displayed the same pattern of increased β-catenin association with ectopic expression of mutant p53-V143A expression. (Figure 23.b)

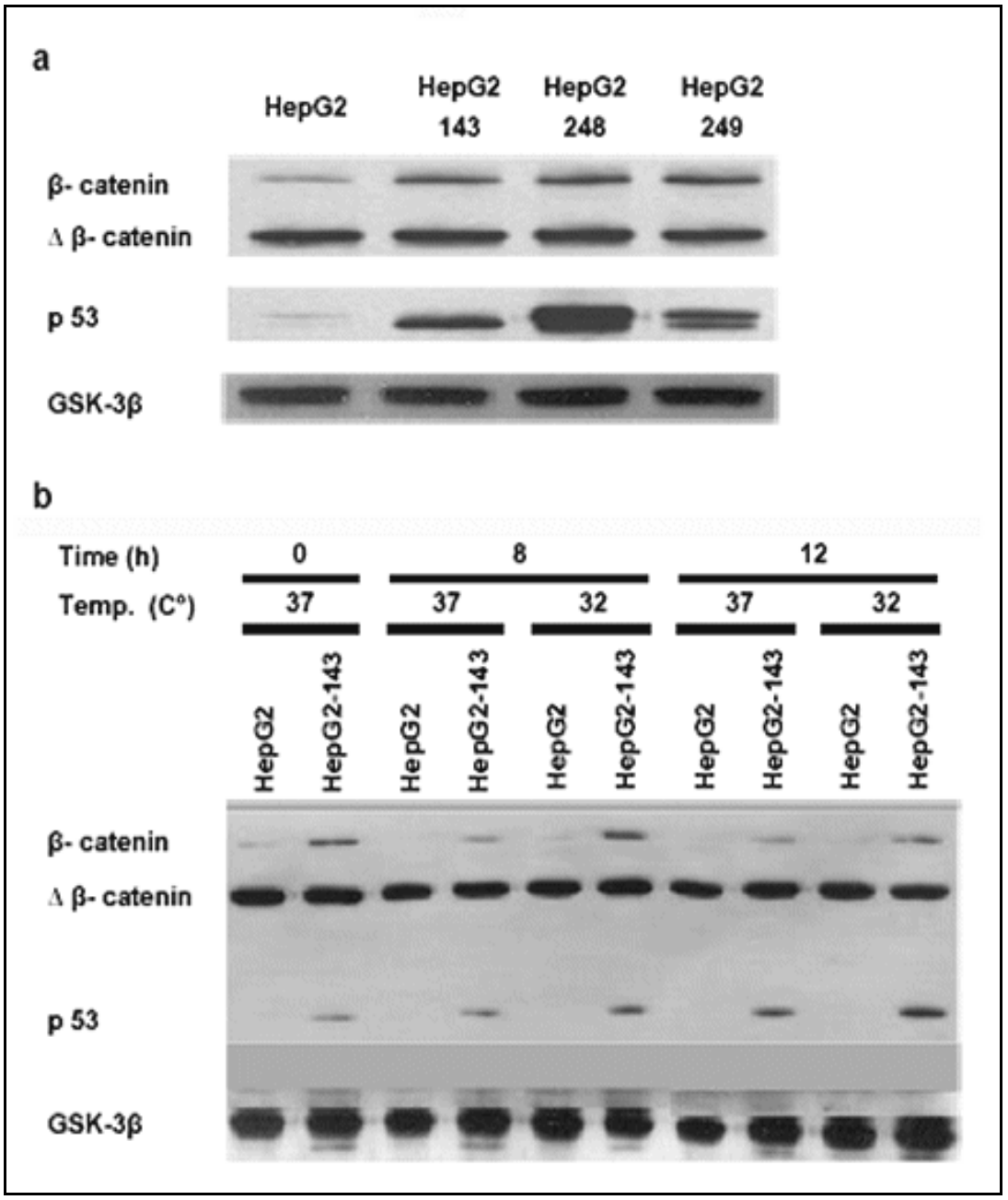


Figure 23 Ectopically expressed mutant p53 proteins cause an increase in the levels of wild-type b-catenin HepG2 stable clones

a. Ectopically expressed mutant p53 proteins cause an increase in the levels of wild-type β-catenin, but not truncated β-catenin (Δ β-catenin) in HepG2-derived stable clones. HepG2-143, HepG2-248 and HepG2-249 express mutant p53-V143A, p53-R248W and p53-R249S proteins, respectively.
b. Increase of wild-type β-catenin levels in clone HepG2-143 is independent of time and temperature.

As shown in Figure 24, wild-type p53-expressing cell line SK-Hep1 which were stably transfected with mutant p53 plasmid (p53-V143A), but not those transfected with a

control plasmid displayed a modest but consistent increase in wild-type β-catenin protein levels. This effect was observed in all three p53-V143A-expressing clones tested.

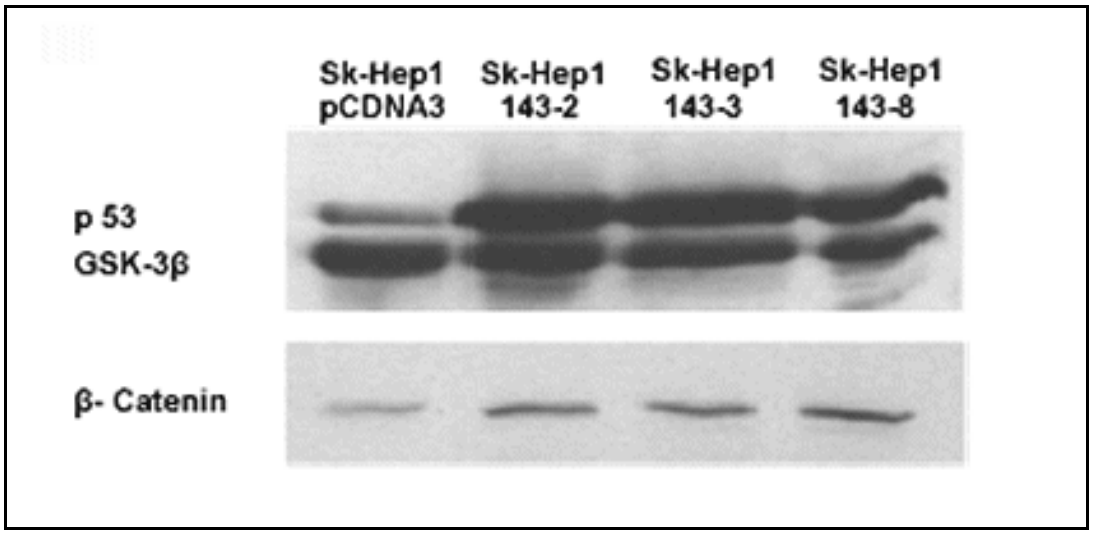


Figure 24 Ectopically expressed mutant p53 proteins cause an increase in the levels of wild-type b-catenin SK-Hep1 stable clones

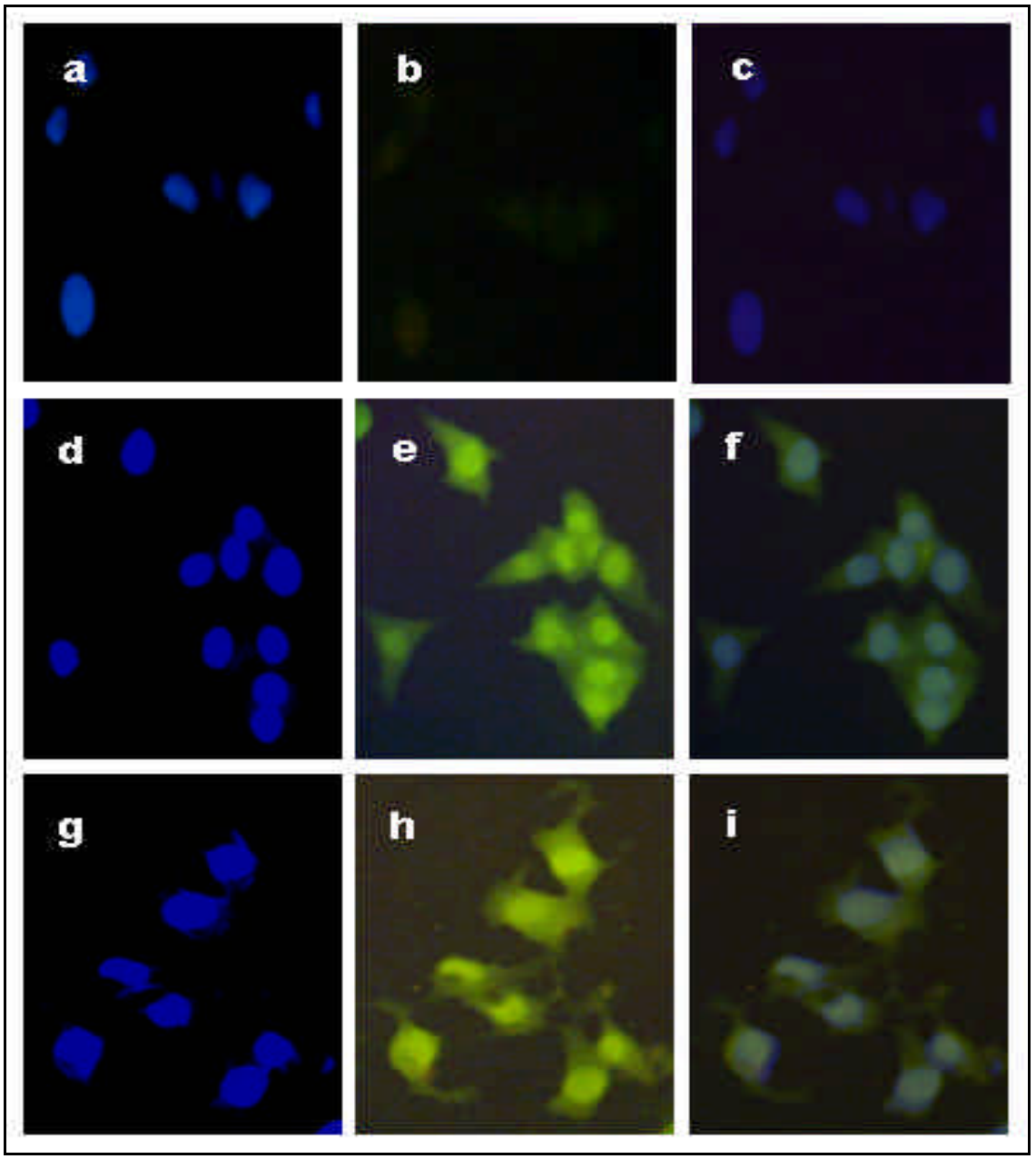
Ectopic expression of mutant p53-V143A leads to an increase in wild-type β-catenin protein levels in SK-Hep1 cells. SK-Hep1-143-2, SK-Hep1-143-3 and SK-Hep1-143-8 clones were generated following stable expression of mutant p53-V143A, whereas SK-Hep1-pCDNA3 clone.

4.6 Subcellular Localization of Accumulated b-catenin Protein in HepG2-derived and SK-Hep1-derived Stable Clones

Cellular distribution of the accumulated β-catenin in HepG2-249 and SK-Hep1-143-8 stable clones was determined by immunofluorescence study as described in the section 3.3.10.

HepG2 cell line which harbors a heterozygous *b-catenin* mutation displayed strongly positive nuclear and positive cytoplasmic staining (Figure 25.e and f), as reported previously (de La Coste et al., 1998; Satoh et al., 2000). The staining pattern did not change in ectopically mutant *p53* expressing HepG2-derived clone; HepG2-249 (Figure 25.h and i).

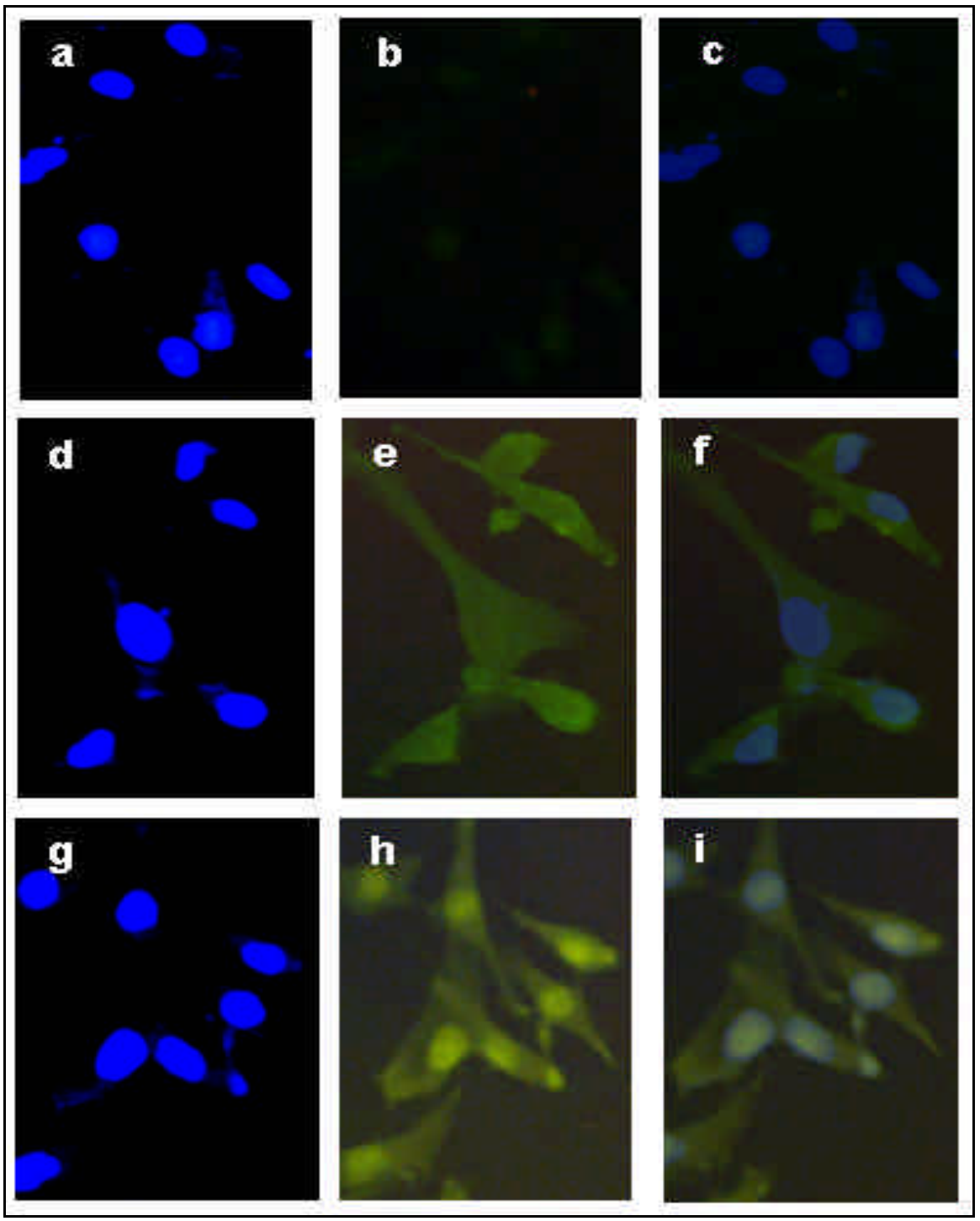
Figure 25 Fluorescent immunocytochemical staining of b-catenin in parental HepG2 cells and HepG2 derived clone 249



Nuclear and cytoplasmic accumulation β-catenin in HepG2 cells following ectopic expression of mutant p53 protein p53-R249S. The negative control clone parental HepG2 cell **(a-f)** and mutant p53 expressing clone HepG2-249 **(g-i)** were subjected to immunofluorescence staining for β-catenin. Compared to antibody negative control **(a-c)**, staining of HepG2 with anti-β-catenin antibody **(d-f)** showed heterogeneous cytoplasmic and nuclear staining **(e, f).** Same profile was observed with HepG2-249 clone **(h, i).** Fixed and permeabilized cells were subjected to nuclear DNA **(a, d, g)** and immunofluorescence staining **(b, e, h)**, and then digital pictures were merged **(c, f, i).**

SK-Hep1-derived negative control clone (SK-Hep1-pCDNA3) displayed diffuse staining for β-catenin (26.e and f), whereas SK-Hep1-derived clone 143-8 showed highly positive nuclear and positive cytoplasmic staining (Figure26 2.h and i).

Figure 26 Fluorescent immunocytochemical staining of b-catenin in tranfection negative control clone SK-Hep1-pCDNA3 and SK-Hep1 derived clone 143-8



Nuclear accumulation of β-catenin in SK-Hep1 cells following ectopic expression of mutant p53 protein p53-V143A. The negative control clone SK-Hep1- pCDNA3 **(a-f)** and mutant p53 expressing clone SK-Hep1-143-8 **(g-i)** were subjected to immunofluorescence staining for β-catenin. Compared to antibody negative control **(a-c)**, staining of SK-Hep1-pCDNA3 with anti-β-catenin antibody **(d-f)** showed diffused cytoplasmic staining with weak nuclear staining **(e, f)**. In contrast, strong nuclear β-catenin staining was evident with SK-Hep1-143-8 clone **(h, i)**. Fixed and permeabilized cells were subjected to nuclear DNA **(a, d, g)** and immunofluorescence staining **(b, e, h)**, and then digital pictures were merged **(c, f, i)**.

4.7 Up-Regulation of Wild-Type b-catenin Transcripts in SK-Hep1 and HepG2 Cells Following Extopic Expression of Mutant p53

To address the question whether β-catenin up-regulation occurs at the transcriptional or post-transcriptional level, we studied *Siah1* and ***b-catenin*** transcript levels by semi-quantitative PCR It has been shown that the inhibitory effect of transcriptionaly active p53 on β-catenin has to be mediated by a p53-induced gene called *Siah1* that is required for ubiquitin-degradation complex formation with β-catenin, and consequently degradation of β-catenin (Liu et al., 2001; Matsuzawa and Reed, 2001).

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First strand cDNA of 12 HCC cell lines and SK-Hep1 and HepG2 stable clones were subjected to cold PCR to determine the cycle number for the *Siah1* and ***b-catenin*** cDNA amplifications. After cold-PCR, amplification products from samples of cycle 14-30 were visualized in agarose gel each transcript (Figure 27). Evaluating the intensity of fragment for corresponding cycle, following optimal cycle numbers were determined;

Optimal cycle number for *Siah1* : 33 (Figure 27 upper-panel)
Optimal cycle number for ***b-catenin*** : 30 (Figure 27 lower-panel)

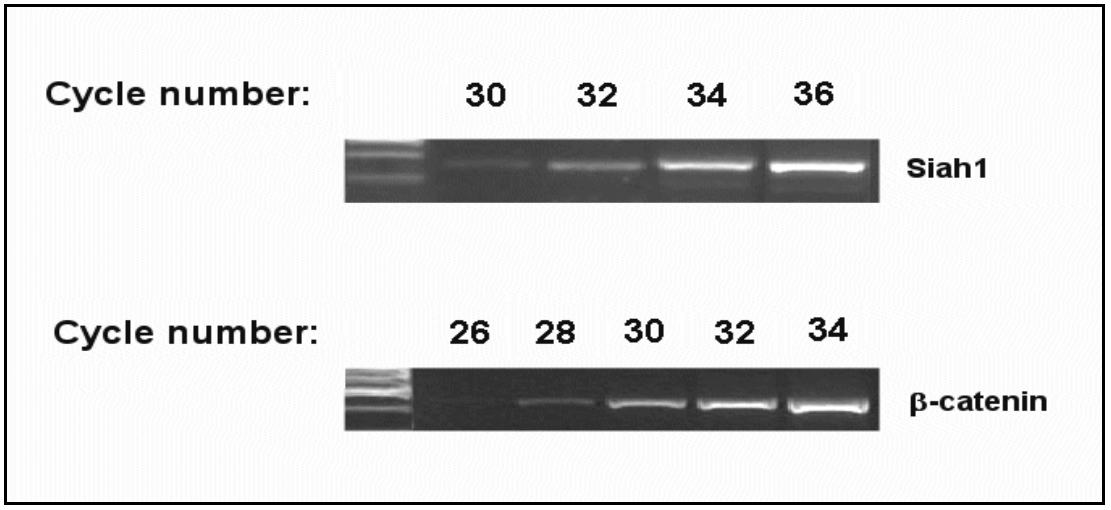

Figure 27 Cycle optimization of *Siah1* and *b-catenin* genes

Amplification of each gene was performed by using the primer pairs given in Table 4. Cold-PCR products of samples were run in 1% agarose gel.

Expression analysis for *Siah1* gene was performed by semi-quantitative PCR. After *GAPDH* normalization as described in section 3.3.6, normalized volume of first strand cDNA for each sample were subjected to cold-PCR amplification of *GAPDH,* and *Siah1*.

As shown in Figure 28.a, expression pattern of *Siah1* gene seems to be independent from the *p53* status of selected 12 HCC cell lines, except extremely low level observed in SK-Hep1 (Lane 1). Additionally, *Siah1* transcripts appear not to be modified in view of absence or presence of mutant p53 in SK-Hep1 and HepG2 derived stable clones (Figure 28.b).

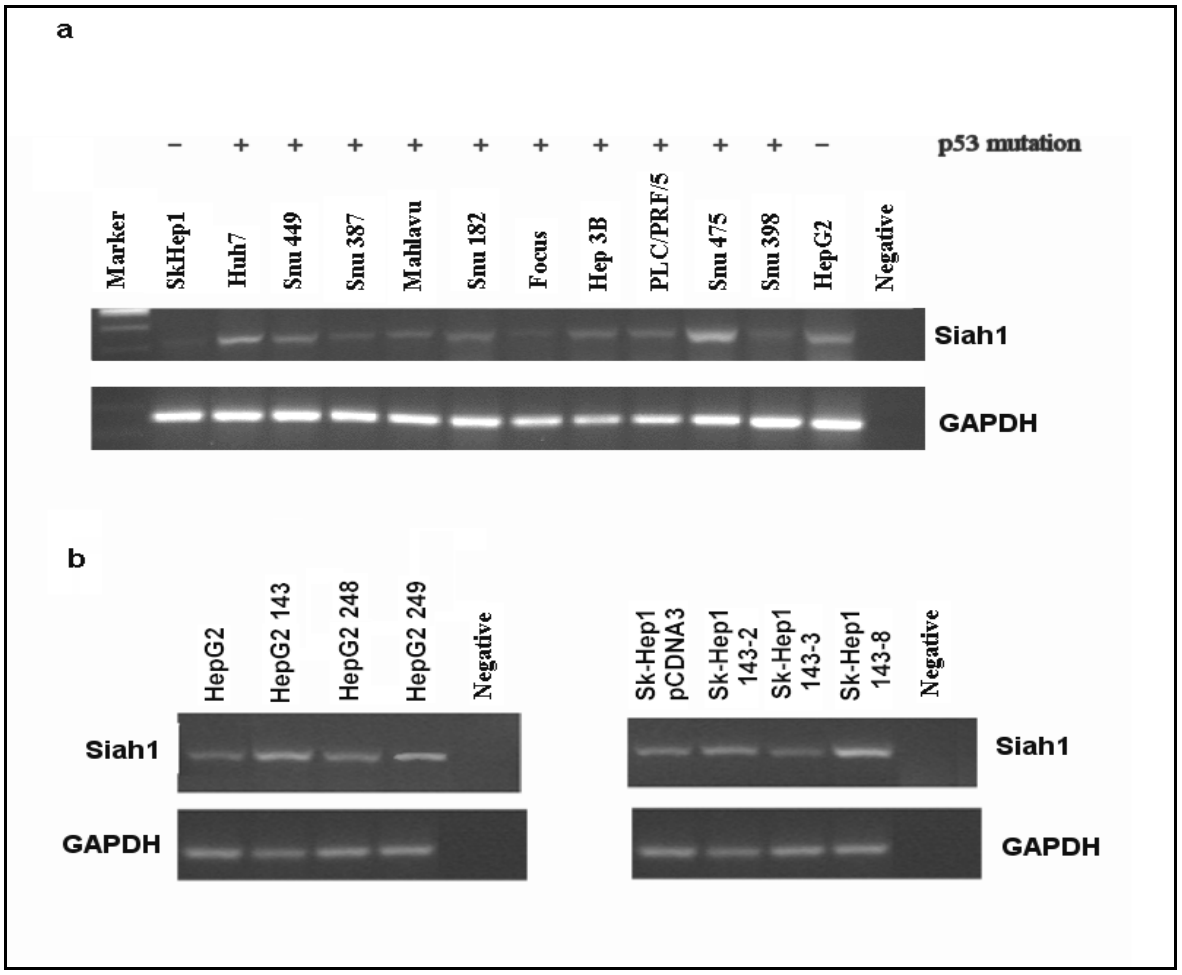

Figure 28 Analysis of *Siah1* transcript by semi-quantitative PCR

Siah1 transcript analysis in selected 12 HCC cell lines **(a)** and SK-Hep1 and HepG2 derived stable clones expressing mutant p53 **(b).** Amplification of each gene was performed by using corresponding optimal cycle (19 cycles for GAPDH, 33 cycles for Siah1) and primer pairs given in Table 4. Cold-PCR products of samples were run in 1% agarose gel.

We also tested whether the increase in β-catenin protein levels following p53 inactivation was due to an increase in gene transcription. As shown in Figure 29.a, semi-quantitative RT-PCR analysis of ***b-catenin*** transcripts indicated that full-length ***b-catenin*** cDNA transcripts encoding for wild-type ***b-catenin*** were higher in HepG2 clones, in comparison with those observed in the parental cell line. Interestingly, we did not observe an increase in truncated ***b-catenin*** cDNA (Δβ-catenin) levels under the same conditions (Figure 29). Thus, it appears that the inhibition of wild-type *p53* in these cells results in an increase in transcripts encoding for wild-type β-catenin protein.

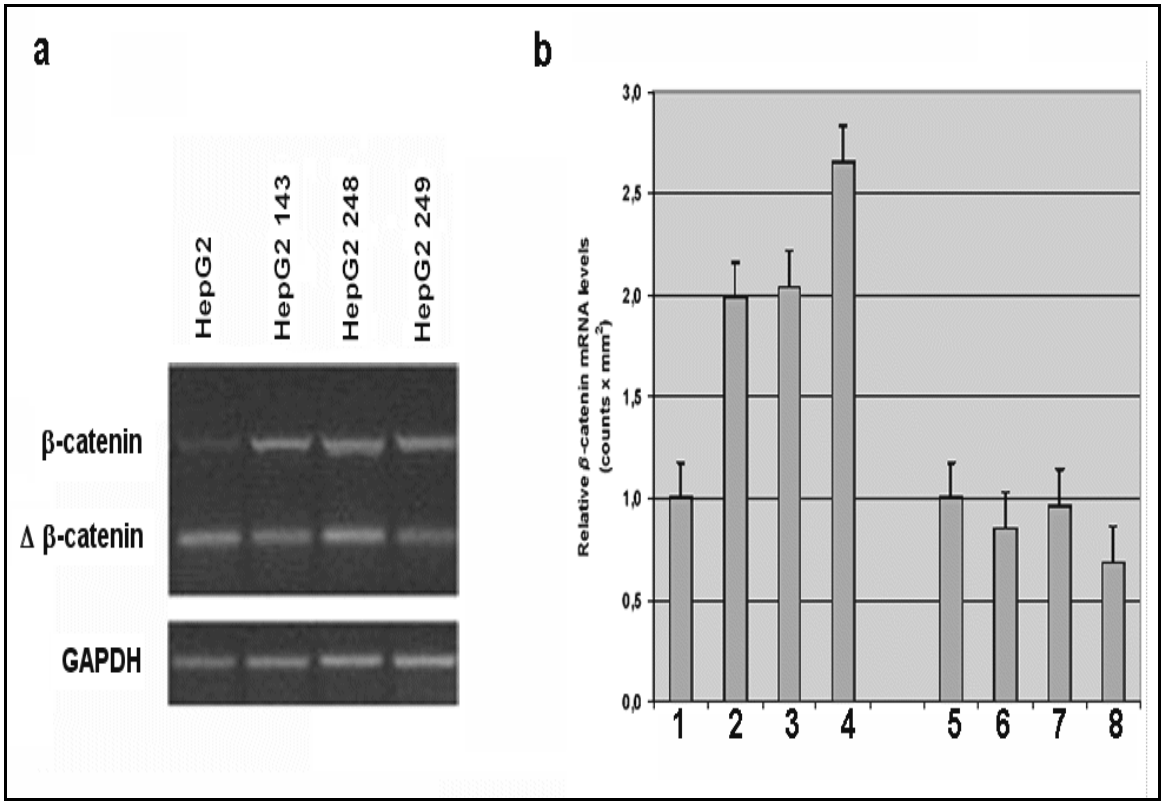

Figure 29 Analysis of *b-catenin* transcript by semi-quantitative PCR in HepG2 clones

(a) Up-regulation of wild-type β-catenin transcripts in HepG2 cells following ectopic expression of mutant p53 proteins p53-V143A, p53-R248W and p53-R249S proteins, respectively. Note that full-length β-catenin (top), but not truncated Δβ-catenin transcripts are increased in mutant p53 expressing clones, in comparison with parental HepG2 cell line. GADPH was used as a normalization control. **(b)** Computerized quantification β-catenin bands on agarose gel image. **(1,2,3,4):**wild-type β-catenin transcript levels in HepG2, HepG2-143, HepG2-248, and HepG2-249 respectively, **(5,6,7,8):** Truncated β-catenin protein levels in HepG2, HepG2-143, HepG2-248, and HepG2-249 respectively.

A modest increase in wild-type ***b-catenin*** transcript levels was also observed in SK-Hep1-derived clones (Figure 30.a). For more precise evaluation of band intensities in semi-quantitative PCR agarose gel, digital gel image were analyzed in the molecular Analyst software (BioRad). Results were summarized as bar graphics in Figure 29.b and 30.b.

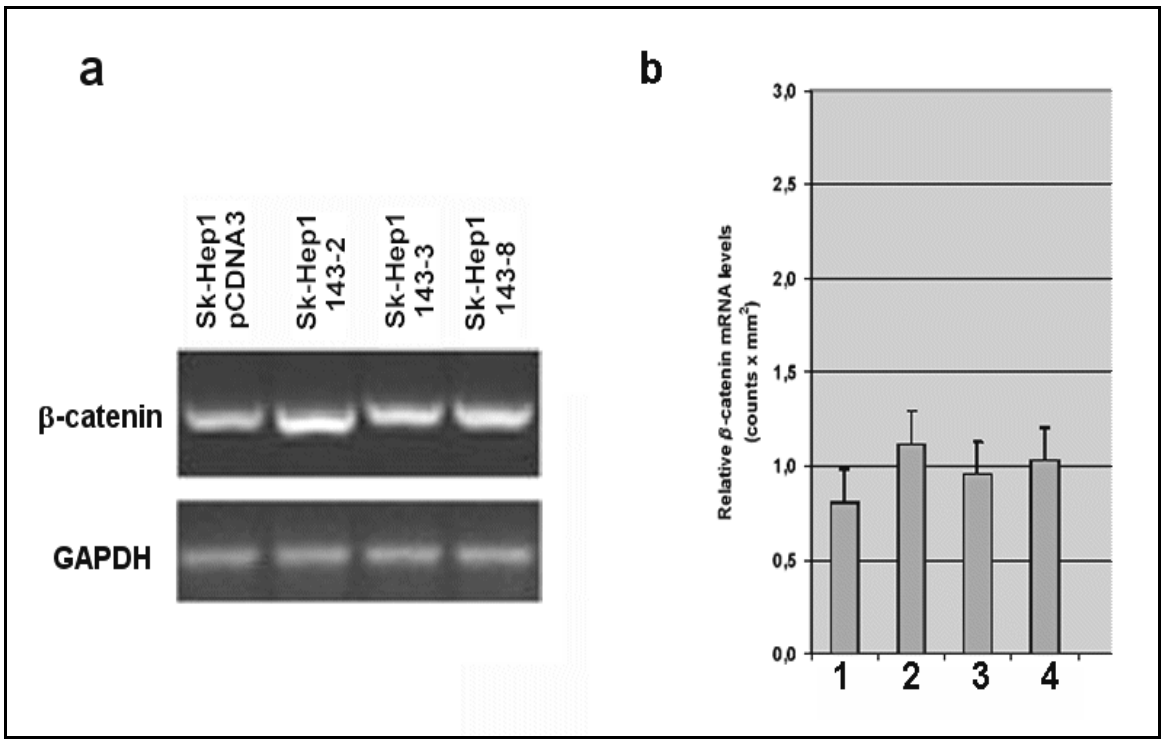

Figure 30 Analysis of *b-catenin* transcript by semi-quantitative PCR in SK-Hep1 clones

(a) Modest up-regulation of β-catenin transcripts in there different SK-Hep1 clones expressing mutant p53 proteins p53-V143A **(b)** Computerized quantification β-catenin bands on agarose gel image. (**1,2,3,4):** wild-type β-catenin transcript levels in SK-Hep1-pCDNA3, SK-Hep1-143-2, SK-Hep1-143-3 and SK-Hep1-143-8 clones respectively.

4.8 Expressional Analysis of Known Target Genes of Activated b-catenin in 12 Selected HCC Cell Lines

It has been shown that oncogenic activation of β-catenin resulted in trancriptional activation of critical targets genes. Among them, *cyclin D1* and *c-myc* are the well-described target genes which are highly activated after oncogenic activation of β-catenin in some tumor cells (Morin et al., 1997; He et al., 1998; Sparks et al., 1998; Tetsu and McCormick, 1999).

To test the response of these two critical genes to *b-catenin* mutation or β-catenin accumulation in the selected 12 HCC cell lines at transcriptional level, we performed semi-quantitave PCR expression analysis. First strand cDNAs were subjected to cold PCR (as described Method section 3.3.6) to determine the cycle number for *the c-myc, cyclinD1* and *GAPDH* gene amplification. After cold-PCR, amplification products from samples of cycle 14-30 were visualized in agarose gel each transcript (Figure30).

The optimal cycle number can be define as the minimum number of cycle necessary for a visible but an unsaturated band for a gene of interest. Evaluating the intensity of fragment for corresponding cycle, following optimal cycle numbers were determined;

Optimal cycle number for *GAPGH* : 19 (Figure 31 upper-panel)
Optimal cycle number for *c-myc* : 30 (Figure 31 mid-panel)
Optimal cycle number for *cyclinD1* : 30 (Figure 31 lower- panel)

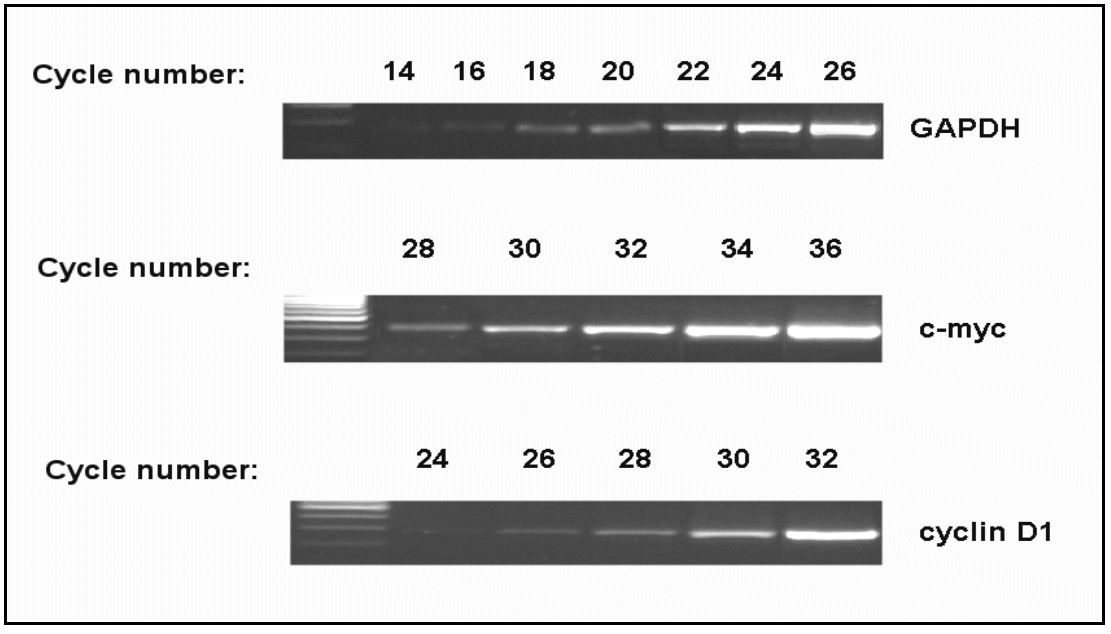

Figure 31 Cycle optimization of *GAPDH, c-myc* and *cyclinD1* genes

Amplification of each gene was performed by using the primer pairs given in Table 4. Cold-PCR products of samples were run in 1% agarose gel.

After determination of the optimal cycle number for both genes, expression analyses for each gene was performed by semi-quantitative PCR. Initially, first stand cDNA for each cell lines were subjected to *GAPDH* normalization as describe in section 3.3.6, then by using the normalized volume of first strand cDNA for each sample, cold-PCR amplification of *GAPDH, c-myc* and *cyclinD1* were performed.

As shown in Figure 32, neither of the *c-myc* and *cyclin D1* genes displayed a correlation with the *b-catenin* mutation or accumulation status of studied 12 HCC cell lines.

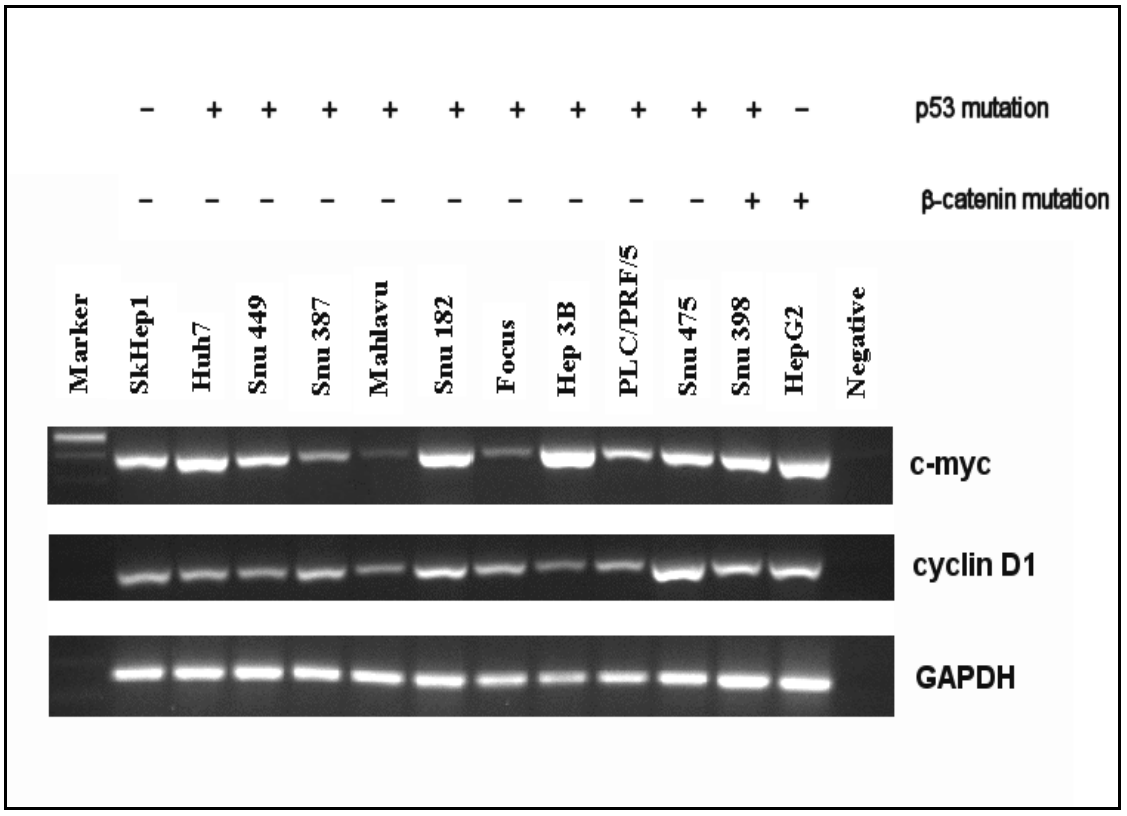

Figure 32 Semi-quantitative PCR results of *c-myc* and *cyclin D1* gene

Amplification of each gene was performed by using corresponding optimal cycle (19 cycles for GAPDH, 30 cycles for both c-myc and cyclin D1) and primer pairs given in Table 4. Cold-PCR products of samples were run in 1% agarose gel.

4.9 Inverse Correlation in the Worldwide Distribution of *b-catenin* and *p53* Gene Mutations in HCC

*In-vitro d*ata we have obtained through this study and other recent reports (McEntee and Brenneman, 1999; Renard et al., 2000; Damalas et al., 2001; Liu et al., 2001; Matsuzawa and Reed, 2001; Sadot et al., 2001; Ueda et al., 2001b; Wetmore et al., 2001) imply that the p53-WNT pathway cross-talk may have an important role in carcinogenesis. To concentrate on this hypothesis, in the course of hepatocarcinogenesis, we compared *p53* and *b-catenin* mutation frequencies in 33 primary HCC tumor from southern Africa and China (Table 7), and showed that these aflatoxin-and –HBV related HCCs maintain wild-type β-catenin gene.

The apparent lack of *b-catenin* mutations in our tumor samples from southern Africa and China was in sharp contrast with high frequency of mutations reported for tumors from other regions of the world (Buendia 2000). Based on the previous work by Unsal *et al.* and Ozturk M, which demonstrated that the mutation rates of *p53* differ significantly from one geographical region to another (Unsal et al.,1994; Ozturk M. 1999), we compared reported *b-catenin* mutation rates in HCCs from different countries.

Using data generated in this work (n=33), as well as data compiled from previous reports by different authors, we calculated mutation frequencies of *b-catenin* in total 1123 HCC from different geographical locations. The overall mutation rate of β-catenin in HCC was 17%, but its geographical distribution was highly heterogeneous. The lowest mutation rate was seen in tumors from Africa (0%), followed by China (9%), Japan (19%) and Europe (24%), which displayed the highest mutation rate. A similar analysis of p53 mutation frequencies (data were taken from study by Ozturk M, 1999) showed that the overall p53 mutation rate in 969 tumors was 27%. The highest mutation frequency was observed in tumors from Africa (42%), followed by China (38%), Japan (27%) and Europe (15%), which displayed the lowest mutation rate (Table 9). As illustrated in Figure 33, *p53* and *b-catenin* mutation rates in HCC follow a perfect but inverse occurrence pattern. This pattern is characterized by a dominant p53 mutation frequency in tumors from

Africa and China, associated with a low rate of ***b-catenin*** mutations. In tumors from other geographical regions, ***b-catenin*** mutations are more prominent, or even more frequent than *p53* mutations, as observed in tumors from Europe (Table 9).

Table 9 Geographical distribution of reported b-catenin and *p53* mutation rates in hepatocellular carcinoma

Region	b-catenin	p53
Africa	0/22 (0%)	11/26 (42 %)
China[a]	12/133 (9%)	50/132 (38 %)
Japan	44/241 (19%)	94/353 (27 %)
Europe	26/108 (24%)	23/151 (15 %)
Other[b]	102/619 (17%)	79/273 (28 %)
All	**184/1123 (17%)**	**263/969 (27 %)**

[a]Mainland China and Hong Kong, [b]Australia, North America, Singapore, South Korea, Taiwan, Thailand, or unidentifiable origin. β-catenin data is a combination of the data presented here (data for Africa and part of China), and those compiled from references Devereux et al. 2001, Wong et al. 2001 (for China); Miyoshi et al., 1998; Kondo et al., 1999; Satoh et al. 2000; Fujie et al., 2001 (for Japan); Nhieu et al., 1999; Terris et al., 1999 (for Europe); Huang et al., 1999; Hsu et al., 2000; Laurent-Puig et al., 2001; De La Coste et al., 1998 (for other). p53 data is from Ozturk M, 1999

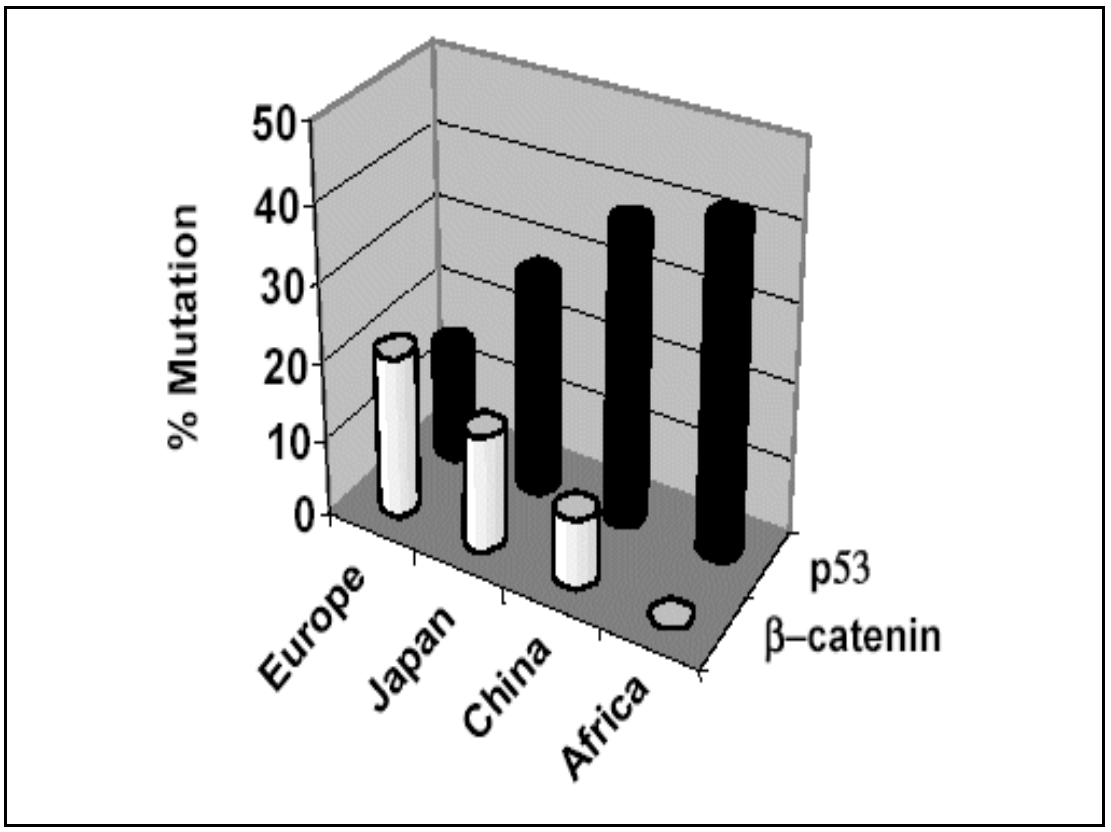

Figure 33 Comparative analysis reveals an inverse relationship between worldwide rates of *p53* and *b-catenin* mutations in hepatocellular carcinoma

p53 and β-catenin mutations in tumors from different geographical locations (reported here and complied from previous reports by others) were calculated as shown in table 2, and represented as a comparative graph here. Note that in tumors from western populations, p53 mutations are rare, while β-catenin mutation rate is high. Inversely, β-catenin mutations are rare in tumors from Africa and China which display high rate of p53 mutations.

CHAPTER 5. DISCUSSION AND PERSPECTIVES

In this study, 14 HCC cell lines were analyzed for *b-catenin* gene mutation by SSCP, fragment length analysis and nucleic acid sequencing. Heterozygous *b-catenin* gene mutations were identified in 2 cell lines (Table 6). HepG2 cell line displayed an interstitial deletion leading to a truncated protein product lacking amino acids from 25 to 140 whereas SNU398 cells displayed a heterozygous TCT->TGT mutation leading to a serine to cystein substitution (S37C) of a GSK-3β phosphorylation site in β-catenin protein. The *b-catenin* gene was wild type in other cell lines. To our knowledge, *b-catenin* gene status of SK-Hep1, Mahlavu, SNU387, SNU182, FOCUS and Hep3B have not been reported previously, but we confirmed wild-type status of *b-catenin* in Huh7, PLC/PRF/5 (Alexander), SNU423 and SNU475 cell lines (Satoh et al., 2000), as well as the interstitial deletion in HepG2 cell line (de La Coste et al., 1998). On the other hand, in our hands, it was SNU398, but not SNU449, which displayed a missense *b-catenin* mutation, in apparent opposition to previously, published data (Satoh et al., 2000). In the same set of 14 HCC-cell lines, PLC/PRF/5, SNU423 and SNU475 have been reported to display *Axin1* mutation, while Huh7 had no mutation (Satoh et al., 2000). So far, we were able to confirm the *Axin1* status of these cell lines, and beside an Arg(454)His substitution (CGC → CAC) in exon 4 of *Axin1* in the Hep40 cell line, we failed to identify any *Axin1* mutation in the remaining 9 cell lines (Table 6). Although, at this stage of our investigation, we did not perform a screening for the Arg(454)His substitution in alleles of control population, occurrence of two altered alleles in the same cell line, and the absence of Arg(454)His (CGC → CAC) alteration in other samples (15 HCC cell lines and 200 HCC/Hepatoblastoma tumors) both in our study and other published studies (Satoh et al., 2000; Taniguchi et al.,

2002) imply that substitution of the highly conserved arginine residue at codon 454 to histidine in the exon 4, which encodes the GSK-3β interaction domain required for formation of the critical Axin-APC-GSK-3β protein complex in proteosomal degradation of β-catenin (Nakamura et al., 1998; Salic et al., 2000), may be involved in the development of liver cancer. These criteria are not adequate to clarify the possible effect of sequence alteration, for that reason, mutational nature of Arg(454)His substitution remains to be demonstrated experimentally. It is also noteworthy that, we did not screen all *Axin1* exons for possible mutations. Previously, published data show that *Axin1* mutations occurs in exon 1 (1.5%), 2 (1.0%), 3 (1.0%), 4 (2.0%), 5 (2.5%), and 6 (0.5%). Since we did not study exons 1, 5, and 6, it is possible that additional HCC cell lines display Axin 1 mutations.

Aberrant accumulation of β-catenin has been observed in different types of tumors. This accumulation is usually due to a defective proteosomal degradation of β-catenin, which may result from mutations affecting *b-catenin*, *APC* or *Axin1* gene, depending on cancer type (Polakis 2000). Here, we report that aberrant accumulation of β-catenin is also associated with *p53* mutations. The reasons for this association are not known presently. However, two types of studies have established the presence of a functional cross-talk between p53 and β-catenin. Recently, it has been reported that wild-type p53 is able to induce the expression of *Siah-1* gene, which encodes a protein involved in the ubiquitin-mediated degradation of β-catenin protein. Accordingly, wild-type p53-induced Siah-1 was shown to down-regulate β-catenin levels (Liu et al., 2001; Matuzawa and Reed, 2001). However, it is unknown whether mutational inactivation of p53 may cause a loss of basal Siah-1 expression leading to defective β-catenin degradation. Using a semi-quantitative RT-PCR assay, we observed that all HCC cell lines tested here express *Siah-1* transcripts independent of p53 status, with the exception of SK-Hep1 cells which display extremely low levels. In addition, *Siah-1* transcripts appeared not to be modified in HepG2 and SK-Hep1 clones expressing mutant p53 protein. Therefore, the accumulation of β-catenin in mutant *p53* cell lines may not be related to loss of Siah-1 activity. On the other hand, oncogenic activation of β-catenin was shown to serve as a stimulus for activation of wild-type p53 activity, which induces cell cycle arrest or apoptosis as a response (Damalas et al., 1999; Damalas et al., 2001). Therefore, the mutant β-catenin and wild type p53 activity may not be

compatible for cell survival, so that *p53* gene is also mutated during tumor progression following ***b-catenin*** mutation. This appears to be the case in colorectal cancers where early oncogenic activation of β-catenin (either directly by ***b-catenin*** mutation, or indirectly by *APC* mutation) is followed by a sharp increase in *p53* mutation frequency (Kinzler and Volgelstein 1998). The same mechanism could also be involved in some HCCs displaying both *p53* and ***b-catenin*** mutations such as SNU398 cell line, but most HCC cell lines have either *p53* or ***b-catenin*** mutation (Table 8). Oncogenic activation of wild-type β-catenin in HCC by mutations affecting *APC* and *Axin1* gene is also conceivable. As stated earlier, *APC* mutation is exceptional in HCC, while Axin1 mutation appears to affect less than 10% of these tumors. In 12 HCC cell lines selected for biochemical studies concerning the p53 and β-catenin relation, PLC/PRF/5, SNU423 and SNU475 have been reported to display *Axin1* mutation, while Huh7 had no mutation (Satoh et al., 2000). We also showed the remaining 8 cell lines contain wild-type *Axin1* sequence encompassing the region exon 2-4 (Table 6). Thus, we believe that in most HCC cell lines, the accumulation of β-catenin occurs as a consequence of functional inactivation of p53. Induced accumulation of wild-type β-catenin in HepG2 and SK-Hep1 cells following ectopic expression of mutant p53 proteins provides additional evidence for our hypothesis. The increase in wild-type β-catenin levels in mutant p53-expressing clones of HepG2 and SK-Hep1 was modest, but this is probably due to the fact that these clones still express wild-type p53, in contrast to other cell lines, which have complete loss of wild-type p53 allele (Table 8). Nuclear accumulation of β-catenin is considered as an indication of its aberrant accumulation in cells, in particular due to *APC* mutations in colorectal cancers (Handerson 2000; Polakis 2000). We have shown wild-type β-catenin accumulation in the nuclei of SK-Hep1 cells, following p53 inactivation. Thus, it appears that p53 inactivation, similar to APC or Axin1 inactivation leads to oncogenic activation of β-catenin in cancer cells. This activation appears to be due, at least in part, to an up-regulation of wild-type ***b-catenin*** transcripts, as demonstrated in mutant p53-expressing clones of HepG2 and SK-Hep1. Interestingly, *p53* status did not affect truncated Δβ-catenin transcripts in the same cell line (Figure 29.a). The reasons of this differential response are presently unknown. However, this could be due to allele-specific interstitial ***b-catenin*** gene deletion in HepG2 cells, which may abolish the ability of this gene to respond to wild-type *p53*. This hypothesis remains to be demonstrated experimentally.

The *p53* gene is one of the most frequently mutated or inactivated genes in hepatocellular, breast, lung and cervix cancers which express aberrantly accumulated β-catenin protein that can not be explained solely by *b-catenin*, *APC* or *Axin1* gene mutations. Thus, it is highly likely that, the loss of p53 function is a considerable cause for aberrant accumulation of β-catenin in these tumors. Presently, it is unclear whether *p53* mutation may serve as an alternative mechanism for oncogenic activation of β-catenin during tumor development. Such a hypothesis could be indirectly verified by testing whether *p53* and *b-catenin* mutations are mutually exclusive, as it has been shown for *APC* and *b-catenin* mutations in colorectal cancers (Sparks et al., 1998). Data presented in Table 8 indicate that *p53* and *b-catenin* mutations are mutually exclusive in 11/12 (92%) of HCC cell lines. We were not able to address this issue in primary tumors, but Figure 33 shows that overall frequencies of *p53* and *b-catenin* mutations show an inverse correlation in HCCs from different geographical locations, as if these mutations are mutually exclusive in these tumors. A careful examination of previously published data on *p53* and *b-catenin* mutations provides additional hints for a close connection between p53 and β-catenin in different HCC models. For example, data published by Laurent-Puig et al.(Laurent-Puig et al., 2001) show that p53 and *b-catenin* mutations are mutually exclusive in 58/62 (94%) of primary HCC tumors, and only 4/62 (7%) of tumors display mutations on both genes. In another study, Renard et al. (Renard et al., 2000) reported that either wild-type *p53* allele was lost or *b-catenin* gene mutated in HCCs observed in N-myc transgenic mice that are heterozygous for *p53* gene. It is also noteworthy that *b-catenin* mutations were shown to be absent in HCCs that occur in SV40 Large T transgenic mice (Umeda et al., 2000), whereas *b-catenin* is mutated frequently in other models of experimentally induced mouse HCCs (de La Coste et al., 1998, Calvisi et al., 2001). Transforming ability of large T antigen is known to be due, at least partly, to functional inactivation of p53 (Pipas and Levine 2001). Therefore, the lack of β-catenin mutation in these tumors could be due to a functional activation of its protein product by large T antigen *via* p53 inactivation.

Taken together, these observations clearly indicate that *b-catenin* mutations occur rarely in cancer cells, which have lost wild-type p53 function, but these cells display aberrant accumulation of wild-type β-catenin protein. These findings may strengthen a hypothesis linking p53 mutations to oncogenic activation of β-catenin, even though this has to be

demonstrated experimentally. If this is the case, critical target genes of activated β-catenin must be induced in cancer cells following p53 inactivation. This demonstration will not be an easy task for HCC, since best described β-catenin targets, namely *cyclin D1* and *c-myc* (He et al., 1998; Tetsu and McCormick 1999) appear not to be involved in β-catenin-mediated hepatocellular carcinogenesis. For example, neither *c-myc* nor *cyclin D1* is overexpressed in hyperplastic livers of transgenic mice, which develop hepatomegaly as a result of oncogenic β-catenin overexpression (Cadoret et al., 2001). Furthermore, Inagawa et al. (Inagawa et al., 2002) did not find any association between accumulation of β-catenin and overexpression of cyclin D1 in human HCCs. In confirmation of these observations, we did not detect any correlation between c-myc/cyclin D1 protein overexpression and β-catenin mutation or β-catenin accumulation in 12 HCC cell lines studied here. Thus, it appears that β-catenin acts on targets other than *c-myc* and *cyclin D1* to exhibit its oncogenic activity in liver cells. Some genes whose over-expression is associated with *p53* mutation in cancer cells could be such targets for β-catenin in liver cells.

In conclusion, both p53 and *wnt* pathways are involved in many cellular processes independent of each other (Barker et al., 2000, Polakis 2000; Ryan et al., 2001). Nevertheless, there is now sufficient evidence for a functional cross-talk between p53 and *wnt* pathways (Damalas et al., 1999; Damalas et al., 2001; Liu et al., 2001; Matsuzawa and Reed 2001; Sadot et al. 2001 and this study). Studies aiming to explore combined effects of p53 and *wnt* pathway deregulations may open new ways to a more comprehensive study of hepatocellular carcinogenesis.

Perspectives

It is apparent that Wnt signaling causes cancer and that tumor promotion by this pathway can proceed through a number of different genetic defects. Additional mechanisms by which defects in the regulation of wnt signaling contribute to tumor progression probably remain undiscovered.

Several lines of evidence demonstrate a role of the Wnt signaling pathway and p53 cross-talk in cancer. Dalamas *et al.* has provided the first evidence for a cross-talk between β-catenin and p53 by demonstrating that aberrant β-catenin accumulation can induce activation of p53 (Damalas et al., 1999). These observations may explain, at least in part, the selective pressure for loss of p53 activity in HCCs harboring deregulated β-catenin, and the occurrence of high frequency of *b-catenin* mutations over p53 inactivation in hepatoblastomas. However, this hypothesis should be demonstrated in HCC tumors lacking mutations in *b-catenin* and other genes implicated in oncogenic activation of β-catenin.

Our results with HCC cell lines and primary tumors show that *b-catenin* mutations occur rarely in cancer cells, which have lost wild-type p53 function, but these cells display aberrant accumulation of wild-type β-catenin protein. This study could indicate an impact of p53 inactivation to activation of canonical wnt pathway during the process of hepatocarcinogenesis. The molecular mechanism by which p53 inactivation induce aberrant β-catenin accumulation is not known yet. Our preliminary results by semi-quantitative RT-PCR reported here did not support the hypothesis of *Siah-1* mediated-regulation of abundance of β-catenin (Liu et al., 2001; Matsuzawa and Reed, 2001). Interestingly, our data indicated that *b-catenin* accumulation appears to be due to, at least in part, to an up-regulation of wild-type *b-catenin* transcripts, as demonstrated in mutant p53-expressing clones of HepG2 and SK-Hep1, and p53 status did not affect truncated Δβ-catenin transcripts in the same cell line. In line with this observation, as a future perspective, we propose to study the luciferase-based transcriptional regulation experiments following the preparation of several deletion constructs of *b-catenin* gene.

The manifestation of cancer by aberrant Wnt signaling most likely results from inappropriate gene activation mediated by stabilized β-catenin. Another exciting question would be opened up for new studies based on observation that was reported in the literature (de La Coste et al., 1998; Cadoret et al., 2001) and this study: although the genes encoding c-myc and cyclin D1 are potential targets of the β-catenin signaling pathway, neither of them showed any correlation with the accumulation of β-catenin. Thus, mutant p53-expressing clones of Sk-Hep1 is a good experimental system to study a gene expression analysis to elucidate other genes that are probably involved in the proliferative stimulus triggered by deregulation of β-catenin signaling in the liver.

Recent data suggest that Axin acts as a crucial negative regulator in Wnt signaling through interaction with CKI, APC, GSK-3β and β-catenin (Nakamura et al., 1998; Satoh et al., 2000; Amit et al., 2002; Taniguchi et al., 2002). Beside this suggestion, Axin also regulates the effects of Smad3 on the transfroming growth factor β (TGF-β) signaling system which has shown to be involved in hepatocarcinogenesis (Yakicier et al., 1999; Furuhashi et al., 2001). Thus, screening of the remaining exons of *Axin 1* will have an important value especially in regard to investigation of a possible role of Axin in TGF-β resistance in human HCC.

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APPENDIX

SNU – 398	
ATCC Number:	CRL-2233
Organism:	Homo sapiens (human)
Designation:	SNU-398
Tissue:	hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-398 was derived in 1990 by J.-G. Park and associates from an anaplastic hepatocellular carcinoma taken from a Korean patient who had been treated by transcatheter arterial embolization with lipoidol plus a combination of doxorubicin and mitomycin-C. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum. Grossly, the original tumor was single nodular with perinodular extensions. Histologically, it was trabecular type. The cultured cells are multinucleated, and maintain the production of intracytoplasmic hyaline globules as seen in the original tumor. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	42 years
Ethnicity:	Oriental
Gender:	male
Growth Properties:	mixed adherent and floating
Antigen Expression:	Blood Type O; Rh +
Karyotype:	aneuploid; modal number = 62
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach. Add fresh medium, aspirate and dispense into new flasks.
Split Ratio:	A subcultivation ratio of 1:3 to 1:6 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	39 hrs

BioSafety Level:	2
Required Forms:	This line is available under the following restrictions: 1.) The cell line was deposited for research purposes only. Neither the cell line nor products derived from it may be sold or used for commercial purposes. Nor can the cells be distributed to third parties for purpose of sale, or producing for sale, cells or their products. The cells are provided as a service to the research community. They are provided without warranty or merchantability of fitness for a particular purpose or any other warranty, express or implied. 2.) Any proposed commercial use of these cells or products produced by them must first be negotiated with Jae-GaHB-Park, Director, Korean Cell Line Bank, 28 Yongon-dong, Chongno-gu, Seoul, 110-744 Korea. Telephone (02) 760-3380, Fax (02) 742-4727. 3.) In all papers reporting any use of these cells or derived products, a direct reference will be made to the original publication (Int. J. Cancer 62:276-282, 1995).

SNU -449	
ATCC Number:	CRL-2234
Organism:	Homo sapiens (human)
Designation:	SNU-449
Tissue:	hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-449 was derived in 1990 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient prior to cytotoxic therapy. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Grossly, the original tumor was single nodular with perinodular extensions. Histologically, it was predominantly compact and minor trabecular type. The cultured cells contain a single or double nucleus. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	52 years; grade II-III/IV
Ethnicity:	Oriental
Gender:	male
Growth Properties:	monolayer of diffusely spreading cells
Antigen Expression:	Blood Type AB; Rh +
Karyotype:	aneuploid; modal number = 57
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach.
Split Ratio:	A subcultivation ratio of 1:5 to 1:10 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	36 hrs
BioSafety Level:	2

SNU - 182	
ATCC Number:	CRL-2235
Organism:	Homo sapiens (human)
Designation:	SNU-182
Tissue:	hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-182 was derived in 1989 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient prior to cytotoxic therapy. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Grossly, the original tumor was single nodular. Histologically, it was predominantly trabecular and minor acinar type. The cultured cells contain a single nucleus. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	24 years; grade III/IV
Ethnicity:	Oriental
Gender:	male
Growth Properties:	adherent
Antigen Expression:	Blood Type O; Rh +
Karyotype:	aneuploid; modal number = 70
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach.
Split Ratio:	A subcultivation ratio of 1:3 to 1:6 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	46 hrs
BioSafety Level:	2

SNU - 475	
ATCC Number:	CRL-2236
Organism:	Homo sapiens (human)
Designation:	SNU-475
Tissue:	hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-475 was derived in 1990 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient prior to cytotoxic therapy. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Grossly, the original tumor was massive growth type. Histologically, it was predominantly compact and minor trabecular type. The cultured cells are multinucleated. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	43 years; grade II-IV/V
Ethnicity:	Oriental
Gender:	male
Growth Properties:	monolayer of diffusely spreading cells
Antigen Expression:	Blood Type AB; Rh +
Karyotype:	aneuploid; modal number = 61
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach.
Split Ratio:	A subcultivation ratio of 1:3 to 1:6 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	66 hrs
BioSafety Level:	2

SNU -387	
ATCC Number:	CRL-2237
Organism:	Homo sapiens (human)
Designation:	SNU-387
Tissue:	pleomorphic hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-387 was derived in 1990 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient who had been treated by transcatheter arterial embolization with lipoidol plus a combination of doxorubicin and mitomycin-C. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum. Grossly, the original tumor was single nodular. Histologically, it was predominantly compact and minor trabecular type. The cultured cells contain a single nucleus. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	41 years; grade IV/V
Ethnicity:	Oriental
Gender:	female
Growth Properties:	adherent
Antigen Expression:	Blood Type O; Rh +
Karyotype:	aneuploid; modal number = 67
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach.
Split Ratio:	A subcultivation ratio of 1:3 to 1:6 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	61 hrs
BioSafety Level:	2

SNU -423	
ATCC Number:	CRL-2238
Organism:	Homo sapiens (human)
Designation:	SNU-423
Tissue:	pleomorphic hepatocellular carcinoma; liver
Morphology:	epithelial
Comments:	SNU-423 was derived in 1990 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient who had been treated by transcatheter arterial embolization with lipoidol plus doxorubicin. Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Grossly, the original tumor was single nodular with perinodular extensions. Histologically, it was trabecular type. The cultured cells are multinucleated. Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization. HBV genomic RNA was not expressed.
Age Stage:	40 years; grade III/IV
Ethnicity:	Oriental
Gender:	male
Growth Properties:	adherent
Antigen Expression:	Blood Type B; Rh +
Karyotype:	aneuploid; modal number = 79
Subculturing:	Remove spent medium, add fresh 0.25% trypsin, 0.03% EDTA solution, rinse and remove trypsin. Add fresh trypsin solution (1 to 2 ml) and let the culture sit at room temperature (or at 37C) until the cells detach.
Split Ratio:	A subcultivation ratio of 1:3 to 1:5 is recommended
Fluid Renewal:	Every 2 to 3 days
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF33286: Park JG et al. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62: 276-282, 1995 PubMed: 95355145
Propagation:	ATCC medium: RPMI 1640 medium, 90%; heat-inactivated fetal bovine serum, 10%
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Doubling Time:	72 hrs
BioSafety Level:	2

Hep 3B	
ATCC Number:	HB-8064
Organism:	Homo sapiens (human)
Designation:	Hep 3B2.1-7 (Hep 3B)
Tissue:	hepatocellular carcinoma; liver
Tumorigenic:	yes, forms ascites in nude mice
Products:	alpha fetoprotein (alpha-fetoprotein); hepatitis B surface antigen (HBsAg); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; complement (C3, C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein); Gc globulin
Morphology:	epithelial
Comments:	This line contains an integrated hepatitis B virus genome. THIS IS NOT A HYBRIDOMA! IT WAS INADVERTANTLY GIVEN AN HB NUMBER.
Age Stage:	8 years
Ethnicity:	Black
Gender:	male
Growth Properties:	adherent
Karyotype:	modal number = 60 with a subtetraploid mode of 82; has a rearranged chromosome 1
Subculturing:	Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks.
Split Ratio:	A subcultivation ratio of 1:4 to 1:6 is recommended
Fluid Renewal:	Twice per week
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF11092: Knowles BB et al. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209: 497-499, 1980 PubMed: 80236294 RF13196: Knowles BB and Aden DP. Human hepatoma derived cell line, process for preparation thereof, and uses therefor. U.S. Pat. 4,393,133 dated July 12, 1983 RF33319: Aden DP et al. Controlled synthesis of HBsAg in a differentiated human liver carcinoma- derived cell line. Nature 282: 615-616, 1979 PubMed: 81012119 RF34776: Darlington GJ et al. Growth and hepatospecific gene expression of human hepatoma cells in a defined medium. In Vitro Cell. Dev. Biol. 23: 349-354, 1987 PubMed: 87222055
Propagation:	ATCC medium: Minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% Temperature: 37C

Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) -- ATCC 30-2003 recommended serum 30-2020
BioSafety:	Handle as potentially biohazardous material under at least Biosafety Level 2 containment.
Patent Statement:	This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims.
BioSafety Level:	2

Hep G2	
ATCC Number:	HB-8065
Organism:	Homo sapiens (human)
Designation:	Hep G2
Tissue:	hepatocellular carcinoma; liver
Tumorigenic:	no
Products:	alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; demonstrates decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress) complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein)
Receptors Expressed:	insulin; insulin-like growth factor II (IGF II)
Morphology:	epithelial
Comments:	There is no evidence of a Hepatitis B virus genome in this cell line. The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities.
Age Stage:	15 years
Ethnicity:	Caucasian
Gender:	male
Growth Properties:	adherent
Karyotype:	modal number = 55 (range = 50 to 60); has a rearranged chromosome 1
Subculturing:	Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks.
Split Ratio:	A subcultivation ratio of 1:4 to 1:6 is recommended
Fluid Renewal:	Twice per week
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF11092: Knowles BB et al. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209: 497-499, 1980 PubMed: 80236294 RF42571: Lieber A et al. Recombinant adenoviruses with large deletions geneerated by cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. J. Virol. 70: 8944-8960, 1996 PubMed: 97126100 RF42601: Dubuisson J and Rice CM. Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. J. Virol. 70: 778-786, 1996 PubMed: 96135186 RF42674: Yamaguchi Y et al. Biochemical characterization and intracellular localization of the Menkes disease protein. Proc. Natl. Acad. Sci. USA 93: 14030-

	14035, 1996 PubMed: 97098515 RF42847: Kounas MZ et al. Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein. J. Biol. Chem. 271: 6523-6529, 1996 PubMed: 96198123 RF42860: Klemm DJ et al. Adenovirus E1A proteins regulate phosphoenolpyruvate carboxykinase gene transcription through multiple mechanisms. J. Biol. Chem. 271: 8082-8088, 1996 PubMed: 96215200 RF42868: Wu X et al. Demonstration of a physical interaction between microsomal triglyceride transfer protein and apolipoprotein B during the assembly of ApoB-containing lipoproteins. J. Biol. Chem. 271: 10277-10281, 1996 PubMed: 96215326 RF42870: Ostlund RE J et al. A sterospecific myo-inositol/D-chiro-inositol transporter in HepG2 liver cells. J. Biol. Chem. 271: 10073-10078, 1996 PubMed: 96215295
Propagation:	ATCC medium: Minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% Temperature: 37C
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) -- ATCC 30-2003 recommended serum 30-2020 derivative CRL-10741 derivative CRL-11997
Patent Statement:	This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims.
BioSafety Level:	1

HuH-7	
Other name:	HuH-7, JTC-39
Animal:	human
Scientific Name:	Homo sapiens
Tissue:	liver
Medium:	Dulbecco's minimal essential medium with 10% fetal bovine serum. Refer RefID1201 for clasical medium
Passage method:	Cells are treated with 0.2% trypsin and 0.02% EDTA.
Cell no. at passage:	$2.5x10^4$ cells/cm^2
Genetics:	hepatoma (differenciated), age 57, (M)
Characteristics:	hepatoma (differenciated), age 57, (M)
Established by	Nakabayshi,H. & Sato,J.
Cell bank maintaining these cells.	OUCI(JCRB)
References:	1.*Nakabayashi,H., Taketa,T., Miyano,K., Yamane,T., and Sato,J.* Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res., 42: 3858-3863, 1982. 2.*Nakabayashi,H., Taketa,K., Yamane,T., Miyazaki,M., Miyano,K., and Sato,J.* Phenotypical stability of a human hepatoma cell line, HuH-7, in long-term culture with chemically defined medium. Gann, 75: 151-158, 1984. 3.*Nakabayashi,H., Taketa,K., Yamane,T., Oda,M., and Sato,J.* Hormonal control of alpha- fetoprotein secretion in human hepatoma cell lines proliferating in chemically defined medium. Cancer Res., 45: 6379-6383, 1985.
Comments:	This cell line can be grown in serum-free medium. Many serum proteins with alpha phyto protein are released into the medium.
Growth temperature	37 C
Passage method	Wash cells with PBS(-) once and treat cells with 0.1% trypsin and 0.02% EDTA for 5 min. at 37 C.
Cell No. at passage:	2.2×10^5 cells/ml
Passage method	Wash cells with PBS(-) once and treat cells with 0.1% trypsin and 0.02% EDTA for 5 min. at 37 C.

PLC/PRF/5	
Other name:	Alexander
Animal:	human
Sex and Age:	MF 24 year-old
Scientific Name:	Homo sapiens
Tissue:	liver
Medium:	Dulbecco's modified Eagle's medium with 10% heat inactivated fetal bovine serum.
Passage method:	Cells are rinsed once with PBS(-) and treat with 0.2% trypsin and 0.02 % EDTA.
Cell no. at passage:	4 x 10^4 cells/cm^2
Case history:	Malignant liver cancer with HBsAg positive.
Genetics:	Loss of D & G group chromosomes, with marker chr.
Life span:	infinite
Morphology:	epithelial-like
Characteristics:	G6PD=Type A. Tumors developed in hamster(imn.sup).
Classification:	tumor
Established by	Alexander,J.J.
Cell bank maintaining these cells.	OUCI(JCRB)
References:	1. *Alexander,J.J., Bey,E.M., Geddes,E.W., and Lecatsas,G.* Establishment of a continuously growing cell line from primary carcinoma of the liver. Sa Medical Journal, 50: 2124-2128, 1976. 2. *Macnab,G.M., Alexander,J.J., Lecatsas,G., Bey,E.M., and Urbanowicz,J.M.* Hepatitis B surface antigen produced by a human hepatoma cell line. Br. J. Cancer, 34: 509-515, 1976. 3. *Alexander,J.J., Macnab,G., and Saunders,R.* Studies on in vitro production of hepatitis B surface antigen by a human hepatoma cell line. In: M.Pollard (ed.), Hepatitis B surface antigenpp. 103-120, New York: Raven Press. 1978
Comments:	Grow in serum free medium. HBs antigen produced.
Growth temperature	37 C
Passage method	Wash cells with PBS(-) once and treat cells with 0.1% trypsin and 0.005% EDTA for 5 min. at 37 C.
Cell No. at passage:	3.8x10^4 cells/cm^2
Anchorage dependency	Yes

Chang Liver	
Animal:	human
Scientific Name:	Homo sapiens
Tissue:	liver
Medium:	Basal medium Eagle with 10% calf serum.
Passage method:	Cells are treated with 0.02% EDTA and 0.25% trypsin.
Cell no. at passage:	split ratio=1/4 - 1/8
Genetics:	liver, HeLa markers
Established by	Chang,R.S.
Cell bank maintaining these cells.	NIHS(JCRB)
References:	1. Chang, R.S. Continuous subcultivation of epithelial-like cells from normal human tissues. Proc.Soc.Exp.Biol.Med., 87: 440-443, 1954. (Ref ID:420). 2. *Murphy, W.H. and Landau, B.J.* Clonal variation and interaction of cells with viurses. Natl. Cancer Inst. Monogr., 7: 249-271, 1962. (Ref ID:1922). 3. The Cell Culture Collection Committee Animal cell strains. Science, 146: 241-243, 1964. (Ref ID:653). 4. Matsuguchi, T., Okamura, S., Kawasaki, C., and Niho, Y. Production of interleukin 6 from human liver cell lines: Production of interleukin 6 is not concurrent with the production of alpha-fetoprotein. Cancer Res., 50: 7457-7459, 1990. (Ref ID:1847).
Comments:	Prepared from CCL 13.
Growth temperature	37 C
Passage method	Wash cells with PBS(-) once and treat cells with 0.1% trypsin and 0.25% EDTA for 5 min. at 37 C.
Cell No. at passage:	$8.3x10^5$ cells/cm^2
Anchorage dependency	Yes

Sk Hep 1	
ATCC Number:	HTB-52
Organism:	Homo sapiens (human)
Designation:	SK-HEP-1
Tissue:	adenocarcinoma; liver; ascites
Tumorigenic:	yes, in nude mice; forms large cell carcinoma consistent with hepatoma
Morphology:	epithelial
Comments:	The SK-HEP-1 line has been identified as being of endothelial origin.
Age Stage:	52 years
Ethnicity:	Caucasian
Gender:	male
Growth Properties:	adherent
Isoenzymes:	Me-2, 1-2; PGM3, 1; PGM1, 2; ES-D, 1; AK-1, 1; GLO-1, 1; G6PD, B; Phenotype Frequency Product: 0.0020
Karyotype:	(P11) hyperdiploid to hypotriploid (+A3, +C, +E, +F, +G, -A, -D) with abnormalities including dicentrics, acrocentric fragments, secondary constrictions, pulverizations, and large subtelocentric and submetacentric markers
Subculturing:	Remove medium, rinse with fresh 0.25% trypsin solution, remove trypsin and let the culture sit at room temperature (or at 37C) until the cells detach (about 10 minutes). Add fresh medium, aspirate and dispense into new flasks. Subculture every 6 to 8 days.
Split Ratio:	A subcultivation ratio of 1:2 to 1:4 is recommended
Fluid Renewal:	2 to 3 times per week
Freeze Medium:	Culture medium, 95%; DMSO, 5%
References:	RF8862: Turner BM and Turner VS. Secretion of alpha1-antitrypsin by an established human hepatoma cell line and by human/mouse hybrids. Somatic Cell Genet. 6: 1-14, 1980 PubMed: 80169372 RF32326: Fogh, J., ed., Human tumor cells in vitro. New York: Plenum Press; 1975:pp. 115-159 RF32969: Fogh J et al. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J. Natl. Cancer Inst. 58: 209-214, 1977 PubMed: 77097006 RF32972: Goodfellow M et al. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. 59: 221-226, 1977 PubMed: 77210034 RF34768: Fogh J. Cultivation, characterization, and identification of human tumor cells with emphasis on kidney, testis, and bladder tumors. Natl. Cancer Inst. Monogr. : 5-9, 1978 PubMed: 79156922 RF34785: Heffelfinger SC et al. SK HEP-1: a human cell line of endothelial origin. In Vitro Cell. Dev. Biol. 28A: 136-148, 1992 PubMed: 92165675
Propagation:	ATCC medium: Minimum essential medium (Eagle) in Earle's BSS with nonessential amino acids and 1 mM sodium pyruvate, 90%; fetal bovine serum, 10%
BioSafety Level:	1

Required Forms:	The cells are distributed for research purposes only. The Memorial Sloan-Kettering Cancer Center releases the line subject to the following: 1.) The cells or their products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of these cells must first be negotiated with The Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-3620; FAX (212) 639-5764.