IDENTIFICATION GENES INVOLVED IN HEPATOCELLULAR CARCINOMA: EVALUATION OF *hCdc4* and *B-Raf* GENES

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By

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September 2003

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ABSTRACT

IDENTIFICATION OF GENES INVOLVED IN HEPATOCELLULAR CARCINOMA; EVALUATION OF *hCdc4* and *B-Raf* GENES

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M.S. in Molecular Biology and Genetics

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Hepatocellular carcinoma (HCC), the major type of primary liver cancer, is one of the most common cancers worldwide. Development and progression of HCC occur as a multistep process, requiring the activation of oncogenes and the inactivation of several tumor suppressor genes. Although, inactivation of tumor suppressor genes, including p53, Axin and activation of oncogene β-catenin, have been shown to be involved in HCC development, the molecular mechanism of hepatocarcinogenesis is still unclear. The identification of additional genes that are involved in hepatocarcinogenesis is, not only, an important task to understand this process, but also for development of novel strategies for prevention or therapy.

The aim of this study was to elucidate a possible function of hCdc4 and B-Raf genes in HCCs.

A gene or genes on chromosome 4q have been implicated in hepatocarcinogenesis by the observation of frequent deletions of this region in HCCs. More recently, *hCdc4*, a gene in this region has been proposed as a candidate tumor suppressor gene. hCdc4 is an F-box protein which is shown to be involved in ubiquitination of cyclin E, thus targeting it for destruction. Cyclin E overexpression, is reported to be a frequent event in different cancers including HCC suggesting a problem in its destruction. 15 HCC cell lines were analysed for expression and mutation of *hCdc4* gene by RT-PCR and direct sequencing of PCR products. No abnormal transcript and mutation observed in HCC cell lines tested. Our findings suggest that alteration of this gene is not a frequent event in hepatocarcinogenesis.

B-Raf, which is one of the human isoforms of RAF, is activated by oncogenic Ras (leading to cooperative effects in cells responding to growth factor signals). B-Raf mutations are found in a wide range of cancers. Eventhough, mutational activation of Ras is not a frequent event in human hepatocarcinogenesis, few Ras mutations were reported in HCC cases. Thus activation of oncogenic MAP kinase pathway by another component of this pathway such as BRAF is worth to analyze. HCC cell lines and tumours were searched for B-Raf mutations. Activating BRAF missense mutations were identified in 2/72 HCCs (3%). Our results suggest that B-Raf may be occasionally involved in hepatocarcinogenesis. Thus MAP kinase pathway might be involved in hepatocarcinogenesis but neither B-Raf nor Ras are the major players of this pathway in this event. For this reason, other members of this pathway should be evaluated for mutations in HCC.

Keywords: HCC, hCdc4, B-Raf, oncogene, tumor suppressor gene, mutation.

ÖZET

HEPATOSELÜLER KARSİNOMDA ROL ALAN GENLERİN TANIMLANMASI: *hCdc4* VE *B-Raf* GENLERİNİN DEĞERLENDİRİLMESİ

Banu SÜRÜCÜ
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Karaciğer kanserinin başlıca türü olan hepatoselüler karsinom (HCC) dünya çapında yaygın kanserlerden biridir. HCC'nin oluşması ve gelişimi çok basamaklı bir süreç olup onkogenlerin aktivasyonunu ve/veya tümör baskılayıcı genlerin inaktivasyonunu gerektirir. Her ne kadar p53, aksin gibi tümör baskılayıcı genlerin inhibisyonu ve β-katenin gibi onkogenlerin aktivasyonu gösterildiyse de hepatoselüler karsinomun moleküler mekanizması belirgin değildir. Bu nedenle, hepatokarsinogenezde rolü olan genlerin tanımlanması sadece bu sürecin anlaşılmasında değil aynı zamanda koruma veya tedavi için yeni stratejiler geliştirilmesinde de önemli bir basamaktır.

Bu çalışmanın amacı *hCdc4* ve *B-Raf* genlerinin HCC'deki olası rollerini değerlendirmektir.

Kromozom 4q üzerinde sık görülen delesyonlar nedeniyle bu bölgede bulunan gen ya da genlerin HCC'de rol alabileceği düşünülmektedir. Yakın zamanda bu bölgede bulunan bir gen olan *hCdc4* klonlanmış ve olası bir tümör baskılayıcı gen olarak gösterilmiştir. hCdc4 bir F-box proteini olup siklin E'nin ubikütinlenmesinde dolayısıyla da yıkımında rol aldığı gösterilmiştir.Yıkımındaki bir bozukluk dolayısıyla olabilecek siklin E ekspresyon seviyesindeki artış, HCC'nin de içinde bulunduğu çeşitli kanserlerde sık görülen bir olaydır. 15 HCC hücre hattında *hCdc4* geninin ekspresyon ve mutasyon çalışmaları RT-PCR ve bunu takiben PCR ürünlerinin direkt DNA dizi analizi yöntemleri kullanılarak incelenmiştir. Test edilen HCC hücre hatlarında mutasyon veya anormal transkript gözlemlenmemiştir. Bu bulgular *hCdc4* genindeki değişimlerin hepatokarsinogenezde sık rastlanan bir olay olmadığına işaret etmektedir.

RAF proteininin insan isoformlarından biri olan B-RAF onkogenik RAS tarafından aktive edilerek hücrelerin büyüme faktörlerine yanıt vermesinde işlev görür.. B-Raf mutasyonları birçok kanser türünde gösterilmiştir. RAS mutasyonları insan hepatokarsinogenezinde sık görülen bir olay olmasa da HCC örneklerinde az sayıda rapor edilmiştir. Bu nedenle onkogenik MAP kinaz yolağının aktivasyonunun B-RAF gibi başka bir bileşeni tarafından aktive edilmesi olasıdır. *B-Raf* mutasyonları HCC hücre hatları ve tümörlerinde araştırılmıştır ve aktive edici *B-Raf* mutasyonları HCC'lerin %3'ünde (2/72) tanımlanmıştır. Bu bulgular *B-Raf* geninin, dolayısıyla bu yolağın hepatokarsinogenezde rol alabileceğini ancak ne *B-Raf* ne de *ras* geninin hepatokarsinogenezde rol oynayan başlıca genler olmadığını göstermektedir. Bu nedenle MAP kinaz yolağının diğer üyelerinin HCC'deki mutasyonları değerlendirilmelidir.

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ABBREVIATIONS

ARMS	Amplification Refractory Mutation System
CDK2	Cyclin dependent kinase
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridization
CRD	Cysteine-rich domain
ddNTP	Dideoxynucleotidetriphosphate
DNA	Deoxyribonucleic acid
ERK-1/2	Extracellular signal regulated kinase
EtBr	Ethidium bromide
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
LOH	Loss Of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK/ERK Kinase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RBD	Ras Binding Domain
RT-PCR	Reverse Transcriptase-PCR
SCF	Skp-Cullin-F box
TBE	Tris-Borate-EDTA
TSG	Tumour Suppressor Gene
UV	Ultraviolet
WD	Tryptophan Aspartic acid

1. INTRODUCTION

1.1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a malignant tumour derived from hepatocytes. HCC is fifth most common malignant disease worldwide with an estimated 564,000 new cases diagnosed annually and fourth in cancer mortality after lung, stomach and colon cancers (Parkin et al., 2001a; Parkin, 2001b). HCC is the predominant cause of cancer mortality in Southern and Sub-Saharan Africa, Taiwan and China. Its incidence is variable and higher in these regions. This geographic pattern overlaps with the distribution of risk factors and probably also reflects genetic characteristics inherited or acquired through oncogenic agents. (Figure 1.1, Table 1.1)

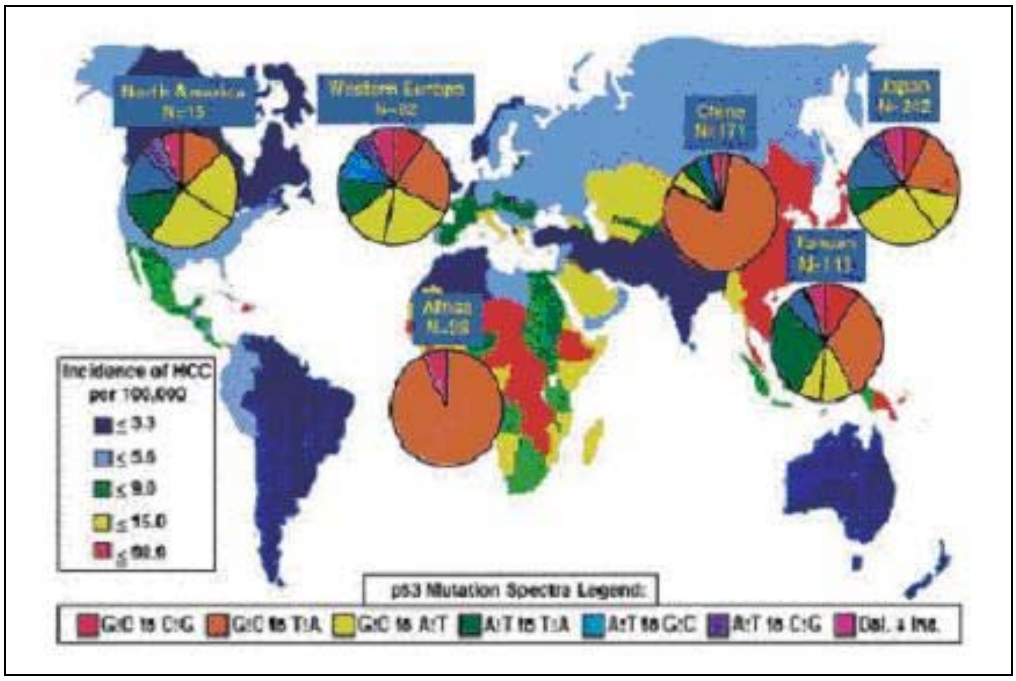


Figure 1.1: Worldwide incidences of HCC. Worldwide incidences of HCC with pie diagrams of p53mutation patterns for various countries and continents according to the IARC p53 mutation database. (www.iarc.fr/p53/index.html). A high frequency of G:C to T:A mutations is found in high incidence areas of both endemic HBV infection and dietary AFB1 exposure. (Staib et al., 2003)

However, several studies have demonstrated a significant increase in the incidence of HCC in many countries in low incidence areas in the last few decades including areas of previously low incidence such as Northern Europe, Northern America and Japan. This increase reflects the strong impact of environmental factors on the pathogenesis of the

tumor. In areas where Hepatitis C Virus (HCV) infection is frequent, particularly in Japan HCC cases are associated with chronic HCV infection. 80% of HCC cases are associated with chronic HCV infection. In contrast, the prevalence of HBV-associated HCC has been rather stable in most parts of the world; therefore the increase of HCC is linked to increased chronic HCV infection. (Okuda K. 2000) (Figure 1.2)

Established and principally suspected risk factors for HCC:
-Associated with chronic liver disease and /or cirrhosis:
Hepatitis B virus
Hepatitis C virus
Alcohol
Cryptogenic cirrhosis
Primary biliary cirrhosis
Autoimmune chronic active hepatitis
Hemochromatosis
Wilson's disease
Glycogen storage disease
Other causes of cirrhosis
-Without necroinflammatory liver disease:
Aflatoxin B1 (p53 codon 249 3rd position G→ T)
Contraceptive and anabolic steroids
Vinyl chloride
Azathioprine
Polychlorinated biphenyls
Arsenic
Other chemical carcinogens

Table 1.1: Risk factors for HCC. (Modified from Ozturk M., 1999)

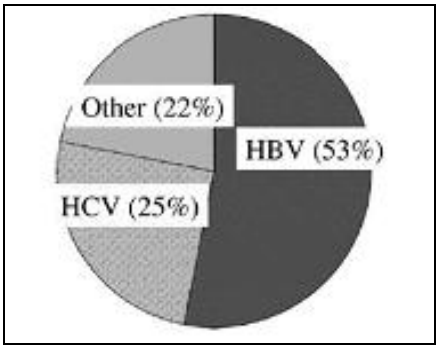


Figure 1.2: Etiology of HCC. The percentage of worldwide HCC associated with HBV, HCV or other causes is shown. Data are from GLOBOCAN 2001 and Parkin *et al*., 2001

1.1.1. HCC Biology

Under normal conditions, adult hepatocytes are non-dividing cells. Only a minor fraction undergoes cell division due to aging or apoptosis. Any type of injury (such as chronic HBV or HCV infection) provokes proliferation of hepatocytes in order to replace defective hepatocytes. (*Regeneration*). Chronic liver regeneration may itself be a source of spontaneous gene mutations as well as other hepatotoxic factors and lead to genomic instability and progression of liver malignancy. (Cetin-Atalay and Ozturk, 2002)

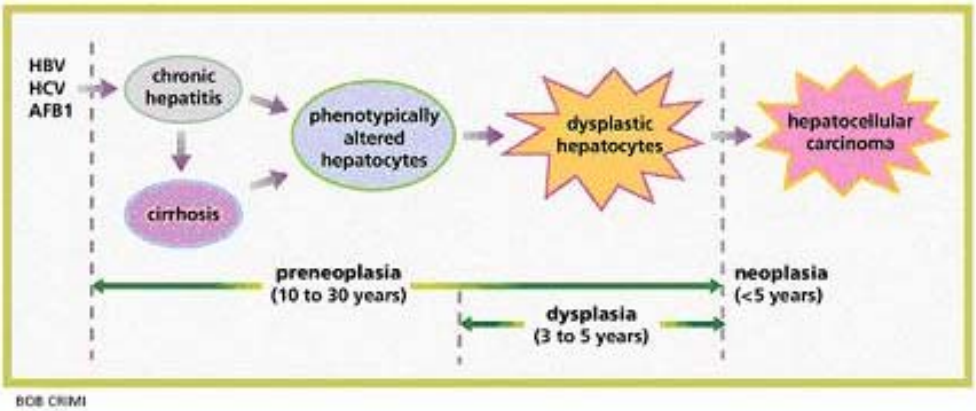


Figure 1.3: Multistage Hepatocarcinogenesis. (HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; AFB1: Aflatoxin B1) (Thorgeirsson and Grisham, 2002)

Hepatocarcinogenesis is a multi-step process, which involves genetic alterations that drive the progressive transformation of normal hepatocytes into highly malignant

derivatives. (Figure 1.3) Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. Six essential alterations in cell physiology that collectively dictate malignant growth are (Hanahan and Weinberg, 2000):

1) Self-sufficiency in growth signals

Instead of being dependent on exogenous growth factors to stimulate growth and cell proliferation, tumour cells can grow and divide without these exogenous signals. This is often caused by mutations in genes that encode components of the signalling pathways that normally regulate these processes. Such mutations lead to constitutively active growth signalling (eg. mutations in Ras).

2) Insensitivity to growth inhibitory (anti-growth) signals

The growth and proliferation of cells is controlled by the balance of growth promoting signals and growth inhibitory (or anti-growth) signals. The loss of growth inhibitory signals or the loss of the ability to respond to these signals is typical of tumour cells (eg. mutations in Smad4 gene in TGF-beta pathway).

3) Evasion of apoptosis

A critical component of normal tissue homeostasis is apoptosis (programmed cell death). This allows the control of cell number and is also a mechanism for the removal of unwanted cells, for example those with significant damage like genetic damage. Tumour cells have often developed mutations in key genes whose products are involved in apoptosis, causing the blockage of cell death (eg. p53 mutations in HCCs). Another mechanism that can lead to the same functional effect is the over-expression of some genes whose normal function is to encode proteins that suppress cell death (eg. bcl-2).

4) Limitless replicative potential

Normal cells have a restricted replicative potential as a consequence of a number of mechanisms. One important mechanism relates to the progressive shortening of chromosome ends (telomeres) at every cell division. Tumour cells have an unrestricted replicative potential, in part, because they overcome the chromosome shortening by the production of an enzyme called telomerase.

5) Sustained angiogenesis

While tumours grow by the above mechanisms at some point they will stop growing because they will outgrow their blood supply, which provides the necessary nutrients and oxygen. Tumours overcome this problem by the production of various factors that stimulate the formation of new blood vessels (eg. VEGF).

6) Tissue invasion and metastasis

Normal cells resides where they belong and tissue boundaries are normally maintained. Tumours are characterised by invasion into nearby tissues and structures (ie. normal boundaries are *not* maintained) and may spread to distant sites (metastasis). These properties are the main reason why tumours are such a significant clinical problem.

As mentioned above these characteristics may be gained by accumulation of mutations in the genes that play crucial roles in cellular homeostasis.

1.1.2. HCC Genetics

HCC may be produced by selection of both genomic and epigenetic alterations that compromise more than one regulatory pathway (Thorgeirsson and Grisham, 2002). It has been shown that HCCs display many genetic alterations including polyploidy, allelic imbalance, amplifications, translocations, inverted duplications, deletions, de novo methylation and loss of heterozygosity (LOH). (Cetin-Atalay and Ozturk, 2002; Buendia, 2000; Puig et al., 2001)

There are more than 20 genes involved in at least 4 carcinogenesis pathways that are shown to be altered in HCC. (Buendia et al., 2000). Most of the genes mutated in HCC are tumour suppressor genes and frequent allelic losses (LOH) leading to biallelic inactivation have been described. (Puig et al., 2001) (Table 1.2)

One of them is p53 TSG (tumour suppressor gene) which is frequently mutated in human cancers and its mutations is found in about 30% of HCCs worldwide. The frequency of all p53 mutations varies between 20% (USA) and 67% (Senegal). A

specific mutation in codon 249 of the p53 gene was found in about 50% of HCC in populations exposed to aflatoxin B1 (Mozambique, Senegal, China) and the presence of this hot spot mutation in patients with HCC from Europe, the USA, Japan and Australia is extremely low. (Bressac et al., 1991; Hsu et al., 1991; Oda et al., 1992; Buetow et al., 1992) Activation of the Wnt/wingless pathway can be caused by a stabilizing mutation of β-catenin gene or by an inactivating mutation of the Axin1 gene (Miyoshi et al., 1998; de La Coste et al., 1998; Legoix et al., 1999; Satoh et al., 2000). Somatic mutations of both β-catenin and Axin1 genes have been observed in 20-25% and 5-10% of HCC cases respectively. Less frequently observed in HCC are the inactivating RB1 and p16 mutations. In contrary, inactivation of p16 by homozygous mutation and methylation are shown to be frequent in HCCs. Overexpression of cyclin D1 and increased RB1 degradation by overexpression of gankyrin lead to the alteration of RB1 pathway, this plays a significant role in HCC development. (Zhang et al., 1994; Higashitsuji et al., 2000). Finally, rare IGF2R, SMAD2, and SMAD4 inactivating gene mutations can alter the transforming growth factor β pathway (De Souza et al., 1995; Kawate et al., 1999; Yakicier et al., 1999).

Some genes mutated in Hepatocellular Carcinoma includes:

Frequently mutated genes	
P53	(10-50 %)
β-catenin	(13-89 %)
Less frequently mutated genes:	
M6P/ IGF2R	(0-33 %)
SMAD2	(0-2 %)
SMAD4	(0-6 %)
Rb1	(~ 15%)
Axin	(~ 5%)
Cmet	(0-30 %)
BRCA2	(~5 %)
PTEN/MMAC1	(0-5 %)

Table1.2: Some genes involved in HCC (modified from Ozturk M., 1999)

LOH is the most common genetic aberration observed in HCCs. Some chromosomes display frequent losses (more than 20%); which implies that either the development or persistence of these changes is not random. It has been suggested that each frequently effected region may contain one or more genes whose loss or altered function contributes to the development and/or progression of HCC. (Figure 1.4)

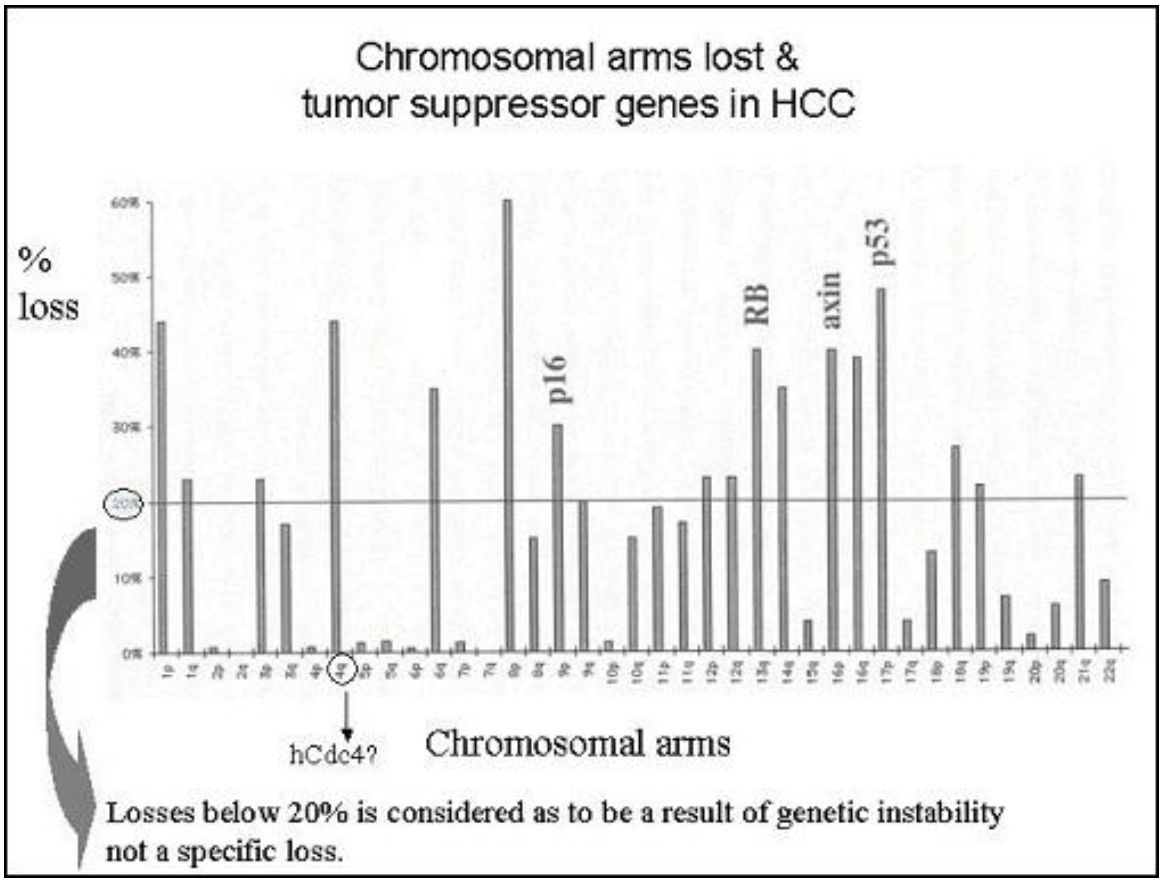


Figure 1.4: Frequency of LOH (Loss of Heterozygosity) **vs. Chromosomal arms lost in HCC**

Although implication of several genes (Table 1.2) have been shown in HCCs, as indicated in figure 1.4 there are still chromosomes that contain unidentified TSGs, In addition, for several chromosomes number of observed mutations or methylations do not correspond to that of LOH. For instance the LOH of several markers on 17p13.3 occurred at a higher frequency than that of *p53* locus, which is located on 17p13.1.

These findings suggest the existence of one or more genes involved in HCC on 17p13 in addition to *p53* gene. (Zhao et al., 2001)

In addition to LOH studies CGH (Comparative Genomic Hybridization) and microarray studies also reveal that there are still more locus involved in HCCs. In contrast to allelic losses and TSGs, some regions (1p, 6p, 8q, 17q) or genes are consistently amplified or over expressed. (Figure 1.5)

Previously, the amplification and/or overexpression of cyclin D1 and c-Myc have been shown in advanced human HCCs. In addition to the human HCC data, in woodchucks, about 50% of hepatocellular carcinomas arising in animals infected chronically with WHV (woodchuck hepatitis virus). Viral DNA sequences were integrated in or adjacent to c-*myc* or N-*myc* genes. On the other hand in a transgenic mice model, overexpression of *cyclin D1* is sufficient to initiate hepatocellular carcinogenesis. (Nishida et al., 1994; Deane et al., 2001)

All these studies with CGH and microarray data suggest that oncogenic gene activation in addition to TSGs inactivation is important in hepatocarcinogenesis. Thus the β-catenin is not the unique oncogene that is involved in human HCC development.

Altogether, epidemiological, genetic, and biological data suggest there are still genes that participate hepatocarcinogenesis to be identified.

The aim of our study is to identify genes that are involved in HCC development. To achieve this aim we searched the literature for possible candidates that may have role in hepatocarcinogenesis.

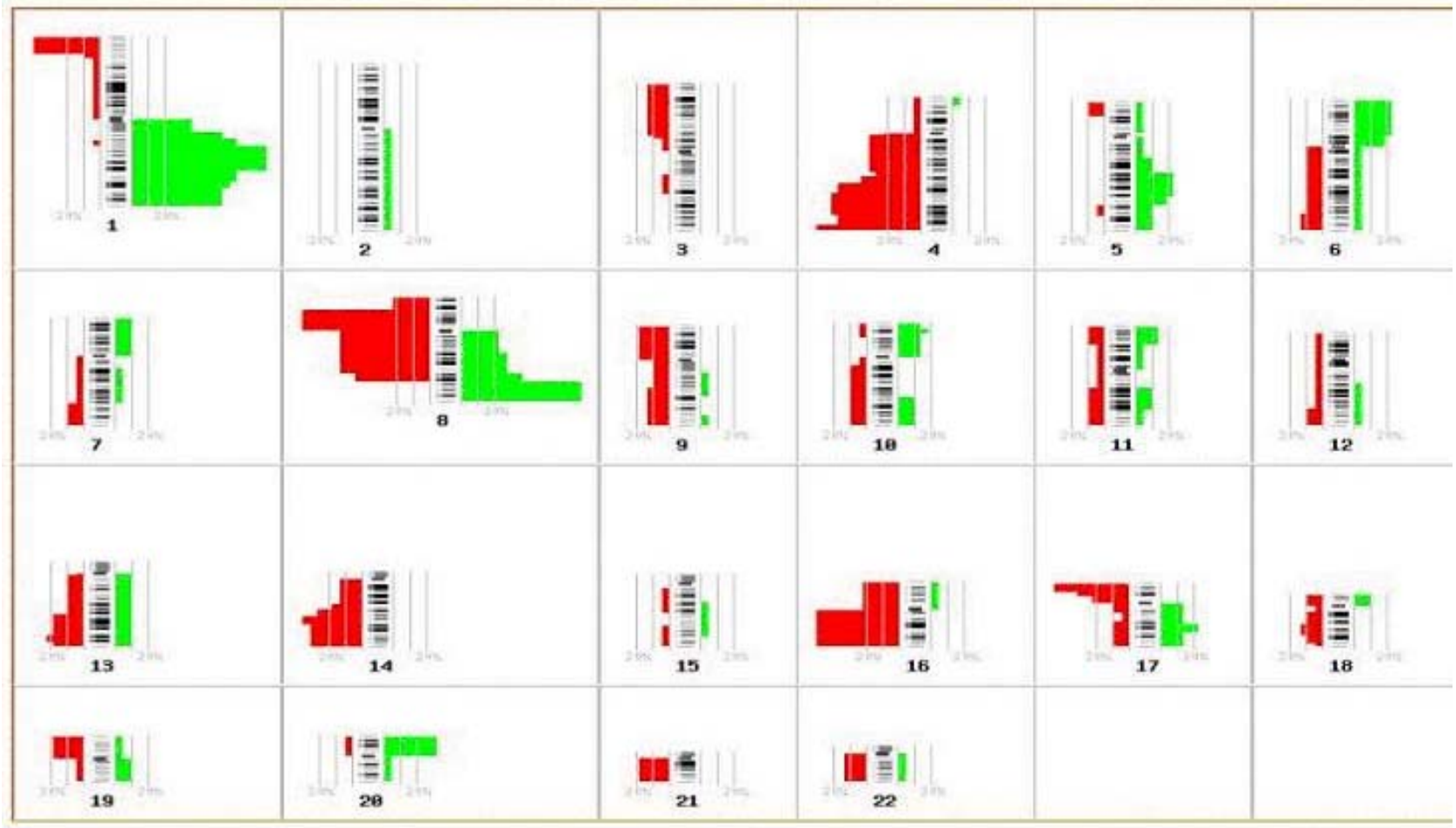


Figure 1.5: Summary of gains and losses detected by CGH. Vertical lines on the left of each chromosome ideogram represent loss of chromosomes, whereas vertical lines on the right correspond to chromosomal gains. Available in http://www.progenetix.de/12048165_P/ideogram.html
(Chang et al., 2002)

1.2. hCdc4 /Archipelago / Fbwx7 / SEL10:

Cyclin dependent kinase 2 (cdk2) activated by Cyclin E is involved in the initiation of DNA replication and other S phase events. In parallel with this role, cyclin E protein is accumulated at the G1→ S phase transition and decreases during early S phase. This increase and decrease is a result of periodic transcription and ubiquitin-mediated proteolysis in control of SCF^{hcdc4}. SCF ubiquitin ligases are composed of a core complex linked to a variable component, which is called F-box protein that provides substrate specificity. hCdc4 (also designated as Fbwx7 or Ago) is identified as the F-box protein that is involved in the ubiquitin mediated degradation of Cyclin E. Defects in proteolysis of Cyclin E can lead to accelerated entry to S phase leading to genetic instability that may result in tumourigenesis. Indeed, many kinds of HCC tumours show elevated levels of cyclin E protein but not mRNA. (Strohmaier et al. 2001, Moberg et al. 2001, Koepp et al., 2001). It has been found that hCdc4 is mutated in human endometrial tumors (16%), ovarian and breast carcinoma cell lines.

Moberg et al. used Drosophila as model organism to identify the loci that are involved in cellular proliferation. By screening the Drosophila mutants, they identified over 23 loci one of which it was termed as archipelago (ago). Within ago mutant clones, the ommatidial clusters of the retina contained extra cells that seemed to be resulted from extra cell divisions. Therefore, ago-mutant cells were examined for increased level of positive cell cycle regulators and it was found that Cyclin E protein, but no other cyclins or cyclin E mRNA level, is increased. Archipelago protein contained seven WD repeats and an F-box, which is a common signature in SCF complexes that have role in selection of proteins for ubiquitination. Moberg et al. showed that Archipelago could bind to Cyclin E, which may indicate the ability of Archipelago to target Cyclin E for ubiquitination.

Two other groups using yeast as model organism started from the assumption that SCF complex may be involved in turnover of Cyclin E. It was found that levels of Cyclin E are stabilized in SCF mutant strains of yeast compared to wild type. And a protein that

was termed as Fbwx7 (Koepp et al., 2001) or Cdc4 (Strohmaier et al., 2001) was identified. Overexpression of Fbwx7 was shown to decrease the levels of Cyclin E while its inhibition resulted in Cyclin E accumulation (Koepp et al., 2001). Human homologue of yeast Cdc4 (hCdc4) was identified and it was confirmed that this protein is a part of SCF complex and it can ubiquitinate Cyclin E in a phosphorylation dependent manner (Strohmaier et al., 2001). Later, it was shown that this protein is mutated in breast and ovarian carcinoma cell lines. (Schwab and Tyers, 2001; Mitchell A., 2001)

The levels of Cyclin E protein in HCC cell lines that we used in our study are shown in Table 1.3:

	Cell lines	Cyclin E protein level
1	Huh 7	*
2	BTR	* *
3	Focus	* * * *
4	MV	* *
5	Hep 40	* * * *
6	Hep 3B	* * * *
7	Hep G2	* * * * *
8	PLC	* * *
9	SK Hep1	* * *
10	Snu 475	* * * * *
11	Snu 449	* * * * *
12	Snu 423	* * * * *
13	Snu 398	* * * *
14	Snu 387	*
15	Snu 182	* *

*	very weak band
* *	weak band
* * *	band
* * * *	strong band
* * * * *	very strong band

Table 1.3: Cyclin E protein levels in HCC cell lines.

1.3. *B-Raf*

Raf genes were first identified as oncogenes in retroviruses that were causative agents of tumours in mice and chickens (Rapp et al., 1983; Jansen et al., 1984; Sutrave et al., 1984). Homologues of these genes are found in higher eukaryotes including *C. elegans*, *D. melanogaster*, *X. laevis*, chicken, mouse, rat and humans. In mammals, three related genes have been identified, namely A-RAF, B-RAF and RAF1 (also known as C-RAF or C-RAF1). The RAF-1 protein is expressed at high levels in most cell types and tissues. By contrast B-RAF has a highly restricted pattern of expression, mainly expressed in neuronal tissue, testis and spleen (Storm et al., 1990; Luckett et al., 2000; Barnier et al., 1995). However, studies to identify activated versions of *raf* genes in human cancers showed no consistent correlation. Instead, the motivation for studying Raf proteins with regard to human cancer was the discovery that they are key effectors of Ras proteins (Sutrave et al., 1984), oncogenic mutations of which are detected in a large percentage of human cancers (Bos et al., 1984). Raf proteins play a major role in the conserved Ras/Raf/MEK/ERK pathway, by acting to relay signals from activated Ras proteins via MAPK/ERK kinase 1/2 (MEK1/2) to the p42/p44 MAP kinases or ERK 1/2, the key effectors of this pathway (Marshall et al., 1994; Marais et al., 1996). Raf proteins are activated in a Ras dependent manner. Once activated, they are able to phosphorylate and activate MEK1/2, which in turn phosphorylates and activates ERK1/2. ERKs have many substrates located in different places in the cell including transcription factors, ribosomal proteins, enzymes and cytoskeletal proteins. B-RAF activity in cancer samples has not been studied in detail so far. However, a recent report has now demonstrated the presence of activating mutations of the *B-Raf* gene in 70% of malignant melanomas and 15% of human colon cancers (Davies et al., 2002). While the *B-Raf* mutations themselves may not be the actual case of cancers, the high correlation in malignant melanomas suggests that the acquisition of *B-Raf* mutations are a likely prerequisite for development of these tumours.

Studies on mice with targeted genetic mutations of the *raf* genes have shown that Raf-1 is dispensable for MEK/ERK activation and that B-RAF appears to be the key MEK/ERK

activator in most tissues and cell types (Wojnowski et al., 2000; Huser et al., 2001; Mikula et al., 2001). In addition, studies on the mechanism of regulation of B-RAF activation have shown that key phosphorylation events take place in B-RAF result to have a higher activity toward MEK than Raf-1 (Mason et al., 1999; Zhang et al., 2000).

It seems that B-RAF isotype has different properties that make it stand out from its counterparts, RAF-1 and A-RAF. These properties can explain why this protein is involved in tumour development rather than the other two RAFs.

1.3.1. *B-Raf* mutations in human cancer:

Missense mutations of the *B-Raf* gene in approximately 70% of human malignant melanomas and 15% of colorectal cancers were detected by sequencing of 923 cancer samples by Davies et al. (Davies et al., 2002). Mutations were also detected at a low frequency in gliomas, lung cancers, sarcomas, ovarian carcinomas, breast cancers. 89 % of the *B-Raf* mutations result in amino acid changes within the activation segment, with the V599E (T→A change at nucleotide 1796) mutation.

Eleven percent of the mutations are found in the glycine residues of the G loop GXGXXG motif in the ATP-binding domain of the kinase domain affecting G463, G465 and G468 (Mercer and Pritchard, 2003).

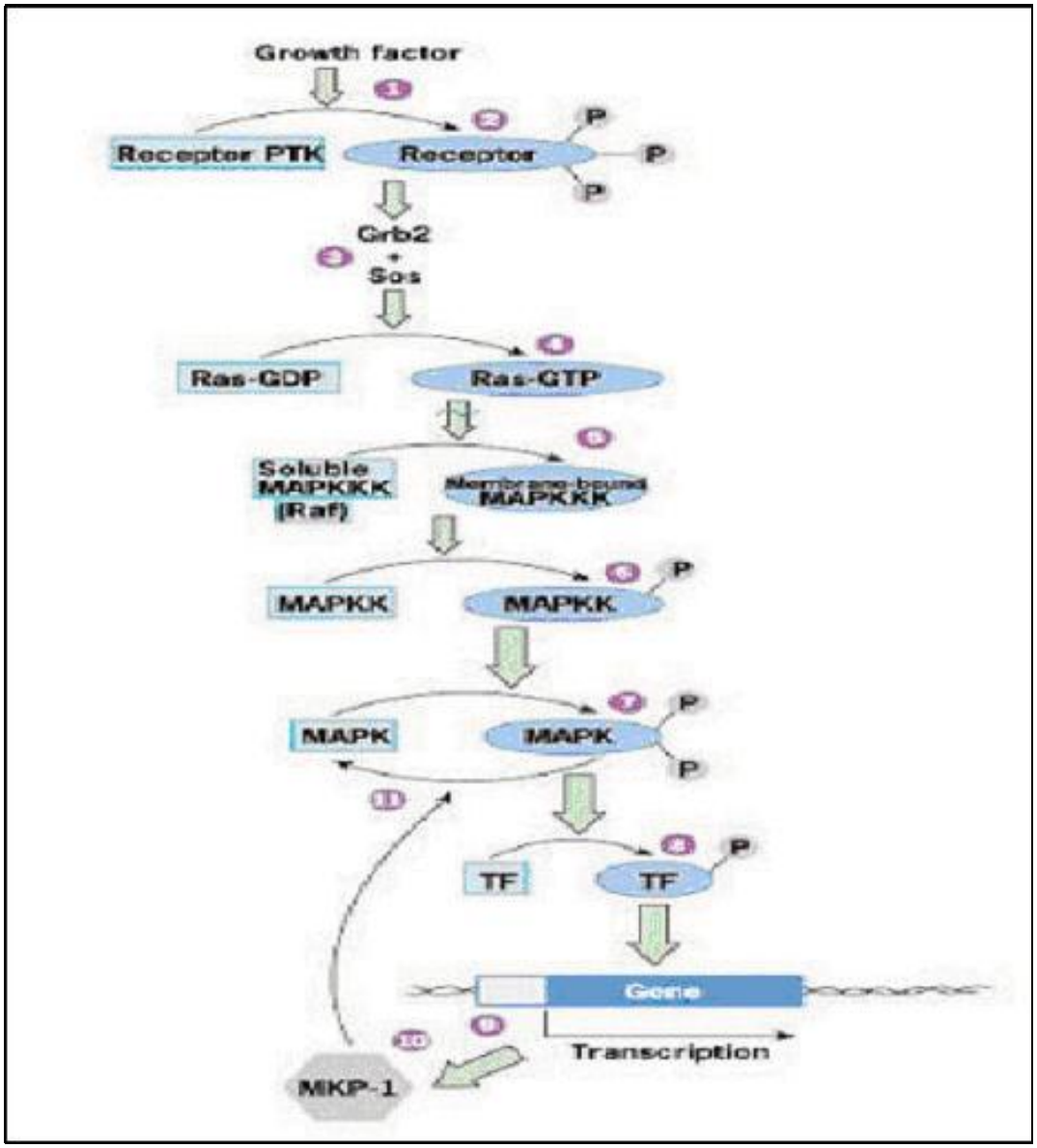


Figure 1.6. MAPK pathway, Karp 2002

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Enzymes

All enzymes or reagents were purchased from the sources below:

Material	Manufacturer
Taq polymerase	MBI Fermentas
dNTPs	MBI Fermentas
Gene ruler DNA ladder mix	MBI Fermentas
Mass Ruler DNA ladder mix	MBI Fermentas
QIAquick Nucleotide Removal Kit	Qiagen
QIAEX II Gel Extraction Kit	Qiagen
Revert Aid First Strand cDNA Synthesis Kit	MBI Fermentas
QIAamp RNA Blood Mini Kit	Qiagen
NucleoSpin C + T	

2.1.2 Equipment and Manufacturers:

Equipment:	Manufacturer
Micropipettes	
P10	Gilson
P20	Eppendorf
P200	Eppendorf
P1000	Eppendorf
GeneAmp PCR System 96000 and 24000	Perkin Elmer
Gel Tanks	
Horizontal Minicell	E-C Apparatus
Horizontal Midicell	E-C Apparatus
Power Supply PAC120	BioRad
UV Transilluminator	BioRad Gel Doc 2000
ABI Prism 377 Automated DNA Sequencer	Perkin Elmer

2.1.3 Plastic Disposables

The list of plastic disposables used and their manufacturers are listed below:

Disposables	Manufacturer
Microcentrifuge Tubes	Costar
0.2 ml	
1.5 ml	
2.0 ml	
Test Tubes	Costar
15 ml	
50 ml	
Micropipette Tips	Costar
Micropipette Filter Tips	Greiner

2.1.4 Solutions

Solutions for Agarose Gel Electrophoresis	
EDTA	0.5 M (stock solution), pH 8.0
1X TBE	45 mM Tris-Borate, 1mM EDTA
1X Loading Buffer	0.25 % bromophenol blue, 0.25 % xylene cyanol, 50 % glycerol, 1 mM EDTA
EtBr	10 mg/ml in water (stock solution)

2.2. METHODS

2.2.1 Cell lines

15 HCC cell lines used in the experiments were kindly provided by Mehmet Ozturk's Group.

	Cell Lines	Mutational Status
1)	Huh 7	P53 mutation
2)	Hep3B-TR	
3)	Focus	P53 mutation
4)	Mahlavu	P53 mutation
5)	Hep 40	
6)	Hep 3B	P53 mutation
7)	Hep G2	B-catenin, Ras mutation
8)	PLC/PRF/5	P53 mutation
9)	SK-Hep1	*B-Raf* mutation
10)	SNU 475	P53 mutation
11)	SNU449	P53 mutation
12)	SNU423	
13)	SNU398	B-catenin, p53 mutation
14)	SNU387	P53 mutation
15)	SNU182	P53 mutation

Table 2.1 :Cell lines used and their mutational status.

2.2.2 Primers

All oligonucleotides were designed by Primer3 program (PCR primer selection tool at Whitehead Institute for Biomedical Research, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and were obtained from IONTEK (Istanbul, Turkey).

hCdc4		PRIMER SEQUENCE		Length
hCdc4-1F		5'-TCTGATAGTCTAGCCAAGGTCCA-3'		23bs
hCdc4-1R		5'-TCCATCTCCTCCTCCTCCTCA-3'		21bs
hCdc4-2F		5'-AGGAAGATGAAGAACATGCTGGT-3'		23bs
hCdc4-2R		5'-TGTAGGTGGCTGGACAGATG-3'		20bs
hCdc4-3F		5'-TGTTCAGCAACACCAACAACT-3'		21bs
hCdc4-3R		5'-TGGTTCATCAATCCCCTCTT-3'		20bs
hCdc4-4F		5'-TCAGACATGTCGCTACTGGAGAA-3'		23bs
hCdc4-4R		5'-CCAGTCTCTGCATTCCACACT-3'		21bs
hCdc4-5F		5'-TGGATCTACAGATCGGACACT-3'		21bs
hCdc4-5R		5'-CTGTCTCCACATCCCAAACA-3'		20bs
hCdc4-6F		5'-TGATGGTATCCATGTGGTGAGT-3'		22bs
hCdc4-6R		5'-CCCGTTTTCAAGTCCCATAG-3'		20bs
hCdc4-7F		5'-ACCAGCTCAGATGATGGAACT-3'		21bs
hCdc4-7R		5'-AAGGGCAGGGAGTATATCGT-3'		20bs

B-Raf		Primer Sequence		Length
BrafG-F		5'-AACACATTTCAAGCCCCAAA-3'		20bs
BrafG-R		5'-GCATCTCAGGGCCAAAAAT-3'		19bs
BAGN-F		5'-AGGTGATTTTGGTCTAGCTACAGT-3'		24bs
BAGM-F		5'-AGGTGATTTTGGTCTAGCTACAGA-3'		24bs
BAG-R		5'-TGTGGATCACACCTGCCTTA-3'		20bs
Bex11-F		5'-CTTTTGGAGGAGTCCTGAAACT-3'		22bs
Bex11-R		5'-ATCCCTCTCAGGCATAAGGT-3'		20bs

Table 2.2 : Sequences of all primers used.

2.2.2.1 Primers for *hCdc4*:

For *hCdc4* gene 7 sets of overlapping primers were designed for PCR using cDNA as template. 1F-3R and 4F-7R primer pairs were used for PCR. 15 HCC cell lines were sequenced using primers below (Figure 2.1).

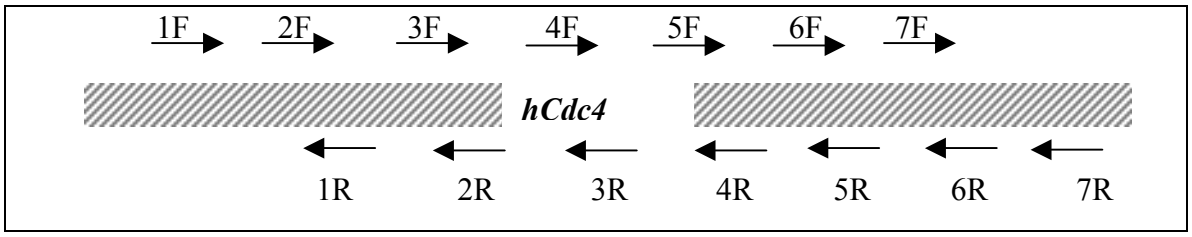


Figure 2.1: Positions of *hCdc4* specific primers

2.2.2.2 Primers for B-Raf

BrafG-F and BrafG-R primers were used for amplification and sequencing of exon 15 of the *B-Raf* gene.

Bex11-F and Bex11-R primers were used for amplification and sequencing of exon 11 of *B-Raf* gene.

For the ARMS test BAG-M and BAG-R genomic primers were used for the mutated allele of the *B-Raf* gene and BAG-N and BAG-R primers were used for the normal allele.

2.2.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of specific desired sequences of DNA. (Mullis and Faloona et al., 1987)
There are three distinct events during a PCR cycle:

1) Denaturation of the template: DNA denaturation occurs when the reaction is heated to 92-96 ºC.
2) Primer annealing: After denaturation the oligonucleotide primers hybridize to their complementary single stranded target sequences. The temperature of this

step varies depending on the homology of the primers for the target sequence as well as the base composition of the oligonucleotides.

3) DNA synthesis by the thermostable Polymerase: The last step is the extension of the oligonucleotide primer by the Polymerase. This step of the reaction is carried out at 72 ºC. The larger the template, the longer is the time required for a proper time for the extension. Also, the time for a proper extension time may be increased if the GC content of the template is high.

2.2.3.1 PCR conditions

PCR conditions	***hCdc4*** (4F-7R 1264bp) (1F-3R 1047bp)
First denaturation	94 ˚C 5'
Denaturation	94 ˚C 30"
Annealing	58 ˚C 30"
Extension	72 ˚C 1' 35"
Last Extension	72 ˚C 5'
Total Cycles	35 cycles

Table 2.3: PCR conditions for *hCdc4*.

PCR conditions	***B-Raf*** exon 15 (399bp)	***B-Raf*** ARMS (532bp)	***B-Raf*** exon 11 (357bp)
First denaturation	94 ˚C 5'	94 ˚C 4'	94 ˚C 5'
Denaturation	94 ˚C 30"	94 ˚C 30"	94 ˚C 30"
Annealing	60 ˚C 30"	68.5 ˚C 30"	58 ˚C 30"
Extension	72 ˚C 30"	72 ˚C 40"	72 ˚C 30"
Last Extension	72 ˚C 5'	72 ˚C 5'	72 ˚C 5'
Total Cycles	35 cycles	35 cycles	35 cycles

Table 2.4: PCR conditions for *hCdc4* and *B-Raf.*

2.2.3.2 PCR mix

PCR mix: (50µL final volume)
1X PCR buffer (MBI Fermentas)
$MgCl_2$ 2 mM (MBI Fermentas)
dNTP 0.2 mM (MBI Fermentas)
20 pmol of each primer
2 units Taq Polymerase (MBI Fermentas)
1-2 ng of genomic DNA or 1 µL cDNA

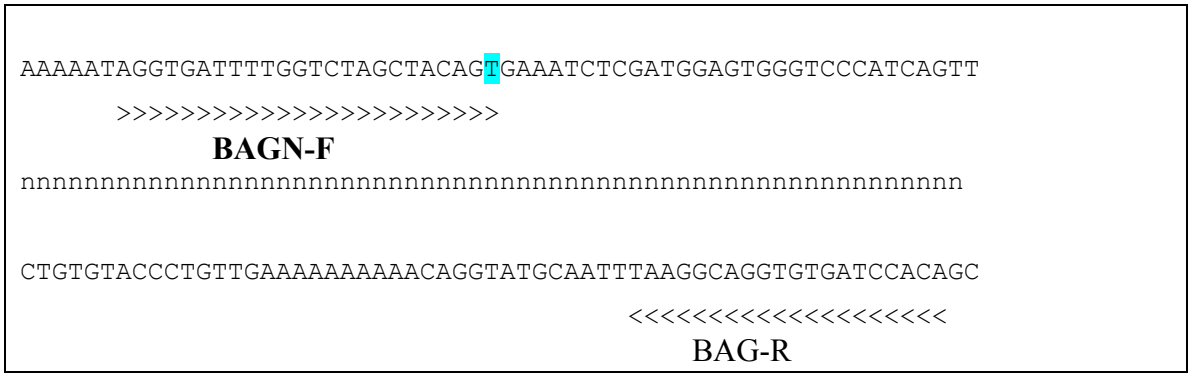
Table 2.5: PCR Mix

2.2.4 ARMS (Amplification Refractory Mutation System)

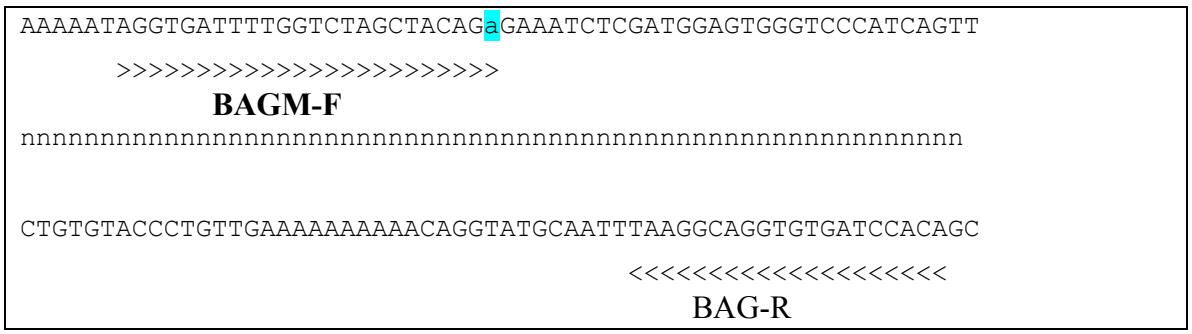
ARMS test was performed in order to detect T1796A transversion resulting in V599E aminoacid missense mutation in exon 15 of the *B-Raf* gene. ARMS is also known as allele-specific PCR (ASPCR) or amplification of specific alleles (PASA). It was first described by Newton et al, 1989. ARMS may be used to detect point mutations and small deletions/insertions. ARMS can generally detect a single copy of the mutant allele in 40 copies of normal allele. This system also leads to fast, reproducible and inexpensive results.

The ARMS primers were designed so that the nucleotide distinguishing between the different alleles was at the 3' terminal base of the primers (when it is specific to the template it will amplify and when it differs it will not). ARMS PCR consisted of two complementary reactions (two tubes) and utilizing 3 primers. One primer is constant and complementary to the template in both reactions (BAG-R), the other primers (BAGM-F and BAGN-F) differed at their 3' terminal residues and are specific to either wild type DNA sequence (BAGN-F) or the mutated sequence at a given base. Only one of these allele specific primers was used per tube. If the sample is homozygous wild type amplification only occurred in one of the tubes, if the sample is heterozygous amplification was seen in both of the tubes. The cell lines and HCC tumour samples that are found to be (+) for the mutation were confirmed by sequencing. The tumour samples were kindly provided from Mehmet Ozturk's group. SK-Hep1 cell line that was found to have the mutation (Davies et al., 2002) was used as (+) control.

2.2.4.1 ARMS primers for normal allele



2.2.4.2 ARMS primers for the mutated allele



2.2.5 DNA Isolation from cell pellets

DNA isolation from cell lines was performed using NucleoSpin C + T kit (Macherey-Nagel) according to manufacturer's manual as follows:

~ 25 mg of pellet was placed in 1.5 ml centrifuge tube and 180 µl of buffer T1 was added. 25 µl of proteinase K solution was added and mixed then incubated at 56ºC in shaking water-bath until complete lysis occurs. 200 µl buffer B3 was added to the sample, mixed and incubated at 70 ºC for 10 minutes. 210 µl ethanol was added and vortexed immediately. Then, the sample was applied to Nucleospin cup that was placed in a 2 ml collection tube and centrifuged for 1 minute at 6000 rpm. The filtrate was discarded the spin cup was washed with 500 µl buffer B5 by centrifugation for 2 minutes at 6000 rpm. and flowthrough was discarded. The washing steps were repeated twice. Then the Nucleospin cup placed in the empty collection tube was centrifuged for 2 minutes at 6000 rpm in order to remove buffer B5, which contains ethanol, completely. The Nucleospin cup was placed in a clean 1.5 ml centrifuge tube and DNA was eluted with 100 µl preheated (70ºC) elution buffer BE.

2.2.6 RNA Isolation from cell pellets

RNA isolation from cell lines was performed using QIAamp RNA Blood Mini Kit (Qiagen, catalogue # 52304) according to manufacturer's manual as follows.

The cell pellet was loosened by flicking the tube and 600 µl buffer RLT (Lysis buffer) was added and pipetted to mix until no cell clumps were visible. The lysate was put into the Qiashredder spin column that was placed in a 2ml collection tube and

centrifuged for 2 minutes at 13000 rpm to homogenize. The column was discarded and 600 µl 70 % ethanol was added to the homogenized lysate then mixed well by pipetting. The sample was pipetted into a QiaAmp spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 10000 rpm. The spin column was transferred into a new collection tube and 700 µl Buffer RW was added into the spin column then centrifuged for 15 seconds at 10000 rpm to wash. Flowthrough was discarded and the column was placed into a new collection tube. 500 µl Buffer RPE was added into the column and centrifuged for 15 seconds at 10000 rpm to wash. 500 µl Buffer RPE was added into the column and centrifuged for 3 minutes at 13000 rpm. The flowthrough was discarded and the column is placed into new collection tube then centrifuged for 1 minute at 13000 rpm to eliminate any chance of possible buffer RPE carryover. To elute, the spin column was placed into a 1.5 ml collection tube and 50 µl of RNase-free water was added directly onto the membrane in the column then centrifuged for 1 minute at 10000 rpm.

2.2.7 Determination of RNA concentration by Spectrophotometry

2 µl of each sample was diluted in 1:500 ratio with sterile ddH_2O. Then OD measurement was done at 260 and 280 nm with the spectrophotometer (Beckman).

Concentration of RNA was calculated according to the formula:

$$[RNA] = OD_{260} \times \text{dilution factor (500)} \times 40$$

OD_{260}/ OD_{280} ratios were calculated in order to check whether there was any protein or DNA contamination in the RNA samples. The expected ratio was between 1.6-2.0.

2.2.8 cDNA synthesis

cDNA synthesis was performed using RevertAid First Strand cDNA synthesis Kit (MBI Fermentas, #K1622) according to manufacturer's manual as follows.

5µg of RNA samples were mixed with 1µl of random hexamer primer and ddH_2O in a total volume of 12 µl. After incubation at 70 ºC for 5 minutes, they are directly chilled on ice. After quick spin, 4µl of 5X reaction buffer, 1 µl of Ribonuclease inhibitor and 2µl of 10mM dNTP mix were added and incubated at

25 ºC for 5 minutes. Afterwards, 1 µl of M-MuLV reverse transcriptase was added and incubated first at 25ºC for 10 minutes, then 42 ºC for 60 minutes and finally 70ºC for 10 minutes. Then they were chilled on ice.

2.2.9 Agarose Gel Electrophoresis of DNA fragments

DNA fragments were separated by gel electrophoresis using agarose at concentrations of 1.5 % w/v in 1X TBE. 0.25-0.50 µg of EtBr/ ml of the gel was used to visualize the DNA. DNA samples were loaded into the wells with 1X loading buffer. Electrophoresis was performed by running the gel for about 1 hour at 95 V. PCR products were visualized by UV illuminometer. The length of the DNA fragments was determined by comparing the fragments with molecular weight marker Gene Ruler DNA Ladder mix (MBI Fermentas, Catalogue # SMO333) that had been loaded and run on the same gel.

2.2.10 Gel Extraction of PCR Products

DNA purification of PCR products from agarose gel was performed using QIAEX II Gel Extraction Kit (Qiagen, Catalogue # 20021) according to manufacturer's manual after running the PCR products on agarose gel and cutting the band from the agarose gel.

1. The DNA bands were excised from the agarose gel with a clean, sharp scalpel by trying to take as little agarose as possible and transferred to a clear microfuge tube.
2. The gel slices were weighed and 3 volumes of Buffer QX1 were added to 1 volume of gel for DNA fragments 100bp-4kb.
3. QIAEX II was resuspended by vortexing for 30 seconds and 20µl QIAEX II was added to the sample.
4. To solubilize the agarose and bind the DNA, the samples were incubated at 50°C for 10 minutes. By vortexing every 2 minutes, QIAEX II was kept in suspension.
5. The sample was centrifuged for 30 seconds and supernatant was carefully removed with a pipette tip.

6. To wash the pellet, 500µl of Buffer QX1 was added, to remove residual agarose contaminants. The pellet was resuspended by vortexing then centrifuged for 30sec and all traces of supernatant were removed.
7. The pellet was washed twice with 500µl of Buffer PE (to remove residual salt contaminants). The pellet was resuspended by vortexing then centrifuged for 30sec and all traces of supernatant were removed.
8. The pellet was air-dried for 10-15 minutes or until the pellet becomes white. Over-drying should be avoided, as this may result in decreased elution efficiency.
9. To elute DNA, 20µl of Buffer EB of Qiagen Nucleotide Removal Kit was added and the pellet was resuspended by vortexing then incubated at RT, 5 minutes.
10. After incubation, the samples were centrifuged for 30 seconds then the supernatant containing the DNA was pipetted into a clean tube avoiding to take the Qiaex beads since the beads interferes with enzymatic reactions.
11.

2.2.11 Purification of PCR Products

The amplified DNA fragments were purified with Nucleotide Removal Kit (Qiagen, Catalogue # 28304) to remove excess dNTPs, $MgCl_2$ etc. according to manufacturer's manual.

10 volumes of buffer PN (binding buffer) to 1 volume of PCR product were added and mixed by pipetting. The samples were loaded into the spin column placed in the 2 ml collection tube. To bind DNA, the samples were centrifuged for 1 minute at 6000 rpm. The flow-throughs were discarded and the columns were placed back into the same tube. To wash the columns, 750 µl of buffer PE (wash buffer) were added and centrifuged for 1 minute at 6000 rpm. The flow-throughs were discarded and additional 250 µl of buffer PE is added then centrifuged for 1 minute at 6000 rpm. The flow-throughs were discarded and the columns were placed back into the same tube and the empty column were centrifuged for 1 minute at 13000 rpm to remove all residual ethanol which was present in buffer PE. The columns were placed in a clean 1.5 ml tube and 50 µl of Buffer EB (elution buffer) were added into the center of the membrane in the column, let the column stand for 1 minute then centrifuged for 1 min at 13000 rpm.

After purification, concentrations of the PCR products were determined by comparing with Mass Ruler DNA Ladder Mix (MBI Fermentas, Catalogue #SMO403) utilizing agarose gel electrophoresis.

2.2.12 Automated DNA Sequencing

PCR products were sequenced with appropriate primer pairs by ABI Prism 377 DNA Sequencer at Bilkent University, Department of Molecular Biology and Genetics by Tulay Arayici and Yeliz Yuva. For sequencing reactions, DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Catalogue # US81050) was used. Cycle Sequencing utilizes thermostable DNA polymerase and dideoxyncleotides (ddNTPs) are used to create a nested set of DNA fragments with ddNTPs at the 3' end of each fragment. Since each of the four ddNTPs is labeled with a different color of fluorescent dye, the reaction can be performed in a single tube and the sequence can be determined easily. Electropherograms of each sample were analyzed and the ambiguities in the sequence data were solved by comparing the results of the both forward and reverse primers as well as by comparing with sequences in the NCBI database.

3. RESULTS

3.1. *hCdc4*

To screen for alterations in *hCdc4* gene, as it contains 13 exons spanning approximately 210 kb of the genome, RT-PCR was performed, followed by direct sequencing on 15 RNAs extracted from HCC cell lines. Two overlapping fragments covering *hCdc4* were amplified using 1F-3R and 4F-7R primer pairs. (Figure 3.1)

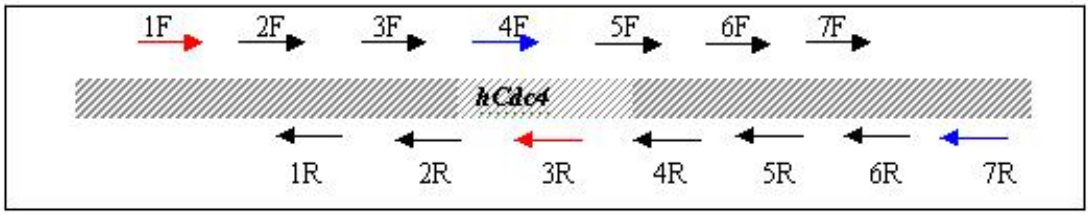


Figure 3.1: Positions of *hCdc4* specific primers. Primers for PCR amplification: 1F-3R and 4F-7R, Primers for sequencing: 1F to 7R and 1R to 7R.

Analysis of PCR products on agarose gel revealed the expected length of bands for 1F3R and 4F7R, 1047 bp and 1264 bp respectively. (Figure 3.2, Figure 3.3) No smaller and/or extra bands were observed, this suggest that there are no deletions or insertions that can be observed by conventional PCR and gel electrophoresis. Although not quantitative, it can be concluded that there is expression of hCdc4 in all of the cell lines tested since PCR was performed using equal amount cDNA synthesized from equal amount of RNA as template. There was no significant difference in the expression level of both products after 35 cycle of PCR (nested PCR was not performed) under the same PCR conditions between the 15 cell lines tested.

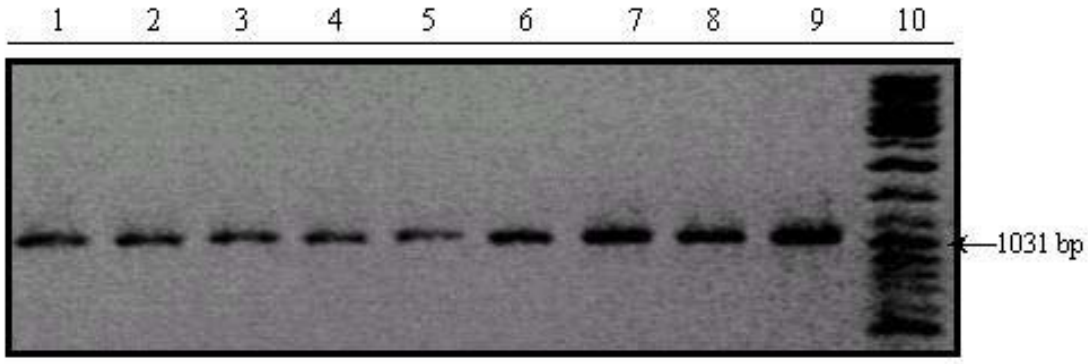


Figure 3.2: RT-PCR analysis of 5' *hCdc4* cDNA using 1F and 3R primers. (1047 bp. product)
Lanes: 1) Huh7 2) Hep3B-TR 3) Focus 4) Mahlavu 5) Hep40 6) Hep3B 7) PLC 8)SK-Hep1
9) Snu475 10) Marker: Gene Ruler DNA Ladder mix

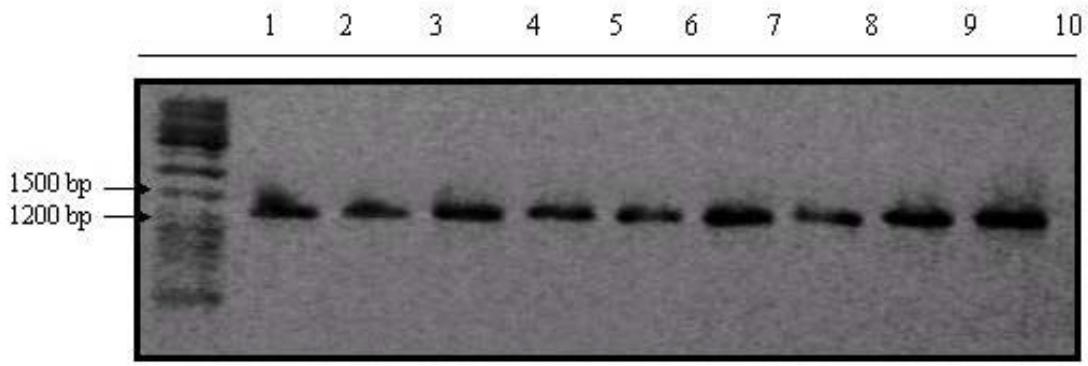


Figure 3.3: RT-PCR analysis of 3' *hCdc4* cDNA using 4F and 7R primers. (1264 bp. product)
Lanes: 1) Marker: Gene Ruler DNA Ladder mix 2) Hep3B 3) HepG2 4) PLC 5) SK-Hep1 6) Snu475 7) Snu449 8)Snu423 9) Snu398 10) Snu387

The PCR products were purified and sequenced using appropriate primers.

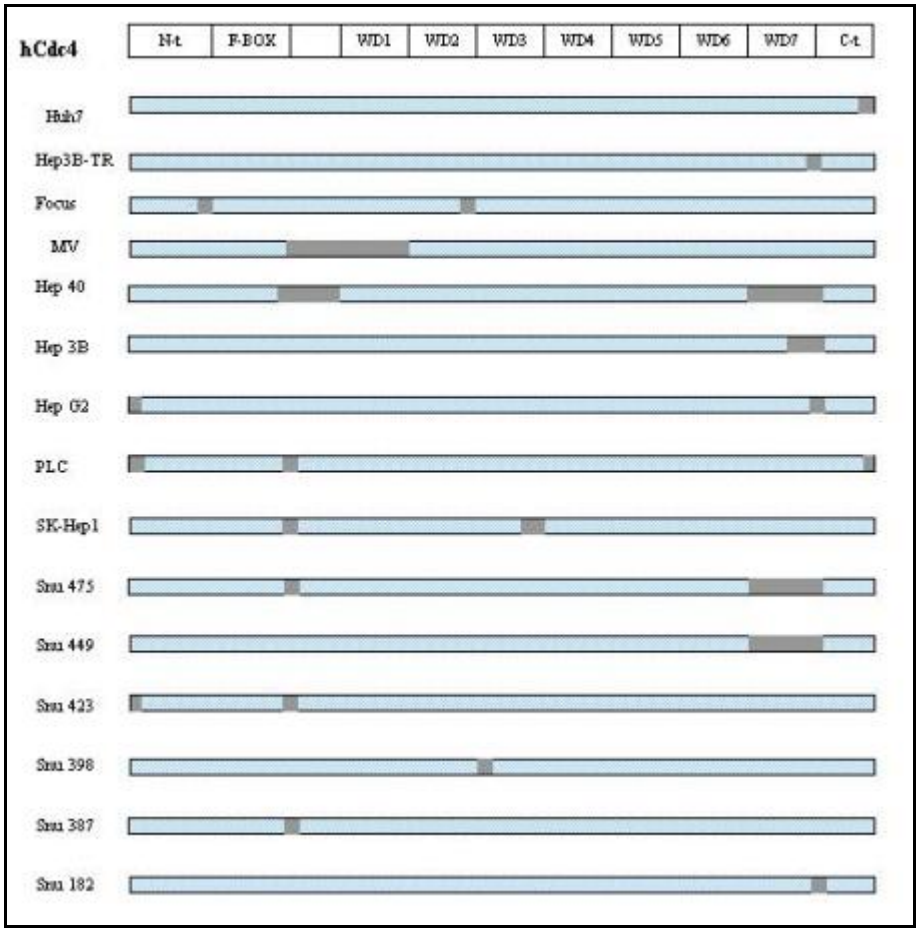


Figure 3.4: The sequenced regions of *hCdc4* in 15 HCC cell lines. The regions shown in blue correspond to clear sequence information, the regions shown in gray represents where the sequence information is not clear or missing.

No mutations (in the F-box, WD domains and previously reported sites where mutations were found in other studies), abnormal transcripts, or no striking change in the transcription level were found among the 15 cell lines tested.

3.2. *B-Raf*

3.2.1. Exon 15 and Exon 11 Mutations

3.2.1.1. Exon 15 mutations

Genomic DNA from 15 HCC cell lines were analyzed by PCR followed by direct sequencing of PCR products. No sequence alterations were detected except T1796A mutation resulting in V599E change in amino acid sequence of B-Raf in SK-Hep1 cell line, which was previously reported. (Davies et al., 2002)

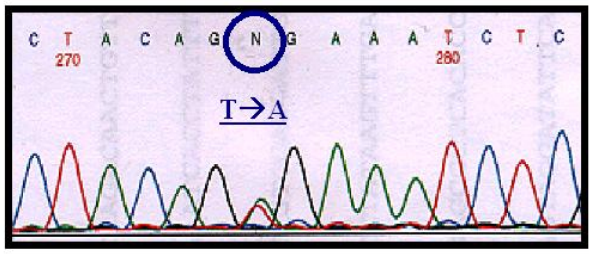


Figure 3.5: Sequencing result of SK-Hep1 with BrafG-R primer showing the mutation T1796A.

3.2.1.2. Exon 11 Mutations

12 HCC cell lines (Huh7, Focus, Mahlavu, HepG2, PLC, SK Hep1, Snu 475, Snu 449, Snu 423, Snu 398, Snu 387, Snu 182) were screened for mutations in exon 11. DNA extracted from 12 HCC cell lines were amplified by PCR (Figure 3.6), purified and sequenced with appropriate primers. No alteration was found in the cell lines in exon 11.

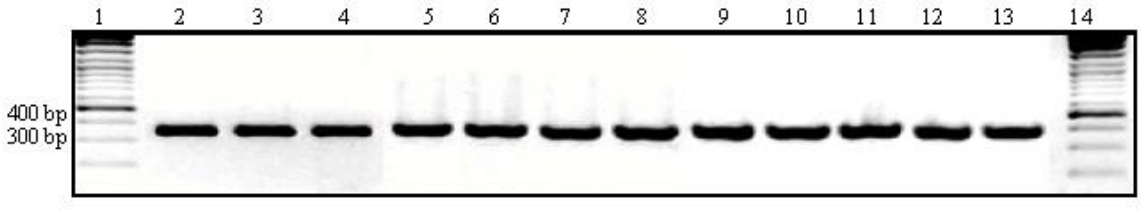


Figure 3.6: *B-Raf* exon 11 PCR products of HCC cell lines. Lanes: 1) Marker (Gene Ruler DNA Ladder Mix) 2) SK-Hep1 3) Focus 4) SNU182 5) Huh7 6) Mahlavu 7) HepG2 8) PLC 9) SNU449 10) SNU423 11) SNU398 12) SNU387 13) SNU475 14) Marker (Gene Ruler DNA Ladder Mix)

3.2.2. ARMS

3.2.2.1 ARMS test for HCC cell lines

15 HCC cell lines and 58 HCC tumours were screened for exon 15 mutations using ARMS test. (Figure 3.7, 3.8) DNA extracted from cell lines and tumours have been subjected to ARMS test and the positive ones were confirmed via sequencing. V599E mutation in SK-Hep1 cell line (Figure 3.5) was used as (+) control in the experiments.

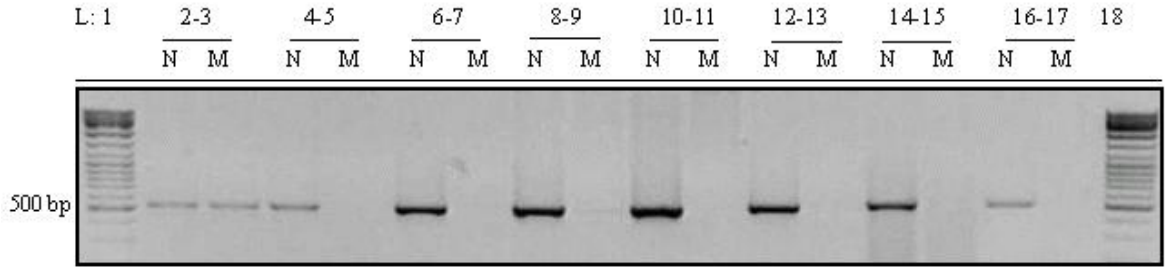


Figure 3.7: ARMS results of exon15 T1796A mutation for HCC cell lines (a). Lanes: 1) Marker (Gene Ruler DNA Ladder Mix), 2-3) SK-Hep1, 4-5) Huh7, 6-7) Focus, 8-9) Hep40, 10-11) HepG2, 12-13) PLC, 14-15) SNU398, 16-17) SNU182 18) Marker (Gene Ruler DNA Ladder Mix)
N: for the normal allele M: for the mutated allele

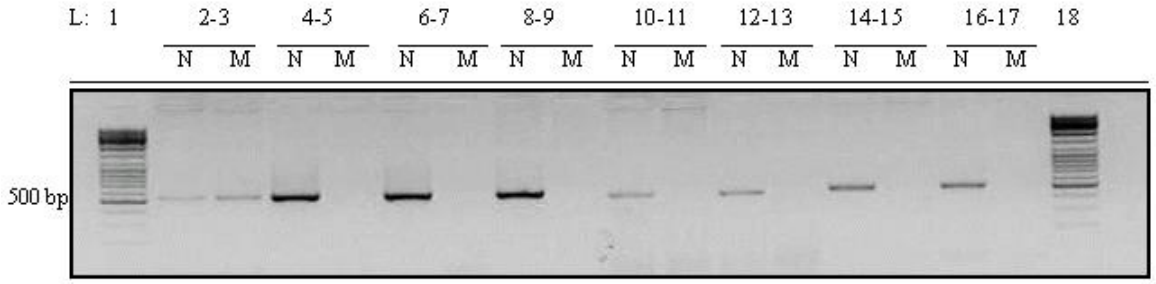


Figure 3.8: ARMS results of exon15 T1796A mutation for HCC cell lines (b). Lanes: 1) Marker (Gene Ruler DNA Ladder Mix), 2-3) SK-Hep1 (+ control) 4-5) SNU387 6-7) Hep3B 8-9) Mahlavu 10-11) SNU475 12-13) SNU423 14-15) SNU449 16-17) SNU182 (- control) 18) Marker (Gene Ruler DNA Ladder Mix)
N: for the normal allele M: for the mutated allele

3.2.2.2. ARMS test for HCC tumours

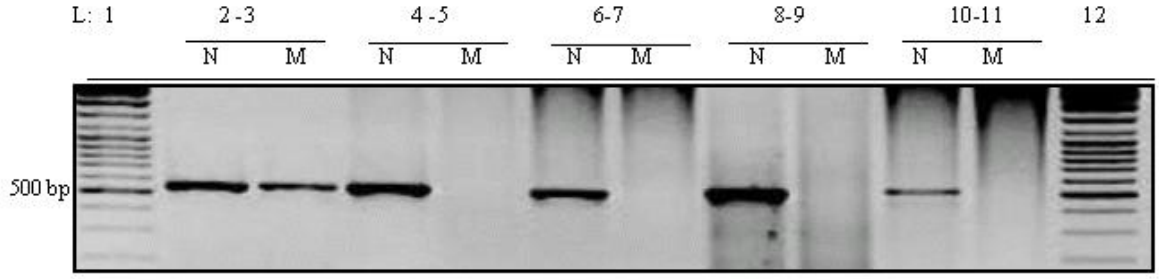


Figure 3.9: ARMS results of exon 15 T1796A mutation for HCC tumours (a). Lanes: 1) Marker (Gene Ruler DNA Ladder Mix), 2-3) SK-Hep1 (+ control) 4-5) C14 6-7) T70 8-9) T75 10-11)T78 12) Marker (Gene Ruler DNA Ladder Mix) N: for the normal allele M: for the mutated allele

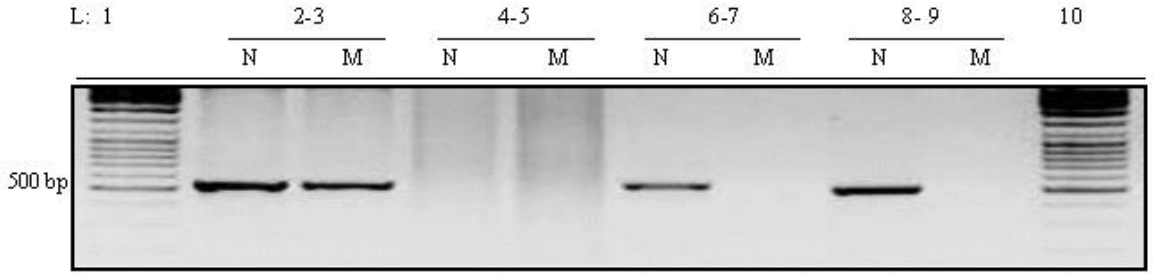


Figure 3.10: ARMS results of exon 15 T1796A mutation for HCC tumours (b). Lanes: 1) Marker (Gene Ruler DNA Ladder Mix), 2-3) T39 4-5) N38 (corresponding normal tissue for T39) 6-7) T83 8-9) SNU182 (- control) 10) Marker (Gene Ruler DNA Ladder Mix) N: for the normal allele M: for the mutated allele.

Within 58 tumours tested for V599E mutation, 1 tumour (T39) was found to have the mutation. (Figure 3.10)

In order to confirm the mutation detected in ARMS result, sequencing for sample T39 is performed. Although the mutation couldn't be seen in the sequencing results, it is obvious in more sensitive ARMS test indicating that proportion of normal cells was high in the sample. (Figure 3.11)

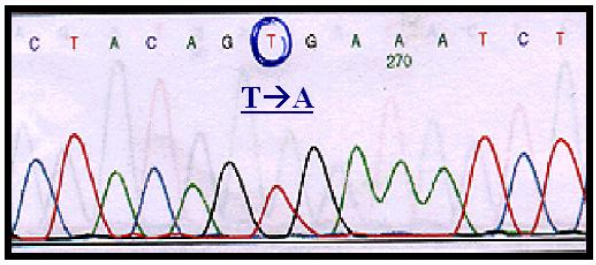


Figure 3.11: Sequencing result with BrafG-R primer for HCC tumour T39. Although mutation cannot be observed in sequencing results it is observable in the more sensitive ARMS test showing that the proportion of the normal cells was high in the sample.

4. DISCUSSION

4.1. *hCdc4*

Several studies have shown that chromosomal deletions at the 4q are associated with human HCCs (76.5% by Shipley et al. and 72.7% by Piao et al.).

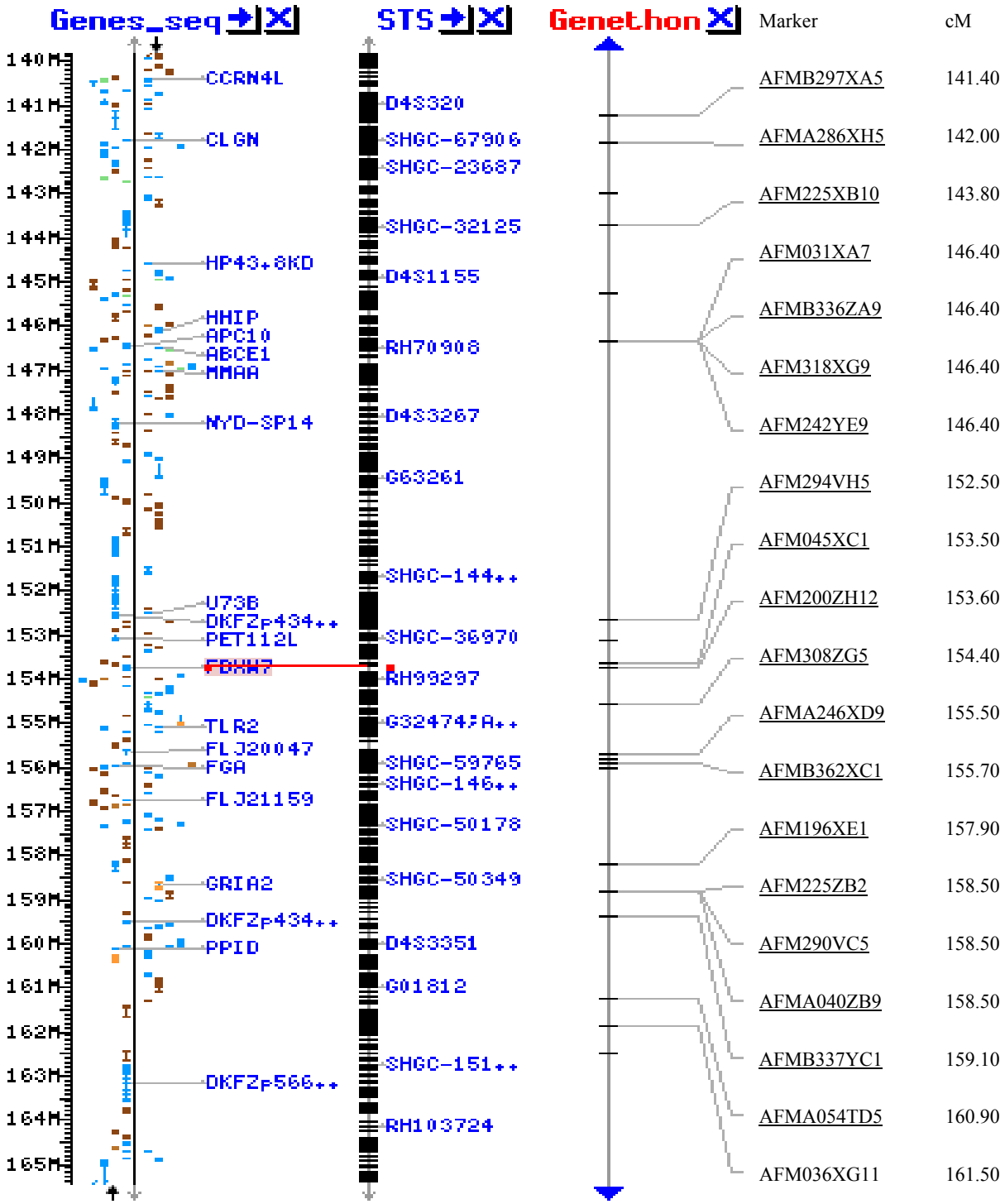


Figure 4.1: *hCdc4/Fbwx7* **gene locus on chromosome 4q**

However no TSG involved in liver carcinogenesis has been identified on this chromosome.

On the other hand, there is strong evidence that cyclin E is involved in carcinogenesis. Genetic analyses have revealed that one of the commonly altered genes in HCC is the cell cycle regulator cyclin E (Malumbres et al., 2001). Overexpression of cyclin E was found in ~70% of HCC patients, which correlated with the poor prognosis of those patients (Jung et al., 2001). In addition, Pascale et al. and Masaki et al. showed that overexpresssion of cyclin E occurred in HCC of chemically induced HCC of Fischer 344 rats and in preneoplastic and neoplastic liver of Long Evans Cinnamon rats (Pascale et al., 2002: Masaki et al., 2000). In fact, overexpression of cyclin E and skp2 (which promotes p27 degradation and thereby activates cyclinE/cdk2) synergistically promote cell cycle progression in cultured hepatocytes in the absence of mitogen or in the presence of growth inhibitors and *in vivo*; hepatocyte replication and hyperplasia of the liver (Nelsen at al., 2001). These previous data suggest the possibility that cyclin E might play a role in the carcinogenesis or progression of human HCC.

Recently, two research teams have identified a gene mutation that leads to persistently elevated levels of Cyclin E protein and cell proliferation in human tumor cells (Moberg et al, 2001; Strohmaier et al., 2001). Having a role in the regulation of cyclin E expression *hCdc4* is proposed to be a TSG and its mutations have been shown in ovarian carcinoma, ALL (acute lymphoblastic leukemia and breast carcinoma cell lines and endometrial tumours. (Moberg et al., 2001; Strohmaier et al. 2001; Spruck et al. 2002). *hCdc4* gene mutations were accompanied by loss of heterozygosity and correlated with aggressive disease. The observation of LOH involving 4q in those tumors suggests that *hCdc4* gene acts as a tumor suppressor, in keeping with the "two-hit" model. Additional evidence suggests that this newly identified gene, *hCdc4*, may also be involved in another cancer related pathway, the Notch signaling pathway (Gupta-Rossi et al., 2001).

Interestingly, *hCdc4* is located on chromosome 4q where the most frequent LOH observed in HCCs.

Based on these facts; we consider *hCdc4* is a good candidate tumour suppressor gene that might be involved in HCC.

The *hCdc4* gene is composed of 13 exons spanning approximately 210 kb of the genome. hCdc4 is an F-box protein that involves an F-box and seven WD repeats which is shown to be involved in protein-protein interactions important in substrate recognition for ubiquitination (Bai et al., 1996; Skowyra et al. 1997) of cyclin E, thus targeting it for destruction. F-boxes and WD40 repeats are typically found in proteins that function as the substrate-recognition components of SCF-type ubiquitin ligase (E3) complexes.

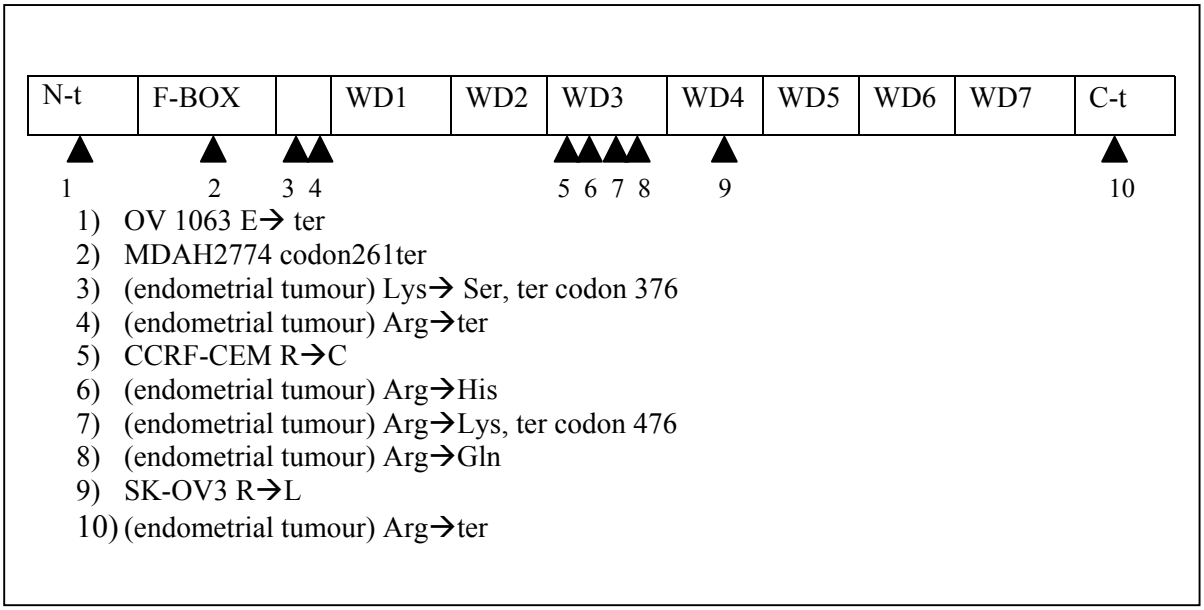


Figure 4.2: *hCdc4* mutations reported previously in cell lines by Moberg et al. (Moberg et al., 2001) and in endometrial tumours reported previously by Spruck et al. (Spruck et al., 2002)

Observed cancer related mutations were localized on functionally important domains of the protein such as F-box or WD domains (Figure 4.2).

Knudson has proposed that a TSG is inactivated by LOH in one allele and by mutation in the other (Knudson, 1996). Since this hypothesis has developed, homozygous deletions, silencing by methylation were reported for several Tumour Suppressor Genes such as p16, PTEN and SOCS1. (Nagai et al., 2003; Schagdarsurengin et al., 2003; Zhang et al., 2002; Yu et al., 2002)

In this study we performed sequence and expression analysis of *hCdc4* gene in 15 HCC cell lines. The transcript levels of hCdc4 were almost the same in all cell lines tested. This suggests that there is no silencing or over expression of *hCdc4* in HCC cell lines. Although our RT-PCR method is not quantitative, we would expect to detect drastic change in expression level. We observed expected length of RT-PCR products for all cell lines, which indicate that there was no detectable deletion or insertion of *hCdc4* gene in these cell lines. Additionally, our mutation analysis on the *hCdc4* using RT-PCR followed by direct sequencing did not reveal any alteration in 15 HCC cell lines. In order to cover all coding region, we sequenced ORF of hCdc4 using primers whose products expected to overlap each other, however we missed some sequencing information for most of the cell lines (see Figure 3.4). But sequencing reads were clear where the most of the mutations described previously (F-box and WD domains). Thus we concluded that there is no mutation on functionally important domains of the protein hCdc4 in HCC cell lines.

Though frequent LOH reported within this region provides the first requirement, in our study no mutation and/or deletion, which may provide the second requirement, or no drastic change in expression level (although our method is not quantitative), which may otherwise indicate silencing by another mechanism, was observed.

The genes that are frequently involved in HCC such as p53, β-catenin and axin are also reported to be frequently mutated in HCC cell lines. (Sayan et al., 2001; Olivier et al., 2002; Satoh et al., 2000; Cagatay and Ozturk, 2002) Since inactivation of *hCdc4* due to

homozygous deletions, mutations or methylation was not observed in any HCC cell lines tested, we have not extend this study to the HCC samples.

Altogether these findings suggest that alteration of this gene is not a frequent event in hepatocarcinogenesis. Other genes that may have role in accumulation of cyclin E in HCC should be searched.

4.2. B-RAF

B-Raf gene is located on chromosome 7q34 and encodes a 765 aa. proto-oncogenic serine-threonine protein involved in transduction of mitogenic signals from the cell membrane to the nucleus via MAP kinase pathway. It belongs to Ser/Thr family of protein kinases.

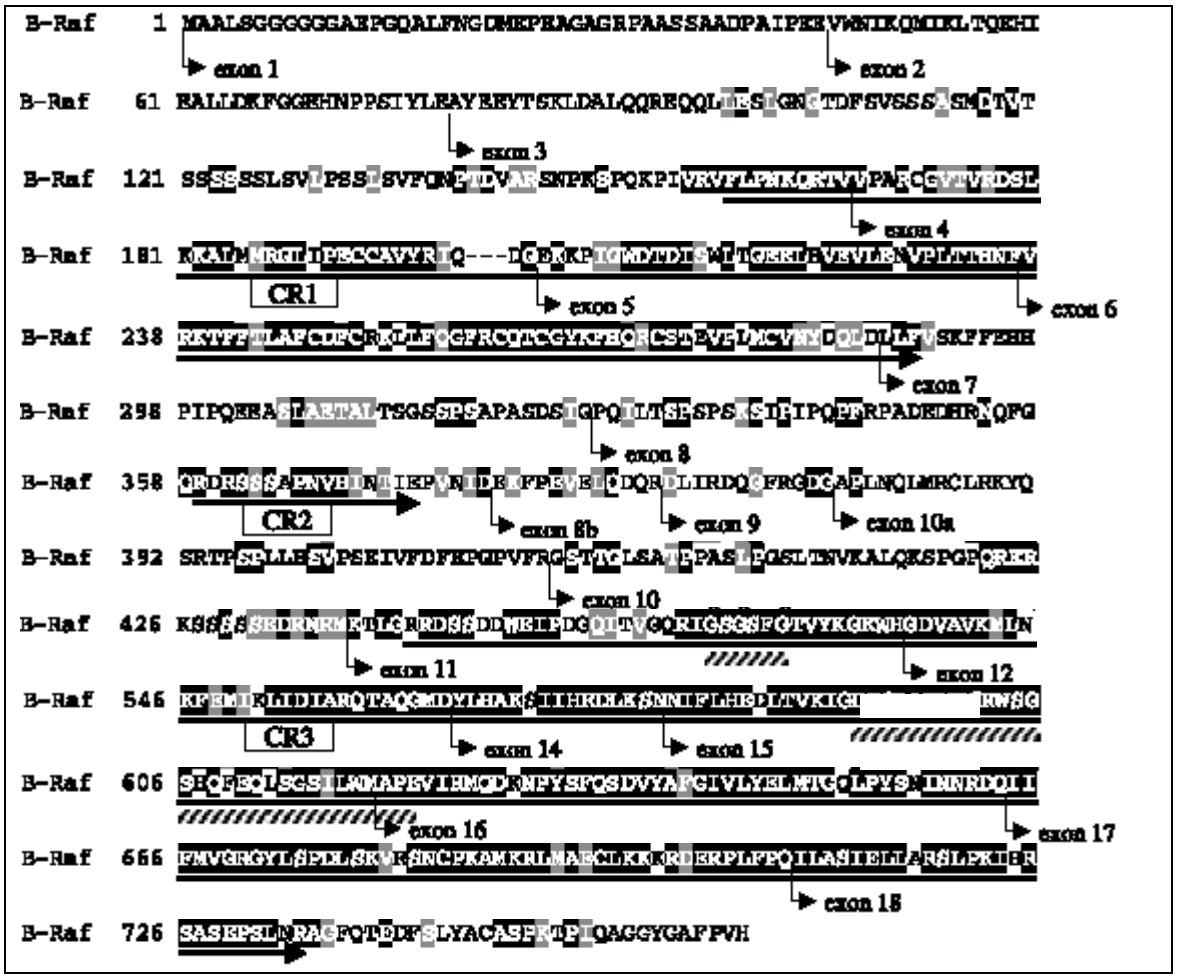


Figure 4.3: Exons and motifs of B-Raf. Amino acids highlighted in black are identical and those highlighted in gray are similar among A-Raf, Raf-1 and B-Raf. The three conserved regions CR1–3 are highlighted by the black arrows. The GXGXXG motif and the activation segment are indicated by the hatched bars.

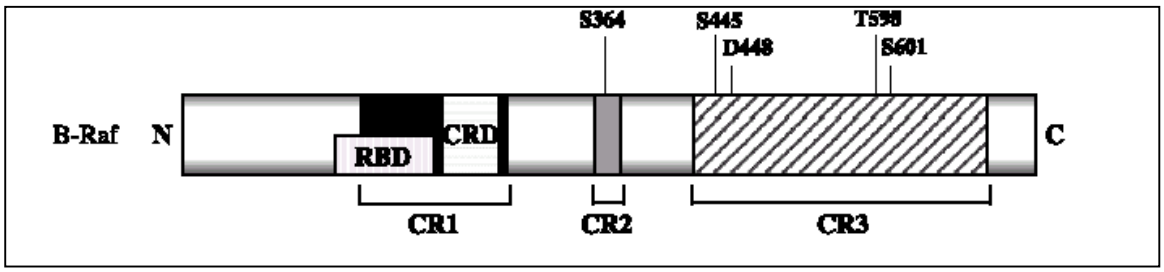


Figure 4.4: B-Raf protein. CR1, CR2, CR3: conserved domains among Raf-1, A-Raf and B-Raf. CR1 contains two domains that bind to Ras-GTP: Ras binding domain (RBD), Cystein rich domain (CRD), and a putative zinc finger domain. CR2 contains regulatory phosphorylation sites (serine threonine rich residues). CR3 contains the kinase domain (with possible phosphorylation sites indicated).

B-Raf mutations are found in a wide range of cancers. Missense mutations of the B-RAF gene in approximately 70% of human malignant melanomas and 15% of colorectal cancers were reported (Davies et al., 2002). High prevalence of B-RAF mutations is described in papillary thyroid cancers (Kimura et al., 2003). Mutations were also detected at a low frequency in gliomas, lung cancers, sarcomas, ovarian carcinomas and breast cancers.

It seems that B-RAF activating mutations play an important role in different organ carcinogenesis. Eventhough mutational activation of Ras is not a frequent event in human hepatocarcinogenesis, Ras mutations in few HCC cases were reported. Some animal models treated with carcinogen display also frequent Ras mutations (Boivin-Angele et al., 2000; Barbin A., 2000; Froment et al., 1994) suggesting that RAS-MAP kinase signaling pathway may be important in hepatocarcinogenesis. Recent work by Davies et al. has identified *B-Raf* mutations in several cancers but no primary HCC samples and only seven HCC cell lines were screened. We therefore decided to determine the incidence of *B-Raf* mutations in our HCC cell lines and patient population.

Genomic DNA from 15 HCC cell lines is screened for sequence variants using PCR and direct sequencing analysis. Mutation analysis was restricted to exon 11 and 15 of B-RAF gene as all previously reported mutations cluster to these two exons within the G

loop in the ATP binding domain of the kinase domain and the activation segment respectively.

We observed only one V599E mutation in SK-Hep1 cell line that has been reported previously. Since we found V599E mutation in one cell line, we extended our study to additional 58 HCC patient samples restricted to this particular mutation. We used ARMS (allele-specific amplification method) to evaluate the incidence of B-RAF activating mutations in patients with HCC. We found one additional mutation in one HCC sample (T39). In order to confirm the mutation detected in T39 sample we amplified exon 15 with the primers covering this exon and sequenced PCR product directly. We were not able to detect this mutation by direct sequencing. Our result is concordant with those reported previously by Pollock et al. (2002), and explained by higher sensitivity of ARMS method compared to the sequencing. Altogether, we found two tumors displaying BRAF mutations out of 72 samples tested (3%).

Eighty-nine percent of the B-RAF mutations encode amino acid changes within the activation segment, with the V599E (T→A change at nucleotide 1796) mutation accounting for 92% of these mutations. Other mutations detected within the activation loop at a lower frequency are V599D, L596V, L596R, G595R and F594L. Eleven percent of the mutations are found in the glycine residues of the G loop GXGXXG motif in the ATP-binding domain of the kinase domain affecting G463, G465 and G468. Thus using direct sequencing method on cell lines we would find if one of these mutations were present.

In a very recent report by Tannapfel et al, (2003) neither BRAF nor KRAS mutations were detected in 25 HCC samples. However in our study we found BRAF V599E mutation in 1/58 HCC tumours and 1/15 HCC cell lines giving an overall percentage of 3 %. The difference in the results might be due to etiological factors meaning that differences in the origins of the tumour samples which in our case was African origin. A minor factor might be the cause of the mutation we found and that factor may not be involved in the region from where the tumour samples were taken by the other group.

Our results suggest that B-Raf may be occasionally involved in hepatocarcinogenesis. This is also the case for Ras which is also rarely mutated in human HCCs. Our results together with previously published HCC mutation studies and reports on HCC animal models suggest that MAP kinase pathway might be involved in hepatocarcinogenesis but neither B-Raf nor Ras are the major player of this pathway in this event. Thus, other members of this pathway should be evaluated for mutations in HCC.

5. FUTURE PERSPECTIVES

Given the importance of cyclin E over expression in HCC as well as other cancers, it would not be surprising to see alteration of the cyclin E degradation pathway in these cancers. Our next step will be to find out if the other components of this pathway such as Skp1 and Cul 1 are altered in cancers. It is likely that multiple cyclin E degradation pathways operate in mammals since the Roberts et al. has recently found that another mammalian cullin, Cul3, also influences cyclin E levels in mice and in cell lines (Singer et al., 1999). It would be also interesting to test this gene and identify unknown members of cyclin E degradation pathway.

There is no doubt that Ras/Raf/MEK/ERK signaling pathway is involved in carcinogenesis. However neither B-Raf nor Ras are the major players of this pathway in this event. Thus, other members of this pathway such as RHEB and Rho-GAP will be evaluated for mutations in HCC in the further study.

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