ANALYSIS OF p73 GENE IN HEPATOCELLULAR CARCINOMA

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

BY
NECATİ FINDIKLI

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I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as thesis for the degree of Master of Science.

Prof. Dr. Mehmet Öztürk

I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as thesis for the degree of Master of Science.

Assoc. Prof. Kuyaş Buğra

I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as thesis for the degree of Master of Science.

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ABSTRACT

ANALYSIS OF p73 GENE IN HEPATOCELLULAR CARCINOMA

Necati Fındıklı

M.S. in Molecular Biology and Genetics

Supervisor: Prof. Dr. Mehmet Öztürk

August 1998

Hepatocellular carcinoma (HCC) is the eighth most frequent cancer worldwide. Epidemiologically-studied risk factors include hepatitis B virus (greater than 80%), hepatitis C virus and aflatoxins. Molecular mechanisms of hepatocarcinogenesis are poorly understood. The only gene known to be consistently involved in these tumors is the p53 tumor suppressor gene. However, this gene was found to be mutated or inactivated in about 30% of HCC. There is a need to study additional genes in order to fully understand hepatocellular carcinogenesis. p73 has been identified recently as a p53-homolog gene. In this study, we analyzed the possible involvement of this gene in HCC. We investigated both the expression and structure of p73 gene in HCC for possible alterations.We first developed a novel method to analyze the expression of alternatively spliced transcripts of p73 (p73α and p73β) simultaneously. This technique, based on RT-PCR, allows the analysis of p73 transcripts semi-quantitatively. We found that p73α was expressed ubiquituously in 8 cell lines derived from normal liver or HCC tumors. Interestingly, p73β was present only in 5 differentiated but not in 3 undifferentiated cell lines. The differentiation status of these cell lines were tested by the analysis of albumin and α-fetoprotein transcripts by RT-PCR. These transcripts were present in 3/5 differentiated but not in 3 undifferentiated cell lines. Next, we screened 25 HCC samples for possible mutations of p73 gene at selected exons with non-radioactive heteroduplex test, radioactive SSCP analysis, restriction enzyme analysis and DNA sequencing. No alterations were found in exons homologous to those of p53 known to harbor mutational hotspots.From these observations, we conclude that p73 gene is not mutated in HCC, but it may play a critical role in hepatocellular differentiation. As p73β was found in differentiated cell lines, this form may be involved in transcriptional regulation of liver-specific genes. Additional studues are needed to confirm this hypothesis.

ÖZET

KARACİĞER KANSERİNDE p73 GENİNİN ANALİZİ

Necati Fındıklı

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Yöneticisi: Prof. Dr. Mehmet Öztürk

Ağustos 1998

Hepatoselüler karsinom (HCC) dünyada sekizinci en yaygın kanserdir. Epidemiyolojik olarak araştırılmış risk faktörleri arasında hepatit B virüsü (%80'den fazla), hepatit C virüsü ve aflatoksinler başta gelmektedir. Hepatoselüler karsinogenezin moleküler mekanizmaları çok iyi bilinmemektedir. Bu tümorlerde sıkça rolü olduğu saptanan tek gen p53 tümor baskılayıcı genidir. Ancak, bu genin mutasyonu ya da inaktivasyonu HCC'lerin yaklaşık %30'unda gözlenmiştir. Hepatoselüler karsinogenezin tam olarak anlaşılabilmesi için diğer genlerin de incelenmesi gerekmektedir. p73 yakın zamanda p53-homologu bir gen olarak bulunmuştur. Bu çalışmada, bu genin hepatoselüler karsinomdaki olası rolü araştırılmıştır. p73 geninin HCC'deki yapısı ve ifadesi, olası değişimler açısından incelenmiştir. Önce p73'ün alternatif olarak ifade edilen iki transkriptini (p73α ve p73β) ayni zamanda incelemek için yeni bir yöntem geliştirilmiştir. RT-PCR'a dayanan bu teknik, p73 transkriptlerinin miktarını kabaca belirleyebilmektedir. Böylece HCC tümörlerden ve normal karaciğerden türetilmiş 8 hücre hattında p73α'nın ifade edildiği gözlenmiştir. Buna karşılık p73β sadece 5 farklılaşmış hücre hattında görüldü. Farklılığını yitirmiş 3 hücre hattında ise p73β bulunamadı. Bu hücrelenin farklılaşma durumları RT-PCR tekniği ile albümin ve alfa-fetoprotein transkriptleri incelenerek test edildi. Bu transkriptler 5 farklılaşmış hücrenin 3'ünde görüldü ancak 3 farklılığını yitirmiş hücre hattında görülmedi. Daha sonra, 25 HCC örneği p73 geninin seçilmiş ekzonlarındaki olası mütasyonları belirlemek amacıyla non-radyoaktif heterodupleks testi, radyoaktif SSCP analizi, restriksiyon enzim analizi ve DNA dizi analizi yöntemleri ile tarandı. p53 geninin en çok mütasyona uğradığı bilinen ekzonlarına benzerlik gösteren p73 eksonlarında mutasyon bulunmadı. Bu gözlemlerden yola çıkarak, p73 geninin HCC'de mutasyona uğramadığı, ama hepatoselüler farklılaşmada kritik bir rol oynayabileceği sonucunu çıkardık. p73β sadece farklılaşmış hücrelerde görüldüğünden, bu form karaciğere-özgün genlerin RNA ifadesinin düzenlenmesinde rol oynayabilir. Bu hipotezin ek çalışmalarla doğrulanması gerekmektedir.

To my parents

Vahit and Fatma

and

to my sisters Zuhal and Hilal...

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ABBREVIATIONS

AGE	Agarose gel electrophoresis
APS	Ammonium per sulfate
cDNA	Complementary DNA
C-terminus	Carboxy terminus
DBD	DNA-binding domain
DEPC	diethyl polycarbonate
DEPC	Diethyl polycarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DS	DNA sequencing
EDTA	Ethylene diamino tetra acetic acid
FCS	Fetal calf serum
F-medium	Freezing medium
G3PDH	Glucose-3-phosphate dehydrogenase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDA	Heteroduplex analysis
HPV	Human papillomavirus
kDa	Kilo dalton
LOH	Loss of heterozygosity
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
N-terminus	Amino terminus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI(3)K	Phosphotidyl inositol 3' kinase
RB1	Retinoblastoma gene
REA	Restriction enzyme analysis
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SSCP	Single strand conformation polymorphism
SV40	Simian virus 40
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TBP	TATA-binding protein

TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
UV	Ultraviolet

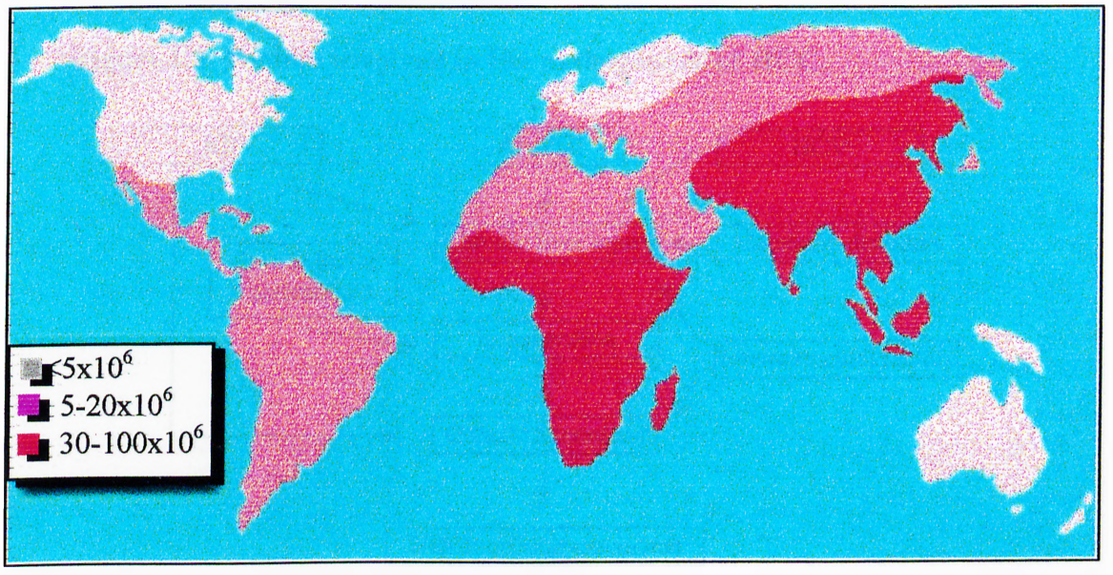
1-INTRODUCTION

1.1. Molecular pathogenesis of HCC and role of p53

Hepatocellular carcinoma (HCC) which stems from the malignant transformation of liver cells called hepatocytes is one of the most common human tumors throughout the world. HCC itself accounts for 85% of primary liver cancers and shows very wide geographical variation in incidence (Figure 1.1.). In the Far East and Sub-Saharan Africa, it is a major public health problem. Surgical cure is not possible for most of the cases and the cancer is associated with poor prognosis (Harris CC., 1990; Okuda K., 1992).

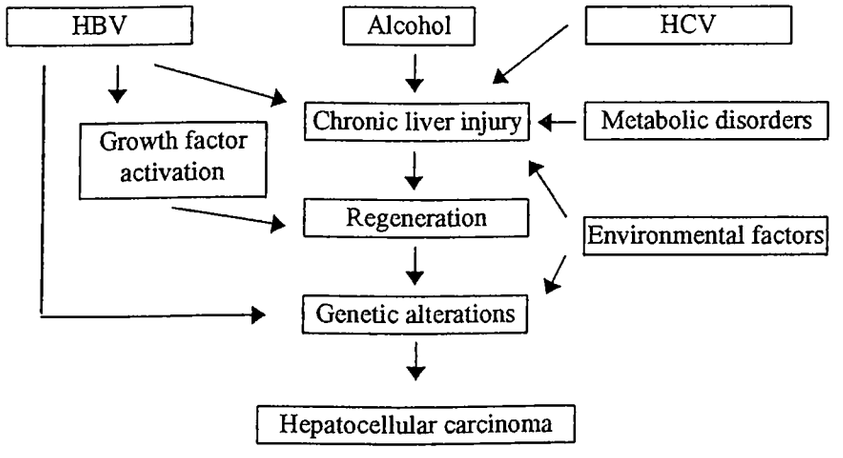
Although the molecular mechanisms of hepatocarcinogenesis are poorly understood, the risk factors for the development of HCC are now well recognized and some of the steps involved in the molecular pathogenesis of HCC have been elucidated in recent years.

Figure 1.1.: Worldwide incidence of hepatocellular carcinoma



Most HCCs develop as a result of chronic liver disease induced by hepatitis B virus (HBV) or hepatitis C virus (HCV). Chronic exposure to aflatoxins and certain metabolic diseases of the liver also cause HCC. (Figure 1.2.). In both cases resulting genetic mutations are common events before hepatocellular carcinoma develops (Wands JR., 1995).So far, the most extensively studied etiological factors are HBV, HCV and certain toxins ingested via contaminated food. In sub-Saharan Africa and certain parts of Asia including Japan relatively high number of the HCC patients carry serological markers of hepatitis B virus (>90% for southern Africa; Wands JR., 1995). Although the mechanisms by which HBV enhances hepatocellular carcinogenesis are not well defined, insertional mutagenesis has been suggested but appears to be important only in the woodchuck model in which insertional activation of N-myc gene is common (Fourel et al., 1990). In human HCC, only a few hepatomas have been found with viral integration near genes associated with growth control (for example cyclin A) and differentiation (for example retinoic acid receptor; Wang et al., 1992; Dejean et al., 1986). It has also been suggested that viral protein HBx, encoded by

Figure 1.2. Factors involved in the pathogenesis of HCC.



hepatitis B virus may act as an effector of cellular transcription factors (Maguire et al., 1991) or may inactivate the function of p53 by sequestering the protein in the cytoplasm (Henkler et al.,1995; Elmore et al., 1997; Ueda et al., 1995).

Like HBV, percentage of HCC patients infected by HCV varies from population to population and depends in part on the exposure of general population to this virus. In that manner, HCV appears to be a major cause of HCC in Japan, Italy, and Spain whereas it seems to play a less important role in South Africa and Taiwan where HBV is predominant. So far, there is no evidence to suggest that HCV has a direct role in the molecular pathogenesis of HCC. HCV is associated with this disease by its ability to produce chronic liver injury, followed by regeneration and the eventual development of cirrhosis (Colombo et al., 1991; Liang et al., 1993; Raedle et al., 1997).

Many attempts have been made to characterize the chromosomal abnormalities in HCC, although karyotyping of primary HCC was difficult and achieved only in limited cases (Bardi et al., 1992; Simon et al., 1990) including HCC cell lines in which a high rate of aneuploidy was detected (Chen et al., 1993). In HCC, chromosome gains especially chromosomes 4, 6, 7, 8, 16 and 17 were observed whereas total chromosome loss was seen more rarely. Multiple abnormalities of chromosome 1 was noticed. Aberrant chromosome number, as well as deletions and translocations that resulted in loss of the distal region were described (Yeh et al., 1994). Chromosomal regions including 4q, 5q, 10q, 11p, 13q, 16q and 17p and 22q have frequently been found to show allelic losses, chromosome 17p in which p53 tumor suppressor gene resides giving the highest rate. As in the case of p53 in 17p, these losses or alterations in chromosome number and the regions may reveal the genes that are important for the

HCC development (Simon et al., 1991; Takahashi et al., 1993; Yeh et al., 1994; Zimmermann et al., 1997).

Activation of known protooncogenes such as Ki-ras, N-ras and IGF2 does not seem to play an important role in HCC, however in several cases, amplification and over expression of cyclin D1 has been reported (Takada et al., 1989; Tsuda et al., 1989; Zhang et al., 1993; Nishida et al., 1994).

Among the known tumor suppressor genes, only a few of them have been tested for the possible involvement in HCC. So far, the most extensively studied tumor suppressor gene is p53 gene, which encodes a nuclear phosphoprotein that acts as an important factor in the control of normal cell proliferation. It has been reported that this gene shows genetic aberrations in 30% of the hepatocellular carcinomas. It has also been reported that HCC-specific codon 249 mutation of p53 is frequent in geographic areas where there is a high risk of exposure to aflatoxin $\beta 1$, which is a known mutagen inducing G→ T transversions (Bressac et al., 1991; Hsu et al., 1991; Ozturk et al., 1991; Aguilar et al., 1993; Fujimoto et al., 1994). HCC samples from other regions show low frequency of p53 mutations and codon 249 mutation is rare or absent. (De Benedetti et al., 1995; Debuire et al., 1993; Kubicka et al., 1995; Kazachkov et al., 1996; Unsal et al., 1994).

As another important tumor suppressor gene, retinoblastoma gene (RB1) has been reported to show frequent loss of heterozygosity (LOH) and loss of expression. RB1 mutations have been described only rarely. This could be due to the very large size

of the gene which is difficult to study systemically (Farshid et al., 1994; Hada et al., 1996; Puisieux et al., 1993; Friedman et al., 1997).

p16/INK4A /MTS1 gene, which was found to show germ-line mutations in half of the familial melanomas, has also been reported to show rare germ-line and somatic mutations in HCC. The loss of p16 protein expression was also reported for several HCC cell lines (Kita et al., 1996; Piao et al., 1997; Biden et al., 1997; Chaubert et al., 1997; Kaino et al., 1997; Bonilla et al., 1998). Finally, p16/INK4A/MTS1 gene promoter was shown to be silenced by *de novo* methylation. This type of inactivation is frequently observed in human cancers including hepatocellular carcinoma (Merlo et al., 1995; Chaubert et al., 1997).

Another gene that is reported to be mutated in HCC is the mannose-6-phosphate/insulin-like growth factor-II receptor (M6P/IGF2R) gene. Its gene product plays a role in the activation of the TGFβ and the degradation of IGF2 (De Souza et al., 1995; Piao et al., 1997).

Compared to other genes, it appears that p53 gene plays an important role in hepatocarcinogenesis. But so far, p53 has been found to be mutated in 30-50% of the cases. The remaining 50% of the cases can be attributable to the other gene(s) that play critical roles in liver carcinogenesis. Other genes with p53-like activity could also be involved in HCC development. p73 has been identified recently as a new relative of p53 gene (Kaghad et al., 1997). Although there is not enough data about its implications in cancer as well as its cellular function, its high homology to p53 suggests that it can also play an important role in cancer. The study of p73 in HCC was interesting because this

gene is located at chromosomal locus 1p36 which is known to be deleted in HCC (Yeh et al., 1994). Before presenting the aim of our work, we will summarize the present state of literature about p53 and p73 with a specific emphasis on HCC-related observations.

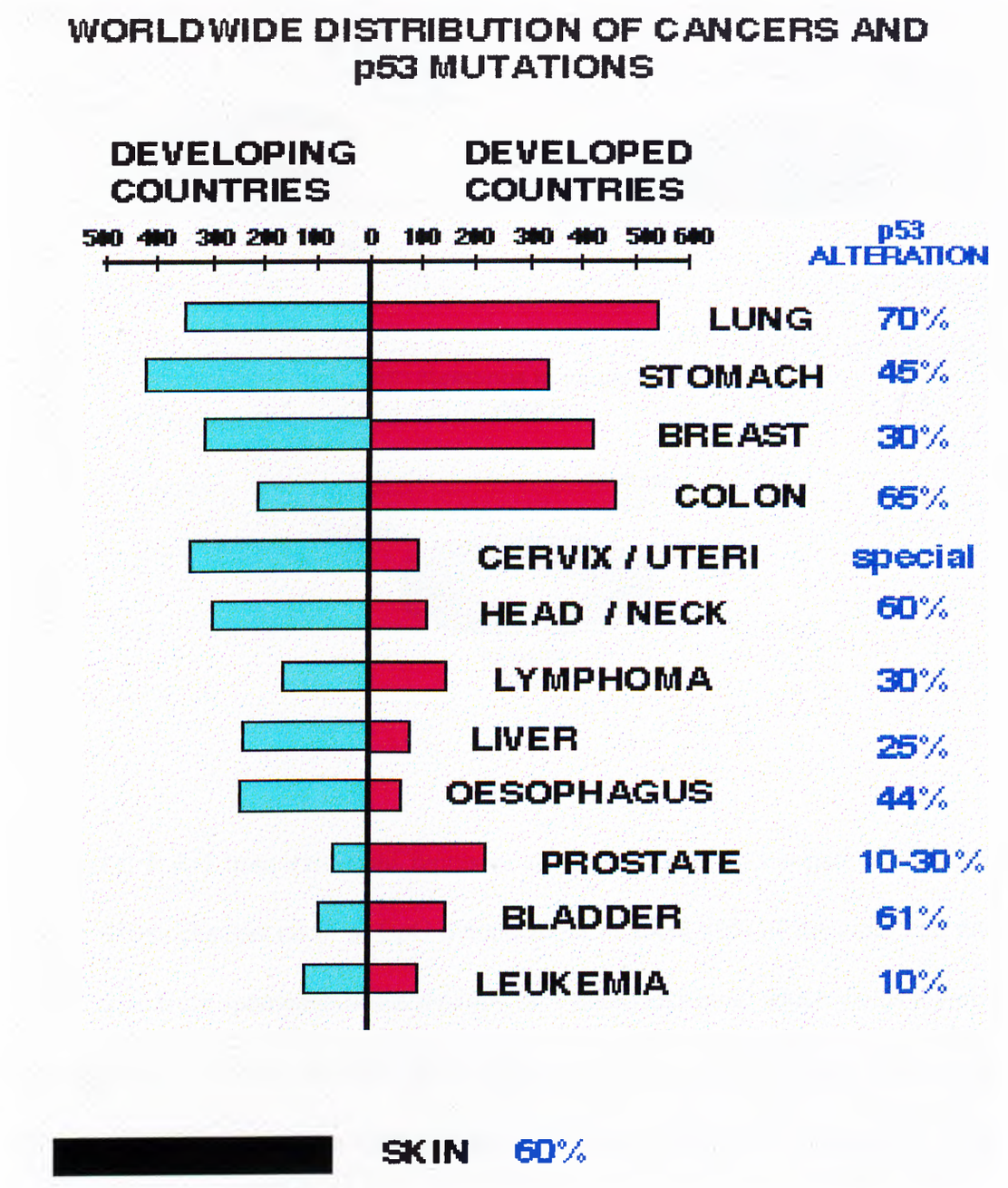
1.2. p53 tumor suppressor gene and cancer

p53 protein was discovered in 1979 by different groups as a cellular antigen associated with the large T viral antigen of the simian 40 tumor virus (Deleo et al., 1979; Linzer et al., 1979). Later, a murine p53 cDNA was cloned and this clone was found to immortalize cells in vitro or transform primary fibroblasts together with ras. These initial observation led to the mis-classification of p53 as a dominantly acting oncogene (Jenkins et al., 1984; Parada et al., 1984). However, subsequent studies showed that this particular mouse cDNA was mutant and that wild-type p53 was a tumor suppressor gene (Eliyahu et al., 1988; Hinds et al., 1989). Also it was showed that normal p53 gene product negatively regulates cell growth of some cells (Mowat et al., 1985) and abolishes the tumorigenic potential of a cell line in culture (Chen et al., 1985). Hovewer, growth advantage has been observed in the presence of wild-type/mutant genotype as compared with the two wild-type possessing controls (Srivastava et al., 1993) indicating that the mutant protein can act as a dominant negative by forming multimeric complexes with wild-type p53 gene product and inhibiting its normal role in a cell (Milner et al., 1991).

During the last ten years, many observations from different laboratories have established the fact that p53 is the most frequently mutated gene in human cancer. The mutation spectra of p53 shows differences in frequency, distribution and nature (Figure

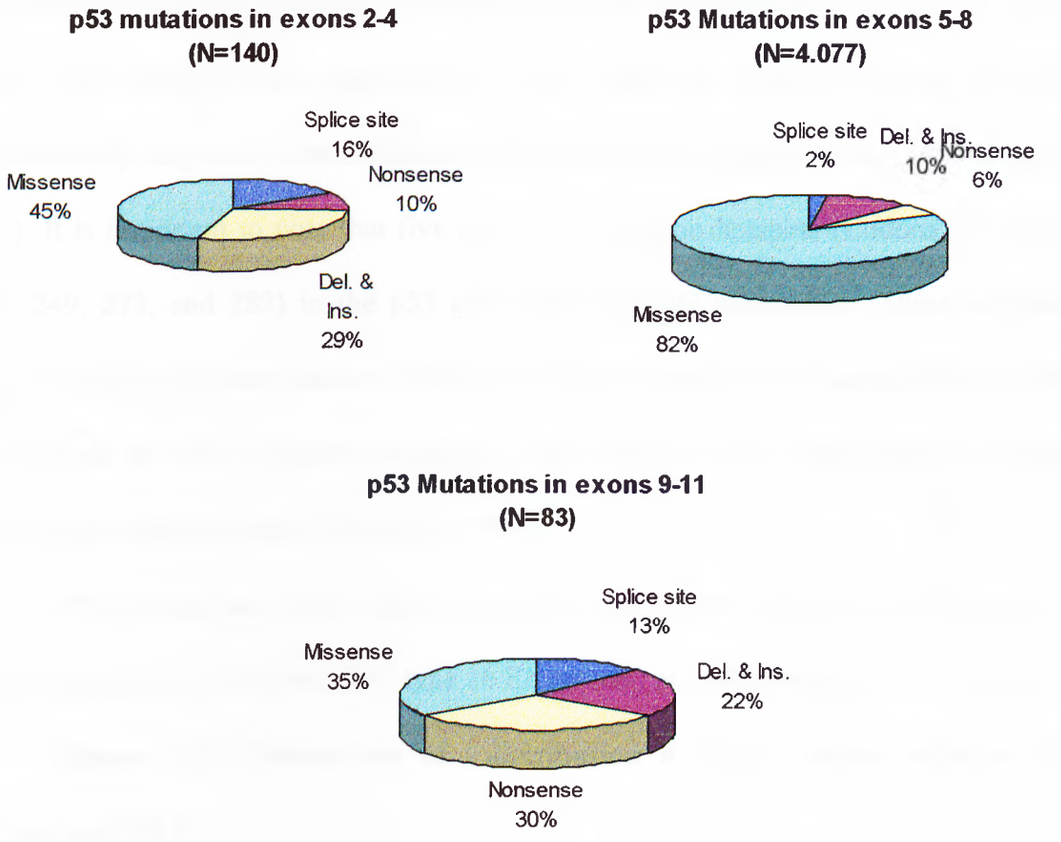
1.3.). Distribution of mutations in different exons is shown in figure 1.4. In this picture, it is clear that the percentage of missense mutations resulting in the inactive protein is relatively higher in the exons that span consensus DNA binding domain (82%) than

Figure 13.: Worldwide distribution of cancers and p53 mutation rates



those encoding the acidic transactivation domain and the basic carboxy terminal domain (Wang et al., 1997).

Figure 1.4. Distribution of p53 mutations in different exons

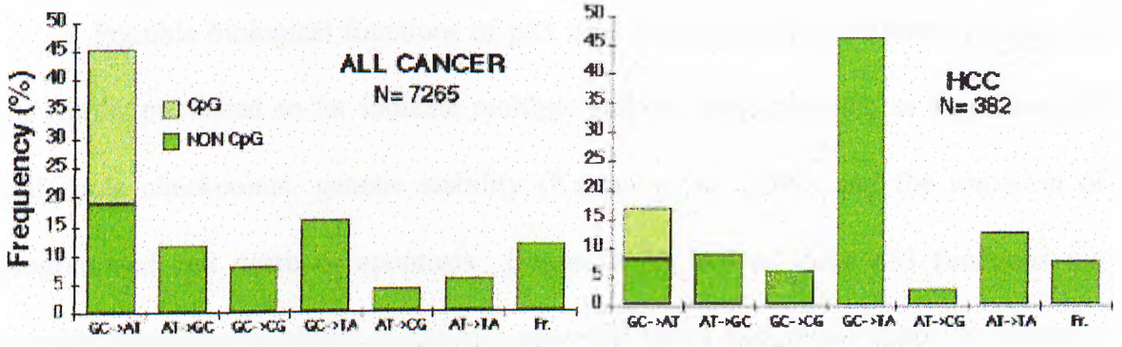


G:C→ A:T base transitions represent about 40% of all p53 mutations in human cancers. These changes occur at CpG dinucleotides in about 60% of the cases and they seem to arise from spontaneous deamination of 5-methylcytosine, which yields thymine (Greenblatt et al., 1994). In HCC, the conversion of G:C→ T:A is found to be much more common than any other base changes as shown in figure 1.5. (Soussi T., 1997). Moreover, this conversion was found to be a specific G→ T transversion of the third

base of codon 249, leading to a substitution of arginine to serine in a significant number of patients from southern Africa and the Qidong area in China (Bressac et al., 1991; Hsu et al., 1991). It was suggested that this mutation was associated with high aflatoxin B1 intake in food and may have contributed to the high incidence of HCC in these areas. This finding is also supported by in vitro studies in which p53 codon 249 was preferentially targeted to form adducts with aflatoxin B1 (Aigular et al., 1993; Figure 1.6.). It is important to note that five of the six mutation hotspots (Codons 175, 245, 248, 249, 273, and 282) in the p53 gene occur at CpG nucleotides. These hotspots encode arginines at sites that are essential for the maintenance of contact between the protein and its DNA consessus sequence: this contact in turn responsible for DNA binding and transactivation (Cho et al., 1994).

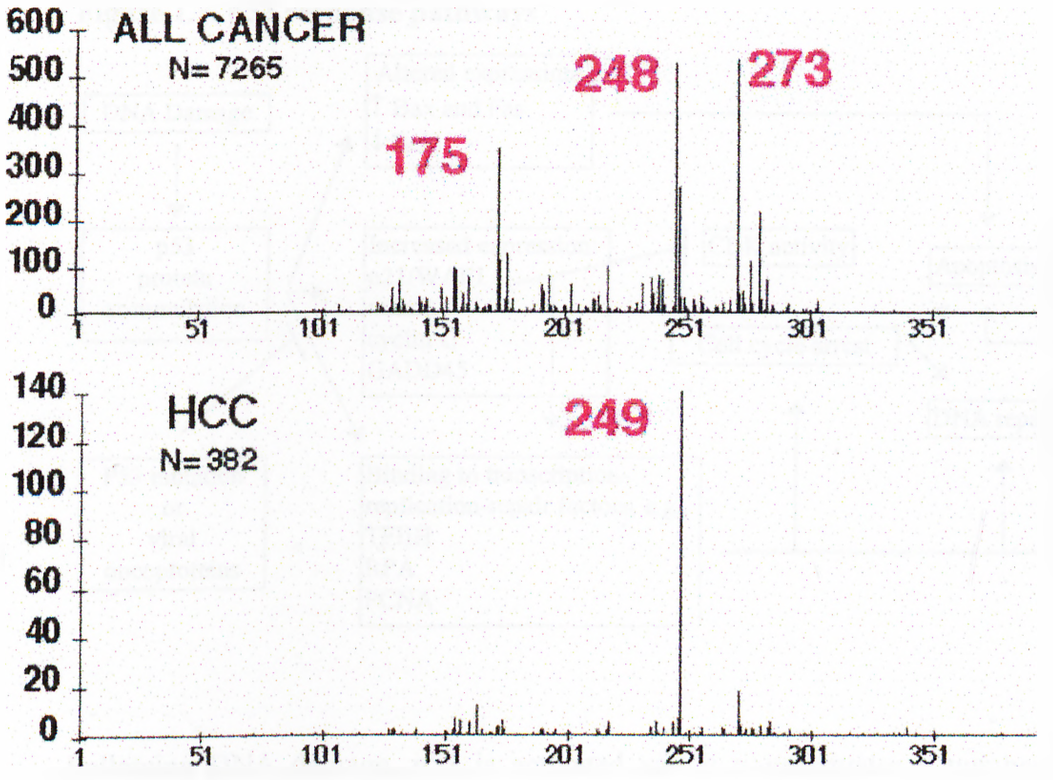
P53 protein was also found to complex with viral proteins (SV40 large T antigen, adenovirus E1B and HPV type 16 E6 protein) and such complexes of p53 were

Figure 1.5.: Comparison of distribution of base changes between all cancers and HCC



found to be inactive (reviewed by zur Hausen H., 1996). Thus, functional inactivations of p53 also appears to be frequent in cancers, as it will be discussed later in detail.

Figure 1.6. p53 hotspots in all cancers and in HCC.

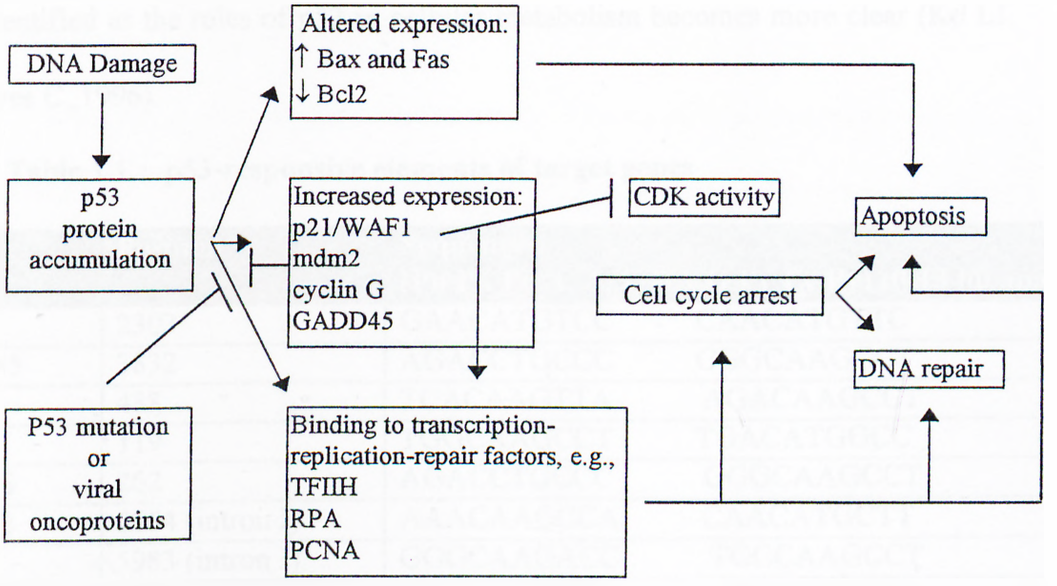


1.3. Cellular functions of p53

Possible biological functions of p53 have been studied by different groups and the results published so far indicate multiple cellular functions such as the control of cell cycle checkpoints, genetic stability (Kastan et al., 1992) and the initiation of programmed cell death or apoptosis (Figure 1.7.). All of these p53 functions are possibly related to its ability to act as a powerful tumor suppressor gene. Experiments show that initiation events which triggers the p53 response can be DNA damage, activated oncogenes or certain stress conditions. It is crucial to point out that p53

response generated by such signals can differ in different cell types or in the presence of different extracellular signals (Lane DP., 1992).

Figure 1.7. p53 response pathways



Following DNA damage, p53 is activated and it transactivates a number of downstream genes whose products contribute to the cellular response to DNA damage with diverse biological activities. The following genes are the most important p53 target genes: p21 (El-Diery et al., 1993), mdm2 (Wu et al., 1993), GADD45 (Kastan et al.,1992; Sun et al.,1995) , HIC (Vales et al.,1995), cyclin G (Okamoto et al.,1995; Zauberman et al.,1995), Bax (Miyashita et al.,1995), proliferating cell nuclear antigen (PCNA; Shivakumar et al.,1995), IGF-BP3 (Buckbinder et al., 1995), hMSH2 (Scherer et al., 1996) and BTG2 (Rouault et al., 1996). The products of these genes were shown to have a direct effect on cellular growth, DNA repair or DNA replication. p53 binds to

specific p53-responsive elements on either the promoter or intronic regions in these genes and these responsive elements are outlined in table 1.1.

Other candidates for the p53 response genes are TGF-α, thrombospondin-1, fas/APO1, Rb, EGF receptor, cyclin D and p53 itself. Other target genes will continue to be identified as the roles of p53 in cellular metabolism becomes more clear (Ko LJ. and Prives C.,1996).

Table 1.1.: p53-responsive elements of target genes

Gene	Position*	Sequences: (Pu Pu Pu C A/T T/A G Py Py Py) (0-13 bp apart)	
p21	2303	GAACATGTCC	CAACATGTTC
GADD45	3832	AGACCTGCCC	GGGCAAGCCT
Bax	488	TCACAAGTTA	AGACAAGCCT
MCK	119	TGGCAAGCCT	TGACATGGCC
Cyclin G	262	AGACCTGCCC	GGGCAAGCCT
IGF-BP3	5064 (intron I)	AAACAAGCCA	CAACATGCTT
	5983 (intron I)	GGGCAAGACC	TGCCAAGCCT
hMSH2	447	AGGCTAGTTT	AAGTTTCCTT
MDM2	139	GGTCAAGTTG	GGACACGTCC
BTG2	97	AGTCCGGGCA	AGCCCGAGCA

*** Position of the response sequence according to Genbank's map positions**

One of these downstream genes is p21, the CDK2 inhibitor (also known as universal CDK inhibitor) that inhibits the activity of cyclin-dependent kinases. Experimentally, a p53-dependent inhibition of cyclin E/CDK2 was observed in fibroblasts and this inhibition was shown to be mediated p21. Recently developed p21 null mice showed a normal developmental pattern but mouse embryo fibroblasts that lack p21 were partially deficient in their ability of G1 arrest in response to DNA damage. This observation is another confirmation of the importance of p21 in G1

checkpoint. It has been implied that contribution of p53 into G1 checkpoint is to preserve the genetic stability. In the absence of p53, genomic instability results from checkpoint loss creating gene amplifications, aneuploidy and other chromosomal aberrations (Smith et al., 1995).

However, p21-independent cell cycle arrest also exists (Deng et al., 1995; Brugarolas et al., 1995). Fibroblasts with elevated p53 expression also causes minor G2 arrest. Furthermore wild-type p53 overexpression in a human ovarian cancer cell line resulted in a transient G2 delay (Vikhanskaya et al., 1994). So it has been suggested that in addition to G1 cell cycle arrest, p53 also participates G2 cell cycle checkpoint and length of this arrest duration is dependent on cell type (Kastan et al., 1991).

p53-dependent apoptosis was first detected in experiments using myeloid leukemic cell lines with overexpressed exogeneous wild-type p53. In these experiments, the cell viability was rapidly lost, in a manner characteristic of apoptosis (Yonish et al., 1991). From the reverse approach, thymocytes isolated from p53-deficient mice did not undergo apoptosis after ionizing radiation as compared with the normal thymocytes confirming the involvement of p53 in physiological apoptosis process (Lowe et al., 1993). Recently it was shown that expression of bax is induced in slow growing apoptotic tumors indicating the role of p53-induced bax gene (Yin et al., 1997). Furthermore tumour growth is accelerated and the apoptotic ratio drops by 50% in bax-deficient mice, suggesting the requirement of bax for a full p53-dependent response (Yin et al.,1997). Under conditions such as highly damaged nuclear DNA and absence of required survival factors, certain cell types are observed to go through p53 dependent programmed cell death with the possible function of elimination of unstable genomes

and inadequately-fed cells, diminishing the probability of having cancerous phenotype. Beside the involvement of Bax gene in the p53-dependent apoptosis, recently, it has been proposed that p85 protein, which is the regulatory subunit of p110 PI(3) kinase, is upregulated by p53 under oxidative stress conditions leading to cell death and this involvement is independent of PI(3)K (Yin et al., 1998).

A recent study utilized the oncogenic ras that was formerly shown to transform immortal rodent cells to a tumorigenic state and in this study it was found that the expression of oncogenic form of the ras in primary human or rodent cells resulted in a permanent G1 arrest, in which the accumulation of p53 and p16 was observed. Also, disruption of either p53 or p16 was shown to abolish the ras induced growth arrest, concluding that oncogenic ras provokes premature senescence in primary rodent and human cells in association with the induction of p53 and p16. This suggests that besides other functions, p53 has an important role in the induction of the senescence program (Serrano et al., 1997).

Most of the experiments, performed to analyze the possible role of p53 in differentiation and development, were done using cells of the haemopoietic lineage. When wild-type p53 is overexpressed in early pre-B cell line, the cytoplasmic μ heavy chain and B-cell specific antigen which are the indicators of more mature stage are expressed (Shaulsky et al., 1991). Likewise, a Friend erythroleukemia cell line overexpressing wild-type p53 is observed to start expression of hemoglobin, a marker of erythroid differentiation (Johnson et al., 1993). Together, these reports can be interpreted that p53 is involved in haemopoietic development. The mechanism may not be through a direct induction of differentiation but the differentiation could be

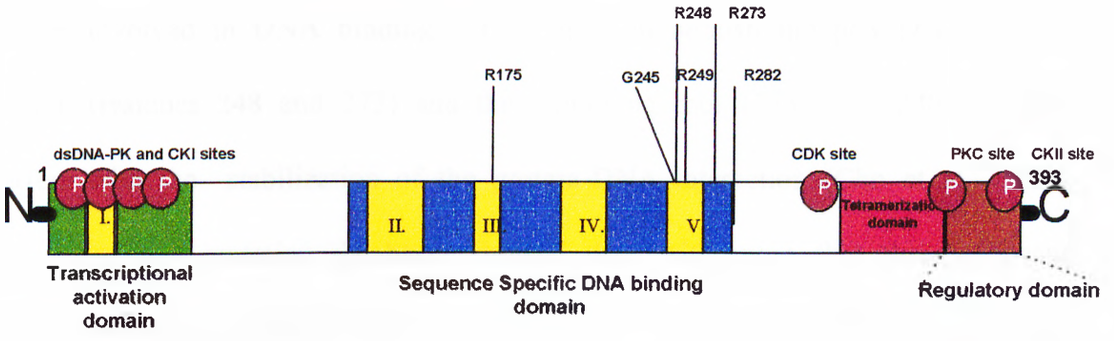
facilitated by the inhibitory action of p53 in cell cycle (Hu et al., 1995). It has been described that p53 knock-out mice shows a normal viable development; but in another experiment a fraction of the p53-deficient embryos displayed neural tube defects (Sah et al., 1995). Thus, p53 may also be involved in the development and differentiation independently of its ability to act as a tumor suppressor.

1.4. Biochemical characterization of the p53

Human p53 gene covers 16-20 kb of DNA in chromosome 17p13, having 11 exons. Exons between 2 and 11 encodes an mRNA of 2,2-2,5 kb that is translated into a protein of 53 kDa with 393 amino acids. The protein contains five distinct evolutionarily conserved boxes numbered as I-V and the residues between II to V are encoded by exons 4 to 9. Most of the p53 mutations occur in this region. p53 protein has several distinct structural domains (Figure 1.8.) one of which resides in N terminus and contains acidic transcriptional activation domain that interacts with the transcription initiation machinery. Next to transcriptional activation domain, there is a proline-rich region. This hydrophobic domain appears to be important for the maintaining the overall structural stability of p53 protein (Levine et al., 1991). Recently, this domain was shown to be involved in p53-mediated apoptosis (Ruaro et al., 1997). The central portion of p53 located between amino acids 120-290 is the DNA binding domain. This domain recognizes a specific DNA motif (5'- Pu Pu Pu C A/T T/A G Py Py Py) which is repeated on target genes and shown in table 1.1.(El-Deiry et al.,1992; Zambetti et al., 1992). Another charged basic domain through C-terminus was found to be essential for the tetramerization (Iwabuchi et al.,1993) and subsequent nuclear transport signal containing region resides between amino acids 316 and 325

(Shaulsky et al.,1990). At the very end of the carboxy-end a regulatory domain is found and this domain regulates the activation of the wild-type TP53. This last domain is absent in protein product of an alternatively spliced mRNA of p53 (Wolkowicz et al., 1995)

Figure 1.8.: Functional domains of TP53



Recent studies about the transcriptional activation and repression functions of p53 have revealed that the consensus p53 response element plays a crucial role. The genes which have this sequence are transcriptionally activated by p53 and the absence of the response element is thought to be sufficient for the repression. The key mechanism that mediates the activation/repression process involves TATA-binding protein (TBP). Experiments show that TBP interacts with both the amino and carboxy terminus of p53 and it is proposed that this difference plays a switching role between p53-dependent transcriptional activation and repression of target genes (Horikoshi et al., 1995).

1.5. Inactivation and activation of p53

p53 function can be inactivated in several ways one of which is the mutation that occur on p53 gene in nearly 60% of all human cancers (Hollstein et al., 1991;

Greenblatt et al., 1994). In most cases, only the mutant allele is detected in tumor cells. Thus, the loss of wild type allele in most cases is followed with a mutation on the other allele. Vast majority of missense mutations are clustered in the sequences encoding the central region of the p53 protein. The analysis of p53 crystal structure and mutation distribution patterns has revealed that p53 mutational hotspots are on amino acid residues involved in DNA binding. Two of them abolish the p53 DNA binding function (residues 248 and 273) and the remaining four (175, 245, 249 and 282) interfere with the stabilization of the protein-DNA interaction (Cho et al., 1994). Analysis of p53 mutation databases showed that, with more that three thousand different mutations collected from the human tumor cell lines, different tumor types have distinct p53 mutation profile. These profiles are very helpful in understanding the cancer aethiology, clinical basis of tumorigenesis and functions of p53 domains.

Another mechanism for the loss of p53 function is the inactivation of the protein by viral proteins. p53 has been shown to form complexes with the SV40 large T antigen (Linzer DI. and Levine AJ..,1979), adenovirus type 5 E1B 58 kDa antigen (Sarnow et al., 1982; Zantema et al., 1985; Braithwaite et al., 1991) and the E6 protein of human papilloma virus type 16 (HPV-16; Werness et al., 1990). The region of p53 bound by large T antigen closely correlates with the conserved core domain and is contained within residues 94 to 293. This large T antigen-p53 complex inhibits p53 mediated transcriptional activation by inhibiting DNA binding as it is shown by in vitro experiments. Similarly E1B, one of the genes encoded by adenovirus, forms a complex with p53 and inhibits transcriptional activator function but binding of this molecule to p53 does not prevent its DNA binding ability. As different from the E1B and large T

antigen, the association of E6 protein with p53 targets it effectively for degradation through ubiquitin pathway, leading to a deficit of p53 activity for the control of normal cell proliferation. (Scheffner et al., 1990).

In addition to these abrogation-in-function states of p53 protein, deregulation of p53 activity by the overexpression of cellular proto-oncogene mdm-2 is reported and characterized by several groups (reviewed by Oliner JD., 1993; Picksley et al., 1993). In certain tumors (particularly sarcomas) with wild-type p53 the amplification of mdm-2 gene was observed suggesting the alternative inactivation pathway of p53 protein. The mdm-2 gene was first identified originally by virtue of its amplification in transformed mouse lines. Overproduction of this protein was shown to be tumorigenic (Fakkarzadeh et al., 1991). Interestingly, mdm-2 gene is also found to be transcriptionally activated by p53 protein and it is proposed that p53 function is controlled by p53/Mdm-2 autoregulatory feedback loop where p53 stimulates mdm-2 expression and its own function is impaired by being sequestered in an inactive p53-Mdm-2 complex (Barak et al., 1993; Wu et al., 1993). The main inhibitory role of mdm-2 is its ability to bind to the transcriptional activation domain of p53 and to block the p53-target gene activation. It was recently reported that mdm-2 also promotes the rapid degradation of p53 (Haupt et al.,1997).

p53 protein is extensively modified by phosphorylation (Meek et al., 1994).The serine/threonine phosphorylations are clustered into two distinct domains at the N and C-termini of the protein and the enzymes that are responsible for this process in vitro are found to be DNA dependent protein kinase (DNA-PK), casein kinase I-like enzyme and casein kinase II-like enzyme (Fiscella et al.,1993).

In most cells, wild-type p53 is present in extremely low quantities and has a short half-life (6-20 minutes). Ionizing radiation or UV light and alkylating agents induce p53 and the presence of DNA strand breaks is critical for this induction (Nelson and Kastan 1994). It is generally agreed that the inductive response is post-transcriptional (Kastan et al., 1991; reviewed by Ko and Prives, 1996).

Also, recently identified factor ref-1, previously named as redox/repair protein, was found to be an extremely potent activator of the latent p53 protein. It was further stated that ref-1 stimulates p53 both in redox-dependent and redox independent manner in vivo and in vitro suggesting that this factor may play a critical sensor role for the genotoxic stress that can activate latent p53 (Jayaraman et al., 1997).

Vaziri et al. investigated the possible relationship between the telomere loss and the p53 activity and they concluded that in ataxia-telengiectasia fibroblasts which show accelerated telomere loss and premature senescence when compared with normal fibroblasts also shows increased specific activity of p53. It has further been shown that p53 can associate with PARP (poly ADP-ribose polymerase) and inhibition of PARP activity leads to abrogation of p21/WAF1 and mdm2 expression in response to DNA damage. So p53 can not only be activated in response to DNA damage but also in response to telomere shortening (Vaziri et al., 1997).

In summary, p53 protein is transcriptional activator which upregulates the functions of several genes involved in the control of cellular growth. Under normal conditions p53 has a short half-life. Following DNA damage (X-rays, DNA alkylating agents etc.) p53 protein is stabilized and accumulates in cells leading to its transcriptional activation. One of the p53 target genes, p21 encodes an inhibitor of

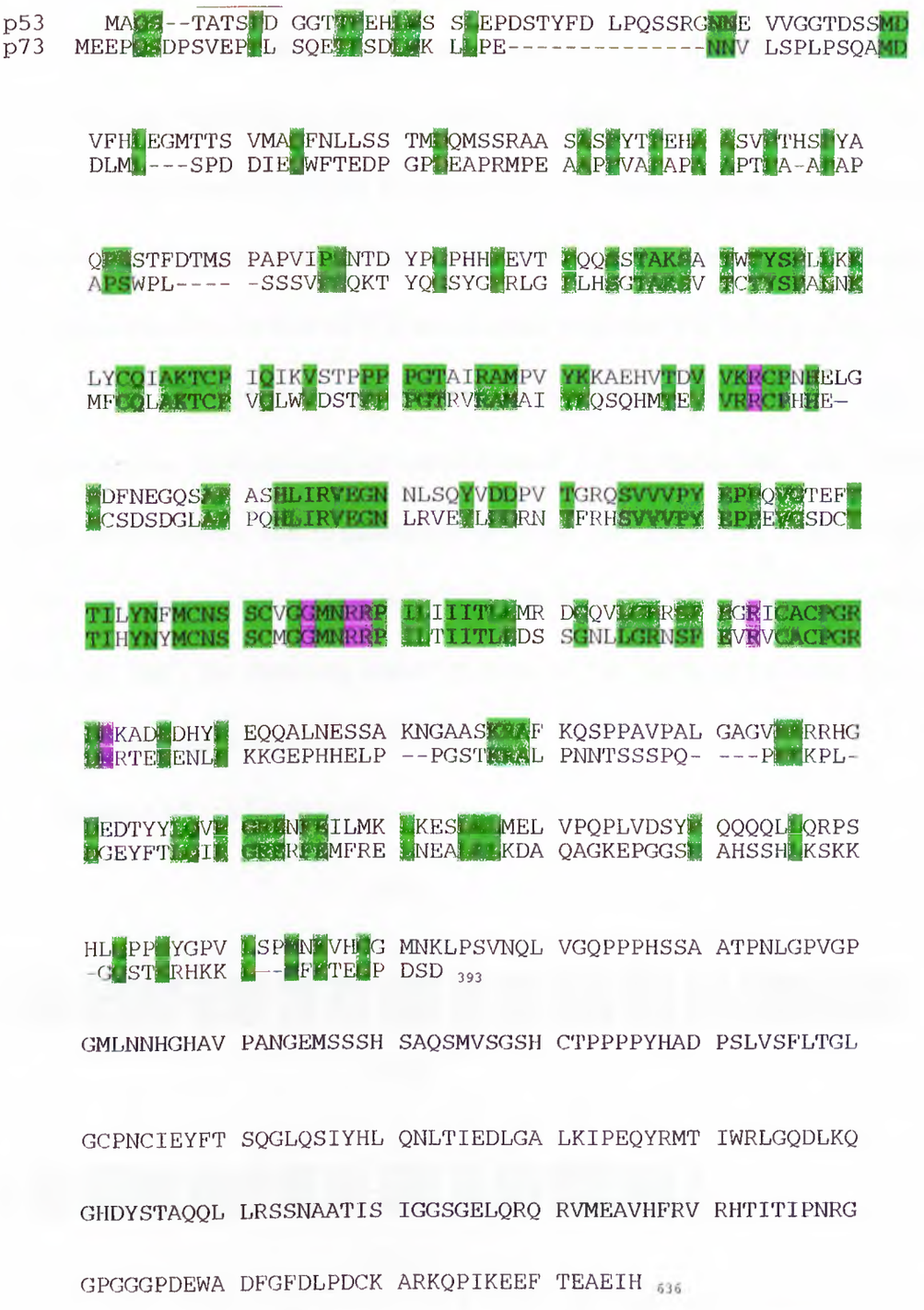
cyclin-dependent kinases, inducing cell cycle arrest at both G1 and G2/M phases. p53 induces apoptosis in certain cells either directly or by the induction of bax expression. In tumor cells, this role of p53 is lost either by mutational inactivation or functional inactivation.

1.8. Identification and characterization of p73 gene

p73 gene is a newly discovered gene that shows considerable homology with p53 tumor suppressor gene (Kaghad et al.,1997). Interestingly, the gene was identified while pursuing research into growth factors called cytokines. The group was performing a hybridization screen of a COS cell cDNA library using degenerate oligonucleotides corresponding to IRS-1 binding domains. Coding sequence gave no considerable homology with IRS-1 binding domain sequence but later it was found that the molecule shows homology with p53 in both aminoacid and DNA sequence (figure 1.9.).

For almost 20 years, many researchers were searching for the possible homologs of p53 since many oncogenes and tumor suppressor genes found so far exist as a family like myc (c-myc, N-myc and L-myc), ras (H-ras, K-ras and N-ras) and Rb (Rb, p105 and p130) family of genes. Suprisingly all the low stringent hybidization and degenerate PCR techniques gave no positive results. This negative results led people to think that p53 function was unique and there was no redundant genes. The discovery of p73 demonstrated that, p53, like many other genes, belong to a family which shows structural and probably functional similarities (Oren M., 1997).

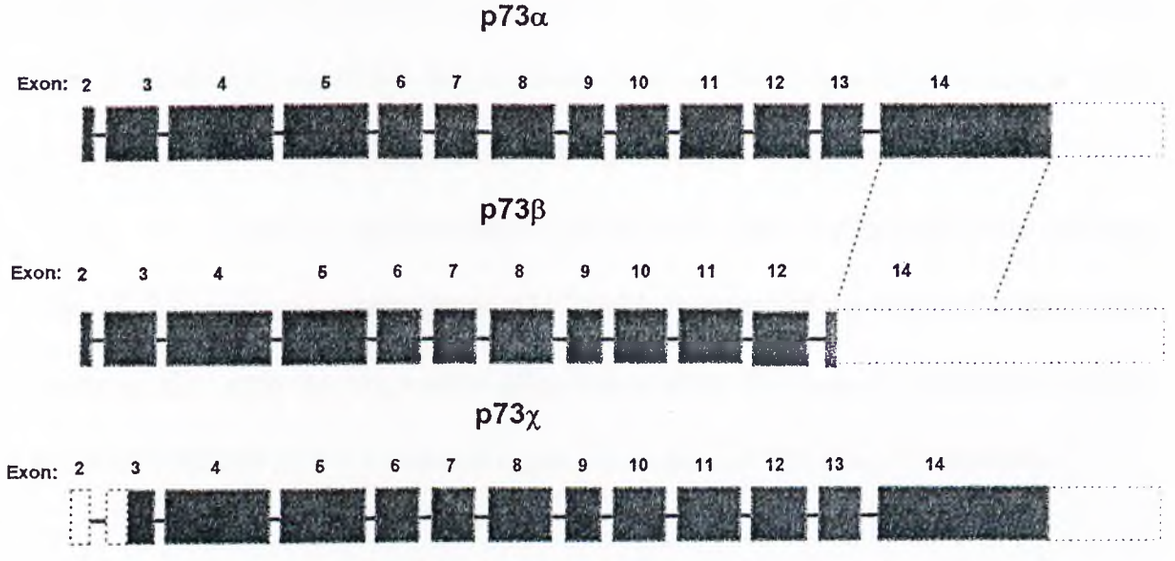
Figure 1.9.: Homology between p73α and p53



1.7. Functional and structural similarities between p53 and p73

p73 gene shows similarities and differences with p53 tumor suppressor gene in several aspects. It is localized in chromosome 1p36 and has 14 exons. It has two splicing variants differing at their C termini, termed as p73α and p73β (lacks 96 nucleotides corresponding to exon 13) respectively. It is also reported that there exists a third form of p73 protein resulting from the alternative start codon at the N terminus. P73α form codes for a protein of 636 amino acids long whereas β form , due to exon 13 deletion, yields a polypeptide with 499 amino acids (Figure 1.10.). On the other hand, p53 gene resides in short arm of chromosome 17. Although they are mapped to different chromosomes, the organization of these two genes are similar in exonic structures and coding / non-coding parts (In both, exon 1 is non-coding e.g.). Since p73 has a longer ORF, the resulting transcript sizes of two forms are 4.4 kb and 2.9 kb whereas p53 has a transcript size of 2.5 kb.

Figure 1.10.: p73 variants.



According to the protein product, p73α and p73β forms, though they are longer than p53, resemble the latter in three regions: N terminus, which is responsible for transcriptional activation, is 29%; DNA binding domains are 63% and oligomerization domain is 38% identical. Furthermore, the amino acid residues that are frequently found to be mutated in variety of cancer types (R175, G245, R248, R249, R273 and R282 which are also shown in purple in figure 1.9.) are also conserved in p73. No significant homology was reported between the C-terminal domain of mammalian p53 and p73 indicating that this unique region might be responsible for the interaction with other factors that cooperate with p73 or regulate its function(s). However, this C terminal region of p73α form is similar to invertebrate p53 homologs and this opens another debate about the evolution pattern of p53 gene. It has now been speculated that p53 may have evolved from a p73-like gene.

Also, p53 and p73β (but not the p73α) have been shown to form homo-oligomers in yeast two-hybrid assays. In these experiments it has also been observed that p73β showed significant but relatively weak interactions with p53 but the actual existance of these mixed oligomers in vivo still needs to be determined.

Another important finding about p73 is that, it transcriptionally activates one of the main p53 target genes p21/WAF1 in transient-transfection experiments. Interestingly, when the Arg residue of p73 at position 292 (which is analogous to Arg residue at position 273 of human p53 gene) is mutated to His, this p73-dependent

activation of p21/WAF1 is abolished. Arg 273 in p53 has been shown to be mutated in variety of cancer types and this change abrogates the ability of p53 to act as a transcriptional activator (Kaghad et al.,1997).

Although overexpressed p73 has been shown to induce apoptosis, the mechanism is not well understood. The analysis of apoptosis-asociated genes such as bax and Fas/Apo1 are needed to clarify the role of p73 in apoptotic response (Jost et al., 1997).

1.8. Aim of the project

Molecular pathogenesis of HCC remains poorly understood. High frequency of p53 mutations in HCC cells and possible functional inactivation by Hbx of HBV virus suggest that one major pathway of hepatocellular carcinogenesis is the inactivation of hepatocellular functions mediated by p53. However, p53 is found to be mutated in 30% of these tumors. The malignant transformation of other HCCs might be due to gene aberrations other than p53 mutations. The chromosome location of p73 (1p36) was known to be frequently altered in HCC. This is why we decided to study whether HCC cells display aberrations of p73 gene. If so, this would help to find another pathway of malignant transformation of HCC mediated by the loss of p73 function. Before starting any functional studies of p73 in hepatocytes and HCC cells, it was important to know whether p73 gene expression is lost or modified by genetic mutation. We decided to study first the expression of p73 in HCC cells and then the primary tumors for possible mutations in p73 gene by using non-radioactive heteroduplex test, radioactive SSCP analysis, restriction enzyme analysis and DNA sequencing.

2-MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Cell lines and primary tumor samples

The following cell lines were used in this study: HT29 (Huet et al., 1987), Chang (Chang RS., 1954), WRL68 (Apostolov K., U.S. Pat.:3,935,066), Mahlavu (Alexander JJ., 1984), Hep3B (Aden et al., 1979), Hep3B-TR (Hasegawa et al., 1995), HepG2 (Aden et al., 1979), FOCUS (He et al., 1984) and PLC/PRF/5 (Alexander et al., 1976). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, glutamine (2 mM), non-essential amino acids, penicillin and streptomycin. All cell lines were negative for mycoplasma contamination except PLC/PRF/5 and Mahlavu.

DNA samples from primary HCCs have also been studied. These samples have been collected from different geographical locations in the world. They have been previously studied for their status of p53 gene and HBV DNA sequences. The data on patients (age, sex, viral markers etc.) was also known (Unsal et al., 1994).

2.1.1. Chemicals

NAME:	PRODUCER:
Agarose	Prona basica
Ammonium persulfate	Carlo Erba
Boric acid	Sigma
Bromo phenol blue	Sigma
DMEM	Sigma

DNA ladder, 1 kb	MBI Fermentas
$MgCl_2$ (25 mM)	Promega
Reverse transcription 10X buffer	Promega
dNTP mixture (10 mM)	Promega
Rec. Rnasin© ribonuclease inhibitor	Promega
AMV reverse transcriptase	Promega
Oligo$(dT)_{15}$ Primer	Promega
DNA ladder, 100 bp	MBI Fermentas
dNTP mix (10 mM)	MBI Fermentas
Dulbecco's PBS	Biological
EDTA	Sigma
Ethidium bromide	Sigma
Formamide	Carlo Erba
Formaldehyde	Carlo Erba
Gel extraction kit	Qiagen
Glycerol	Carlo Erba
Guanidium thiocyanate	Merck
L-Glutamine	Biochrom-Seromed
Non-essential amino acids	Biological
Nusieve agarose	FMC
^{32}P dCTP	Izotop
Penicillin streptomycin	Biochrom-Seromed

Phenol	Merck
Sodium acetate	Carlo Erba
Sodium chloride	Carlo Erba
Sodium Hydroxide	Carlo Erba
Taq DNA Polymerase	MBI Fermentas
TEMED	Sigma
Trypsin-EDTA	Sigma
Xylene cyanol FF	Sigma

2.1.3. Stock solutions and buffers

SOLUTION	METHOD OF PREPARATION
For Agarose Gel Electrophoresis:	
5X formaldehyde gel running buffer	Add 10 ml of 2M sodium acetate and 10.3 gr of MOPS into 390 ml of DEPC-treated water. Dissolve and adjust the pH to 7.0 with sodium hydroxide. Add 5 ml of 0.5M EDTA (DEPC-treated) pH:8.0 and fill up to 500 ml with DEPC-water.
EDTA 0.5 M (pH:8.0)	Add 186.1 g of disodium ethylenediaminetetraacetate- $2H_2O$ to 800 ml of dH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH.

Ethidium Bromide (10 mg/ml)	Add 1 g of ethidium bromide to 100 ml of dH_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.
Formaldehyde gel loading buffer	Prepare appropriate amount that contains 50% Glycerol, 1 mM EDTA (pH:8.0), 0.25% Brom Phenol Blue and 0.25% Xylene cyanol.
TBE 10X	Dissolve 108 g Tris base and 55 g of Boric acid in 800 ml dH_2O. Then add 40 ml of 0.5 M EDTA and adjust volume to 1 liter with dH_2O.

For Polyacrylamide Gel Electrophoresis:

Acrylamide 30%	Dissolve 29 g of acrylamide and 1 g of N,N'-methylenebisacrylamide in a total volume of 60 ml of dH_2O. Heat the solution to 37^0C to dissolve the chemicals. Adjust the volume to 100 ml with dH_2O. Check that the Ph is 7.0 or less and sterilize the solution by filtration. Store the solution in dark bottles at room temperature.

Acrylamide 50%	Dissolve 49.5 g of acrylamide and 0.5 g of N,N'-methylenebisacrylamide in a total volume of 60 ml of dH_2O. Heat the solution to 37°C to dissolve the chemicals. Adjust the volume to 100 ml with dH_2O. Check that the Ph is 7.0 or less and sterilize the solution by filtration. Store the solution in dark bottles at room temperature.
SSCP Loading buffer	In an 50 ml falcon tube add the following in an appropriate volume: Formamide 95% (v/v), bromophenol blue 0,01 (w/v) and NaOH (final conc. 10 mM). Store at -20 °C.
Ammonium persulfate 10%	For fresh use: Dissolve 0.1 g of ammonium persulfate with 1 ml dH_2O.
For RNA Extraction:	
Denaturation Solution (stock)	Prepare a stock solution by dissolving 250 g guanidium thiocyanate in a solution of 293 ml dH_2O, 17.6 ml of 0.75 M sodium citrate, pH:7.0, and 26.4 ml of 10% Sarkosyl at 60°C to 65°C with stirring. The stock solution can be stored up to 3 moths at room temperature.

Denaturation Solution (working)	Prepare working solution by adding 0.35 ml of 2-ME per 50 ml of stock solution. The working denaturing solution can be sotred 1 month at room temperature.
Phenol, water-saturated	Dissolve 100 g phenol crystals in dH_2O at 60°C to 65°C. Aspire the upper water phase and store up to 1 month at 4°C
Sodium acetate 2M	Add 16.42 g of sodium acetate (anhydrous) to 40 ml dH_2O and 35 ml of glacial acetic acid. Adjust solution to pH:4.0 with glacial acetic acid and the final volume to 100 ml with dH_2O. The solution is 2 M with respect to the sodium ions.
Sodium dodecyl sulfate 10% (SDS)	Dissolve 100 g of electrophoresis-grade SDS in 900 ml of dH_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 liter with dH_2O.

2.1.4. Plastics and disposables

1.5 ml Eppendorf tubes	Eppendorf
2.0 ml Eppendorf tubes	Eppendorf

0.2 ml PCR tubes	Perkin Elmer
10 ul white pipette tips	LP
200-1000 ul blue pipette tips	LP
5-200 ul yellow pipette tips	LP
Falcon tubes (50 ml)	Costar
Falcon tubes (15 ml)	Costar
Gloves	Beybi plastics
Parafilm	American National Can
Sterile plastic pipettes	LP
Bottle filter system (500 ml)	Costar
75 cm^2 straight neck flask	Costar
162 cm^2 straight neck flask	Costar
225 cm^2 straight neck flask	Costar

2.1.5. Apparatus

APPARATUS:	PRODUCER:
Φ40 pH meter	Beckman
Axiovert 25 Microscope	Zeiss
Biofuge *pico* bench centrifuge	Heraeus instruments
CS-15R Centrifuge	Beckman
DU640 Spectrophotometer	Beckman

EC175 Adjustable vertical gel system	E-C apparatus corporation
EC250-90 Power supply	E-C apparatus corporation
EC350 Midicell Electrophoretic gel system	E-C apparatus corporation
EC370M Minicell Electrophoretic gel system	E-C apparatus corporation
Geneamp PCR system 9600 thermal cycler	Perkin-Elmer
Locator 6 *plus* Liquid nitrogen tank	Thermolyne
Micro chef microwave	Moulinex
Mirak ™ magmetic stirrer	Thermolyne
Oligo 1000M DNA synthesizer	Beckman
Pipette 1000 ul	Eppendorf
Pipette 20 ul	Eppendorf
Pipette 200 ul	Eppendorf
Slab gel drier SGD2000	Savant
SpeedVac SC110	Savant
Tempette™ TE-8D water bath	Techne
The butterfly Cell House 170	Heto

2.2. METHODS

2.2.1. Growth Medium Preparation

DMEM is prepared from 10X concentrated stock solution as follows:

800 ml	dH_2O
100 ml	10X DMEM

49.3 ml Sodium bicarbonate 7.5% (pH:7.3)

fill up to 1 liter with dH_2O

At that stage, DMEM solution can be stored at 4°C.

Add:

10 ml non-essential amino acids

10 ml penicillin/streptomycin

10 ml L-glutamine (200 mM)

100 ml Fetal calf serum

Sterilize the solution by filtration and store at +4°C.

DMEM solution at that state can be used up to 30 days.

2.2.2.Cyropreservation of stock cells and thawing frozen stock of cells

About 1-2x10^6 of cells were recovered by 10 ml of DMEM after trypsin treatment. They were then spinned at 1500 rpm for 5 minutes. After washing with 10 ml of PBS, cells were again spinned down at 1500 rpm for 5 minutes. 2 ml of F-medium containing 80% DMEM, 10% DMSO and 20% FCS were added and suspension was transferred into freezing vials. Cells were stored at -70 °C for one day and then transferred into liquid nitrogen for cyropreservation.

In order to achieve rapid thawing, vials containing frozen cells were transferred directly to a 37°C water bath without submerging the cap. Once thawed, vials were then sterilized by swapping the outer surface with 70% ethanol. The contents were transferred into a sterile plate containing pre-warmed, fresh growth medium with the help of a pipette and plated into small flasks. Thawed cells were examined daily using a microscope and subcultured as soon as confluency was reached.

2.2.3. Subculturing of cells

Old medium was removed by the help of an aspirator and cells were washed with 1-3 ml of PBS. In order to detach the cells from the plate, 0.5-1 ml of trypsin was added onto the plate and the plate was incubated at 37 ^{0}C for 2-3 minutes to quicken the detachment. Cells were then examined under microscope to make sure that all the cells were efficiently detached. DMEM was added to a new plate that was sterile and labeled (8 ml for 75 cm^2 plates, 25 ml for 225 cm^2 plates). 2-3 ml of DMEM was placed over the detached cells. After the cells were properly suspended with newly-added medium, they were removed, and placed into the new plate.

2.2.4. Total RNA Preparation

Guanidium thiocyanate total RNA isolation method was used (Adapted from Chomczynski P., Sacchi N., 1987). The procedure is as follows:

- Cells were washed with 10 ml PBS two times
- 4 ml of denaturation solution was added
- Cell lysis is aided by mixing with pipette.
- Lysate was then transferred into 15 ml sterile tube
- 0.1 volume of 2M sodium acetate was added
- Mixture was aliquoted into four tubes (4 X 1 ml)
- 1 ml of water-saturated phenol was added and mixed
- 0.2 volume of 49:1 chloroform/isoamyl alcohol was added and mixed
- Tubes were stored at 4^{0}C for 15 minutes, being resuspended frequently

- Tubes were then centrifuged at 10.000 rpm for 15 minutes at 4°C.
- Aqueous phase was taken to a fresh tube
- 1 volume of 100% isopropanol was added and mixture was stored at -20°C overnight
- Samples were centrifuged at 10.000 rpm for 10 minutes at 4°C
- Supernatant was discarded, pellet was dissolved in 300 ul of denaturation solution
- Precipitation was carried out using 300 ul 100% isopropanol
- Samples were stored again at -20°C overnight
- Tubes were centrifuged at 10.000 rpm for 15 minutes at 4°C
- After discarding the supernatant, pellet was dissolved in 75% EtOH
- Tubes were mixed and left for 10-15 minutes at room temperature
- They were then centrifuged again at 10.000 rpm for 15 minutes at 4°C
- Pellet was dried with Speedvac
- Finally, pellet was resuspended in 100-200 ul DEPC-treated water
- Samples were aliquoted and were stored at -70°C by adding 1 ml of EtOH and 10 ul of sodium acetate.

2.2.5. Electrophoresis of RNA

Electrophoresis of RNA was performed according to the procedure from Molecular Cloning (Sambrook et al., 1989) in which total RNA was loaded and run on the agarose gel containing formaldehyde. This method was adapted from those of Lehrach et al. (1977), Goldberg (1980) and Seed (1982).

2.2.5.1. Formaldehyde-containing 1% Agarose Gel

After the casting step, 11 ml of 5X formaldehyde gel running buffer, 35 ml of DEPC-treated water and 0.5 g of agarose were added into a beaker and the solution was heated in microwave oven until the agarose was melted completely. When the solution cooled down to approximately 65°C, 10 ml of formaldehyde was added and the mixture was poured into the casted apparatus.

2.2.5.2. Sample Preparation

Extracted total RNAs were diluted to 5ug/ul with DEPC-treated water. Into fresh tubes:

4.5 ul	RNA
2.0 ul	5X formaldehyde gel running buffer
3.5 ul	formaldehyde
10.0 ul	formamide

were added and the tubes were incubated at 65°C for 15 minutes for the denaturation of RNA, chilled on ice for 5 minutes and instantly centrifuged . Samples were then loaded into the corresponding wells on the gel with the addition of 2.0 ul formaldehyde gel loading buffer.

Electrophoresis was then carried out at 70 V for 2 hours at room temperature.

2.2.5.3. Staining and Visualization

After the run, the gel was put into the 0.1 M ammonium acetate containing 0.5 ug/ml ethidium bromide for 30-45 minutes. Then the gel was destained with DEPC-treated water for at least 30 minutes and the gel was visualized under UV light.

2.2.6. cDNA Preparation

For cDNA preparation, Promega Reverse Transcription System was used. According to the original protocol, into each tube:

1 ug	RNA
4 ul	$MgCl_2$ (25 mM)
2 ul	Reverse transcription 10X buffer
2 ul	dNTP mixture (10 mM)
0.5 ul	Recombinant Rnasin© Ribonuclease Inhibitor
15 u	AMV Reverse Transcriptase
0.5 ug	Oligo$(dT)_{15}$ Primer

and nuclease-free water was added to a final volume of 20 ul.

The reaction was incubated at 42°C for 15 minutes and the AMV Reverse transcriptase was inactivated by heating the sample at 99°C for 5 minutes followed by a 5 minute incubation at 0-5°C.

2.2.7. PCR Amplification

The PCR which was invented by Kary Mullis (Mullis et al., 1987) is an *in vitro* method for enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers.

In this study, in order to amplify the p73 cDNA or gene fragments this method was applied by using different sets of primers given in table 2.1. and 2.3.

A standard 50 ul reaction set up in a 0.2 ml PCR tube with the amounts listed below:

Primers (10 pmol/ul):	1 ul of each primer (reverse and forward)
dNTPs (10 nM):	1 ul
Buffer:	5 ul from the 10X stock
$MgCl_2$ (25 mM):	3 ul
Template DNA:	1 ul of cDNA
Enzyme:	3 unit of Taq Polymerase enyzme
MiliQ water:	adjusted to the final volume of 50 ul.

In order to amplify the desired region, a temperature profile was set to be:

Initial denaturation:	95 ^{0}C, 3 minutes
For 30 cycles:	
Denaturation:	95 ^{0}C, 30 seconds,
Primer annealing:	60 ^{0}C, 30 seconds,
Primer Extension:	72 ^{0}C, 30 seconds,
For final extension:	72 ^{0}C, 10 minutes
Termination:	4 ^{0}C

2.2.8. Agarose Gel Electrophoresis

To check the amplification status, the amplified fragments are tested with the Agarose Gel Electrophoresis (AGE) technique. In this technique, within the linearly

Table-2.1.: Synthetic oligonuclotide primers used for RT-PCR analysis

Name	Sequence 5'→ 3'	Location Strand Position*		Size	Tm ^{0}C
GA317	cggaattcac caccatcctg	sense	850	20	63.5
TC160	gcgcgagttc tactgctgcg cggtgctgta	antisense	1790C	30	80.5
GA313	tcctttgagg gccgcatctg	sense	975	20	68.0
TC162	gcgcgaattc tcagtggatc tcggcctccg	antisense	2012C	30	78.3
TC183	cgcacagttc ggcagctaca	sense	1417	20	65.8
TC182	gtttggcaccc ccaatcctgt	antisense	1617C	20	67.4
TC406	agggccccca ggtcctgacg	antisense	1696C	20	72.2
TC382	tgaaggtcgg agtcaacgga tttggt	sense	-	26	71.4
TC383	catgtgggcc atgaggtcca ccac	antisense	-	24	73.9
TC226	tgcagccaaa gtgaagaggg aaga	sense	-	24	68.5
TC227	catagcgagc agcccaaaga agaa	antisense	-	24	67.8
TC224	cacagcattc cttcagttta ctggagatcg	sense	-	30	70.2
TC225	cagcatcgat cccacttttc caagttccag	antisense	-	30	74.6

* Denotes the base pair positions of primers on the p73α cDNA sequence

polymerized medium like agarose and a suitable electrolytic buffer, charged molecules such as DNA or RNA are oriented and migrate towards the corresponding attractive node when electric current is applied into the system. Since charge per unit mass is the same for these molecules, each DNA or RNA fragment is migrated towards the opposite pole with the rate proportional to its molecular mass, that is, the longer the fragment , the slower the migration rate, resulting the effectively separation of the molecules that differ in size. Moreover, by changing the concentration of the agarose, one can change the effective range of separation in the electrophoresis system as shown in the table 2.2.

Table 2.2.: Agarose concentration vs. effective range of separation of DNA

Effective range of separation of DNA (in kb)	Amount of agarose (w/v)
5-60	0.3
1-20	0.6
0.8-10	0.7
0.5-7	0.9
0.4-6	1.2
0.2-3	1.5
0.1-2	2.0

Generally TAE (Tris-acetic acid-EDTA) and TBE (Tris-Boric acid-EDTA) buffers are used as conductive media. TBE buffer is recommended for high voltage and short runs whereas TAE buffer is most suitable for the procedures that require low voltage and long run time.

In this study, TBE buffer was used as an electrolyte with the final concentration of 1X. Agarose, which is produced as powdered form form the seaweed, was added to the 1X TBE containing flask and is heated until all the agarose powders were completely dissolved as the mixture gets transparent. Heated mixture was allowed to cool down to approximately 60^{0}C, then appropriate amount of ethidium bromide dye (10 mg/ml) was added into the heated agarose solution. After mixing and evenly distributing the dye in the mixture, the gel was poured into the previously-set gel casting system defined by the comb and the clamps. The gel was solidified and ready to be loaded. After the solid gel was removed from the casting apparatus, the comb was removed, leaving the wells in which the DNA in question was loaded. Samples were mixed with the loading buffer containing xylene cyanol and bromo phenol blue and loaded into the wells.

A typical gel run was carried out at 80-110 V . After the run, bands corresponding to the expected products were visualized under the UV illluminator.

2.2.9. Isolation of genomic DNA

As stated earlier, tumor DNA samples were extracted previously by phenol/chloroform DNA extraction method and stored at -20 °C.

2.2.10. PCR amplification of p73 gene

The basis and the parameters of this method described in part 2.2.7. were also the same for the amplification of p73 exonic fragments except genomic DNA was used as a template. The exon-specific primers used in this study are outlined in table 2.3.

Table 2.3. Synthetic oligonucleotide primers of p73 DBD exons used for heteroduplex and radioactive SSCP analysis

Name	Sequence 5'→ 3'	Location Strand	Position*	Size	Tm °C
GA307	ctccccgctc ttgaagaaac	sense	542	20	61.1
GA322	tgtccttcgt tgaagtccct	antisense	721C	20	59.5
GA323	ctgctccagc cagccacctc	sense	733	20	68.1
GA324	accacgacgc tctgcctggcc	antisense	814C	21	73.4
GA317	cggaattcac caccatcctg	sense	850	20	63.5
GA318	actcccgcat ctccagg	antisense	959C	17	59.4
GA313	tcctttgagg gccgcatctg	sense	975	20	68
GA320	gcacgcttgc tggcggcccc	antisense	1087C	20	78.4

2.2.11. Agarose Gel Electrophoresis

The basis of this method is explained in part 2.2.8. and no longer will be discussed here. In this study, amplifications were visualized in 2% agarose gel.

2.2.12. Non-radioactive Heteroduplex Analysis

The basis of this technique depends on the fact that two complementary DNA strands derived from alleles that differ in sequence will include mismatched positions when base paired. Such double-stranded heteroduplex molecules may show altered migration in native gels when compared to homoduplexes of either allele. (White et al.,1993). With this concept, heteroduplex analysis has been applied for most of the mutation detection experiments so far. The method was further modified and the use of ^{32}P was replaced with ethidium bromide fluorescence to detect re-annealed heteroduplexes (Xing et al., 1996).

In this study, we applied this technique in order to detect possible mutations in the p73 DNA-binding domain.

10% Non-denaturing Polyacrylamide Gel is prepared as follows:

30% Acrylamide stock :	16.7 ml
Glycerol:	5.0 ml
10X TBE:	5.0 ml
Agarose:	250 mg
dH_2O:	23.3 ml
APS:	350 ul
TEMED:	20 ul
Final Concentration:	50 ml

In an appropriate flask, the indicated amounts of 10X TBE, Glycerol, dH_2O and agarose were added and put into microwave in order to melt the agarose. After the

solution cooled down to 65°C, Acrylamide stock , APS and TEMED were added, mixed and the resulting solution was poured into previously casted apparatus.

In order to prepare the samples, equal amounts of amplified products from control and tumor samples were put into a 0.2 ml PCR tube. Denaturation has carried out as described by Xing et al. :

95 ° C	8 min.
90 ° C	1 min.
80 ° C	1 min.
70 ° C	1 min.

Room Temperature

After the samples have been loaded properly, PAGE was set to run at 200 V for 10 minutes, 180 V for 60 minutes and 140 V for 22 hours. After the removal of the gel from the apparatus, ethidium bromide staining was applied. Gel was put into distilled water containing 0.5 ug/ml ethidium bromide for 40 - 60 minutes and then visualized under UV light.

2.2.13. Radioactive SSCP analysis:

The basis of the SSCP technique is that: Two single-stranded DNA molecules from each denatured PCR product assume a three dimensional conformation which depends on the primary nucleotide sequence. So, a sequence change that exist on one or both strands may result in the mobility difference of single-stranded DNA molecules.

In this study, 25 ul of PCR reaction was set with the following amounts:

10X PCr buffer:	2.5 ul
dNTP (10 mM):	0.1 ul
$MgCl_2$ (25 mM):	1.5 ul
Primer (10 pmol) forward:	1.0 ul
Primer (10 pmol) reverse:	1.0 ul
Taq DNA Pol (4u/ul):	0.5 ul
^{32}P dCTP:	0.01 ul
Template DNA:	1.0 ul
ddH_2O:	complete to 25 ul

The same temperature profile explained in section 2.2.7. was used. The amplified products were mixed (18ul loading buffer/2 ul PCR product) with SSCP-loading buffer containing 95% (v/v) formamide, 0.01% (w/v) bromophenol blue and NaOH (10 mM). Single-strand products were obtained by incubating them at 95 ^{0} C for 5 minutes and then immediately placing them in the ice for 5 min.

Prepared samples were loaded into polyacrylamide gel that was prepared as follows:

10X TBE:	5 ml
Acry/bisacry.(49.5%/0.5%):	6 ml
Glycerol:	5 ml
APS:	0.7 ml

TEMED:	30 ul
Water:	up to 50 ml

PAGE was performed at 200V. Running time was between 6 and 8 hrs depending on the size of the product. After the run, the gel was dried and placed in a casette for autoradiography.

2.2.14. Restriction Enzyme analysis:

In order to screen for p73 mutations at residues homologous to p53 mutational hotspots, restriction enzyme analysis was done.

In restriction enzyme analysis (REA), known sequences are digested with restriction enzymes that specifically recognize and cleave the sequence. Since any base change in the sequence abolishes the activity of enzyme, resulting digestion pattern can reveal the nature of the sequence. If there is an alteration (if ATGCTA is changed to ATTCTA e.g.), the enzyme can not recognize the sequence and the product remains undigested. After the digestion products are resolved in an agarose gel, the migration pattern can give information about the digestion status of the product, hence the nature of the sequence.

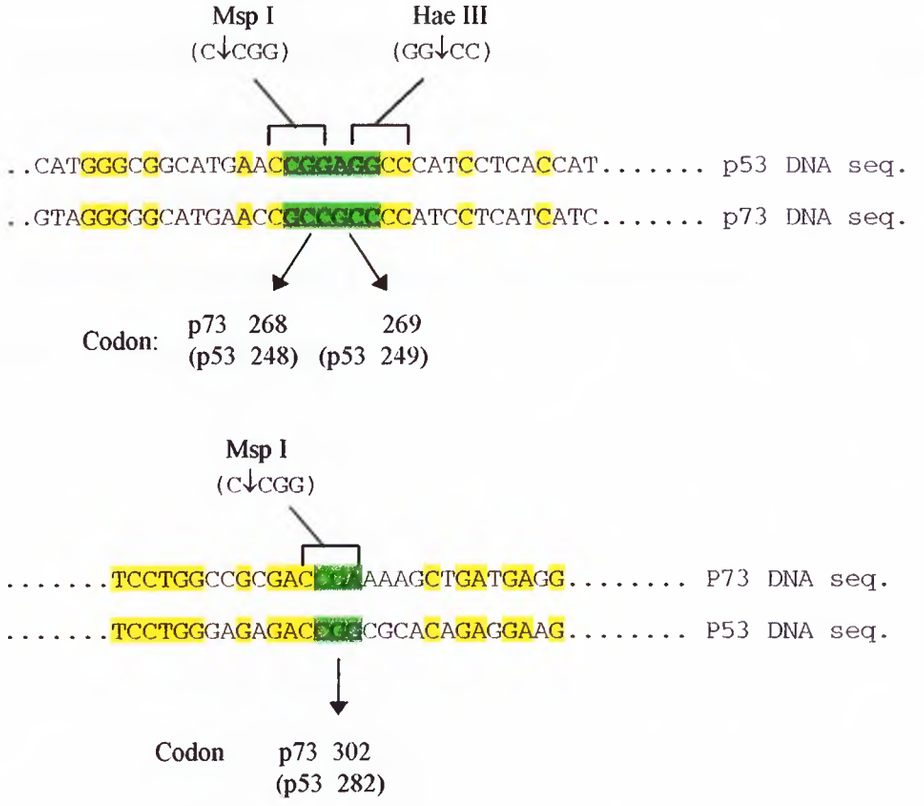
Restriction enzyme analysis was performed by Kezban Unsal using Msp I and Hae III enzymes. It was possible to screen for possible mutations at codons 268 (homolog of p53-248), 269 (homolog of p53-249) and 302 (homolog of p53-282) of p73 gene (Figure 2.1). A typical digestion reaction was prepared in a total volume of 30 ul as follows:

p73 gene (Figure 2.1). A typical digestion reaction was prepared in a total volume of 30 ul as follows:

DNA template:	20 ul
10X RE buffer:	3 ul
Enzyme:	1 ul
dH_2O:	6 ul

Resulting digestion fragments are resolved in 2.5% agarose-NuSieve agarose gel.

Figure 2.1: Mutational hotspots that are conserved also in p73 and the enzymes used for the REA:



2.2.14. Direct DNA sequencing

DNA sequencing (DS) refers to the direct sequence analysis by PCR products without prior sub-cloning into sequencing vectors. All the methods are capable of detecting mutations with varying efficiencies, but none defines precisely the nature of the change except direct DNA sequencing. In this method, the template, which is a previously amplified PCR product, is simultaneously amplified and sequenced by the addition of dideoxy terminators to a PCR reaction. Fluorescently labeled dideoxy terminators with different fuorescent dye-coupled to each of the four ddNTPs, the sequencing product is labeled at the site of the dideoxy termination rather than at the sequencing primer end. The bands giving different colors and different peak values are analyzed by an automated sequencing machine resulting the primary DNA sequence. These analyses were performed by Marie Ricciardone and Hilal Ozdag. The fragments previously amplified were prepared for the sequencing by using ABI PRISM™ Dye terminator cycle sequencing core kit and 3.2 pmol of exonic forward and reverse primers previously shown in table 2.3.Then, samples were sequenced with the help of ABI PRISM™ 377 DNA sequencer.

3-RESULTS

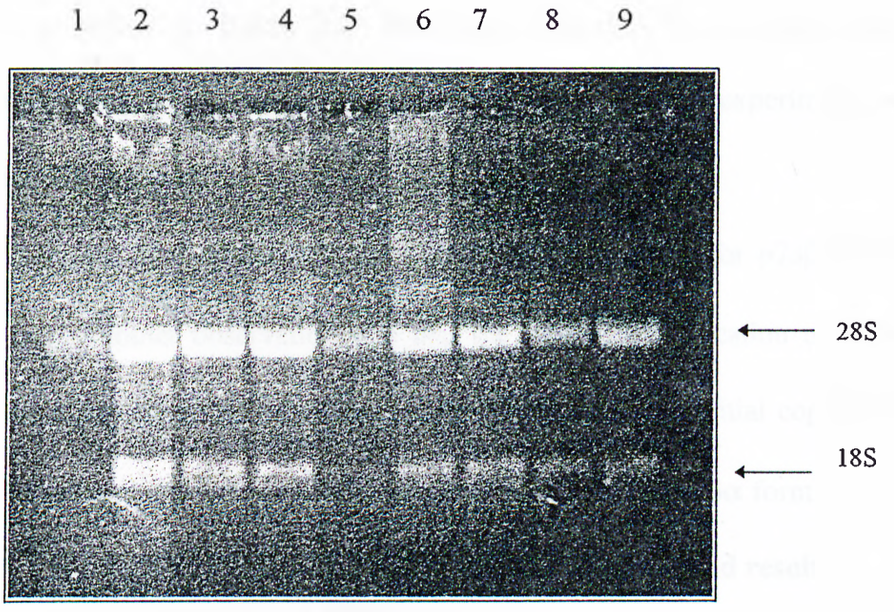
3.1. The expression of p73 in HCC cell lines

p73 gene, which is a newly-discovered p53 homolog, has two distinct alternatively spliced forms namely p73α and p73β. Former one encodes a protein product that is 636 amino acids long whereas the latter results from alternative splicing that skips exon 13 hence creates a truncated 499 amino acids-long protein. So, these two forms are almost identical except the last 5 residues of p73β (Kaghad et al., 1997). It has been reported that these two forms are expressed in all the human tissues tested including brain, kidney, placenta, colon, heart, liver, spleen and skeletal muscle, indicating a widespread but low level of expression of these forms (Kaghad et al., 1997). In transfection experiments p73α form was found to induce p21/WAF1 gene transcription (Kaghad et al., 1997) and apoptosis (Just et al., 1997). Although it has also been shown that these two forms differ in their homotropic interaction and interaction with p53 in yeast two hybrid assays (Kaghad et al., 1997), there are no data so far that explains their mode of regulation and possible implications in cellular mechanisms. Kaghad and his coworkers examined the expression of these forms in neuroblastoma-derived and several other cell lines including HT-29 (colorectal cancer cell line) and MCF-7 (breast cancer cell line). The loss of both α and β transcript was shown in SK-N-AS neuroblastoma cell line (Kaghad et al.,1997). At the time we began this study, there was no other published information about the expression pattern and the possible implications of these forms in normal and malignant cells. So we aimed to analyze the expression of these two alternatively-spliced forms in liver and HCC-derived cell lines.

Eight different cell lines and one control cell line, HT-29 that was reported to express both forms (Kaghad et al., 1997), were chosen and grown under the conditions explained in materials and methods part.

Total RNA was extracted and tested for integrity by gel electrophoresis. Formaldehyde-containing 1% agarose gel was prepared and samples were run under 70 V for 2 hours. As shown in figure 3.1., all RNA samples were good quality. There was no degradation.

Figure 3.1. Total RNAs as tested by Et-Br staining



Lanes: Total RNAs extracted from cell lines: 1: HT29, 2: Hep3B-TR, 3: Hep3B, 4: Mahlavu, 5: Chang, 6: WRL68, 7: FOCUS, 8: HepG2 and 9: PLC/PRF/5 respectively.

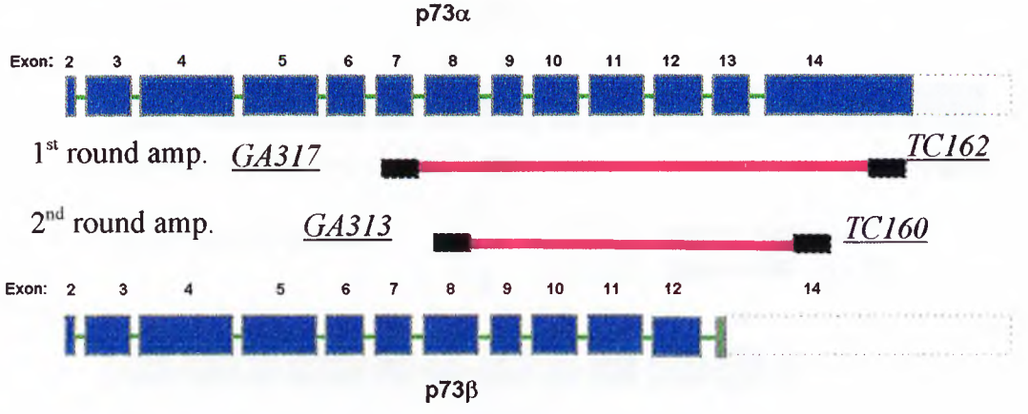
3.1.1. Establishment of a new method for combined analysis of alternatively spliced p73 transcripts

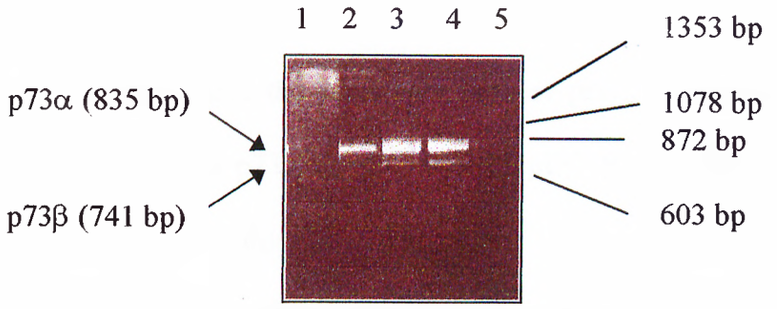
cDNAs corresponding to each cell line was synthesized using Promega Reverse transcription system with 1 ug of extracted RNAs.

In this study, in order to analyze the alternatively-spliced forms of p73, we first applied nested-PCR technique with the primer pairs GA317-TC162 (Exon 7 forward-exon 14/2 reverse) for the first round and GA313-TC160 (Exon 8 forward-exon 14/1 reverse) for the second round of amplification. For the second round PCR, 1 ul of the first round product was used as a template. The resulting band pattern for three cell lines is shown in figure 3.2. We chose nested-PCR technique since no bands corresponding to p73 forms were observed in the control experiments with a single round PCR reaction.

In the control amplification, we observed no band for p73β form in Mahlavu cell line. Another observation was that the intense amplification of p73α form with respect to the other, probably due to the difference in the initial copy number of these two forms or the initial preference of the primers towards p73α form.

Figure 3.2.: Nested-PCR amplification diagram and results

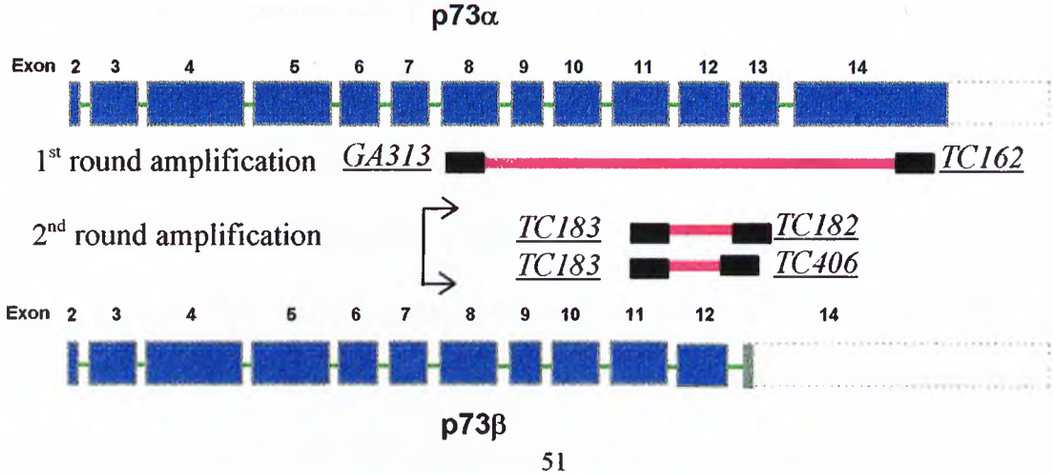




Lanes: 2nd round amplification results. Lane 1 is a negative control; lane 2 to 4 are RT-PCR results using cDNAs of Mahlavu, HepG2 and Hep3B cells. Lane 5 is ϕX174/Hae III marker. The four bands seen correspond to 1353, 1078, 872 and 603 bp respectively.

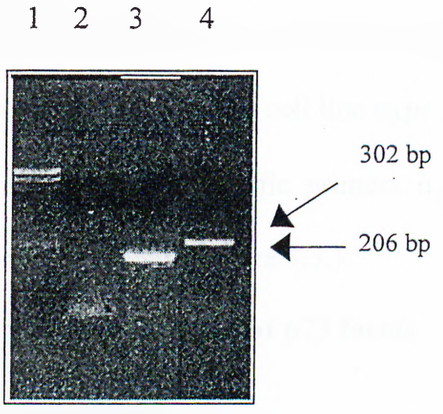
So, in order to increase both the specificity and sensitivity of the technique, we established a new method to analyze these alternatively-spliced forms. Two transcript-specific antisense primers were designed for each p73 variants. Exon 13 is missing in p73β form, so one of the primers were selected form exon 13 sequence, being specific for p73α form. The other was chosen from the exon12-14 boundary since in p73β transcript exon12 is spliced with exon14. The schematic illustration of these primers is shown in figure 3.3.

Figure 3.3. Amplification diagram of p73 forms.



In order to check the specificity of these primers, a standard PCR reaction was performed by using a p73α cDNA clone in pUC19 as a template. The result of this amplification is seen in figure 3.4.

Figure 3.4.: Specificity test of p73α and p73β-specific PCR primer



Lanes: Lane 1 is ϕX174/Hae III marker; 2 is negative control; 3 is amplification with p73α-specific primers (206 bp); and lane 4 is amplification with p73β-specific primers.

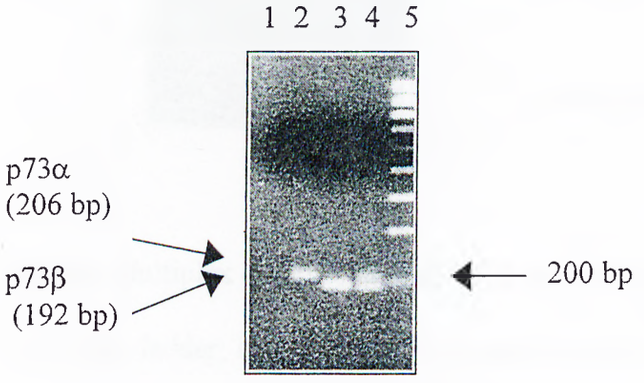
The expected sizes of p73α and p73β PCR products are respectively 206 and 192 bp. p73α-specific PCR reaction yielded a 206 bp product from the p73α cDNA template. We also expected that p73β-specific primer pair does not give a 192 bp product because the template is p73α cDNA. As expected, p73β-specific PCR did not produce a 192 bp fragment. Instead, a 302 bp fragment was amplified. This fragment is probably a product of non-specific priming with the p73β specific primer. This is probably due to the fact that we used a cloned cDNA (at high levels) as a template.

Next, we tested both primer pairs using Hep3B cDNA as a template. As a 1st round PCR, both forms of p73 cDNAs were amplified by using GA313 (exon 8

forward) and TC 162 (exon 14 reverse) primers and 1 ul of Hep3B cDNA. In the 2nd round, 1 ul of the 1st round product, one common forward primer (TC183 from exon 11), two transcipt-specific reverse primers (TC182 from exon 13 of p73α and TC406 from exon 12-14 boundary of p73β) were used together.

In that manner, we were able to detect both α and β-specific PCR fragments in Hep3B cDNA. This was expected because this cell line express p73β transcripts (Figure 3.2.).When using both p73α and p73β-specific primers in the same test tube, both transcripts were detected simultaneously (Figure 3.5.).

Figure 3.5.: Multiplex amplification of p73 forms

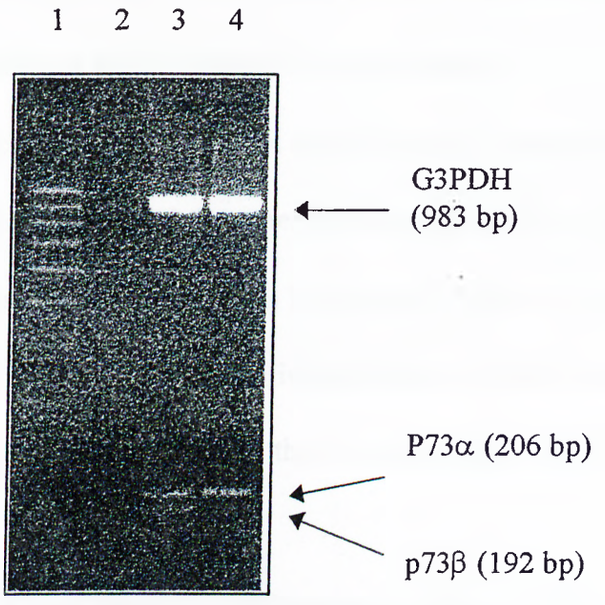


Lanes: Multiplex amplification of p73 forms. Lane 1: Negative control; lane 2: p73α; lane 3: p73β; lane 4: multiplex amplification of p73α and β forms in the same reaction; lane 5: marker (100 bp ladder).

As a next step, we included glucose-3-phosphate dehydrogenase (G3PDH) primers TC382 and TC383 (10 pmol for each) into the 1st and the 2nd round PCR

reaction mixes. The amplification level of this gene was used as an internal control. The result was shown in figure 3.6.

Figure 3.6. Multiplex amplification of p73 forms and G3PDH



Lanes: Multiplex amplification of p73 forms and G3PDH internal control. Lane 1: 100 bp ladder; lane 2: Negative control; lane 3: 2nd round amplification using HT29 cDNA as a template; and lane 4: 2nd round amplification from Mahlavu cDNA as a template.

It is important to note that with this new method, with the help of G3PDH amplification, we were able to assess the quality control of the cDNAs and the semi-quantitative expression level of the p73 forms since each alternatively-spliced variant has its own specific primer, indicating the initial expression level of these two forms. So, although this new method requires two consecutive PCR amplifications, the

resulting multiplex nature allowed us to analyze the expression of both forms in both qualitative and quantitative manner.

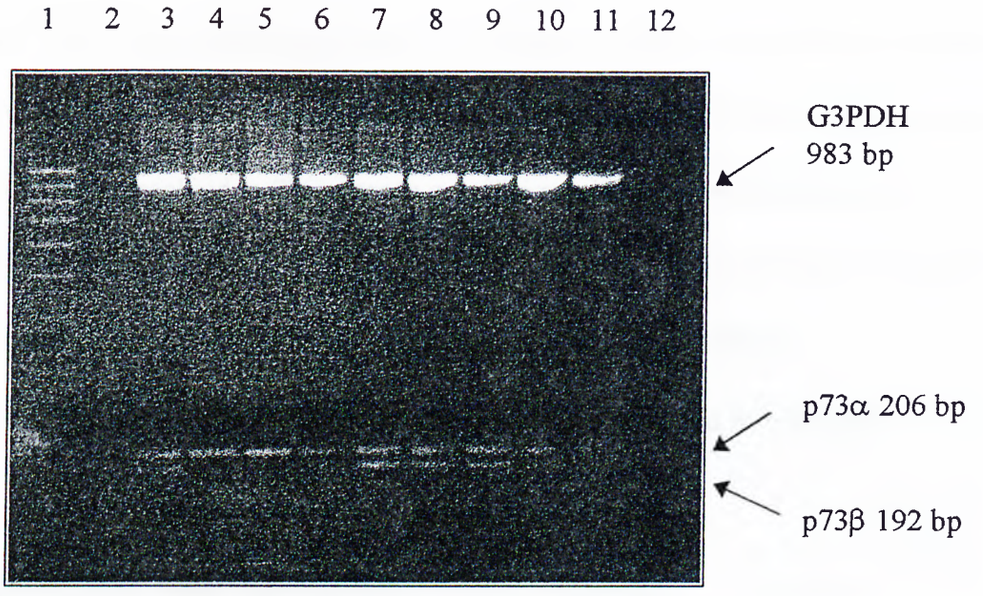
3.1.2. p73α and p73β form in HCC and liver-derived cell lines

Then, we applied this new method in HCC and liver-derived cell lines. Figure 3.7. shows the result of the 2nd round PCR amplification.

In this picture, It is clearly seen that all the cells tested showed p73α expression, irrespective of their origin and the other criteria that are shown in table 3.1., indicating the constitutive expression of this form in these cell lines. On the other hand, p73β was not detected in 3 out of 8 HCC and liver-derived cell lines, namely WRL68, FOCUS and Mahlavu. These studies showed that the expression of p73 could be affected in HCC.

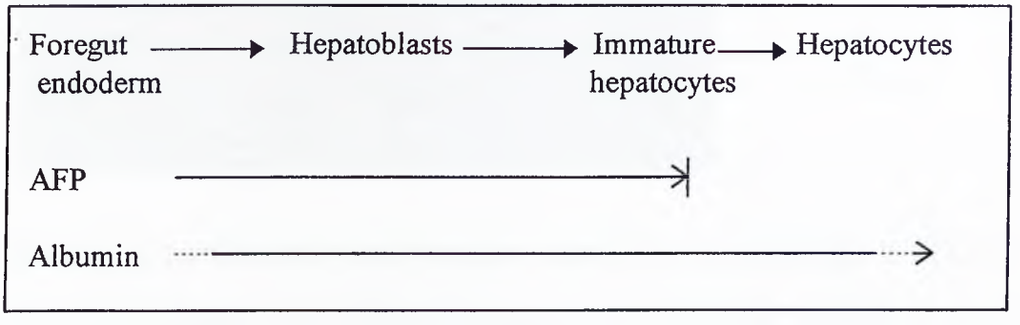
We next tested the differentiation status of cells using two markers α-fetoprotein and albumin. AFP and albumin are used as liver-specific markers. Precursor cells from foregut endoderm starts to produce AFP at about 10 days. The major 2.1 kb AFP mRNA is abundantly expressed in fetal liver. This expression ceases when mature hepatocytes are formed. Albumin expression is also seen at about the same time as AFP or a day later. This expression then gradually increases after birth. This gene is continuously expressed during the life time of hepatocytes and it is the most abundant mRNA expressed only in liver (Figure 3.8).

Figure 3.7. RT-PCR results of two p73 forms.



Lanes: Lanes 1 and 12 are 100 bp DNA size marker. Lane 2 is negative control. Lanes 3 to 11 corresponds to amplifications from cell lines HT29, Mahlavu, FOCUS, WRL68, PLC/PRF/5, Chang, HepG2, Hep3B and Hep3B-TR respectively.

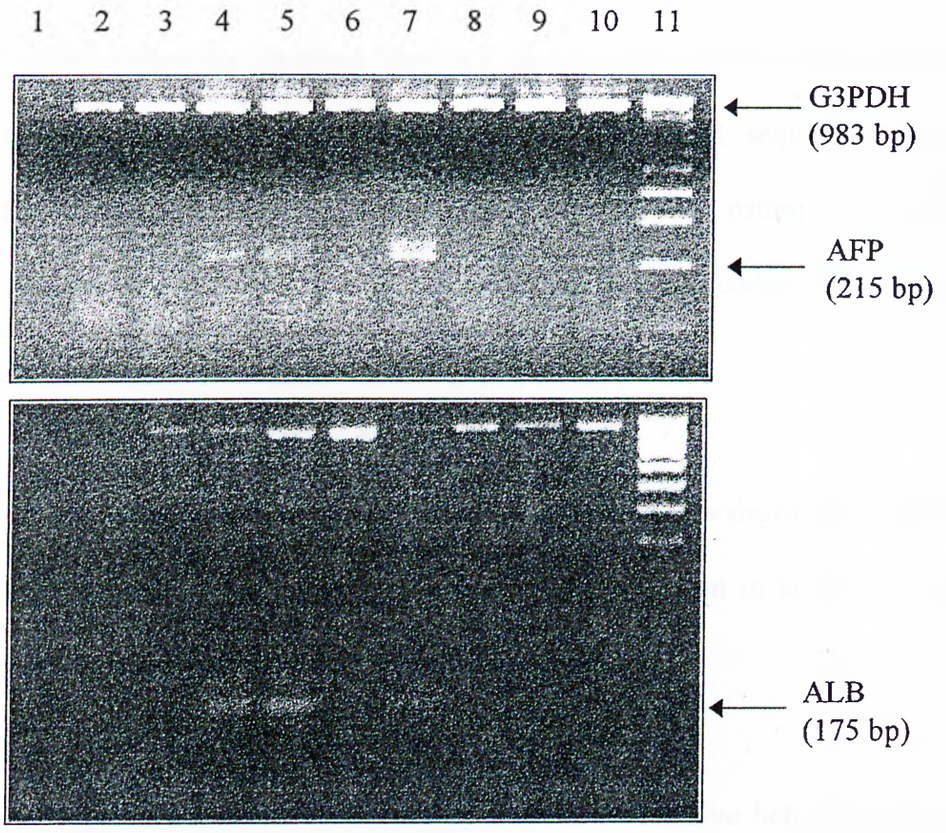
Figure 3.8.: AFP and albumin expression during hepatocyte development



In our study, two separate two-round-PCR was performed by using primer pairs TC226 and TC227 for α-fetoprotein and TC224 and TC225 for albumin. G3PDH primers (TC382 and TC383) were also included in both rounds of PCRs. The resulting amplifications were resolved in 2% agarose gel. The results are shown in figure 3.8.

we observed amplification of both markers in three cell lines Hep3B, Hep3B-TR and HepG2. Next, we studied primary tumors for possible mutations.

Figure 3.9.: Amplification of α-fetoprotein and albumin in cell lines



Lanes: Amplification of liver markers. Lane 1: Negative control; 2: HT29; 3: Chang; 4: Hep3B; 5: Hep3B-TR; 6: Mahlavu; 7: HepG2; 8: WRL68; 9: Focus; 10: PLC/PRF/5; and 11: Marker (100 bp ladder)

3.2. p73 gene mutation analysis in primary tumors

Due to its homology nature to p53, we next screened 25 primary HCC tumor samples with non-radioactive heteroduplex test and radioactive SSCP analysis for the DNA-binding region exons (exons 5, 6, 7, and 8) of p73 gene in which the greatest percentage of homology was shown in accordance with the conserved mutational hotspots that are frequently found to be mutated in variety of cancers. We have chosen non-radioactive HDA technique due to its convenient nature for the rapid screening of unknown mutations, although its sensitivity is reported to be around %70. By using HDA, we simultaneously screened the DBD-region exons for any possible alteration(s). At the same time, restriction enzyme analysis and direct sequencing were also performed in the department in order to detect any alteration pattern in the conserved codon 248, 249 and 282 (identical to p53 mutational hotspots codon 268, 269 and 302) of p73 gene.

3.2.1. PCR amplification of p73 exons

The primers, length of the PCR product and the figures showing the gel pictures for amplification of corresponding gene fragments are shown in table 3.1. Resulting fragments were resolved in 2% agarose gels.

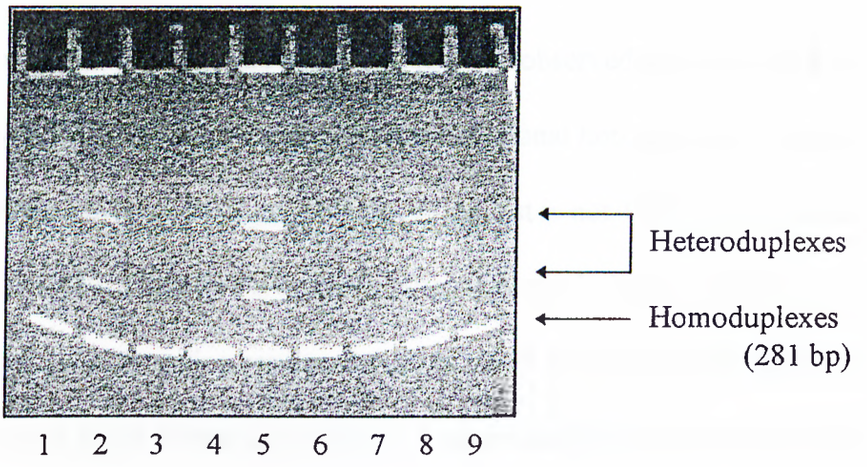
3.2.2. Results p73 mutation analysis

In order to check the sensitivity of the non-radioactive heteroduplex analysis technique, a sample gel was prepared and previously identified mutations were analysed. The result is illustrated in figure 3.10.

Table 3.1. Reference figures of p73 exonic amplifications

Exon:	Primers:		Product length:	Reference figure:
5	GA307 (F)	GA322 (R)	179 bp	Figure 3.11.
6	GA323 (F)	GA324 (R)	91 bp	Figure 3.12.
7	GA317 (F)	GA318 (R)	109 bp	Figure 3.13.
8	GA313 (F)	GA320 (R)	112 bp	Figure 3.14.

Figure 3.10: 10% non-denaturing PAGE



Lanes: Lanes 1, 4 and 7 is amplified from normal template; lanes 2, 5 and 8 has 4 bp deletion; and lanes 3, 6 and 9 has G→T transversion.

In this test gel, we were unable to detect a single base change that is G → T transversion whereas a sample with 4 bp deletion gave a good heteroduplex pattern. Of course, the rate of detecting single nucleotide changes greatly depends to the nature of the base change, and this can increase or decrease the sensitivity of the technique.

Samples that were amplified by PCR as described in section 2.2.12. were then analyzed in 10% non-denaturing polyacrylamide gels that were prepared as described in methods part. Gel pictures showing the heteroduplex analysis results of exons 5, 6, 7 and 8 for 9 tumors are shown in figures 3.15 through 3.18 as an example.

Table 3.2. shows the previously identified characteristics of the tumor samples and the p73 mutation analysis results. These samples were collected from Mozambique and other parts of the South Africa where HBV infection and daily exposure to mycotoxins are high. It has been previously reported that in these areas, codon 249 of p53 gene was found to be mutated with a high frequency (rewieved by Puisieux and Ozturk., 1997).

In our study, interestingly, no p73 alteration was observed for four exons that span the conserved DNA-binding domain and the mutational hotspots of p73 gene by non-radioactive heteroduplex analysis. This screening test is not 100% sensitive. Its sensitivity is about 70%. Therefore we used an alternative technique (radioactive SSCP) to confirm heteroduplex screening results. A total of 28 PCR fragments obtained by amplification of exon 5 and 6 from 20 tumors. An example of SSCP result is shown in figure 3.19. There was no band shift in any of these samples tested. As described in materials and methods part, Restriction Enzyme Analysis (REA) and DNA sequencing (DS) were also performed for these samples in our department by Ph.D. students Kezban Unsal and Hilal Özdağ respectively. The DNA nucleotides encoding codons that are homologous to mutational hotspots for p53 gene, namely codons 268, 269 and 302 (corresponding to codons 248, 249 and 282 of p53 gene) were analyzed and no

change that abolish the restriction enzyme site was observed in REA. For DNA sequencing, although several samples could not be sequenced, all the other samples gave the sequence identical to wild-type (Table 3.2.; for sequencing data, see appendix I.).

Taken together, these studies indicated that p73 gene is not frequently mutated in HCC. This contrasts with high frequency of p53 mutations. However, it is important to emphasize that we screened tumors only for mutations at the DNA binding region exons of p73 gene. In addition, DNA sequence analysis did not give clear data on all samples. We can exclude only the presence of mutations at codon 268, 269 and 302 of p73 which are homologous to p53 mutational hotspots 248, 249 and 282. It is of particular interest that we did not detect mutation at codon 269 of p73 which is homologous to codon 249 of p53 which is an HCC-specific mutational hotspot. For other codons of exons 5, 6, 7 and 8, we can say that the probabilty of wild-type sequence is high. In other words, we may have missed about 30% of possible mutation by the techniques used here. In addition, other exons of p73 (exons 1-4 and 8-14) were not tested. Although the p53 gene does not display high rate of mutation outside the DNA binding region (exons 5-8), this may not be the case for p73.

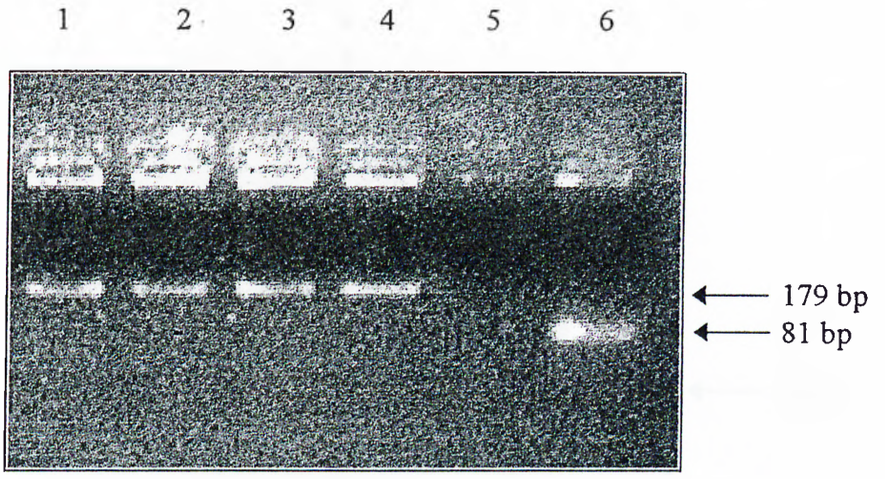
In conclusion, our observations indicate that 3 codons of p73 (268, 269 and 302) which are homologous to 3 of the five p53 mutational hotspots (codons 248, 249, 282) do not display mutations in 25 tumors tested. In addition, mutations at exons 5, 6, 7 and 8 of p73 appear to be rare or inexistent because a rapid mutation screening technique (heteroduplex analysis) failed to detect any mutations. However, p73 may display mutations in other exons which are not tested during this study.

Table 3.2.: Results mutation analysis of p73 DBD exons and conserved hotspots

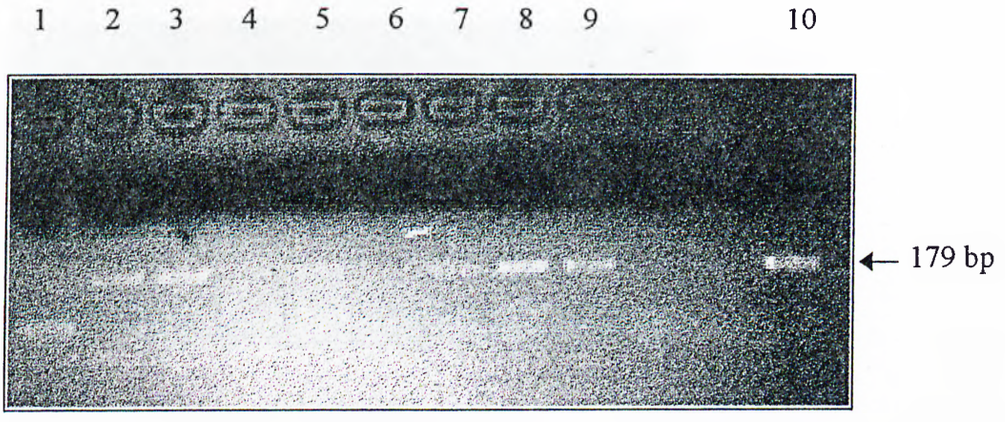
DNA (Country)	p53 mut.	Nucleic acid	amino acid	HBV status	HDA DBD exons				SSCP DBD exons		Mut. Hotspots					
					5	6	7	8	5	6	268		269		302	
											REA	DS	REA	DS	REA	DS
T47 (M)	157	GTC → TTC	Val → Phe	P	--	--	--	--	ND	--	--	--	--	--	--	ND
T15 (M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	ND	--	--	--	--	--	--	ND
K3T (M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	--	ND	--	--	--	--	--	ND
K11T(M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	--	ND	--	ND	--	ND	--	ND
K15T(M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	ND	ND	--	ND	--	ND	--	ND
T27(M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	--	ND	--	--	--	--	--	ND
T29(M)	249	AGG → AGT	Arg → Ser	N	--	--	--	--	--	ND	--	--	--	--	--	ND
T37(M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	--	--	--	--	--	--	--	ND
T55(M)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	--	--	--	--	--	--	--	ND
T13(M)	wt			P	--	--	--	--	--	--	--	--	--	--	--	ND
T51(M)	wt			P	--	--	--	--	ND	ND	--	--	--	--	--	ND
T9(M)	wt			P	--	--	--	--	--	ND	--	--	--	--	--	ND
K7T(SA-T)	249	AGG → AGT	Arg → Ser	P	--	--	--	--	ND	ND	--	ND	--	ND	--	ND
T43(SA-T)	286	8 bp del.	Frame shift	P	--	--	--	--	ND	ND	--	--	--	--	--	ND
T25(SA-T)	wt			N	--	--	--	--	--	--	--	ND	--	ND	--	ND
T31(SA-T)	wt			P	--	--	--	--	--	--	--	ND	--	--	--	ND
T33(SA-T)	wt			P	--	--	--	--	ND	--	--	--	--	--	--	ND
T23(SA-T)	wt			P	--	--	--	--	--	--	--	--	--	--	--	ND
T12(SA-T)	wt			N	--	--	--	--	ND	ND	--	--	--	--	--	ND
T19(SA-T)	wt			N	--	--	--	--	--	ND	--	ND	--	ND	--	ND
T49(SA-C)	wt			P	--	--	--	--	--	ND	--	--	--	--	--	ND
T41(SA-L)	wt			P	--	--	--	--	--	--	--	--	--	--	--	ND
T39(SA-S)	wt			P	--	--	--	--	--	--	--	ND	--	ND	--	ND
T8(SA-L)	wt			P	--	--	--	--	--	ND	--	ND	--	ND	--	ND
T17(SA-L)	wt			N	--	--	--	--	--	ND	--	ND	--	ND	--	ND

REA: Restriction enzyme analysis; HDA: Heteroduplex analysis test; SSCP: Single strand conformation polymorphism test; ND: Not done; DS: DNA sequencing

Figure 3.11: PCR Amplification of p73 exon 5

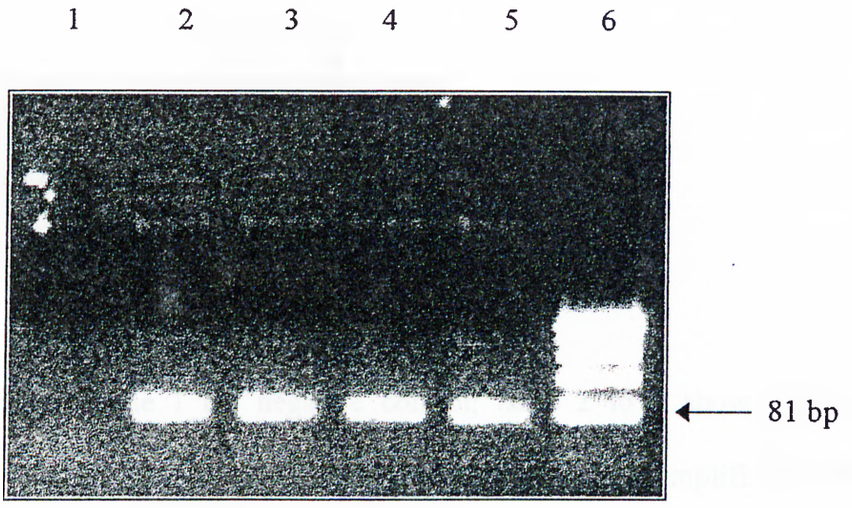


Lanes: Lane 1, 2, 3 and 4 were amplified from control samples; lane 5 is negative control; lane 6 is 81 bp exon 6 product that was used as a marker.

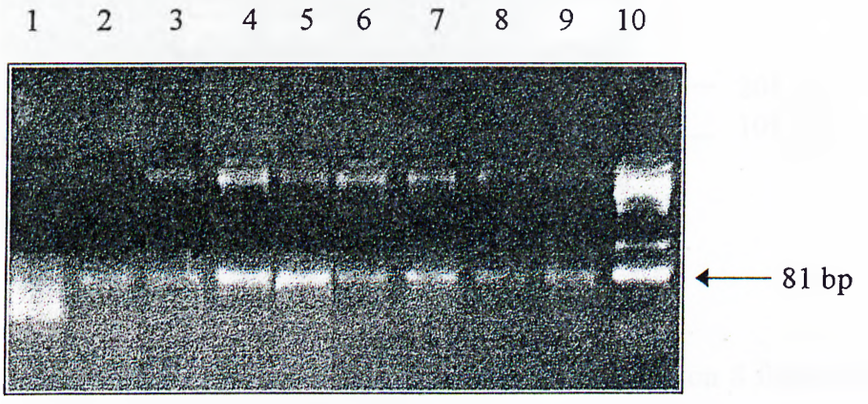


Lanes: Lane 1 : negative control; lanes 2 through 9 are amplified from tumor samples T47, T15, K15T, K3T, K11T, T55, T51 and T9 respectively. Lane 10 is previously amplified exon 5 product that serves as a marker.

Figure 3.12: PCR Amplification of p73 exon 6

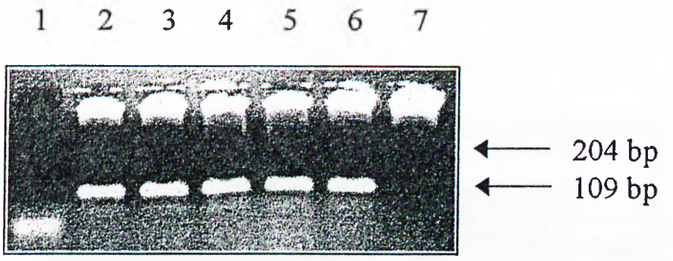


Lanes: Lane 1 is negative control; lanes 2 through 5 is exon 7 fragments amplified using control DNA; lane 6 is 100 bp DNA ladder used as size marker.

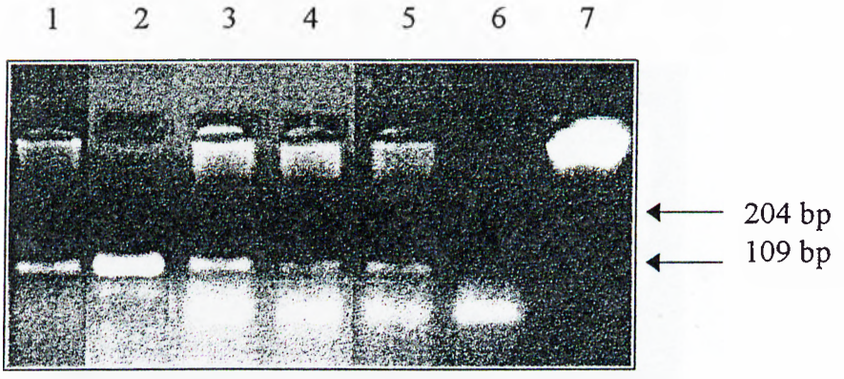


Lanes: Lane 1 is negative control; Lanes 2 through 9 is exon 7 fragment amplified from tumor samples T15, K3T, T27, K3T, T29, T37, T55 and T13 respectively. Lane 10 is 100 bp DNA size marker.

Figure 3.13.: PCR Amplification of p73 exon 7

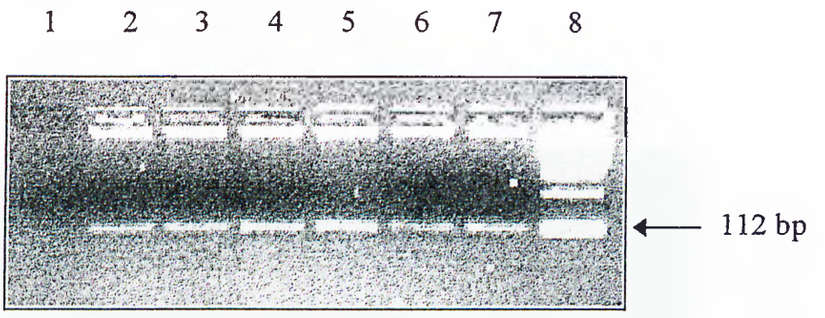


Lanes: Lane 1 is negative control; lanes 2 to 6 show exon 8 fragments amplified using control DNA; lane 7 is previously amplified 204 bp $p21^{WAF1}$ promoter fragment used as a marker.

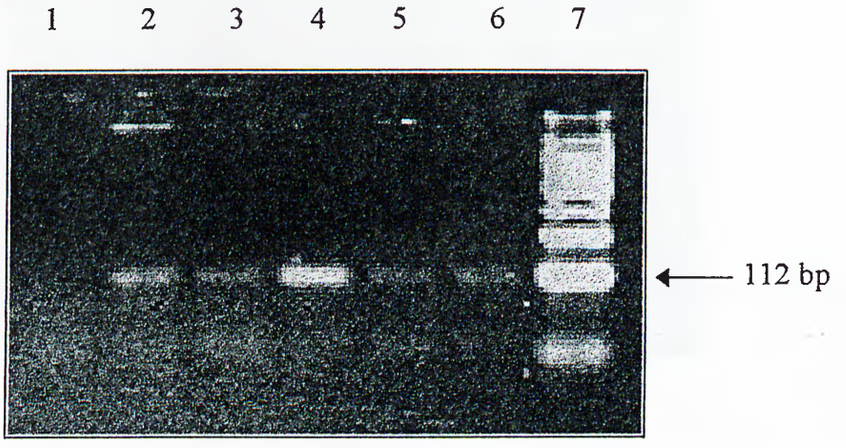


Lanes: Lanes 1 through 5 correspond to amplified exon 8 fragments amplified from tumor samples T13, T29, T9, T37 and T15 respectively. Lane 6 is negative control; lane 7 is previously amplified 204 bp $p21^{WAF1}$ promoter fragment used as a marker.

Figure 3.14.: PCR Amplification of p73 exon 8

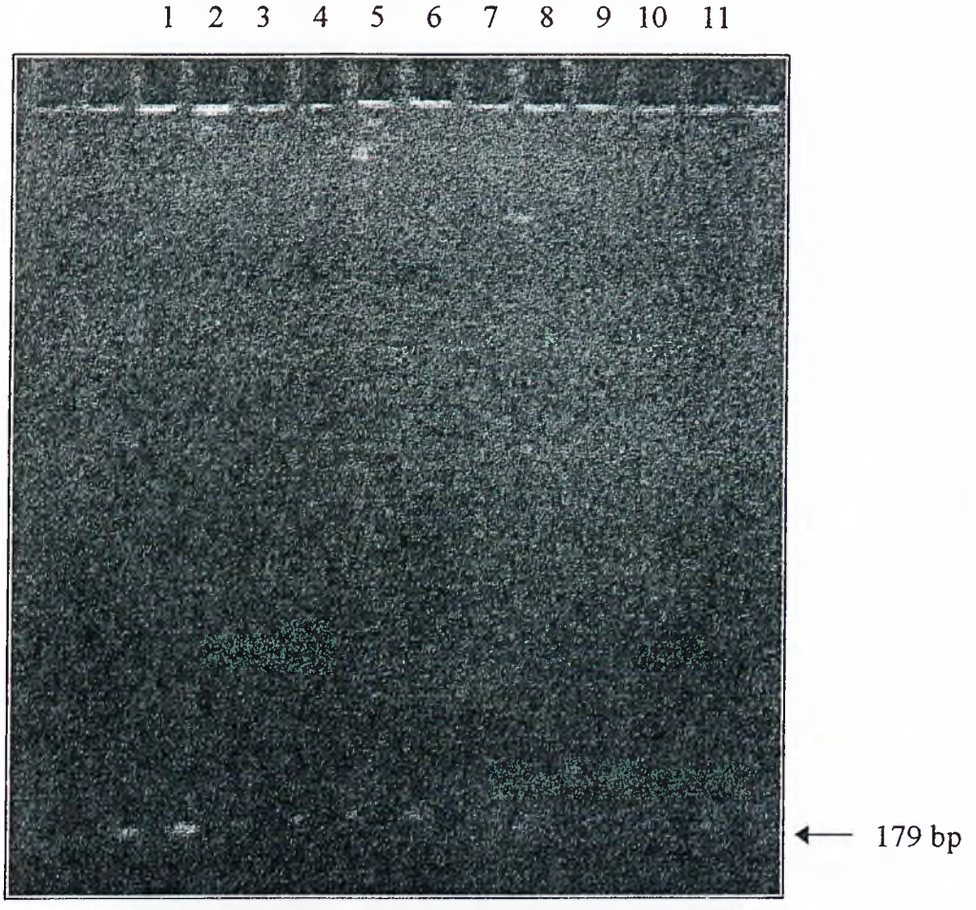


Lanes: Lane 1 is negative control; lanes 2 to 7 are PCR products using control DNA as a template; lane 8 is 100 bp DNA size marker.



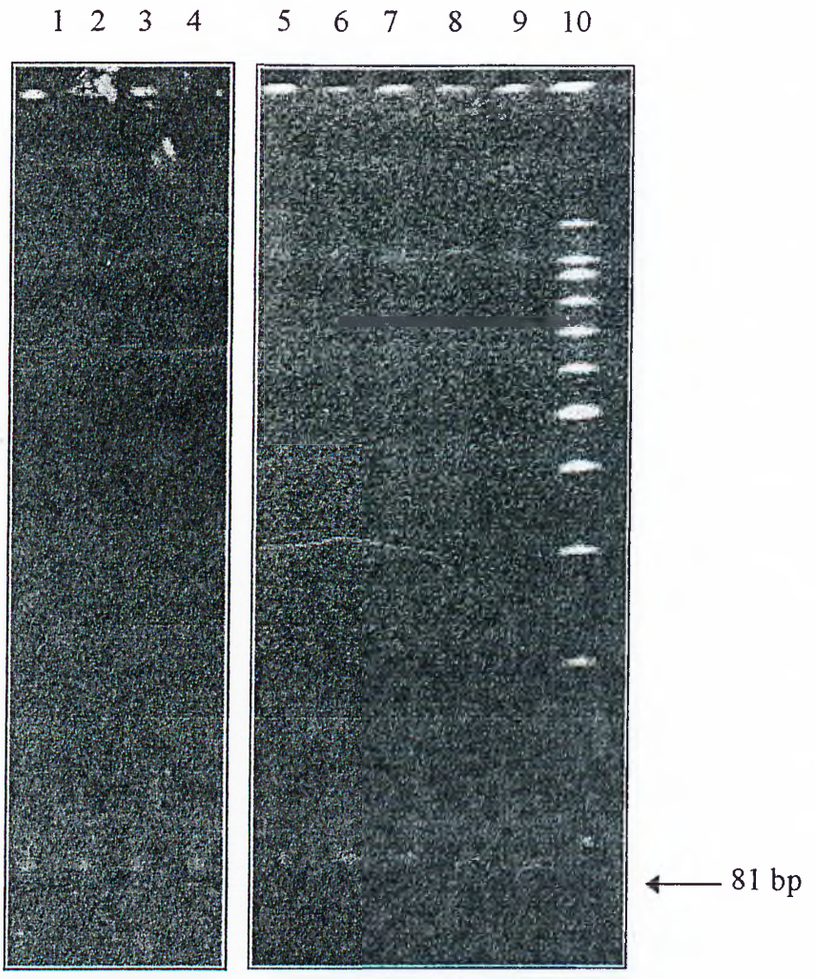
Lanes: Lane 1 is negative control; lanes 2 through 6 are PCR products from tumor samples T27, T29, T37, T55 and T13 respectively. Lane 7 is 100 bp DNA size marker.

Figure 3.15.: Heteroduplex analysis of p73 exon 5.



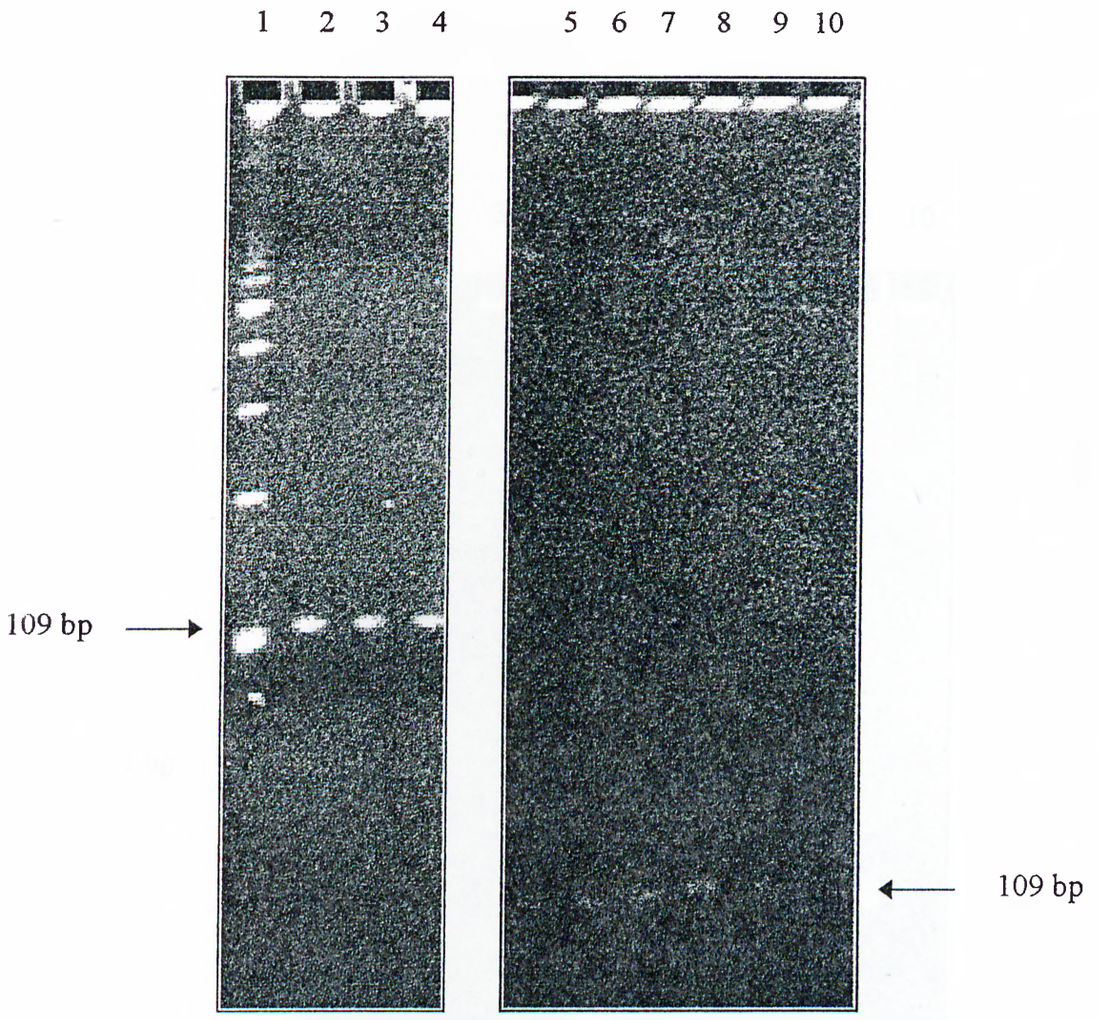
Lanes: Lanes 1 through 9 corresponds to fragments amplified from tumor samples T23, T33, T43, T29, T37, T13, T8, T39 and T41. Lane 10 is used 179 bp exon 6 fragment used as marker.

Figure 3.16.: Heteroduplex analysis of p73 exon 6.



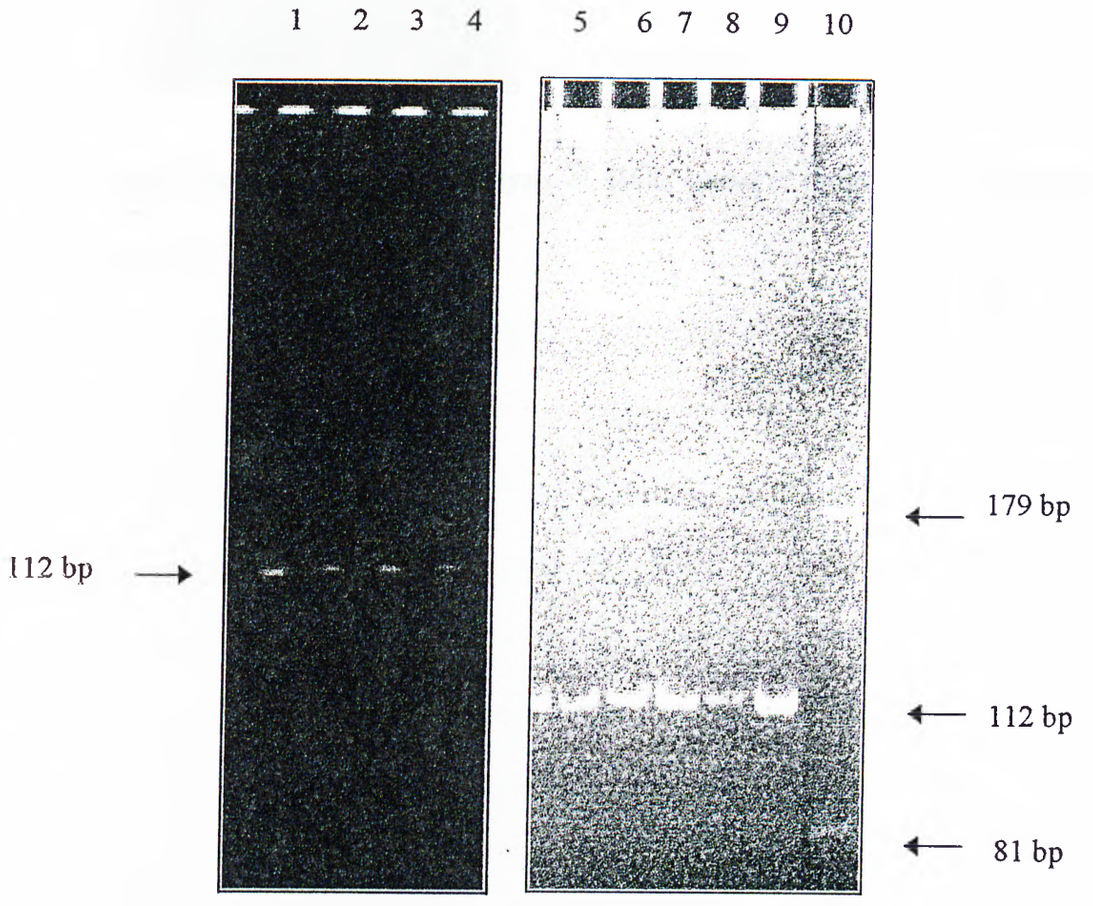
Lanes: Lanes 1 through 9 corresponds to HA samples of T43, T29, T37, T13, T23, T33, T8, T39 and T41. Lane 10 is 100 bp DNA size marker.

Figure 3.17.: Heteroduplex analysis of p73 exon 7.



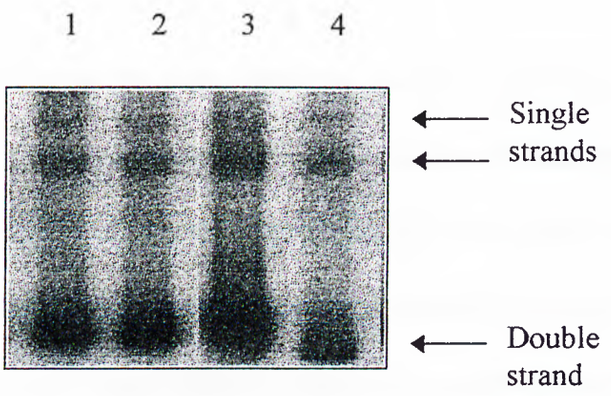
Lanes: Lane1 is 100 bp DNA size marker; Lanes 2 to 10 are HA samples of T37, T13, T29, T23, T33, T8, T41, T39 and T43 respectively.

Figure 3.18.: Heteroduplex analysis of p73 exon 8.



3.2.3. Lanes: Lane 1 to 9 are HA samples of T23, T29, T37, T13, T33, T43, T8, T39 and T41 respectively; lane 10 is PCR products of exons 5 (179 bp) and 6 (81 bp).

Figure 3.19.: Radioactive SSCP analysis of exon 5



Lanes: Radioactive-SSCP analysis of HCC tumors: Lanes 1 to 4 represents tumors T17, T31, T39 and T55.

4-DISCUSSION

For almost 20 years after its discovery, p53 gene has become a center of scientific interest for its exceptional role in tumor development (reviewed in Oren M., 1997). Its variety of functions including cell cycle arrest, apoptosis and induction of cell senescence in response to genotoxic stress led scientists to search for its possible homolog(s) but these intentions gave no positive results until Kaghad et al. had discovered the p73 gene which shows mono-allelic expression and considerable homology to p53 in both structural and functional manner (Kaghad et al., 1997). p73, when over-expressed, was observed to induce p21/WAF1 transcription and apoptosis (Kaghad et al., 1997; Jost et al., 1997). Moreover, all the hotspots whose alterations result in the altered or abolished response of p53 were found to be conserved in the p73 gene. It has also been reported by Kaghad et al. that unlike p73α form, p73β form shows high binding affinity to p53. It has been suggested that disruption of normal p53 function may result in compensatory or deleterious up-regulation of p73 (Kaghad et al., 1997). These results, taken together, increase the possible tumor suppressor role of p73 gene.

In this study, our aim was to test whether p73 gene was altered in hepatocellular carcinoma. So we decided to study both the expression and the structure of p73 gene for possible abnormalities. We first analyzed the expression pattern of p73 forms in HCC and liver-derived cell lines. A sensitive two-step RT-PCR method was developed for

simultaneous analysis of p73α and p73β transcripts. In order to avoid the mis-interpretations and sampling errors, with the help of nested-PCR technique and transcript-specific primers, two p73 forms, together with the internal control G3PDH were amplified. In the 1st round PCR, in addition to G3PDH primer pairs, a set of p73 primers that are flanking exon 9 and exon 14 were used to amplify both forms. In the second round PCR, a standard reaction was set with 5 different primers (one p73 forward primer, one p73α and one p73β-specific reverse primer and two G3PDH primers) and three transcripts (p73α, p73β and G3PDH) were co-amplified in the same reaction mixture by using 1 ul of the first PCR products. This method, owing to its multiplex nature and specificity, is very convenient for simultaneous analysis of α and β forms. The set of specific primers for p73α and p73β allowed us to avoid false positive or false negative results due to preferential amplifications. The addition of an internal control (G3PDH) to PCR reaction allowed to demonstrate that equal amounts of cDNA products were used in different experiments.

We next analyzed p73 expression pattern with this newly-established technique. p73α expression was found to be constitutive, since it was found to be expressed in all the cell lines examined. However, the loss of expression of p73β form was detected in 3/8 of the cell lines. Known characteristics of the cell lines together with the p73 status was shown in table 4.1. The p73β was not detectable in 2 HCC cell lines (Focus and Mahlavu) and one cell line derived form embryonic liver (WRL68). Thus, there was no specific loss due to malignancy of the primary tissue. There was also no correlation between p53 status and the absence of p73β. Similarly, there was no apparent relation

to find was the differentiation status of the cell lines. The published data on the differentiation status of these cells indicated that 5 cell lines (Chang, PLC/PRF/5, Hep3B, Hep3B-TR and HepG2) were differentiated, and 3 (WRL68, Focus and Mahlavu) were undifferentiated.

The differentiation status of these cell lines was checked by using two markers (albumin and α-fetoprotein) transcripts by RT-PCR. Amplification of both markers were detected in 3/5 of differentiated (namely Hep3B, Hep3B-TR and HepG2) but not in 3 undifferentiated cell lines.

In contrast to these observations, we failed to detect p73 gene mutations at the DNA binding region (exons 5-8) in 25 primary tumors. Thus, p73 gene, does not appear to be mutated in HCC. In the light of recent studies on the status of p73 in lung, prostate and colon cancers, this is not unexpected (Nomoto et al., 1998; Takahashi et al., 1998; Mai et al., 1998; Sunahara et al., 1998). Indeed, It becomes now clear that p73 gene is not frequently mutated in human cancers. Thus, unlike p53, the p73 gene appears not to be a target for mutations causing malignant transformation of cells.

However, our observation indicate that the expression of p73 gene is modulated according to the differentiation status of hepatocyte-derived cell lines. The loss of p73β in undifferentiated cell lines can be a cause or a result of the loss of cellular differentiation.

Table 4.1.: Data collected from previous reports and p73 expression status of cell lines

Name	Origin	Diff.	HBV	p53	Rb	p73α	p73β	Alb	AFP
Chang	Adult liver	+	-	wt	wt	+	+	-	-
WRL68	Emb. Liver	-	-	wt	wt	+	-	-	-
FOCUS	HCC	-	+	-/-	wt	+	-	-	-
mahlavu	HCC	-	-	m/-	wt	+	-	-	-
Hep3B	HCC	+	+	-/-	wt	+	+	+	+
Hep3B-TR	HCC	+	+	-/-	wt	+	+	+	+
PLC/PRF/5	HCC	+	+	m/-	-/-	+	+	-	-
HepG2	Hepatoblastoma	+	-	wt	wt	+	+	+	+

p73α and β can work on different sets of genes. As β is lost in undifferentiated cells, it could act specifically for the induction of expression of liver-specific genes.

Our studies started almost a year ago, just after the discovery of p73. During the last months more data about p73 and other p53-homologue genes became available. Similar to our observations, the lack of p73 mutations was reported for different cancers, including lung, prostate and colon carcinomas (Nomoto et al., 1998; Takahashi et al., 1998; Mai et al., 1998; Sunahara et al., 1998). Thus, it appears that, unlike p53, p73 gene is not mutated in cancer. The lack of tumors in p73 knock-out mice also favor the hypothesis that p73 may not a classical tumor suppressor gene (Kaelin GW., 1998). However, these observations do not rule out the possibility of aberration other than mutation of p73 in cancer. In this regard, the expression pattern of p73β may indicate a subtle role of alternative splicing of p73 in tumor development and/or dedifferentiation. Tumor progression is often accompanied by the loss of differentiation in cancer cells. Alternatively p73 and other p53 like genes may play a direct role in differentiation and/or in specific cell types. For example, specific expression of p73 in neural type

cells has been reported (Osada et al., 1998). The data about two other p53-like genes, namely p51 (human homolog of ket gene in rat) and p40 is very scanty (Osada et al., 1998; Trink et al., 1998). The only noticeable data is the presence of p51 mutations in some rare cases of epidermal cancers (Osada et al., 1998). It is possible that p53 is a house-keeping gene present in all cells while p53-like genes have more specific functions. Alternatively, p53-like genes may have redundant functions, explaining why they are not a target for mutation. Indeed, there are several examples of gene families in which only one gene is mutated in cancer, but not others. For example, among Rb-family genes (p110, p130 and p107) onlyRb1 is mutated. Similarly, among cyclin-dependent kinase inhibitors (p15, p16, p21, p27, p57), only p16 is known to be frequently mutated in cancers (Kaelin GW., 1998). The lack of mutation does not necessarily indicate that such genes are not important in maintaining normal phenotype.

The era of p53-like genes is just starting. The future will tell how they are involved in normal cellular function.

5-PERSPECTIVES

In this study, we found that in three out of eight cell lines, there was a loss of expression of p73β form in correlation with their undifferentiated status. Up to now, many inactivation pathways such as mutational inactivation, change in the half-life in both the protein and mRNA level, promoter-specific methylation as well as regulation pathways have been described or proposed, resulting in an aberrant phenotype (reviewed by Liggett WH. and Sidransky D., 1998; Guinn BA. and Mills KI., 1997; Ko LJ. And Prives C., 1996). Differential expression of p73β may also be due to other unknown mechanism. For example, the proliferation state of cell lines may correlate with cell cycle phase-specific expression of p73β.

Our future analysis should be concentrated on a more careful analysis of p73β transcripts in cell lines. Cells could be synchronized in cell cycle and the expression of p73β could be tested at selected phases such as G_1, S and G_2/M.

If our observation with p73β are confirmed with additional studies, It will be interesting to compare specific functions of p73α and p73β transcripts. As the expression of p73β appears to correlate with cellular differentiation, it may be a transcription factor acting on tissue-specific gene expression. With regard to our experimental model, we plan to construct p73β expression vector and then transfect hepatoma cell lines. Such transfected cell lines will be tested to know whether p73β expression can induce the expression of liver-specific genes such as albumin and α-

fetoprotein. The differentiation is often accompanied by an exit from cell cycle. Then the effects of p73β on cellular proliferation should also be studied.

Additionally, since we analyzed the expression of p73 only in cell lines, this expression analysis should be performed also in primary tumor samples as well as their normal counterparts. In that manner, p73 expression should also be analyzed with larger sampling size, probably showing the relevance of this change in tumor development.

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7-APPENDIX:

DNA-Sequencing results of p73 exons (exons 5, 6, 7, 8) and the homologues of p53 mutational hotspots:

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                    10        20 (302)  30        40        50        60        70
1 p73_08            CGCCTGTCCT GGCCGCGACC GAAAAGCTGA TGAGGACCAC TACCGGGAGC AGCAGGCCCT GAACGAGAGC
2 N/ga313           --------CT GGCCGCGACC GAANAGCTGW TGAGGACCAC TWCCGGGAGC AGCAGGCCCT GAACGAGAGC
3 K3T/ga313         ---------- ---CGCKAYT GaaNAGCTGa TgAgGACCcAC TWCCGGKASC AGCAGGCY-T GARCGAGAGC
4 T27/ga313         ---------- GSCCGCGACN GAAAAGCTGA TGAGGACCAC TACCGGGASC AGCAGGCY-T GARCGAGAGC
5 T29/ga313         ---------- GGSCGCGACN GAAAAGCTGA TGAGGACCAC TACCGGGASC AGCAGGCY-T GARCGAGAGC
6 T47/ga313         ---------- ---------- ----GCTGa TgAGGACCAC TWCCGGGASC AGCAGGCY-T GARCGAGAGC
7 T55/ga313         ---------- ---------- ---------- -GAGGACCAC TACCGGGNGC AGCAGGCCCT GAACGAGAGC

                    80        90        100       110       120       130       140
1 p73_08            TCCGCCAAGA AC
2 N/ga313           TCCGCCAARA AC
3 K3T/ga313         TCCGCNAAGA AC
4 T27/ga313         TCCGCMAAGA AC
5 T29/ga313         TCCGCMAAGA AC
6 T47/ga313         TCCGCMAAGA AC
7 T55/ga313         TCCGCCAAGA AC
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