ACQUIRED EXPRESSION OF TRANSCRIPTIONALLY ACTIVE P73 IN HEPATOCELLULAR CARCINOMA CELLS

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 DOCTOR OF PHILOSOPHY

BY
A. EMRE SAYAN
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TO MY WIFE,

BERNA

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

Prof. Dr. Mehmet Öztürk

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ABSTRACT

Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells

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Ph.D. in Molecular Biology and Genetics
Supervisor: Prof. Dr. Mehmet Öztürk
2002, 127 pages

P53 gene is the most common mutated tumor suppressor gene during tumorigenesis. From its description till 1997, p53 gene was thought to stand alone in the human genome. In 1997, p73 gene and in 1998, p63 gene was identified which are encoding functional homologues of p53 protein. Unlike p53, the knock out mice for p73 and p63 genes did not yield a tumor prone phenotype and the mutation frequency of these genes is very low compared to p53 gene. There is also extensive alternative splicing and changes in the expression pattern of p73 and p63, unlike p53. Thus the new p53 homologues were considered as non-classical and non-Knudson type tumor suppressor genes. A codon specific, aflatoxin ingestion related p53 mutation was shown to be important in the ethiopathology of HCC so loss of p53 function is a major factor during HCC development. The rate of p53 functional inactivation was determined by lots of studies in HCC but the knowledge for new p53 homologues is scarce. We aimed to define the probable function of the new p53 homologue, p73 in HCC development. For this purpose, we have analyzed the 3' alternative splicing and expression pattern of p73 in a series of HCC derived cell lines. Our results showed the alteration of splicing and expression in HCC cell lines compared to normal liver. After the completion of human genome project, the contig harboring the p73 gene was entered to the public database. With the hints of the presence of an alternative promoter in the p63 gene and the description of the alternative promoter in mouse p73 gene, we have made an *in silico* analysis to identify the probable promoter and exon within p73 gene. Our studies revealed the *in vivo* description of a new human p73 encoded transcript. The proposed protein product of the transcript was lacking the transactivation domain so it was named as Dominant Negative p73 (DN-p73) and

the former p73 was renamed as Transactivating p73 (TA-p73). Since the promoters of these two transcripts are different and probably under the regulation of different transcription factors, we studied the expression pattern of them by semi quantitative RT-PCR method. We have shown the presence of only DN-p73 in normal in normal liver. HCC derived cell lines and primary HCC tumors also express DN-p73 together with the acquired expression of TA-p73 in most of the cell lines and some of the primary HCC tumors. The promoter of TA-p73 was shown contain E2F1 transcription factor binding sites. The Retinobastoma protein (pRb) is the most potent inhibitor of the E2F1 transcription factor and the dysregulation of the Rb pathway components is a common event in HCC development (Rb gene mutations and proteolytic dysregulation of pRb and mutational and epigenetic inactivation of p16). We have shown the expression of TA-p73 in some of the HCC derived cell lines and primary HCC tumors so the acquired expression of TA-p73 in HCC cells might be the indicator and the effect of of Rb pathway dysregulation. We tested this hypothesis by analyzing the expression of pRb and p16, together with the endogenous E2F1 transcription factor targets such as cyclin E, $p14^{ARF}$ and TA-p73. Our results showed a 75% inactivation of Rb pathway components and a partial correlation of TA-p73 expression in HCC cells. The acquired expression of TA-p73 in HCC cells is unfavorable during tumorigenesis since TA-p73 mimics the pro-apoptotic and cell cycle regulatory function of wild type p53. Mutant p53 proteins were shown to inhibit the pro-apoptotic fuction of wild-type p53 and TA-p73. We have analyzed the p53 protein status of 15 HCC derived cell lines and defined the presence of mutant p53 or no functional p53 protein in 87% of the HCC derived cell lines. As a summary, we have identified the human homologue of mouse DN-p73 and defined the 3' alternative splicing and 5' differential promoter initiation products of p73 gene encoded products in normal liver versus a series of HCC derived cell lines and primary tumors. Moreover we have correlated the expression of TA-p73 with Rb pathway inactivation and expression of mutant p53 proteins.

ÖZET

Karaciğer kanseri hücrelerinde p73'ün transkripsiyonel olarak aktif formunun edinilmiş ifadesi

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2002, 127 sayfa

P53 geni tümör oluşumu sırasında en sık mutasyona uğrayan gendir. Tanımlanmasından 1997 yılına kadar, p53 geninin insan genomunda yalnız olduğu düşünülüyordu. p53 proteininin fonksiyonel homologları kodlayan p73 ve p63 genleri sırasıyla 1997'de ve 1998'de bulunmuştur. p53'ün aksine, p73 veya p63 geni olmayan farelerin tümör oluşumuna yatkınlıklarında bir değişiklik olmadığı gibi, bu genlerde görülen mutasyon sıklığı da p53'e oranla çok düşüktür. Ayrıca yine p53'ün aksine, p73 ve p63'te kapsamlı bir alternatif kırpılma ve gen ifadesi değişimleri bulunmaktadır. Bu sebeplerden dolayı, bu yeni p53 homologları atipik ve Knudson hipoyezine uymayan tümör baskılayıcı genler olarak değerlendirilmektedir. Aflatoksine bağlı, bir kodona özgül p53 mutasyonunun HCC etiyopatolojisindeki önemi, p53'ün fonksiyonel kaybının HCC gelişiminde önemli bir faktör olduğunu vurgulamaktadır. p53 mutasyonlarının HCC'deki önemi ile ilgili pek çok çalışma olduğu halde, p53 homologları ile ilgili çalışma çok azdır. Bu nedenle biz, yeni p53 homologlarından biri olan p73'ün HCC gelişimindeki olası rolünü araştırdık. Bunun için p73'ün 3' alternatif kırpılma ve ifade profilini bir seri HCC kökenli hücre hattında araştırdık. Sonuçlarımız, p73'ün normal karaciğere kıyasla HCC hücre hatlarında kırpılma ve ifade değişimlerine uğradığını gösterdi. İnsan genom projesinin tamamlanmasının ardından p73 genini taşıyan kontig databaza sunuldu. p63 geninde bir alternatif promotorun varlığı ve fare p73'ünde bir alternatif promotorun tanımlanması üzerine p73 genininde olası bir promotor ve ekson tanımlamak üzere *in siliko* bir analiz yaptık. Çalışmalarımız sonucunda insan p73 geninden kodlanan yeni bir transkripti *in vivo* olarak tanımladık. Bu transkriptten oluşacak olası proteinde transaktivasyon bölgesi olmayacağından bu proteini

'Dominant Negatif p73' (DN-p73) ve önceden bilinen p73 formunu da 'Transaktive Edebilen p73' (TA-p73) olarak adlandırdık. Bu iki transkriptin promotorları farklı olduğundan ve muhtemelen farklı transkripsiyon faktörleri tarafından regüle edildiklerinden, bunların ifadesini yarı-kuantitatif RT-PCR metoduyla çalıştık. Normal karaciğerde sadece DN-p73 ifadesine rastlarken; HCC kökenli hücre hatlarının çoğunun ve primer HCC tümörlerinden bazılarının DN-p73 ifadesini korurken, TA-p73 ifadesini edindiklerini gösterdik. TA-p73 promotorunda E2F1 transkripsiyon faktörü bağlanma noktaları bulunmaktadır. Retinoblastoma proteini (pRB) E2F1 transkripsiyon faktörünün en güçlü inhibitörlerinden biridir ve Rb yolu bileşenlerinin regülasyonundaki bozukluklara HCC'de sıklıkla rastlanmaktadır (Rb geni mutasyonları ve pRB proteini yıkımıyla ilgili bozukluklar ile p16'nın mutasyonları ve epigenetik mekanizmalar ile inaktivasyonu). Biz TA-p73'ün bazı HCC kökenli hücre hatları ve primer HCC tümörlerinde edinilmiş ifadesi gösterdiğimizden, bu ekspresyonun Rb yolu regülasyonundaki bozuklukların indikatörü ve sebebi olabileceğini düşündük. Bu hipotezi pRb, p16 ve endojen E2F1 transkripsiyon faktörü hedeflerinin (cyclin E, $p14^{ARF}$ and TA-p73) ifadesi analizini yaparak test ettik. Sonuçlarımız Rb yolu bileşenlerinde %75 inaktivasyon ve TA-p73 ifadesi ile de kısmi bağdaşma olduğunu gösterdi. TA-p73, p53'ün pro-apoptotik ve hücre döngüsü düzenleyicisi fonksyonlarını taklit ettiğinden, aslında TA-p73'ün HCC hücrelerindeki edinilmiş ifadesi tumor gelişimini desteklememektedir. Mutant p53 proteinin, p53 ve TA-p73 proteinlerinin pro-apoptotik fonksiyonlarını engellediği bilindiğinden, 15 HCC kökenli hücre hattında p53 proteininin durumunu kontrol ettik. Bu hücre hatlarının %87'sinde mutant veya fonksiyonel olmayan p53 olduğunu tesbit ettik. Özetle, fare DN-p73'ünün insan homoloğunu tanımladık ve p73 geninden sentezlenen 3' alternatif kırpılma ve 5' farklı promotor sonucu oluşan değişik gen ürünlerinin, normal karaciğere kıyasla primer tümörler ve HCC kökenli hücre hatlarındaki ifadesinin analizini yaptık. Ayrıca TA-p73 ifadesini Rb yolu inaktivasyonu ve mutant p53 expresyonu varlığı ile bağdaştırdık.

ACKNOWLEDGEMENTS

Firstly, I would like to thank to my supervisor, Prof. Dr. Mehmet Ozturk for chosing me as his Ph.D. student and making me his collegue. He opened up my vision, thought me how to make research and how to make science. Dr. Ozturk is the scientist that I have taken as a model and want to be in the future. He is also my model in humaniterian aspect as being modest, patient, diligent and reliable. I have great intentions to be his collegue again.

I would also like to thank to my wife, Berna, for being the person she is. Without her, this thesis will not come into existence. She helped me a lot in my and it is impossible to be inaffirmative when she is around. She is a very "handy minded" person so she makes life and experiments easier.

I would also like to thank to my parents Erol and Esin Sayan for supporting me to be a scientist from the beginning of my education. I also would like to thank to Keriman Ozcelik as my new mother in Ankara. I can never forget the support of "Erkilic Family", especially Ali, Revman, Cenan, Saide and Burhanettin. My special thanks go to Eser, Korcan and Aysin as sisters and brother. They are ready when I ask their help.

I would like to express my deepest regards to the Bilkent MBG department faculty sharing their experiences with me. They have supported and positively critisized me, so helped me to evaluate and improve my scientific thinking.

The junior members of the Molecular Oncology Group, especially Esra, Tolga, Nuri and Ozgur made my time memorable here. I would like to thank to them for their great friendship, help and support.

I specially want to thank Ahmet Ucar and Abdullah Yalcin for being my friends. I have my best times in Bilkent University with them.

Special thanks to Tuba, Ebru, Cemaliye, Hani and Belhaj as friends and grad students of MBG Department. I also wish to express my deepest graditudes to Tulay, Fusun Hn, Sevim Hn, Abdullah bey and Yavuz bey for supporting MBG depatment and for their constant efforts.

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ABBREVIATIONS

ARF	Alternative Reading Frame
BSA	Bovine Serum Albumin
CDK	Cyclin Dependent Kinase
CKI	Cyclin Dependent Kinase Inhibitor
CO_2	Carbon Dioxide
C-terminus	carboxy terminus
DNA	deoxyribonucleic acid
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
HRP	Horse Reddish Peroxidase
LB	Luria-Bertani media
MDM2	Mouse Double Minute 2
MMLV	Murine Maloney Leukemia Virus
MMP	Matrix MetalloProtease
MTS	Multiple Tumor Suppresor
N-terminus	amino terminus
O/N	Over Night
OD	Optical Density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
pRb	Retinoblastoma protein
Rb	Retinoblastoma gene
RNA	ribonucleic acid
S/N	Supernatant
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween-20
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane

TGFβ	Transforming Growth Factor beta
Tris	tris (hydroxymethyl)-methylamine
TSG	Tumor Suppressor gene
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

CHAPTER I

1-1 CANCER

Cancer is the clonal expansion of a cell that gained specific properties to serve for replicate its DNA, in an uncontrolled manner. This phenomenon is achieved by a multistage process involving the inactivation of tumor suppressor genes (TSGs) and activation of proto-oncogenes. Thus each step in carcinogenesis can be directly (genetic changes) or indirectly (epigenetic changes) linked to the genetic material, DNA. In this scheme, oncogenes act as dominant players of the game, since their mutations serve as a dominant phenotype, whereas TSGs act as recessive players, as the loss of both alleles is necessary to produce the loss of function phenotype.

The risk of cancer can be directly associated with occupational (Vinyl chloride, asbestos), environmental (aflatoxin B1, UV light) and being willfull to the exposure (smoking, alcohol consumption, tanning) to chemical or physical carcinogens (Hussain and Harris, 2000). On the other hand, some viruses interrupt the normal cellular processes to favor cell replication or resistance to apoptosis. Carcinogens, viruses or combinations of them produce a series of changes to favor the best adopting and fast dividing cell to be selected among others. These changes produce gross phenotypic results, which can be summarized as below:

GROWTH FACTOR INDEPENDANCY: Growth factors are essential for a cell to replicate, and more importantly, to survive. As a first step in carcinogenesis, a cell gains a phenotype to divide and survive without the need of a extracellular stimuli. For obtaining this phenotype, the cell surface receptors (EGF-R, HER2) or the downstream pathway molecules (ras, MAP kinase pathway elements) must be mutated (Hanahan and Weinberg, 2000).

UNCONTROLLED DIVISION: Most of the cells in an organism is in quiescent state, which is G0. Under appropriate conditions these cells divide, but return to the quiescent state. The negative regulators of cell cycle progression, such as Rb, $p16^{INK4A}$ and p53 must be inactivated or positive regulators, such as cyclins and cyclin dependent kinases, must be activated so as to abolish the control over cell cycle (Hanahan and Weinberg, 2000). Continuous replicating eukaryotic cells face a problem of telomere shortening, which leads to a crisis, so that, chromosomes are lost, recombined or rearranged. The cell must overcome this problem, firstly, by overexpressing the components of telomerase enzyme complex, which contains RNA and proteins. Most of the time the RNA subunit of the complex, tERT is expressed to activate this enzyme. One in a million cell overcomes this crisis by tERT expression, so now inherited the ability of uncontrolled division (Drayton and Peters, 2002).

RESISTANCE TO APOPTOSIS: Together with uncontrolled division and growth factor independancy, the apoptotic mechanisms begin working to eliminate the cell that is dividing in an infinite manner. Pro-apoptotic genes, such as p53, $p14^{ARF}$, bax, bak, apaf1 and CD95 and anti-apoptotic genes such as bcl-2, mdm2 and PTEN are mutated in favor of a cell to be resistant to apoptotic signals (Hanahan and Weinberg, 2000).

ANGIOGENESIS AND METASTATIS: Tumor cells can replicate without the need of extracellular stimuli, but can not live without enough metabolites. The metabolite source for a cell is a blood vessel, so tumor cells attract blood vessels to their vicinity, by expressing angiogenic factors like, Vascular Endothelial Growth Factor (VEGF). Without angiogenesis, a tumor can only get as big as 2-5 mm in diameter, but after getting enough nutrients, it can grow bigger. The blood vessels also serve for a tumor cell to migrate through it, get into the blood circulation and settle in a new tissue, a process, collectively named as metastasis. For a tumor cell to make metastasis, the expression of Matrix Metalloproteases (MMP) is essential as well as the modulation in the levels of adhesion molecules like integrins and cadherins (Hanahan and Weinberg, 2000).

Every year millions of people develop cancer, for which there is no well defined cure for now. Figure 1-1 summarizes the common pathways that are dysregulated during tumorigenesis. Blocking any of the steps that is described above is a probable cure for cancer development.

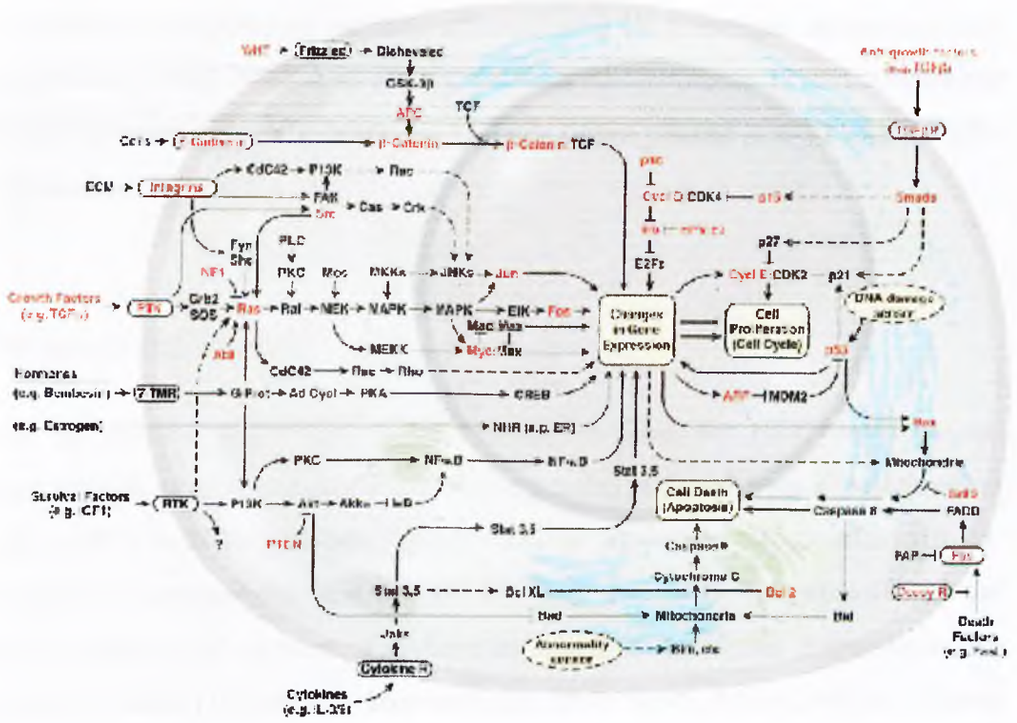

Figure 1-1: Common effected pathways during tumorigenesis.
The red names indicate either a tumor suppressor gene or an oncogene that are mutated in human cancers. (Hanahan and Weinberg, 2000)

1-2 HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the sixth most common cancer of men and eleventh most common cancer of women world-wide (Hussain et al., 2001). However, because almost every individual who develops liver cancer dies of the disease, HCC is

the third most common cause of the cancer deaths in men and seventh most common in women (Hussain et al., 2001). The major causes of HCC are chronic Hepatitis C Virus (HCV) and/or Hepatitis B Virus (HBV) infection, chronic alcohol intake and exposure to some chemicals, such as aflatoxins. At diagnosis 60% of cases, the liver contains multiple nodules of cancer. In 30% of cases there is a large single mass of cancer often with surrounding satellite lesion, and in the remaining cases, the liver is diffusely infiltrated. Common symptoms of patients affected with HCC include abdominal pain (91%), weight loss (35%), weakness (31%), fullness and anorexia (27%), abdominal swelling (43%). If untreated, HCC leads to the death of the patient in 6-12 months after diagnosis (Hussain et al., 2001).

1-2.1 ETHILOGY OF HCC

There are several major risk factors, clearly defined for HCC development. These factors include chronic alcohol consumption, being infected with hepathotrophic viruses such as HCV or HBV and being exposed to the hepatocarcinogen aflatoxin B1. The heterogeneous geographical distribution of HCC has been instrumental in the identification of major risk factors. Sub-Saharan and Eastern Asian populations are under high risk for HCC (104 men in Mozambique, 29 in South Africa, 168 in Haimen City in China per 10 000) (Hussain et al., 2001). The high risk of HCC in these regions of the world is closely associated with a fungal toxin, aflatoxin B1 intake with contaminated food (Bressac et al., 1991). Aflatoxins are mycotoxins produced by the Fungi Aspergillus *flavus* and Aspergillus *parasiticus*. Aflavus mould and aflatoxin can be found in a variety of stored grains, particularly in hot, humid parts of the world where grains such as rice are stored in unrefrigerated conditions. Aflatoxin B1 is the first carcinogen defined to hit a hotspot in the human genome. Specific p53 mutations were shown to correlate with aflatoxin related HCC (Bressac et al., 1991, Hsu et al., 1991). Data on aflatoxin exposure by contamination of food correlates well with incidence rates in Africa and to some extent in China. In hyperendemic areas of China, even farm animals such as ducks have HCC (Hussain et al., 2001).

Chronic Viral Hepatitis is another major cause of HCC. 85% of all HCC cases are seen in Sub-Saharan and Eastern Asia populations, where chronic infection with HBV is endemic in these regions (Brechot et al., 2000). The HCC patients without

chronic HBV infection and not being exposed to aflatoxin B1 are mostly infected by HCV. HCV infection is also seen in developed countries since there is no available vaccine against this virus. It is a major source for HCC in Japan, USA and Western Europe. Chronic infection with both of these hepathotrophic viruses is also seen occasionally (Koike et al., 2002).

Chronic alcohol consumption produces cirrhosis, which may lead to HCC, but it is not closely associated with HCC development alone, since USA and Western Europe countries, where alcohol consumption is high, HCC incidences are low (1.8 per 10 000 for men and 0.7 per 10 000 women in USA and Britain) (Di Bisceglie AM, 1997). Alcohol intake and HCC development are closely linked with concomitant chronic HCV infection. Patients with alcoholic liver disease have a higher sero-prevalence of HCV, but not of HBV disease. Patients with chronic hepatitis C drinking 60 gm of ethanol or more daily for five years have higher titters of serum HCV RNA and lower levels of serum neopterin, a marker of activation of cell mediated immunity, linking impaired cellular immunity in chronic alcoholics to severity of viral infection (Oshita et al., 1994).

As a result, African and Asian countries are under high risk of HCC, since viral hepatitis is endemic in these regions. Moreover, the environmental and food storage conditions favor the growth of aflatoxin B1 producing fungi, which is a carcinogen for liver.

1-2.2 GENETICS OF HCC

Initial genetics studies relied on gross chromosome gains or losses during HCC development, which gave a brief information about the locus of tumor suppressor genes (TSGs) and oncogenes involved in this pathogenesis. After the development of Comperative Genomic Hybridization (CGH), Restriction Fragment Length Polymorphism (RFLP) and Micro Satellite Analysis (MSA) techniques, DNA gains and losses as small as 10 Kb are detected. The ethiopathology of HCC is very well defined, so the minimal DNA gains or losses are being refined every day. Now, there is a clear picture for at least a minimum set of chromosomal arms that are affected in most of the cases (Buendia MA., 2000). Common chromosomal losses associated with HCC are 1p, 2q, 4q, 6q, 8p, 9p, 10q, 13q, 16p, 16q and 17p. Common chromosomal gains include 1q,

6p, 8q and 17q. Some of the candidate genes for HCC development are listed below under the headings "oncogenes" and "tumor suppressor genes" with their proposed functions and chromosomal locations.

1-2.2.1 ONCOGENES

β-Catenin: β-catenin gene was localized to chromosome 3p21, which is a somatic effected chromosomal region for different cancer types (Kraus et al., 1994). β-catenin is a member of the 'armadillo repeat' family of proteins and a central player in a number of important but distinct cellular processes. Through its central armadillo repeat region, β-catenin forms mutually exclusive complexes with adhesion molecules like cadherin, transcription factors such as members of the LEF/TCF-family, and the tumor suppressor gene product APC. Recent studies showed that expression of APC decreased the stability of β-catenin while the Wnt-1 proto-oncogene had the opposing effect. The mutations in the β-catenin gene, that increased the half-life of the protein, were identified in human cancers (reviewed by Polakis et al., 2001). Mutation detection studies showed that β-catenin is a very important mutation target for Hepatoblastoma and HCC development. A review of literature by Ozturk M. and Cetin-Atalay R. had revealed that a total of 97 out of 485 (20%) HCC tumors and 45 out of 79 (58%) Hepatoblastomas displayed β-catenin mutations (Ozturk M. and Cetin-Atalay R., in press). The mutations of β-catenin generally occur at the exon 3 of the gene, affecting the serine-threonine phosphorylation motifs, so the degradation of β-catenin protein. When accumulated, β-catenin translocates to the nucleus and become a transcriptional co-activator of LEF-TCF family of transcription factors which in turn transactivate a series of cell cycle progression genes such as cyclin D and myc (Calvisi et al., 2001).

Mouse Double Minute 2 (MDM2): The mouse homologue of human MDM2 gene was identified as a genomic DNA segment producing minichromosomes in mouse cancers (Cahilly-Snyder et al., 1987). Later the protein product of this gene was defined to be an interacting protein of p53. The main function of this protein was defined to control p53 protein levels by binding and ligating p53 protein to ubiquitin proteasomes (Honda et al., 1997). The overexpression of human MDM2 (hMDM2) was reported to

be present at 5-40% in different cancer types, especially soft tissue sarcomas (Oliner et al., 1992). An immunohistochemistry based study reported 26% positivity of hMDM2 in HCC samples, which correlated with poorer prognosis (Endo et al., 2000).

Cyclin genes: Cell cycle regulatory kinases, cyclin dependent kinases (CDK) are tightly controlled by cyclin family of proteins throughout the cell cycle (Ho and Dowdy, 2002). Cyclin genes are candidate proto-oncogenes for this purpose. Their expression is increased usually during carcinogenesis, but few of them were proven to be oncogenes. Cyclin A2 gene is the first cyclin gene found to be mutated in HCC (Wang et al., 1992), which was altered by an integration of HBV genome to the promoter of cyclin A2 gene in a single tumor. Cyclin A gene amplifications were reported to occur at 19% of HCCs (Ozturk M., 1999). Cyclin D is another target altered during HCC development. Cyclin D amplification is seen at 10-13% of HCCs. (Ozturk M., 1999, Nishida et al., 1994).

1-2.2.2 TUMOR SUPRESSOR GENES

MTS locus encoded genes: The chromosomal localization for this locus is 9p. Multiple Tumor Suppressor (MTS) locus encode for a cdk inhibitor protein ($p16^{INK4A}$) and a p53 regulating protein ($p14^{ARF}$). These two genes share 2 exons (exon 2 and 3) and a portion of the promoter region. The first exon of $p16^{INK4A}$ is named as exon 1α and $p14^{ARF}$ named as exon 1β (Serrano M., 2000). $p14^{ARF}$ protein is translated in a different frame so the protein products of these two gene isoforms are totally different. MTS locus is a hotspot for mutations and epigenetic regulations since it harbors two TSGs (Serrano M., 2000). Up to 60% of HCC tumor samples were shown to have either genetic or epigenetic (methylation) defects at MTS locus, proving the evidence that the loss of these proteins is very important in HCC development (Jin et al., 2000).

Retinoblastoma (Rb): The Rb protein (pRb) is the universal inhibitor of the cell cycle progression and a gatekeeper of G1 (Nevins JR., 2001). The inactivation of Rb is a key event in tumor progression. The Rb gene is localized to chromosome 13q, which is a common deletion site for different types of cancers including HCC (Ozturk M. 1999). Rb mutations are also observed at 15% of HCCs (Ozturk M., 1999).

Retinoblastoma protein is a target for ubiquitin dependent degradation and the degradation mechanism was shown to be dysregulated in HCCs by overexpression of a pRb specific ubiquitin ligase, gankyrin (Higashitsuji et al., 2000).

Transforming growth factor receptor beta (TGF-β) Type II Receptor: TGFβ1 is a negative regulator of cell proliferation for some cell types, including hepatocytes. In a study by Furuta *et al.* (Furuta et al., 1999), frameshift mutations at a poly-A tract of TGF-β Type II Receptor gene was described at up to 50% of HCC samples of different differentiation status, increasing through dedifferentiation. Such studies must be done to confirm the role of TGF-β Type II Receptor in HCC development.

Insulin-Like Growth Factor Type 2 Receptor Gene (IGF2R): IGF2R gene, also so called mannose-6-phosphate receptor, M6PR gene) was localized to chromosomal region, 6q24 (Rao et al., 1994). The LOH at this chromosomal region was shown to be 50-80% by several studies (De Souza et al., 1995, Kishimoto et al., 2001). IGF2R plays a crucial role in regulating cell growth by facilitating the activation of the growth inhibitor transforming growth factor beta (TGF-β) and inactivating the growth stimulator insulin-like growth factor-II (IGF2). Aberrations of this gene are therefore predicted to result in both increased cell proliferation and reduced apoptosis, consistent with the M6P:IGF2R protein, functioning as a tumor suppressor (Dennis and Rifkin, 1991, Kornfeld S., 1992). The description of mutations by several reports at frequencies of 18-30% also supported the role of M6P:IGF2R gene in HCC development (see the review of Ozturk M. and Cetin-Atalay R.).

p53: p53 gene is the most mutated tumor suppressor gene known for all cancer types (Hanahan and Weinberg, 2000) and localized to chromosome 17p, which is a common site for deletion. The protein product of p53 gene is activated by different stimuli such as oncogenic activation, DNA damage, decrease in nucleotide pools and oxidative stress (Blagosklonny MV., 2002, Wang XW., 1999) and induce cell cycle arrest or apoptosis, depending on the cell contex (Blagosklonny MV, 2002. The frequency of p53 mutations in HCC is 28% worldwide (Ozturk M., 1999). There is a strong correlation between p53 mutations, large tumor size, and poor differentiation

state (Ng et al., 1994). Consequently, patients with p53 mutations have a poor prognosis and experience a short tumor free survival following surgery. A HCC specific p53 mutation was found (Bressac et al., 1991, Hsu et al., 1991) and ethiologically associated with aflatoxin B1 ingestion. This finding is a proof for the importance of p53 mutations in the development of HCC.

Above, I have mentioned the genes commonly mutated in human tumors. This information provided by geneticists was correlated with *in vitro* and *in vivo* studies that were done by molecular biologists so that the individual tumor suppressor proteins and onco-proteins are now members of pathways that are dysregulated during tumorigenesis.

The results proposed in this thesis revealed a partial correlation with transcriptional regulation of p73 with Rb pathway dysregulation, so I also want to mention about the Rb pathway under a separate heading. The main subject of this thesis is the contribution of p73, the new p53 family member to liver carcinogenesis so I want to mention about p53 pathway specifically. The components in a pathway are mutated in a mutually exclusive manner and when the mutation frequencies of these two pathways (p53 pathway and Rb pathway) are added up, the sum is nearly 100%, indicating most, if not all cancers have lost the regulation on the components of these two pathways.

1-3 Retinoblastoma Pathway

Cellular senescence and quiescence is strictly controlled by the balance of proliferative and anti-proliferative signals that a cell acquires. The study of senescence and quiescence and their related aspects of cellular life-span uncontrolled division and immortalization have a great importance in understanding tumorigenesis. The molecular machinery that controls cell division is based on the sequential activation of a family of protein kinases, known as cyclin dependent kinases (CDKs) (Sherr and Roberts 1999). These kinases require to be associated with an activating protein which is named cyclin so as to phosphorylate their substrates. The expression of cdks are generally ubiquitous, whereas the transcriptional induction of cyclins govern the association with a specific CDK and progression of cell cycle. When quiescent cells are stimulated, first CDK4 and CDK6 are activated by association with D type cyclins. The activation of CDK4 and CDK6 were followed by the subsequent activation of CDK2 by cyclin E and cyclin A, which in turn initiates DNA replication. As DNA replication process finishes, CDK1-cyclin B complex is activated leading to mitosis. Thus the uncontrolled activity of CDK family proteins and cyclins may lead to tumorigenesis, which in turn makes them potential oncoproteins. This hypothesis was proven to be true by the description of an

activating mutation in CDK4 and the description of overexpression of CDK4 and cyclin D (reviewed by Ortega et al., 2002).

The process of sequential association of cyclins with their kinase parthers was strictly controlled by 2 families of proteins, both regulating the kinase unit of this partnership so they are collectively named as cyclin dependent kinase inhibitors (CKIs). First group of CKIs is the INK4 family. This family is composed of 4 members ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$), having negative effect on CDK4 and CDK6, so regulating the G1-S transition of cell cycle mainly. The second group is Cip/Kip family of CKIs, which is composed of 3 members ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$). Interestingly, this group of CKIs have an activating effect on CDK4/6-cyclin D complex by facilitating and stabilizing the association of cyclin and CDKs. In contrast the effect of Cip/Kip family of CKIs is negative on CDK2 and cyclin E/cyclin A complex, so they are effective on late G1 to G2 phases of cell cycle. Since the Cip/Kip family of CKIs have both positive and negative effects on cell cycle progression, inactivating mutations in this family is unexpected. Mutations of CKIs or epigenetic downregulation of CKI expression yields in a loss of the control on cell cycle thus the CKIs, especially INK4 family are potential tumor suppressor genes (TSGs). The positive and negative regulators of cell cycle are described in figure 1-2.

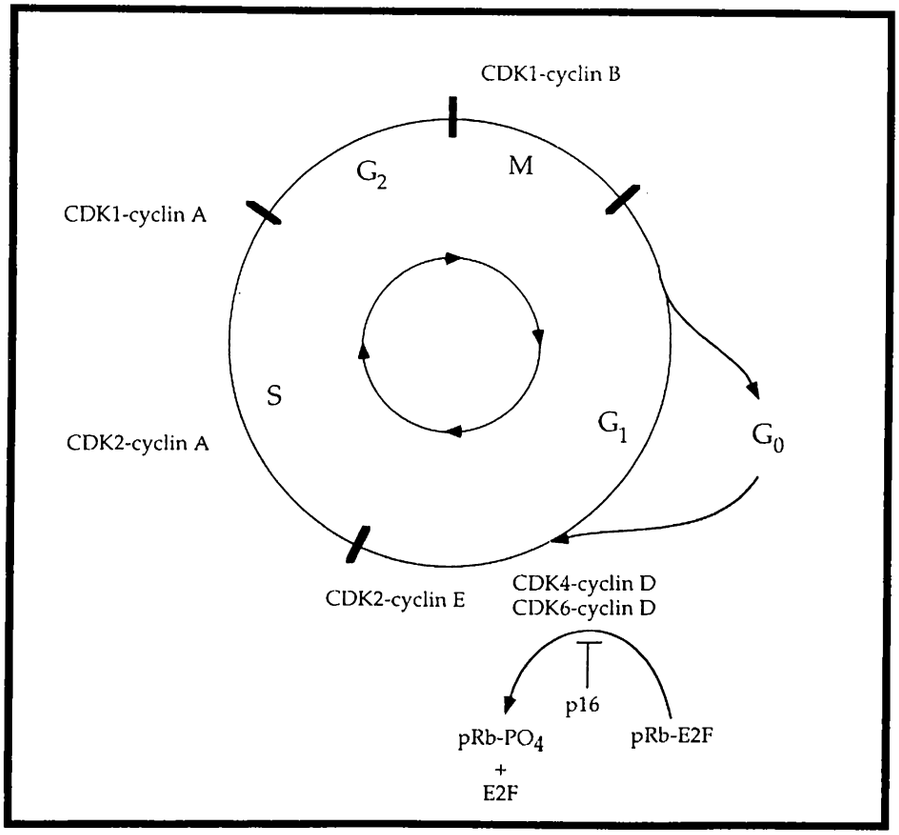

Figure 1-2 : Cell cycle and cell cycle regulators (Collins et al. 1997)

The Retinoblastoma protein (pRb) is the major substrate for CDK4/cyclin D and CDK6/cyclin D complex. In unphosphorylated or hypophosphorylated form pRb is bound to E2F family of transcription factors, so silencing their transactivation functions. The E2F family of transcription factors activate the transcription of several important cell cycle proteins such as cyclin E and cyclin A. pRb must be phosphorylated by CDK4/cyclin D so as to free E2F1 transcription factor, which in turn transactivates cyclin E gene and induces DNA synthesis (Collins et al. 1997).

The major components of Rb pathway are cyclins (cyclin D, cyclin E and cylin A), cyclin dependent kinases (CDK4, CDK6 and CDK2), cell cycle inducing transcription factors (E2F1, E2F2, E2F3, and E2F4 and E2F5), CDK inhibitors of INK4 family and of course the key holder Rb. Frequently altered tumor suppressor genes and oncogenes in HCC were summarized at part 1-2.2.1 and 1-2.2.2. In these sections, $p16^{INK4A}$ (as a MTS locus encoded transcript) and Rb genes were defined to be frequently altered tumor suppressor genes and cyclin D gene was defined to be a frequently altered oncogene in HCC development. These data suggests that Rb pathway inactivation is a key and a frequent event in HCC development.

1-4 p53 Pathway

The p53 tumor suppressor protein is inactivated in approximately half of human cancers. The inactivation of p53 protein is most of the time a result of missense point mutations in p53 gene, but functional inactivation of p53 protein by rapid degradation or by viral oncoproteins is also present in some cancer types. In normal conditions p53 is expressed as a latent transcription factor with a short half life. DNA damage or oncogenic activation provokes p53 protein to be post-translationally modified, thus stabilized. The modification mechanisms include phosphorylation at N-terminal residues and acetylation at C-terminal residues. The modifications not only induce an increased half life of p53 protein but also cause conformational changes to be competent for oligomerization and DNA binding (Giaccia and Kastan 1998). Upon activation, p53 protein transactivates several target having roles in cell cycle control ($p21^{Cip1}$) and apoptosis (Bax). The activation of p53 and the probable effects of p53 are schematized in fig 1-3.

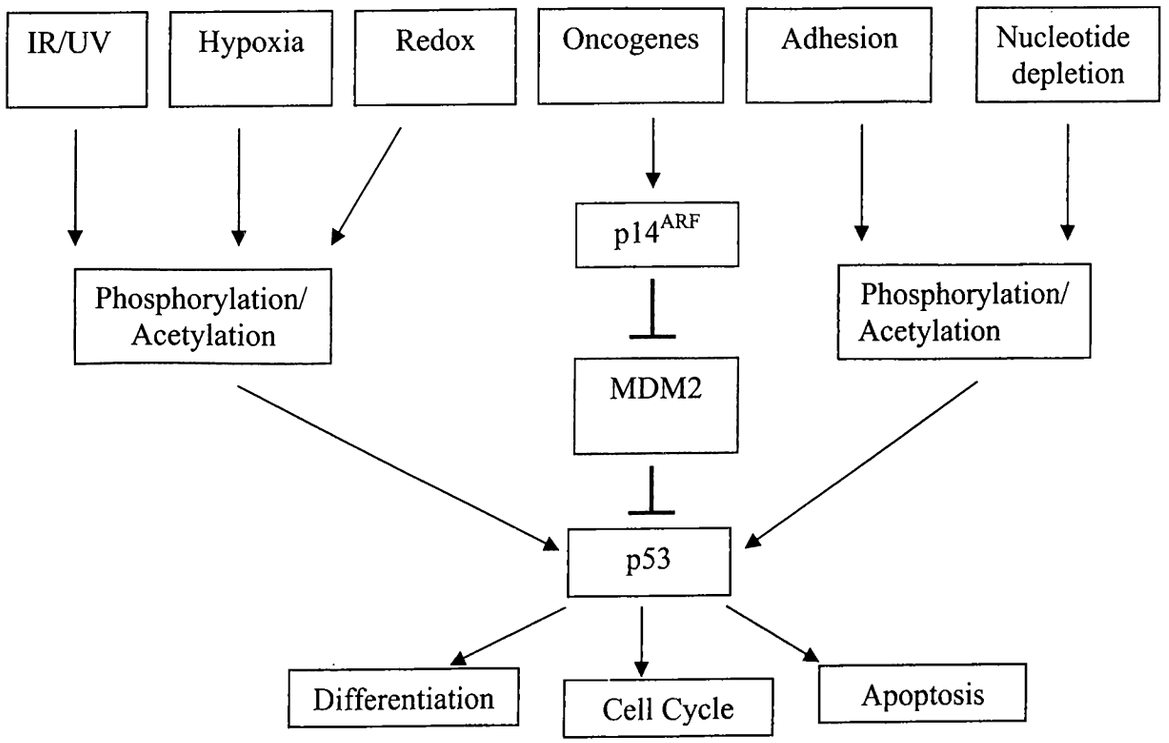

Figure 1-3 : The activation and the effects of p53 protein

Active and accumulated p53 protein is dangerous for the cell since it induces cell cycle arrest or apoptosis, so p53 protein levels are strictly controlled by ubiquitin dependent proteolysis. The main ubiquitin ligase for p53 protein is the MDM2 protein. The N-terminal phosphorylation of p53 disables the MDM2 binding, so increases the half life of p53 protein. As a negative regulator of p53 tumor suppressor, MDM2 was considered as a candidate oncogene. This hypothesis was proven to be true by the detection of amplified or overexpressed MDM2 in sarcomas and some other types of cancers (Momand et al. 1998). It is not very surprising that the MDM2 expression is under the regulation of p53 transcriptional regulation. So p53 protein levels are defined by a auto-regulatory feedback loop of transcriptional activation of MDM2 expression.

One of the most interesting discoveries of tumor biology in recent years is the description of an alternative promoter and exon within $p16^{INK4A}$ gene (exon 1β) (Serrano M., 2000). Since this gene product has been initiated from an alternative exon, the reading frame of the transcript differs from $p16^{INK4A}$ although the two transcripts of this locus are sharing 2 exons (exon 2 and exon 3). The protein product of this gene was 14 kilodaltons in humans, so this gene is named as p14 Alternative Reading Frame ($p14^{ARF}$). The function of this protein was defined to be the inhibition of MDM2 oncoprotein. Overexpression of $p14^{ARF}$ was shown to stabilize p53 and activate its downstream targets and the phenotype of knock-out mice of $p14^{ARF}$ was defined to be developing tumors in the spectrum of p53 deficient mice. Interestingly, the promoter of $p14^{ARF}$ is under the regulation of E2F1, which is a pRb regulated transcription factor. The activation of E2F1 is essential for the proceeding of cell cycle, since the endogenous E2F1 targets also include cyclin E, which is an essential cyclin for S phase progression. Rb pathway dysregulation is a common event in cancer development as described in section 1-3, leading to release of E2F1. These results propose the negative feedback regulation of uncontrolled division through p53 activation as schematized in figure 1-4.

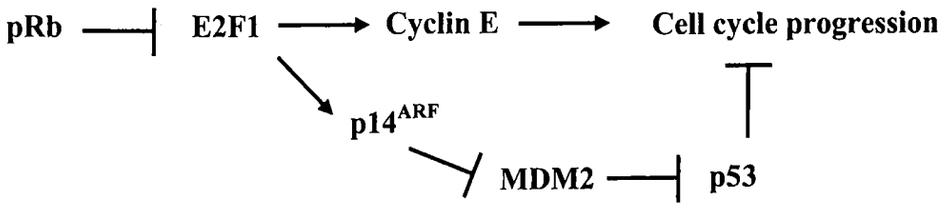

Figure 1-4 : The anti-mitotic effect of p53 on uncontrolled division

The description of gross deletions covering both genes and tumor specific epigenetic down-regulation of $p16^{INK4A}$ and/or $p14^{ARF}$ defined this locus to contain two tumor suppressor genes. Thus the INK4A/$p14^{ARF}$ locus was renamed as Multiple Tumor Suppressor (MTS) locus. This locus encodes for an Rb pathway component ($p16^{INK4A}$) and a p53 pathway component ($p14^{ARF}$). As summarized at part 1-2.2.1 and 1-2.2.2, p53 gene and p14ARF gene are commonly deregulated tumor suppressor genes and MDM2 is a commonly deregulated oncogene in HCC. The pathway covering p14ARF, MDM2 and p53 covers the activation of p53 as a result of oncogenic response and uncontrolled division. Although the mutation frequencies of all components of a pathway must add up to 100% in a specific cancer type, the frequencies of p53 pathway components do not add up to 100%, since p53 activation follows the route of both oncogenic activation and DNA damage response (figures 1-3 and 1-4) and the components of these pathways must be studied more extensively.

CHAPTER II

THE P53 HOMOLOGUE P73

2-1 IDENTIFICATION OF P73

P73 gene was identified by Kagdad et al., at 1997 (Kagdad et al 1997). The compementary DNA (cDNA) encoding p73 was discovered in a hybridization screen of COS cell line cDNA library using degenerate IRS-1 binding nucleotides by fortune. Subsequently, normal human colon cDNA library also revealed two alternative splice variants for p73 and named as p73α (full length p73) and p73β (exon 13 splice out form) (figure 2-1).

The gene encoding p73 was localized by Flourescence In Situ Hybridization (FISH) analysis, to the sub-telomeric, small arm of chromosome 1 (Kagdad et al 1997). This region is a common deletion site for different types of tumors, as also shown for neuroblastoma (Kagdad et al 1997), for lung (Nomoto et al., 1998), for non-astrocytic brain tumors (Alonso et al., 2001), and for HCC (Mihara et al., 1999). Refined chromosomal location of p73 gene was defined by Lo Cunsolo et al. (Lo Cunsolo et al., 1998) and by Perri et al. (Perri et al., 1999) to be 1p36.33 between markers, D1S47 and D1S243.

A Northern blot using the p73 central domain as a probe revealed 3 discrete transcripts, at 4.4 kb, 2.7 kb and 1.5 kb (Stiewe and Putzer, 2000). The major transcript was defined to be the 4.4 kb, which is the product of all 14 exons of the p73 gene (Kaghad et al., 1997). There is an extensive alternative splicing at exons at the 3' end of the p73 transcript, which is not a common feature of p53 gene encoded transcript (Figure 2-1). Full length p73 (p73α) and the transcripts, lacking exon 13 (p73β), are the most common p73 transcripts in normal tissues, but cancer related changes in p73 alternative splicing and accumulation of different transcripts were reported for several types of cancers (table 2-1). A descriptive study on p73 expression analysis and mutation detection on primary breast tumors and cell lines showed the presence of at

least six 3' splicing isoforms as described at figure 2-1 (Zaika et al., 1999). In this study normal breast was shown to be expressing low amounts of p73α and p73β, but 5 of 7 cell lines and 30 of 77 primary breast tumors were expressing higher amounts of all six p73 isoforms (p73α, β, γ, ϕ, ε, δ) or combinations of these isoforms. The functions of some of the p73 3' splicing variants were defined by Ueda *et al.* (Ueda et al., 1999). When the transactivation activity of p73α , β, γ and ε are compared with p53, p73β was shown to be as effective as p53, followed by p73γ, p73α and p73ε. The latter three p73 forms (α, γ, ε) were shown to decrease endogenous p53 activity when transfected to p53 wild-type (wt) cell line like MCF-7 or HepG2. Another study by Ueda *et al.* (Ueda et al., 2001) also showed that, inter-association of alternative splicing products of p73 may determine the transcription activity of p73 as a tetramer. The p73 isoforms with low transactivation ability (p73γ, ε), also decrease the transactivation ability of p53, p73α and p73β, since they make oligomers and tetramers with each other. These results provided some information to the involvement of p73 to breast carcinogenesis by an unknown mechanism.

A 5' splicing product is also defined (Kaghad et al., 1997) (figure 2-1), which is lacking exon 2. This product is named as p73 Δ exon 2. The protein of this transcript is initiated from an alternative in-frame methionine at exon 3, so the protein is lacking the N-terminal 48 amino acids. Thus, this protein is deficient for the critical amino acid residues for the transactivation ability of p73, but DNA binding domain and oligomerization domains are intact (Figure 2-2). The domain structure of p73 Δ exon 2 protein leads p73 investigators to propose a dominant negative function. This protein product of p73 gene can make tetramers with other p73 gene products and decrease the transactivation capability of the tetramer, or directly bind to the natural targets of p73 gene on the genomic DNA, so occupy the promoter regions without transactivating them. The dominant negative function of this from has been defined recently by Fillipovich et al. (Fillipovich et al., 2001) and the expression was shown to be present at RNA level for breast cancer cell lines (Fillipovich et al., 2001) and for vulval cancer (O'Nions et al., 2001).

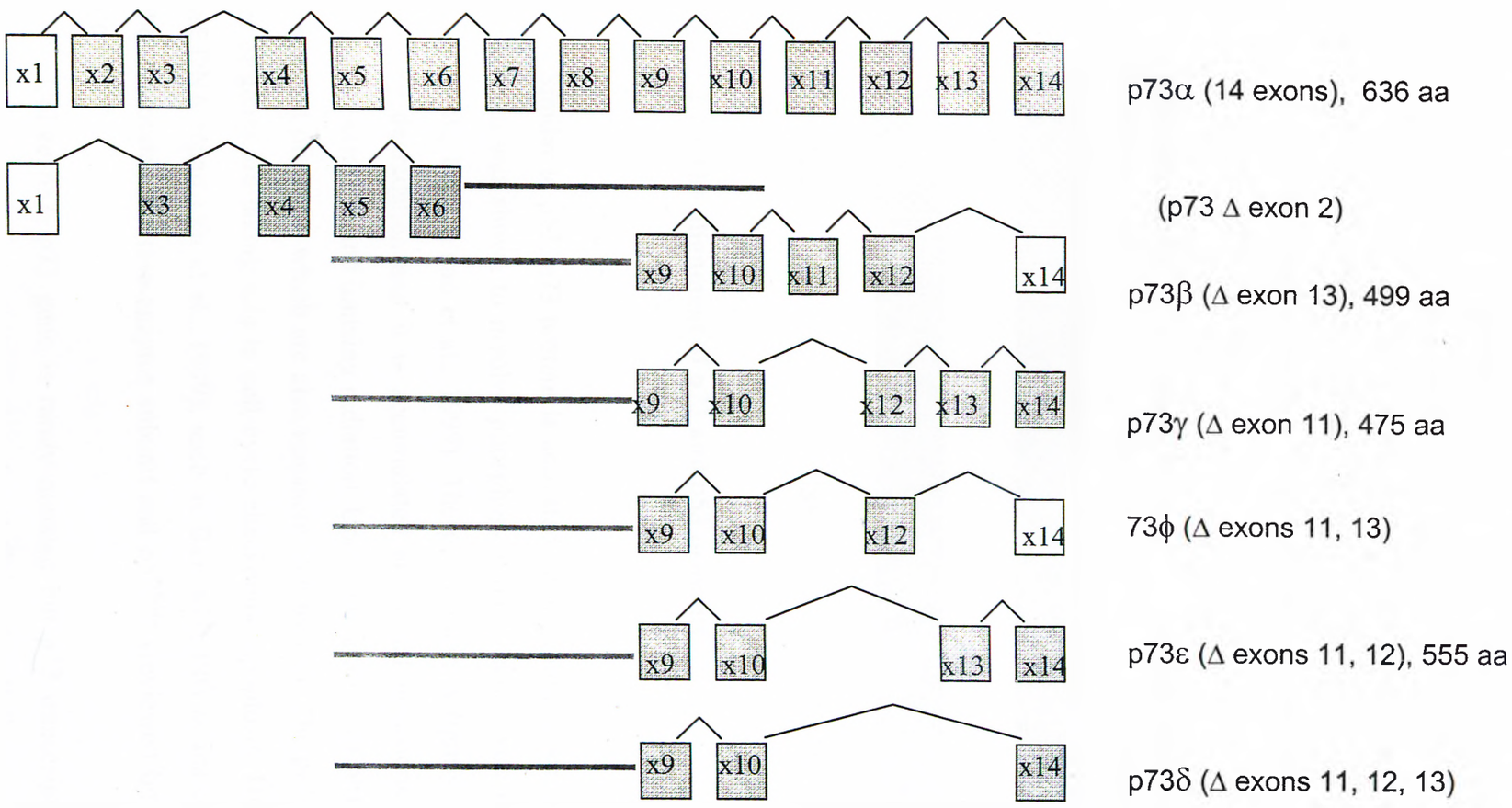

Figure 2-1: p73 gene encoded transcripts and their alternative splicing products

p73 cDNA encodes for a protein having structural and functional homologous domains with p53 (Kagdad et al 1997). P73 and p53 share a 60% conserved DNA binding domain, at which most of the p53 mutations reside (Kagdad et al 1997) (Figure 2-2). This domain of p53 is also well conserved from drosophila to men (Ollmann et al., 2000). Moreover, p53 and p73 proteins also share conserved oligomerization (38% identity) and transactivation (29% identity) domains (Figure 2-2), providing some evidence about the function of p73, similar to p53 (Kagdad et al 1997).

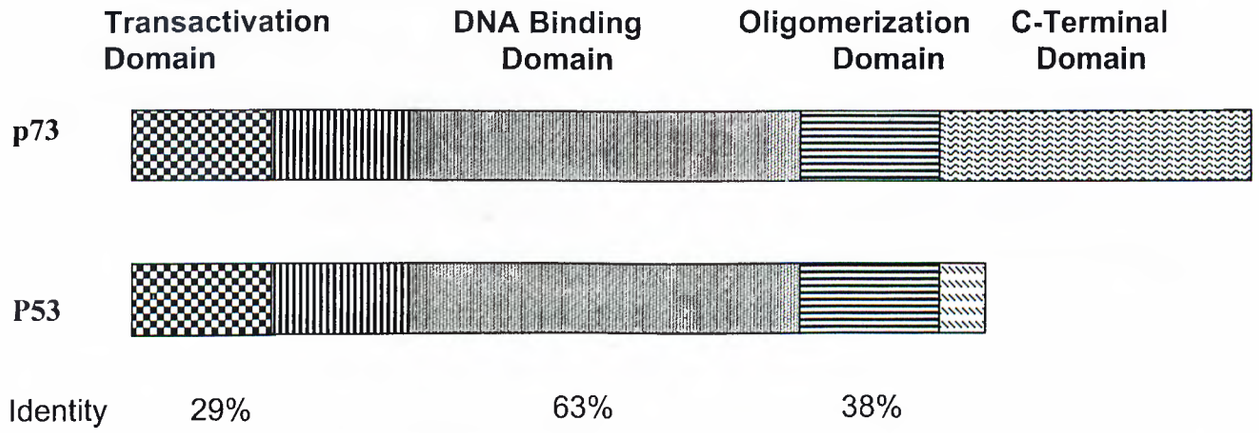

Figure 2-2 : The alignment of p73 and p53 proteins

Similar to p53, p73 protein is activated upon genotoxic stress and the activation mechanism was shown to involve phosphorylation by abl kinase (Agami et al., 1999, Gong et al., 1999, Yuan et al., 1999). The p73 protein is tightly regulated at protein level by degradation and it is accumulated at special occasions like DNA damage caused by cisplatin and ionizing radiation. Upon activation, p73 protein transactivates a similar set of genes, which are also transactivated by p53. The protein products of this set of genes are taking role in cell cycle checkpoint, apoptosis, DNA damage sensing and DNA repair (Yu et al., 1999), such as Bax, p21, PIG series of genes, 14-3-3σ, a ribonucleotide reductase enzyme subunit and $p57^{KIP2}$ (reviewed by Stiewe and Putzer, 2002).

Unlike p53, p73 gene is rarely mutated but p73 transcription is regulated at epigenetic levels. There are lots of studies defining the allelic expression of p73 gene, but there are contradictory results. It seems that, p73 gene is expressed monoallelicly or

bialellicly depending on the tissue, person and the tumor (reviewed in Stiewe and Putzer, 2002). Some expression studies defined that there is no correlation between allele number and the p73 transcript levels. Cells retaining only one allele of p73 express comparable levels of p73 with the cells having two or even three alleles of p73 gene (Kaghad et al., 1997, Stiewe and Putzer, 2002).

Many cellular oncogenes, inducing c-myc, ras and E2F1, induce the stabilization and the accumulation of p53. This phenomenon is achieved, at least partially by the induction of $p14^{ARF}$, which in turn binds and blocks the ubiquitin ligase MDM2. In the absence of MDM2, p53 is accumulated at protein levels. Moreover, MDM2 binds to the transactivation domain of p53 (aminoacids: 1-27), suggesting another level of regulation of MDM2 on p53 activity. The amino-acid residues of p53 which are important for MDM2 binding are also conserved in p73. Several groups have shown that MDM2 and the closely related protein MDMX can bind to p73 (Ongkeko et al., 1999, Zeng et al., 1999, Balint et al., 1999). Interestingly, MDM2 or MDMX binding doesn't lead to degradation of p73, but stabilization of p73 (Zeng et al., 1999). However, MDM2 binding to p73α and p73β strongly reduces the transactivation capacity of these protein isoforms (Balint et al., 1999, Dobbelstein et al., 1999). These results provide some information about another type of regulation of MDM family of proteins on the function of p53 family proteins.

So, p73 gene encoded products are regulated at transcriptional and translational levels.

2-2 MUTATION DETECTION STUDIES OF P73

There are lots of studies, defining the LOH at 1p36 region, mutations at p73 gene or describing the expression pattern of this gene. More than 3000 samples were analyzed from different types of tumors or tumor derived cell lines, but the mutation frequency turned to be roughly 0.5%. A high incidence of LOH at 1p36 locus has been described especially at neuroblastomas, lung cancers, astrocytic and non-astrocytic brain tumors and hepatocellular carcinomas (see table 2-1).

A total of 11 polymorphisms were defined recurrently in different types of cancers (table 2-2, figure 2-3). Only two asssociations of tumor progression with p73

allelic presence was proposed. The study by Ryan *et al.* (Ryan et al.,2001), the p73 polymorphisms at the 5' of the start codon is genotyped in oesophageal carcinoma and the presence of AT/AT allele (nucleotide 4 of exon2: A or G, nucleotide 14 of exon 2: T or C) of p73 gene was shown to be significantly less prevalent in the oesophageal cancer patients when compared with the normal population. Ryan et al. proposed the probable presence of stem like secondary structures in p73 mRNA and the structural changes at this stem like structure by the presence of the polymorphisms affecting the translational efficiency of the p73 mRNA. Another study by Hamajima et al. (Hamajima et al., 2002) examined the allelic frequencies of the same polymorphisms and described no association of these polymorphisms with the risk of digestive tract cancers in Japanese patients.

A total of 15 p73 gene have been mutations identified up to 15 June 2002 (table 2-1, table 2-2, figure 2-3). Most of these mutations are point mutations leading to amino acid substitution. There are 3 deletion type mutations, two of which does not affect the reading frame. The mutation profile of p73 gene and p53 gene are quite similar. High percentile of point mutations leading to amino acid substitutions and microdeletions occuring outside of DNA biding domain are common features of p53 gene mutations (Greenblat et al., 1994). Interestingly, mutational hotspots of p53 gene (codons 175, 248, 249, etc) are not mutational targets for p73 gene. Two of the mutations are affecting the far carboxyl-terminal portion of the p73 protein which is only present in p73α, indicating a specific role for this domain against tumor development. There is no mutation affecting the N-terminal transactivation domain, suggesting the strong regulation of p73 by MDM family of proteins or other factors, which reduces the selection for mutants at this region.

Table 2-1 : Summary of the studies defining the LOH at 1p36, mutation of p73 gene, polymorphisms of p73 gene and the expression of p73 RNA or protein (if otherwise is not indicated, it is RNA).

The bold lines are the references that we could obtain. The data for others are gathered from abstracts and the articles that cite them.

NT: Not Tested, T: tumor, N: Normal, +: positive, +/-: slightly positive, -: negative, prot: protein

Ref.	Samples	Number	LOH	Mutation	Polymorp.	Expression
Kaghad etal. 1997	**various**	**17**	**NT**	**1(neuroblastoma)**	**2**	**NT**
Nomoto et al. 1998	lung ca.	62	42%(11/26)	none	6	NT
Takahashi et al. 1998	**prostatic ca.**	**106**	**6%**	**none**	**found**	**T>N (α>β)**
Mai et al. 1998 (a)	**lung ca.**	**21**	**NT**	**none**	**6**	**T>N**
Sunahara et al. 1998	**colorectal ca.**	**82**	**17%(8/46)**	**none**	**3**	**T>N**
Mai et al. (b) 1998	oligodendrioma	20	NT	none	found	
Nimura et al. 1998	**esophageal ca.**	**48**	**8%**	**none**	**found**	**T=+(α>β)**
Koyaley et al. 1998	neuroblastoma	42	NT	none	4	
Tsao et al. 1999	**melanoma**	**24**	**NT**	**none**	**9**	**NT**
Kroiss et al. 1998	melanoma	17	NT	none		
Ichiyama et al. 1999	**neuroblastoma**	**151**	**19%**	**2**	**4**	**T=+/-(α>β)**
Yokomizo et al. (a) 1999	**bladder ca.**	**30**	**NT**	**none**	**6**	**T>N (α, β)**
Han et al. 1999	**various**	**185**	**NT**	**1(breast)**	**4**	**NT**
Yokomizo et al. (b) 1999	prostate	31	NT	none		T=N
Yoshikawa et al. 1999	**various**	**54**	**NT**	**3(lung)**	**5**	
Herbst et al. 1999	**melanoma**	**56**	**6%(/17)**	**NT**	**NT**	

Shishikura et al. 1999	breast	87	13%	none		T=N
Chi et al. 1999	**bladder**	**45**	**NT**	**none**	**found**	**T>N(x1-x3)**
Zaika et al. 1999	**breast**	**8**	**NT**	**none**	**2**	**T>N(x2-x5)**
Stirewalt et al. 1999	leukemia	60		none	found	
Schittek et al. 1999	**melanoma**	**68**	**20%**	**none**	**NT**	**T>N**
Mihara et al. 1999	**HCC**	**48**	**20%**	**none**	**4**	**T=N(α>β)**
Corn et al. 1999	**leukemia/lymphoma**	**35**	**NT**	**none**	**4**	**T<N(x1-x3)**
Kawano et al. 1999	**leukemia/lymphoma**	**115**	**NT**	**none**	**2**	
Yokozaki et al. 1999	**gastric adenoca**	**95**	**38%**	**none**	**found**	**NT**
Liu et al. 2000	neuroblastoma	31	NT	none	found	
Schwartz et al. 1999	**breast**	**77**	**NT**	**none**		**NT**
Van Gele et al. 2000	**Merkel cell ca.**	**15**	**NT**	**1**	**4**	**NT**
Cai et al. 2000	**esophageal**	**15**	**64%**	**none**	**1**	**T>N**
Ng et al. 2000	**ovarian**	**70**	**50%(5/10)**	**none**	**NT**	**T>N(prot)**
Kang et al. 2000	**gastric adenoca.**	**80**	**-**	**none**	**NT**	**T>N**
Peng et al. 2000	HCC	22	18%	1(5bp del)		
Kong et al. 2000	neuroblastoma	50	38%	none	NT	
Tsujimoto et al. 2000	oligodendroglioma	10	NT	none	found	
Dominguez et al. 2000	breast	193	27%	NT	NT	
Ahomadegbe et al. 2000	**breast**	**59**	**32%**	**none**	**found**	**T>N**
Fukushima et al. 2001	HCC	36		none		
Shan et al. 2001	Parathyroid adenoma	32	37%	none		

El-Naggar et al. 2001	**oral/laryngeal ca.**	**67**	**30-40**	**2**	**1**	**N=T(prot)**
Nozaki et al. 2001	meningioma	27	NT	none		T>N
Alonso et al. 2001	**non-astrocytic**	**65**	**50%**	**1**	**3**	
Ichiyama et al. 2001	neuroblastoma	272	28/151	2 (1 germline)	ND	
Barrois et al. 2001	neuroblastoma	61	7/20	NT	NT	
Alonso et al. 2001	astrocytic	60	20%	none	5	
Dominguez et al. 2001	**breast**	**70**	**17%**	**NT**	**NT**	**T>N**
Lomas et al. 2001	**meningioma**	**30**	**NT**	**1**	**NT**	**NT**
F-Laurens et al. 2001	**HNSCC**	**17**	**NT**	**none**	**1**	**N=T**
Peters et al 2001	fam. prostate-brain	49	NT	none	found	
Momoiet al.2001	cholangiocarcinoma	23	high			
Ryan et al. 2001	oesoophageal	84	14/37			
Araki et al. 2002	squamus	41	73%	none		
Weber et al. 2002	HNSCC	68	ND	none		
Dong et al. 2002	**Oligodendroglioma**	**44**	**NT**	**1**	**5**	**T<N**

Table 2-2: The summary of all identified mutations and polymorphisms of the p73 gene with references.

5'UTR-ATG :	**A/G at nt 4 of ex 2**	: Kaghad et al. 1997, Nomoto et al. 1998, Mai et al. 1998 (a), Tsao et al. 1999, Yokomizo et al. (a) 1999
	T/C at nt 14 of ex 2	: Kaghad et al. 1997, Nomoto et al. 1998, Mai et al. 1998 (a), Tsao et al. 1999, Yokomizo et al. (a) 1999
Codons 101-200:	**S110L**	: Van Gele et al. 2000
	173(ACT/ACC)	: Mai et al. 1998 (a), Tsao et al. 1999, Yoshikawa et al. 1999, Cai et al. 2000, Lomas et al. 2001
	146(CCG/CCA)	: Alonso et al. 2001 (a), Lomas et al. 2001
Codons 201-300:	**R269Q**	: Han et al. 1999
	245(GTG/GTA)	: Yoshikawa et al. 1999, Zaika et al. 1999, Corn et al. 1999
	G264W	: Yoshikawa et al. 1999
	Q291K	: Alonso et al. 2001 (a)
	204(AAC/AAT)	: Alonso et al. 2001 (a), Lomas et al. 2001, F-Laurens et al. 2001
	N204S	: Lomas et al. 2001

Codons 301-400 : **336(GCC/GCT)** : Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichiyama et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Kawano et al. 1999, Van Gele et al. 2000, Lomas et al. 2001

349(CAT/CAC) : Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichiyama et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Corn et al. 1999, Kawano et al. 1999, Van Gele et al. 2000, El-Naggar et al. 2001, Lomas et al. 2001

Codons 4010500: **P405R** : Ichiyama et al. 1999, Zaika et al. 1999

P425L : Ichiyama et al. 1999

Del 2 and 4 bp in coding exon 10 affecting codons 417-420 : Yoshikawa et al. 1999

S469R : El-Naggar et al. 2001

A472T : Kaghad et al. 1997

S477W : El-Naggar et al. 2001

Codons 501-636 : **557(GCG/GCA)** : Nomoto et al. 1998, Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichiyama et al. 1999, Yoshikawa et al. 1999, Corn et al. 1999, Van Gele et al. 2000

563(TCT/TCC) : Yoshikawa et al. 1999

610(GCG/GCA) : Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichiyama et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Corn et al. 1999, Van Gele et al. 2000

Del 12 bp at coding exon 13, so deletion of codons 604-606 : Yoshikawa et al. 1999

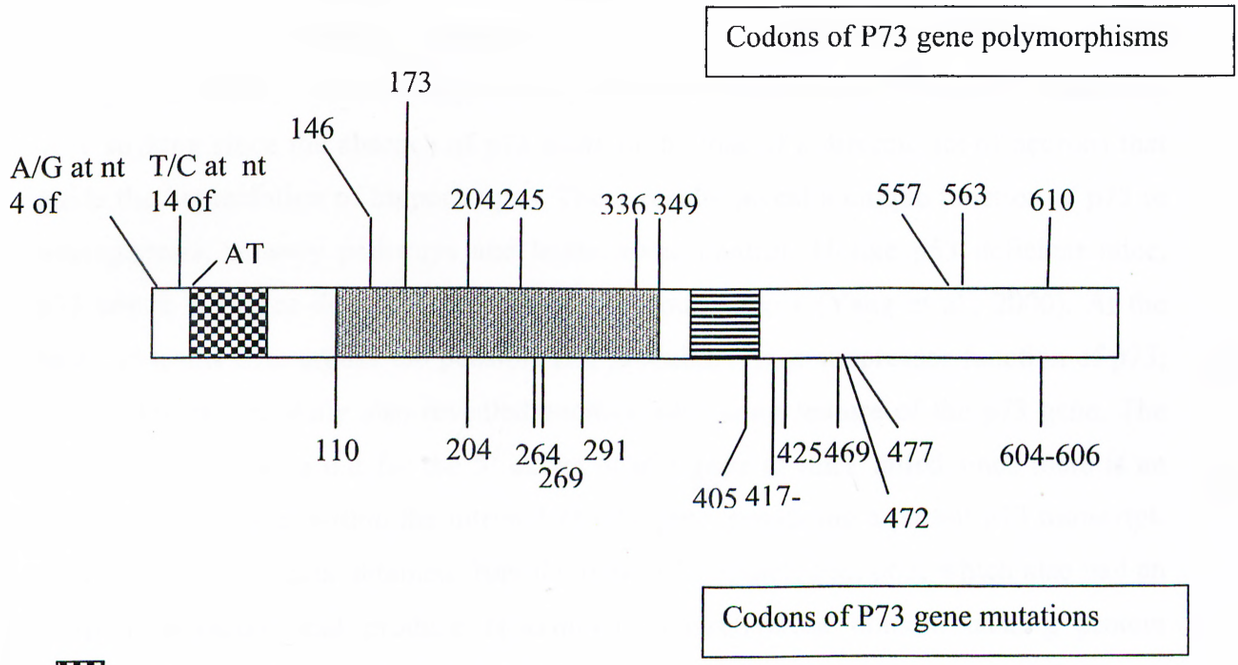

Figure 2-3: The schematic representation of all identified mutations and polymorphisms of p73 gene together with corresponding domains of p73 protein.

2-3 KNOCK OUT STUDIES OF P73

P73 gene deficiency causes developmental defects including, chronic infections, inflamations, hydrocephalus, hippocampal dysgenesis and probably some secondary effects like abnormalities in pheromone sensory pathway in mouse knock out model (Yang et al., 2000). The role of p73 in hippocampal formation of the brain is especially very striking since the absence of p73 leads to the loss of a discrete set of neurons that guide the organization of hippocampus. These results reveal a unique function of p73 in neurogenesis, sensory pathways and homeostatic control. Unlike p53 deficient mice, p73 knock out mice does not develop spontaneous tumors (Yang et al., 2000). At the first sight, this data argues the possible and probable tumor suppressor function of p73, but the knock out study also revealed another interesting feature of the p73 gene. The attempts of knocking out for the 5' exons of p73 gene in mice failed since there is an alternative promoter within the intron 3 of p73 gene, producing a partial p73 transcript. With regard to the data obtained from the other p53 homologue, p63, which also had an internal promoter, and produce N-terminal transactivation domain lacking protein products, the new promoter and exon in the mouse p73 gene was characterized (Figure 2-4) (Yang et al., 2000, Yang et al., 1998). This isoform was named as Dominant Negative p73 (DN-p73) and its mRNA was described to be the major transcript in developing and adult mouse tissues (Yang et al., 2000). The former p73 transcript was renamed as TA-p73, since it contains a transcription activation domain.

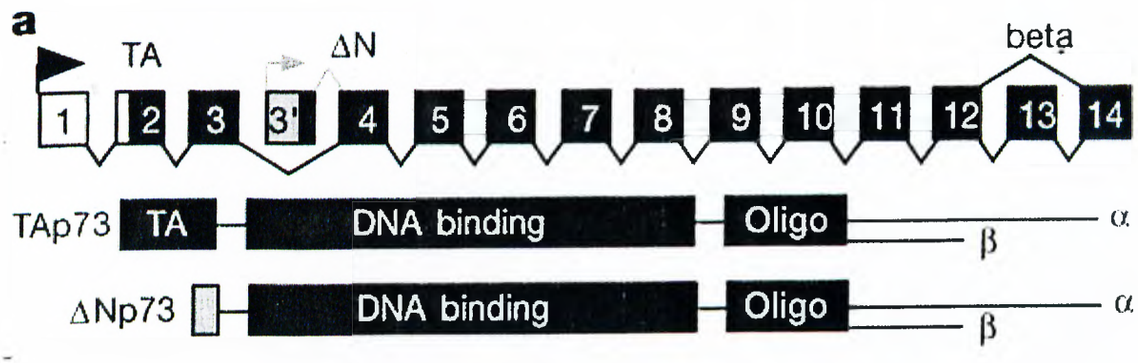

Figure 2-4 : The mouse p73 gene and transcription initiation architecture.

The full length of two transcripts share 11 exons but the DN-p73 lacks the first 3 exons of TA-p73, encoding for a p53 like transactivation domain, which is necessary to induce cell cycle arrest and apoptosis in the presence of appropriate signals. The data obtained from DN-p63 suggest that DN-p73 may function as an antagonist of TA-73 so mouse p73 loci contains a candidate oncogene (DN-p73) and a tumor suppressor (TA-p73) gene isoforms. Since these two transcripts are under the regulation of two distinct and different promoters, their transcriptions are probably independent. The knock out mice for both transcripts has also partially kept the relative balance of TA and DN isoform. Specific knock out studies for TA and/or DN-p73 must be done to specifically assign the role of both of the p73 loci harboring isoforms.

2-4 FUNCTIONAL STUDIES OF P73

Due the structural similarity of p73 and p53, they are regarded to be redundant at some extent, however, DN-p73, the other product of the p73 gene, can be considered as a anti-p53 and anti-TA-p73 protein. From the identification of p73 gene (TA-p73, Kaghad et al., 1997) to the description of mouse DN-p73 isotype (Yang et al., 2000), all of the functional studies assigned the probable functions of TA-p73 and its alternative splicing products. However, the lack of mutations in the p73 gene and the increased expression of TA-p73 isoforms in different types of cancers, together with the identification of a probable oncogenic form of p73 (DN-p73) in mice which is encoded from p73 locus, significantly draw the attention of tumor biologists to this interesting gene architecture. Now, it is quite clear that this locus produces essentially two classes of proteins: Those containing a transactivation domain (TA-p73 and 3' alternative splicing forms) and those lacking a transactivation domain (Δ exon 2 splice form of TA-p73 together with DN-p73 and their 3' alternative splicing forms).

2-4.1 TA-p73

When endogenously expressed, TA-p73 and its alternative splicing products mostly resemble p53. TA-p73 protein mimics the biological function of p53 by making dimers and tetramers, binding to canonical p53 response elements at the target promoters and transactivating a series of genes taking role in cell cycle control and apoptosis. These genes involve p21, 14-3-3-σ, PIG series (PIG3, PIG6, PIG7 and PIG11), MDM2 (Zhu et al 1998) and a ribonucleotide reductase (p53R2) (Nakano et al., 2000). A colony formation assay, with p53 and TA-p73 revealed no colonies after 3 weeks, whereas mutant p53 or p53 mutant mimicking p73 harboring cells produce similar number of colonies, suggesting a p53 like function of TA-p73α in SK-N-AS cells (Kaghad et al., 1997).

The probable pro-apoptotic and cell cycle regulatory role of TA-p73 is cell type dependent. TA-p73, like some other cell cycle regulatory proteins also regulates the differentiation state of different cell types.

In a neuroblastoma cell line, overexpression of transcriptionally active forms of p73 (TA-p73) together with retinoic acid treatment, induced both morphological (neurite outgrowth) and biochemical (expression of neurofilaments and neural cell adhesion molecule (N-CAM); down-regulation of N-MYC and up-regulation of pRB) markers of neuronal differentiation (Del-Laurenzi et al., 2000). The induction of differentiation is not achieved by p53 or dominant negative forms of p73 transfection together with retinoic acid treatment (Del-Laurenzi et al., 2000).

The effects of TA-p73α and TA-p73β are quite similar of neuroblastoma overexpression results in bladder cells. The overexpression of these gene isoforms produced irreversable growth arrest together with the markers of replicative senescence in EJ bladder carcinoma cells (Fang et al., 1999).

One of the most striking results about TA-p73 may be the definition of the E2F1 binding sites at the TA-p73 promoter and the description of the induction of TA-p73 transcription by the E2F1 transcription factor (Seelan et al., 2002, Stiewe and Putzer, 2000). Deregulation of E2F1 by some cellular (Ras and c-myc) and viral (E1A and v-abl) oncogenes have been reported (Hickman et al., 2002). Moreover, mutations at the Rb pathway (Rb, p16, cdk4, cyclin D) give rise to the activation of the E2F1 transcription factor and promotes cell cycle progression (Harbour JW and Dean DC, 2000). As a general rule such oncogenic activities are also sensed by the general guardian p53 (via blocking the "p53 ubiquitination ligase, MDM2" depletion by $p14^{ARF}$) (Sherr CJ. and Weber JD. 2000).

2-4.2 DN-p73

There are several proposed functions for DN-p73, speculated for a dominant negative role of this protein on TA-p73 and/or p53. Thus DN-p73 is a candidate oncogene. However, the human homologue of DN-p73 was not described and there was only one publication describing a possible function to mouse DN-p73 isotype during the progression of this thesis. Since the knock out study described a discrete set of changes in neuronal tissue, the function of mouse DN-p73 was investigated in neuronal cells of mouse.

Ectopic expression of DN-p73 in cell culture provided evidence for the several proposed *in vivo* functions of DN-p73. Withdrawal of Nerve Growth Factor (NGF)

induces a p53 dependent apoptosis in sympathetic neurons. Conversely, NGF withdrawal leads to a decrease of DN-p73 protein levels (Pozniak et al., 2000). This crude data had suggested a balance between apoptotic activity of p53 and anti-apoptotic activity of DN-p73. Adenoviral transfer of DN-p73 rescued the cells from apoptosis after NGF withdrawal. Likewise, neuronal cells didn't under go apoptosis when infected with both p53 and DN-p73 adenoviral vectors. Also *in vivo* complexes of DN-p73 and p53 were shown to be present by pull down assays (Pozniak et al., 2000).

2-5 INHIBITION OF P73 BY P53 MUTANTS

The E2F1 mediated upregulation, c-abl dependent protein stabilization or ectopic overexpression of TA-p73 leads to apoptosis (Stiewe et al., 2000, Jost et al., 1997, Gong et al., 1999). Rb pathway dysregulation (Rb gene mutations, pRb degradation, p16 gene mutations and promoter methylation related p16 expression downregulation) is very common events in malignant transformation (Ho and Dowdy, 2002, Serrano M. 2000) These two results propose a probable upregulation of TA-p73, together with p53, during tumorigenesis as a result of oncogenic activation. Except for leukemias and lymphomas, overexpression of TA-p73 was shown to be true for lot of cancer types (see table 2-1). Prolonged overexpression of TA-p73 must, therefore, is a common event in tumorigenesis, which is unfavorable for cancer cells. The cells must overcome the pro-apoptotic activity of TA-p73. This could be achieved by p53 mutants, which could inhibit the putative tumor suppressor activity of TA-p73 in a dominant negative fashion by generating transactivation defective hetero-oligomers with TA-p73. In cotransfection assays, R175H and R248W mutants of p53 were shown to coimmunoprecipitate with TA-p73α (Di Como et al., 1999). Moreover, transactivation activity of TA-p73 is reduced, when cotransfected with p53 mutants, in a reporter assay (Di Como et al., 1999) which is partially correlated with p73 dependent apoptosis. Direct protein-protein interaction of TA-p73 isoforms and some p53 mutants have been described (Di Como et al., 1999, Gaiddon et al., 2001, Marin et al., 2000). The hetero-oligomerization of p53 family members was shown to be mediated not to occur by the oligomerization domain (Davison et al., 1999). Interestingly, the peptide motif, crucial for this interaction was identified to be in the DNA binding domain of p73, together with the oligomerization domain (Strano et al., 2000, Gaiddon et al., 2001). Thus, the former, non-functional DNA binding domain of some p53 mutants is necessary for interaction with TA-p73.

The protein-protein interaction between p73 (TA and DN) and p53 (wild type and mutant), would probably be a decisive event for the cell fate during tumorigenesis. A common p53 polymorphism at codon 72 of p53 giving rise to an arginine or a proline residue is characterized and proposed to take role in papiloma virus induced cervical cancer (Storey et al., 1998). The presence of a proline or an arginine at codon 72 was also proposed to affect the protein stability and development of auto-antibodies against p53 (Soussi T. and Beroud C. 2001). Wild type and mutant p53 containing arginine at

codon 72 was shown to bind to TA-p73 protein more efficiently than p53 containing a proline (Marin et al., 2000). It seems that the conformation of mutant p53 which unable to bind DNA, together with the presence of an arginine at codon 72, is crucial and very effective for the functional inactivation of TA-p73 by mutant p53. A recent report by Tada et al. (Tada et al., 2001), described and classified p53 mutants in two categories. The first category involves the mutant p53 proteins having dominant activity on the remaining wild type p53 (transdominant mutants). The second category involves mutant p53 proteins having recessive activity on wild type p53 protein, so does not affect the activity of p53 in tumor cells (recessive mutants). Interestingly, 85% of the mutant p53 alleles at the first category have arginine at codon 72, whereas, the percentage of arginine and proline for the second category is about 50-50. These results clearly describe a dominant negative function for some of the mutant p53 mutants on the remaining, wild-type p53 (transdominant mutants), whereas the other p53 mutants, especially having a proline at codon 72 on p53 (recessive mutants).

AIM AND STRATEGY OF THE STUDY

CHAPTER III

AIM AND STRATEGY OF THE STUDY

p53 is the most common mutated tumor suppressor gene (TSG) for most of the cancer types. Till august 1997, p53 stood alone in the tumor suppessor heaven without a close family member. p73 gene was identified accidentally by a group screening for IRS binding proteins. First studies, trying to assign a role to 73 protein defined p53 like functions, such as, being induced by phosphorylation following DNA damage, transactivation of a similar set of target genes of which the protein products have role in cell cycle progression, induction of apoptosis and DNA repair. Mutation detection studies revealed a very low mutation rate of p73 gene, at the levels of 0.5%, for different cancer types. Expression studies, most of which done by RT-PCR, yielded contradictory results, even for the same cancer type. Some of them described elevated, some decreased and others unchanged levels of p73 transcripts in normal-tumor paired tissues. The results of mutation detection studies, together with the descriptions of alterations in the expression pattern, proposed a non-Knudson type tumor suppressor function to p73.

In May 2000, together with the description of tumor-free phenotype of p73 knock out mice, an alternative exon and an alternative promoter within p73 gene was described. These two products of p73 locus share 11 exons in the same reading frame, so most of their protein sequence is common, except for the N-terminal transactivation domain. Since, the second promoter is different than the first one, the expression pattern of the new p73 gene encoded transcript is totally different. Moreover, this product of the new gene of p73 locus was shown to be the prevalent product in developing and adult mouse tissues. The new transcript of the p73 locus can not encode for a p53 like transactivation domain, so, it was named as dominant-negative p73 (DN-p73), regarding the possible inhibitory function of the protein on p53 and former p73, which was renamed as TA-p73, representing the presence of transactivation domain.

From the date of the description of p73 gene, we were concentrated to assign the probable function to p73 gene products in Hepatocellular Carcinoma (HCC) development. First of all a mutation screening study was done in Bilkent University Department of Molecular Biology and Genetics, which yielded to describe no mutations, but several polymorphisms in aflatoxin related HCC samples. This result, together with other studies in other cancers and HCC proved p73 gene to be an uncommon target for mutations during cancer development. However, the description of complex 3' alternative splicing of p73 mRNA and the description of a dominant negative form of p73 transcript in mice provoked us to uncover the 3' alternative splicing of p73 transcripts and to define the human homologue of mouse DN-p73 for understanding the function of p73 gene encoded products in HCC development.

For these reasons, we first aimed to make *in silico* analysis of the contig covering p73 gene for the descriptions of the promoter regions of TA and DN-p73, so as to compare the transcription factors binding to these promoters. As a second step we aimed to make expression analysis studies of p73 gene encoded transcript isotypes using a series of hepatoma derived cell lines and primary tumors, compared with non-tumor and normal liver tissues. The description of TA/DN-p73 and the alterations of 3' alternative splicing isotypes of p73 transcripts may correlate with other known genetic or epigenetic aberrations during HCC development so may enlighten the changes in expression patterns of p73 in normal and tumor cells as the effect or the result of malignant transformation.

Thus, the description and association of specific p73 transcripts with HCC development may have prognostic or diagnostic values. Also, correlation of the presence or absence of some specific p73 transcript with a described cancer related abnormality may enable us to identify alternative pathways that are dysregulated during tumorigenesis.

MATERIALS AND METHODS

CHAPTER IV

MATERIALS AND METHODS

4-1 Tissue Culture Studies

All cells (listed in table 4-1) were grown in media supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 100 ug/ml penicillin/streptomycin at 37°C and 5% CO_2. The cells were routinely subcultured at 2-4 day intervals depending on the growth rate.

4-1.1 Defrosting Cells

One vial of the frozen cell line from the nitrogen tank was taken and immediately put into ice. The vial was left 1 minute on the bench to allow excess nitrogen to evaporate and then placed into 37°C water bath until the external part of the cell solution is thawed (takes approximately 1-2 minutes). The cells were resuspended gently using a pipette and transfered immediately into a 15 ml. sterile tube containing 10 ml cold medium. The cells were centrifuged at 1500 rpm at 4°C for 5 minutes. Supernatant was discarded and the pellet was resuspended in 10 ml 37°C culture medium to be plated into 100 mm dish. Cells were left O/N in culture. The following morning culture medium was refreshed.

Table 4-1: The cell lines used in this thesis

NAME	SPECIES	TISSUE/ORIGIN	MEDIUM	SUPP. CHEM.
COS-7	Monkey	Kidney	DMEM	-
Huh7	Human	HCC	DMEM	-
Hep3-BTR	Human	HCC	DMEM	-
FOCUS	Human	HCC	DMEM	-
Mahlavu	Human	HCC	DMEM	-
Hep 40	Human	HCC	DMEM	-
Hep3B	Human	HCC	DMEM	
HepG2	Human	HCC	DMEM	-
PLC/PRF/5	Human	HCC	DMEM	-
SK-Hep1	Human	HCC	DMEM	-
SNU 182	Human	HCC	DMEM	-
SNU 387	Human	HCC	DMEM	-
SNU 398	Human	HCC	DMEM	-
SNU 423	Human	HCC	DMEM	-
SNU 449	Human	HCC	DMEM	-
SNU 475	Human	HCC	DMEM	-
BC-1	Human	HCC	DMEM	Insulin and methylprednisol

4-1.2 Subculturing Of Cells

All cells were splitted once-per-week. Culture renewal was done every three days. For splitting, the medium was aspirated and the cells were washed with PBS pH: 7.4 once. PBS was removed and trypsin was added to the plates. Plates were incubated in the incubator for 3-10 minutes until the cells are detached. Cells were plated in the desired dilution into new plates. For susupension cultures, cells were centrifuged at 1500 rpm for 3 minutes and desired number of cells was resusupended in fresh medium.

4-1.3 Freezing Cells

After defrosting a vial of cell, a few new fresh vials are immediately frozen for futher studies. The cells to be frozen were grown in a 150 mm cell culture dish to 70-80% confluency. After washed two times with PBS, the cells are trypsinized and detached from the plate. The suspension of cells are centrifuged at 1500 rpm for 5 minutes and resuspended in 1.5 ml freeze medium unless otherwise indicated at ATCC definition. Then the cells are immediately placed to -20°C, and kept 5 hours at this temperature. Consequently, the cells are moved to -70°C and kept O/N at this temperature and next day transferred to liquid nitrogen tank.

Freeze medium:

10% Foetal Calf Serum

10% DMSO

80% DMEM

4-2 Protein Studies

4-2.1 Protein Extraction from Cells or Tissues

4-2.1.1 Protein Extraction from Cultured Cells

Cells were grown to 70-80% confluency and washed two times with ice-cold PBS. Cells were scraped in ice-cold PBS and centrifuged at 1500 rpm for 5 minutes at 4°C. Pellet was either frozen in liquid nitrigen or lysed immediately in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris (pH 8.0)). For lysis, the pellet was resuspended in 4-5 volume of NP-40 lysis buffer and incubated in ice for 30 minutes. The lysate was centrifuged at 13.000 rpm for 30 minutes at 4°C. Supernatant was taken to a fresh tube and following protein quantitation, aliquoted and stored at –70°C.

4-2.1.2 Protein Extraction from Tissues

The tissue is weighed and washed two times with ice-cold PBS. Four to five times the weight of the tissue is considered the amount of lysis buffer to be added to the sample (lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris (pH 8.0)). For lysis, the tissue is homogenized with a polytron on ice for 1-2 minutes in lysis buffer. The lysate was centrifuged at 13.000 rpm for 30 minutes at 4°C. Supernatant was taken to a fresh tube and following protein quantitation, aliquoted and stored at –70°C.

4-2.2 Bradford Assay for Protein Quantitation

A standard curve was prepared by using BSA as described below:

Tubes nb.s	1	2	3	4	5	6	7	8
BSA (ul)	0	2.5	5	7.5	10	12.5	15	20
DH_2O (ul)	100	97.5	95	92.5	90	87.5	85	80
Bradford (ul)	900	900	900	900	900	900	900	900

Protein samples were prepared as described below:

tubes numbers	1	2	3	4	5	6
sample(ul)	0	2	2	2	2	2
dH_2O(ul)	98	98	98	98	98	98
Bradford (ul)	900	900	900	900	900	900
lysis buffer	2	-	-	-	-	-

(1= blank in both tables)

OD_{595} were read after samples were incubated at room temperature in dark for 5 minutes.

Bradford Stock Solution:

4.75ml ethanol + 250 ul dH_2O (95 % ethanol)

10 ml 85% phosphoric acid

17.5 mg Coomassie Brilliant Blue

Bradford Working Solution:

21.25 ml dH_2O

0.75 ml 95% ethanol

1.5 ml 85% phosphoric acid

1.5 ml Bradford stock solution

4-2.3 SDS-Polyacrylamide Gel Electrophoresis of Proteins

The glass plates were assembled according to the manufacturer's instructions (EC). The volume of the gel mold was determined according to the information provided by the manufacturer (EC). In an Erlenmeyer flask, the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel was prepared. Effective range of separation of SDS-PAGE gels due to different acrylamide concentrations are summarized in table 4-2 and concentrations of components of the resolving gel at different concentrations are summarized in table 4-3.

Table 4-2: Effective range of separation of SDS-PAGE gels

Acrylamide concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Without delay, the mixture was swirled rapidly and the acrylamide solution was poured into the gap between the glass plates. Sufficient space (the length of the teeth of the comb plus 1 cm.) for the stacking gel was left. The acrylamide solution was overlayed by using a pasteur pipette with isobutanol. The gel was placed in a vertical position at room temperature. After polymerization was complete, the overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. As much fluid as possible was drained from the top of the gel and then any remaining water was removed with the edge of a paper towel. Stacking gel was prepared in a disposable plastic tube at an appropriate volume and at desired acrylamide concentration. Concentrations of components of the stacking gel at different volumes are summarized in table 4-4. Without delay, the mixture was swirled rapidly and the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. The comb was immediately inserted into the stacking gel, being careful to avoid trapping air bubbles. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerizing, the samples to be loaded were prepared by heating them to 100^{o}C for 5 minutes in 1X SDS gel-loading buffer to denaturate the proteins. After polymerization was complete, the comb was removed carefully. By using a squirt bottle, the wells were washed with deionized water to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The bubbles that were trapped at the bottom of the gel between the glass plates were removed by a bent hypodermic needle attached to a syringe. 30-200 ug of protein was loaded in a predetermined order into the wells. The electrophoresis apparatus was attached to an electric power supply and the gel was run at 80 volts until the dye front has moved to the resolving gel, after the voltage was increased to 15 V/cm, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off. The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. By using a spatula, the plates were pried apart. Orientation of the gel was marked by cutting a corner from the bottom.

Tris-glycine electrophoresis buffer:

25 mM Tris

250 mM glycine (electrophoresis grade)

0.1% SDS

30% mix (Acrylamide and bis-acrylamide solution)

A stock solution composed of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide, prepared in water. Solution was stored in dark bottles at 4°C.

10% SDS

A 10% (w/v) stock solution was prepared in deionized water

APS

A small amount of 10% stock solution was prepared in deionized water and stored at 4°C.

5X gel loading buffer

3.8 ml distilled water

1.0 ml 0.5 M Tris-HCl

0.8 ml glycerol

1.6 ml 10% SDS

0.4 ml 0.05% BPB

400 ul β-Me ethanol (added freshly)

Table 4-3 Solution of preparing resolving gels for Tris-glycine SDS-PAGE

Solution components	Component Volumes (ml)							
6%	**5 ml**	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
dH_2O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.004	0.008	0.012	0.016	0.020	0.024	0.032	0.040
8%								
dH_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.030
10%								
dH_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
12%								
dH_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5

10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
15%								
dH_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Table 4-4: Solution of preparing 5% stacking gels for Tris-glycine SDS-PAGE

Solution components	**Component Volumes (ml)**							
5% gel	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
dH_2O	0.68	1.4	2.1	2,7	3.4	4.1	5.5	6.8
30% mix	0.17	0.33	0.50	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0,25	0.38	0.50	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

4-2.4 Transfer of Proteins from SDS-Polyacrylamide Gels to Solid Supports

As the SDS-polyacrylamide gel was approaching the end of its run, four pieces of Whatman 3MM paper and one piece of transfer membrane (PVDF or nitrocellulose) was cut to the exact size of the SDS-SDS-polyacrylamide gel by wearing gloves. One corner of the membrane was marked with a soft-lead pencil. The membrane was left in methanol for 1 minutes and then washed with deionized water and soaked into transfer buffer for 15-20 minutes. Meanwhile the Whatman 3MM papers were soaked into a shallow tray containing a small amount of transfer buffer and kept shaking for 1 minute.

The transfer apparatus was set as follows:

- 2 layers of Whatman 3MM paper that have been soaked in transfer buffer was put onto the plate which will be positively charged (anode). All air bubbles were squeezed.

- The membrane was placed onto the Whatman 3MM papers. (The transfer membrane should be exactly aligned and the air bubbles trapped between it and the Whatman 3MM paper should be squeezed out.)

- The glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank, and the gel was transferred to a tray of deionized water.

- SDS-polyacrylamide gel was placed onto the transfer membrane. Any trapped air bubbles were squeezed out with a gloved hand.

- 2 layers of Whatman 3MM paper were placed to the top of the sandwich (this side will be negatively charged during the transfer (cathode side)).

The upper plate of the apparatus will be the cathode during the transfer. The electrical leads of the apparatus were connected to the power supply and the transfer was carried out at a current of 3.5 mA/cm^2 of the gel for a period of 30-45 minutes. The electric current was turned of at the end of the run time and the transfer apparatus was disassembled from top downward, peeling off each layer in turn. The gel was transferred to a tray containing Coomassie Brilliant Blue and stained in order check if the transfer is complete or not. The top left-hand corner of the membrane was cut as insurance against obliteration of the pencil mark.

Transfer Buffer:

2.9 g Glycine

5.8 g Trisma base

0.37 g SDS

200 ml methanol

Adjust volume to 1 lt with ddH_20

4-2.5 Staining proteins immobilized on solid surfaces with Ponceau S

The membrane onto which proteins were transferred was washed with deionized water and then soaked into Ponceau S. When bands of proteins become visible, the membrane was washed in several changes of deionized water at room temperature. The positions of proteins used as molecular-weight standards were marked.

Ponceau S:

10% glacial acidic acid

5% Ponceau S

dH_2O

4-2.6 Immunological detection of immobilized proteins (Western Blotting)

After staining the proteins immobilized on transfer membrane with Ponceau S, the membrane was washed gently with deionized water and neutralized with the blocking buffer for 5 minutes. In order to inhibit non-specific binding sites, the membrane was immersed in the blocking solution for one hour. Primary antibody (table 4-5) was diluted according to instructions in blocking solution and incubated with the membrane at room temperature for one hour or at 4°C O/N on a slowly rotating platform. Afterwards the membrane was washed three times, once for 15 minutes and twice for 5 minutes, with TBS-T. Following the washes, the membrane was incubated with an appropriate secondary antibody (HRP-conjugated) which is diluted as recommended by the supplier in blocking solution for 1 hour at room temperature and

then washed three times with TBS-T. Then the membrane becomes ready for incubation with substrate and development

Table 4-5: The primary antibodies used in this thesis

Name	Origin	Against	Clonality	Dilution
6B10	Mouse	P53	Monoclonal	1/1
15B9	Mouse	P33ING1	Monoclonal	1/1
AF18	Mouse	pRb	Monoclonal	1/500
Ab1	Mouse	P73	Monoclonal	1/200

Blocking Solution:

3% milk powder in 0.1% Tween 20-TBS solution

4-2.7 Detection of proteins immobilized on membranes by using the ECL Western Blotting kit (Amersham Life Science) (Catalog no: RPN 2106)

Detection of proteins immobilized on membranes was done by using the ECL Western Blotting kit (Amersham Life Science) (Catalog no: RPN 2106)

4-3 Extraction of RNA from Cells and Tissues

For the initial studies, RNA extraction was done using conventional method, then, faster and more reliable methods, such as TriPure and column based RNA extraction methods following DNAse treatment were optimized and used.

4-3.1 RNA extraction by conventional method

Conventional RNA extraction method relies on isolation of small molecular weight nucleic acids from a tissue or cell lysate. In this method cells were harvested with a guanidium thiocycnide containing high salt lysis buffer.Total cellular proteins and high molecular nucleic acids (DNA) were extracted by precipitation and extraction

with phenol-chloroform-isoamyl alcohol based extraction method. The un-precipitated material in the soluble phase is RNA and other low molecular weight nucleic acids, such as plasmids. This phase is collected and nucleic acids were precipitated with isopropanol. This method is appropriate for extraction of high amounts of un-pure RNA. Since this method includes phase seperation step, contamination is possible and must be taken into cosideration.

The basic steps of this extraction-precipitation are as follows:

1. Cells or tissue was washed 3 times with PBS
2. 4 ml denaturation solution was added for 10^6 cells or 50mg tissue

   Denaturation solution:

   Stock : 250 gr Guanidium thiocyanide is added into
   293 ml dH2O and
   17.6 ml 0.75M Sodium Citrate (pH: 7.0) and
   26.4 ml 10% Sarkosyl is added to the mixture.

   The solution is stirred to mix at 65°C and stored for 3 months at RT.

   Working : For 50 ml stock add 0.35 ml β-mercapto ethanol

   The solution must be used immediately
3. Lysate was prepared using a pipette or homogenized with a polytron (the solution must be not viscous)
4. The lysate was trasfared to a 15 ml falcon
5. 0.1 volume of 2M Na-Ac was added
6. Mixture was aliquoted into 5 tubes (5x0.9 ml)
7. 1 ml of water saturated phenol was added
8. 0.2 volume of 49:1 chloroform/isoamyl alcohol is added
9. Tubes were stored at 4 C for 15 min, resuspended frequently.
10. Tubes were centrifuged at 10,000 rpm for 15 min at 4 °C
11. Aqueous phase was taken to a fresh tube
12. 1 volume of 100 % isopropanol is added and the mixture is stored at -20 °C overnight (O/N)
13. The tubes are centrifuged at 10 000 rpm for 10 min at 4°C

14. Supernatant (S/N) was discarded, pellet was dissolved in 300 ul denturation solution
15. 300 ul isopropanol is added
16. Samples were stored at -20 °C O/N
17. Tubes were centrifuged at 10 000 rpm for 10 min at 4 °C
18. S/N is discarded
19. Pellet is resuspended in 75 % ET-OH and left at RT for 15 min
20. The tubes are cenrifuged for 15 min at 4 C
21. The pellets were dried in speedvac
22. The pellets are resuspended in 100-200 ul DEPC H2O
23. Samples are aliquoted and stored at -70°C

The RNA isolated with this method contains high amounts of salt and probably sheared genomic DNA. For these reasons, spectrophotometric analysis and formaldehyde containing agarose gel electrophoresis results does not give parallel results. For example spectrophometrically, very concentrated RNA might be unvisible on RNA agarose gel. Another handicap of this protocol is the time consumption. This method takes 3 days with only 4 hours of hands-on time.

4-3.2 RNA Isolation with TriPure Reagent

TriPure isolation reagent is a monophasic solution of phenol and guanidine thiocyanate for RNA, DNA and protein isolation at the same time. TriPure isolation reagent allows the isolation of total RNA, DNA and protein from the same sample in a single-step liquid-phase separation. The expected yield of RNA and DNA from various starting material are as follows:

RNA from tissues: liver or spleen, 6–10 μg/mg tissue; kidney, 3–4 μg/mg tissue; skeletal muscle or brain, 1–1.5 μg/mg tissue; placenta, 1–4 μg/mg tissue.

RNA from cultured cells: epithelial cells, 8–15 μg/106 cells; fibroblasts, 5–7 μg/106 cells.

DNA from tissues: Liver or kidney, 3–4 μg/mg tissue; skeletal muscle, brain, or placenta, 2–3 μg/mg tissue.

DNA from Cultured cells: human, rat, or mouse cells, 5–7 μg/106 cells.

The protocol for nucleic acid and protein extraction differs slightly for tissue and cultured cells as indicated below.

4-3.2.1 Phase Seperation Step

For 50-100 mg of tissue (or 1-5*106 cultured cells) 1 ml of TriPure reagent is used. Basically, the tissue is homogenized using a polytron (the cultured cells are lysed by pipetting or by passing though a 20 gouge 0.9 mm needle) and let stand for 5 min at room temperature. For each 1 ml of TriPure reagent used 0.3 ml of chloroform is added and mixed vigorously by shaking (not vortexing). For complete dissassociation of proteins and lipids, the mixture is let stand for 5 min at room temperature. The sample is then centrifuged for 13000 rpm at 4 degrees for 15 minutes. After this centrifugation there must be 3 layers formed in the tube. The upper layer is colorless and aqeous, containing small molecular weight nucleic acids like RNA and plasmids. The middle, white phase, between red, organic phenol and colorless, aqeous phase is containing some proteins, lipids and DNA. The lower, red phase is made of phenol and containing lipids and proteins. If the middle phase is thicker that 2 mm, 500 ul TriPure and 200 ul chloroform is added to the sample and the sample is mixed vigorously and then centrifuged again at 13000 rpm at 4 degrees for 15 minutes to achieve a clear 3 phase.

4-3.2.2 RNA Isolation Step

The upper aqeous phase containing small molecular weight nucleic acids are taken to a new tube and for each 1 ml of TriPure reagent used as starting material, 0.5 ml of isopropanol is added to the sample. The sample is mixed briefly by inversion a few times and then incubated at -20 degrees for 30 min. Then the sample is centrifuged at 13000 rpm at 4 degrees for 10 minutes.

A white pellet containing salts and RNA must be formed after this centrifugation. The pellet is washed with 1 ml 80% ethanol once and recentrifuged at 10000 rpm at 4 degrees for 10 minutes. The supernatant is discarded and the pellets were air dried for 5-10 minutes. The pellet is resuspended in Rnase free (DEPC treated) water and spectrophometrically analysed. A 260/280 ratio < 1.5 reveals the presence of salts, >2.0 reveals the presence of phenol or spectrophotometre dysfunction. A 260/280

ratio >1.9 and <1.6 reveals good quality of RNA, but DNA contamination must always be suspected and analysed by RNA gel electrophoresis and RT-PCR.

4-3.3 RNA Isolation by Nucleospin-II kit

Nucleospin II RNA purification system allows the purification and extraction of total RNA from various sources like, cells, tissues, yeast cells, bacterial cells, body fluids and RNA contaminated with DNA or proteins in an aqeous solution. The purification system of this kit relies on the affinity of nucleic acid to positively charged glass beads. Briefly, the cells are lysed with 400 ul RA1 (a solution containing Guanidine Thiocyanide and b-mercapto ethanol) and the lysate is loaded to a columnn containing charged glass beads after the addition of 250 ul 100% ethanol. The nucleic acid components of the cell bind to the columnn material and then the columnn is trated with DNase (15 minutes at room temperature) so as to get rid of DNA. After the inactivation of DNase (by addition of 500 ul RA2) and several washes (twice with 350 ul RA3), the only resident of the columnn, RNA, is eluted with RNase free water.

4-3.4 Formaldehyde Containing RNA Gel and RNA Electrophoresis

1% Formaldehyde containing agarose gel is prepared as follows:
11 ml 5X Forrmaldehyde gel running buffer (10 ml of 2M Sodium Acetate, 10.3 gr MOPS and 390 ml DEPC treated distilled water is mixed and pH is adjusted to 7.0 with Sodium Hydroxide (~15 ml 5M NaOH). 5 ml, 0.5 M, pH. 8.0 EDTA is added and the solution is added up to 500 ml), 35 ml DEPC water and 0.5 gr agarose is added and the sample is heated to boil in a microwave owen. When the solution is cooled to 60-70 degrees, 10 ml Formaldehyde is added and the gel is immediately poured into the casted apparatus.

RNA samples for loading are prepared as follows:
15 ul of RNA loading buffer (50% formamide, 20% Formaldehyde, 15% 5X running buffer, 15% glycerol-dye, premixed and stored at -20 degrees) and 5 ul of RNA sample is mixed, heated at 70 degrees for 5 minutes, chilled on ice.

The prepared samples are loaded to the gel and run at 80V for 45 minutes and stained in Et-Br containing water for 5 min and destained overnight in distilled water.

4-4 Extraction of DNA from Cells and Tissues

The middle and bottom phases from RNA isolation (Phase seperation-as explained in section 4-3.2.1) were used for DNA extraction. Briefly, 0.5 m of ethanol is added to the middle and bottom phases. The tube is inverted several times for complete dissassociation of middle layer. The mixture is centrifuged for 10K rpm for 5 minutes. The pellet containing DNA and other impurities must be washed twice with 30% ethanol, 10 mM Sodium Citrate solution.

Then the pellet is resuspended in 1 ml freshly prepared digestion buffer (100 mM Sodium Chloride, 10 mM Tris pH : 8.0, 25 mM EDTA pH : 8.0, 0.5% SDS and 0.1 mg/ml proteinase K) and incubated overnight at 65 degrees. The protein components of the pellet is disassociated after 16 hours and DNA is extracted using phenol/ chloroform/isoamyl alcohol and precipitated with the addition of 200 ul of 7.5 M Ammonium acetate and 2.5 volumes of absolute ethanol. The DNA precipitate is resuspended in 100-200 ul of dH2O at 55 degrees for 1 hour with occasional mixing. The presence and the quality of DNA must be investigated with PCR using 1-2 ul of DNA as template or direct agarose gel electrophoresis.

4-5 cDNA Preparation from Total RNA:

cDNA is prepared from 3-5 ug total RNA. X ul (3-5 ug) RNA, 1 ul of oligo-dT primer and (11-x) ul Rnase free dH_2O are placed into an Rnase free tube. The tube is incubated at 70 degrees for 5 minutes so as to disrupt the secondary structure of RNA. The tube is chilled on ice and then placed to a 37 degree incubator for 5 minutes with the addition of 4 ul of 5X reaction buffer, 2 ul of 10mM dNTP mix and 1 ul of Rnase inhibitor. Then the reaction is first equilibrated and after the addition of 1 ul RT enzyme, incubated at 42 degrees for 1 hour. After a final incubation at 70 degrees for 10 minutes so as to heat inactivate MMLV reverse transcriptase enzyme, 20 ul of cDNA is ready to use for PCR amplification.

4-6 PCR Amplification Using cDNA:

For a common PCR reaction with Taq polymerase, 8 components must be mixed and incubated at specific temperatures at specific time periods in a thermal cycler device. For a representative 50 ul PCR reaction, the following components must be mixed in a 200 ul PCR tube (Table 4-6). Please note that optimization indicates the probable need for optimization.

Table 4-6: The reagents of a common PCR reaction

The components of a common PCR reaction using cDNA as template:				
REAGENT	**STOCK**	**USED**	**VOLUME**	**COMMENTS**
dH_2O			32.6	
PCR buffer	10X	1X	5 ul	
dNTP mix	2 mM	200 uM	5 ul	
$MgCl_2$	25 mM	1.5 mM	3 ul	optimization
F-Primer	25 pm/ul	25 pm	1 ul	optimization
F-Primer	25 pm/ul	25 pm	1 ul	optimization
Taq polimerase	5U/ul	2U	0.4 ul	
Template DNA (cDNA)			2 ul	optimization

After mixing the components, the PCR tubes containing the reaction mixtures are carrried rapidly to a thermal cycler which incubates the samples at specific temperatures for specific time periods. A representative, 30 cycle PCR program for amplification of up to 1000 bp fragments is as follows:

	TEMPERATURE	TIME	COMMENTS
	94	5 min.	Initial denaturation
30X	94	30 sec.	Cycle denaturation
	62	30 sec.	Cycle annealing
	72	30 sec.	Cycle extension
	72	5 min.	Final extension
	4	forever	Waiting

4-7 Definition of the Fidelity of Reverse Transcription and Genomic DNA Contamination in the cDNA:

The fidelity and genomic DNA contamination of freshly prepared cDNA must be checked before making expression analysis. This can be achieved by making PCR for a ubiquitously expressed transcript such as GAPDH, cyclophilin or beta-actin. The primers for this analysis must be carefully selected, so that, they must anneal to different exons, and produce a longer PCR product from genomic DNA covering a small intron. We have used GAPDH primers for this analysis, producing a 151 bp fragment from cDNA and 250 bp fragment from genomic DNA (for sequence of GAPDH primers look 'primers used in this thesis' section.). Making a 19-24 cycle GAPDH PCR using 1 ul of cDNA reveals the efficiency of cDNA synthesis and genomic DNA contamination.

4-8 Expression analysis of RNA using cDNA:

20 ul reaction mixture of the prepared cDNA contains 3-5 ug of total RNA, converted to cDNA. Using an equal amount of cDNA (such as 0.5-3 ul) for PCR amplifications gives comparable results at a specific number of PCR cycles. The number of PCR cycles is determined by an initial study for each transcript by making 20-35 cycles of PCR with 2-4 cycle intervals. By doing such a analysis, for example, 20, 23, 26, 29, 32, 35 cycles of PCR with an equal cDNA load defines the minimum number of cycle to visualize the product on agarose gel and the saturation cycle. Moreover, if the analysis covers a few sources, such as different cell lines and tumors, all samples should be analyzed initially at a high number of PCR so as to be sure at the presence or absence of the transcript in all samples. Semi quantitative RT-PCR can be achieved in 2 different approaches: comparison with GAPDH or equalizing GAPDH

4-8.1 Comparison with GAPDH

All samples are initially analyzed for their GAPDH expression at a unsaturated cycle, such as 19-24. Then the gene of interest is analyzed again at an unsaturated cycle. The band intensities of each sample is compared that of GAPDH using an image-analysis software. For example, p73 gene expression in sample A, B and C are compared. If, sample A has a GAPDH intensity of 10 and p73 intensity of 5, sample B has GAPDH intensity of 20 and p73 intensity of 10 and sample C has GAPDH intensity of 5 and p73 intensity of 10. The GAPDH versus p73 intensities are : sample A: 0.5, sample B: 0.5 and sample C: 2. This results mean that p73 expression levels are same and low in sample A and B but increased 4 times (0.5 versus 2) in sample C, although p73 band intensity is not 4 times increased.

4-8.2 Equalizing GAPDH

Like method 1, all samples are initially analysed at an unsaturated cycle for GAPDH. IF the results are the same for samples A, B and C are 10, 20 and 5 respectively. This means that either the RNA content or the fidelity of reverse transcription reaction is differing among these samples. In this method, equalizing

GAPDH is necessary, so cDNA is diluted accordingly (A: ½, B: ¼ and C 1/1). Using equal volumes of cDNA, such as 2 ul will give the same band intensities for GAPDH and probably will show equal and low intensity for p73 in sample A and B, but 4 times increase in sample C.

4-8.3 PCR amplification of selected transcripts using the optimized cDNA

The PCR reactions were carried out as described above in section 4-6. The transcript specific primers are summarized in table 4-7.

Table 4-7: Synthetic Oligonucleotides Used In This Study

NAME	SEQUENCE	Tm (°C)
p73L-F	(5'-CGGCCATATTGGTGCCGCAGCCACTGGTG-3')	64
p73-X14-R	(5'-CCTCAGTGGATCTCGGCCTC-3')	63
p73α–R	(5'-GTTTGGCACCCCCAATCCTGT-3')	61
p73β–R	(5'-AGGGCCCCCAGGTCCTGAGC-3')	63
GAPD-F	(5'- GGCTGAGAACGGGAAGCTTGTCAT-3')	64
GAPD-R	(5'- CAGCCTTCTCCATGGTGGTGAAGA-3')	64
TA-p73-F	(5'-AACCAGACAGCACCTACTTCGACC-3')	62
DN-p73-F	(5'-ACCATGCTGTACGTCGGTGACCCC-3')	62
p73-X4-R	(5'-GCGACATGGTGTCGAAGGTGGAGC-3')	62
16^{INK4a}-F	(5'- CGGAGAGGGGGAGAACAGAC-3')	61
$p14^{ARF}$-F	(5'- TCACCTCTGGTGCCAAAGGG -3')	62
C-R	5'- GGCAGTTGTGGCCCTGTAGG -3')	63
cyclin E-F	(5'-TTGACCGGTATATGGCGACACAAG -3')	64
cyclin E-R	(5'-ATGATACAAGGCCGAAGCAGCAAG -3')	64
Filip-F	(5'-GGACGGACGCCGATGCC-3')	63
Filip-R	(5'-GGTCCATGGTGCTGCTCAGC-3')	63
β-actin-F	(5'-TGATATCGCCGCGCTC-3')	59
β-actin-R	(5'-CAGTCAGGTCCCGGCC-3')	59
p53-F	(5'-TCCCCCTTGCCGTCCCAA -3')	58
p53-R	(5'- CGTGCAAGTCACAGACTT -3')	55

4-9 CLONING for SEQUENCING

PCR amplified DNA fragments are cloned for sequencing for the description of splice acceptor and donor sites of alternative splicing products and for the description of a new p73 gene encoded transcript. As a vector, pGEM-T easy, T/A cloning vector was used (Promega). As a host for the plasmid, DH5α strain of *E.coli* was used.

THE GENOTYPE OF DH5α

Strain	Genotype	usage	reference
DH5α	*supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Host for plasmid DNA	Hanahan (1983)

4-9.1 Growth of *E. coli* strains

All strains were grown in LB medium or on LA medium supplemented with the appropriate antibiotic if necessary.

Solid and liquid mediums:

LB medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl.
LA medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl and 1% agar.

Antibiotics:

Ampicillin with a final concentration of 50 ug/ml was used in this study.

4-9.2 Preparation of competent bacteria

Cells were made competent using a modification of the CaCl2 method described by Maniatis *et al.* (Maniatis et al.,, 1982). In brief, 5 ml LB was inoculated using a single colony from a freshly grown plate of the *E. coli* strain to be used, and was incubated at 37°C for approximately 2 hours, until the OD_{600} of the culture is 0.3-0.4. The culture was then cooled on ice for 5 minutes, 1 ml aliquots were added to microcentrifuge tubes and then the cells were pelleted by centrifugation (1 minute at 13,000rpm). The cells were resuspended in 0.5 ml of 50 mM CaCl2 by gentle vortexing, before being placed on ice for 30 minutes. The cells were pelleted by centrifugation (1 minute at 13,000 rpm), and the supernatant was discarded. The pellet was resuspended in 0.1 ml of CaCl2 with gentle vortexing. The cells were then stored on ice until required for transformation. Alternatively competent cells were prepared and stored at -70°C until required. 500 ml of LB was seeded with a 10 ml of overnight culture and grown to an OD600= 0.6. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, before being incubated on ice for 20 minutes. The cells were harvested as before, resuspended in 25 ml of 50 mM CaCl2 /20% glycerol and aliquoted into microcentrifuge tubes before being frozen. Samples were stored at -70°C and were viable for at least 2 months. Cells were thawed on ice prior to the addition of DNA.

4-9.3 Ligation

Ligation reaction is done as described in the technical manual of pGEM-T easy vector system handbook. This system allows the usage of unpurified PCR products in the ligation. Briefly, the following components were mixed and the reaction was incubated at room temperature for 1 hour for the ligation to proceed.

2X ligation buffer	5 ul
vector	1 ul
Insert	X ul
Ligase	1 ul
dH2O	3-X ul

4-9.4 Transformation of plasmid DNA in bacterial cells

The DNA to be transformed (usually a 1-10 ul of ligation mixture) was added to the 100 ul of competent cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 90 seconds and chilled by placing on ice for 2 minutes. 1 ml of pre-warmed LB was then added and the suspension was incubated at 37°C for 1 hour. Each sample was pelleted by centrifugation at 13,000 rpm for 2 minutes, resuspended in 100-200 ul of LB and plated onto selective medium and incubated overnight at 37 °C to allow the growth of the transformants.

4-9.5 Isolation of plasmid DNA from bacteria: Small scale preparation of plasmid DNA

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979).

The transformed bacterial strain containing the plasmid of interest was grown at 37°C overnight in 5 ml of LB+antibiotic. 1.5 ml of the bacterial culture was pelleted by centrifugation for 1 minute (bench-top microfuge, 13,000 rpm) in a 1.5 ml microfuge tube. After the removal of the supernatant, the cells were resuspended in 0.1 ml of ice-cold solution I and stored for 5 minutes at room temperature. 0.2 ml of solution II was mixed by inversion, the tube was then stored on ice for 5 minutes. Bacterial chromosomal DNA and proteins were precipitated by the addition of 0.15 ml of ice-cold solution III. The mixture was left on ice for 5 minutes, then centrifuged at 13000 rpm in a bench-top centrifuge for 5 minutes to pellet the host DNA and proteins. The supernatant was mixed with an equal volume of phenol-chloroform (1:1) and centrifuged in a bench-top microfuge at 13000 rpm for 3 minutes to separate the two phases. The top phase was removed and plasmid DNA was precipitated by mixing it with 2.5 volumes of 95% ethanol, and pelleted by centrifugation for 10 minutes (bench-top microfuge, 13,000 rpm) after keeping the mixture at 4°C for 15 minutes. The supernatant was discarded and the pellet was left for 15-20 minutes at room temperature

to dry and then resuspended in 20-30 ul of TE buffer containing 10 ug/ml RNaseA. Samples were stored at -20 °C.

Solution I

50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/square in. on liquid cycle, and stored at 4oC.

Solution II

0.2M NaOH (freshly diluted from 1 M stock)
1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

TE buffer

pH 7.4	10mM Tris.Cl (pH 7.4)
	1mM EDTA (pH 8.0)

The results of the miniprep was quantified by spectrophotometric analysis at 260/280nm and by agarose gel electrophoresis before the sequencing reaction.

4-9.6 Restriction enzyme analysis

Restriction enzyme digestion was either used to confirm the presence of an insert in a plasmid or for genotyping p53 codon 72 polymorphism. DNA restriction reactions were carried out in a total volume of 20-100ul with 5-10 units of restriction enzyme. The volume of the reaction buffers were arranged to be 1X and the enzyme volume was never greater than 1/10th of the reaction volume so as to prevent star activity due to the high glycerol concentration. Analytical studies for detection of the presence of an insert in a plasmid was achieved with a two hour restriction, whereas, the genotyping studies was achieved with an overnight digestion of the PCR amplified DNA so as to achieve 100% restriction. A typical 20 ul restriction reaction was described below.

Component	volume	Unit
H2O	13 ul	-
DNA	5 ul	-
10X buffer	2 ul	-
Enzyme	1 ul	10U

The restriction enzymes were obtained from MBI Fermentas (Lithuania).

4-10 Analysis of DNA using agarose gele electrophoresis

The agarose gel electrophoresis of DNA was carried out using the instructions defined by Maniatis *et al.* (Maniatis et al., 1988) and using the mini, midi and maxi systems of EC.

RESULTS

CHAPTER V

THE DESCRIPTION AND ANALYSIS OF P73 GENE ENCODED TRANSCRIPTS AND PROTEINS

We have started to investigate the probable role of p73 in liver carcinogenesis, just after the description of the p73 gene, in late 1997. The role of p53 in the ethiopathology of HCC is well defined so its structural and functional homologue p73 gene was suspected to be structurally inactivated during HCC development. A group of scientists, under the supervision of Prof. Mehmet Ozturk were organized to look for mutations in the DNA binding domain of p73, especially to the codons corresponding to the mutational hotspots of the p53 tumor suppressor. The results of this study revealed no mutations but several polymorphisms in a set of primary liver cancer samples so the role of p73 on liver carcinogenesis was described to be different than the other family member, p53 (Ozdag et al., unpublished data).

With the clues of this initial study, we have suspected changes in the expression pattern of p73 gene during liver carcinogenesis so we planned to work with HCC derived cell lines for characterization of p73 gene encoded transcripts. We knew that p73 gene encodes for at least 2 major transcripts, namely p73α and p73β. The difference of these two transcripts is that p73β, lacking exon 13. Since other groups stated that p73 mRNA levels are very low (Zaika et al., 1999), we planned to describe all 3' alternative splicing forms by a 2 step PCR as described in figure 5-2. Thus we have extracted total RNA from 10 HCC cell lines, namely Huh7, Hep3-BTR, Focus, Mahlavu, Hep40, Hep3B, HepG2, PLC/PRF/5, BC-1 and SK-Hep1 and a normal liver by conventional method. The 260/280 nm, Optical Density (OD) of these samples were between 1.8 and 2.0, and the concentrations were between 0.8 to 1.5 ug/ul.

5-1 cDNA preparation and β-actin PCR results

cDNA from these samples were prepared by using 5 ug of total RNA, by oligo-dT primer. The reaction volume of cDNA preparation is 20 ul and 1 ul from each sample is used for β-actin amplification for the control of the quality and the quantity of cDNA (Figure 5-1).

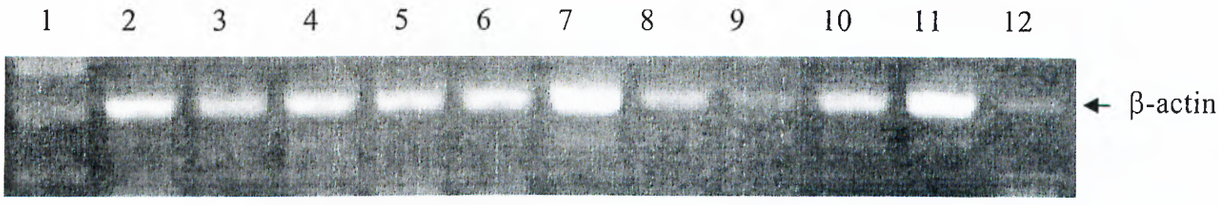

Figure 5-1: The RT-PCR picture of β-actin of 10 HCC cell lines and a normal liver. Lanes: 1: marker, 2: Huh7, 3: Hep3-BTR, 4: Focus, 5: Mahlavu, 6: Hep40, 7: Hep3B, 8: HepG2, 9: PLC/PRF/5, 10: BC-1, 11: SK-Hep1 and 12: Normal Liver

5-2 Description of 3' end alternative splicing forms of p73 transcripts by 2 round PCR amplification

The two round PCR strategy was used to detect the p73 encoded transcripts is schematized in figure 5-2. First, a PCR using p73L-F and p73-X14-R was performed from all samples, using 2.5 ul of cDNA as a template. The result of this amplification did not produce a visible band on ethidium bromide stained agarose gel. However, the second round PCR, using 5 ul of product from first PCR as template and p73L-F as the forward and p73α-R and p73β-R as the specific reverse primers yielded positive and comparable results, as shown in figures 5-3 and 5-4.

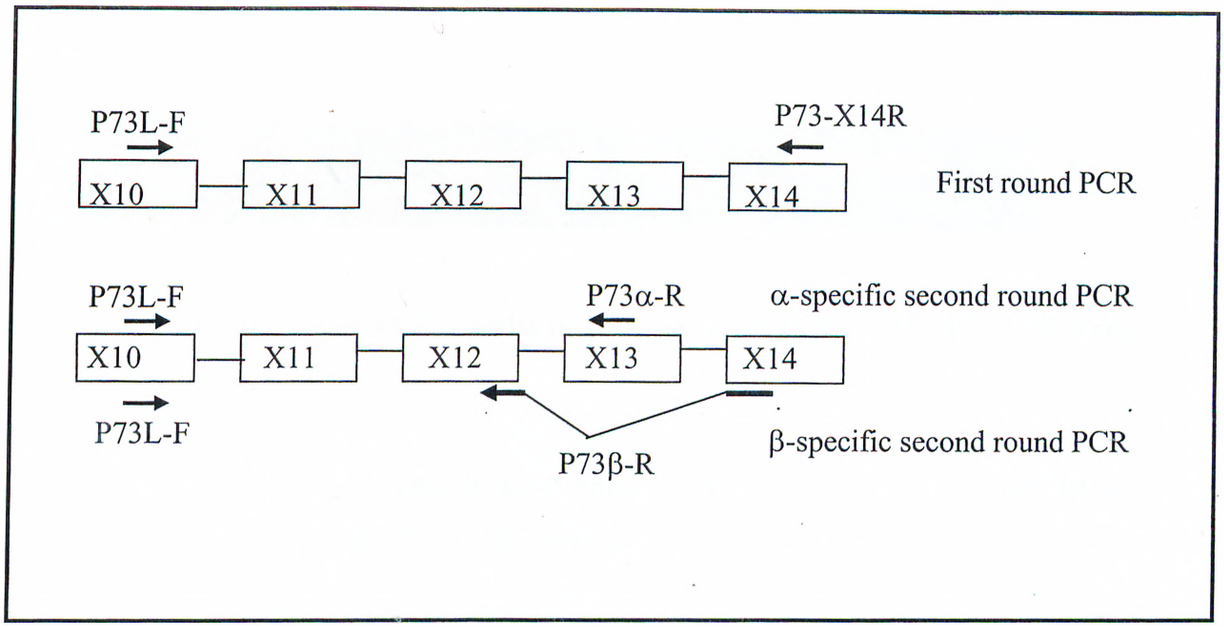

Figure 5-2 : The schematic representation of the approach: Two round PCR based detection of 3' alternative splicing products of p73 transcripts

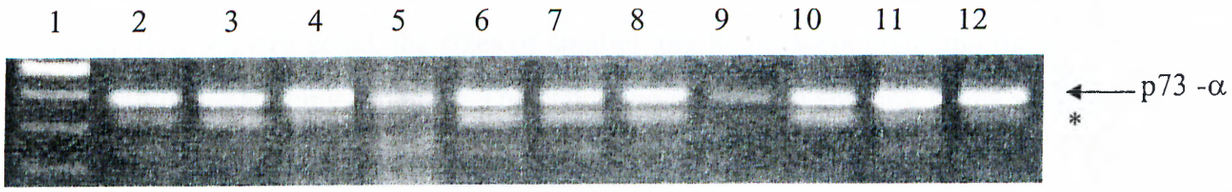

Figure 5-3 : Second round PCR results of p73α specific PCR of 10 HCC cell lines and a normal liver.

Lanes: 1: marker, 2: Huh7, 3: Hep3-BTR, 4: Focus, 5: Mahlavu, 6: Hep40, 7: Hep3B, 8: HepG2, 9: PLC/PRF/5, 10: BC-1, 11: SK-Hep1 and 12: Normal Liver. The band indicated with "*" below the α transcript is a non-specific band

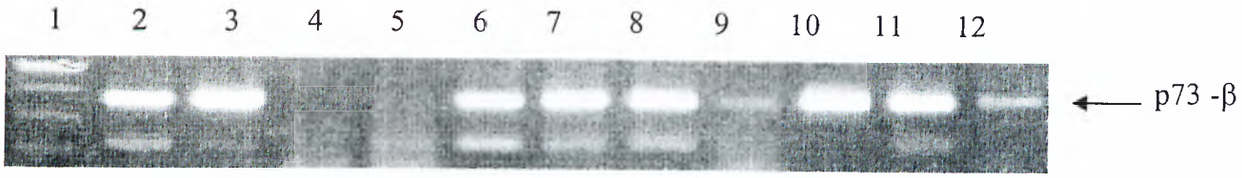

Figure 5-4 : Second round PCR results of p73β specific PCR of 10 HCC cell lines and a normal liver.

Lanes: 1: marker, 2: Huh7, 3: Hep3-BTR, 4: Focus, 5: Mahlavu, 6: Hep40, 7: Hep3B, 8: HepG2, 9: PLC/PRF/5, 10: BC-1, 11: SK-Hep1 and 12: Normal Liver

5-4 Identification of consistently amplified smaller bands as new transcript isoforms and cloning of the new transcripts

The PCR results for some cell lines, produced consistent and small amplification products, which can not be diminished by changing the annealing temperature and magnesium concentration (Figure 5-4, lane 6). Therefore these products were suspected to be specific products of the PCR reaction, so alternative splicing was taken under consideration. As suspected, the sizes of smaller amplicons were matching the probable size of candidate splicing products. The candidate alternative splicing forms are lacking one or more exons so with the approach defined in figure 5-2 the relative sizes of the probable bands that can be obtained with our experimental system are summarized in table 5-1.

Table 5-1: The probable product size of α and β specific second round PCR reactions, as calculated by the lack of one or a combination of more than one exons.

α specific second round PCR produce	β specific second round PCR produces
p73α : 383 bp	p73β: 369 bp
p73γ : 235 bp	p73ϕ : 221 bp
p73ε : 97 bp	

All of the PCR products from α and β specific reactions were excised from agarose gel, purified and ligated to pGEM-T-easy, T/A cloning vector. The ligation products were transformed to $DH_5\alpha$ strain of E.*coli* and selected for ampicilin resistancy on LA plates. The colonies formed were inoculated into LB-ampicilin medium and minipreps were prepared (Figure 5-5). Candidate clones were digested with EcoRI, which takes the insert out (Figure 5-6).

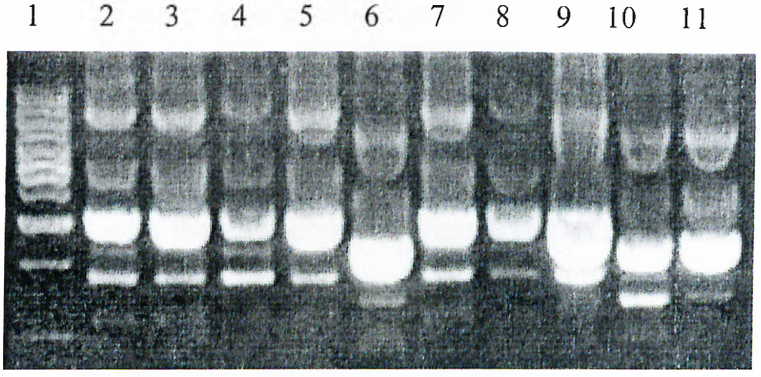

Figure 5-5: A representative miniprep results of several p73 alternative splicing products

Lanes: 1:marker, 2,3,4,5: candidate clones from p73α, 6: A blue colony, 7,8,9,10,11: candidate clones from p73β

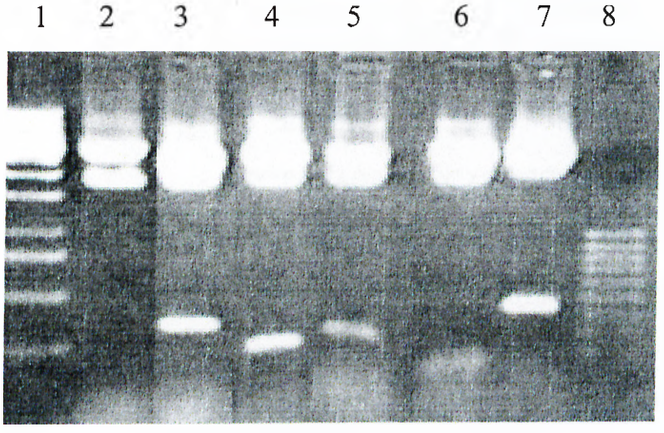

Figure 5-6 : Restriction of miniprep results of several p73 alternative splicing products with EcoRI

Lanes: 1: 1kb marker, 2: empty pGEM-T-easy, 3: p73β, 4:p73ϕ, 5: p73γ, 6: p73ε, 7: p73α, 8: 100bp DNA ladder

5-5 Sequencing results and the description of exon-exon boundaries of alternative splicing products

New minipreps were prepared and phenol-chloroform treatment was done so as to be sure that plasmids are ready for sequencing. Sequencing results showed the authenticity of α, β, γ, ϕ and ε forms with exon-exon junctions (Figure 5-7). Through the progression of these studies, Zaika *et al.* (Zaika et al., 1999), described the presence of all probable splicing forms of p73 transcripts in breast cancer cell lines and primary breast cancer tissues, including the ones we have described (Figure 2-1). Our results correlate well with the data obtained from breast cancer samples, which proposed the expression of p73α and p73β as major transcripts in normal breast tissue, but cancer related changes in the alternative splicing give rise to different p73 gene encoded transcripts through the progression of carcinogenesis. Our results showed the presence of p73α and p73β in normal liver and the acquired expression of a combination of least 5 different p73 3' alternative splicing forms in HCC derived cell lines.

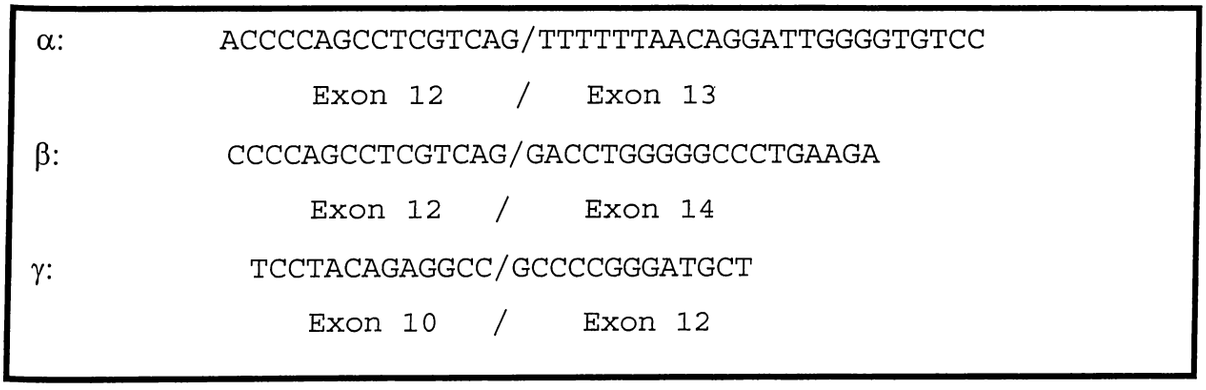

Figure 5-7: The sequencing results of the plasmid clones containing the different splicing products of p73.

5-6 Identification of a new exon and a promoter region within intron 3 of p73 gene

After the Human Genome Project was finished, all the sequence of human chromosomes entered to the public database. *In silico* analysis of the contig harboring human p73 gene yielded the identification of a promoter like DNA fragment, at which different transcription factors bind. Moreover, the promoter like DNA fragment contained a putative exon, transcription initiation site, translation initiation site, an open reading frame at considerable length and a splicing acceptor site (Figure 5-8). These results lead us to propose the initiation of a new transcript from intron 3 of human p73 gene. The new exon in the intron 3 of p73 was called as exon 3', regarding the nomenclature of p63 and mouse DN-p73 (Yang et al., 1998, Yang et al., 2000)

```
1__CCTGCTTCCAGGGGGACTCGGGCCCCTCTGCCAGGGTCAACTTTGTACCCAAGACGGCTGAAATACAATG
71__GAAATTCAGACGGCCCAACAGGGAGTGGCAGTCACCTCAAAGGCCCCACTAGACGGGTGCGGGGCACCA
140__CTGCAGAGCCCCTCCCTGGCTGTGCCAAGGCCGTCCACGCCTGCAGGGGGCCCCACTGCCGGGCTGTT
219__CTTTGGCAACAGTGGCTTGTCCCTGTTTCCTGGGGGCTTGGCCAGTGCCAGGGTGGGCTCCAAACGCA
287__CGGCTCTGGGCTCTTGGACTCACCCCTGCTTTGGGCAGGCAGTGGAAGGCAGGCCCCACAAGAGCTGC
355__TCACTCCCGTCACCTGTCTCCCTCGGGGGTCTAGGGTCGAACCTCCTGTGAGCCCCTCCTCTCCATGC
423__AGCCCTTGGACTGGTCCTGGCGGACCACCGAGTTCCCCGCGCAGGGGGCAGGTGCGCCCCACCTGGGT
491__GCCAAGGGAGGCGACACCATCTCTCCCCCTTGGGGTGGCCCAGCCTTGCCTACCATGATCTCCAGGGC
559__CGGGGCTCAGCCCTCATGCCTGGGAACAGAGGCTGCTTTACGGGGTGAGGGCCTGGGGCCCCCCGAGC
627__CTTCCCCAGGCAGGCAGCATCTCGGAAGGAGCCCTGGTGGGTTTAATTATGGAGCCGGCGCTGACCGG
695__CGTCCCGGCCCTCCCCACGCAGCCTCCTTGGTGCGGT
                                                    predicted DN-p73 promoter
732__CCAACACATCACCGGGCAAGCTGAGGCCTGCCCCGGACTTGGATGAATACTCATGAGGAATAAAGGGG
800__TGGGCCGCGGGTTTTGTTGTTGGATTCAGCCAGTTGACAGAACTAAGGGAGATGGGAAAAGCGAAAAT
868__GCCAACAAACGGCCCGCATGTTCCCCAGCATCCTCGGCTCCTGCCTCACTAGCTGCGGAGCCTCTCCC
936__GCTCGGTCCACGCTGCCGGGCGGCCACGACCGTGACCCTTCCCCTCGGGCCGCCCAGATCCATGCCTC
1004__GTCCCACGGGACACCGTTCCCTGGCGTGTGCAGACCCCCCGGCGCCTACCATGCTGTACGTCGGTGG
1071__ACCCCGCACGGCACCTCGCCACGGTAGGTGTGACGCGCCATTCATAGGATCTCTTCGGGGACTTTG
```

underlines= probable DN-p73 promoter
bold "**T**" in the underlined region= probable transcription initiation site
letters on grey background= probable first exon of DN-p73 (exon 3')
the bold "**GT**" at the end of exon= splicing signal

blue highlights= probable STAT binding site (100% match)
green highlights=probable sp1 binding site((100% match)also found in mouse e2f1 promoter). Hsiao et al., 1994
yellow highlights=probable sp1 binding site((100% match)also found in human glycophorin B promoter). Rahuel et al., 1992.
purple highlights=probable sp1 binding site((100% match)also found in human cdc2 promoter). Shimizu et al., 1995.

Figure 5-8 : The analysis of the contig, covering a putative promoter, binding sites for transcription factors, a putative exon, transcription and translation initiation sites and a splice donor site.

5-7 Description of the transcript from the new promoter containing the new exon

Primers were designed from exon 3' and exon 4 to amplify the putative p73 transcript (now called as DN-p73), but not the former p73 transcript (now called as TA-p73). The initial amplification trials using SNU398 cell line cDNA revealed a band at expected size (Figure 5-9).

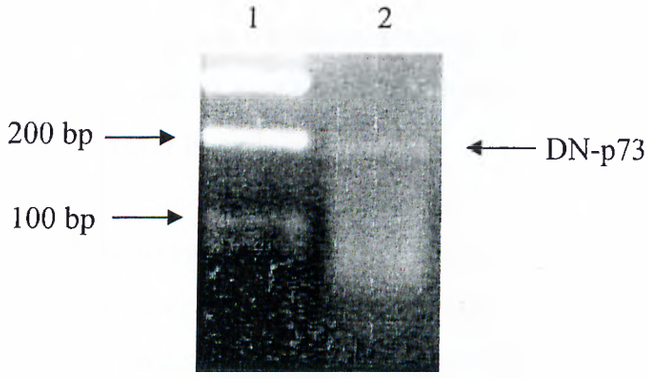

Figure 5-9: The PCR result of exon 3'-exon 4 which is specific for DN-p73 but not TA-p73.

Lanes: 1: 100 bp marker, 2: DN-p73 PCR result of SNU 398 (expected to be 186 bp)

5-8 Cloning and sequencing of the new transcript

The new transcript was ligated to pGEM-T-easy, T/A cloning vector. The ligation products were transformed to $DH_5\alpha$ strain of E.*coli* and selected for ampicilin resistancy on LA plates. The colonies formed were inoculated into LB-ampicilin medium and minipreps were prepared for sequencing. The results of sequencing revealed the exon-exon boundries of exon 3' to exon 4 as shown in Figure 5-10.

CCATGCTGTACGTCGGTGACCCCGCACGGCACCTCGCCACGGCCCAGTTC
AATCTGCTGAGCAGCACCATGGACCAGATGAGCAGCCG

Figure 5-10: The sequencing result of a DN-p73 clone

The bold letters indicate the putative exon 3' of DN-p73 and the underlined letters indicate the former exon 4 of p73

5-9 RNA isolation by TriPure reagent and purification by MN Nucleospin II kit from 15 HCC derived cell line, 7 primary HCC tumor, 1 correspoding non-tumor liver and 1 normal liver

The expression profile of the new transcript, together with the former one and the 3' end alternative splicing isoforms must be studied in a series of HCC cell lines and primary tumors in comparison with non-tumor liver tissue and normal liver, in order to assign a role to p73 in hepatocarcinogenesis. The most common method for such studies is semi-quantitative RT-PCR method. Since this method is very sensitive, it requires highly pure, DNAse treated RNA. For this reason, 15 HCC derived cell lines, namely Huh7, Hep3BTR, FOCUS, Mahlavu, Hep40, Hep3B, HepG2, PLC/PRF/5, SK-Hep1, SNU 182, SNU 387, SNU 398, SNU 423, SNU 449 and SNU 475 were grown to 70% confluency in 150 mm tissue culture plates and their RNA is isolated by TriPure RNA isolation reagent and then purified with MN Nucleospin II RNA isolation kit, including DNAse treatment. RNA from a normal liver sample was used previously in the description of 3' alternative splicing products. This RNA was further purified by MN Necleospin II RNA isolation kit and DNAse treatment is done. All the samples from cell lines and normal liver were subjected to spectrophotometric analysis and RNA gel electrophoresis (Figure 5-11).

The isolated RNA is aliquoted and snap frozen with liquid nitrogen immediately except for 1 aliquot. 5 ul of RNA from the unfrozen aliquot was mixed with RNA loading buffer, heated at 70°C for 5 min, cooled on ice and loaded to the formaldehyde containing agarose gel. 4 ul of RNA from the unfrozen aliquot was diluted in 396 ul of DEPC treated water and spectrophotometrically analyzed.

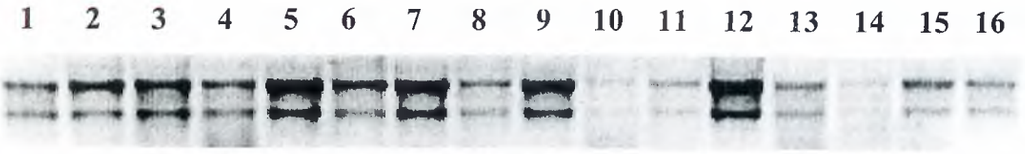

Figure 5-11 RNA electrophoresis of purified RNA from 15 cell lines and normal liver

Lanes: 1: Huh7, 2: Hep3BTR, 3: FOCUS, 4: Mahlavu, 5: Hep40, 6: Hep3B, 7: HepG2, 8: PLC/PRF/5, 9: SK-Hep1, 10: SNU 182, 11: SNU 387, 12: SNU 398, 13: SNU 423, 14: SNU 449, 15: SNU 475, 16: Normal liver

7 primary HCC tumors and 1 corresponding non-tumor tissue were chosen from the archives of Prof. Mehmet Ozturk, homogenized with a polytron in TriPure RNA isolation reagent and their RNA is isolated. Following the purification of RNA by MN Necleospin II RNA isolation kit and DNAse treatment, the samples were subjected to RNA electrophoresis, together with spectrophotometric analysis (Figure 5-12).

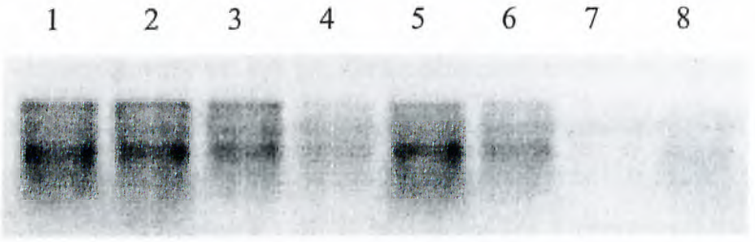

Figure 5-12 : The purified total RNA of the 7 archival HCC tumors and 1 corresponding non-tumor tissue

Lanes: 1: non-tumor 1, 2: tumor1, 3: tumor 2, 4: tumor3, 5: tumor 4, 6: tumor 5, 7: tumor 6, 8: tumor 7

5-10 cDNA preparation and GAPDH PCR results

cDNA is prepared from 3-5 ug of total RNA by oligo-dT primer following the instructons of the cDNA preparation kit (MBI, #K1622). The quality of cDNA, for semi-quantitative PCR analysis can only be described by a semi-quantitative PCR for a ubiquotously expressed transcript like GAPDH, β-actin or cyclophilin. We have previously used β-actin to analyze cDNA synthesis efficiency, but, we have been informed the presence of pseudo-genes of actin in human genome which does not contain any introns (genbank no: D50658). If RNA is not treated with DNAse, the sample will contain some DNA contamination. The contaminant DNA not only will interfere with the cDNA preparation reaction, but also the pseudo-genes from the genomic DNA will produce fake amplicons, which can not be distinguished from the amplifications from cDNA by their size. For this purpose, we have switched to a 2 step RNA isolation and purification step with DNAse treatment, and we have also switched to GAPDH as the internal control, for which no pseudo-gene reported to be present in human genome. The primers for amplification of GAPDH were designed from different exons, so the PCR reaction will produce a band at 250bp from the genomic DNA, covering a small intron and 151 bp from the cDNA. This also allowed us to detect the probable genomic DNA contamination of our samples.

Semi-quantitative RT-PCR is optimized according to a constant load of cDNA (such as 1 ul/ 50 ul PCR reaction) and increasing number of PCR cycles. The aim of this optimization is to obtain an unsaturated but visible PCR yield. For this reason, an initial study of 30 cycles RT-PCR was done for 5 representative samples. The samples with the minimum and the maximum yield were taken to a further analysis. These samples were tested for increasing number of PCR cycles as described in figure 5-13.

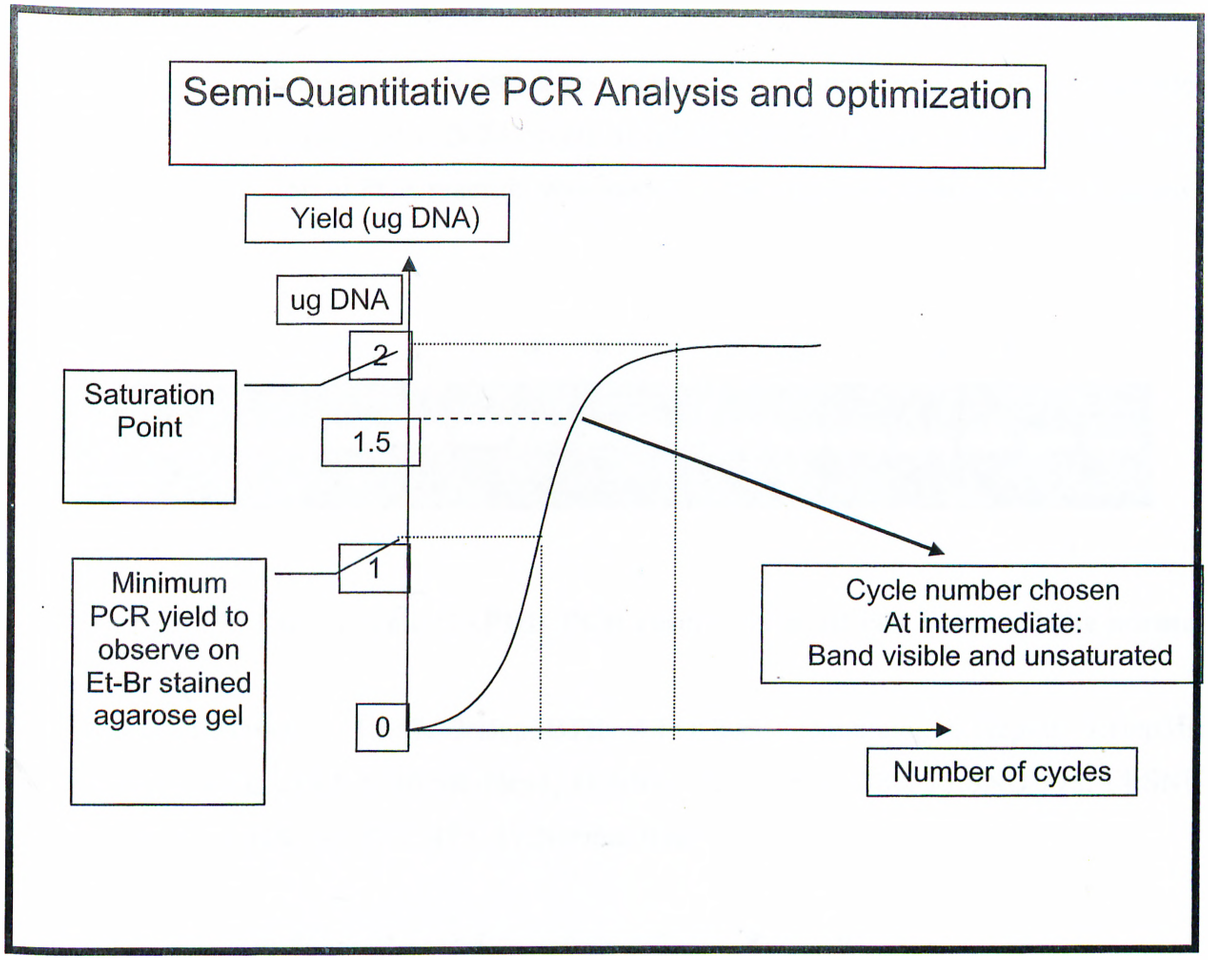

Figure 5-13: The strategy of semi-quantitative PCR optimization

The cycle number of the minimum number of cycle necessary for a visible but an unsaturated band is obtained for each transcript. First and most importantly, such a study for GAPDH was done and the optimum cycle range for GAPDH was determined to be 22-24.

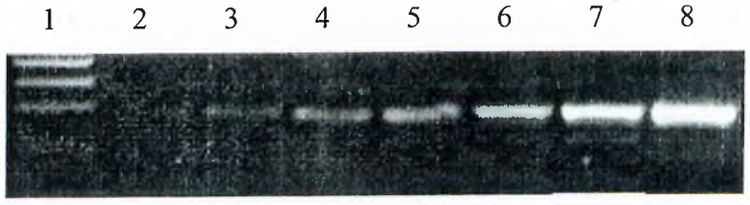

Figure 5-14: Cycle optimization of GAPDH RT-PCR

Lanes: 1: Marker, 2: 19 cycle, 3: 20 cycle, 4: 21 cycle, 5: 22 cycle, 6: 23 cycle, 7: 24 cycle, 8: 25 cycle

After optimization, all cell lines, normal liver, tumor and non-tumor samples were tested and equalized with 24 cycles of GAPDH PCR (Figure 5-15 and 5-16). 20% of the yield of a 50 ul PCR reaction was loaded to the ethidium bromide stained agarose gel.

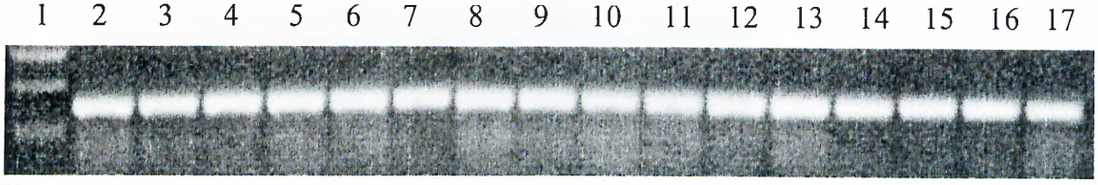

Figure 5-15: The 24 cycle GAPDH PCR results from all cell lines and the normal liver

Lanes : 1:Marker, 2:Huh7, 3:Hep3BTR, 4:FOCUS, 5:Mahlavu, 6:Hep40, 7:Hep3B, 8:HepG2, 9:PLC/PRF/5, 10:SK-Hep1, 11:SNU 182, 12:SNU 387, 13:SNU 398, 14:SNU 423, 15:SNU 449, 16: SNU 475, 17:Normal liver

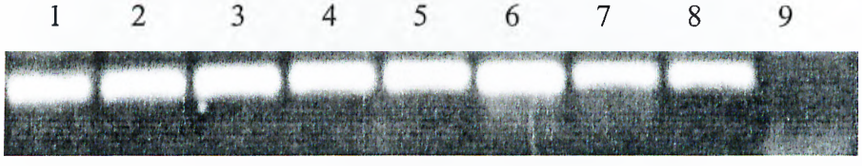

Figure 5-16: The GAPDH PCR results of archival tumors and the non-tumor

Lanes: 1: non-tumor1, 2: tumor 1, 3: tumor 2, 4: tumor 3, 5: tumor 4, 6:tumor 5, 7: tumor 6, 8: tumor 7, 9: negative control

5-11 Description of 3' end alternative splicing forms of p73 in 15 HCC cell lines compared to a normal liver by semi-quantitative PCR

After the cDNA is qualified for semi quantitative PCR, we have optimized the conditions for p73α and p73β specific semi quantitative PCR reactions (The second step PCR of Figure 5-2). Since the two step PCR was incompatible for a semi-quantitative PCR analysis, we tried to optimize our conditions for a single step PCR to detect p73α, p73γ, p73ε with p73L-F-p73α–R and p73β and p73ϕ with p73L-F-p73β–R

primer pairs (Figure 5-2). The optimum cycle for p73α specific PCR appeared to be 33 and p73β specific PCR to be 34 cycles (Figure 5-17 and Figure 5-18).

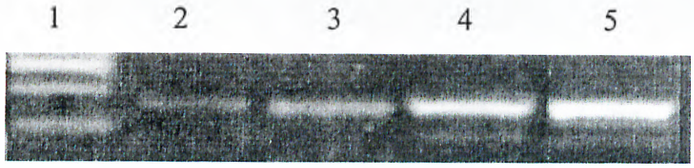

Figure 5-17 : Cycle optimization of p73α semi-quantitative RT-PCR

Lanes: 1: Marker, 2: 32 cycle, 3: 34 cycle, 4: 36 cycle, 5: 38 cycle

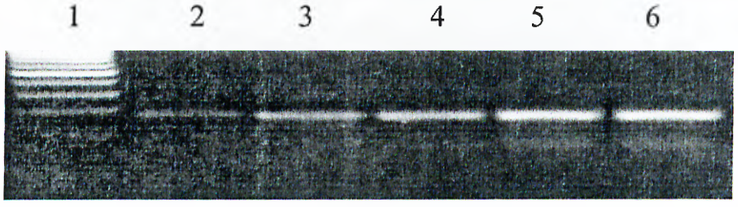

Figure 5-18 : Cycle optimization of p73β semi-quantitative RT-PCR

Lanes: 1: Marker, 2: 30, 3: 32 cycle, 3: 34 cycle, 4: 36 cycle, 5: 38 cycle

The set of 15 HCC cell lines and a normal liver was analyzed at the optimized conditions. The p73α specific PCR resulted with the description of p73α and p73γ increased in most of the cell lines, when compered with normal liver (Figure 5-19). The result of p73β specific PCR was also very striking since the expression of p73β and p73ϕ was deviated in positive (Figure 5-20, lane 7) and negative manner (Figure 5-20, lane 5) when compared with normal liver, depending on the cell line (Figure 5-20).

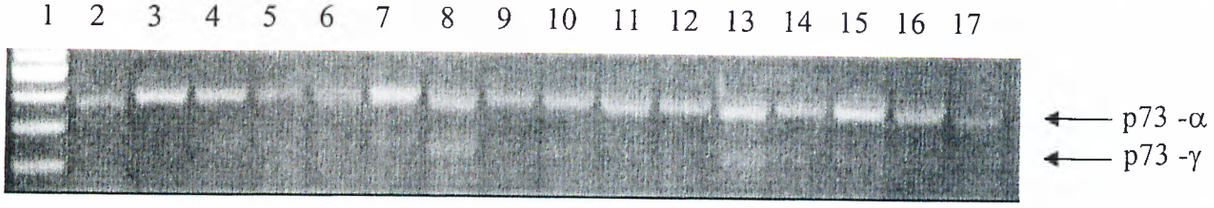

Figure 5-19 : Semi quantitative PCR results of p73α specific PCR

Lanes : 1:Marker, 2:Huh7, 3:Hep3BTR, 4:FOCUS, 5:Mahlavu, 6:Hep40, 7:Hep3B, 8:HepG2, 9:PLC/PRF/5, 10:SK-Hep1, 11:SNU 182, 12:SNU 387, 13:SNU 398, 14:SNU 423, 15:SNU 449, 16: SNU 475, 17:Normal liver

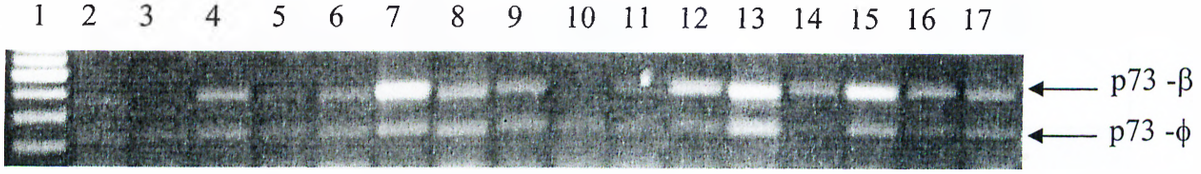

Figure 5-20 : Semi quantitative PCR results of p73β specific PCR

Lanes : 1:Marker, 2:Huh7, 3:Hep3BTR, 4:FOCUS, 5:Mahlavu, 6:Hep40, 7:Hep3B, 8:HepG2, 9:PLC/PRF/5, 10:SK-Hep1, 11:SNU 182, 12:SNU 387, 13:SNU 398, 14:SNU 423, 15:SNU 449, 16: SNU 475, 17:Normal liver

5-12 Densitometric analysis of the p73, 3' end splicing isoforms, compared to normal liver.

The agarose gel pictures were analysed with an image analysis software (Multi-Analyst, Beckman Instruments), and then bands are compared with their relative intensities. The area under the curve provides information of total band intertsity, whereas the peak point of the curve defines the maximum intensity. The intensity can get values between 0-255, 0 to be extreme white and 255 to be the extreme black. The interval of 1-254 yields different intensities of gray. Each picture contains 3 windows. The first window is the result of α–specific PCR, which yields p73α and p73γ. The second window shows the result of β-specific PCR, ehich yields p73β and p73ϕ. The

third window provides the result of equal template loading control, GAPDH. Each picture is background subtracted before the image analysis, meaning the reduction of a constant number of intensity from all the pixels of a figure. This is done to normalize all the samples to a common background and setting "0" to be the absolute white. Figure 5-21 describes the analysis of 3 representative samples (Huh7, Hep40 and Hep3B) and the normal liver.

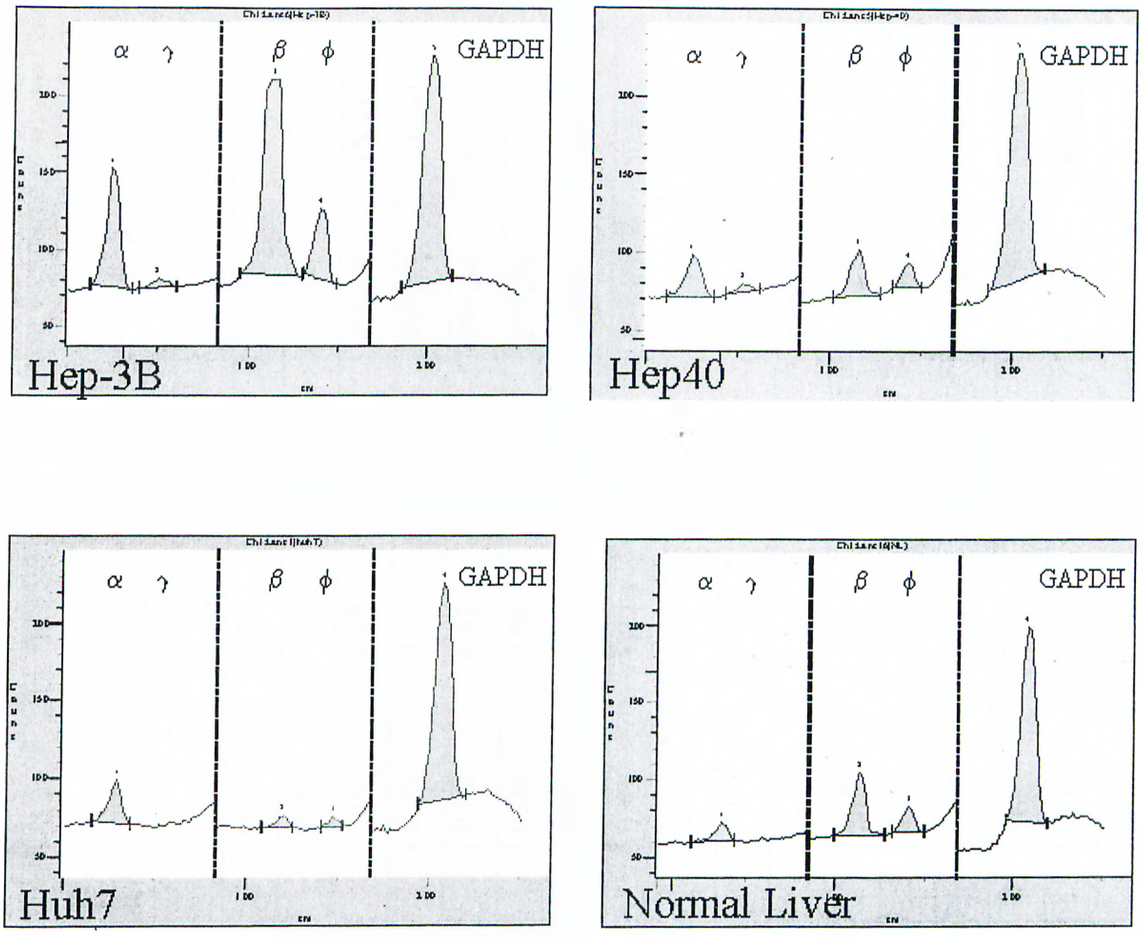

Figure 5-21: The densitometric analysis of three representative HCC cell lines of p73 3' end alternative splicing isoforms compared to normal liver

Table 5-2: The densitometric analysis of p73, 3' splicing isoforms in numeric form compared to normal liver (bold numbers indicate 2 times increase or decrease, compared to normal liver)

SOURCE	p73alfa	p73beta	p73gamma	P73phi	p73 total	p73alfa/NL	p73beta/NL	p73 total /NL
Normal Liver	1,06	3,57	0,00	1,23	5,86	100,00	100,00	100,00
Mahlavu	1,43	0,53	0,00	**0,21**	**2,17**	134,91	**14,85**	**37,03**
Huh-7	2,21	0,55	0,00	**0,39**	3,15	**208,49**	**15,41**	53,75
SK-Hep1	3,81	0,37	0,35	0,64	5,17	**359,43**	**10,36**	88,23
SNU 182	4,18	0,79	0,00	**0,51**	5,48	**394,34**	**22,13**	93,52
SNU 423	2,95	2,76	0,00	**0,59**	6,30	**278,30**	77,31	107,51
Hep3B-TR	6,03	0,15	0,00	0,67	6,85	**568,87**	**4,20**	116,89
Hep40	2,67	2,77	0,41	1,27	7,12	**251,89**	77,59	121,50
PLC/PRF/5	2,39	3,75	0,09	1,83	8,06	**225,47**	105,04	137,54
SNU 475	3,65	3,51	0,00	0,96	8,12	**344,34**	98,32	138,57
FOCUS	4,67	3,18	0,28	1,60	9,73	**440,57**	89,08	166,04
SNU 387	2,79	8,15	0,00	1,55	**12,49**	**263,21**	**228,29**	**213,14**
HepG2	3,62	6,33	1,29	**3,92**	**15,16**	**341,51**	177,31	**258,70**
SNU 449	7,31	13,34	0,43	**2,63**	**23,71**	**689,62**	**373,67**	**404,61**
SNU 398	5,09	14,10	0,54	**7,23**	**26,96**	**480,19**	**394,96**	**460,07**
Hep3B	6,82	16,68	0,60	**3,94**	**28,04**	**643,40**	**467,23**	**478,50**

5-13 Description of TA and DN-p73 transcript isotypes by semi-quantitative PCR in 15 HCC cell lines and primary HCC samples

The 5' end differing transcripts of p73, namely TA-p73 and DN-p73 were also detected by semi-quantitative PCR. For each analysis, an initial study of 30-38 cycles were done and optimum cycle was determined using an adequate sample cDNA. Although these two products are under the regulation of different promoters, the optimum cycle number for TA-p73 and DN-p73 PCR appeared to be both 35 (Figures 5-22 and 5-23).

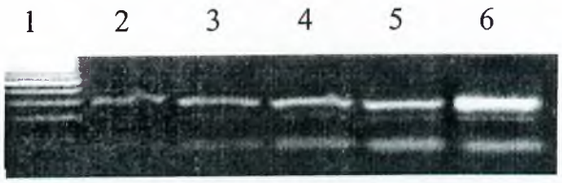

Figure 5-22 : Cycle optimization of TA-p73 semi-quantitative RT-PCR
Lanes : 1: marker, 2: 32 cycle, 3: 33 cycle, 4: 34 cycle, 5: 35 cycle, 6 : 36 cycle

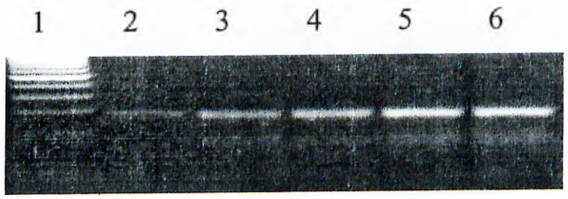

Figure 5-23 : Cycle optimization of DN-p73 semi-quantitative RT-PCR
Lanes : 1: marker, 2: 33 cycle, 3: 34 cycle, 4: 35 cycle, 5: 36 cycle, 6 : 37 cycle

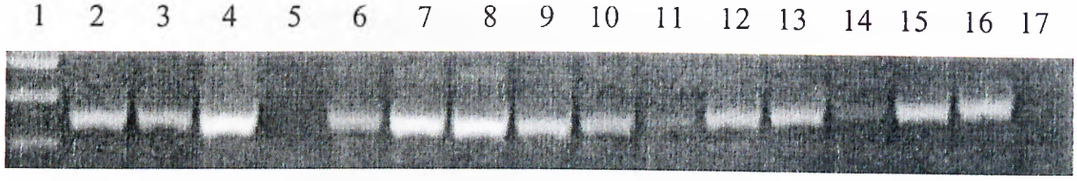

Figure 5-24: Semi quantitative PCR results of TA-p73 specific PCR for 15 HCC cell lines and a normal liver

Lanes : 1:Marker, 2:Huh7, 3:Hep3BTR, 4:FOCUS, 5:Mahlavu, 6:Hep40, 7:Hep3B, 8:HepG2, 9:PLC/PRF/5, 10:SK-Hep1, 11:SNU 182, 12:SNU 387, 13:SNU 398, 14:SNU 423, 15:SNU 449, 16: SNU 475, 17:Normal liver

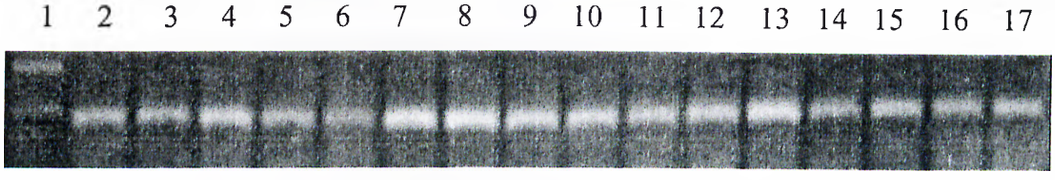

Figure 5-25: Semi quantitative PCR results of DN-p73 specific PCR for 15 HCC cell lines and a normal liver

Lanes : 1:Marker, 2:Huh7, 3:Hep3BTR, 4:FOCUS, 5:Mahlavu, 6:Hep40, 7:Hep3B, 8:HepG2, 9:PLC/PRF/5, 10:SK-Hep1, 11:SNU 182, 12:SNU 387, 13:SNU 398, 14:SNU 423, 15:SNU 449, 16: SNU 475, 17:Normal liver

The results of TA-p73 and DN-p73 expression in HCC derived cell lines showed that DN-p73 is expressed at a uniform level in all samples. However, we could not detect any TA-p73 expression in normal liver. So the major p73 transcript in normal liver appeared to be DN-p73. There is an acquired expression of TA-p73 in 14/15 HCC derived cell lines. These results are the first description of the TA and DN p73-p73 gene isoform expression in a cancer. This data have been partially confirmed by earlier studies which showed an increased expression of p73 (now named as TA-p73) in different cancer types, as summarized at table 2-1. With the lack of the knowledge of the presence of DN-p73, the former studies could only detect TA-p73 transcript (if they have amplified exon 1-3 of

TA-p73) or the total levels of TA and DN-p73 (if they have amplified the exons 4-14 of p73). A very nice example to this discrepency could be the contradictory results obtained from HCC samples. The study of Mihara *et al.* (Mihara et al., 1999) described similar levels of p73 transcripts in non-tumor and tumor liver samples. Hovewer, the studies of Herath *et al.* (Herath et al., 2000) and Tannapfel *et al.* (Tannapfel et al., 1999) described an elevation in p73 transcript levels. These results were in apparent contradiction before the description of TA-p73 and DN-p73 but they are all true. Mihara *et al.* had studied p73 expression using primers located at the 3' end of transcript so described TA-p73 and DN-p73 together. Our results showed the presence of DN-p73 in normal liver and the presence of DN-p73 together with TA-p73 in HCC tumors. Thus it is plausible that the total DN and TA-p73 levels are similar in non-tumor and tumor liver tissue respectively. Herath *et al.* and Tannapfel *et al.* have described the expression of p73 using oligomers to the extreme 5' end of the transcript, so detected only the expression of TA-p73, but not DN-p73. Their results were also correct and they described the acquired expression of TA-p73 in tumor cells but not in the normal cells of liver.

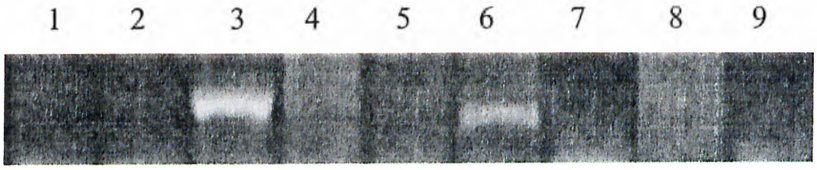

Figure 5-26: Semi quantitative PCR results of TA-p73 specific PCR for 7 primary HCC samples and a non-tumor liver

Lanes: 1:non-tumor 1, 2: tumor 1, 3: tumor 2, 4: tumor 3, 5: tumor 4, 6: tumor 5, 7: tumor 6, 8: tumor 7. 9: negative control

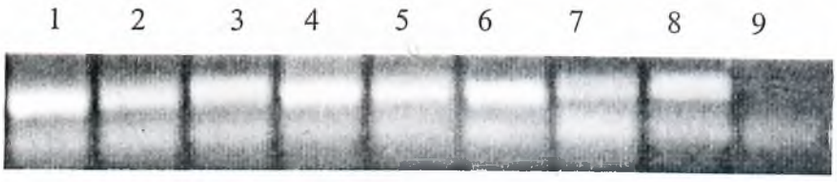

Figure 5-27: Semi quantitative PCR results of DN-p73 specific PCR for 7 primary HCC samples and a non-tumor liver

Lanes: 1:non-tumor 1, 2: tumor 1, 3: tumor 2, 4: tumor 3, 5:tumor 4, 6:tumor 5, 7:tumor 6, 8:tumor 7. 9: negative control (no template DNA)

The results of TA-p73 and DN-p73 expression in primary HCC tumors and the non-tumor tissue were in correlation with the results of HCC derived cell lines. DN-p73 was expressed at similar and comparable levels in tumor and non-tumor liver tissues but TA-p73 was not expressed in the non-tumor liver. Interestingly, the percentile of TA-p73 expression changed significantly when cell lines (14/15) were compared with primary tumors (3/7). This alteration could be the results of the heterogeneity of the tumorous cells in the total tumor cell population since the whole population of a cell line is composed of rapidly proliferating cells, whereas primary tumors contain a portion of normal or non-tumor cells.

5-14 : The description of the presence of endogenous DN-p73 protein in a HCC derived cell line.

The presence of the DN-p73 transcript was described in RNA level by semi-quantitative PCR, but the presence of RNA does not prove evidence for the presence of DN-p73 protein. We have analyzed the mouse homologue of DN-p73 together with the predicted amino acid composition of human DN-p73 and calculated the molecular weight of DN-p73 to be 62 kDa. Since there is an extensive alternative splicing of p73 encoded transcripts, we have chosen an antibody recognizing only p73α. The western blot using 300-500 ug of total protein yielded 2 bands coresponding to the molecular weight of TA and DN-p73α proteins (Figure 5-28). The lysate of COS7 cell line was used as a positive control for TA-p73α protein.

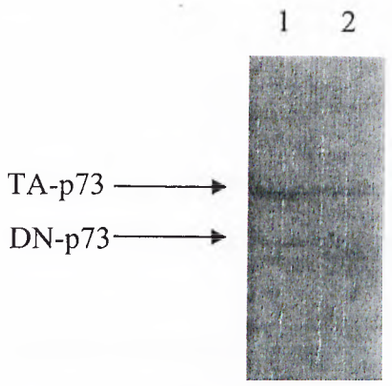

Figure 5-28: Western blot using the ER13 antibody, which recognizes a epitope against the carboxyl-end of the p73 protein.
Lanes: 1: SNU 398, 2: COS7

This result is the first description of endogenous human DN-p73 protein. This result also provided information about the importance of DN-p73 and TA-p73 during tumorigenesis. First of all, TA-p73 expression was shown to be a cancer related event for HCC. Of great interest, the acquired expression of TA-p73 is an unexpected event during carcinogenesis when the structural and functional similarity of p53 tumor suppressor and TA-p73 are concerned. The description of E2F1 transcription factor binding sites in TA-p73 promoter and the induction of TA-p73 by exogenous E2F1 expression (Stiewe and Putzer, 2000) lead us to propose a relationship between the Rb pathway dysregulation and TA-p73 expression. The results of the experiments for the description of the status of Rb pathway components and the evidence for the transcriptional activity of E2F1 transcription factor are provided in the next chapter.

CHAPTER VI

THE STATUS OF RB PATHWAY COMPONENTS AND THE EVIDENCE FOR THE ACTIVITY OF E2F1 TRANSCRIPTION FACTOR

The retinoblastoma gene was identified as a locus involved with the development of an inherited eye tumor (Knudson AG, 1971). Further studies revealed the presence of mutations in this gene in different cancer types and the proof of the tumor suppressor idea (Knudson AG., 1971, Friend et al., 1986). The E2F family of transcription factors were defined to be the key molecules associated with the growth suppressive activity of Rb protein (pRb) (Chelappan et al., 1991). E2F proteins are a family of transcription factors, composed of at least 5 members, having role in the transactivation of cell cycle regulatory genes, that are especially necessary for S phase progression (Nevins JR, 1998). Thus, the main role of pRb was the sequestering of E2F family of proteins.

pRB is the natural substrate of cyclin dependent kinases-cyclin complexes. As the name implies, cyclin dependent kinases are phosphorylating enzymes that are under the regulation of cyclin proteins (reviewed by Sherr and Roberts 1999). The sequestering activity of pRb on E2F proteins is regulated by phosphorylation of pRb by cyclin D-CDK4 complex at multiple positions (reviewed by Sherr and Roberts 1999). Upon phosphorylation, pRb releases E2F proteins to initiate the cell cycle. The restriction point or point of no-return was described as the key point in the G1 phase-S phase progression of cell cycle, which was associated with the release of E2F1 transcription factor from pRb (reviewed by Sherr and Roberts 1999).

The initiation of the cell cycle, thus the phosphorylation status of pRb was strictly controlled by cyclin dependent kinase inhibitors (CKI) (Lundberg and Weinberg, 1998). The major inhibitors of CDK4-cyclin D complex are the INK4 family of CKIs especially $p16^{INK4A}$ (Nevins JR, 2001).

Thus, there are positive regulators (cyclin D, CDK4, E2F1) and negative regulators (pRb, p16) of cell cycle initiation and S phase progression, collectively

named as the Rb pathway. There are mutations, virus related interferences and epigenetic regulations of the components of Rb pathway, defined for almost each component in different types of cancers (Nevins JR, 2001). With the description of E2F1, as a transactivator of TA-p73, we have suspected the acquired expression of TA-p73 to be a result of Rb pathway inactivation.

E2F1 dysregulation is the ultimate result of Rb pathway disruption, but demonstration of pRb bound and unbound fraction of E2F1 protein is out of our technical limits so we aimed to look at pRb protein levels and $p16^{INK4A}$ transcript levels for characterization of Rb pathway. We would also like to prove the Rb pathway inactivation by detection of endogenous targets of E2F1 transcription factor, such as $p14^{ARF}$, cyclin E and correlate the results with the expression of the recently described p53 homologue, TA-p73.

6-1 Description of p16 transcripts by semi-quantitative PCR in 15 HCC cell lines

$p16^{INK4A}$ gene resides in the MTS locus which is harboring both the $p16^{INK4A}$ and $p14^{ARF}$ genes. These two genes share 2 of their 3 exons, but the first exons, thus the promoter regions are totally different. Since this locus harbors two tumor suppressor genes, the allelic or total deletions are commonly seen in cancers. Inherited mutations of $p16^{INK4A}$ gene were described in the familial cases of Melanoma, with the absence of Rb gene mutations, suggesting the functional loss of $p16^{INK4A}$ as a functionally equivalent event for tumor progression (reviewed by Nevins JR, 2001). Moreover epigenetic modifications of promoter region of $p16^{INK4A}$ gene is also seen through the progression of almost all cancers at different frequencies (Serrano M, 2000). For the reasons I have described above, we aimed to analyze the $p16^{INK4A}$ expression in RNA level by semi quantitative RT-PCR method. RNA isolation, pufication and cDNA synthesis were done as described for TA-p73 expression analysis. GAPDH amplification was done prior to this analysis as an equal template loading control. After an initial study for identification of the logarithmic phase of amplification for p16 PCR (Figure 6-1), we have decided to make the final experiment at 34 cycles (Figure 6-2). This result indicates a probable deletion of MTS locus in 3 of 15

(SNU 387, SNU 449 and SK-Hep1) and probably methylation related decreased expression of $p16^{INK4A}$ expression in 4/15 (SNU 398, SNU 423, SNU 475 and PLC/PRF/5) HCC derived cell lines.

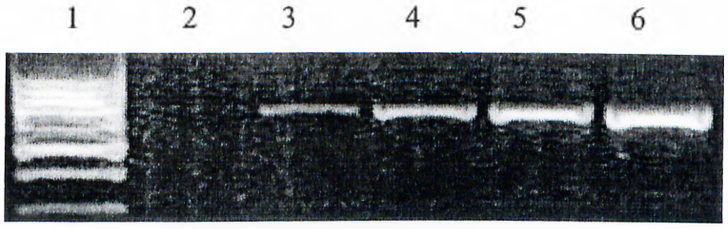

Figure 6-1 : Cycle optimization for p16, semi quantitative RT-PCR.

Lanes : 1: marker, 2: 30 cycle, 3: 32 cycle, 4: 34 cycle, 5: 36 cycle, 6 : 38 cycle

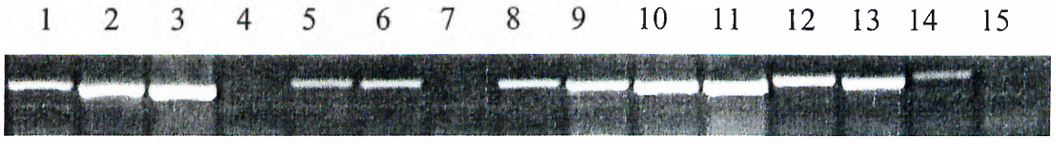

Figure 6-2 : Semi-quantitative p16 PCR results of 15 HCC cell lines

Lanes : 1: Mahlavu, 2: Hep3B, 3:SNU 182, 4: SNU 387, 5: SNU 398, 6: SNU 423, 7: SNU 449, 8: SNU 475, 9: Huh7, 10: Hep3BTR, 11: FOCUS, 12: Hep40, 13: HepG2, 14: PLC/PRF/5, 15: SK-Hep1

6-2 The description of retinoblastoma protein (pRb) in HCC cell lines

Retinoblastoma gene product is the major component of Rb pathway. The epigenetic modifications leading to decreased Rb mRNA expression, truncation mutations leading to small and unstable pRb derived peptide and increased expression of pRb-protease targetting molecules such as gankryn are common events leading to the loss of functional Rb protein in HCC. Thus pRb is the ultimate Rb gene encoded product to describe for detection of functional

inactivation. For these purposes, we have extracted protein from all cell lines and made western blots for the detection of pRb. Initially, we have tested the presence of proteins by Ponceau S staining after the transfer to nitrocellulose or PVDF membrane, and then make an initial western blot for an equal loading control. Our previous studies yielded p33ING1 as a good internal control for equal protein loading (Figure 6-3).

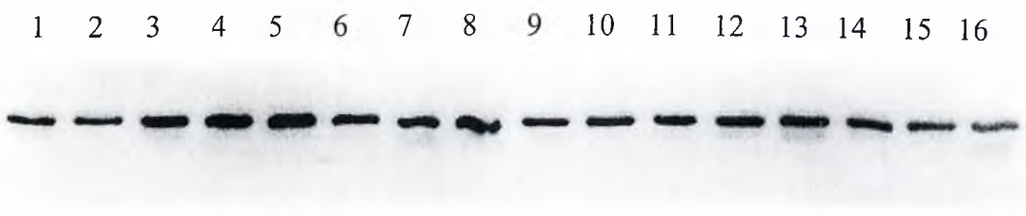

Figure 6-3 : Anti-p33ING1 western blot results of 15 HCC cell lines

Lanes : 1:Huh7, 2:Hep3BTR, 3:FOCUS, 4:Mahlavu, 5:Hep40, 6:Hep3B, 7:HepG2, 8:PLC/PRF/5, 9:SK-Hep1, 10:SNU 182, 11:SNU 387, 12:SNU 398, 13:SNU 423, 14:SNU 449, 15: SNU 475, 16:positive control

After an initial optimization study for antibody dilution and protein concentration, we made the pRb western by using 50 ug of total protein (Figure 6-4). AF18, anti pRb antibody was used to probe the membranes at a dilution of 1/500. The upper band in the western blot is the pRB and the band denoted with an asterix (*) is a previously defined non-specific band (Morel et al., 2000). There is a lost or decreased pRb expression in 6/15 (40%) of HCC derived cell lines (Hep3B, SNU 423, SNU 449, Hep3BTR, FOCUS, Hep40).

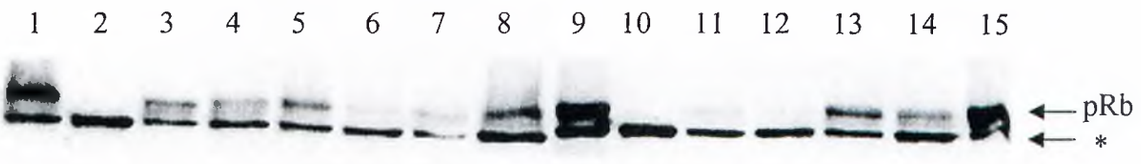

Figure 6-4 : Anti pRb western blot results of 15 HCC cell lines

Lanes: 1: Mahlavu, 2: Hep3B, 3:SNU 182, 4:SNU 387, 5:SNU 398, 6:SNU 423, 7:SNU 449, 8: SNU 475, 9:Huh7, 10:Hep3BTR, 11:FOCUS, 12: Hep40, 13:HepG2, 14:PLC/PRF/5, 15:SK-Hep1

These data together with $p16^{INK4A}$ RT-PCR results provide us the information of Rb pathway dysregulation. We have observed Rb pathway dysregulation in 11/15 HCC cell lines (Table 6-1). The result of RB pathway inactivation is the activation of E2F1 responsive genes thus this correlation was also described with the data of cyclin E, p14 and TA-p73 RT-PCR results in table 6-1 at the end of this chapter.

6-3 THE DESCRIPTION OF ENDOGENOUS E2F1 TARGETS, $p14^{ARF}$ and CYCLIN E

The activation of endogenous E2F1 transcription factor targets is a clue for Rb pathway dysregulation. These genes include, Matrix Metalloprotease 6 (MMP6), Bak, Bad, Bid (Apoptotic genes), TA-p73 and cyclin E and many others (Stanelle et al., 2002). We have chosen to study the expression of cyclin E and p14ARF as the proof of active E2F1. Cyclin E is a late G1 phase to late S phase cyclin and the presence of cyclin E is necessary for the activation of CDK2, thus the progression of cell cycle. $p14^{ARF}$ is the other product of MTS locus, which is functioning as p53-ubiqutin ligase, MDM2 blocker, so p53 activator (p14Alternaive Reading Frame) (Kamijo et al., 1998). This gene was reported to be upregulated by E2F1 transcription factor. TA-p73 was also defined to be a responsive gene for E2F1 mediated transactivation. We have made expression analysis of several transcripts, namely $p14^{ARF}$ and cyclin E, to correlate with TA-p73 expression the results of TA-p73 expression and other E2F1 targets are co-summarized with Rb pathway components in table 6-1, at the end of this chapter.

We have initially studied for the description of the logarithmic phase of amplification for cyclin E and p14ARF transcripts In this study, 32-33 cycle was observed to be in the logaritmic phase of amplification (Figure 6-5), so 33 cycles was chosen for semi-quantitative PCR of cyclin E experiments. Such a study also revealed 34 cycles for the semi-quantitative analysis of $p14^{ARF}$ PCR experiments (Figure 6-6).

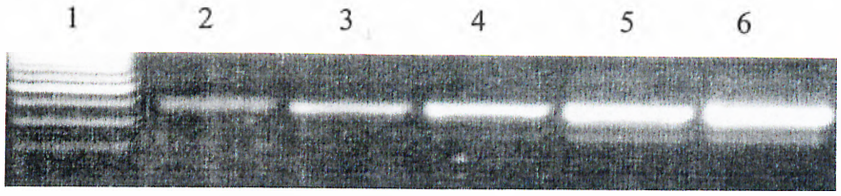

Figure 6-5 : Cycle optimization for cyclin E, semi quantitative RT-PCR.
Lanes : 1: marker, 2: 29 cycle, 3: 31 cycle, 4: 33 cycle, 5: 35 cycle, 6 : 37 cycle

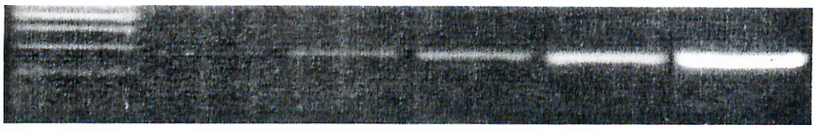

Figure 6-6 : Cycle optimization for p14, semi quantitative RT-PCR.
Lanes : 1: marker, 2: 28 cycle, 3: 30 cycle, 4: 32 cycle, 5: 34 cycle, 6 : 36 cycle

The cDNA synthesized and equalized for the description of TA-p73, DN-p73 expression was used as a template to test the semi-quantitative expression of cyclin E and p14ARF transcripts in HCC derived cell lines, normal liver, primary tumors and non-tumor tissue. The results of cyclin E PCR of HCC cell lines revealed a similar and comparable of cyclin E transcripts in all cell lines, which is slightly more than that of normal liver (Figure 6-7). Since cell lines are homogenous populations of dividing cells, this result is not unexpected. Interestingly, the semi-quantitative RT-PCR results of primary tumors and the non tumor liver tissue pointed out the dysregulation of Rb pathway to be present in 3 of 7 tumors (43%).

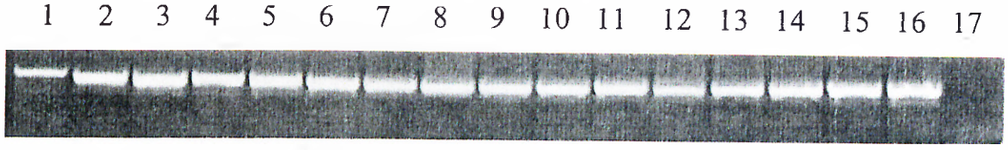

Figure 6-7 : Semi-quantitative Cyclin E PCR results of 15 HCC cell lines and a normal liver

Lanes: 1: Normal liver, 2: SNU 475, 3: SNU 449, 4: SNU 423, 5: SNU 398, 6: SNU 387, 7: SNU 182, 8: SK-Hep1, 9: PLC/PRF/5, 10: HepG2, 11: Hep3B, 12: Hep40, 13: Mahlavu, 14: FOCUS, 15: Hep3BTR, 16: Huh7, 17: negative control (no template DNA)

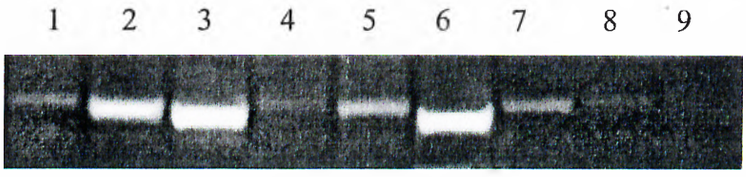

Figure 6-8 : Semi-quantitative cyclin E PCR results of a non-tumor liver and 7 HCC tumors

Lanes: 1: non-tumor1, 2: tumor1, 3: tumor 2, 4: tumor3, 5:tumor4, 6:tumor5, 7:tumor6, 8:tumor7. 9: negative control (no template DNA)

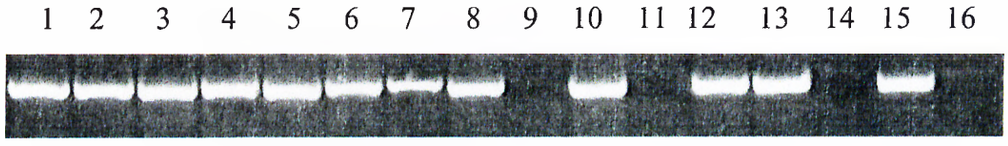

Figure 6-9 : Semi-quantitative p14 PCR results of 15 HCC cell lines and a normal liver

Lanes : 1:Huh7, 2:Hep3BTR, 3:FOCUS, 4:Mahlavu, 5:Hep40, 6:Hep3B, 7:HepG2, 8:PLC/PRF/5, 9:SK-Hep1, 10:SNU 182, 11:SNU 387, 12:SNU 398, 13:SNU 423, 14:SNU 449, 15: SNU 475, 16:Normal liver

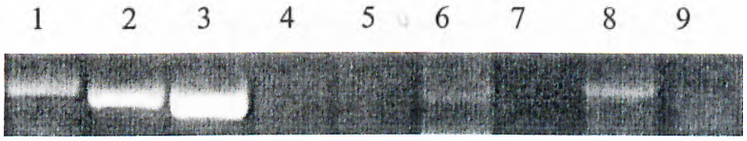

Figure 6-10: Semi-quantitative p14 PCR results of a non-tumor liver and 7 HCC tumors

Lanes: 1: non-tumor1, 2: tumor 1, 3: tumor 2, 4: tumor 3, 5: tumor 4, 6: tumor 5, 7: tumor 6, 8: tumor 7. 9: negative control (no template DNA)

Table 6-1: Correlation of TA-p73 induction with E2F1 target gene activation and the Rb pathway inactivation in HCC cell lines.

		E2F1 Target Genes		*RB1* Pathway Genes	
Cell Lines	**TA-p73**	**$p14^{ARF}$**	**Cyclin E**	**pRb**	**$p16^{INK4a}$**
Group I: Inactivated RB1 Pathway					
Hep3B	Positive	Positive	Positive	ND*	Positive
Hep3B-TR	Positive	Positive	Positive	ND	Positive
Hep40	Positive	Positive	Positive	ND	Positive
FOCUS	Positive	Positive	Positive	Trace	Positive
SNU-449	Positive	ND	Positive	Trace	ND
SNU-387	Positive	ND	Positive	Positive	ND
SK-Hep1	Positive	ND	Positive	Positive	ND
PLC/PRF/5	Positive	Positive	Positive	Positive	Trace.
SNU-475	Positive	Positive	Positive	Positive	Trace.
SNU-398	Positive	Positive	Positive	Positive	Trace.
SNU-423	Trace	Positive	Positive	Trace	Trace
Group II: Normal RB1 Patwhay					
HepG2	Positive	Positive	Positive	Positive	Positive
Huh-7	Positive	Positive	Positive	Positive	Positive
SNU-182	Trace	Positive	Positive	Positive	Positive
Mahlavu	ND	Positive	Positive	Positive	Positive

*ND: Not detected.

CHAPTER VII

THE PROTEIN STATUS AND CODON 72 GENOTYPING OF P53 GENE

Mutation of the p53 gene is the most common event in cancer development. As the central player of the tumor suppressor team, p53 protein has interesting properties. Unlike the other tumor suppressor genes, most of the mutations of p53 gene give rise to functionally inactive but more stable protein products (Blagosklonny MV, 2000). Thus the presence of a mutant p53 protein is favorable for a tumor cell. Such mutant proteins were thought to be functioning in a dominant negative manner to inactivate the remaining p53 protein by hetero-oligomerization (Blagosklonny MV., 2000). However, the descriptions of a mutant p53 gene together with the deletion of the other p53 allele raised the question of the function of the "mutant p53". A codon specific p53 mutation was identified as the first carcinogen specific hotspot mutation, and associated with ingestion of a fungal toxin (Bressac et al., 1991, Hsu et al., 1991). Therefore, the presences of mutant p53 proteins are specifically important for HCC cells.

The probable function of mutant p53 proteins was proposed to interact with TA-p73 and regulate its death inducing activity (Marin et al., 2000). Generally p53 mutations give rise to conformationally improper or DNA binding deficient p53 proteins. Such proteins can not bind to DNA and transactivate its negative feedback loop component MDM2. The downregulation of MDM2 expression results in the accumulation of mutant p53 proteins. The activation mechanisms of both p53 and p73 rely on tetramerization so accumulated p53 mutant proteins could easily take few role of the p73 tetramer.

The study of Marin *et al.* defined the presence of a hetero-oligomerization of TA-p73 with different mutant forms of p53 proteins (Marin et al., 2000). Moreover, this study also provided evidence for the strength of this interaction to

be regulated by a common polymorphism of p53 gene (arginine or proline at codon 72, R72P). Thus we aimed to describe the relevance of these data in HCC samples.

7-1 Western blot for p53 protein for 15 HCC cell lines

After an initial study for the definition of optimum antibody dilution and antigen concentration, p53 western is done by using 30 ug uf total protein to discriminate mutant and wild type p53 proteins. 6B10, anti-p53 antibody (hybridoma supernatant) was used to probe the membranes at a dilution of 1/1 (Figure 7-1).

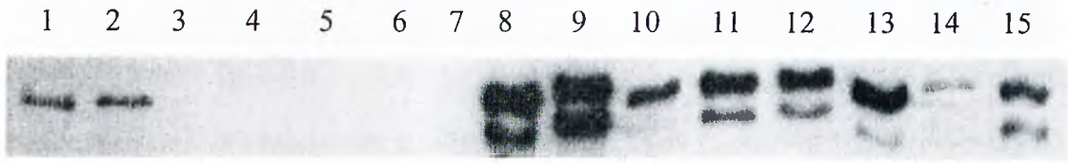

Figure 7-1 : Anti p53 western blot results of 15 HCC cell lines
Lanes : 1: HepG2, 2: SK-Hep1, 3: FOCUS, 4: SNU 387, 5: SNU 398, 6: Hep3B, 7: Hep3BTR, 8: Hep40, 9: Mahlavu, 10: PLC/PRF/5, 11: SNU 423, 12: SNU 449, 13: Huh7, 14: SNU 182, 15: SNU 475

Previous published data (Puisieux et al., 1993, Hsu et al., 1993) together with figure 7-1 suggests the presence of p53 gene mutations at least 13/15 HCC derived cell lines (with the exception of HepG2 and Sk-Hep1). We could not detect p53 protein in 5 cell lines (Hep3B, Hep3BTR, FOCUS, SNU 387 and SNU 398), probably because of gene deletions.

7-2 Genotyping the codon 72 Arg/Pro polymorphisms of p53 from HCC cell lines

Marin et al. (Marin et al., 2000) suggested a possible role of mutant p53 for the inhibition of the apoptotic activity of TA-p73. Moreover, the inhibition was shown to be dependent to a polymorphism at codon 72 of p53. It was also

suggested that mutant p53 proteins having an arginine at codon 72 instead of a proline was more capable of inactivating the apoptotic activity of TA-p73. Therefore, we have decided to genotype the p53 expressing HCC cell lines for the codon 72 polymorphism. This genotyping strategy is based on amplification of the 5' 470bp of p53 and restriction of the PCR product with an enzyme (FnuDII, MBI Fermentas) recognizing the polymorphic site if it encodes for an arginine. The arginine restriction yields 250 and 220bp products and the proline restriction produces the unaffected 470 bp band (Figure 7-2)

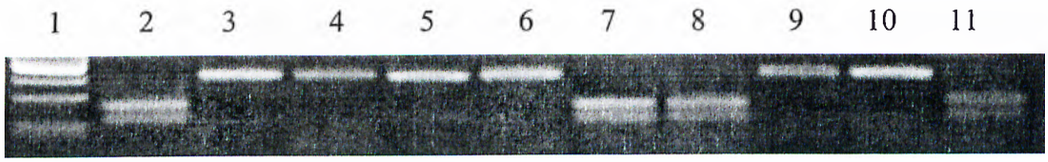

Figure 7-2 : The restriction products of p53 PCR revealing the R/P status of codon72

Lanes: 1: 100 bp DNA ladder, 2: Huh7, 3: Mahlavu, 4: Hep40, 5: HepG2, 6: PLC/PRF/5, 7: SK-Hep1, 8: SNU 182, 9: SNU 423, 10: SNU 449, 11: SNU 475

Our results showed that 6/10 (60%) of the p53 protein expressing HCC cell lines encode for a p53, having a proline at codon 72 and 4/10 (40%) encode for a p53 having an arginine at codon 72. These results are in contradiction with the data presented by Marin *et al.*, since the number of cell lines with a mutant p53 containing a proline (n=5; Mahlavu, Hep40, PLC/PRF/5, SNU 423, SNU 449) are less abundant than the number of cell lines with a mutant p53 containing an arginine (n=3; Huh7, SNU 182, Snu 475). If mutant p53 with arginine at codon 72 had an antagonistic effect of TA-73, most of the cell lines will have arginine, instead of proline.

CHAPTER VIII

THE SUMMARY OF RESULTS

In this thesis we firstly aimed to define a function for the p73 gene encoded transcripts in Hepatocellular Carcinoma. As a second step, we tried to correlate our results with a known cancer related dysregulation, so make p73 a component of a commonly dysregulated pathway.

For this reason, we have tried to describe all 3' alternative splicing forms of p73 transcripts. We have identified p73γ, p73ϕ and p73ε in addition to the known p73 forms p73α and p73β. We also described the cancer related alteration at the 3' alternative splicing of p73 by semi-quantitative RT-PCR method, with comparison to a normal liver sample.

With the description of an alternative promoter within the mouse p73 gene and the clues of the other p53 homologue, p63, we made an *in silico* study to define the human form of mouse DN-p73. Our studies revealed the *in vivo* presence of the *in silico* data. Thus we have specifically analyzed the expression of DN-p73 and TA-p73 by semi-quantitative RT-PCR method in HCC cells. Our results were very interesting since we have detected an acquired and cancer related expression of TA-p73 in HCC cells, whereas the DN-p73 expression was moderate in tumor and non-tumor liver cells. If the pro-apoptotic and cell cycle regulatory function of TA-p73 was concerned, the cancer related TA-p73 expression could easily be illustrated as an unwanted event for tumor cells. However, the identification of E2F1 transcription factor binding sites in the promoter region of TA-p73 and the description of the induced TA-p73 expression by ectopic E2F1 expression rerouted us to correlate our data with a probable Rb pathway inactivation. The expression and status of Rb pathway (pRb and $p16^{INK4A}$) components were investigated together with the E2F1 activation related changes in gene expression ($p14^{ARF}$ and cyclin E expression). Our

results revealed a partial correlation with Rb pathway inactivation and induction of TA-p73 expression in HCC derived cell lines and primary HCC tumors.

Mutant p53 proteins are retained in cancer cells, thus they acquire a function favoring the tumor cells. The probability of hetero-oligomerization of mutant p53 and TA-p73 which would result in functional inactivation of TA-p73 thus could be a result of the presence of mutant p53 proteins in cancer cells. We have investigated the levels of p53 protein and the presence of the incidence of a p53 gene polymorphism, which was proposed to take a role in p53-p73 interaction. Our results showed the presence of mutant p53 in more than half and showed the diminished p53 expression in more than 30% of our samples. We have also defined the p53 polymorphism as an irrelevant correlation to justify the functional inactivation of TA-p73 expression.

The description of new human p73 gene encoded product (DN-p73), the expression analysis of DN-p73 and TA-p73, p53, Rb pathway components and E2F1 transcription factor regulated genes were published in the journal "Oncogene" at 23 August 2001.

DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER IX

DISCUSSION

The studies presented in this thesis describe the identification of the human homologue of mouse DN-p73, and the cancer related changes in the expression profile of p73 locus harboring genes.

p73 gene was identified at 1997 to encode for a new structural and functional homologue of the p53 protein, which is considered the guardian of the genome. p73 gene was localized to 1p36.3, which is a common site for deletions for different cancer types, especially neuroblastoma and Hepatocellular Carcinoma (HCC). The studies trying to assign a "p53 like" inactivation profile to p73 failed because of the lack of a significant number of mutations in the p73 gene coding region (see table 2-1). However, functional studies, done either by inducing the endogenous p73 or ectopic expression of p73 containing plasmid in mammalian cells described the structural homologues having similar effects on cell fate. The decisive events induced by p73 on cell fate are cell cycle arrest and apoptosis, just like p53. Expression analysis studies mostly defined increased expression of p73 when compared with the corresponding non-tumor counterpart, which is an unexpected situation, when the p53 like function of p73 is regarded. Another difference of p53 and p73 was identified with the description of extensive alternative splicing at 3' end of p73 gene encoded transcripts, which is not a common feature of p53. These transcripts were shown to have different transactivation capabilities.

The mouse knock-out study of p73 gene led the identification of a new transcript from the same locus. This transcript was shown to be originated from an alternative promoter and the transcription was initiated from a hidden exon (exon 3') within the intron 3 of mouse p73 gene. Since the protein of this new mRNA was lacking the p53 transactivation homology domain, it was named as

DN-p73. The former p73 isoform, which was only called as "p73" was renamed as TA-p73. TA and DN-p73 gene isotypes share in-frame 11 exons, so most of their protein products were the same. So, their real function can only be discriminated by the help of their different N-Terminal domains.

The function of TA-p73 (former p73) was defined to be similar with that of p53 (Figure 9-1). When activated, TA-p73 transactivates a set of genes having role in cell cycly regulation and induction of apoptosis. The function of DN-p73 however, was defined recently, just before the submission of this thesis to be an anti-apoptotic protein (Stiewe et al. 2002).

There were several proposed functions for human DN-p73, which of most describing a dominant negative role of this protein on TA-p73 and/or p53. Thus DN-p73 was a candidate oncogene. The oncogenic capacity of this protein was proposed to happen in two different approaches, but the role of DN-p73 in neutralizing TA-p73 or p53 might involve both (Figure 9-1). The first approach relied on the possibility that DN-p73 tetramers can bind to the p53 responsive elements, so p53 or TA-p73 can not bind to them. The second approach relied on the probability of oligomerization of different p73 isoforms (α, β, ϕ, etc) and p73 isotypes (TA-p73 and DN-p73) within each other or with p53. The final tranactivation ability of p53 family aggregates will be defined by the transactivation domain containing (TA-p73 or p53) and transactivation deficient (DN-p73 or Δ exon 2-p73) composition of the hetero-tetramer.

Both of these possibilities were proven to be true by Stiewe *et al.* (Stiewe et al., 2002). For the description of the first hypothesis, Stiewe *et al.* tested the DNA binding ability of DN-p73 by Electro Mobility Shift Assay (EMSA), since DN-p73 was a protein containing DNA binding domain. The labeled p53 consensus oligonucleotides were bound to and supershifted with DN-p73 at a comparable affinity and this binding was reversed with competing oligonucleotides. Secondly, Stiewe *et al.* tested the transactivation ability of TA and DN p73 isoforms and described the incapability of DN-p73, but not TA-p73 or p53 on p53 responsive-luciferase reporter activity. These results show that DN-p73 alone could bind to the promoters of p53 responsive genes and block their

transactivation. Stiewe *et al.* described the second hypothesis of hetero-oligomerization, by cotransfection of p53-responsive luciferase reporter and DN-p73, together with TA-p73 or p53 at increasing plasmid concentrations and assaying for the luciferase activity. The results of this study revealed the dose dependent inhibition of wild type p53 activity by DN-p73, so the second hypothesis.

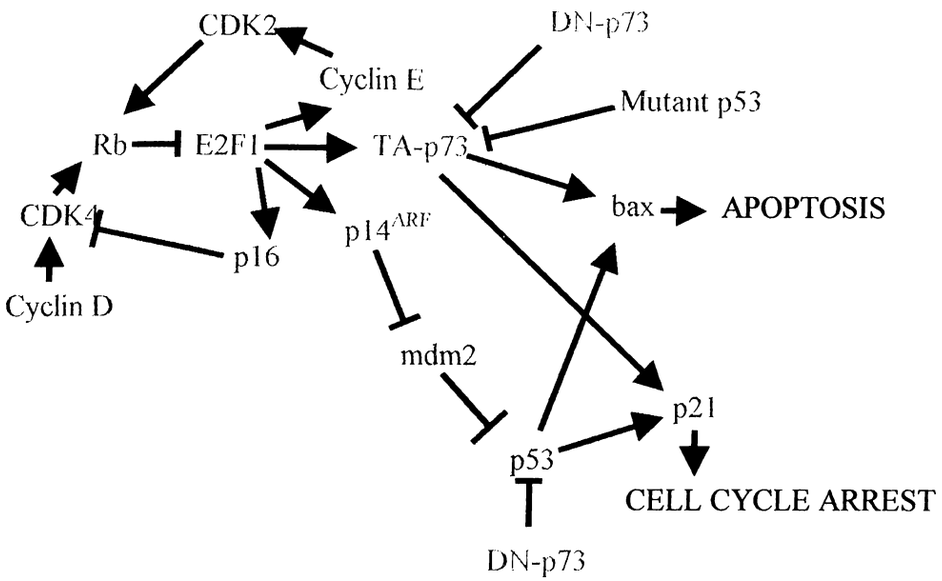

Figure 9-1 : Linking the Rb pathway and p53 pathway components through $p14^{ARF}$ and TA-p73.
The proteins indicated with red are commonly mutated genes during tumor progression. TA-p73 is indicated with purple color and DN-p73 is indicated with green color.

Our first aim was to define the alterations in the p73 locus originated transcripts in HCC as compared with non-tumor and normal liver cells. The differences in the expression profile of TA and DN-p73, in combinations of 3' alternative splicing forms may lead to the production of different p53 like proteins under the name of TA and DN-p73 with different transactivation capabilities. The complexity of the scheme is increased when the probability of these proteins is considered to make heterodimers and heterotetramers with each other and with mutant p53.

Our results support the presence of a complex 3' alternative splicing, which may produce at least 5 proteins with different carboxyl-ends. In normal liver we have only observed the presence of α, β and ϕ forms, whereas we have detected a combination of α, β, γ, ϕ and ε forms but mainly an increase in α, γ and ε forms and either increase or decrease in β and ϕ forms in HCC derived cell lines. Except for the transcriptionally very active β and active α forms, all other splicing variants have very low or no transactivation activity.

In additions to the alterations in the 3' alternative splicing pattern of p73, the expression changes due to direct transcriptional regulation of TA and DN promoter, together with the changes in 5' alternative splicing of TA-p73 produces more decisive transactivation capable and transactivation deficient p73 gene derived proteins. In fact, the studies concerning the 5' region of p73 must be the first choice for description of the transactivation abilities of p73 gene encoded proteins, since the 3' alternative splicing does not give a DN-transcript the transactivation ability. This is the case for normal liver, which produces DN-p73 and its alternative splicing products, but no TA-p73. So the function of p73 gene encoded proteins in normal liver is mainly anti-apoptotic.

As a conclusion, there are gross changes in the expression profile of p73 in HCC derived samples. First of all 3' alternative splicing pattern of p73 was altered to produce different levels of α, β and ϕ forms in addition to cancer related expression of γ and ε forms. Human DN-p73 is defined to be expressed at comparable levels in normal, non-tumor and tumor tissues of liver. The computer based promoter analysis revealed the binding of ubiquitous transcription factors to the promoter of DN-p73, such as Sp-1, so we have confirmed our *in silico* data which correlates with our *in vivo* data obtained from liver derived tissues. Interestingly, we have also defined the induction of TA-p73 expression at transcription level in 14 of 15 HCC cell lines and 3 of 7 primary HCC tumors. The earlier promoter analysis studies described the presence of E2F1 transcription binding sites in the TA-p73 promoter and TA-p73 was shown to be induced by *in vitro* ectopic E2F1 expression by several groups (Selean et al., 2002, Stiewe and Putzer, 2000). The absence of TA-p73 in normal and

non-tumor liver as well as the cancer related expression of TA-p73 led us to correlate the *in vitro* data obtained by other groups by induction of TA-p73 by overexpression of E2F1 transcription factor and *in vivo* data obtained by us about the description of the induction of TA-p73 in a panel of HCC samples. Since the Rb protein functions to block E2F1 transcription factor, we have proposed the acquired expression of TA-p73 as an indicator of Rb pathway dysregulation, which is a common event for HCC. The Rb pathway dysregulation includes the mutations in Rb gene, functional inactivation of Rb protein, mutational inactivation of p16 gene and epigenetic downregulation of p16 mRNA expression (see Figure 9-1). So, we aimed to prove that Rb pathway is dysregulated, both by showing the overexpression of endogenous targets of E2F1 (TA-p73, p14 and cyclin E) and describing the p16 and Rb status of our HCC derived samples (Figure 9-1). We planned to experiment our aim by studying the expression natural E2F1 target genes, namely p16, p14, cyclin E and TA-p73 by semi quantitative RT-PCR and describing the cellular status of Rb protein by western blot.

Our results clearly showed that Rb pathway is non-functional in at least 80% of HCC cell lines and 45% of primary tumors (table 6-1). Moreover, the acquired expression of TA-p73 was partially correlated with activation of endogenous E2F1. These results are the first correlation study concerning the description of Rb pathway dysregulation and expression of TA-p73 in cancer samples.

This data is very important when the therapeutic approaches are concerned. First of all, p53 gene mutations and Rb pathway dysregulation (which induces TA-p73) are common events in cancer development. It seems that p53 is the most specialized member of the family, being mutated in 50% of all cancer types, being induced by a variety of oncogenic signals and genotoxic drugs. Thus, most of the therapeutic approaches for cancer involve the activation of endogenous, wild type p53. The first leakage of this approach is the possibility of the status of p53. Since p53 gene is mutated in 50% of the tumors, such therapies are unlikely to produce a curative result in the tumors with mutant p53, so 50% of the patients. The remaining 50% of tumors with wild type p53 respond to the high genotoxic stress induced by drugs such as cisplatin or by γ-irradiation. The second leakage of this approach appears in this step, at which, not only the normal cells are killed, but also a selection

on cancer cells, for the ones containing mutant p53 was induced. So even the tumor of the patient contains a wild type p53, a very strong selection is introduced to select the tumor cells with mutant p53. This is why, the relapse of tumor is followed by a gain of a more aggressive tumor, which is resistant to a variety of genotoxic drugs most of the time. Thus the cancer therapy approaches are not convenient if they are only relying on the function of wild type p53.

When p73 gene encoded proteins are regarded as target molecules for tumor therapy, there are two aspects, defined by the two major transcripts of this gene, TA-p73 and DN-p73. The proteins of these two transcripts have opposing functions. TA-p73 can be regarded to function as a tumor suppressor and DN-p73 as an oncogene. I will mention the strategies for both of them separately.

In the absence of functional p53, TA-p73 may compensate for the function of it partially. The presence of TA-p73 transcript is not an indicator of a functional p73 protein, but the induction and stabilization of TA-p73 protein by genotoxic drugs such as cisplatin or by inducing the cellular tyrosine kinase, c-abl may be good approaches to selectively eliminate tumor cells. Moreover, the identification of new drugs that are stabilizing and activating TA-p73 protein will be an important improvement of tumor pharmacology. Another approach for inducing TA-p73, even in the absence of RB pathway dysregulation may be introduction and expression of the E2F1 transcription factor to the tumor cells by means of gene therapy approaches. Such a transfer, together with treatment with a TA-p73 protein inducing genotoxic agent, will define the fate of cancer cells, independent of the p53 status of tumor cell population. The strategy presented in this thesis must also be reproduced for other cancer types, so as to describe alternative pathways to eliminate cancer cells in the absence of p53.

The oncogenic product of p73 locus, DN-p73, may also be targeted as an anti-p53 protein in cancer therapy. DN-p73 was shown to be major p73 gene product in normal physiological condition in developing and adult mouse tissues. Moreover, we have shown the presence of DN, but not TA form of p73 mRNA in normal and non-tumor liver by semi-quantitative RT-PCR method. These data, together with the knowledge of the ubiquitous and low levels of expression of p53 in normal tissues

propose a balance between DN-p73 and p53. Since the main function of DN-p73 was shown to block the apoptotic activity of p53 and TA-p73, its activity must be down-regulated together with the activation of p53 or TA-p73 for inducing a higher degree of apoptosis. This could be achieved by targeting the DN-p73 promoter for downregulation of mRNA expression, or targeting the very N-terminal of DN-p73 protein for rapid degradation. We have also shown the presence of similar levels of DN and TA-p73 proteins in a HCC derived cell line with no functional p53 protein. This result may be the answer of how acquired expression of TA-p73 is tolerated by tumor cells. The presence of low and ubiquitous levels of DN-p73 might be the major opposing force against the tumor related presence of TA-p73.

A common polymorphism at codon 72 of p53 was proposed to alter the binding ability of p53 mutants to wild type p53 proteins or TA-p73. The p53 molecule having an arginine at codon 72 was shown to be binding to TA-p73 more efficiently than the one having a proline at this position (Marin et al., 2000). This binding was also shown to inhibit the apoptotic activity of TA-p73 and wild type p53 (Marin et al., 2000). We have shown the presence of TA-p73 transcript in 14/15 HCC cell lines. If this hypothesis is true, then most of the HCC derived cell lines would have mutant p53 molecule with an arginine at codon 72, instead of a proline. However, our data on p53 Arginine/Proline genotyping and protein expression did not support for the selection of mutant p53 with the arginine allele. This discrepancy could be for few reasons. Firstly, our cell lines were originated from different regional and etiological background. We have some cell lines originated from Korea, Japan, USA and African countries. Since the allelic frequency of Arginine/Proline changes dramatically with race background, the data from cell lines may not be used as a random population. Secondly, the ethiology of HCC, so the timing of p53 mutation may be important. P53 gene mutations in aflatoxin related HCC is a very early event, however, the functional inactivation of p53 by viral proteins followed by structural inactivation of p53 is seen in virus related HCC development, which is a late event. Thus the etiology of HCC may be decisive for the selection of p53 alleles. Unfortunately, we do not have enough HCC samples from different etiological backgrounds to make a correlation study.

The description of p73 isoforms in different tissues, cancers and pathologies will allow us to make reasoning for the physiological functions of these isoforms and the phenotype – cell contex correlation for combinations of p73 gene products. Such studies, together with the functional description of p73 gene derived products and identification of p73 targeting molecules may provide different approaches for the cure of different pathologies.

CHAPTER X

FUTURE PERSPECTIVES

This study was the first in vivo correlation study for the demostration of the Rb pathway dysregulation and TA-p73 transactivation. Our results were promising since we have defined the cancer related expression of a p53 functional homologue (TA-p73). If activated properly, TA-p73 protein would accumulate, and drive the cancerous cells to apoptosis. One of the major implications of the use of TA-p73 in tumor therapy is the tumor specific effect of TA-p73, unlike other approaches such as the use of cysplatin and radiation to induce p53 dependent apoptosis. The TA-p73 protein would be present in only Rb pathway dysregulated cells, which are the cancer cells of a tumor mass. Therefore new drugs that are specifically inducing the stability of TA-p73 protein must be discovered as soon as possible.

The expression of DN-p73 was described to be at similar levels in all normal and tumor derived cell populations studied. Also DN-p73 was defined to be present in all developing and adult tissues of mouse. Thus it is anticipated to be present in most of the human tissues. It is currently not known whether DN-p73 mRNA expression or protein levels are regulated. Moreover, due to the presence of DN-p73 at almost all cell types make things harder, since the description of a model system lacking DN and/or TA-p73 expression but harboring a wild type p73 gene is necessary. In our department, such a study is proceeding under the supervision of Prof. Mehmet Ozturk, paralel with our studies. We have initally described 2 breast cancer cell lines with the absence of any p73 gene product. Our initial results of E2F1 transcription factor transfection recovered TA-p73 expression, together with a lower DN-p73 expression. My collegue Ozgur Karakuzu is now investigating the time dependent activation of TA and DN-p73 in response to E2F1 activation. These results are important for the description of TA and DN-p73 regulation by ectopic E2F1 expression.

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CHAPTER XI

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APPENDIX

Oncogene (2001) 20, 5111-5117

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Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells

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p53 and p73 proteins activate similar target genes and induce apoptosis and cell cycle arrest. However, *p53*, but not *p73* is considered a tumour-suppressor gene. Unlike *p53*, *p73* deficiency in mice does not lead to a cancer-prone phenotype, and *p73* gene is not mutated in human cancers, including hepatocellular carcinoma. Here we report that normal liver cells express only ΔN-p73 transcript forms giving rise to the synthesis of N-terminally truncated, transcriptionally inactive and dominant negative p73 proteins. In contrast, most hepatocellular carcinoma cells express TA-p73 transcript forms encoding full-length and transcriptionally active p73 proteins, in addition to ΔN-p73. We also show that together with the acquired expression of TA-p73, the 'retinoblastoma pathway' is inactivated, and E2F1-target genes including *cyclin E* and *p14^{ARF}* are activated in hepatocellular carcinoma. However, there was no full correlation between 'retinoblastoma pathway' inactivation and TA-p73 expression. Most TA-p73-expressing hepatocellular carcinoma cells have also lost p53 function either by lack of expression or missense mutations. The *p73* gene, encoding only ΔN-p73 protein, may function as a tumour promoter rather than a tumour suppressor in liver tissue. This may be one reason why p73 is not a mutation target in hepatocellular carcinoma. *Oncogene* (2001) **20,** 5111-5117.

Keywords: liver cancer; p73; retinoblastoma; p16^{INK4a}; p14^{ARF}; cyclin E

Introduction

The *p53* and its newly discovered homologues, namely *p73* and *p63* form a family of genes that activate similar target genes, and induce apoptosis and cell cycle arrest (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). However, *p53*, but not the other family members, is considered a tumour-suppressor gene. For example, unlike *p53*, *p73* deficiency in mice does not lead to a cancer-prone phenotype (Yang *et al.*, 2000), and *p73* gene is not mutated in human tumours, including hepatocellular carcinoma (HCC) (Marin and Kaelin, 2000; Mihara *et al.*, 1999). Presently, the reasons for this discrepancy are not known. One of the major differences between *p53* and the two other family members is the ability of *p63* and *p73* genes to encode multiple transcript isoforms (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). Different *p63* and *p73* C-termini are generated as a result of alternative splicing between exons 10-15 and 10-14, respectively (Marin and Kaelin, 2000). In addition, *p63* utilizes a cryptic promoter located in intron 3 to generate additional transcripts called ΔN-p63 isoforms (Yang *et al.*, 1998).

Our knowledge on truncated ΔN-p73 isoforms is scarce, but ΔN-p73 transcripts lacking transactivation domain were proposed to be predominant *p73* gene products in some mouse tissues (Yang *et al.*, 2000). Pozniak *et al.* (2000) have recently demonstrated that *p73* is primarily present in developing neurons as a truncated ΔN-p73 isoform in mouse. Like the transcripts encoding ΔN-p63 (Yang *et al.*, 1998), murine ΔN-p73 messages were derived from an alternative promoter located in intron 3, and ΔN-p73 failed to activate transcription from a p53-reporter gene, but suppressed the transactivation activity of both TA-p73α and wild-type p53 (Yang *et al.*, 2000; Pozniak *et al.*, 2000). On the other hand, most, if not all reported studies on the expression of p73 in different cancers did not distinguish between ΔN-p73 and TA-p73 isoforms (reviewed in Marin and Kaelin, 2000; Lohrum and Vousden, 2000).

With regard to p73 implications in liver malignancy, Tannapfel *et al.* (1999a,b) have reported that both p73 transcripts and protein were undetectable in most normal liver cells, but p73 protein was over-expressed in a subset of HCCs, and could serve as an indicator of poor prognosis (Tannapfel *et al.*, 1999b). On the other hand, Mihara *et al.* (1999) reported the absence of mutation, as well as absence of over-expression of *p73* gene in HCC. These observations were in apparent contradiction and they did not provide evidence about the mechanisms of p73 involvement in liver malignancy. These reports, together with the identification ΔN-p73 in some mouse tissues (Yang *et al.*, 2000; Pozniak *et al.*, 2000), led us to test whether p73 expression in normal liver and HCC differs in terms of ΔN-p73 and TA-p73 isoforms. We also compared p73

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Received 27 March 2001; revised 8 May 2001; accepted 24 May 2001

expression in HCC cells with the status of 'retinoblastoma pathway' and p53.

Results

By comparison of murine p73 cDNA sequences with that of human *p73* gene, we developed assays for identification and detection of ΔN-p73 and TA-p73 transcript isoforms (Figure 1a). Initial RT–PCR assays and nucleic acid sequencing identified ΔN-p73 isoform in SNU 398 HCC cell line (Figure 1b). The ΔN-p73 was also detected uniformly in mouse, rat (data not shown) and human liver tissues, as well as HCC cell lines (Figure 1c). In contrast, TA-p73 was not detectable in normal liver, but it was present in 14/15 (93%) of HCC cell lines. However, TA-p73 bands displayed various intensities. They were at the limit of detection in cDNAs from SNU 182 and SNU 423 cell lines, and there was no detectable TA-p73 expression in Mahlavu cell line (Figure 1c). p73 transcripts also differ in their 3′ regions because of alternative splicing between exons 10 to 14, giving rise to α, β, γ, δ, ε and φ isoforms (Zaika *et al*., 1999). p73 transcripts expressed in normal liver and HCC cell lines were mainly α, β and φ isoforms (data not shown). To confirm the expression of ΔN-p73 and TA-p73, we tested the presence of these protein isoforms in SNU 398 as compared to COS7 (Figure 1d), using ER 13 monoclonal antibody against amino acids 495–636 of p73-α (Oncogene Research Products, MA, USA). This antibody was shown to react with the α, but not β isoform of p73 protein (Marin *et al*., 1998). As shown in Figure 1d, ER13 antibody reacted with full-length p73-α (i.e. TA-p73-α) in COS7 cell line which was used as a positive control (Marin *et al*., 1998). SNU 398 cell line expressed TA-p73-α similar to COS7, but also a shorter polypeptide with an apparent molecular weight of 62 kD. This polypeptide can not be p73-β because of ER 13 specificity. Its apparent molecular weight correlates with that of mouse ΔN-p73-α described by Pozniak *et al*. (2000). Since, SNU 398 express both TA-p73 and ΔN-p73 transcripts (Figure 1c), the presence of both TA-p73 and ΔN-p73 proteins in this cell line is expected.

Next, p73 expression was studied in primary HCC tumours. As shown in Figure 1e, all tumours, as well as non tumour liver samples expressed ΔN-p73 isoform. In addition, two of seven (29%) HCC tumour samples expressed TA-p73 isoform. An additional tumour (T1) displayed a weakly positive TA-p73 band. There was no detectable TA-p73 in the remaining T3, T4, T6 and T7 tumours, as well as in non tumour liver NT1. Thus, acquired expression of TA-p73 in HCC was demonstrated in both primary HCC tumours and HCC-derived cell lines. However, the presence of TA-p73 was more frequent in cell lines than primary tumours. The TA-p73 transcripts shown in Figure 1 were tested with a primer pair located on exons 3 and 4, respectively. It has recently been described that, in some breast carcinoma cell lines overexpressing TA-p73, a transactivation-deficient splice variant lacking exon 2 (p73Δexon2) is also detected (Fillippovich *et al*., 2001). Since our TA-p73 transcript test system would not distinguish between TA-p73 and p73Δexon2, we performed additional RT–PCR experiments. Using a forward primer located on exon 1 together with a reverse primer located on exon 4 (Fillippovich *et al*., 2001), we detected mainly TA-p73 transcripts in both primary HCC and cell lines (data not shown). This confirms that TA-p73 forms described in Figure 1 are indeed full-length forms, even though one can not rule out a weak expression of p73Δexon2 form in some cell lines.

Induced expression of TA-p73 in some HCCs suggested that its expression is acquired during malignant transformation of hepatocytes. Recently, E2F1 transcription factor has been shown to activate p73 transcription through E2F1-binding motifs around non-coding exon 1 of p73 gene (Stiewe and Putzer 2000; Lissy *et al*., 2000; Irwin *et al*., 2000; Zaika *et al*., 2001). E2F1-responsive *p73* gene products have been identified as full-length p73α and p73β proteins (Stiewe and Putzer, 2000; Lissy *et al*., 2000; Irwin *et al*., 2000) that can be encoded by TA-p73, but not ΔN-p73 transcript isoforms (Figure 1a). Thus, we hypothesized that the selective induction of TA-p73 expression in HCC tumours is due to E2F1 activation. Therefore, we also tested the expression of E2F1 target genes $p14^{ARF}$ and *CCNE1* encoding cyclin E (Dyson, 1998). As shown in Figure 1e, the expression of cyclin E was induced in three tumours (T1, T2, T5), as compared to non tumour liver tissue NT1. The expression of $p14^{ARF}$ was also induced in two tumours (T1 and T2). It was also noteworthy that in TA-p73-positive T2 and T5 tumours, cyclin E expression was also induced, but only T2 showed $p14^{ARF}$ induction. Thus, three E2F1 target genes (i.e. *p73, Cyclin E* and $p14^{ARF}$) were induced in some HCC tumours. However, there was no full correlation between their expression patterns in different tumours. This could be due to the quality of RNA extracted from these archival tumour tissues. (We were not able to test additional samples, because HCC is a rarely operated tumour). Alternatively, the expression of these E2F1 target genes in HCC may be under the influence of additional factors.

As the activity of E2F1 is controlled by the protein product of retinoblastoma (RB1) gene (pRb) (Dyson, 1998), we studied whether the induction of TA-p73 expression is related to the inactivation of 'retinoblastoma pathway' in HCC cells. The 'retinoblastoma pathway' in cancer cells is altered mainly by inactivation of either *RB1* or $p16^{INK4a}$ gene (Dyson, 1998). In HCC, *RB1* mutations are rare, but allelic loss and decreased pRb levels occur frequently (reviewed in Ozturk, 1999), which may be due to overexpression of gankyrin that reduces the stability of pRb, and releases 'free E2F1' (Higashitsuji *et al*., 2000). Loss of $p16^{INK4a}$ expression by gene deletion or promoter methylation is also common in HCC (see Ozturk, 1999; Baek *et al*., 2000). Therefore, we compared the expression of TA-p73 with the expression of pRb protein and $p16^{INK4a}$

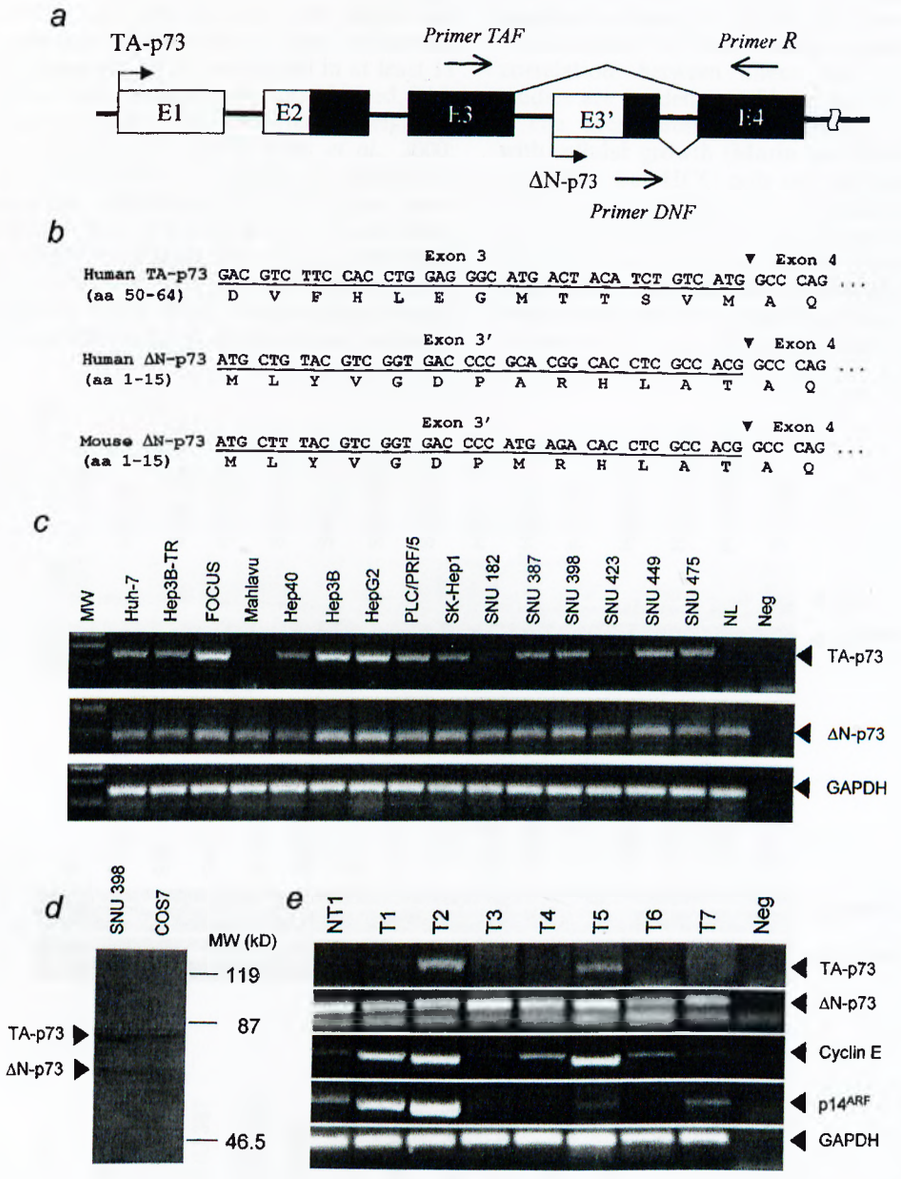

Figure 1 Identification of TA-p73 and ΔN-p73 transcripts and their expression in normal liver, HCC cell lines and primary HCC tumours, as compared to E2F1 target genes cyclin E and $p14^{ARF}$. (**a**) Exon-intron structure of 5′ coding region of human p73 gene showing the transcription start sites and splicing events leading to TA-p73 and ΔN-p73 isoforms. Initiation of transcription in exon 1 produces the TA-isoforms, containing the transactivation (TA) domain (previously called p73), while initiation in exon 3′ gives rise to the ΔN-isoforms without the TA domain. The putative transcriptional start site and exon 3′ for ΔN-p73 were identified by *in silico* analysis of human p73 gene, as compared to mouse ΔN-p73 transcript sequence. Primers TAF and DNF indicate the positions of isoform-specific forward primers used to identify respectively TA-p73 and ΔN-p73 transcripts, in combination with Primer R common to both isoforms. (**b**) Partial nucleotide and amino acid sequences of human TA-p73 and ΔN-p73 transcript isoforms as compared to mouse ΔN-p73 isoform. Data for human ΔN-p73 was obtained from direct sequencing of a 186 bp RT–PCR product obtained from SNU 398 HCC cell line, using DNF and R primer pair. (**c**) As compared to normal liver (NL), the expression of TA-p73, but not ΔN-p73 is induced strongly in 14 out of 15 (93%) HCC cell lines. TA-p73 and ΔN-p73 isoforms were tested as described in **a**. GAPDH was used as a control for equal template loading in PCR. Neg; negative control. (**d**) Western blot analysis of p73 protein in SNU 398 and COS7 reveals the presence of a ~62 kD polypeptide (presumably ΔN-p73 according to Pozniak *et al*., 2000) in SNU 398, in addition to TA-p73 protein present in both cell lines. Five-hundred μg total protein was analysed with anti-p73 ER13 monoclonal antibody which recognizes p73-α, but not p73-β isoforms (Marin *et al*., 1998). (**e**) Expression of TA-p73, ΔN-p73, cyclin E and $p14^{ARF}$ transcripts in primary HCC tumours. RT–PCR analysis using oligonucleotide primers shown in **a**, reveals the expression of ΔN-p73 in all samples tested. TA-p73 is easily detected in two out of seven tumours (T2, T5), T1 shows only weak expression, non tumour liver NT1 (counterpart of T1) is negative. Similarly, cyclin E expression is also induced in T1, T2, and T5, but $p14^{ARF}$ transcripts are induced only in T1 and T2. GAPDH was used as a control for equal template loading in PCR. Neg; negative control

transcripts in HCC cell lines. The expression of either pRb or $p16^{INK4a}$ was lost in six cell lines, and significantly decreased in five others. Thus, 'retinoblastoma pathway' appeared to be inactivated in at least 11 out of 15 (73%) cell lines (Figure 2a). Indeed, this inactivation has previously been reported for eight of these cell lines (Morel *et al.*, 2000; Baek *et al.*, 2000; Puisieux *et al.*, 1993; Suh *et al.*, 2000). In support of this conclusion, the expression of E2F1 target genes *cyclin E* and $p14^{ARF}$ were also induced in all cell lines, except three for $p14^{ARF}$ (Figure 2b), due to CDKN2A gene deletion in SNU 387 and SNU 449 (Baek *et al.*, 2000), and probably in SK-Hep1. These studies showed that both the inactivation of 'retinoblastoma pathway' and the induction of TA-p73 expression are relatively common events in HCC cell lines. However, our observations do not allow establishing a direct correlation between these two events. Additional studies are needed to address this issue.

The overexpression of TA-p73 is not compatible with cellular growth (Marin and Kaelin, 2000; Fang *et al.*, 1999), but HCC cells can apparently tolerate TA-p73 expression (Figure 1c). The ΔN-p73, co-expressed in these cells, is known to suppress both TA-p73 and p53 activities (Yang *et al.*, 2000; Pozniak *et al.*, 2000). In addition, some tumour-derived p53 mutant proteins were shown to bind and inactivate TA-p73 protein (Marin and Kaelin, 2000; Di Como *et al.*, 1999; Strano

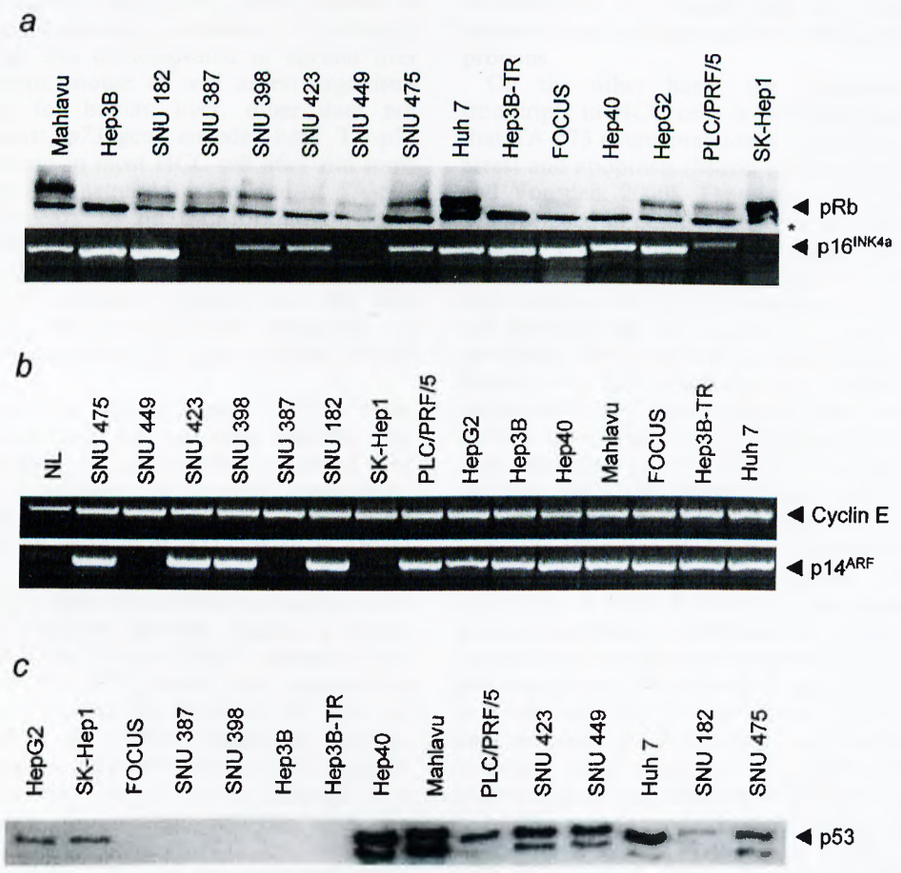

Figure 2 Comparative analysis of the status of retinoblastoma pathway (pRb and $p16^{INK4a}$), E2F1 target (cyclin E and $p14^{ARF}$) and p53 genes in HCC cell lines. (**a**) Either pRb or $p16^{INK4a}$ expression is totally lost (Hep3B, SNU 387, SNU 449, Hep3B-TR, Hep40, SK-Hep1), or significantly decreased (SNU 398, SNU 423, SNU 475, FOCUS, PLC/PRF/5) in 11 out of 15 (73%) cell lines tested. Western blot analysis using antibody IF8 reveals that pRb protein is not detectable in HepB3, Hep3B-TR and Hep40, or weakly positive in SNU 423, SNU 449 and FOCUS. The asterix (*) denotes a non-pRb cross-reactive antigen (Morel *et al.*, 2000), serving as a loading control for Western blot assay. RT–PCR analysis shows that $p16^{INK4a}$ is not detectable in SNU 387, SNU 449 and SK-Hep1, or weakly positive in SNU 398, SNU 423, SNU 475 and PLC/PRF/5. See Figure 1c for template loading control using GAPDH. Negative control for $p16^{INK4a}$ RT–PCR is not shown. (**b**) The expression of E2F1 target cyclin E and $p14^{ARF}$ genes is induced in HCC cell lines. RT–PCR analysis of cyclin E transcripts reveals that their expression is uniformly induced in all cell lines tested, as compared to normal liver (NL). The induction of $p14^{ARF}$ expression is detectable in all but three cell lines. The lack of expression in SNU 449 and SNU 387 (see Baek *et al.*, 2000), and probably SK-Hep1 (notice the lack of both $p16^{INK4a}$ and $p14^{ARF}$ expression) is due to CDKN2A gene deletion. See Figure 1c for the use of GAPDH as template loading control. Negative control is not shown. (**c**) Western blot assay for p53 shows no expression in FOCUS, SNU 387, SNU 398, Hep3B, Hep3B-TR, and high levels in Hep40, Mahlavu, PLC/PRF/5, SNU 423, SNU 449, Huh7, SNU 475 cell lines. Mahlavu and PLC/PRF/5 display Arg249Ser, and Huh7 Tyr220Cys mutations. HepG2 and SK-Hep1 express wild-type p53 (Puisieux *et al.*, 1993; Hsu *et al.*, 1993). pRb Western blot data was used as a loading control (as in **a**)

et al., 2000; Marin *et al.*, 2000; Gaiddon *et al.*, 2001). Therefore, we tested whether there was a correlation between TA-p73 expression and *p53* gene status in HCC cells. As shown in Figure 2c, p53 protein was either lost or mutant in at least eight out of 15 (53%) cell lines tested.

Discussion

Our observations demonstrate that *p73* gene in normal liver cells encodes only truncated ΔN-p73 transcript isoform. In mouse, this isoform was shown to code for an inactive p73 protein, acting as a dominant negative form on the activities of full-length TA-p73 as well as wild-type p53 proteins (Yang *et al.*, 2000; Pozniak *et al.*, 2000). The dominant expression of ΔN-p73 transcript isoform was demonstrated in normal liver tissues from human, mouse as well as rat organisms (see Figure 1c,e for human liver, other data not shown). In contrast, *p73* gene encodes both TA-p73 and ΔN-p73 isoforms in most HCC cell lines and some primary tumours. The acquired expression of TA-p73 in HCC cells appears to be transformation-related rather than proliferation-related, since Mahlavu cells did not express detectable TA-p73 transcripts, while expressing ΔN-p73 isoform (Figure 1c). We also detected ΔN-p73, but not TA-p73 transcripts in proliferating normal human fibroblast cell line MRC5 (data not shown).

Separate analysis of ΔN-p73 and TA-p73 transcripts in liver and HCC has not been reported yet. However, Tannapfel *et al.* (1999a) reported the absence of p73 expression in normal hepatocytes, using an antibody directed against amino acid residues encoded by exon 3 of p73, which is absent in ΔN-p73, but present in TA-p73 transcripts (see Figure 1b). Studies with this antibody (together with another anti-p73 antibody directed against a region common to both TA-p73 and ΔN-p73 proteins) also demonstrated that the p73 protein was negative in non tumour liver tissues, but positive in 32% of HCCs (Tannapfel *et al.*, 1999b). Based on observations described here, it now appears that p73 detected in these HCCs is the TA-p73 form, although this requires a separate confirmation analysis with the TA-p73-specific antibody alone.

The induction of TA-p73 and the 'retinoblastoma pathway' inactivation was common in HCC cell lines (Table 1). However, comparative studies did not allow us to establish a link between the induction of TA-p73 expression and the inactivation of 'retinoblastoma pathway'. For example, the expression of E2F1-target genes was also induced in cell lines with apparently normal pRb and $p16^{INK4a}$ expression. Nevertheless, our results indicate that 'retinoblastoma pathway' inactivation is common in HCC cell lines and it is accompanied by an induction of E2F1 target genes (i.e. *p73*, *cyclin E* and $p14^{ARF}$) in most HCCs. This may suggest that the acquired expression of TA-p73 in some HCC cell lines is due to the inactivation of 'retinoblastoma pathway'. However, this remains to be demonstrated with further studies.

The expression of ΔN-p73 in normal liver, and the acquired expression of TA-p73 in HCC reveal new implications for p73 in tumour biology. The *p73* gene, encoding only ΔN-p73 transcripts in normal liver, may function as a tumour promoter rather than a tumour suppressor in this tissue, by interfering with wild-type p53 function (Yang *et al.*, 2000; Pozniak *et al.*, 2000). This could explain why *p73* is not mutated in HCC (Mihara *et al.*, 1999). However, *p53* gene is frequently mutated in HCC (Ozturk, 1999), as also demonstrated here in HCC-derived cell lines (Figure 2c). Thus, ΔN-p73 protein may not completely neutralize wild-type p53 activity. Alternatively, the induction of TA-p73 transcription in tumour cells may redirect ΔN-p73 protein from wild-type p53 to newly expressed TA-p73 proteins.

On the other hand, the expression of TA-p73 transcripts in HCC cells is puzzling, based on the fact that TA-p73 overexpression is known to induce growth arrest and apoptosis (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). Therefore, HCC cells expressing TA-p73 need to equip themselves with mechanism(s) to tolerate TA-p73 protein expression. In addition to a possible role of ΔN-p73 protein for TA-p73 inactivation (Yang *et al.*, 2000; Pozniak *et al.*, 2000), mutant p53 proteins may also inactivate TA-p73, as reported previously (Di Como *et al.*, 1999; Strano *et al.*, 2000; Marin *et al.*, 2000; Gaiddon *et al.*, 2001). Accordingly, several HCC cell lines reported here (Mahlavu, PLC/PRF/5, Huh7 etc.) express mutant p53 proteins that may inactivate TA-p73 protein. However, some other cell lines (FOCUS, Hep3B, SNU 387, SNU 398 etc.) have lost p53 expression. In this later group of cell lines, the tolerance of TA-p73 expression may require mutant p53-independent mechanisms. One additional hypothesis is that TA-p73 protein levels and/or its growth suppressive activities are regulated by post-translational mechanisms, independent of both ΔN-p73 and mutant p53. For example, endogenous p73 protein in a tumour cell line was shown to be stabilized by cisplatin and by c-Abl kinase, with no change in p73 transcript levels (Gong *et al.*, 1999). Moreover, the apoptosis-inducing function of p73 was demonstrated to be dependent to or enhanced by the c-Abl kinase in different cell types (Yuan *et al.*, 1999; Agami *et al.*, 1999; Gong *et al.*, 1999). Thus, some HCC cell lines may tolerate TA-p73 expression, if, for example, they are deficient in c-Abl kinase activity.

Materials and methods

Tissues and cell lines

Normal liver tissues from Balb/c mice and Sprague-Dawley rats were obtained after ether anaesthesia. Normal human liver tissue was obtained from discarded surgical material from a patient operated for hepatic hydatid cyst. Primary HCC tumours and non tumour liver were freshly frozen

Table 1 Comparative analysis of TA-p73 induction, E2F1 target gene activation and the retinoblastoma (*RB1*) pathway inactivation in HCC cell lines

Cell lines	*RB1 pathway genes*		*EF21 target genes*		
	pRb	*$p16^{INK4a}$*	*TA-p73*	*$p14^{ARF}$*	*Cyclin E*
Group I: Inactivated RB1 pathway					
hep3B	ND	Positive	Positive	Positive	Positive
Hep3B-TR	ND	Positive	Positive	Positive	Positive
hep40	ND	Positive	Positive	Positive	Positive
FOCUS	Traces	Positive	Positive	Positive	Positive
SNU-449	Positive	ND	Positive	ND	Positive
SNU-387	Positive	ND	Positive	ND	Positive
SK-hep1	Positive	ND	Positive	ND	Positive
PLC/PRF/5	Positive	Traces	Positive	Positive	Positive
SNU-475	Positive	Traces	Positive	Positive	Positive
SNU-398	Positive	Traces	Positive	Positive	Positive
SNU-423	Traces	Traces	Traces	Positive	Positive
Group II: Normal RB1 pathway					
Mahlavu	Positive	Positive	ND	Positive	Positive
SNU-182	Positive	Positive	Traces	Positive	Positive
HepG2	Positive	Positive	Positive	Positive	Positive
Huh-7	Positive	Positive	Positive	Positive	Positive

ND: not detected

archival materials. Fifteen human hepatoma-derived cell lines (14 HCC and one hepatoblastoma), MRC5 and COS7 were grown in culture as described (Morel *et al.*, 2000). Most cell lines were from ATCC, the others have been previously described (Morel *et al.*, 2000; Baek *et al.*, 2000; Puisieux *et al.*, 1993; Bouzahzah *et al.*, 1995).

RNA and cDNA preparations

Total RNA from cell lines and tissues was extracted using NucleoSpin RNA II Kit (MN Macherey-Nagel, Duren, Germany) and TriPure reagent (Boehringer Mannheim, Indianapolis, IN, USA), respectively. The cDNAs were prepared from total RNA (5 μg) using RevertAid First Strand cDNA Synthesis Kit (MBI-Fermentas, Vilnius, Lithuania).

Transcript analysis by RT–PCR

For the identification of TA-p73 and ΔN-p73 forms, we used the available human p73 genomic sequence and mouse ΔN-p73 cDNA sequence data and designed two forward and one reverse (Primer R; 5′-GCGACATGGTGTCGAAGGTG-GAGC-3′) primers to specifically amplify these forms from human, mouse and rat tissues. These sequences were selected on the basis of highest homology between mouse and human DNA sequences (maximum one mismatch, data not shown). The forward primers TAF (5′- AACCAGACAGCACCT-ACTTCGACC-3′) and DNF (5′-ACCATGCTG TAC-GTCGGTGACCCC-3′) were used for specific amplification of TA-p73 and ΔN-p73 forms, respectively. For the detection of p73 isoforms generated by differential splicing between exons 10 to 14, a forward primer from exon 10 (5′-CGGCCATATT GGTGCCGCAGCCACTGGTG-3′) and two different reverse primers were used. A reverse primer from exon 13 (5′-GTTTGGCACCCCCAATCCTGT-3′) was used for specific amplification of transcripts containing this exon, i.e. p73α, p73ε, and p73γ (see Zaika *et al.*, 1999 for terminology). Another reverse primer, encompassing sequences from exon 12 followed by exon 14 (5′-AGGGCCCC-CAGGTCCTGAGC-3′), was used for specific amplification of p73β and p73φ isoforms (Zaika *et al.*, 1999) that contain this particular sequence. $p16^{INK4a}$ and $p14^{ARF}$ RT–PCRs were done using a specific forward primer for each transcript (5′-CGGAGAGGGGGAGAACAGAC-3′ for $p16^{INK4a}$ and 5′-TCACCTCTGGTGCCAAAGGG-3′ for $p14^{ARF}$) and a common reverse primer (5′-GGCAGTTGTGGCCCTG-TAGG-3′). For the detection of cyclin E mRNA levels, cyclin E-F (5′-TTGACCGGTATATGGCGACACAAG-3′) and cyclin E-R (5′-ATGATACAAGGCCGAAGCAG-CAAG-3′) primers were used. All RT–PCR reactions were done using appropriate annealing and extension conditions (additional information is available upon request). Equal amount of RNA was used in cDNA synthesis and the quality of cDNA was initially tested by GAPDH RT–PCR amplification with primer pair F (5′-GGCTGAGAACGG-GAAGCTTGTCAT-3′) and R (5′-CAGCCTTCTCCAT-GGTGGTGAAGA-3′), using 1/40 vol-ume of cDNA preparation. Further PCR studies were performed with cDNA preparations yielding equal amounts of GAPDH amplification products. Total PCR cycle numbers have been defined following an initial study at 22, 26, 30 and 34 cycles, in order to remain in the logarithmic phase of amplification. All RT–PCR results have been repeated several times from different batches of RNA preparations except primary tumours for which single RNA preparations were used. The expression of TA-p73 isoform was confirmed with an additional pair of primers, as described by Fillippovich *et al.* (2001). The identity of different p73 isoforms (TA-p73, ΔN-p73, p73α, p73β, p73γ, p73φ) has been confirmed by restriction enzyme mapping and automated sequencing (PE, ABI PRISM 377 automated sequencer) techniques.

Western blotting

The expression of retinoblastoma (pRb) and p53 proteins in different cell lines was studied by Western blotting, using anti-pRb IF8 (SC102, Santa Cruz Biotechnology) and anti-p53 6B10 (Yolcu *et al.*, 2001) monoclonal antibodies, respectively, as described previously (Morel *et al.*, 2000). Equal protein loading was confirmed by blotting with control antibody against cytokeratin 18 (JAR13 clone, gift from D Bellet, Institut Gustave Roussy, France). p73 protein Western

blot assays were done with ER13 (Ab-1, Oncogene Research Products, MA, USA) using the experimental procedure described by Marin *et al.* (1998).

Abbreviations
HCC, hepatocellular carcinoma; RB1, retinoblastoma gene; pRb, retinoblastoma protein.

Acknowledgements
This work was supported by a grant from TUBITAK (Turkey). We would like to thank B Carr (University of Pittsburgh, PA, USA) for providing Hep40 and Hep3B-TR cell lines, D Bellet (Institut Gustave Roussy, France) for JAR13 antibody, R Cetin-Atalay for critical reading of the manuscript, T Cagatay for DNA sequencing work, C Akcali's group for animal surgery, and G Tuncman for help in RNA studies.

Accession numbers
The following sequences were deposited in the GenBank database. Human DNA sequence from clone RP5-1092A11 on chromosome 1p36.2-36.33 containing p73 gene: AL136528; Mouse mRNA for p73 delta-N protein: Y19235; Homo sapiens p73 gene: AH007820.

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Report

NAPO as a novel marker for apoptosis

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Apoptosis or programmed cell death plays a pivotal role in embryonic development and maintenance of homeostasis. It is also involved in the etiology of pathophysiological conditions such as cancer, neurodegenerative, autoimmune, infectious, and heart diseases. Consequently, the study of apoptosis is now at center of both basic and clinical research applications. Therefore, sensitive and simple apoptosis detection techniques are required. Here we describe a monoclonal antibody–defined novel antigen, namely *NAPO* (negative in apoptosis), which is specifically lost during apoptosis. The anti-*NAPO* antibody recognizes two nuclear polypeptides of 60 and 70 kD. The antigen is maintained in quiescent and senescent cells, as well as in different phases of the cell cycle, including mitosis. Thus, immunodetection of *NAPO* antigen provides a specific, sensitive, and easy method for differential identification of apoptotic and nonapoptotic cells.

Introduction

During the last decade, apoptosis has become a major focus of interest for many fields of biomedical research. Programmed cell death is required for proper embryonic development as well as for the maintenance of homeostasis in adult tissues (Vaux and Korsmeyer, 1999; Wyllie and Golstein, 2001). Moreover, apoptosis is involved in the etiology and pathophysiology of a variety of diseases, such as cancer, neurodegenerative, autoimmune, infectious, and heart diseases (Chervonsky, 1999; Roulston et al., 1999; Mattson, 2000; Narula et al., 2000; Reed, 2000). Apoptotic cell death is characterized by a series of morphological changes, including cell shrinkage, nuclear condensation, chromatin segregation, membrane blebbing, formation of membrane-bound apoptotic bodies, and internucleosomal DNA cleavage (Saraste and Pulkki, 2000). These morphological changes result from a series of genetically programmed biochemical changes initiated by either the activation of death receptors or intracellular stress conditions such as DNA damage. These proapoptotic signals are conveyed to mitochondria to cause the release of caspase-activating factors from this organelle, followed by a cascade of caspase activation which leads to cell death (Earnshaw et al., 1999; Gottlieb, 2000).

Apoptosis, as a critical component of life in multicellular organisms, is a target subject for understanding cellular mechanisms of many diseases, as well as for developing new drugs that interfere with either proapoptotic or antiapoptotic molecular networks. Consequently, it has become important to develop reliable assays to measure cell death. Techniques currently available for apoptosis detection are based on the study of morphology of apoptotic cells (light and fluorescence microscopy coupled to nuclear staining with specific dyes and electron microscopy), DNA fragmentation detected by terminal transferase-mediated dUTP nick-end labeling (TUNEL)* and similar techniques, membrane changes detected by annexin V in vivo labeling, and on immunological assays using antibodies directed to apoptosis-related proteins (Stadelmann and Lassmann, 2000). Essential requirements for apoptosis detection techniques include high sensitivity for apoptotic cells, the ability to differentiate between apoptosis and other forms of cellular changes, as well as distinction between different stages of the cell death process. However, we are facing a relative paucity for simple techniques fulfilling these requirements, and furthermore allowing quantitative analysis (van Heerde et al., 2000). Immunological detection of apoptosis-related proteins is probably the best approach to overcome this obstacle, but there are only a few known apoptosis marker antigens (Stadelmann and Lassmann, 2000).

Here we describe a mouse monoclonal antibody–defined nuclear antigen composed of two polypeptides that we call *NAPO* (for negative in apoptosis), which is strongly expressed in cells under many conditions (proliferation, quiescence, mitosis, and senescence) except apoptosis. The immunoreactivity of the antigen, as tested by immunofluorescence technique,

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Key words: apoptosis; apoptotic cell death; apoptotic marker; quiescence; senescence

*Abbreviations used in this paper: TUNEL, terminal transferase–mediated dUTP nick-end labeling.

The Journal of Cell Biology, Volume 155, Number 5, November 26, 2001 719–724
http://www.jcb.org/cgi/doi/10.1083/jcb.200106044

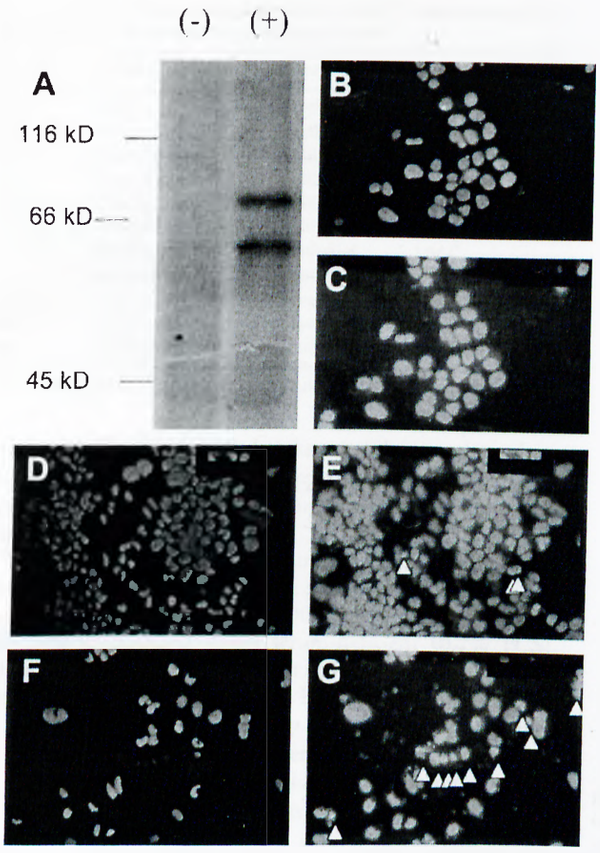

Figure 1. **Initial characterization of *NAPO* antigen.** Anti-*NAPO* monoclonal antibody recognizes two bands migrating at ~60 and 70 kD. [^{35}S]methionine-labeled Huh7 cells were subjected to immunoprecipitation with anti-*NAPO* antibody (+). (−) is a negative control (A). Immunofluorescence staining of Huh7 cells with anti-*NAPO* antibody indicates that *NAPO* is a nuclear antigen (B). Hoechst 33258 counterstain for nuclear DNA (C). *NAPO* immunofluorescence staining of SNU 398 cells growing in standard culture medium indicates that the majority of cell nuclei are positive, but occasionally some cells with small nuclei (presumably apoptotic) are negative (D), as indicated by white arrows in nuclear DNA staining (E). *NAPO* antigen is negative in apoptotic SNU 398 cells which are induced by growth in serum-free medium (F). Apoptotic cells are indicated by white arrows in Hoechst 33258 counterstaining (G).

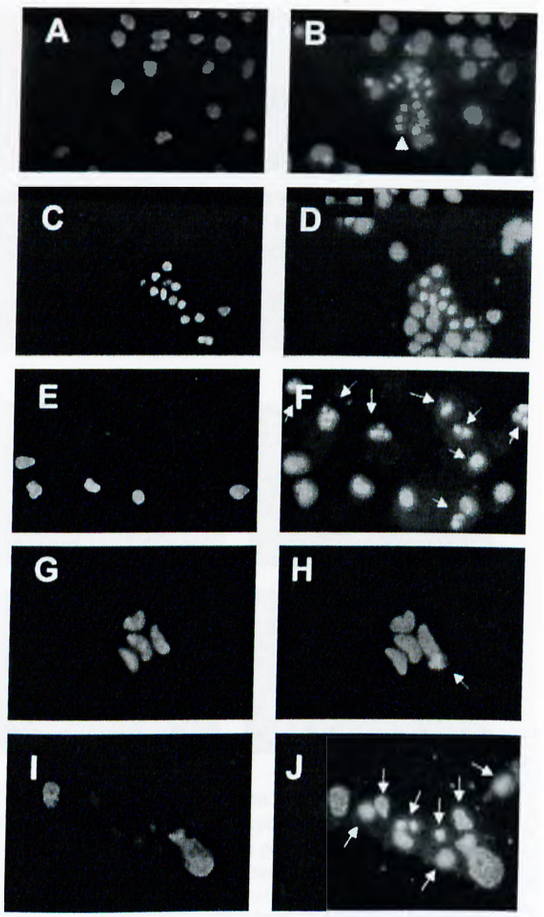

Figure 2. **Identification of *NAPO* as a common apoptosis marker.** *NAPO* is negative in 100 μM H_2O_2-treated apoptotic Huh7 cells (A), in contrast to positive staining with TUNEL (C). NAPO is also lost in Fas-mediated apoptosis in Jurkat cells (E), H_2O_2-mediated apoptosis in 293 cells (G), and UV-C–mediated apoptosis in MRC-5 cells (I). B, D, F, H, and J show Hoechst 33258 counterstaining.

is lost in apoptotic cells in a way opposite to TUNEL and annexin V staining. Thus, *NAPO* antigen may serve as a reliable marker for apoptosis.

Results and discussion

Biochemical characterization of the *NAPO* antigen

A mouse IgG monoclonal antibody (named anti-*NAPO* antibody) was generated against a nuclear antigen after immunization with human colorectal cell line COLO 320. Detergent-soluble proteins were prepared from metabolically labeled Huh7 cells and subjected to immunoprecipitation with anti-*NAPO* antibody. As shown in Fig. 1 A, anti-*NAPO* antibody recognized two proteins migrating at ~60 and 70 kD, respectively. Immunofluorescence studies revealed with the same cell line indicated that *NAPO* was a nuclear antigen (Fig. 1, B and C). This antibody was specific for human and monkey *NAPO*, since all human cell lines and monkey COS-7 showed positive immunostaining, but mouse (HC 11), rat (IAR-6), and hamster (CHOK-I) cell lines were negative (unpublished data).

Although *NAPO* antigen was ubiquitously present in all human cell lines so far tested, small nuclear fragments which are occasionally observed with some cell lines under normal culture conditions were negative (Fig. 1, D and E as an example). This suggested to us that *NAPO* antigen could be lost during apoptosis.

Identification of *NAPO* as an apoptotic marker

Hepatocellular carcinoma–derived SNU 398 cells, which undergo apoptosis when grown under serum-free conditions were serum starved for three days and tested for *NAPO* antigen immunoreactivity. Cells displaying morphological char-

Table I. **List of cell lines tested for loss of *NAPO* immunoreactivity after induction of apoptosis by various stimuli**

Cell line	Origin	Morphology	Apoptosis stimuli
Huh 7	HCC	Epithelial	H_2O_2
SNU 398	HCC	Epithelial	Serum starvation
MCF7	Breast cancer	Epithelial	TNF-α, UV-C
HeLa	Cervix cancer	Epithelial	UV-C
SW480	Colon cancer	Epithelial	UV-C
LNCaP	Prostate cancer	Epithelial	UV-C
U2OS	Osteosarcoma	Epithelial	UV-C
A375	Melanoma	Epithelial	UV-C
Jurkat	TCL	Lymphoid	Anti-Fas, UV-C
MRC-5	Lung	Fibroblastic	UV-C
293	Embryonal kidney	Epithelial	Cisplatin, H_2O_2

HCC, hepatocellular carcinoma; TCL, acute T cell leukemia.

acteristics of apoptosis (cell shrinkage, nuclear condensation, and fragmentation) displayed negative *NAPO* staining in contrast to positive nuclear staining of all nonapoptotic cells (Fig. 1, F and G).

To confirm the loss of *NAPO* antigen during apoptosis in another cellular system, hepatocellular carcinoma–derived Huh7 cells were used. H_2O_2 (100 μM) treatment of these cells induce apoptosis under serum-deficient (0.1% FCS) conditions (unpublished data). As shown in Fig. 2 A, *NAPO* antigen was negative in apoptotic Huh7 cells that are identified as cells with small nuclei by Hoechst 33258 counterstaining (Fig. 2 B). To test whether the loss of *NAPO* expression is specific to this antigen, rather than a common feature shared by nuclear proteins, we also tested Huh7 cells for p53 protein immunoreactivity under similar conditions. Huh7 cells express a mutant p53 protein that accumulate in their nuclei (Volkmann et al., 1994). Both apoptotic and nonapoptotic Huh7 cells displayed positive staining for p53 protein. Indeed, apoptotic cells displayed a stronger p53 immunoreactivity when compared with nonapoptotic cells (unpublished data). This indicated that the loss of *NAPO* immunoreactivity in apoptotic Huh7 cells was specific to this antigen rather than a common feature of nuclear proteins.

For further characterization of NAPO as an apoptosis marker, additional studies were performed in different cell lines treated with different apoptosis stimuli. For all experiments NAPO tests were run in parallel to TUNEL or annexin V staining (TUNEL data for Huh7 shown in Fig. 2 C as an example). To show whether NAPO antigen is lost during death receptor–mediated apoptosis, TNF-α–treated MCF 7 and anti-Fas antibody–treated Jurkat cells were used. NAPO was lost in apoptotic Jurkat (Fig. 2 E) as well as MCF-7 cells (unpublished data). To test whether *NAPO* loss during apoptosis was common to cells of different origin, additional tumor-derived (HeLa, U2OS, A375, SW480, LNCaP) as well as normal tissue–derived (293 and MRC-5) cell lines were induced to undergo apoptosis by H_2O_2, UV-C, or cisplatin treatment (Table I). NAPO staining was lost in all apoptotic cells in contrast to strong nuclear staining of the nonapoptotic counterparts (example data on 293 and MRC-5 cells are shown in Fig. 2, G and I, respectively).

These results demonstrate that NAPO is ubiquitously expressed in living cells, but lost during apoptosis independent of the apoptosis activating pathway (Table I). The loss of

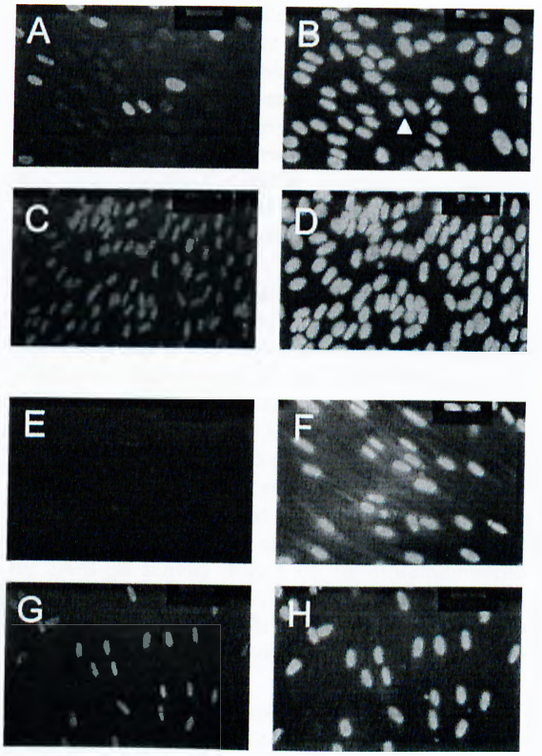

Figure 3. ***NAPO* antigen is positive in quiescent cells.** MRC-5 cells were tested in parallel for BrdU incorporation (A and E) or *NAPO* antigen (C and G). Cells in A–D were grown under standard culture conditions. Cells in panels E–H were serum starved for 3 d to induce a quiescent state, as indicated by negative BrdU staining in E. Note that both actively growing (C) and quiescent cells (G) are positive for *NAPO*. B, D, F, and H show Hoechst 33258 counterstaining.

NAPO during apoptosis strongly suggests that this antigen is a nuclear caspase substrate. The epitope recognized by anti-*NAPO* antibody on this antigen is probably lost as a result of caspase-mediated protein cleavage. However, it is presently unclear whether any of 60- and 70-kD polypeptides of the *NAPO* antigen are known caspase substrates. To our knowledge, proteins with similar molecular weight have not been described previously as apoptosis-associated proteins. Thus, *NAPO* appears to be a novel marker for apoptosis that could serve to distinguish apoptotic from nonapoptotic cells. However, it was important to know whether the antigenic reactivity is modified under different growth conditions such as quiescence, cell cycle (especially mitosis), and senescence.

Expression of the *NAPO* antigen in quiescent cells

MRC-5 human embryonic lung fibroblast cells (passage 18) were grown to confluency and serum starved for 3 d to induce quiescence. To show that these cells are indeed quiescent, BrdU incorporation was also tested. Our results indicate that ~15% of asynchronously growing MRC-5 cells are positive for BrdU i.e., in S phase (Fig. 3 A), whereas no BrdU labeling was observed in quiescent cells (Fig. 3 E). Under both conditions, all cells displayed a similarly positive nuclear staining for *NAPO* (Fig. 3, C and G). These obser-

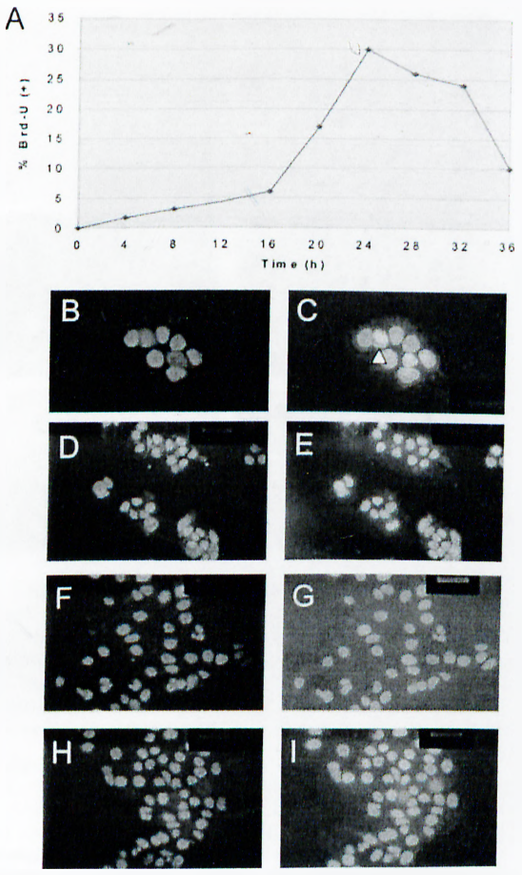

Figure 4. ***NAPO* expression during cell cycle.** Huh7 cells were synchronized by nocodazole treatment, followed by mitotic shake-off. Freshly collected cells were then grown in culture for up to 36 h. The S phase was identified by BrdU incorporation assay. Time points between 0–16, 20–32, and 36 h were evaluated respectively as G1, S, and G2 phases according to the BrdU incorporation index (A). *NAPO* and BrdU staining were performed at 4 h intervals. B, D, F, and H illustrate *NAPO* staining of cells at mitotic arrest (B), 8 h (D), 24 h (F), and 36 h (H), respectively. C, E, G, and I show Hoechst 33258 counterstaining. Note diffused *NAPO* staining in mitotically arrested Huh7 cells (B and C) which were digitally magnified threefold for better visualization.

vations indicated that *NAPO* expression is not lost in nondividing quiescent cells.

Expression of the *NAPO* antigen during cell cycle

We also analyzed the expression pattern of *NAPO* in synchronized cells in order to follow its positivity during different phases of the cell cycle. For this purpose Huh7 cells were treated with nocodazole and mitotic cells were collected by mitotic shake-off and plated onto coverslips. Synchronized Huh7 cells were tested every 4 h for 36 h of culture for both BrdU incorporation and *NAPO* staining. BrdU incorporation was minimal until 16 h after the release from mitotic arrest with a maximum of BrdU incorporation at 24 h, followed by a significant decrease at 36 h (Fig. 4 A). According to BrdU incorporation index, cells at time points before 16 h were evaluated as G1 phase cells, cells between time points 20 and 32 h as S phase cells, and those at time point 36 h as G2 phase cells. Mitotically arrested cells showed a diffusely positive (nuclear and cytoplasmic) *NAPO* staining (Fig. 4 B). *NAPO*-staining pattern was nuclear throughout the cell cycle, at all time points (time points 8, 24, and 36 h are shown in Figs. 4, D, F, and H, respectively). Thus, *NAPO* staining was always positive during the cell cycle, the only noticeable change being a diffuse staining during mitosis, in contrast to strictly nuclear staining in other phases of the cell cycle.

Expression of the *NAPO* antigen in senescent cells

To test whether *NAPO* antigen expression is modified during senescence, MRC-5 cells were grown until passage 40, at which point they remain alive and attached to cell plate, but they stop dividing, a characteristic feature of senescence (Fulder and Holliday, 1975).The senescence is often accompanied by a positive SA-β-gal activity, which is negative in presenescent cells (Dimri et al., 1995). As shown in Fig. 5, in contrast to presenescent MRC-5 cells at passage 18 (Fig. 5 A), senescent MRC-5 cells at passage 40 were positive for SA-β-gal activity (Fig. 5 B). Immunofluorescence data shown in Fig. 5, C and D, indicated that both presenescent and senescent MRC-5 cells were positive for *NAPO* antigen immunoreactivity, demonstrating that *NAPO* expression is not lost in senescent cells.

Our observations demonstrate that *NAPO* is present in living cells in all phases of the cell cycle as well as during senescence and quiescence, getting lost only during apoptosis. When compared with other available apoptosis detection systems, *NAPO* test is highly specific for apoptosis and offers the simplicity of antibody-based assays. The anti-*NAPO* antibody can be used for detection of apoptotic cells under different conditions, such as in situ staining of cells and tissue sections, and for flow cytometry. TUNEL assay (Gavrieli et al., 1992) is widely used for the identification of apoptotic cells, even though it requires several cumbersome experimental steps. As *NAPO* and TUNEL assays provide exclusive nuclear staining of alive and apoptotic cells, respectively, we believe that both assays may be combined for better identification of apoptosis. Moreover, *NAPO* assay may detect apoptotic cells before DNA fragmentation and it does not require special pretreatment of assay samples. *NAPO* may also be used in combination with annexin V staining (Martin et al., 1995). *NAPO* differs from previously identified and antibody-defined apoptosis markers (Grand et al., 1995; Zhang et al., 1996; Hammond et al., 1998; Srinivasan et al., 1998; Leers et al., 1999) by its exclusive loss in apoptotic cells, but not in quiescent, proliferating, senescent, or even mitotic cells. We believe that this antibody will be very helpful for development of simple and easy immunoassays for measurement of apoptosis in both cell lines and tissue samples.

Materials and methods

Monoclonal antibody production

10,000,000 COLO 320 cells were lysed in 2 ml PBS and 0.5 ml of lysate was injected into tail vein of Balb/c mice. 1 mo later, mice were immunized twice more at 1 wk intervals, hybridomas were prepared from splenic cells, and antibody-producing clones were selected as described previously (Ozturk et al., 1989). One of the antibodies of IgG isotype, named anti-*NAPO*, was used for further studies.

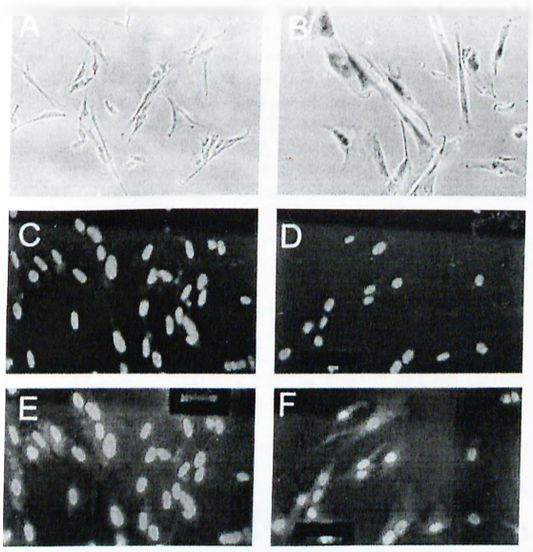

Figure 5. ***NAPO* antigen is positive in senescent cells.** Presenescent (A, C, and E) and senescent (B, D, and F) MRC-5 cells were stained for senescence-associated β-galactosidase activity (A and B), *NAPO* immunoreactivity (C and D), and Hoechst 33258 DNA staining (E and F). Note that senescence-associated β-galactosidase-positive cells are also positive for *NAPO* antigen.

Tissue culture

Huh7, SNU 398, COLO 320, MCF-7, HeLa, U2OS, SW480, A375, 293, MRC-5, COS7, IAR-6, and CHOK-I cells were grown in DME (Biochrome or GIBCO BRL). HC11 was grown in RPMI 1640 (Biological Industries) supplemented with 10 ng/ml EGF (Sigma-Aldrich) and 5 μg/ml insulin (Sigma-Aldrich). Jurkat and LNCaP cells were grown in RPMI 1640. All cells were grown in media supplemented with 10% FCS, 1% nonessential amino acids, 100 μg/ml penicillin/streptomycin at 37°C and 5% CO_2.

Induction of apoptosis

Apoptotic cell death was induced by either serum starvation or treatment with H_2O_2, UV-C, cisplatin, anti-Fas antibody or TNF-α treatment. SNU 398 hepatocellular carcinoma cells were induced in serum-free medium for 3 d and tested for apoptosis. For oxidative stress–induced apoptosis, Huh7 cells were incubated in a culture medium containing 0.1% FCS for 72 h, and treated with freshly prepared 100 μM H_2O_2 for at least 4 h before apoptosis assay. 293 cells were treated with 200 μM H_2O_2 or 100 μM cisplatin. MCF-7, HeLa, U2OS, A375, SW480, LNCaP, Jurkat, and MRC-5 cells were treated with UV-C irradiation (60–120 mJ/cm^2). For physiologically induced apoptosis studies, TNF-α–treated (Boehringer; 50 ng/ml for 72 h) MCF-7 and anti-fas antibody–treated (Upstate Biotechnology; clone CH11, 25 ng/ml for 24 h) Jurkat cells were used.

Induction of quiescence

Presenescent MRC-5 cells (passage 18) were grown to confluency on coverslips and serum starved for 3 d. At the end of 3 d, one set of cells was tested for BrdU labeling and the other set was subjected to immunofluorescence for the expression of the *NAPO* antigen as described later. Asynchronously growing MRC-5 cells of the same passage were used as a control.

Mitotic arrest and cell cycle synchronization

Huh7 cells were grown to 60% confluency and incubated with 50 ng/ml nocodazole (Sigma-Aldrich) for 18 h. Mitotic cells were collected by mitotic shake-off and replated onto coverslips. At indicated time points (between 4 and 36 h), one set of cells was tested for BrdU labeling, and the other set was subjected to immunofluorescence for the expression of the *NAPO* antigen.

Immunoprecipitation

Huh7 cells grown to 70% confluency were starved in DME lacking methionine (Sigma-Aldrich) and labeled with 200 μCi [^{35}S]methionine (Amersham Pharmacia Biotech) per 4 ml medium for 2 h. Cells were scraped in ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris pH 8.0, protease inhibitor cocktail; Roche), and centrifuged at 13,000 rpm at 4°C for 30 min. The cell lysate was incubated with anti-*NAPO* antibody for 2 h and the *NAPO* antigen was immunoprecipitated by using protein G sepharose (Amersham Pharmacia Biotech).

Immunofluorescence

Cells were grown on coverslips and fixed with 100% ice-cold acetone for 1 min or by 4% paraformaldehyde for 1 h. When paraformaldehyde was used, cells were permeabilized for 3 min with 0.1% Triton X-100 in 0.1% sodium citrate. After saturation with 3% BSA in PBS-T (0.1%) for 15 min, fixed cells were incubated with anti-*NAPO* antibody for 1 h at room temperature. FITC-conjugated goat anti–mouse antibody (Dako) was used as the secondary antibody and diluted as recommended by the supplier. The immunofluorescence staining of Huh7 cells for p53 protein was tested using 6B10 monoclonal antibody (Yolcu et al., 2001). Nuclear DNA was visualized by incubation with 3 μg/ml Hoechst 33258 (Sigma-Aldrich) for 5 min in the dark. Cover slips were then rinsed with distilled water, mounted on glass microscopic slides in 50% glycerol, and examined under fluorescent microscope (ZEISS). Jurkat cells were cytospinned (Shandon) for 3 min at 200 rpm before immunofluorescence procedures.

TUNEL and annexin V stainings

The TUNEL assay was performed using an in situ cell death detection kit (Roche), according to manufacturer's recommendations. The annexin V assay was performed by annexin V-PE reagent (PharMingen), according to manufacturer's recommendations, and cells were fixed in ethanol. After TUNEL and annexin V assays, cells were counterstained with Hoechst 33258 and examined as described.

BrdU labeling and identification of S phase cells

For BrdU incorporation, cells were incubated with 30 μM BrdU for 1 h before fixation with ice-cold 70% ethanol for 10 min. After DNA denaturation in 2 N HCl for 20 min, cells were incubated with FITC-conjugated anti-BrdU antibody (Dako) in the dilution as recommended by the supplier, cells were counterstained with Hoechst 33258 and examined as described.

Senescence-associated β-galactosidase assay

MRC-5 cells were grown to passage 40 and subjected to senescence-associated β-galactosidase (SA β-gal) assay, as described by Dimri et al. (1995). Briefly, cells were fixed in 3% formaldehyde for 5 min and incubated with SA β-gal solution (40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM potassium ferro cyanide, 5 mM potassium ferric cyanide, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/ml X-Gal) for up to 12 h, and examined under light microscope.

We thank Rolf I. Carlson for his technical help in antibody production and Gokhan S. Hotamisligil for providing some of the cell lines used here. The present work was supported by a grant from Bilkent University.

The initial stage of this project (monoclonal antibody production) was performed at Massachusetts General Hospital Cancer Center (Charlestown, MA) and supported by a grant (CA-54567) from the National Institutes of Health to M. Ozturk.

Submitted: 6 August 2001
Revised: 14 September 2001
Accepted: 17 October 2001

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