GENETIC ANALYSIS OF SMAD4 GENE IN TGF-β SIGNALLING PATHWAY IN HUMAN LIVER CANCER

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

By
MELİHA BURCU IRMAK
JULY, 1998

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

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Prof. Dr. Mehmet Öztürk

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ABSTRACT

GENETIC ANALYSIS OF SMAD4 GENE IN TGF-β SIGNALLING PATHWAY IN HUMAN LIVER CANCER

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M.S. in Molecular Biology and Genetics
Advisor: Dr. M. Cengiz Yakıcıer
July, 1998

HCC is a multistep genetic disease in which many genomic changes occur as a result of uncontrolled proliferation of hepatocytes. Molecular events leading to HCC is still unclear. Until now, neither an oncogene nor a tumor suppressor gene has been shown to be prefentially altered in HCC. Genetic alterations other than p53, p16, BRCA2 (Breast Carcinoma Associated Protein), M6P/IGFIIR (Mannose 6 Phosphate/ Insulin Like Growth Factor II Receptor), Rb (Retinoblastoma), PRLTS (Platelet Derived Growth Factor Receptor-β-Like Tumor Suppressor Gene), and Tg737 (Candidate polycyctic kidney disease gene) genes remain unknown. TGF-β is a strong inhibitor of hepatocyte proliferation. In HCC and cirrhosis increased levels of TGF-β is observed, so this shows that the presence of high levels of TGF-β does not avoid hepatocyte proliferation. Thus, there may be a disruption in the signalling pathway of TGF-β. The common mediator Smad4 gene, which is among the genes located in TGF-β signalling pathway, is found to be mutated in many cancer types. We decided to do the mutational analysis of Smad4 gene, which is located in the signalling pathway of the hepatocyte antiproliferative factor, TGF-β. Exons 8, 9, 10, and 11 which are in MH2 region, and exon 2 which is in MH1 region of Smad4 is mutationally analysed by SSCP for 35 HCC cases. In the 35 HCC tumors, 5 alterations were observed (14%), 3 of them being in exon 8, one of them being in exon 9a, and the last one being in exon 10 of Smad4 gene. In the samples we tested, no big deletions were observed, but the alterations observed are probably single base changes. Also HCC cell lines namely, HepG2, Hep3B, Huh-7, FOCUS, Mahlavu, and PLC/PRF/5 were checked for their mutations and cell lines other then PLC/PRF/5 were analysed for their mRNA transcription. There were no big deletions or alterations in N- and C- terminals of the cell lines and we have shown mRNA transcription for all cell lines except Hep3B in which PCR has revealed very weak amplification. Our results suggest that Smad4 might be involved in at least a part of primary HCC tumor development.

Key words: HCC, TGF-β, Mutation, Smad4, Cell line, SSCP, PCR.

ÖZET

TGF-β SİNYAL YOLUNDA YER ALAN SMAD4 GENİNİN İNSAN KARACİĞER KANSERİNDE GENETİK ANALİZİ

Meliha Burcu Irmak
Moleküler Biyoloji ve Genetik Bölümü Yüksek Lisans
Tez Yöneticisi: Dr. M. Cengiz Yakıcıer
Temmuz, 1998

Hepatosellüler karsinoma, hepatositlerin kontrolsüz çoğalması sonucu, değişik genetik bozuklukların birikmesiyle oluşan çok basamaklı bir hastalıktır. Hepatosellüler karsinomaya yol açan moleküler olaylar henüz bilinmemektedir. Bu güne kadar, ne belli bir onkogenin, ne de tümör baskılayıcı genin, HCC'ye neden olduğu gösterilememiştir. HCC vakalarında p53, p16, BRCA2, M6P/IGFIIR, Rb, PRLTS, ve Tg737 genlerinde gösterilen mutasyonların dışında gen bozuklukları hala bilinmemektedir. TGF-β, hepatosit çoğalmasını önleyici çok güçlü bir moleküldür. Hepatosellüler karsinoma ve siroz sırasında TGF-β seviyesinde artma gözlenmiştir, buna rağmen hepatositler çoğalmaya devam etmiştir. Bu sebeple, karaciğer kanserlerinde TGF-β sinyal yolunda bir bozukluk olabileceği düşünülebilir. TGF-β sinyal yolunda yer alan genlerin arasında bulunan, ortak aracı, Smad4 geninin çeşitli kanser tiplerinde mute olduğu bulunmuştur. Biz de, hepatosit çoğalmasını engelleyici bir faktör olan TGF-β'nın sinyal yolunda yer alan Smad4 geninin mutasyon analizini yapmaya karar verdik. 35 HCC vakası için, SSCP analizi ile Smad4 geninin, mutasyonların çok yoğun olduğu MH2 bölgesine denk gelen, kodlayan bölgeler 8, 9, 10, ve 11 ile MH1 bölgesine denk gelen kodlayan bölge 2'nin mutasyonları tarandı. 3 tanesi kodlayan bölge 8, 1 tanesi kodlayan bölge 9 ve sonuncusu da kodlayan bölge 10'da olmak üzere, 35 HCC vakasinda 5 tane (14%) değişiklik göründü. Test edilen örneklerde büyük delesyonlara rastlanmadı, ama görülen değişiklikler büyük bir ihtimalle tek baz değişiklikleridir. Bununla birlikte, HCC hücre hatları olan, HepG2, Hep3B, Huh7, FOCUS, Mahlavu, ve PLC/PRF/5 mutasyonları ve PLC/PRF/5 hariç diğer hücre hatları mRNA transkripsiyonu için kontrol edildi. Hücre hatlarının N- ve C-uçlarında büyük delesyon ve değişikliklere rastlanmadı, ve Hep3B dışında bütün hücre hatlarının mRNA yazılımı gösterildi. Elde ettiğimiz sonuçlar Smad4 geninin en azından bazı HCC vakalarının gelişiminde rol oynayabileceğini düşündürmektedir.

Anahtar kelimeler: Hepatosellüler karsinoma, TGF-β, Mutasyon, Smad4, Hücre hattı, SSCP, PCR.

To my family

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ann	Annealing
APS	Ammonium Persulphate
Arg	Arginine
Bisacrylamide	N, N, methylene bis-acrylamide
BMP	Bromephenol blue
bp	Base pair
BRCA2	Breast Carcinoma Associated Protein 2
CMC	Chemical Mismatch Cleavage
DDGE	Denaturing Gradient Gel Electrophoresis
del	Deletion
DPC4	Deleted in Pancreatic Carcinoma 4
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
Dpp	Drosophila gene decapentaplegenic
EDTA	Ethylene Diamine Tetra Acetic Acid
E2F	Elongation Factor 2
f	Familial
Freq	Frequency
G	Glysine
HA	Heteroduplex Analysis
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
IGFII	Insuline-like Growth Factor II
IGFIIR	Insuline-like Growth Factor II Receptor
KD	Kilodalton
L	Leucine
LOH	Loss of Heterozygosity
M	Molar
MAD	Mother Against dpp
MADR	Mother Against dpp Related
MAP	Mitogen Activated Protein
mCi	Millicuri
$MgCl_2$	Magnesium Chloride
MH1	Mad Homology region 1
MH2	Mad Homology region 2
MI	Microsatellite Instability
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messanger Ribonucleic Acid
M6P	Mannose 6 Phosphate
MTSI	Multi Tumor Suppressor I
N-terminal	Amino-terminal

P	Proline
^{33}P-dATP	Phosphate 33- deoxyadenosine three phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	picomol
PRLTS	Platelet Derived Growth Factor Receptor-β-like Tumor Suppressor Gene
PTT	Protein Truncation Test
RB	Retinoblastoma
RNA	Ribonucleic Acid
S	Serine
SAD	Smad Activating Domain
sec	Second
Ser	Serine
Smad	Sma and mad related
SSCP	Single Stranded Conformation Polymorphism
STK	Serine/Threonine Kinase
TAK-1	TGF-β Activated Kinase-1
TBE	Tris-Boric acid-EDTA
temp	Temperature
TGF-β	Transforming Growth Factor-β
TGF-β1	Transforming Growth Factor-β1
TGF-β2	Transforming Growth Factor-β2
TGF-β3	Transforming Growth Factor-β3
Tg737	Candidate Polycystic Kidney Disease Gene
Thr	Threonine
TSG	Tumor Suppressor Gene
TβR I	Transforming Growth Factor-β Receptor I
TβR II	Transforming Growth Factor-β Receptor II
µl	Microliter
µM	Micromolar
µg	Microgram
UV	Ultraviolet

Chapter 1

I. Introduction

I.1. Hepatocellular Carcinoma

Multiple cell types such as hepatocytes, bile duct epithelia, neuroendocrine, and mesodermal cells within the liver may cause primary hepatic tumors. However, the hepatocellular carcinomas (HCC) constitute the great majority of primary liver cancers which derive from hepatocytes. HCC is the 7^{th} most frequent cancer occurring worldwide. However, it is the 4^{th} most common cause of death from cancer. The overall ratio of mortality to incidence is 99% because of its poor prognosis. When compared to other parts of the world, the incidence of HCC is significantly higher in Southern Africa and Southeast Asia. Like many adult tumors, HCC is a multistep disease in which many genomic changes occur as a result of uncontrolled proliferation of hepatocytes. Under normal physiological conditions, adult hepatocytes are nondividing cells. Infact, as a response to a minor cell loss due to aging or apoptosis (programmed cell death), only a minor fraction of hepatocytes appears to undergo cell division. During liver injury, substantial cell death can occur as a result of exposure to viral (i.e., hepatitis B virus,

hepatitis C virus), chemical (i.e., aflatoxin) or even cellular (i.e., iron or copper) factors. Liver injury provokes an increase in the proliferating fraction of hepatocytes. Chronic state of cell death and regeneration is probably the main source of genetic errors which lead to malignant transformation of hepatocytes and probably explains the big time lapse (10s of years) between the first exposure to etiologic factors and the clinical manifestation of HCC (Ding *et al.* 1991[1], Öztürk 1994 [2], Wands *et al.* 1995 [3]).

Chronic hepatitis B virus (HBV) infection is the major etiology of hepatocellular carcinoma. The integration of viral sequences into the host genome is a relatively common event in HCC. However, viral integration is neither necessary nor sufficient for hepatocyte transformation. The genes coding for cyclin A (Wang *et al.* 1990 [4], 1992 [5]), and retinoic acid receptor (Dejean *et al.* 1986 [6]) which are involved in cellular proliferation and cellular differentiation respectively, are the two examples where HBV integration has interrupted the integrity of the genes. In addition to these two genes, the most recent HBV DNA integration characterized occurred in the cellular mevalonate kinase gene (Graef *et al.* 1994 [7], 1995 [8]) which is the key enzyme of cholestrol biosynthesis pathway, and is indirectly involved in the farnesylation of growth-related proteins like the ras oncoprotein. Overexpression of this gene may contribute to the dysregulation of hepatocyte proliferation. These integrations are extremely rare and unique. Another hepatotrop virus, hepatitis C virus (HCV), antibodies and viral RNA have been also detected respectively in the serum and in the liver of the patients with HCC. In contrast to HBV, because of the fact that HCV is an RNA virus, insertion to

host genome is not a mechanism for HCV directed hepatocarcinogenesis (Ding *et al.* 1991[1], Öztürk 1994 [2], Wands *et al.* 1995 [3]).

I.2. Genetics of Hepatocellular Carcinoma

Even though increased expression (H-ras, myc, and IGF-II genes have been observed to be overexpressed in livers of mice, rats and woodchucks) and some mutations of several oncogenes (H-ras is mutated in 30-60% of the spontaneous HCC in B6CFI and C3H mice) are frequently observed in animals, in human HCC, no known oncogene has been shown to be consistently activated by a structural change (Grisham *et al.* 1996 [9]). However, c-myc (Arbuthnol *et al.* 1991 [10]), c-fos (Arbuthnol et al. 1991 [10]), cyclin D1 (Nishida *et al.* 1994 [11]), c-met (Boix *et al.* 1994 [12]), and retinoic acid receptor (Sever *et al.* 1991 [13]) genes has been shown to be amplified in a limited number of cases in human HCC.

The analysis of chromosomal changes in HCC permitted the identification of several loci deleted in HCC, the short arm of chromosome 17 (17p13) being the most frequently deleted one which harbors the p53 gene. It was later reported that "the p53 gene is a frequent target in HCCs". Most of the mutations clustered to a single hotspot and converted an arginine residue at codon 249 of the p53 gene to a serine residue. Worldwide, the presence of codon 249 mutation in HCC's correlated with high risk of exposure to aflatoxins and HBV with high incidence in the countries such as Mozambique, Transkie, China, and Vietnam and with no incidence of codon 249

mutation in other locations such as North America, Europe, the Middle East, and Japan (Even though HBV infection is frequently seen in the Middle East and Japan) (Öztürk 1994 [2]). Even though, p53 mutations are the most frequent genetic abnormalities observed in HCCs, tumors from low aflatoxin exposure areas show infrequent p53 mutations.

In addition to 17p13, other chromosomal arms are found to display loss of heterozygosity (LOH) in more than 20% of HCCs studied (16p, 16q, 5p, 4q, 1p, 5q, 8q, 13q). Chromosomal deletions that occur at low frequency in HCCs (11 p, 10q, 14q, 17q, 4p, 15q) are random deletions which are not probably related to the progression of malignant transformation but related to the increased genomic instability of cancer cells (Öztürk 1994 [2], Grisham *et al.* 1996 [9]).

Recently, another frequently deleted region at the mannose 6-phosphate/insulin-like growth factor receptor II (M6P/IGF-IIR) locus located at chromosome 6q26-27 has been observed, and frequent mutations of this gene were published (Souza *et al.* 1995 [14]). LOH at the chromosome 9p21 (54%) has also been added to the list recently. However, multi tumor suppressor I/p16 (MTSI/p16) gene located at chromosome 9p21 was mutated infrequently in the samples tested (Hui *et al.* 1996 [15], Chaubert *et al.* 1997 [16], Qin *et al.* 1996 [17], Biden *et al.* 1996 [18]). In a study, it is also stated that, p16 in HCC is inactivated predominantly by post-transcriptional regulation rather than by genomic aberrations and lack of transcription (Hui *et al.* 1996 [15]). Another tumor suppressor gene (TSG), breast cancer associated protein 2 (BRCA2) gene which is

located close to the retinoblastoma (RB) gene on chromosome 13q was also found to be mutated in HCCs. Two out of three mutations observed in BRCA2 gene were germline mutations (Katagiri *et al.* 1996 [19]). Also, Rb was shown to have tumor specific small deletions in the remaining corresponding allele of the lost allele (Zhang *et al.* 1994 [20]).

I.3. Transforming Growth Factor-β

In most of the cases, tumor cells develop when normal progenitor cells lose control of signal transduction pathways that regulate responses to soluble growth factors. Some growth factors such as epidermal growth factor and platelet-derived growth factor stimulate the signaling pathways, others lead to inhibition of cell growth presumably through mechanisms that target the cell-cycle-regulated events. Among the growth inhibitory factors, the autocrine/paracrine effectors of the type β transforming growth factors, TGF-β1 TGF-β2, and TGF-β3 are the ones of key interest, TGF-β1 being the most frequently studied one (Alexandrow *et al.* 1995 [21]).

The TGF-β superfamily of cytokines consists of at least 25 different peptides that have been implicated as multifunctional regulators of cell growth, differentiation, and function. On the basis of sequence similarity, this cytokines can be grouped into three, TGF-βs, activins, and bone morphological proteins (BMP) (Brand *et al.* 1996 [22]). TGF-βs control cell fate by regulating the expression of genes encoding cell cycle regulators, differentiation factors, cell adhesion molecules, matrix products, and many other

components that are key determinants of cell phenotype and apoptosis (Attisano *et al.* 1994 [23], Heldin *et al.*1997 [24]).

TGF-β1 is excreted as inactive preproTGF-β1 containing a latency peptide which blocks its binding to its receptors. By proteolytic cleavage of the latency peptide prepro TGF-β1 is activated. This process can be accomplished by plasmin in the presence of transglutaminase when the preproTGF-β1 is bound to the M6P/IGF-IIR through phosphomannosyl residues on the growth factor. Following the cleavage, active ligand can bind to TGF-β receptor II (Souza *et al.* 1996 [25]).

I.4. Transforming Growth Factor-β Signalling

Cellular action of TGF-β family members is initiated by their binding to receptor complexes of type I (TβRI) and type II (TβRII) with intrinsic serine/threonine kinase (STK) activity. There is also a third receptor called type III (betaglycan). It binds to all three TGF-β isoforms with high affinity but has no apparent signaling function. Even though it serves an auxiliary role by presenting TGF-β to TβRII, the cells lacking betaglycan can respond to TGF-β. TβRI and TβRII are similar in structure with small cysteine-rich extracellular regions and intracellular parts consisting mainly of the STK domains. The ligand binding domain contains six to nine variable cysteines and an invariant cysteine box close to the transmembrane domain in all STK receptors. Positioning of cysteine residues determines ligand binding and is conserved in receptors with the same ligand specificity. Although TβRI is quite similar to TβRII, it has three

distinguishing features. The extracellular ligand binding domains of all TβRIs contain seven cysteine residues at nearly the same positions and the cysteine box. At the carboxyl terminus, only a very short nonkinase sequence is present lacking serine/threonine (ser/thr) residues. Most important, the kinase domain is immediately preceded by a type I receptor-specific domain that is rich in serine (SGSGSGLP, the GS box) (Brand *et al.* 1996 [22], Heldin *et al.* 1997 [24]).

Receptor activation occurs upon binding of the ligand to TβRII. Ligand-sitimulated autophophorylation of tyrosine kinase receptors is not observed in this ser/thr kinase receptor. TβRII is constitutively autophosphorylated. Recently, it was reported that phosphorylation of Ser213 and Ser409 is required for TβRII activity, whereas phosphorylation of Ser416 inhibits the receptor signaling and autophosphorylation on tyrosine residues, as well as on serine and threonine residues occur, even though the importance of tyrosine autophoshorylation remains to be determined. Unlike TβRII, TβRI is unable to recognize and bind to the free ligand, TGF-β, in the free medium. However, it can recognize ligand-bound type II receptor forming an oligomeric complex, a heterotetramer consisting of two TβRI and two TβRII, shown by the analysis of ^{125}I-labelled TGF-β1 crosslinked to its receptor. This complex formation is strongly correlated with the signaling process. Cells defective in one of the two receptors are refractory to any TGF-β effects. Phosphorylation of receptor I occurs upon its recruitment into the ligand-TβRII complex and it is a central event in the signal transduction pathway of receptor kinases. TβRI phosphorylation occurs in a cluster of

serine (Ser165) and threonine residues on the GS domain, a highly conserved region next to the N-terminus of the kinase domain in all type I receptors (Heldin *et al.* 1997 [24], Eppert *et al.* 1996 [26], Massague 1996 [27], Wrana *et al.* 1994 [28]). The phosphorylation process is likely to be catalyzed by the type II receptor which can be concluded from the experiments that compare the phosphorylation state of these receptors when cotransfected with wild-type or kinase-defective mutants of each other such as when TβRI was coexpressed with the kinase defective TβRII, it bound ligand and formed a complex with mutant receptor, but did not become phosphorylated (Wrana *et al.* 1994 [28]). The assembly of the receptor complex is triggered by ligand binding, but the complex is also stabilized by direct interaction between the cytoplasmic parts of the receptors. All these things predict that the type II and type I receptors act in sequence, which is supported by the finding that a constitutively active type I receptor harboring a mutation that results in an aspartate or glutamic acid in GS domain endows TβRI with elevated kinase activity which makes it exert TGF-β signals in the absence of TβRII and the ligand (Heldin *et al.* 1997 [24], Souza *et al.* 1996 [25]). In short, in the case of TGF-β signaling pathway, receptor activation occurs upon binding of ligand to TβRII which then recruits and phoshorylates TβRI that propagates the signal to downstream targets. A general model of TGF-β/SMAD signalling is provided in Figure A.1 in Appendix A.

Nowadays, more is known about the nuclear targets for TGF-β cascades involved in both growth control and gene transcription. The RB, $p15^{INK4a}$, $p21^{CipI}$, and $p27^{KipI}$ are found to be implicated in TGF-β signaling. Proliferation of eukaryotic cells is regulated by the balance of positive and negative cell cycle regulators-cyclins, cyclin dependent kinases

(Cdks), Cdk inhibitors, and cyclin-activating kinases. Rb, a tumor suppressor 'pocket protein', is a key substrate for G1 Cdks. It complexes with E2F transcription factors which regulate DNA polymerase-alpha, dihydrofolate reductase, and cdc2 expression that are required for DNA synthesis. Rb phosphorylation is increased upon activation of Cdks by mitogenic growth factors which results in releasing of E2F , sequential binding of E2F to differing pocket proteins and ultimately cell-cycle progression. For many cell types TGF-β acts as an anti-mitogen. It has been reported that, in TGF-β treated cells, the activity (p27) or abundance (p15 and p21) of several key Cdk inhibitors is increased. Conversely, TGF-β suppresses the synthesis of the late G1- and S-phase cyclin E and A along with Cdk2 and Cdk4 resulting in the control of Rb phosphorylation and acting as secondary adaptations to the growth-arrested state. This is the pivotal event to the antiproliferative pathway since deregulated E2F expression can overcome growth suppression by TGF-β. The events that couple receptor activation to increased expression of Cdk inhibitors and reduced cyclin/Cdk expression still remains undefined (Alexandrow *et al.* 1995 [21], Brand *et al.* 1996 [22], Ravitz *et al.* 1997 [29]).

I.5. Smads

Until today, very little was known about TGF-β signal transduction from the cell membrane to the nucleus. The first clue came from the studies with Drosophila (fruit fly) resulting in the identification of some components of the pathway. Recently, a cytoplasmic candidate interacting with Dpp (Drosophila gene decapentaplegenic that encodes a fly homologue of BMP) has been identified and called mothers against dpp, or

MAD. Later it has been shown that the Drosophila MAD protein is present in the cytoplasm and is necessary for Dpp, which is a Drosophila TGF-β family member, mediated response. Shortly after that, MAD related genes (MADR=Smad) have been described in Xenopous (Xmad1 and Xmad2) and Caenorhabditis elegans (sma-2, sma-3, and sma-4 genes) (Eppert *et al.* 1996 [26], Lagna *et al.* 1996 [30], Baker *et al.* 1997 [31]).

A recent paper describes the identification of a novel candidate tumor suppressor gene in human, Deleted in Pancreatic Cancer (DPC4=Smad4) with sequence homology to MAD. Up to 85% similarity to these proteins was observed in exons 1, 2, and 11 and up to 75% similarity was seen in exon 8 (Hahn *et al.* 1996 [32]). Further other homologues of MAD (JV18-1, JV15-1, JV15-2, JV5-1, and JV4-1) were identified based on homology in the expressed sequence databases (Riggins *et al.* 1996 [33]). Not to be confused in terminology, all the human MADRs are named as Smad (Smad1, Smad2, Smad3, Smad4, and Smad5). Very recently identification of Smad6, Smad7, and Smad8 have also been described (Derynck *et al.* 1996 [34], Imamura *et al.* 1997 [35], Nakao *et al.* 1997 [36], Chen *et al.* 1997 [37]).

Smad family genes encode proteins of about 42KD-60KD and these proteins are about 450 amino acids long. They show great homology at the amino and the carboxyl terminals termed Mad-homology domains, MH1 and MH2, respectively, which are linked by a proline-rich linker sequence. However, they have no specific protein motif that gives

clue to their function. Thus, it is still unclear how Smad proteins elicit information (Heldin *et al.* 1997 [24], Massague 1996 [27], Niehrs *et al.* 1996 [38]) . Analysis of Smad1 suggests that the protein resides predominantly in the cytoplasm in unstimulated cells, but accumulates in the nucleus upon activation of BMP signaling pathways. So controlling of Smad retention in the cytoplasm can be an attractive hypothesis for the control of TGF-β signalling pathway as this is the case recurring in other signal transduction pathways (Niehrs *et al.* 1996 [38]).

Members of the Smad family have different roles in signaling which depends on the type of the ligand (TGF-β, BMP, or activin) and their phosphorylation. They can be categorized into two depending on their phosphorylation fashion as pathway-restricted or common mediator Smads. Smad1, Smad2, Smad3, Smad5, and Smad8 interact with and become phosphorylated by specific type I serine/threonine kinase receptors and thereby act in a pathway-restricted manner. Activation of these Smads is triggered by their phosphorylation by TβRI at SSXS (Ser-Ser-X-Ser) motif at their most C-terminal regions. Upon phosphorylation , they are released from the receptor (Wrana *et al.* 1997 [39], Whitman 1997 [40]). On the other hand, Smad4, which is distantly related to Smad2 and Smad3, differs in the mode of action. Smad4 does not enter into contact with TβRI. Smad4 is not phosphorylated by TβRI, since it lacks the C-terminal SSXS motif. However, Smad4 was found to be constitutively phosphorylated in Mv1Lu cells, and the phosphorylation levels remained unchanged upon TGF-β1 stimulation. The phosphorylation of Smad4 has been reported to increase after activin stimulation, but the functional importance of this remains to be determined (Lagna *et al.* 1996 [30]). Upon

ligand stimulation and phosphorylation of pathway-restricted Smads, Smad4 forms hetero-oligomeric complex with them (Wrana *et al* 1997 [39], Whitman 1997 [40]). In mammalian cells, Smad4 forms hetero-oligomers with Smad2 and Smad3 after activation of TGF-β or activin type I receptors, whereas it forms complexes with Smad1 and possibly with Smad5 after activation of BMP type I receptors (Lagna *et al.* 1996 [30], Baker *et al.* 1997 [31]). It has been reported that a possible candidate for an activating kinase of Smad4 is TGF-β activated kinase, TAK-1, which is a mitogen activated protein (MAP) kinase kinase homologue. Interestingly, both TAK-1 and Smad4 were shown to act in both TGF-β and BMP signalling pathway (Lagna *et al.* 1996 [30], Nakao *et al.* 1997 [41]).

Very recently identified inhibitory Smads, Smad6 and Smad7, lack carboxyl-terminal phosphorylation site like Smad4, but unlike Smad4, they interact with type I receptors and probably more stable than pathway restricted Smads. This may be the reason of competitive inhibition of the Smad binding site on the type I receptor. Since they are not phosphorylated, inhibitory Smads are not released from the receptor occupying the binding site for pathway-restricted Smads (Whitman 1997 [40], Wu *et al.* 1997 [42]).

In the case of TGF-β-mediated Smad activation, Smad2, Smad3, and Smad4 exist as homomeric complexes in the absence of ligand. Upon activation of receptors and their phosphorylation, the three Smads assemble in a common complex, or possibly in several types of complexes, of which the stoichiometry between the components is unknown (Nakao *et al.* 1997 [41]). Smad4 plays a central role in signalling by different TGF-β

related factors and receptor systems, distinct from the other Smads. It synergizes with Smad1, Smad2, and Smad3 depending on the signal, while Smad1, Smad2, and Smad3 do not synergize with each other to induce gene expression. In addition, the fact that it does not associate with the receptor and it does not become phosphorylated by the receptor is consistent with its central function in the signaling pathway (Wu *et al.* 1997 [42]). It has been pointed out that Smad phosphorylation is important in homomerization and heteromerization of the Smads (Whitman 1997 [40], Nakao *et al.* 1997 [41]). It has been reported that the effector function of Smad4 is located in the conserved C-terminal domain and is inhibited by its N-terminal domain. This inhibition is achieved by the interaction of the N-terminal domain with the C-terminal domain which prevents the association with other Smads. It was observed that this inhibitory function is increased in tumor-derived forms of Smad4, having mutant Arg100 (exon 2) in their N-domains with higher affinity to their respective C-domains. However, still it is the C-terminal domain that harbors most of the mutations in Smad4 resulting in Smad4 which is not able to homomerize or heteromerize. The three dimensional structure of the Smad4 C-terminal domain predicts that some of these mutations destabilize the core structure, others disrupt the C-domain homomerization, and others disrupt a putative Smad4-Smad2 interface (Hata *et al.* 1997 [43], Liu *et al.* 1996 [44], Baker *et al.* 1996 [45]). The crystal structure of C-terminal domain of Smad4 reveals that it forms a crystallographic trimer through a conserved pro-protein interface, and the tumor derived missense mutations map to this region of the protein (Shi *et al.* 1997 [46]). Separate activation and ligand-response domains within Smad4 have been characterized using a functional assay based on the restoration of TGF-β responsiveness in a Smad4 null cell line by transient transfection

with Smad4. It is shown that amino acid residues 274-321 in Smad4 is necessary for transducing signal downstream from the receptor and thus is referred as Smad activating domain (SAD). SAD may be unique to Smad4 in Smad family members. The ligand response domain of Smad4 is located to N-terminal domain of the gene and is thought to obscure the SAD located at the C-terminal of the middle linker region in the absence of ligand either by direct blockage of this region, or by conformational interference. However, following ligand activation, SAD is exposed most probably due to unfolding of the molecular structure to interact with other Smads (Caestecker *et al.* 1997 [47]).

After ligand activation, Smad4, Smad2, and possibly Smad3 translocate to the nucleus as a heteromeric complex which contains trimers of each and perform their transcriptional activation role there by specifically interacting with DNA-binding proteins or directly with DNA. One example of transcriptional activation by Smad complex is the activation of Mix.2 gene (immediate early response gene) in Xenopous which is activin sensitive. This activation occurs consequent to Smad2-Smad4 and FAST-1 (DNA binding protein) complex binding to activin responsive element in the promoter of Mix.2 gene (Chen *et al.* 1996 [48], 1997 [49]). Another example is that Drosophila Mad binds to DNA and directly mediated activation of an enhancer within vestigial wing-patterning gene in cells across the entire developing wing blade (Kim *et al.* 1997 [50]). A general model of TGF-β/SMAD signalling is provided in Figure A.1 in Appendix A.

Consequently, the nature of the signals that controls Smad protein localization is still unclear, but the results suggest that Smad proteins can function in the pathway to

transmit signals from the cytoplasm into the nucleus. There are many potential targets for Smads in the nucleus, and the possibility that Smad proteins are transcription factors is suggested by the observation that the protein can function as a transcriptional activator, when fused to a heterologous DNA-binding domain (Niehres *et al.* 1996 [38]).

I.6. Aim

Taking all the information above into consideration, what may be the relation between HCC development and TGF-β signaling pathway and what may be the reason for choosing Smad4 for performing mutational analysis in HCC?

As it has been discussed above, HCC usually develops after a 20-50 year period of HBV infection, often subsequent to cirrhosis. This long latent period before the clinical symptoms indicates they are the result of a multistep process. Several studies have been directed toward the identification of common genetic alterations. Frequent deletions in at least ten chromosomal locations might indicate that there are at least ten different genes involved in HCC. There are few tumor suppressor genes known to be mutated and inactivated in human HCC. Genetic alterations other than p53 (Öztürk 1994 [2], Ünsal *et al.* 1994 [51], Puisieux & Öztürk 1997 [52], Kishimoto *et al.* 1997 [53]), p16 (Hui *et al.* 1996 [15], Chaubert *et al.* 1997 [16], Qin *et al.* 1996 [17], Biden *et al.* 1996 [18]), BRCA2 (Katagiri *et al* 1996 [19]), M6P/IGF-IIR (Souza *et al.* 1995 [14]), RB (Zhang *et al* 1994 [20], Puisieux *et al.* 1993 [54]), PRLTS (Fujiwara *et al.* 1995 [55]), and Tg737

(Isfort *et al.* 1997 [56]) remain highly unknown. The alterations reported in these genes in HCC cases are summarized in the table below, Table 1.1.

Gene	Alteration	Freq.	Reference
p16	GC deletion Homozygous deletion G→C No protein expression Hemizygous germ-line mutation	8/24 2/24 1/23 11/32 4/26	Qin *et al.*, 1996 Biden *et al.*, 1997 Hui *et al.*, 1996 Chaubert *et al.*, 1997
Rb	Loss of heterozygosity (LOH) No protein expression No protein expression Small deletion	9/21 8/9 of LOH 3/12 of non-LOH 2/9 of LOH	Zhang *et al.*, 1994
BRCA2	6 base pair deletion in intron Germ-line mutation • G→T • A→C	1/60 2/60	Katagiri *et al.*, 1996
M6P/IGF2R	LOH C:G→A:T	70/100 25/100 of LOH	Souza *et al.*, 1995
p53	Point mutation • High aflatoxin • Low aflatoxin	 55% 28%	Puisieux & Öztürk., 1997
PRLTS	Val302Ala TT deletion CTTTC175CTG	2/48	Fujiwara *et al.*, 1995
Tg737	Deletion of exons 3, 5, 14, 22	4/11	Isfort *et al.*, 1997

Table 1.1: HCC Mutations

TGF-β1 is a strong inhibitor of hepatocyte proliferation and it has been proposed as guardian of quiescence in liver. Certain data indicate that TGF-β1 can actively suppress tumor formation in vitro (Fausto *et al.* 1995 [57]). Loss of growth inhibitory response to

TGF-β at the cellular level is probably an important step in malign progression. Recently, several lines of evidence indicate that TGF-β receptors and proteins involved in signaling by TGF-βs act as tumor suppressor genes. The prediction was born out with the finding that the TβRII is inactivated by mutation in $(A)_{10}$ repeat in colon cancer with microsatellite instability (MI) (Markowitz *et al.* 1995 [58], *Akiyoma et al.* 1996 [59], Lu *et al.* 1995 [60]). This idea was further supported by the mutations in this gene in gastrointestinal cancer (Renault *et al.* 1996 [61]). When we have a look at other genes in the pathway, we see that IGF-IIR is inactivated by mutation in $(G)_8$ repeat in gastrointestinal tumors with MI (Souza *et al.* 1996 [25]), Smad2 is mutated in lung (Uchida *et al.* 1996 [62]), and colon cancers (Eppert *et al.* 1996 [26], Riggins *et al.* 1996 [33]), and common mediator Smad, Smad4, is mutated in colon (MacGrogan *et al.* 1997 [63], Thiagalingam *et al.* 1996 [64]), pancreas (Hahn *et al.* 1996 [32]), lung (*Nagatake et al.* 1996 [65]), biliary tract (Hahn *et al.* 1998 [66]), and juvenile polyposis (Howe *et al.* 1998 [67]). The list of the published mutations, and alterations in Smad4 gene in various tumor types are summarized in Table 1.2.

Tumor	Codon (Exon)	Alteration	Freq.	Reference
Biliary tract Biliary tract Biliary tract Biliary tract Biliary tract	343(8) 433(9) 497(11) 502(11) 523(11)	TCA→TGA Ser→stop GCA→ACA Ala→Thr CGC→CAC Arg→His AGG→GGG Arg→Gly TGC→TGG Cys→Trp	5/32	Hahn et al., 1998
Lung Lung Lung	202-203(4) 420(9) 441(10)	2 base pair deletion (CC) frameshift CGT→CAT Arg→His CGT→CCT Arg→Pro	3/42	Nagatake et al., 1996
Colorectal	64(1)	GCA→GTA Gly→Val	1/9	MacGrogan et al., 1997
Pancreatic Pancreatic Pancreatic Pancreatic Pancreatic Pancreatic	358(8) 412(9) 483(10) 493(11) 515(11) 516-518(11)	GGA→TGA Gly→stop TAC→TAG Tyr→stop AGT→AAT aberrant splicing GAT→CAT Asp→His AGA→TGA Arg→stop CAGAGCATC→C frameshift	6/27	Hahn et al., 1996
Neuroblastoma	-	Altered protein expression	6/32	Kog et al., 1997
Colon Colon Colon Colon Colon Colon	1-11 1-11 358(8) 130(2) 361(8) 370(8)	Homozygous deletion Homozygous deletion TCTGGA→TTCCTGA Gly→stop C→T Pro→Ser C→T Arg→Cys T→A Val→Asp	6/100	Thiagalingam et al., 1996
Juvenile polyposis Juvenile polyposis Juvenile polyposis Juvenile polyposis Juvenile polyposis	414-416(9) 414-416(9) 414-416(9) 348(8) 229-231(5)	4 base pair deletion 4 base pair deletion 4 base pair deletion 2 base pair deletion 1 base pair insertion	5/9 3/5(f) 2/5(s)	Howe et al., 1998

Table 1.2: Smad4 Mutations and Alterations in Tumors

Breast, ovarian, pancreas (Schuttle *et al.* 1996 [68]), stomach (Nishizuka *et al.* 1997 [69]), head and neck squamous cell (Kim *et al.* 1996 [70]), and colon (MacGrogan *et al.* 1997 [63]) carcinoma cell lines also show alterations in Smad4 gene. The below table, Table 1.3, lists the mutations, and altareations found in those cell lines.

Cell line	Tumor	Codon (Exon)	Alteration	Freq.	Reference
CACO-2	Colon	351(8)	GAT→CAT	3/12	Macgrogan et al., 1997
COLO205	Colon	1-4	Homozygous deletion		
SW403	Colon	10-11	Homozygous deletion		
MNK-45	Stomach		Homozygous deletion	1/5	Nishizuka et al., 1997
LAN-5	Neuroblastoma		Altered protein expression		Kong et al., 1997
CHP-134	Neuroblastoma		Altered protein expression		
LAN-1	Neuroblastoma		Altered protein expression		
LAN-2	Neuroblastoma		Altered protein expression		
STBN-1	Neuroblastoma		Altered protein expression		
STBN-8	Neuroblastoma		Altered protein expression		
UMSCC22A	Head & Neck Squamous Cell	526(11)	GAA→TAA Glu→stop	2/16	Kim et al., 1996
UMSCC22B	Head & Neck Squamous Cell	526(11)	GAA→TAA Glu→stop		
MDA-MB468	Breast		Homozygous deletion	1/8	Schutte et al., 1996
SW626	Ovarian	351(8)	GAT→CAT Asp→His	1/8	
Colo357	Pancreas	1-4	Homozygous deletion	4/8	
AsPc1	Pancreas	100(2)	AGG→ACG Arg→Thr		
Capan1	Pancreas	343(8)	Ser→stop		
MX36	Pancreas	343(8)	Frameshift		

Table 1.3: Cell Line Mutation and Alterations of Smad4

Also 3' untranslated region (UTR) nucleotide substitution in esophageal squamous cell carcinoma (Barrett *et al.* 1996 [71], Maesawa *et al.* 1997 [72]), and altered expression in neuroblastoma (Kong *et al.* 1997 [73]), in Smad4 gene have been observed.

Eventhough, there is a recent study for the mutational analysis of other Smads in TGF-β signalling pathway, no mutation was characterized for Smad1, Smad3, Smad5, and Smad6 (Riggins *et al.* 1997 [74]).

In HCC, decreased levels of TβRII and IGF-IIR mRNA and protein levels have been observed (Sue *et al.* 1995 [75]). Mutations of IGF-IIR gene in HCC was also published (Souza *et al.* 1995 [14]). These alterations may result in the disruption of TGF-β signaling pathway. Depending on the type and the region of the mutation homomerization or heteromerization of the Smad family members is prevented, thus they can not oligomerize and translocate to the nucleus to transactivate the target gene. As it is discussed before, cancer cells lose responsiveness to TGF-β which is an antiproliferative agent. Even though increased levels of TGF-β has been observed in liver cancers and in cirrhosis, cellular proliferation occurs which can be explained by the possibility of disruption of the genes in the TGF-β signalling pathway.

Smad4, which is a common mediator Smad, is shown to be mutated in many cancer types. Interestingly, there is no study concerning genetic analysis of recently identified Smad4 gene in HCCs has been performed. Consequently, we decided to do the mutational analysis of Smad4 gene in human HCCs.

Smad4 gene consists of 11 exons and codes for 552 amino acids long protein. As it has been discussed before the mutations are mostly localized to MH1 and MH2 regions. Comparing the exons based on the number of the mutations, exon8 in MH2 region comes

first. It is followed by exon 11, exon 9, and exon 10 which are all located in MH2 region. Then comes exon 2 in MH1 region. Finally other exons in Smad4 gene namely exon 1, 4, and 5 take place. In correlation with this data, we decided to concentrate on the exons located in MH2 region of the gene, exons 8, 9, 10, and 11, and exon 2 which is in MH1 region. The structure of Smad4 gene is provided in Figure A.2 in Appendix A.

Chapter 2

II. Materials and Methods

II.1. Materials

II.1.1. Samples

We investigated 35 tumor DNAs that were obtained from hepatocellular carcinoma patients living in South Africa, Mozambique, China, Japan, and Germany. These tumors were tested for their p53 gene mutation and HBV status which is summarized in Table 2.1. These DNA samples were studied in France before, so no DNA extraction was performed by us. We both had the tumor DNAs, which were isolated from the liver tissue of the patients, and the normal DNA of the patients, which were isolated from the normal part of the liver of the patients.

Tumors	p53	HBV
1. T8	Wild type	+
2. T12	Wild type	-
3. T15	Codon 249 Mutation	-
4. T17	Wild type	-
5. T19	Wild type	-
6. T21	Wild type (Exon 5-7)	+
7. T27	Codon 249 Mutation	+
8. T29	Codon 249 Mutation	-
9. T31	Wild type	+
10. T33	Wild type	+
11. T35	Wild type	+
12. T37	Codon 249 Mutation	+
13. T39	Wild type	+
14. T43	Codon 286-8 base pair deletion	+
15. T47	Codon 157 Mutation	+
16. T49	Wild type	+
17. T51	Wild type	+
18. T53	Wild type	+
19. T73	Wild type (Exon 7-9)	+
20. T80	Wild type	+
21. C2	Wild type	+
22. C3	Wild type	+
23. C6	Wild type	+
24. C9	Wild type	+
25. C10	Codon 249 Mutation	+
26. C12	Wild type	+
27. C15	Wild type	+
28. C23	Wild type	+
29. J1T		
30. J3T		
31. J4T		
32. J8T		
33. J9T		
34. J10T		
35. G5	Wild type (Exon 5-7)	+

Table 2.1: p53 Gene Mutations and HBV Status of 35 Tumor Samples (Ünsal *et al.*, 1994)

We also investigated six hepatocellular cell lines for the mutations and the trancription of Smad4 gene. The examined cell lines are HepG2 (Aden et al., 1979 [76]), Hep3B (Aden

et al., 1979 [76]), Huh-7 (Nakabayashi et al., 1982 [77]), FOCUS (He et al., 1984 [78]), Mahlavu (Alexander et al., 1984 [79]), and PLC/PRF/5 (Alexander et al., 1976 [80]). The information about the cell lines are given in Table 2.2.

Name	Origin	P53	HBV
HepG2	Hepatoblastoma	Wild type	-
Hep3B	HCC	Deleted	+
Huh-7	HCC	No gross change	-
FOCUS	HCC	Deleted	+
Mahlavu	HCC	No gross change	-
PLC/PRF/5	HCC	No gross change	+

Table 2.2: The origins, p53 Gene, and HCV Integration Status of the Cell Lines

II.1.2. Solutions

Tris-borate	0.5X:0.045 M Tris-borate (TBE), 0.001 M EDTA
Loading Buffer	6X:0.25% bromphenol blue 30% glycerol in water
Ethidium bromide	10 mg/ml in water (stock solution), 0.5 μg/ml (working solution)
SSCP loading buffer	95% formamide, 0.25% BPB bromphenol blue
Acrylamide-bisacrylamide	49.5% acrylamide and 0,5 % N-N-methylene bis-acrylamide

All the solutions are prepared according to the procedures of Maniatis

II.2. Methods

II.2.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed for the amplification of the 5 exons of Smad4 gene and cell lines, and full length cDNAs of hepatoma cell lines. It is an in vitro enzymatic method for the amplification of the sequence of interest.

PCR is based on three main steps and these three steps are called a "cycle". The cycle involves a denaturation step, in which template DNA is denatured, an annealing step, during which primers which are short oligonucleotides complementary to the sequence to be amplified, are allowed to bind to the template DNA and a polymerization step, during which a thermostable enzyme uses deoxyribonucleotide three phosphates to synthesize the complementary strands of the strands the primers annealed in 5' to 3' direction.

II.2.1.1. Procedure

PCR for the exons of Smad4 gene was performed in 25 µl working mixture containing 0.005 mCi ^{33}P-dATP, 1.5 mM $MgCl_2$, 40 µM dNTP, 20 pmol forward and 20 pmol reverse primer, 2.5 µl 10X buffer and 1.5 unit Taq polymerase and the mixture was completed to 25 µl with autoclaved ddH_2O. The conditions for the amplification of the exons of the cell lines were the same.

The samples were initially denatured at 95 °C (3 min). Then 30 cycles of denaturation at 95 °C (30 sec), annealing at 56 or 57 °C (30 min), and extension at 72 °C (45 sec) follows the initial denaturation step. Lastly, a final extension at 72 °C (5 min) completes the reaction.

Full length cDNAs of the hepatoma cell lines are amplified by PCR in a 25 µl working solution containing 1.5 mM $MgCl_2$, 400 µM dNTP, 20 pmol forward primer, 20 pmol reverse primer, 2.5 µl 10X buffer, and 1.5 unit Taq polymerase.. The mixture is completed to 25 µl with autoclaved ddH_2O.

Before performing radioactive PCR, the PCR conditions were set up nonradioactively and the amplification products were checked with 1.5% agarose gel, and visualized with ethidium bromide staining under UV.

Perkin Elmer thermal cycler model 9600 was used for the amplification of the exons. The primers have been synthesized in house using Beckman 1000M oligosynthesizer. The primer sequences, their anneealing temperatures, the length of the amplified DNA sequence and the exon numbers are given in Table 2.2. The primers used for the amplification of full length cDNA of the hepatoma cell lines are given in Table 2.3.

Primer Sequence	Ann. temp	Exon	Length	Reference
F TTCTAGGTGGCTGGTCGGAA R CAGGTGATACAACTCGTTCG	56°C	2	175 bp	Designed by us
F TGTTTTGGGTGCATTACATTTC R CAATTTTTTAAAGTAACTATCTGA	56°C	8	344 bp	Moskaluk et al., 1996
F TATTAAGCATGCTATACAATCTG R CTGTCTAAGTAGTAACTCTG	57°C	9a	194 bp	Hahn et al., 1998
F CAAAGGTGTGCAGTTGGAATG R CTTCCACCCAGATTTCAATTC	57°C	9b	234 bp	Hahn et al., 1998
F GAATTTTCTTTATGAACTCATAG R TTTAAAAAAGAATGAAAAGCATAC	57°C	10	213 bp	Hahn et al., 1998
F CTGATGTCTTCCAAACTCTTTTCTG R TGTATTTTGTAGTCCACCATC	57°C	11	299 bp	Hahn et al., 1998

Table 2.3 : The List of the Primers Used for Tumors

Primer Sequence	Ann temp	Length	Reference
F AAGCGGATCCGCTTCAGAAATTGGAGA R AAGCAAGCTTCCATCCTGATAAGGTTAAGGG	55 °C	1769 bp	Designed by us

Table 2.4: The List of cDNA Primers

II.2.2. Single Stranded Conformation Polymorphism Analysis

Single stranded conformation polymorphism (SSCP) analysis is used as a mutation detection technique while investigating Smad4 gene.

This technique is based on denaturation of the PCR product and running each sample, which is now composed of the denatured single strands, on vertical electrophoresis side

by side through a non-denaturing polyacrylamide gel. The idea behind denaturing the amplified sequence is that, the two single strands of the DNA molecule assume a three dimensional conformation which is dependent on the primary sequence. The single strands migrate according to the conformation they acquire. In the case of a sequence difference, differential migration of the single strands is expected when compared to the migration of the single strands of the normal DNA sequence.

II.2.2.1. Procedure

In order to prepare the PCR products for SSCP analysis, 8 µl of SSCP buffer containing formamide is mixed with 2 µl of PCR product and denatured at 96 °C for 2 min in heat block. Formamide avoids renaturation of the denaturated single strands. Denatured sample is then kept in ice for 5 min before SSCP analysis which will be observed by polyacrylamide gel electrophoresis.

II.2.3. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis is a high resolution technique used to observe the DNA bands by using different staining methods, such as, ethidum bromide, sybrgreen and silver staining or autoradiography. Due to the negative charges of the phosphates of the DNA molecule, upon voltage application DNA migrates from negative to the positive pole of the gel through the porous structure of the polyacrylamide gel.

II.2.3.1. Procedure

10% 100 ml polyacrylamide gel solution, containing 20 ml 49.5:0.5 acrylamide-bisacrylamide, 10 ml 10XTBE, and 10 ml 10% glycerol is prepared, filtered, and degassed. Then, 500 µl 10% APS and 50 µl TEMED, is added to the mixture. After filtering and degasing it, it is poured to EC160 vertical gel electrophorase. Following 2 hours of polymerization and 30 min of pre-run at 50 watt in cold room (5 °C), 10 µl of formamide and denatured sample mixture is loaded and run at 50 watt for 11 to 14 hours depending on the conditions set for each exon.

II.2.4. Gel Drying

The gel is mounted on a 3M Wattman paper, covered with stretch film, and dried for two hours at 80 °C with a gel drier in the presence of vacuum.

II.2.5. Autoradiography

A film is put over the dried gel, and together they are put in a cassette with intensifier for exposure during 3 days. Following the exposure time, the film is developed with a developer in a dark room. In the case of faint signals, the same procedure is repeated for a longer time.

II.2.6. Restriction Enzyme Digestion

Restriction endonuclease, MnlI is used to detect the arg100thr (AGG→ACG) mutation in exon 2 and the recognition site of the enzyme is shown in Table 2.4. If there is no mutation in the exon, the enzyme digests at the given sequence by recognizing the GG sequence of exon 2 which is at the 3' recognition site of the enzyme. However, in the case of mutation, it can not recognize the base change from G to C, thus can not digest the sequence.

Restriction Endonuclease	Recognition site
Mnl1	5'...CCTC$(N)_7$'...3' 3'...GGAG$(N)_6$'...5'

Table 2.5: Restriction Enzyme Cutting Sites

Chapter 3

III. Results

We sucsessfully amplified the exons 2, 8, 9, 10, and 11 by PCR, and performed radioactive SSCP analysis to all 35 HCC samples for each exon.

In SSCP, after exposure and development of the film, in most of the cases, we were able to detect two bands which are close to each other, and another third band which is far from the other two bands. With the help of a control nondenatured DNA sample, we were able to conclude that the lower band corresponds to the double-stranded DNA, and the other closely migrated bands correspond to denatured single strands.

In the gels with longer migration time to increase the resolution, the bands of double-stranded DNA were not observed since they migrate faster than the single strands.

In order to test our gel conditions, we performed SSCP analysis for the p53 gene codon 248-249 mutations. We were able to observe very clear shifts in the mutant samples compared to their wild-types as can be seen from Figure 3.1.

The two upper bands correspond to single strands, and the lower bands, which migrated the same in both the wild type and the mutant p53 correspond to the nondenatured double strand of the amplified DNA.

wt-p53 and mut-p53 Analysis in SSCP

Figure 3.1: SSCP Analysis of p53 Gene Codon 248-249 Mutations.

In most of the cases, no alterations were observed following SSCP analysis as can be seen from Figure 3.2.

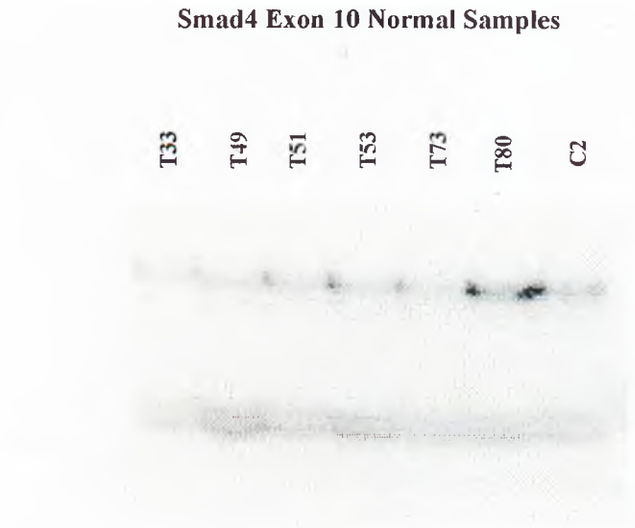


Figure 3.2: SSCP Analysis of Exon 10 Tumor DNAs with No Alteration

In the cases in which differential migration has been observed, the shifted bands have been observed to migrate slower than the other normal bands. Sometimes in addition to the shifted band, a single strand with a same migration pattern with the normal denatured DNA strand can be observed in the shifted tumor DNA. This result may be due to the fact that, the tumor DNA may not be homogeneous i.e., some normal cell contamination such as lymphocytes and endothelial cells to the cancer liver tissue may have lead to normal DNA contamination.

In the 35 tumors we analyzed, we were able to observe three shifted samples in exon 8. The below figure, Figure 3.3 shows SSCP analysis of exon 8 of Smad4 gene for some tumor samples.

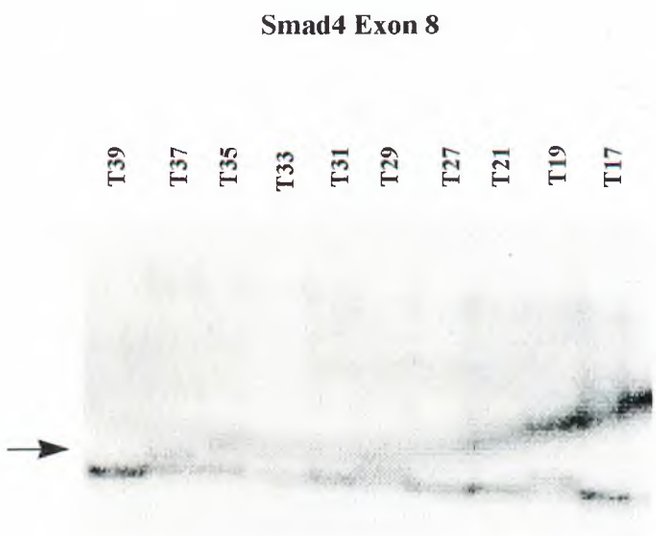


Figure 3.3: SSCP Analysis of Exon 8

In addition to the shifts observed in exon 8, SSCP analysis resulted in two more shifts, one in exon 9a (to cover the coding region of exon 9, it was divided into two parts, namely, exon 9a and exon 9b), and in exon 10. SSCP analysis of some tumor samples together with the shifted samples can be seen from Figure 3.4 and 3.5, respectively. The samples, in which differential migration has been observed, were amplified together with their DNA extracted from the normal part of the liver. We also observed similar migration pattern (two bands instead of one). However, in one of the tumors, one of these two bands was weaker than the other one, contrary to two similar band pattern in normal DNA from the same patient.

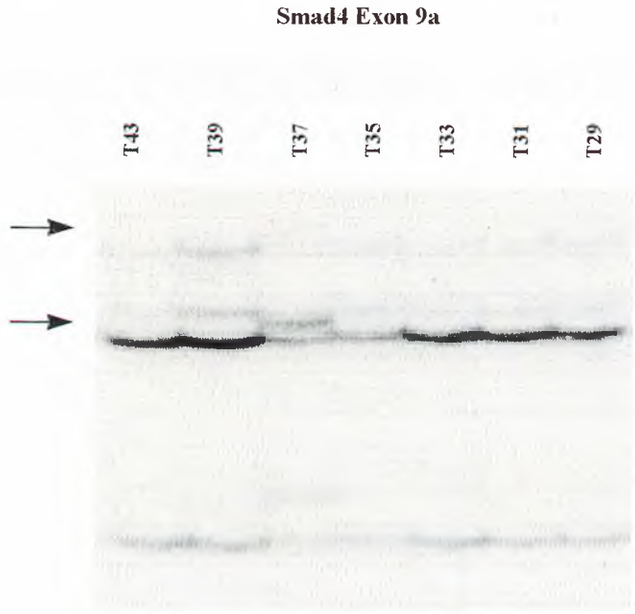


Figure 3.4: SSCP Analysis of Exon 9a

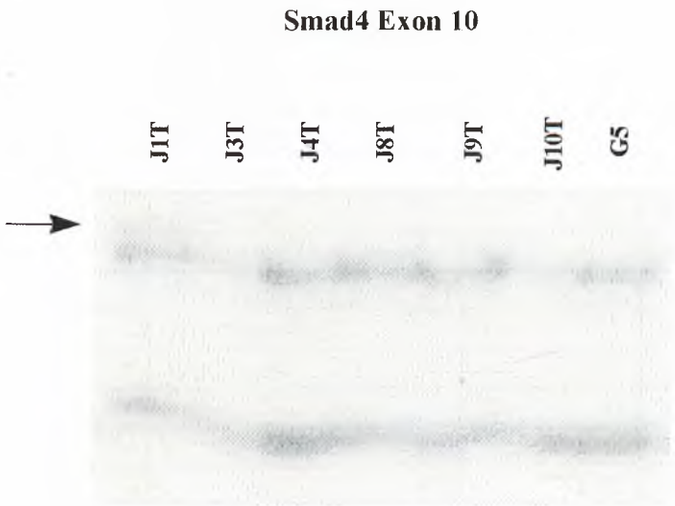


Figure 3.5: SSCP Analysis of Exon 10

The results of SSCP analysis of all the samples can be seen from the following table, Table 3.1 associated with their coutries, p53 gene mutation, and HBV integration status.

Tumors	p53	HBV	Smad4
1. T8 (SA-L)	Wild type	+	Wild type
2. T12 (SA-T)	Wild type	-	Wild type
3. T15 (M)	Codon 249 Mutation	-	Wild type
4. T17 (SA-L)	Wild type	-	Wild type
5. T19 (SA-T)	Wild type	-	Shift (Exon 8)
6. T21 (SA-T)	Wild type (Exon 5-7)	+	Wild type
7. T27 (M)	Codon 249 Mutation	+	Wild type
8. T29 (M)	Codon 249 Mutation	-	Shift (Exon 8)
9. T31 (SA-T)	Wild type	+	Wild type
10. T33 (SA-T)	Wild type	+	Wild type
11. T35 (SA-T)	Wild type	+	Wild type
12. T37 (M)	Codon 249 Mutation	+	Shift (Exon 8, 9)
13. T39 (SA-S)	Wild type	+	Wild type
14. T43 (SA-T)	Codon 286-8 bp. del.	+	Wild type
15. T47 (M)	Codon 157 Mutation	+	Wild type
16. T49 (SA-C)	Wild type	+	Wild type
17. T51 (M)	Wild type	+	Wild type
18. T53 (M)	Wild type	+	Wild type
19. T73 (G)	Wild type (Exon 7-9)	+	Wild type
20. T80 (G)	Wild type	+	Wild type
21. C2 (C)	Wild type	+	Wild type
22. C3 (C)	Wild type	+	Wild type
23. C6 (C)	Wild type	+	Wild type
24. C9 (C)	Wild type	+	Wild type
25. C10 (C)	Codon 249 Mutation	+	Wild type
26. C12 (C)	Wild type	+	Wild type
27. C15 (C)	Wild type	+	Wild type
28. C23 (C)	Wild type	+	Wild type
29. J1T (J)			Shift (Exon 10)
30. J3T (J)			Wild type
31. J4T (J)			Wild type
32. J8T (J)			Wild type
33. J9T (J)			Wild type
34. J10T (J)			Wild type
35. G5 (G)	Wild type (Exon 5-7)	+	Wild type

Table 3.1: SSCP Results of the Tumors for Smad4 Gene

The samples were from Sa-L, South Africa (Lesonto); SA-T, South Africa (Transkei); M, Mozambique; SA-S, South Africa (Swaziland); SA-C, South Africa (Caucasian); C, China; J, Japan; and G, Germany.

In order to test whether the normal DNAs of the samples shift or not, we also performed SSCP analysis to them, and we loaded the normals of the shifted samples with their tumors to the SSCP gel. Four of the normals (exon 8 and exon 9a) showed shifts like their tumors. We are still trying to confirm the shift in exon 10.

While analyzing the inhibitory mutation in codon 100 which corresponds to exon 2 and results in arginine (AGG) to threonine (ACG) change, we expected to have a digestion at 3'...GGAG$(N)_6$'...5' unless there is a mutation. We observed that all samples were digested successfully with the enzyme, revealing that, codon 100 is not mutated.

In addition to the patient tumor samples, SSCP analysis were also performed for the genomic hepatoma cell lines. However, no alteration was observed in the cell lines in any of the tested exons.

Consequent to the results of SSCP analysis for the cell lines, we checked Smad4 gene expression for cell lines HepG2, Hep3B, Huh-7, FOCUS, and Mahlavu by amplifying the cDNAs of them. All cell line cDNAs were amplified as can be seen from Figure 3.6. However, the band corresponding to Hep3B was very faint. There was no large deletion detectable with 2% agarose.

Smad4 Expression In Hepatoma Cell Lines

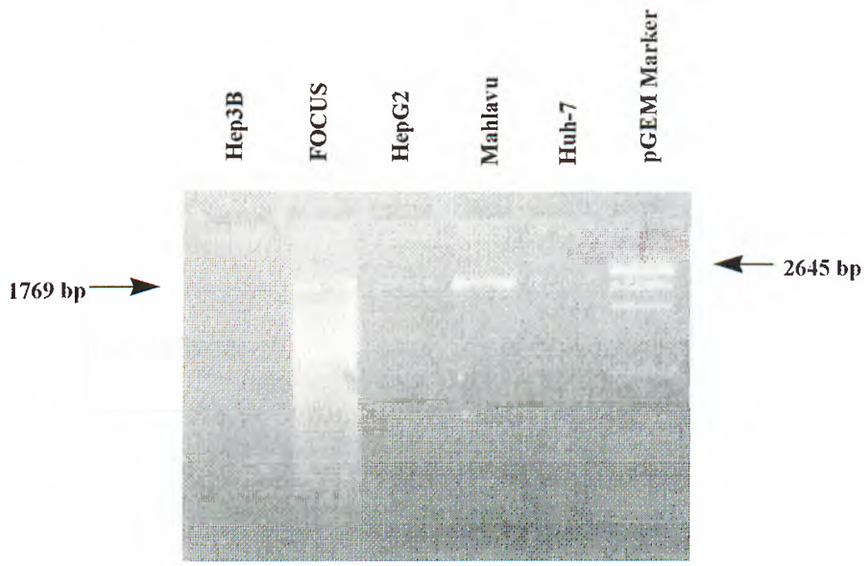


Figure 3.6: PCR Analysis of Cell Line cDNAs

We are trying to amplify Smad4 cDNA in the presence of a house keeping gene primers, as an internal control in order to confirm this result.

Chapter 4

IV. Discussion

It has been indicated that, HCC usually develops after a 20-50 year period of HBV infection, often subsequent to cirrhosis (Ding *et al.* 1991[1], Öztürk 1994 [2]). This long latent period before the clinical symptoms indicates that they are the result of a multistep process. However, the molecular events leading to HCC still remains to be established. In contrast to other tumors, in human HCC, neither a single oncogene nor a tumor suppressor gene has been shown to be preferentially altered nor show a characteristic of HCC pattern. Even though viral HBV-DNA integrations resulted in altered expression of some genes namely cyclin A, retinoic acid receptor gene, and mevalonate kinase gene (Wang *et al.* 1990[5], Dejean *et al.* 1986[6], Graef *et al.* 1994[7]), there is no HBV-DNA specific locus in human hepatocyte genome which will result in altered expression of an oncogene or a tumor suppressor gene. Several studies on human HCC tissues suggest that overexpressed c-myc, c-fos, cyclin D1, c-met and retinoic acid receptor genes may play a role in human hepatocarcinogenesis (Arbuthnol *et al.* 1991[10], Nishida *et al.* 1994[11], Boix et al. 1994[12], Sever *et al.* 1991[13]).

There is no evidence of a familial predisposition to HCC, and HCC is not associated with the development of other malignancies.

Until now, several studies have been directed toward the identification of the genetic alterations in HCC. However, genetic alterations other than p53, p16, BRCA2, M6P/IGF-IIR, Rb, PRLTS, and Tg737 remain highly unknown. In high aflatoxin exposure areas , p53 mutations are 55% and most of them are codon 249 mutations. In the remaining seven genes, mutations appear very infrequently.

In most cases, tumor cells develop when normal progenitor cells lose control of signal transduction pathways that regulate responses to soluble growth factors. Some growth factors inhibit cellular proliferation and TGF-β is among them.

TGF-β is a strong inhibitor of hepatocyte proliferation. The signal from the ligand, TGF-β, to the nucleus is mediated by serine-threonine kinase receptor pathway. In this pathway TβRII is found to show MI in gastrointestinal and colon cancers. Also, IGF-IIR shows MI in gastrointestinal tumors. Smad2 is mutated in lung, and colon cancers. Smad4, the common mediator Smad is shown to be mutated in breast, ovarian, pancreas, colon, head and neck squamous cell, lung, stomach, biliary tract, and juvenile polyposis cancers. On the other, hand other Smads namely Smad1, Smad3, Smad5, and Smad6 in the pathway were not found to be mutated. Smad7 and Smad8 are not mutationally analyzed until now.

The mutations in the genes that participate in the pathway may lead to unresponsiveness to TGF-β resulting in hepatocyte proliferation. In TGF-β signalling pathway, only TβRII and IGF-IIR mRNA and protein levels have been tested for HCC patients and shown to be decreased in level. This altered levels of expression result in the disruption of TGF-β signalling pathway. No mutational analysis of the pathway genes in HCC has been done until now.

Single base alterations are the most common type of mutations. Hetereduplex analysis (HA), denaturing gradient gel electrohporesis (DGGE), RNase cleavage, chemical mismatch cleavage (CMC), protein truncation test (PTT), restriction enzyme digestion, direct sequencing, and single stranded conformation polymorphism (SSCP) analysis are the methods that are used to detect single base alterations in the genome in literature.

HA is based on the idea that denatured and renatured PCR products will form both homoduplexes of the wild type and the mutant DNA strands, or heteroduplexes of the mutant and wild type DNA strands and homoduplexes will have differential migration pattern on polyacrylamide gel.

DGGE also relies on differential electrophoretic migration of wild type and mutant DNA when they are electrophorased through a gradient of increasing concentration of a denaturing agent. As the PCR product migrates, it denatures and single base change exhibits a differential mobility.

The principle of HA mismatch analysis applies both to RNase cleavage and chemical mismatch cleavage analysis. In the former, RNA-DNA hetereduplex, generated by PCR, is subjected to cleavage by RNase A. This enzyme cleaves the single stranded RNA at the sites of mismatch and the reaction is analyzed by electrophoresis. In CMC, a heteroduplex between a radiolabelled wild type RNA and mutant DNA (or RNA) is modified at the sites of the mismatch. Maxam-Gilbert sequencing chemistry is used. Labelled DNA is cleaved by piperidine at the sites of the modification, and this is followed by denaturing PAGE.

PTT rapidly detects mutations that interrupt the reading frames of the genes. This technique is based on in vitro-coupled transcription and translation of T7 promoter-modified PCR or reverse transcription-PCR-amplified coding sequence.

Restriction enzyme digestion is an easy and rapid method if the normal or the mutated sequence of interest is recognized by a restriction endonuclease. However, this method is mostly applied when there is a hot spot region for the mutation.

Direct sequencing both detects and locates the single base change. Direct sequencing refers to the direct sequence analysis of PCR products without prior subcloning into sequencing vector and can be primary method of mutation detection as this method becomes more efficient with the use of automation and new fluorescence detection technology. Unfortunately, its cost is really high when compared with the other methods.

SSCP analysis has become the most widely used mutation detection technique. It relies on the idea that, when denatured normal and tumor DNAs are electrophorased side by side in a polyacrylamide gel, if a base change is present in the tumor DNA, the conformation that the single strand acquires as a result of the base change will consequently lead to differential migration of the mutant strand, so a shift will be observed in the polyacrylamide gel.

Radioactive SSCP analysis is performed by us for the mutational analysis of Smad4 gene. SSCP analysis is the widely used mutation detection technique as it can be understood from the literature. It is a rapid, easy, efficient, and cheap method. The apparatus is easy to handle. The efficiency of the method is proportional to the length of the PCR product. SSCP is 80-90% sensitive when the fragment length is 250 base pair or less. The sensitivity of the method decreases as the length of the fragment increases. Once the conditions are set up it is a reliable method. In order to set up the experimental procedure, we used different conditions. The gels were run at room temperature, in cold room, with or without glycerol, and with a short and long electrophorase apparatus. In literature one realizes that different base changes are detected in different conditions. The best results we obtained were the experiments done in the cold room, in the presence of 10% glycerol, and a long distance electrophorase. To be sure that with our conditions the one can detect shifts for the mutant samples in the polyacrylamide gel, known p53 codon 248 and 249 mutations were also loaded with our samples as control. In the cold room, in the presence of 10% glycerol, and with a long distance migration, the shifts were detected in mutant samples when compared to their normals.

During our analysis of 35 HCC tumor DNA samples, five shifts are observed when all 35 tumors are loaded side by side for electrophoresis. Three of them are visualized in exon 8, one of them is seen in exon 9a, and the last one was from exon 10. To confirm the shift, the normals of the shifted samples were loaded together with their tumor DNAs. If there is a somatic mutation, the one expects no shift in the normal DNA, so a shift in the tumor DNA when compared to its normal. However similar shift was observed in normal DNA of four out of five tumors, which can lead us to think that it is a polymorphism or a germ-line mutation. In the literature, germ-line mutations of Smad4 gene have been shown in the families with juvenile polyposis syndrom. Moreover, germ-line mutations of p16 and BRCA2 genes have been reported in HCC cases. For further analysis, to locate the base change and understand the nature of the change, the PCR products of shifted samples have to be sequenced.

As it can be seen from Table 3.1, Smad4 gene showed 5 alterations in 35 HCC samples (14%). The shifts found in exon 8 belong to the tumor samples T19 (SA-T), T29 (M), and T37 (M). In the perivious studies, it has been reported that T19 has wild type p53 gene, and has no HBV integration. On the other hand, T29, and T37 has codon 249 mutation of p53 gene. In addition to this, T37 has HBV integration, whereas T29 does not have. These are all from African patients who are exposed to high levels of aflatoxin. As it has been previously mentioned, in high aflatoxin areas, the incidence of codon 249 mutation is considerably high. The shift observed in exon 9a also belongs to the tumor, T37. Among the 35 samples I have been studying, 7 of them found to have p53 mutation, five of them being codon 249 mutation, in the literature. In two of the

shifted samples (T29 and T37), codon 249 mutation is observed which is really a high percentage, and thus interesting. Consequently, Smad4 alterations also can be due to high aflatoxin exposure, like in the sase of p53 mutations in HCC. However, the shift observed in exon 10 is from a Japanese patient, and in Japan low aflotoxin exposure is observed. This tumor was not tested for its p53 mutation, and HBV integration. However, for these results to be statistically significant more HCC samples have to be tested.

In this study, we analyzed the C-terminal region of Smad4 since it is the highly mutated region of the gene in other cancer types, and this region belongs to the Mad homology region. In addition to that, we tested exon 2 which is in the N-terminal region of the gene and in literature Arg100Thr mutation in this exon is found to have an inhibitory effect on Smad4 function since mutations in this region leads to binding of N-terminal to C-terminal of the protein inhibiting homomerization and heteromerization of the protein. So since the effect of Arg100 mutation is known, we also analyzed this exon by firstly MnlI digestion of the codon 100 and SSCP. However, no mutation was detected. The interesting thing in exon 2 was that, in SSCP analysis instead of observing two bands by autoradiography, we were able to observe only one band, and this may be due to the same conformation acquired by the two single strands, and therefore same migration pattern.

SSCP analysis and sequence analysis were done at least two times for each sample to confirm the results.

In this study, we have shown,

1. Transcription of mRNA of Smad4 gene in the hepatoma cell lines, HepG2, Huh-7, FOCUS, and Mahlavu, except Hep3B, which showed very low amplification after nested PCR.

2. Genetic alterations of the Smad4 gene in five patients with SSCP analysis. In addition, we were not able to show any big deletions and alterations in N- and C- terminals of Smad4 gene in the cell lines tested.

Perspectives

Currently, we are investigating the sequencing data of the altered bands in order to lighten the reason of these alterations.

It would be interesting to do mutational analysis of other Smads in the pathway, and finally test Smad4 protein expression in the hepatoma cell lines that have been worked for Smad4 cDNA amplification.

APPENDIX A

A General Model of TGF-β/SMAD Signalling

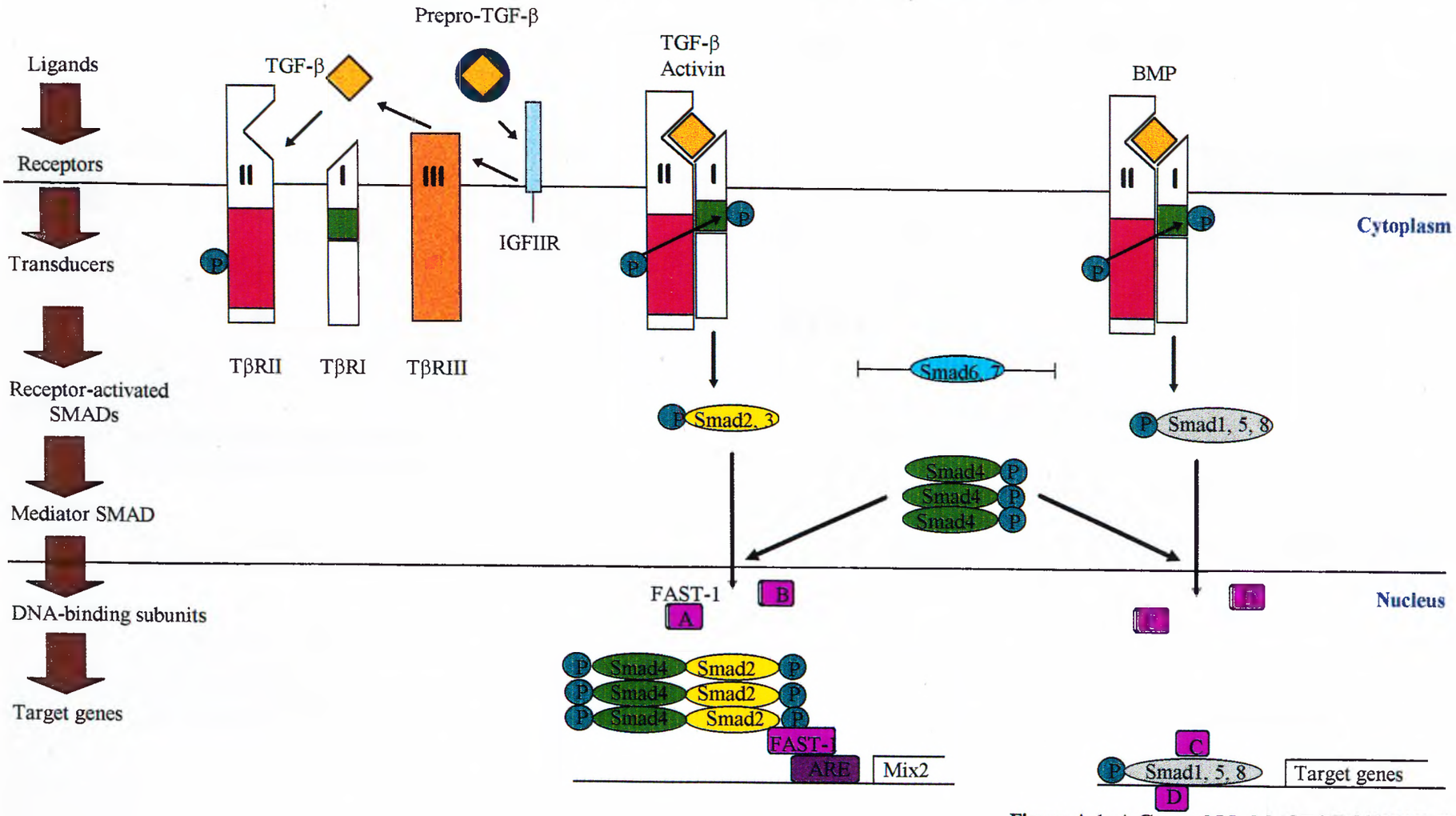
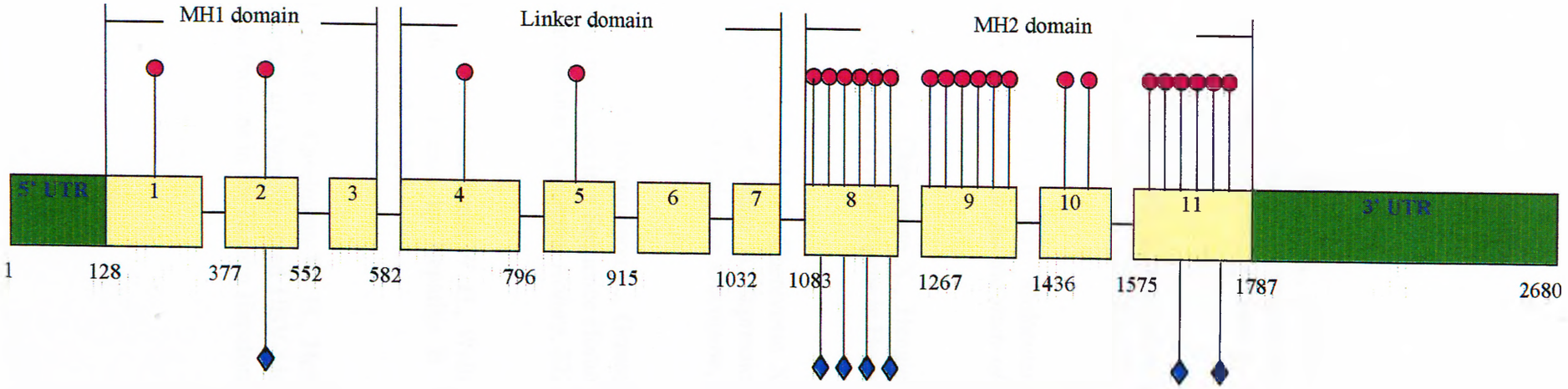


Figure A.1: A General Model of TGF-β/SMAD signalling

The Structure of Smad4 Gene



MH1 domain:

- N-terminal Smad homology domain.
- Repressor of transcription activation.

Linker domain:

- Repressor of transactivation (N-terminal).
- Effector for Transactivation (End of C-terminal).

MH2 domain:

- C-terminal Smad homology domain.
- Effector for transcription.
- Homo-oligomer formation.
- Hetero-oligomer formation.

1, 2, .., 11 stands for the exon numbers.
1, 128, ..,2680 stands for the length of the regions in base pairs.

Stands for the different type of mutations in Smad4 gene in cancer patients.

Stands for the different type of mutations in Smad4 gene in different cancer cell lines.

Figure A.2: The structure of Smad4 Gene.

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