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Analysis of Clonality and Vascular Biology in Hepatocellular Carcinoma

A thesis submitted for the degree of Doctor of Philosophy

University of London

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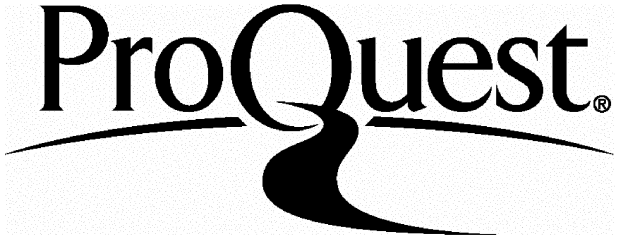

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With deepest love and affection to my wife,

Dr Vorapan Sirivatanauksorn

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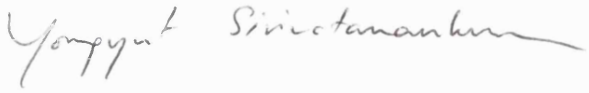

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Abstract

Hepatocellular carcinoma (HCC) arising in cirrhosis is frequently multifocal. Whether HCC develops monoclonally or multiclonally is an unresolved question. Of the multiple tumour nodules which often develop in cirrhotic, it has not been established whether the smaller lesions represent intrahepatic metastases or *de novo* cancers. The degree of genomic heterogeneity was therefore assessed, and the clonal evolution was studied in synchronous HCCs in cirrhosis from patients undergoing elective orthotopic liver transplantation. All samples studied were negative on p53 immunohistochemistry, and no mutation in exons 5-9 of the p53 gene was found by performing polymerase chain reaction and direct DNA sequencing analysis. Using arbitrarily primed-polymerase chain reaction (AP-PCR) technique, reproducible and interpretable fingerprinting patterns of amplified genomic DNA isolated from microdissected paraffin-embedded tissues were generated. The fingerprints were highly polymorphic amongst HCCs and regenerative nodules. Clonal evolution was found to occur in HCCs more than 6 mm in diameter which consistently contain multiple clones. In addition, the genomic fingerprint patterns of the extra-hepatic metastatic lesions were polymorphic compared to those of the corresponding primary HCCs. By contrast, DNA fingerprints of multiple areas of primary fibrolamellar carcinomas (FLCs) and all their metastatic lesions were identical. The advent of laser capture microdissection (LCM) technology allowed analysis of selected cell groups within a single tumour much more rapid and simple to apply.

The control of neovascularisation in tumours, particularly in HCCs, depends on a net balance of positive and negative angiogenic factors. Transfection of thrombospondin-1, an important negative factor, into Sk-Hep-1 cells was associated with decreased thymidine incorporation *in vitro* and reduced primary tumour growth *in vivo*. Antiangiogenic therapy after removal of a dominant HCC might be a useful treatment to suppress the growth of residual or synchronous tumours.

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Abbreviations

AFB1	Aflatoxin B1
aFGF	Acidic Fibroblast Growth Factor
AP-PCR	Arbitrarily-Primed Polymerase Chain Reaction
AR	Amphiregulin
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
bp	Base-pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curie
cpm	Counts Per Minute
CTP	Cytosine Triphosphate
Da	Dalton
dATP	Deoxyadenine Triphosphate
dCTP	Deoxycytosine Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
DEPC	Diethyl Pyrocarbonate
dGTP	Deoxyguanine Triphosphate
ddH_2O	Deionised Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate

DTT	Dithiothreitol
dTTP	Deoxythymine Triphosphate
EDTA	Ethylenediaminetetra Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMBL	European Molecular Biology Laboratory
Et Br	Ethidium Bromide
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FLC	Fibrolamellar carcinoma
GDB	Genome Data Base
GDF	Growth and Differentiation Factor
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HB-EGF	Heparin-Binding EGF-like Growth Factor
HBV	Hepatitis B Virus
HbsAg	Hepatitis B Surface Antigen
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HGF	Hepatocyte Growth Factor
HGMP	Human Genome Mapping Project
ICRF	Imperial Cancer Research Fund
IGF	Insulin-like Growth Factor
kb	Kilobase
kDa	KiloDalton
KRAS	Kirsten-ras

Mb	Megabase
mg	Milligram
ml	Millilitre
MRN	Macroregenerative nodule
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
ng	Nanogram
NP-40	Nonidet P-40
OD	Optical Density
OLT	Orthotopic Liver Transplantation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNK	Polynucleotide Kinase
RB	Retinoblastoma
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SDS	Sodium Dodecyl Sulphate
SF	Scatter Factor
SSC	Standard Saline Citrate
TBE	Tris-Borate EDTA
TE	Tris EDTA
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TGFα	Transforming Growth Factor Alpha
TGFβ	Transforming Growth Factor Beta

TGFβR	Transforming Growth Factor Beta Receptor
Tris.Cl	Tris(hydroxymethyl) Aminomethane Hydrochloride
TSP1	Thrombospondin-1
µg	Microgram
µg	Microlitre
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VNTR	Varying Number of Tandem Repeat
VPF	Vascular Permeability Factor

CHAPTER 1

INTRODUCTION

I. INTRODUCTION

1.1 Hepatocellular carcinoma: a dismal malignant disease

Hepatocellular carcinoma (HCC) is the eighth commonest malignant neoplasm worldwide and the sixth among men (Bosch, 1997). It is responsible for more than 4% of all cancer cases and is the third commonest cause of death from cancer throughout the world (315,000 deaths per year). It has an estimated annual incidence between 250,000 and over 1 million cases worldwide (Wands & Blum, 1991). HCC is more frequent in men than in women, with ratios between 3:1 to 8:1. The incidence of HCC varies considerably with the geographic region because of differences in the major causative factors and their incidence. Whereas the incidence is low among whites in the United Kingdom, the United States and Australia, it is much higher among blacks in Mozambique, other African natives and in the Far East. In the developing world, HCC is very common, accounting for more than 80% of the global cases. The cases in China account for 55% of the worldwide incidence. Interestingly, the incidence of histologically proved HCC in the United States increased from 1.4 per 100,000 population for the period from 1976-1980 to 2.4 per 100,000 for the period from 1991-1995, and the disease has progressively shifted toward younger people (El-Serag & Mason, 1999).

1.2 Risk factors

Although cirrhosis is considered a major risk factor, it is not an absolute prerequisite for the development of HCC, as shown by the occurrence of

HCCs both in non-cirrhotic chronic liver diseases and in otherwise apparently normal livers. Abundant epidemiological and experimental evidence indicates that HCC is of multifactorial aetiology. The relative roles of risk factors in HCC vary considerably among populations (Table 1).

	Prevalence (%)				
Risk factor	South Africa	China and Taiwan	Japan	USA	Southern Europe
Hepatitis B virus	50	60-90	20	15	5-10
Hepatitis C virus	30	30	70	30-60	60-80
Alcohol	<10	<10	<10	<15	15-30

Table 1. Prevalence of risk factors in HCC patients: geographical differences

1.2.1 Cirrhosis

Cirrhosis is the most important risk factor associated with HCC. About 70%-90% of HCCs develop on a background of cirrhosis, and approximately 20% of all patients who die of cirrhosis have evidence of associated HCC at autopsy (Okuda, 1992). Non-alcoholic cirrhosis is more frequently associated with HCC than alcoholic cirrhosis. In patients with cirrhosis, the annual incidence of HCC is 2-7% (Okuda *et al*, 1982; Liaw *et al*, 1986).

1.2.2 Hepatitis B virus

Hepatitis B virus (HBV) is an enveloped virus with a very compact, incompletely double-stranded DNA genome. It has a propensity to persist after perinatal infection by a carrier mother or after infection in early life, and after 10-15% of acute HBV infections in adults. In Southeast Asia and central Africa, where more than 10% of the population are HBV carriers, chronic active hepatitis and cirrhosis are major causes of death. Intensive epidemiological studies have supported a correlation between high prevalence of chronic HBV infection and the high incidence of HCC in the Far East and Africa (Simonetti *et al*, 1991). In prospective studies, the risk of developing HCC for 3,454 hepatitis B surface antigen (HBsAg) carriers was 102 times greater than that of non-carriers (Beasley *et al*, 1981). The carriers at especially high risk of developing HCC were those with actively replicating infection (HBeAg positive) and those with cirrhosis. In case control studies, the relative risk of HCC associated with HBsAg ranges from 10 to 20 (Lu *et al*, 1988).

HBV could contribute to hepatocarcinogenesis both directly and indirectly, though the mechanisms remain largely unknown. A well-known direct mechanism is viral genome integration into the hepatocyte chromosomal DNA during the typically long period of infection. HBV replicates *via* reverse transcription of pregenomic RNA, and the viral genome frequently integrates into multiple sites of the host genome. This process may cause or contribute to genomic instability as a result of point mutations, inversions, translocations and deletions (Brechot *et al*, 1980). About 80% of HCCs contain multiple HBV DNA integration sites in their chromosomes (Chen *et*

al, 1982). The number of integration sites varies from 1 to 12, and the HBV sequences can be either complete genomes or rearranged subgenomic fragments (Nagaya *et al*, 1987). The integration sites are at random, suggesting that this process does not lead to insertional mutagenesis in most HCCs. However, the sites are sometimes located in or close to the growth-regulating genes (such as cyclin A gene) (Wang *et al*, 1990) or hormone receptors (such as retinoic acid receptor gene) (Dejean *et al*, 1986), which regulate cell differentiation. By contrast, studies in woodchuck HCC revealed integrations of the woodchuck hepatitis virus near the *myc* gene, *win* and *b3n* loci in 50% to 75% of cases (Hsu *et al*, 1988; Fourel *et al*, 1990; Bruni *et al*, 1995). HBV integrations are commonly associated with microdeletions of about 10 base pairs (Nakamura *et al*, 1988). They can also trigger large chromosome deletions in the cellular DNA at the integration site (Rogler *et al*, 1985), which may result in the loss of important tumour suppressor genes. Some of the viral gene products, such as HBX protein, activate transcription and may increase the expression of growth-regulating genes involved in the malignant transformation of hepatocytes.

As an indirect mechanism, persistent HBV infection (but not HBV itself) predisposes the hepatocyte to genetic changes resulting from other causes. The virus continuously replicates and causes recurrent episodes of hepatitis. The liver responds to persistent inflammation with continuous regeneration and fibrosis that eventually results in cirrhosis.

1.2.3 Hepatitis C virus

Hepatitis C virus (HCV) is usually transmitted by the parenteral route, the commonest mode of transmission being *via* blood transfusion (before 1991) and the use of intravenous drugs. Unlike antibodies to hepatitis A and B viruses, antibodies to HCV are not protective and, in most cases, are markers for disease. Recognition of the importance of HCV in the development of HCC was initially made by studies in Japan (Liver Cancer Study Group of Japan, 1988). Whereas HBV is a major risk factor for the disease in Africa and Asia, HCV is of major importance in Japan, Europe and the United States. Long term follow-up studies indicate that up to 30% of HCV-infected patients will develop cirrhosis and liver tumours within 30-40 years of infection (Tong *et al*, 1995). The mean times from viral acquisition to the development of clinically important chronic hepatitis, cirrhosis, and HCC were 18, 21, and 28 years, respectively. Cirrhosis is found in over 80% of anti-HCV positive (HBsAg negative) HCC patients (Hasan *et al*, 1990), and HCC develops in 18% of patients with HCV-associated cirrhosis within 2 years and in 75% of patients within 15 years (Foster *et al*, 1997; Ikeda *et al*, 1993). The risk for HCC is increased in patients with HCV who are chronic carriers of HBV or who concomitantly use alcohol.

The aetiological role of HCV infection in terms of molecular events in hepatocarcinogenesis is unknown, but it is clearly different from that of HBV infection. HCV is a positive-sense, single-stranded RNA virus and, unlike HBV, is not reverse transcribed to DNA; hence it is not integrated into the host cell DNA. Moreover, the activation of specific protooncogenes or

inactivation of tumour suppressor genes has not been documented. Continuous inflammation, liver cell death and ongoing viral replication seem to underlie HCV-related hepatocarcinogenesis. Generally, cases of HBV-associated HCC occur in younger patients than do cases of HCV-associated HCC (Shiratori *et al*, 1995). Several clinical and pathological studies have suggested that inflammation is more active in the liver in HCV-associated HCC compared with HBV-associated HCC (Jeng & Tsai, 1991; Shiratori *et al*, 1995), and liver cell proliferation also is active (Taroa *et al*, 1994). Seventy to ninety-four percent of anti-HCV-positive HCC patients have HCV-RNA in their sera, suggesting active HCV infection (Tabor & Kobayashi, 1992).

1.2.4 Aflatoxins

Aflatoxin contamination of food probably increases the risk of HCC. Aflatoxins are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and are chemically divided into two groups, aflatoxin B1 (AFB1), which is the most widely studied compound, and its derivatives and aflatoxin G1 and its series. AFB1 contaminates foods such as corn, peanuts, milo, sorghum and rice in some areas of the world (Van Rensburg *et al*, 1985).

The oncogenic role of AFB1 has been clearly established in experimental animal models in which it has reproducibly led to HCC (Groopman *et al*, 1980). It is quickly converted to its reactive 2,3-oxide by liver microsomes and is trapped as an RNA adduct. This AFB1 epoxide reacts extensively with the guanine residue of the DNA molecule, forming a covalent bond.

More than 90% of AFB1 is excreted in various forms into the urine within 24 hours. It has been shown to cause a specific mutation, transversion of guanine to thymine in codon 249 of the p53 tumour suppressor gene. Epidemiological investigations have revealed a linear relationship between the content of AFB1 in the diet and the risk of HCC (Van Rensburg *et al*, 1985; Yeh *et al*, 1989). However, two studies in China (Campbell *et al*, 1990) and Thailand (Srivatanakul *et al*, 1991) have found no association between aflatoxin metabolites in urine or serum and the risk of HCC.

1.2.5 Miscellaneous factors

Alcohol is a risk factor both for cirrhosis and for HCC. In the United States, where the prevalence of HBV is low, the HCC risk is increased up to 40% by heavy alcohol consumption (Okuda, 1992). In London, HCC was seen in 30% of alcoholics with cirrhosis compared with 11% of non-alcoholics with cirrhosis (Lee, 1966). The 10-year cumulative occurrence of HCC in patients with alcoholic cirrhosis is 18.5% (Yamauchi *et al*, 1993). Several mechanisms have been postulated to explain the role of ethanol in promoting the development of HCC: activation of chemical carcinogens through the induction of the microsomal P450-dependent biotransformation system, direct hepatocellular injury, reduction in the activity of the enzymes involved in the repair of carcinogen-mediated DNA alkylation and suppression of the immune system. A recent study demonstrated that the activity of ornithine decarboxylase, the rate-limiting enzyme for polyamine metabolism in cell proliferation, in both noncancerous and HCC tissues of heavy drinkers was significantly higher than those of nondrinkers or occasional drinkers (Kubo *et al*, 1998).

Cigarette smoking is generally considered to pose a 'weakly or modestly positive' association with HCC, according to a review of 14 (mostly case-control) studies which show, on average, that heavy smokers have a relative risk for HCC of about 1.5 compared to non-smokers (Austin, 1991). However, tobacco may be a contributing factor in areas where HCC is not induced by virus. Case-control studies in developed countries show a two-fold to five-fold risk of HCC with oral contraceptive use (Schlesselman, 1995). However, this estimate is not entirely supported by the time trends in incidence or mortality rate, which suggest that oral contraceptives may act as a co-factor or a promoter of the carcinogenic process (Mant & Vessey, 1995). The risk of HCC in patients with haemochromatosis increases by a factor of approximately 200 with the onset of cirrhosis whereas that in patients with autoimmune chronic hepatitis, Wilson's disease, primary biliary cirrhosis, or alcohol abuse without coexisting HCV infection increases by a factor of only 2 to 5.

1.3 Molecular patterns of HCC

1.3.1 Growth factors

The interaction of growth factors with specific membrane receptors triggers a cascade of intracellular signals, resulting in the activation or repression of genes associated with cell growth. Unfortunately, little is known about the cellular activity of growth factors during hepatic proliferation and transformation. Some of the growth factors that may be involved in one or more steps in the development of HCC include epidermal growth factor

(EGF), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), insulin, insulin-like growth factor-II (IGF-II) and acidic fibroblast growth factor (aFGF).

1.3.1.1 Epidermal growth factor

Epidermal growth factor (EGF) receptor (EGFR) (also known as c-erbB) is a transmembrane protein with intrinsic tyrosine kinase activity that is involved in normal cellular growth and differentiation. EGFR belongs to a family of closely related transmembrane proteins that include HER-2/neu (also known as c-erbB-2), HER3 (c-erbB-3), and HER4 (c-erbB-4). HER-2/neu is required to act in heterodimers with other family member, whereas HER3 and HER4 bind to the family of heregulins. To date, at least 15 EGF-like ligands have been described including TGF-α, amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin and epiregulin. Epiregulin, a novel EGF family member, exhibits bifunctional regulatory properties as it stimulates the growth of many types of cells including fibroblasts and hepatocytes, while inhibiting the growth of several epithelial tumour cells (Toyoda *et al*, 1995). These ligands form a complex series of interactions with different members of the EGFR family that promote either homo- or hetero-dimerisation (Carraway & Cantley, 1994).

Expression of EGF was demonstrated in 14 of 56 (25%) HCCs, while it was totally negative in non-cancerous hepatic tissues (Motoo *et al*, 1991). The amount of HB-EGF mRNA expression was increased in HCCs compared with the surrounding liver tissues (Inui *et al*, 1994). These findings suggest

a role for the exclusively produced EGF in HCC cells themselves during the development and progression of the tumour mass. An immunohistochemical study showed a linear localisation of EGFR along the cell membrane of the HCC cells in 21 of 30 cases of HCC (Yamaguchi *et al*, 1995). In addition, concurrent localisation of TGF-α and EGFR in 12 of the 30 cases supports the theory of an autocrine, paracrine and endocrine mechanism of TGF-α and EGFR on the proliferation of human HCC. However, no statistically significant differences in EGFR expression were observed between the HCCs and the adjacent non-neoplastic parenchyma (Nakopoulou *et al*, 1994). Thus, EGFR did not seem to be predominantly involved in the transformation of hepatocytes to the malignant phenotype.

1.3.1.2 Hepatocyte growth factor

Hepatocyte growth factor (HGF), otherwise known as scatter factor (SF), is a ligand for the c-met protooncogene, which encodes the tyrosine kinase receptor. It is a mesenchymal- or stromal-derived multipotent polypeptide, which mediates epithelial-mesenchymal interactions. HGF has been implicated in the regulation of mitogenesis, motogenesis and morphogenesis (Weidner *et al*, 1993). *In vivo*, it plays a role in tissue regeneration, tumour progression and embryological processes that generally require both cell motility and cell proliferation. Over the past few years, the structure, function and signal transduction pathways of HGF and its receptor have become clearer. It is now known to take important parts in the regulation of both normal physiological processes as well as pathological ones (Jiang & Hiscox, 1997).

Serum HGF levels in patients with HCC were significantly higher than those in normal individuals in a recent study (Shiota *et al*, 1995). A similar result was reported from the rat model of chemically induced hepatocarcinogenesis (Burr *et al*, 1996). There was no correlation between HGF concentration in the tumour tissue, clinico-pathological factors and patient survival (Ueki *et al*, 1997). However, HGF receptor, c-met, was overexpressed in HCC compared with non-cancerous tissues, and it correlated with an increased incidence of intrahepatic metastases. Furthermore, HCC patients with high c-met in their tumour tissue had a significantly shorter 5-year survival than those with low c-met (Ueki *et al*, 1997).

1.3.1.3 Transforming growth factor-α

Transforming growth factor-α (TGF-α) is a member of a family of structurally related polypeptide growth factors that includes EGF. It acts in an autocrine or paracrine fashion to stimulate directly hepatocyte DNA synthesis (Mead & Fausto, 1989). TGF-α plays a role in the establishment and maintenance of the malignant phenotype. Increased production of TGF-α and its receptor has been frequently found in human tumours and transformed HCC cell lines in tissue culture (Derynck *et al*, 1987), but not in normal liver tissue (Hsia *et al*, 1992). Moreover, 65% of patients with HCC have elevated TGF-α levels in urine (Yeh *et al*, 1987). The detection of greater quantities of TGF-α in the more differentiated portions of HCCs suggests that its increased expression may be an early event in human hepatocarcinogenesis (Morimitsu *et al*, 1995).

1.3.1.4 Transforming growth factor-β

The transforming growth factor beta (TGF-β) superfamily consists of large numbers of regulatory polypeptides, which include several TGF-β isoforms as well as activin, inhibin, bone morphogenetic proteins, Mullerian inhibiting substance, and growth and differentiation factors (Massague *et al*, 1992). Three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) exist in mammals and possess three major activities: they inhibit proliferation of most cells, but can stimulate the growth of some mesenchymal cells; they exert immunosuppressive effects; and they enhance extracellular matrix formation (Lawrence, 1996). There are three major receptors: TGF-βRI, TGF-βRII, and TGF-βRIII. TGF-βRI and TGF-βRII possess a serine/threonine kinase activity within their cytoplasmic domains and are involved in signal transduction. TGF-βRIII lacks an intracellular signalling domain and functions primarily as a binding and presentation molecule for presenting TGF-β to the TGF-βRI and TGF-βRII (Segarini, 1993).

During liver regeneration, there is increased expression of all three TGF-β isoforms (Fausto & Mead, 1989). By contrast, all TGF-β receptors are rapidly downregulated following partial hepatectomy (Chari *et al*, 1995). Russell and colleagues showed that hepatic regeneration was inhibited by intravenous administration of TGF-β to partially hepatectomised rats (Russell *et al*, 1988). The negative regulation of proliferation is mediated by the induction of apoptosis in hepatocytes and altered cells in pre-neoplastic hepatic foci (Bursch *et al*, 1993). TGF-β has been shown to be a potent inhibitor of DNA synthesis in primary hepatocyte cultures and HCC cell lines

such as HepG2 and Hep3B (Inagaki *et al*, 1993). Increased expression of TGF-β is found in HCC tissue (Unsal *et al*, 1994), and elevated serum levels of TGF-β have been found in patients with HCC (Shirai *et al*, 1992).

1.3.1.5 Insulin-like growth factor-II

Insulin-like growth factor-II (IGF-II) is a polypeptide hormone produced during fetal development and is structurally and functionally related to insulin and insulin-like growth factor-I (IGF-I) (Froesch *et al*, 1985). In the liver, IGF-II- mediated signal transduction occurs through binding to either the insulin receptor or the mannose 6 phosphate/insulin-like growth factor-II receptor (M6P/IGF-IIR) since IGF-I receptor is not present in this tissue (Cohick & Clemmons, 1993). IGF-II is expressed at high levels in fetal liver, and the rate of IGF-II gene transcription declines after birth, to reach the very low level found in normal adult liver. Re-expression of IGF-II has been found in chemically induced hepatocarcinogenesis (Ueno *et al*, 1988), in transgenic mouse models of hepatocarcinogenesis (Cariani *et al*, 1991), in human HCC and in adjacent uninvolved cirrhotic tissue (Cariani *et al*, 1988).

1.3.2 Oncogenes

Activation of cellular oncogenes such as ras has been reported in spontaneous and chemically induced pre-neoplastic lesions of HCC in animal models (Buchmann *et al*, 1991; Dragani *et al*, 1991). However, in contrast to other human tumours, activation of cellular oncogenes is infrequent in human HCC. Several recent studies have found little evidence of activated oncogenes in human HCC (Tada *et al*, 1990; Collier *et al*,

*The ras genes encode 21-kDa proteins with guanosine triphosphatase (GTPase) activity. ras proteins which are synthesised in the cytosol and attached to the inner side of the plasma membrane after posttranslational modifications, have functional and structural resemblance to the regulatory (G) proteins. It has now been shown that they play a role in transduction of signals from the cell surface. The incidence of mutated ras genes varies widely among different tumour types. The highest incidence is found in pancreatic cancers whereas the frequent is low in HCCs. Somatic alterations of the ras gene were demonstrated in 2 of 21 HCCs (Ogata *et al*, 1991). A study has shown an abnormally high level (90%) of ras transcripts in cirrhotic liver tissues compared with the level in normal liver tissues. However, the overexpression is not correlated to the liver-cell proliferation (Liu *et al*, 1994). Expression of the proto-oncogene c-myc has been implicated in liver regeneration and hepatocarcinogenesis. The c-myc gene was amplified in 14 of 42 (33.3%) human HCCs (Kawate *et al*, 1999). Amplification of c-myc was more frequent in larger and less differentiated tumours. Moreover, the disease-free survival in patients showing c-myc amplification was significantly shorter than in those without amplification. The study suggested that c-myc amplification is an indicator of malignant potential and poor prognosis in HCC. β-catenin, a protein associated with the cytoplasmic region of E-cadherin, has been shown to play a role in transcriptional regulation and in cell-to-cell interactions. It is now apparent that deregulation of beta-catenin signalling is an important event in the genesis of a number of malignancies. Somatic mutations of β-catenin were observed in 18-26% of HCCs (de La Coste *et al*, 1998; Legoix *et al*, 1999). These mutations that occur at the N-terminal region of β-catenin lead to an accumulation of aberrant β-catenin proteins that stimulate the activity of a transcription factor.

1992). Moreover, no structural nor functional changes have been found in a large panel of oncogenes in a transgenic mouse model that develops HCC (Pasquinelli *et al*, 1992).

*

1.3.3 Tumour suppressor genes

1.3.3.1 p53

The p53 gene is located on the short arm of human chromosome 17 (17p13.1) and is approximately 20 kb in length. It consists of 11 exons, of which the first is a non-coding exon. Exons 2-4 code for the acidic *N*-terminal domain of the p53 protein, which is involved in the transcriptional control of other cellular genes. Exons 5-9 code for the central hydrophobic core of the protein, which is characterised by its conformational flexibility and ability to fold into a domain with sequence-specific DNA-binding activity. Exons 10-11 specify the more basic *C*-terminus of the molecule, containing motifs involved in nuclear localisation and in oligomerisation. This gene yields a 2.8 kb mRNA transcript and encodes for a 53 kDa phosphorylated 393-amino acid nuclear protein (p53 protein). In response to DNA damage, p53 appears to direct the transcription of the p21 WAF1 gene, which is an inhibitor of cyclin-dependent kinases. This fact suggests that p53-dependent p21 induction is responsible for arresting the cell cycle in G1 following DNA damage. (G1, or gap 1, designates the quiescent phase of the cell cycle between the phases of mitosis and DNA synthesis.) Moreover, p53 protein is also required to direct cells into apoptosis (programmed cell death) when the level of DNA damage is too great. Mutant forms of the p53 protein fail to block the progression of cell division and in some cases may even promote cellular proliferation. p53 protein has

therefore been described as the "guardian of the genome" as it prevents entry into S phase unless the genome has been purged of potentially damaging mutations. The cellular level of p53 is regulated by an autocrine feedback system involving the cellular protein mdm2. When the mdm2 protein binds to p53, p53-directed transactivation is inhibited.

Sporadic mutations in the p53 tumour suppressor gene are the single most common genetic alterations found in human malignancies (Levine *et al*, 1991); 100% of small-cell lung cancers, 70% of colon cancers and 30-50% of breast cancers contain these mutations (Hollstein *et al*, 1991). The mutations occur over a large area of the gene but are most commonly restricted to exons 5-8, which contain the conserved regions. The p53 mutational spectrum in HCC shows a striking geographic variation (Aguilar *et al*, 1994). In some parts of the world, mutations are consistent with those caused by aflatoxin exposure and possibly by HBV infection. Highly specific genetic alterations in the p53 gene were described by two independent groups of investigators in human HCC. There appeared to be a unique association between a specific p53 mutation in HCC derived from two different regions of the world. Bressac *et al* (1991) found three G:C to T:A transversions at the third base of codon 249 of the p53 gene leading to a substitution of arginine to serine in ten tumours from southern Africa. Hsu *et al* (1991) also described seven G:C to T:A mutations in codon 249 of the p53 gene and one G:C to C:G substitution in 16 HCCs derived from the Qidong area of China. HCC biopsies from patients in 14 countries showed that among 72 specimens from South Africa and Southeast Asia (high consumption of aflatoxin), 12 (17%) showed a specific G to T mutation at

codon 249 of the p53 gene (Ozturk *et al*, 1991). The mutation was not found in any of 95 HCCs from countries where aflatoxin exposure is rare. However, one study showed a low frequency of p53 mutations in HCC patients from Thailand, where aflatoxin contamination of foods has been documented (Hollstein *et al*, 1993). Additionally, one recent study from Taiwan and Japan did not support the hypothesis that codon 249 of the p53 gene is a hotspot for aflatoxin-induced mutagenesis (Hsieh & Atkinson, 1995).

In studies of HCC with codon 249 mutations, a substantial proportion of tumours retain the presence of the wild-type p53 allele, suggesting that the first step in p53 inactivation is a point mutation in one allele followed by subsequent loss of the wild-type allele by a deletional event (Scorsone *et al*, 1992). This hypothesis was supported by the recent observation of codon 249 mutations in the p53 gene in the normal liver of aflatoxin-exposed individuals (Aguilar *et al*, 1994).

Mutational hotspots in the p53 gene other than at codon 249, such as at codon 166 in exon 5 and at codon 286 in exon 8, have also been described in HCCs (Diamantis *et al*, 1994). It remains possible either that each mutation was a result of a different carcinogenic influence or that the different mutations reflect a general genetic instability or repair defect. In the absence of p53 mutation, the functions of normal p53 can be inactivated when p53 protein forms stable complexes with protein of DNA tumour viruses (SV40 large T antigen, adenovirus E1B antigen, Epstein-Barr virus

EBNA-5 protein) or shows increased degradation by human papillomavirus E6 protein.

P53 mutations may also be late occurrences in hepatocarcinogenesis and may occur in association with dedifferentiation to a more aggressive histological type or the development of metastasis. Various reports showed that the p53 mutations were closely related to the progression of HCC. These mutations were found in 44-50% poorly differentiated HCCs and in 22-36% moderately differentiated HCCs, but in none of the well differentiated HCCs (Murakami *et al*, 1991; Yumoto *et al*, 1995). One patient with a long history of chronic HCV hepatitis and ethanol use has been reported in whom two HCC grade II or III nodules containing two different p53 mutations were surrounded by a grade I nodule containing only wild-type p53 (Oda *et al*, 1994). A similar case has been reported of two different p53 mutations among three HCC nodules of different histological grades in one patient (Yumoto *et al*, 1995). Furthermore, p53 mutations are usually found only in one of the nodules in multifocal HCC (Oda *et al*, 1992), suggesting a role in tumour progression rather than in tumour initiation. It was noteworthy that similar findings of involvement of p53 gene in high-grade or advanced tumours were also seen in studies on breast, colon and endometrial cancers (Cattoretti *et al*, 1988; Baker *et al*, 1990; Kohler *et al*, 1992).

1.3.3.2 Retinoblastoma

The retinoblastoma (RB1) gene, located at 13q14, encodes a 110 kD, 928 amino acids, phosphoprotein (pRB). It is an important tumour suppressor

*1.3.3.3 **Other tumour suppressor genes**

p16 (CDKN2/MTS1/p16INK4A) gene is located at chromosome 9p. It is one of the inhibitors for cyclin-dependent kinases (CDKs) which complexes with cyclin D1 and drives a cell's progression through the division cycle. p16 acts on RB1 to form a negative feedback loop that regulates the ability of RB1 to prevent cell proliferation. Deletions or mutations in the p16 gene may affect the relative balance of functional p16 and cyclin D, resulting in abnormal cell growth. Both germline and somatic mutations of p16 were found in HCC patients. Mutations of the p16 gene occur in HCC at a rate of 5-55% (Kita *et al*, 1996; Kim *et al*, 1998). APC (adenomatour polyposis coli) gene mutations have been demonstrated not only in colorectal carcinoma but also in a variety of human cancers. However, the possibility of APC as the gene defect in the genesis of human hepatocellular carcinoma may be very rare (Chen *et al*, 1998). E-cadherin gene which is located on 16q22.1 was shown to display frequent LOH and methylation in HCCs. Immunohistochemical examination revealed reduced E-cadherin expression in 56-59% of HCCs. Downregulation of E-cadherin correlated with the size of tumours, high nuclear grade, the mitotic index and survival (Kanai *et al*, 1997; Garcia *et al*, 1998). M6P/IGF2R (mannose-6-phosphate/insulin-like growth factor 2 receptor) gene is located on chromosome 6q26-q27 and is frequently inactivated during carcinogenesis. It is postulated to be a tumour suppressor gene due to its ability to bind and degrade the mitogen IGF-II, promote activation of the growth inhibitor TGF-β, and regulate the targeting of lysosomal enzymes. The M6P/IGF2R gene is mutated in 18-33% of HCCs (De Souza *et al*, 1995; Yamada *et al*, 1997).

gene that is found to be mutated in numerous tumour types. Normally, the underphosphorylated-pRB localises in the nucleus and prevents cell growth beyond the G1 phase of the cell cycle, whereas the phosphorylated form appears to localise in the cytoplasm and cell growth can occur.

Abnormalities of the RB gene in human HCCs have been indicated by finding loss of homozygosity (LOH) of the RB gene in 10-44% of cases (Murakami *et al*, 1991; Zhang *et al*, 1994; Yumoto *et al*, 1995) and by immunohistochemistry in 20-29% (Kawakita *et al*, 1994; Hsia *et al*, 1994). These findings were common in moderately or poorly differentiated HCCs. An additive effect of p53 and RB genetic alterations in hepatocarcinogenesis has been suggested. LOH at the RB gene was detected in six of seven (86%) HCCs with a p53 mutation compared to none of 17 HCCs without a p53 mutation (Murakami *et al*, 1991).

1.3.3.3 Other tumour suppressor genes*

1.4 Clinical problem of HCC

Studies on the natural history of untreated HCC have shown that survival can reach 3 years in 12.8% of patients (Sheu *et al*, 1985), but most patients die within 4 months from the onset of symptoms (Nagasue *et al*, 1984). The prognosis of HCC largely depends on the size of tumour, the growth rate, the histological grade, the degree of cirrhotic changes and the presence of underlying diseases. Early detection and early resection may afford a permanent cure or a worthwhile prolongation of survival. An analysis made in Japan demonstrated that 1306 patients who had had curative hepatic resections (with sufficient clear margins) had a median survival of 3.6 years and a 7-year survival rate of 46% (Liver Cancer Study Group of Japan,

1990). However, the remarkable advances in diagnostic imaging techniques and the introduction and popularisation of ultrasound-guided fine needle biopsy have led to the early detection and resection of smaller HCCs. Approximately 20% of all nodules less than 1 cm in diameter when first detected already show microscopic signs of intrahepatic metastasis (invasion of the fibrotic capsule or portal vein) (Ebara *et al*, 1986). The number of tumours showing microscopic metastases increases with increasing tumour size and approaches 80% for nodules larger than 3 cm. In advanced HCC, the clinical course is relentlessly progressive, and the patient succumbs within a few months.

Although hepatic resection offers the only opportunity for cure of HCC, it is feasible only in a minority of patients due to local tumour spread and the severity of pre-existing cirrhosis. Resectability in cirrhotic patients is limited by the diminished functional reserve of the cirrhotic liver, with the attendant risks of intraoperative bleeding and postoperative liver failure. In the absence of cirrhosis, the tumour is usually diagnosed at a very late stage with the median tumour diameter of 8.8 cm (Bismuth *et al*, 1995). Surgical procedures consisted of a major hepatectomy (three segments or more) in 72% of patients with 3-year survivals of 52% (Bismuth *et al*, 1995). Liver transplantation has become widely accepted as therapy for HCC when tumours are not suitable for resection because of anatomical restrictions (location, size or number of intrahepatic lesions) or functional impairment (underlying cirrhosis). For transplantation, the 3-year survival without recurrence in the case of small uninodular or binodular tumors (< 3 cm) is significantly better than that for resection (83% *vs.* 18%). However in terms

of overall survival rates, resection and transplantation yield the same results (50% *vs.* 47%) (Bismuth *et al*, 1993). Despite evidence for long-term survival after transplantation in some patients with large tumours, most of these patients die of recurrent disease. The growth rate of HCC is accelerated with mean tumour doubling times of 37 days in recurrent tumours after transplantation, compared with 274 days in patients with recurrence after resection (Yokoyama *et al*, 1991). One of the most frequent sites for recurrence is the transplanted liver. The tumour size is a very important variable in predicting recurrence.

Because of the low resectability rate in patients with HCC, various non-surgical treatment modalities have been developed and used extensively, but their efficacy is far from satisfactory and the prospect for the development of more effective treatments is dismal. Systemic chemotherapy and radiotherapy have a very limited role in the treatment of HCC. Percutaneous ethanol injection (PEI) is considered the most effective form of direct ablation therapy for HCC. It is suitable for HCC less than 3 cm in size and fewer than three in number. Absolute alcohol induces cellular dehydration, coagulative necrosis and vascular thrombosis causing destruction of the tumour cells. Though a 3-year survival rates of 55%-70% have been reported, more than half of these patients develop recurrent tumour within 2 years (Castells *et al*, 1993; Isobe *et al*, 1994). Transcatheter arterial chemoembolisation (TACE) is a combination of chemotherapy and arterial embolisation that has both selective ischaemic and chemotherapeutic effects on HCC. The patients treated by TACE have often had larger tumours and worse tumour staging than those treated by

PEI. The 3-year survival rate after TACE is about 20% (Mondazzi *et al*, 1994), but as yet there has been no prospective randomised controlled study to prove its efficacy. Hormonal therapy by tamoxifen may be a convenient treatment modality for patients with advanced HCC who are not suitable for PEI or TACE. Results from small randomised trials on tamoxifen in the treatment of hepatocellular carcinoma (HCC) are conflicting. Some prospective randomised studies demonstrated significant prolongation of survival in the tamoxifen group with 1-year survival rates 35%-49% compared with 0%-9% in the control group (Farinati *et al*, 1992; Martinez-Cerezo *et al*, 1994). Recent randomised controlled studies showed that tamoxifen is not effective in prolonging survival of patients with cirrhosis and HCC (Riestra *et al*, 1998; CLIP Group, 1998). Multidisciplinary treatment such as the combination therapy of PEI and TACE appears to be the current trend of management for nonresectional HCC, and improvement of survival can be achieved compared to a single mode of treatment.

1.4.1 Recurrence of HCC

Most HCCs develop on a background of cirrhosis, and they are frequently multifocal (Bhattacharya *et al*, 1997). The underlying cirrhosis poses a constant threat of *de novo* emergence of HCC. A new HCC may develop from the cirrhotic nodules after resection of the original tumour and be considered a recurrence. Such recurrence has been reported at a rate of 80% at 5 years and is the commonest cause of death (Chen *et al*, 1994). Survival rates after recurrence were 71%, 42% and 25% at 1, 2 and 3 years respectively (Nagao *et al*, 1990). Recurrences that appear in the liver remnant could result from incomplete removal of tumour, pre-existing

intrahepatic metastases or undetectable synchronous tumours. Less than 27% of recurrences occur near the resection site, suggesting that incomplete removal of the tumour is responsible for only a minority (Chen *et al*, 1994; Matsumata *et al*, 1989). An early recurrence of multiple lesions probably represents undetected synchronous lesions (Chen *et al*, 1994; Matsumata *et al*, 1989). The accuracy of radiological procedures in detecting small tumours is poor. Ultrasonography, computed tomographic scanning and angiography failed to demonstrate multiple tumours in 80% of patients who were discovered to have them when the explanted livers were examined (Rizzi *et al*, 1994). Late recurrence (3 to 6 years after resection) is unlikely to be due to the presence of occult intrahepatic metastases but to non-synchronous multifocal tumourigenesis (Ouchi *et al*, 1993). In addition, recurrence in the cirrhotic liver often occurs in areas of the liver not targeted by ablation of the primary tumour, suggesting multifocal hepatocarcinogenesis (Adachi *et al*, 1995).

1.4.2 Extrahepatic metastasis in HCC

Surgical excision of a primary neoplasm is not curative because metastasis has occurred by the time of resection. Often these metastases are too small to be detected. Most extrahepatic metastases are multiple and occur at a relatively late stage. The incidence of extrahepatic metastasis in HCC ranges from 50% to 70% in Western countries (MacSween, 1974). Haematogeneous metastases are much more common than lymphatic metastases in HCC, occurring in 51% as opposed to 26% of patients (Watanabe *et al*, 1994). Metastases spread to the lung in the majority of

patients, followed by the kidneys, the skeleton and the diaphragm (in order of frequency).

1.5 Fibrolamellar carcinoma

Fibrolamellar carcinoma (FLC) is a variant of HCC with distinctive histological and clinical features. It has been known by various names such as eosinophilic HCC with lamellar fibrosis, polygonal cell carcinoma with fibrous stroma, eosinophilic glassy cell hepatoma and fibrolamellar oncocytic hepatoma. This tumour is relatively common among whites, occurring with equal frequency in men and women and accounting for 40% of HCC cases in those younger than 35 years. It is nearly non-existent in Asia (Farhi *et al*, 1983). It constitutes about 7% of all primary liver cancer cases (Craig *et al*, 1980). The clinical symptoms are usually non-specific and are often related to the presence of a large palpable hepatic mass and abdominal pain. The tumour typically arises in the non-cirrhotic liver and has serum biochemical markers that differ from the usual HCC. Alpha-fetoprotein is usually normal in FLC (Berman *et al*, 1988), but the levels of serum vitamin B12-binding globulin (Paradinas *et al*, 1982) and neurotensin (Collier *et al*, 1984) are often increased. The presence of copper and copper-binding protein in tumour cells is found not only in FLCs, as previously suggested (Lefkowitch *et al*, 1983), but also in all variants of HCCs and benign hepatic tumours (Guigui *et al*, 1988). There is no recognised association of FLC with HBV, alcohol, cirrhosis, oral contraceptives or genetic disorders such as Wilson's disease.

Histologically, FLC is characterised by large polygonal-shaped cells with eosinophilic granular cytoplasm separated into nests and sheets by lamellar bands of collagen. The nuclei are vesicular and contained prominent eosinophilic nucleoli. Pleomorphism and mitoses are minimal. At the ultrastructural level, the cytoplasm is packed with abundant swollen mitochondria which are relatively uniform in size and shape. The radiographic and macroscopic appearance of this tumour may closely mimic focal nodular hyperplasia. Flow cytometric analysis shows no differences in modal DNA content of FLC from normal liver (Saul *et al*, 1987).

Complete surgical resection results in a cure in 50% to 75% of cases (Starzl *et al*, 1986). Extensive surgical resection is warranted in FLC even with very large tumours (Soreide *et al*, 1986). In a large study of 41 patients with FLC, long-term survival frequently was achieved with aggressive surgical treatment; cumulative survival at 1, 3, 5, and 10 years was 98%, 72%, 66%, and 47% respectively (Pinna *et al*, 1997). Tumour-free survival at these times was 80%, 49%, 33%, and 29% respectively. The involvement of regional lymph nodes, the presence of metastasis and advanced TNM stage were significant prognostic factors reducing tumour-free survival, whereas the presence of macrovascular invasion of the tumour was a significant prognostic factor reducing overall survival. Neoadjuvant chemotherapy with 5-fluorouracil *via* hepatic artery infusion for 1 month can result in tumour shrinkage, allowing surgical resection (Ringe *et al*, 1991). Tumour recurrences in lungs, lymph nodes, peritoneum and liver are reported after intervals ranging from a few months to 10 years (Ang *et al*,

1991). However, resection of recurrent and metastatic tumour is commonly performed and may be beneficial (Soreide *et al*, 1986).

1.6 Clonal analysis of HCC

Tumour cells have been known to be less stable genetically than normal cells, and this instability leads to the development of more mutations in tumour cell genes that are associated with possible regulators of the cell cycle (Nicolson, 1987). The increased genetic instability could produce new subpopulations within the progressing tumour to form clonal heterogeneity. HCC often contains subpopulations of heterogeneous cellular differentiation within each tumour. Most HCCs first appear as well differentiated cancers, and as they proliferate they undergo gradual dedifferentiation. Cancer nodules of less than 1 cm diameter have a uniform distribution of well-differentiated malignant tissue, whereas approximately 40% of cancer nodules 1.1 to 3 cm in diameter consist of more than two malignant components of varying differentiation levels (Kenmochi *et al*, 1987). In such an HCC nodule, moderately or poorly differentiated tissues are always found inside the lesion, and well-differentiated tissues outside. Once the tumour becomes detectable by imaging, however, it is usually already about 1 cm in diameter, representing a total of 10^9 cells. In the evolving process of hepato-carcinogenesis, this is a late event.

When multiple small HCC nodules appear in cirrhotic livers, it is very difficult to decide whether they are attributable to multicentric primary tumours or to intrahepatic metastasis from a single primary nodule. To assess the clonality in tumours, various techniques have been developed such as the

detection of oncogene or tumour suppressor genetic alterations (Tada *et al*, 1990; Hsu *et al*, 1991), the integration of HBV into the cellular genome (Esumi *et al*, 1986; Sakamoto *et al*, 1989; Hsu *et al*, 1991), X-linked restriction fragment length polymorphisms (RFLPs) within the tumour (Vogelstein *et al*, 1985), the analysis of DNA ploidy pattern (Nagasue *et al*, 1992) and the demonstration of a specific cytogenetic abnormality. However, these techniques have some limitations in assessing the clonality in cancers.

The presence of discrete HBV-hybridising bands found in individual tumours suggests clonal expansion of a single transformed hepatocyte. Molecular evidence for a monoclonal origin has been provided by identical Southern blot hybridisation patterns of tumour DNA obtained at different locations within the liver (Imazeki *et al*, 1986; Esumi *et al*, 1986; Blum *et al*, 1987). However, integration sites may occasionally vary in different tumour nodules within the same liver, indicating that some HCCs are multicentric in origin (Esumi *et al*, 1986; Hsu *et al*, 1991). After surgical removal of a primary HCC, recurrent tumours can be shown to arise from the original residual clone in some cases and to represent *de novo* neoplasms in other cases (Chen *et al*, 1989).

1.7 Arbitrarily primed-polymerase chain reaction

DNA fingerprinting technology is a powerful technique that has the ability to distinguish genomic DNA within the same species and has shown forensic and medical applications. The classic approach to DNA fingerprinting relies on enzymes that digest DNA or break it apart. In this way a single

chromosome, which is made up of two complementary strands of DNA containing millions of nucleotide bases, is broken into many smaller fragments. The actual number and size of the DNA fragments depends on the structure of the chromosome. The DNA fragments can be separated on electrophoresis gels according to their molecular size. The fragments appear in the gel as a pattern of bands, referred to as the DNA fingerprint, which visually resembles the "bar codes" used by stores to identify merchandise. Therefore, the clonality of cells can be distinguished by this principle.

An arbitrary primer-based DNA amplification technique has been recently proposed as an alternative targeting tool for genetic typing and mapping. This strategy uses randomly selected primers to initiate amplification of discrete but arbitrary portions of the genome. It has been called by a plethora of terms such as random amplified polymorphic DNA (RAPD) (Williams *et al*, 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al*, 1991), multiple arbitrarily amplicon profiling (MAAP) (Caetano-Anolles *et al*, 1992), and arbitrarily primed-polymerase chain reaction (AP-PCR) (Welsh & McClelland, 1990).

AP-PCR is one of the novel fingerprinting techniques described by Welsh and McClelland in 1990 (Welsh & McClelland, 1990). It was originally used to distinguish strains of *Staphylococcus sp.* and *Streptococcus sp.* by comparing polymorphisms in AP-PCR genomic fingerprints using PCR-length primers (18 to 32 nucleotides). This technique is a modification of PCR, a method that is widely used to copy sections of DNA for identifying

gene structure or matching tissue specimens. PCR uses two primers whose complementary sequences flank the desired sequence to amplify a region of DNA. The primers usually have specific nucleotide sequences that bind to previously identified segments of DNA. They bind to specific sites on opposing strands of the double-stranded DNA and are extended by a thermostable DNA polymerase to make millions of copies of the intervening stretch of DNA. Normally, the primers are annealed to the template DNA at relatively high stringency. High stringency during the primer-annealing step ensures that the primers do not interact with the template DNA at positions where they do not match. By contrast, AP-PCR allows the detection of polymorphisms without prior knowledge of nucleotide sequence. It is based on the selective amplification of genomic sequences that, by chance, are flanked by adequate matches to an arbitrarily chosen primer. The method utilises short primers of arbitrary nucleotide sequence (10 to 20 bases) that are annealed in the first few cycles of PCR at low stringency. The low stringency of the early cycles ensures the generation of products by allowing priming with fortuitous matches or near-matches between primers and template. This approach results in a high number of products having the original primer sequence at both ends. After a few low-stringency cycles, the annealing temperature is raised and the reaction is allowed to continue under standard, high-stringency PCR conditions. This step will amplify a discrete number of sequences among those initially targeted and permits the unbiased analysis of the cell genome (Figure 1). Alternatively, an intermediate stringency primer-annealing step may be used throughout the PCR to achieve the same outcome. AP-PCR products are resolved on polyacrylamide gels and are detected by autoradiography. If two template

genomic DNA sequences are different, their AP-PCR products display different banding patterns. Such differences can be exploited in ways largely analogous to the uses of restriction fragment length polymorphisms, including genetic mapping, taxonomy, phylogenetics and the detection of mutations.

Figure 1. Principle of arbitrarily primed-polymerase chain reaction (AP-PCR)

(a) An arbitrary sequence primer.

(b) Mismatched annealing of primer to partially complementary sequences within total DNA sample under low-stringency conditions.

(c) Primer incorporated into PCR products - bottom strands are now fully complementary to the primer - PCR continues under higher stringency annealing conditions.

1.7.1 The applications of AP-PCR

As no laborious cloning, nucleotide sequencing nor Southern blot hybridisation are required, AP-PCR permits the rapid and cost-effective detection of polymorphisms and genetic markers in a variety of plants and animals. The most frequent use of AP-PCR has been in the detection of dominant polymorphic markers in genetic mapping experiments (Williams *et al*, 1990). Welsh *et al* (1991) applied the technique of AP-PCR to genetic mapping in the mouse. They noted that AP-PCR is, in many respects, dramatically easier and faster than established methods of genetic mapping. Polymorphisms detected by AP-PCR can also be used as taxonomic markers in population studies of a wide variety of organisms (Welsh *et al*, 1992). The method has been applied extensively in plant breeding studies and in the differentiation of the strains of microorganisms (Preus *et al*, 1993). DNA fingerprinting of different strains has shown polymorphic sequences that can be used to identify the different genomes.

The reproducible and semi-quantitative amplification of multiple sequences provides a powerful tool to study somatic genetic alterations in tumourigenesis. Peinado and colleagues showed the ability of AP-PCR to detect both qualitatively and quantitatively and to isolate, in a single step, DNA sequences representing two of the genetic alterations that underlie the aneuploidy of colorectal cancer cell, *ie.* losses of heterozygosity and chromosomal gains (Peinado *et al*, 1992). Moreover, they confirmed that AP-PCR could yield information on the overall chromosomal composition of the cell. The intensities of the bands derived from single-copy sequences were proportional to the concentration of the target sequences. The

outstanding result using AP-PCR fingerprinting in the field of cancer research was the discovery of the microsatellite mutator phenotype mechanism for carcinogenesis in sporadic and hereditary colon cancers (Innov *et al*, 1993). AP-PCR is also useful for the detection and isolation of DNA sequences to levels well below the minimum levels required by other available methods (Roninson *et al*, 1986), and the products can be used to clone or hybridise back to digested genomic DNA (Wesley *et al*, 1990). In addition, AP-PCR can be applied to RNA to detect differentially expressed genes (Welsh *et al*, 1992).

1.8 Tumour angiogenesis

The development of a complex network of blood vessels is essential not only during embryological development, but during a variety of normal and pathological conditions in the adult including ovulation, implantation, bone formation, inflammatory wound repair and tumour growth. Tumour establishment, growth and metastasis are angiogenesis-dependent. It has been suggested that microvessel density may represent a new predictor of survival and metastases in patients with breast cancer (Weidner *et al*, 1991), melanoma (Srivastava *et al*, 1988), lung cancer (Macchiarini *et al*, 1992), prostate cancer (Fregene *et al*, 1993) and squamous cell carcinoma of the head and neck (Albo *et al*, 1994). Human tumours are not usually angiogenic at the beginning of their development. Studies of the vascularisation of hepatic metastases have demonstrated that tumour metastases are avascular up to 1 mm in diameter and are consistently vascularised beyond this (Lien & Ackerman, 1970). The switch to the angiogenic phenotype depends on a net balance of positive and negative

angiogenesis factors (Table 2), which are based i) on the activity of tumour cells, inflammatory cells and extracellular matrix components, ii) on the activity of endothelial cells and iii) on the influx of circulating factors (Figure 2) (Folkman, 1995). Experiments in transgenic mice bearing spontaneous tumours have indicated that not all the cells of a tumour switch to the angiogenic phenotype. Only a subset of cells (as few as 4-10%), become angiogenic (Folkman *et al*, 1989; Kandel *et al*, 1991). In tumourigenesis, the highly angiogenic cells and nonangiogenic cells can reside within the same tumour. Only part of fibrosarcomas arising in transgenic mice underwent rapid expansion whereas the nonangiogenic lesions remained flat and pale (Kandel *et al*, 1991). The onset of angiogenic activity appeared only in a local area of a thin preangiogenic nevus (Srivastava *et al*, 1988). Furthermore, staining of microvessels revealed nonneovascularised as well as neovascularised carcinomas *in situ* in the same histological sections of breast and prostate cancers (Weidner *et al*, 1991, 1993). This suggests a heterogeneity of angiogenic activity within a tumour. In a primary tumour, the areas with the highest microvessel density contain the cells that are more likely to disseminate systemically and to express the angiogenic phenotype than cells escaping from areas with fewer microvessels. Tumour cells that do not express the angiogenic phenotype may become dormant micrometastases. Breakdown of the basement membrane and microinvasion has been observed with and without neovascularisation (Folkman, 1994).

Positive angiogenic factors	Negative angiogenic factors
Vascular endothelial growth factor	Thrombospondin
Acidic/basic fibroblast growth factor	Platelet factor-4
Angiogenin	Heparin/cortisone
Transforming growth factors	Angiostatin
Tumour necrosis factor alpha	Endostatin
Hepatocyte growth factor	Interferon
Granulocyte colony-stimulating factor	Tissue inhibitor of metalloproteinases
Interleukin 8	Cartilage-derived inhibitor
Platelet-derived endothelial growth factor	Prolactin
	Methoxyoestradiol

Table 2. Examples of common positive and negative angiogenesis factors

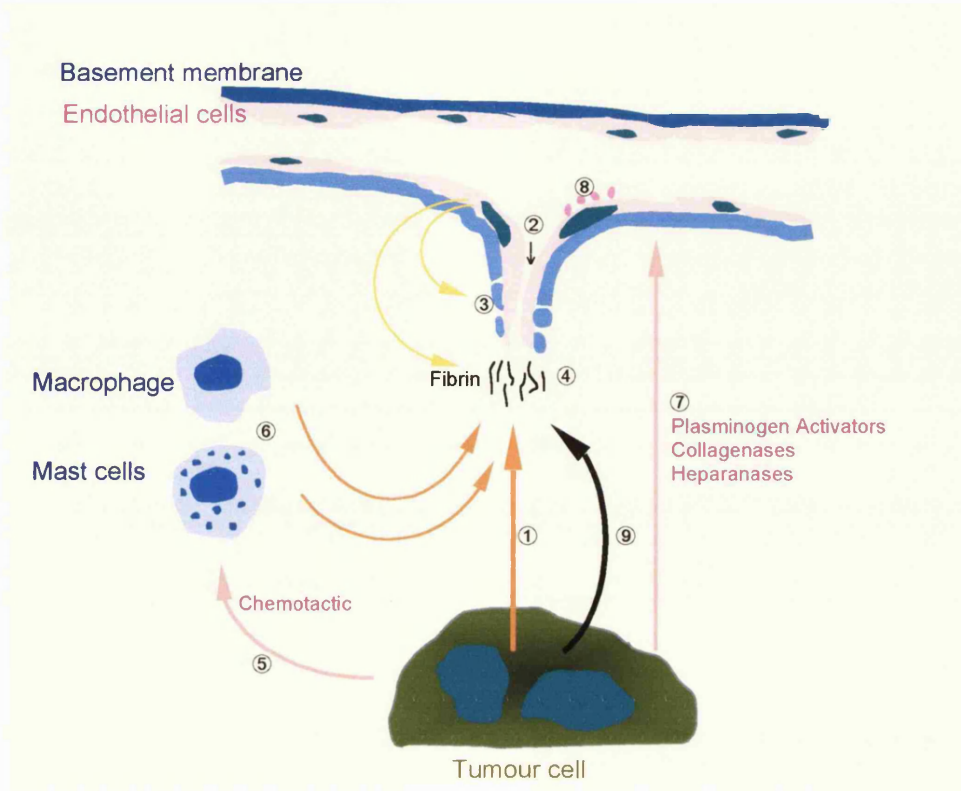


Figure 2. Diagrammatic representation of different mechanisms that may be involved in the switch to the angiogenic phenotype

Some angiogenic molecules are exported out of tumour cells and diffuse through tissue to reach the vasculature ①, stimulate endothelial cell migration ②, stimulate production of collagenases and plasminogen activators by endothelial cells causing the degradation of the basement membrane of the parent venule ③ and cause increased permeability of the capillary bed leading to leakage of fibrin products into the extracellular space ④. Tumours may recruit macrophages and mast cells, which themselves can promote tumour angiogenesis ⑤ and may release specific molecules which can recruit more macrophages ⑥. Tumours may secrete proteases which contribute to degradation of the basement membrane ⑦. In some tumours, endothelial cells may contain significantly more angiogenic molecules than the tumour cells ⑧. Endogenous inhibitors of endothelial growth secreted by tumours may need to be downregulated before the release of angiogenic molecules can be sufficiently unopposed so as to induce neovascularisation ⑨.

1.8.1 The concept of tumour dormancy

In cancer patients, tumour deposits can undergo a period of dormancy for months or years followed by rapid growth during relapse. The processes controlling malignant tumour cells during dormancy may involved a prolonged state of Go (Gunduz *et al*, 1979) or the depletion of hormones in hormone-dependent tumours (Wheelock *et al*, 1981). Recent evidence suggests that angiogenesis will not only enhance tumour growth by supplying more nutrients to the tumour, but also work through paracrine effects of endothelial cells (Folkman, 1994). According to this theory, endothelial cells in the dominant tumours are capable of releasing angiogenic growth factors that promote their own neovascularisation in excess of angiogenesis inhibitors. The angiogenic inhibitor, by virtue of its longer half-life in the circulation, reaches the vascular bed of a smaller synchronous tumour in greater quantities than the angiogenic stimulator that escapes from the dominant tumour or is generated by the smaller tumour. In the absence of circulating inhibitor, angiogenesis could reappear in a smaller tumour because of the same excess of angiogenic stimulator in the local vascular bed. Thus, removal or destruction of the dominant lesion alone leads to accelerated growth of the remaining, previously dormant tumours. Favouring this view, a study has demonstrated that removal of a primary Lewis lung carcinoma tumour in mice results in the exponential growth of its lung metastases (O'Reilly *et al*, 1993). The presence of the primary tumour leads to increased circulating levels of angiostatin, which inhibits growth of Lewis lung metastases *in vivo* by suppressing tumour angiogenesis (O'Reilly *et al*, 1994). Although the metastases exhibited rapid growth when the inhibition of angiogenesis was removed, tumour cell

proliferation was not significantly different in dormant and growing metastases (Holmgren *et al*, 1995). Nevertheless, metastatic-tumour cells exhibited a more than threefold higher incidence of apoptosis. The data suggested that angiogenesis inhibitors control metastatic growth by indirectly increasing apoptosis in tumour cells.

Antiangiogenic therapy is a new approach to the treatment of cancer and one that may be used to augment conventional therapy. Treating neoplasms by targeting both the tumour cells (chemotherapy) and the organ environment (angiogenesis inhibitor) has been shown to produce additive or synergistic therapeutic effects in mice bearing 3LL tumours (Teicher *et al*, 1998). In cancer patients, further therapy with angiogenesis inhibitors after removal of a dominant tumour could suppress the growth of residual or synchronous tumours and maintain them in a state of dormancy.

1.9 Thrombospondin-1 and cancer

Thrombospondin-1 (TSP1) is a multifunctional glycoprotein originally identified as a major component of platelet α granules and with a molecular weight of 450,000 Da. TSP1 gene is located on chromosome 15q15 (Good *et al*, 1990). TSP1 is regulated by several growth factors, oncogenes and tumour suppressor genes including p53, ras, c-jun, v-src, TGF-β, platelet-derived growth factor and FGF-2. For the control of p53, fibroblasts from patients with the Li-Fraumeni syndrome possess one wild-type and one mutant allele of p53 gene, release normal levels of TSP1, but are neither angiogenic nor tumourigenic in immunodeficient mice. After repeated

passage *in vitro*, the fibroblasts lose their remaining wild-type p53, decrease their production of TSP1, become angiogenic and produce neovascularised tumours (Dameron *et al*, 1994). Moreover, whereas TSP1 can be regulated by various factors, its secretion from glioblastoma cells is stimulated not by p53, but by an unidentified gene on chromosome 10q (Hsu *et al*, 1996). In addition to platelets, a variety of other cells have been found to contain and secrete TSP1, including endothelial cells, smooth muscle cells, fibroblasts, pneumocytes, macrophages, monocytes and several tumour cell lines such as squamous carcinoma cells and melanoma cells (Mosher *et al*, 1982; Jaffe *et al*, 1983, 1985; Sage *et al*, 1983; Riser *et al*, 1988).

Human TSP1 was able to block neovascularisation in the rat corneal model and inhibit chemotaxis of capillary endothelial cells towards angiogenic factors (Good *et al*, 1990). The role of TSP1 as an angiogenic inhibitor is further supported by the inability of endothelial cells in fast-growing haemangiomas to produce TSP1 and the ability of antibodies to TSP1 to increase angiogenesis *in vitro* (Bornstein & Sage, 1982). Moreover, TSP1 mRNA has been shown to be downregulated in endothelial cells forming tubes in culture (Canfield *et al*, 1990). TSP1 associated with cancer was first studied in BHK21/CL-13 cells, which are incompletely transformed. During the final step in their transformation, they became tumourigenic and gain the ability to induce angiogenesis (Rastinejad *et al*, 1989). The results have shown that normal BHK cells produce an inhibitor of angiogenesis, which is able to inhibit both endothelial cell migration *in vitro* and neovascularisation in the rat cornea assay. By the time of their transformation to angiogenic neoplastic cells, they produce only 4-6% of the

level of inhibitor originally generated by their normal precursor cells. Later, this angiogenesis inhibitor isolated from BHK cells was reported to be immunologically and functionally indistinguishable from a fragment of TSP1 (Good *et al*, 1990).

The normal TSP1 concentration in circulation is about 0.05-0.4 μg/ml (Kao & Klein, 1986; Tuszynski *et al*, 1992), whereas after the activation of the coagulation system, it increases to 10-30 μg/ml (Switalska *et al*, 1985). Under certain pathological conditions such as in the presence of malignant tumours, the plasma TSP1 concentration increases significantly as well as the amount of TSP1 expressed in tumour tissues (Tuszynski *et al*, 1992; Pratt *et al*, 1989). In cancer, the mean levels of TSP1 in the blood of gastrointestinal, breast and lung cancer patients were two- to three- fold greater than those of controls (Tuszynski *et al*, 1992). These increases were independent of platelet counts and were not seen in nonmalignant disease. The localisation and expression of TSP1 in human malignant tissues has mostly been studied in breast tumours. The cytosolic homogenates of malignant breast tumours contain 10-150 times more TSP1 than those from benign tumours (Pratt *et al*, 1989). Wong *et al* (1992) demonstrated that 96% of malignant breast tumours showed strong TSP1 staining in the desmoplastic stroma juxtaposed to tumour cells or at the basement membrane associated with the malignant ductal epithelium. By contrast, normal breast tissue and benign breast lesions showed no TSP1 staining. This may be a protective effect of TSP1 on tumour invasiveness and metastasis. TSP1 production from human breast, human lung and

murine melanoma carcinoma cells correlated inversely with tumourigenicity and metastatic potential (Zabrenetzky *et al*, 1994). The level of TSP1 expression can provide prognostic information with respect to disease recurrence and overall survival in patients with invasive bladder cancer (Grossfeld *et al*, 1997). Patients with low TSP1 expression exhibit an assume increased risk of disease recurrence and a decreased overall survival when compared with patients with moderate or high TSP1 expression. The association is independent of tumour stage, histologic grade, and lymph node status.

Few studies have been reported about TSP1 in HCCs. TSP1 was observed on the rough endoplasmic reticulum (RER), a major organelle in protein synthesis and secretion, of the KIM-1 HCC cell line (Yamashita *et al*, 1998). TSP1 was expressed in tumour cells of 31/60 (51.7%) HCC cases, and in 23 of these 31 cases, TSP1-positive tumour cells comprised less than 5% of all tumour cells. In noncancerous areas, hepatocytes were negative for TSP1. Fibrous tissues and vascular endothelial cells in HCC and surrounding nonmalignant tissues were diffusely positive for TSP1 in all cases, with markedly stronger staining intensity than in distal tissues (Hayashi *et al*, 1997). Moreover, the positive reaction was observed on the RER and on the microvilli of the HCC cells on the side of the blood space, while the microvilli on the side of the bile canaliculus-like structure were negative. However, in cholangiocarcinoma (CCC) which was found to be hypovascular compared with HCC, the TSP1 expression was more strongly in cancer cells and fibroblasts of CCC than of HCC (Kawahara *et al*, 1998).

The ratio between TSP1 mRNA levels in cancer cells and in adjacent noncancerous cells were higher in CCC than in HCC.

Research Aims

To understand the genetic basis for the development of hepatocellular carcinomas by:

1. Determining the incidence of p53 mutation in synchronous hepatocellular carcinomas.
2. Establishing the DNA fingerprinting technique, arbitrarily primed-polymerase chain reaction, to generate genomic DNA fingerprints from tissue samples.
3. Determining the genotypic aspects of synchronous hepatocellular carcinomas, metastatic hepatocellular carcinomas and fibrolamellar carcinomas.
4. Studying the genomic fingerprints from tissues prepared by the novel technique of laser capture microdissection.
5. Studying the role of an antiangiogenic factor, thrombospondin-1, in the proliferation and invasiveness of hepatocellular carcinoma cells *in vitro*, and tumour growth *in vivo*.

CHAPTER II

MATERIALS & METHODS

II. MATERIALS AND METHODS

2.1 Reagents and solutions

Most of the stock solutions used in this study were prepared according to instructions in Molecular Cloning: A Laboratory Manual (Sambrook *et al*, 1989), unless otherwise stated. The radiolabelled chemicals [γ-^{33}P]-dATP, [α-^{32}P] dCTP, [^{3}H]-thymidine were supplied as aqueous solutions by Amersham International. All oligonucleotides were synthesised by the Department of Virology, Hammersmith Hospital. *Taq* DNA polymerase (BioTaq) and T4 polynucleotide kinase were obtained from Bioline, UK. DNA restriction enzymes were supplied by Boehringer Mannheim, Life Technologies or New England Biolabs. The reaction conditions for the digests were according to the manufacturer's instructions in the appropriate buffer. Ethidium bromide solution at 10 mg/ml, proteinase K, and DNA and RNA size markers were supplied by Gibco-BRL, Life Technologies, UK. Phenol/chloroform/isoamyl alcohol (25:24:1, by volume), Phenol buffered with 0.1 M Tris, diethyl pyrocarbonate (DEPC), ampicillin and NP-40 were obtained from Sigma, UK. Glyoxal solution was from BDH. Hybond N membrane was from Amersham. Whatman chromatography 3MM paper was obtained from Whatman, Ltd. Agarose, acrylamide:bisacrylamide, ultra-pure sequagel, urea, ammonium persulfate and TEMED (*N,N,N',N'*-tetramethylethylenediamine) were supplied by Flowgen, UK. X-OMAT XAR-5 X-ray film was obtained from Eastman Kodak, NY, USA.

All chemicals used for RNA preparation were of Molecular Biology grade with no detectable RNase or DNase activities. All solutions employed for the preparation and manipulation of nucleic acids were made up using distilled deionised water (MilliQ). All solutions were autoclaved before use or, in the case of thermolabile substances, filter-sterilised using a 0.22 μm filter and stored in a sterile container. The materials used in each experiment, whether cell culture or molecular biology techniques, were carefully selected to conform to standard laboratory safety procedures. The processes of cleansing or sterilisation either before or after *in vitro* or *in vivo* experiments were strictly adhered to according to the manufacturers' instructions and/or the good local laboratory codes of practice. The details of materials and recipes of solutions commonly used in the experiments are described in Appendix I.

2.2 Tissue microdissection and DNA preparation

The majority of specimens used in this study are formalin-fixed, paraffin-embedded tissues. One 5-μm section and 10-μm sections were serially cut from each block by microtome. Sections were collected onto glass slides and oven dried overnight at 37°C. The 5-μm section was stained with Haematoxylin and Eosin (H&E) for histological confirmation. The remaining 10-μm sections, tissue samples were separately prepared by microdissection using sterile 27-gauge needles with the assistance of a dissecting microscope and reference to a section stained with H&E. Deparaffinisation was achieved by incubating in 400 μl xylene for 20

minutes at 55°C and then washing twice with 500 µl absolute ethanol. DNA was prepared by incubating tissue in lysis buffer (10 mM Tris.Cl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, Proteinase K 500 µg/ml) overnight at 55°C. Phenol/chloroform/isoamyl alcohol (25:24:1, by volume) (Sigma) was added and mixed with the lysate. The two phases were separated by centrifugation for 10 minutes, and the upper phase was transferred to a fresh microcentrifuge-tube. Extraction with phenol was repeated twice. DNA was precipitated from the aqueous phase by adding two volumes of absolute ethanol and 0.1 volume 3M sodium acetate (pH 7.0), and the solution was left at -20°C for a few hours or longer for the DNA to precipitate. Precipitated DNA was pelleted by centrifugation at 10000 g for 10 minutes. The pellet was washed with 70% ethanol and dried by evaporation, then dissolved in sterile water or Tris/Ethylenediaminetetra-acetic acid (TE) buffer. The concentration of DNA was obtained by measuring its absorbance in solution at a wavelength of 260 nm in an UV spectrophotometer. The reading at this wavelength allows calculation of the DNA concentration. An optical density (OD) of 1 corresponds to approximately 50 µg/ml. The ratio between the readings at 260 nm and 280 nm (OD260:OD280 ratio) provides the estimate of the purity of DNA. When this ratio was about 1.8, the DNA was considered sufficiently pure for analysis. Samples were stored at 4°C or -20°C.

2.3 p53 mutational analysis

2.3.1 PCR amplification of p53

Polymerase chain reaction (PCR) was performed by cycling samples containing template DNA mixed with sequence-specific oligonucleotide primers through three temperature incubations in the presence of *Thermus aquaticus* (*Taq*) DNA polymerase. These cycles are required to: 1) denature double stranded DNA; 2) anneal primers to DNA; and 3) extend target sequences by *Taq* DNA polymerase. Each PCR reaction consist of: 200 ng of genomic DNA, 100 pmol of each primer, 1 unit of *Taq* DNA polymerase, 200 μM of dNTPs (Bioline), PCR buffer (50 mM Tris.Cl pH 9, 20 mM ammonium sulphate, 1.5 mM $MgCl_2$) and sterile-distilled water added to a total volume of 50 μl. Thirty-five cycles of amplification were performed consisting of one minute denaturation at 94°C, one minute annealing temperature and one minute extension at 72°C after an initial 5 minutes denaturation step at 94°C. A final extension for 10 minutes at 72°C was also performed. The incubations were performed in a Hybaid Thermoreactor (Hybaid).

DNA amplification of each sample was performed with a pair of outboard primers around either exon 5, exon 6, exon 7, exon 8 or exon 9. Primer locations and strategy are shown in figure 3, and the details of primers are shown in table 3 (the sequence of these primers and of all other oligonucleotides is shown in 5′ to 3′ orientation):

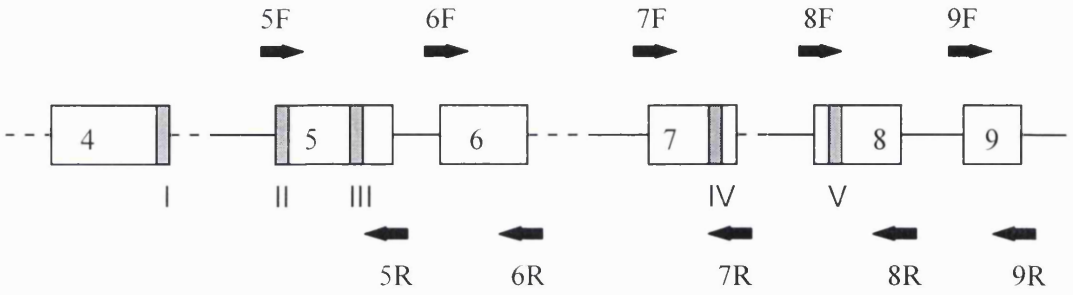


Figure 3. Regions of p53 gene from exon 4 to exon 9. Each sample was analysed with specific primer of exons 5, 6, 7, 8, and 9. Conserved regions I, II, III, IV and V of the p53 gene are indicated in exons 4 to 8. The numbered arrows represent the position of the primers used for DNA amplification and sequencing.

Exon	Primers	Sequence (5′ to 3′)	Position	Size (bp)
5	5F	CAACTCTGTCTCCTTCCTCT	13005-13024	310
	5R	ATCAGTGAGGAATCAGAGGC	13314-13293	
6	6F	CCTCACTGATTGCTCTTAGG	13257-13276	229
	6R	ACTGACAACCACCCTTAACC	13485-13465	
7	7F	TGCTTGCCACAGGTCTCCCCAA	13939-13960	234
	7R	TCAGCGGCAAGCAGAGGCTGG	14172-14152	
8	8F	CTTACTGCCTCTTGCTTCTC	14403-14422	239
	8R	GCTTCTTGTCCTGCTTGCTT	14641-14622	
9	9F	AAGCAAGCAGGACAAGAAGC	14622-14641	192
	9R	CCACTTGATAAGAGGTCCCA	14813-14793	

Table 3. Summary of the specific primers used to study the p53 (exons 5-9) mutation

2.3.2 Agarose gel electrophoresis

Agarose electrophoresis was performed in a horizontal submerged gel apparatus supplied by Flowgen. Gels were prepared by adding agarose (Seakem, Flowgen) to 200 ml 1X TBE to make a solution of 2% and heating to dissolve in a microwave oven. On cooling to below 50°C, 2 µl of ethidium bromide stock solution (10 µg/ml) was added. Gels were poured into a gel former with a well-comb in place. After setting, the gel was submerged in an electrophoresis tank containing 1X TBE buffer. Loading buffer (1/6 volume of 6X stock solution: 0.25% bromophenol blue, 0.25% xylene cyanolFF, 30% glycerol in water) was added to the PCR products which were then transferred into the wells, and electrophoresis was performed at constant voltage (80 volts) for 2-4 h. The gel was transilluminated with short wave ultraviolet light generated by a FotoPrepI transilluminator (Fotodyne, USA), and the DNA was visualised by photography using a UV camera with Polaroid 667 instant film. DNA fragments were sized by reference to a standard DNA ladder (Gibco-BRL, Life Technologies) which was run concurrently.

2.3.3 Recovery of DNA product

The amplified fragment was run on 2.0% agarose gel and visualised by UV light. The bands of interest were excised using a sterile scalpel blade. The DNA was purified from the gel using Qiagen's gel extraction kit (QIAGEN) following the instructions provided by the manufacturer. The method is based on the binding of DNA to silica under high salt conditions. The DNA was eluted from the column with 50 µl sterile-distilled water into a clean 1.5-

ml microfuge tube. One microlitre of the elute was run on an agarose gel to confirm successful purification of the DNA fragment.

2.3.4 Sequencing analysis

Sequencing of the purified PCR product was carried out using the new Thermo Sequenase ^{33}P labelled terminator cycle sequencing kit from USB-Amersham (Amersham, USA). This new kit launched in January 1997 combines Amersham's new thermostable Sequenase with a cycle-sequencing protocol and the concept of labelling the dideoxy-nucleotide mixes, so that only when an amplification product is terminated with one of these isotopic ddNTPs does the fragment get labelled. Briefly, 2 µl dGTP Termination master mix (7.5 µM dATP, dCTP, dTTP, dGTP) was mixed with 0.5 µl of [α-^{33}P] ddNTP (dATP, dCTP, dTTP, dGTP) to produce a termination mix for each ddNTP. Sequencing reactions were carried out by adding 4.5 µl of the reaction mixture containing 100 ng template DNA product, 260 mM Tris.Cl pH 9.5, 65 mM $MgCl_2$, and 2 units of Thermo Sequenase polymerase (0.0006 U/µl Thermoplasma acidophilum inorganic pyrophosphatase, 50 mM Tris.Cl pH 8, 1 mM DTT, 0.1 mM EDTA, 0.5% Tween-20, 0.5% NP-40, 50% glycerol). The reactions were overlaid with 10 µl mineral oil and were cycled through the following temperature profile: 95°C for 30 seconds for denaturing, 55°C for 30 seconds for annealing of primer and 72°C for 1 minute for extension for 35 cycles. All amplifications were performed in a Hybaid Thermoreactor (Hybaid). After finishing, 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each termination reaction.

For sequencing, a denaturing 6% acrylamide gel matrix was used with an acrylamide:bisacrylamide ratio of 19:1 in Tris/Boric acid/Ethylenediamine-tetraacetic acid (TBE) buffer equilibrated in 8M Urea. Samples were denatured by heating at 70°C-80°C for 2-10 minutes immediately before loading onto a glycerol-tolerant sequencing gel. A standard pipette tip was used to load 3-5 µl in each lane. Electrophoresis was carried out at 50°C-55°C in a Model S2 Sequencing Gel Electrophoresis Apparatus (Gibco-BRL, Life Technologies). After electrophoresis, the apparatus was dismantled and the gel was soaked in 10% acetic acid, 10% methanol for 10 minutes to remove the urea. Then it was mounted wet on a piece of a Whatman 3MM paper and covered with Saran-wrap™. Because the lower energy of emission of ^{33}P, the gels have to be dried down on a slab dryer (Flowgen) with a vacuum pump drawing off the moisture at 80°C for 2 h. The dried gel was then exposed to X-ray film (BioMax MR, Kodak) at room temperature overnight or for 2-3 days as required.

2.3.5 Immunohistochemical analysis of p53

A mouse monoclonal antibody, DO-7 (DAKO-p53, Dakopatts, Denmark) recognises an epitope in the N-terminus of the human p53 protein. The epitope for the antibody is known to reside between amino acids 35-45, and it reacts with the wild type and mutant type of p53 protein. Five-micron thick sections were dewaxed in xylene, passed through alcohol and washed in PBS. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in PBS for 30 minutes at room temperature. The slides must not dry out during staining, as this could cause gross nonspecific

staining. The section was then incubated for 1 h in 1:50 dilution of primary antibody, which was detected using the avidin-biotin complex method (ABC system, DAKO Ltd) with diaminobenzidine tetrachloride (DAB, Sigma Biosciences) (125 μg/ml) as the chromogen. Slides were washed in water and lightly counterstained with haematoxylin for 5 seconds. They were washed briefly in water, dehydrated by taking through graded alcohol (*ie.* 70% and 100% methanol for 2 minutes each) and finally cleared by placing in xylene. Each slide was mounted in Pertex synthetic-mounting medium (Histolab AB, Sweden), then dried and viewed under a light microscope (Olympus, Japan). The immunoreactivity for each antigen was assessed semiquantitively (0 = no positive staining, to +++ = intense membrane immunostaining). A section of colonic adenocarcinoma which was previously shown to express a p53 gene with an activating point mutation was included as a positive control. The negative control comprised the omission of the first antibody from the reaction. Cytoplasmic staining was regarded as non-specific. Sections were read as positive if nuclear p53 was detected in more than 10% of the tumour cells.

2.4 Arbitrarily primed-polymerase chain reaction (AP-PCR)

2.4.1 Polynucleotide Kinase reaction

This procedure is useful for radioactive labelling of the 5′ end of oligonucleotide primers. The kinase enzyme requires that the 5′ end of the oligonucleotide has been previously dephosphorylated with alkaline phosphatase. The forward kinase reaction catalyses the exchange of the

terminal γ–phosphate, which is labelled with ^{33}P from the ATP to the terminal phosphate on the oligonucleotide. Conditions are as follows: 50 mM Tris.Cl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM spermidine, 100 pM of oligonucleotide, 50 μCi of [γ-^{33}P]-dATP and 10 units of T4 polynucleotide kinase (T4 PNK) enzyme. The reaction is allowed to proceed for 1 h at 37°C.

2.4.2 AP-PCR amplification

The amount of DNA template was equalised in the PCR reaction mixture by diluting the DNA stocks to comparable concentrations. PCR amplifications were carried out using 200 ng template DNA in a reaction mixture containing [γ^{33}P]-ATP-labelled and kinased arbitrary primer, 0.2 mM each dNTP (Bioline), 10 mM Tris.Cl pH 9.2, 3.5 mM $MgCl_2$, 75 mM KCl, and 0.5 units of *Taq* DNA Polymerase (Bioline) in a final volume of 25 μl. The reactions were overlaid with mineral oil. For the thermocycling profile, we used a two-step protocol with 5 low-stringency steps, followed by 35 high-stringency steps as follows:

- 5 low-stringency cycles (94°C, 1 min; 50°C, 5 min; 72°C, 5 min) then
- 35 high-stringency cycles (94°C, 1 min; 60°C, 1 min; 72°C, 2 min)
- A final chase cycle of 72°C for 5 min was added to allow complete elongation of all products.

After five low-stringency cycles, exact copies of the primer sequence flank a handful of anonymous sequences. Thus, the annealing temperature can be raised after a few cycles and the reaction allowed to continue under

standard, high-stringency PCR conditions. The two-step low-high stringency protocol was designed to avoid internal priming within a larger amplifying product. To ensure reproducibility, all amplifications were performed in duplicate on each sample with a Hybaid Thermoreactor (Hybaid).

2.4.3 Polyacrylamide gel electrophoresis

The PCR product was added to 5 µl of dye mixture and subsequently run on polyacrylamide gel electrophoresis. Polyacrylamide gels are made from acrylamide and *N,N*-methylene bisacrylamide mixtures dissolved in electrolyte and polymerised by the addition of a chemical catalyst (10% ammonium persulfate and TEMED). The acrylamide concentration and the proportion of bisacrylamide added as cross-linker determine the physical properties and resolving power of the gels. The wide range of pore sizes that can be obtained by varying the ratio of acrylamide:bisacrylamide makes this a very popular choice, and gels can be made for many different purposes. For the purposes of displaying the AP-PCR generated fingerprints, a non-denaturing 8% acrylamide gel matrix was used with an acrylamide: bisacrylamide ratio of 29:1 in TBE electrolyte. For the most part the gels were run relatively slowly overnight at 400V constant voltage supply in a Model S2 Sequencing Gel Electrophoresis Apparatus (Gibco-BRL, Life Technologies). An overnight run meant the AP-PCR fingerprint was well displayed under these conditions.

2.4.4 Autoradiography

After electrophoresis, the apparatus was dismantled and the gel was mounted wet on a piece of a Whatman 3MM paper and covered with Saran-wrap. Because of the lower energy of emission of ^{33}P, the gels have to be dried down on a slab dryer (Flowgen) with a vacuum pump drawing off the moisture at 80°C for 2 h. The dried gel was then exposed to X-ray film (BioMax MR, Kodak) at room temperature overnight or for 2-3 days as required.

2.5 Thrombospondin-1 cDNA construct

2.5.1 Thrombospondin-1 preparation and transformation

Thrombospondin-1 cDNA cloned into the *HindIII* and *XbaI* sites of pcDNA1/Neo plasmid vector (Figure 4) was kindly supplied by Dr NP Bouck, Department of Microbiology-Immunology and Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, Illinois, USA. The transformation of TSP1/pcDNA1/Neo into competent *Escherichia coli* bacteria, DH5α strain (Gibco-BRL, Life Technologies) was performed by the heat-shock technique.

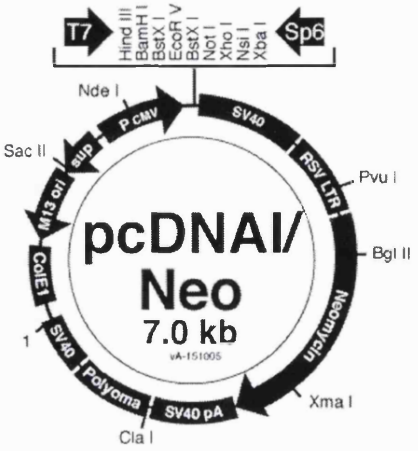


Figure 4. pcDNA1/Neo plasmid vector

2.5.2 Bacterial transformation

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognised by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation.

The DH5α strain of *E.coli* competent cells were thawed on ice, and 50 µl aliquots were placed in cold 1.5 ml-microcentrifuge tubes. One microlitre of DNA was added to the competent cells and gently mixed, then incubated on ice for 30 minutes. Cells were heat-shocked in a 37°C water bath tank for 45 seconds exactly, immediately placed on ice for 2 minutes, and 950 µl of Luria-Bertani (LB) medium (5 g/l bacto yeast extract, 10 g/l bacto tryptone, 10 g/l NaCl pH 7.0) were added. Cell mixture was placed in an

orbital shaker at 250 rpm at 37°C for 1 h. 150 µl cell mixture was plated out on an LB agar plates (5 g/l bacto yeast extract, 10 g/l bacto tryptone, 10 g/l NaCl, 15 g/l bacto agar) containing 100 µg/ml ampicillin. After incubation at 37°C overnight, only bacteria which possess the plasmid DNA will have the ability to metabolise ampicillin and form colonies. Single bacterial colonies were inoculated in 10 ml of LB medium containing 50 µg/ml ampicillin and incubated overnight in a shaking incubator at 37°C. Plasmid DNA was prepared using kits supplied by Qiagen and followed the manufacturer's guidelines.

2.5.3 Plasmid DNA preparation

Plasmids are small circular double-stranded DNA molecules of bacterial origin which replicate independently of the host cell chromosome. Plasmid DNA exists in a covalently closed circular form and is smaller than bacterial chromosomal DNA. The lysis of bacterial cells in the preparation of plasmid DNA may be achieved by boiling the bacterial culture or by treatment with alkali (which denatures chromosomal and plasmid DNA) and SDS (which denatures bacterial proteins). The mixture is then neutralised with potassium acetate, which causes the covalently-closed plasmid to reanneal. The chromosomal DNA and proteins precipitate along with a potassium-SDS complex and are removed by centrifugation. The plasmid DNA may be concentrated from the supernatant by ethanol precipitation.

2.5.3.1 Small scale preparations of plasmid DNA

Plasmid DNA was prepared from small cultures of bacteria using a QIAprep plasmid minipreparation kit (QIAGEN). This procedure was based on the alkaline lysis method for rapid extraction of plasmid DNA from bacterial cells followed by the adsorption of DNA onto silica in the presence of high salt. Three millilitres of the overnight cultures were centrifuged at 10,000 g for 1 minute and the bacteria were then resuspended in 250 µl of resuspension buffer P1 (50 mM Tris.Cl pH 8.0, 10 mM EDTA, 100 µg/ml Rnase A). 250 µl of lysis buffer P2 (200 mM NaOH, 1% SDS) was then added and mixed gently, followed by adding 350 µl of neutralisation buffer N3 (3 M potassium acetate pH 5.5) which adjusts the sample to high salt binding conditions and causes precipitation of denatured proteins, SDS, cellular debris and chromosomal DNA. The samples were then centrifuged at 10000 g for 10 minutes and the supernatants were then transferred to individual columns. Further centrifugation at 10000 g for 2 minutes caused flow through the silica membrane which forms the floor of the columns. After washing with 0.75 ml of buffer PE (200 mM NaCl, 20 mM Tris.Cl pH 7.5, 5 mM EDTA, 95% ethanol) to remove salts, the DNA was eluted by applying 50 µl of sterile-distilled waster to the silica membrane.

2.5.3.2 Large scale preparation of plasmid DNA

The Qiagen Plasmid Maxi kit was used which was based on the modified alkaline procedure followed by binding of plasmid DNA to an anion-exchange resin. A single bacterial colony was used to inoculate a 5 ml volume of L-broth containing ampicillin which was incubated for 8 h in a

shaking incubator at 37°C. Then 1 ml of this culture was used to inoculate 250 ml of L-broth containing ampicillin which was then incubated overnight. The bacteria were pelleted by centrifugation at 6000 g for 20 minutes (J2-H2 centrifuge, Beckman) and resuspended in 10 ml of resuspension buffer P1. Ten millilitres of lysis buffer P2 was then added and left at room temperature for 5 minutes. Ten millilitres of neutralisation buffer N3 pre-chilled to 4°C was added and the sample was centrifuged at 20000 g for 30 minutes. The resulting cell lysate was then filtered onto a QIAGEN-tip which had been pre-equilibrated with 10 ml buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% ethanol, 0.15% TritionX-100) and allowed to enter the anion-exchange resin by gravity flow. Under these conditions, the plasmid DNA binds to the anion-exchange resin. The resin was then washed with 60 ml of medium salt buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% ethanol) to remove RNA, proteins and low molecular weight impurities. The DNA was eluted with 15 ml of high salt buffer QF (1.25 M NaCl, 50 mM Tris.Cl pH 8.5, 15% ethanol), and was then desalted by precipitation with 10.5 ml isopropanol. The DNA was pelleted by centrifugation at 15000 g for 30 minutes at 4°C, washed with 70% v/v ethanol, air-dried and then dissolved in sterile distilled water.

2.5.4 Digestion of DNA with restriction enzyme

Plasmid DNA was digested in volumes of 30 µl using 1-2 units of enzyme per µg of DNA for 60 minutes at 37°C. Appropriate buffers supplied by the manufacturer were used. Then DNA electrophoresis was carried out on 1 %

agarose gel with ethidium bromide. The correct band of TSP1 cDNA was purified using Qiagen's gel extraction kit (section 2.3.3).

2.6 Cell line and cell culture

The human hepatic adenocarcinoma cell line SK-Hep-1 is an aneuploid adenocarcinoma isolated from ascitic fluid in a 52-year-old Caucasian male; it forms a large-cell carcinoma consistent with hepatoma in nude mice.

To maintain healthy and contamination-free cultures, all procedures were carried out in a Class II laminar flow cabinet and all materials used were sterile. Cells were routinely cultured in RPMI-1640 medium, R0883, (Gibco-BRL, Life Technologies) containing 10% heat-inactivated foetal calf serum (FCS; PAA Laboratories GmbH) and 2mM L-glutamine (Gibco-BRL, Life Technologies). Adherent cells were grown as monolayers in an atmosphere of 5% CO_2 in a 37°C humidified incubator and grown to confluence in plastic tissue culture flasks (Falcon, Becton Dickinson). Exhausted media, due to cell metabolism and some spontaneous degradation of constituents needed to be changed periodically to ensure optimum growing conditions. Cells had to be subcultured into new flasks when they had either covered the surface available for growth or depleted the nutrients in the medium. Prior to subculturing, the medium was removed and cells were washed once with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS). Then cells were detached from culture flasks using trypsin (0.05% w/v)-5 mM EDTA (Gibco-BRL, Life Technologies) solution, resuspended in fresh medium containing

10% foetal calf serum, and spun at 1500 g for 5 minutes. The supernatant was removed and the cells were resuspended in the fresh medium. The appropriate volume of cells was replated in new tissue culture flasks contained fresh medium. Cell counts were performed using an Improved Neubauer haemocytometer and an inverted microscope. Cells were regularly screened for and were free of *Mycoplasma* contamination. For storage, cells were trypsinised, pelleted and resuspended at approximately $5x10^6$ cells/ml in FCS containing 10% v/v dimethylsulphoxide (AnalaR, BDH), the presence of which prevented the formation of damaging ice crystals. One-millilitre aliquots were transferred to 1.5 ml Nunc cryotubes which were then frozen slowly and stored immersed in liquid nitrogen (-196°C). Recovery of cells from liquid nitrogen storage was performed by rapid thawing in a 37°C water bath. Thawed cells were washed in 10 ml of medium, harvested by centrifugation and were then transferred to 25 cm^2 flasks containing fresh culture medium.

2.7 Transfection of SK-Hep-1 cell line

2.7.1 Calcium phosphate cell transfection

The human hepatocellular carcinoma cell line SK-Hep-1 was transfected with TSP1 cDNA cloned using the calcium phosphate technique. This method involves mixing DNA with $CaCl_2$ and a phosphate buffer to form a fine precipitate which is deposited onto the cultured cells. Reagents provided in a calcium phosphate mammalian cell transfection kit (5 prime→3 prime, Colorado, USA) were used. The SK-Hep-1 cells were

grown to 30% confluency in a well of 6-well plates. Two hours prior to transfection, the medium was replaced with fresh medium supplemented with 10% FCS. In one sterile microfuge tube, 10 μg of the plasmid DNA, 62 μl of 2 M calcium phosphate and sterile water to a total volume of 500 μl were added. In the other microfuge tube, 500 μl of 2X DNA precipitation buffer (50 mM Hepes pH 7.05, 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM glucose) was added. Air was bubbled with a pipette through the 2X DNA precipitation buffer while slowly adding the $CaCl_2$/DNA solution from the first tube. This was then allowed to stand at room temperature for 30 minutes. The solution was vortexed prior to adding it dropwise to the cells with constant swirling of the plate to mix. Within 15 minutes, a fine precipitate could be seen on top of the cells by light microscopy. The cells were incubated with the calcium phosphate/DNA in 37°C incubator for 4 h. The medium was then removed, the cells were washed once with serum-free medium, and 1 ml of 15% glycerol shocking buffer (2X DNA precipitation buffer:sterile glycerol, 2.5:1 v/v) was added. The glycerol shocking period was allowed for 1 minute at room temperature. Then the contents were removed and the cells were washed once with serum-free medium. The fresh medium supplemented with 10% FCS was finally added and the cells were incubated in 37°C for 48 h.

2.7.2 Selection of the transfected cells

After 48 h of incubation, the transfected cells were almost confluent. The medium was removed and the cells were trypsinised from the plate and split onto 10-cm dishes. Medium supplemented with 10% FCS was added

to a total of 10 ml. At this stage, selection was commenced for cells which had been successfully transfected with TSP1/pcDNA1/Neo by the addition of the antibiotic geneticin (G418, 1 mg/ml) (Gibco-BRL, Life Technologies). Medium plus geneticin was changed every three days. After approximately 10 days, only dispersed colonies of resistant SK-Hep-1 cells were seen. Twenty-four of these colonies were picked by placing a sterile cloning ring over the colony and then adding 100 µl of trypsin to detach the cells. The colonies of cells were then transferred to individual wells of a 6-well plate, fed with fresh medium and allowed to grow to subconfluency before being expanded onto 10-cm plates and then 80 cm^2 culture flasks, respectively.

2.8 Flow cytometry (FACS) analysis

The expression of TSP1 on SK-Hep-1 cells was detected using flow cytometry. Cells were trypsinised and washed once in growth medium and twice in ice-cold wash buffer (PBS with 0.1% w/v BSA and 0.1% sodium azide) and then resuspended in wash buffer at a concentration of $5x10^5$ cells/ml. 150 µl cell suspension (8.0-1.2 x 10^5 cells) was transferred to a clean Falcon 2052 tube. Three tubes were prepared for each sample: unstained (PBS only), negative control (mouse IgG) and positive stained. Primary antibodies (1:500) and mouse IgG (1:1000) were diluted in FACS washing buffer. Fifty microlitres of PBS or mouse IgG or suitably diluted primary antibody was applied to cells labelled as unstained, negative control and positive stained cells, respectively. Tubes were incubated at 4°C for 1 h. Cells were spun at 1000 rpm for 5 minutes and were then

washed twice with 150 µl ice-cold FACS washing buffer. Fifty microlitres of the secondary antibody (FITC - conjugated swine anti-rabbit antibody: Dako F 252 - diluted 1/40 with FACS washing buffer) was applied to cells and incubated at 4°C for 45 minutes. Cells were spun at 1000 rpm for 5 minutes and were then washed twice with 150 µl ice-cold FACS washing buffer. Finally, cells were resuspended in 400 µl ice-cold FACS washing buffer ready for analysis by the FACS Machine (Coulter, USA). Alternatively, cells were fixed in 150 µl 4% fresh formaldehyde solution (4% in PBS) at 4°C overnight, resuspended in 400 µl ice-cold FACS washing buffer and stored at 4°C until analysing.

2.9 Lysis of cultured cells

Cells were detached by a cell dissociation buffer or cell scraper and were washed twice with cold PBS. $1x10^7$cells were resuspended in 1 ml ice-cold single detergent cell lysis buffer (150 mM NaCl, 50 mM Tris.Cl, 1% triton-X, 1µg/ml aprotinin, 100 µg/ml PMSF, 0.02% sodium azide) and were incubated on ice for 30 minutes. The solution was transferred to a microcentrifuge tube and was centrifuged at 12000 g for 10 minutes. The supernatant was carefully transferred to a new microcentrifuge tube and immediately stored at -20°C.

2.10 Measurement of protein concentration

The Lowry procedure has been found to be the most reliable and satisfactory method for quantitation of soluble proteins. The procedure is based on Peterson's modification of the micro-Lowry method and utilises sodium dodecylsulphate, included in the Lowry Reagent, to facilitate the dissolution of relatively insoluble lipoprotein. The principle of this technique is an alkaline cupric tartrate reagent which complexes with the peptide bonds and forms a purple-blue colour when the phenol reagent is added. Absorbance is read at a suitable wavelength between 500-800 nm. The protein concentration is determined from a calibration curve.

The protein assay kit (Sigma Diagnostics, USA) was used. The protein standard solution was diluted by water to a volume of 1 ml in appropriately labelled test tubes to give protein concentrations of 50, 100, 200, 300 and 400 μg/ml. For the blank test tube, 1 ml of distilled water was added. After adding samples to appropriately labelled test tubes and diluting to 1 ml with water, 1 ml Lowry Reagent solution was added to standard, blank and sample tubes and was mixed well. All solutions were allowed to stand at room temperature for 20 minutes. With rapid and immediate mixing, 0.5 ml Folin & Ciocalteu's phenol reagent working solution was added to each tube. The colour was developed within 30 minutes before transferring solutions to cuvettes and measuring the absorbance of the standard and sample tubes *vs.* the blank at a wavelength of 570 nm. Readings were completed within 30 minutes. The absorbance values of the standards were plotted against corresponding protein concentrations to prepare a

calibration curve. Determination of the protein concentration of the sample tube was derived from the calibration curve and multiplying the results by the appropriate dilution factor to obtain the protein concentration in the original sample.

2.11 Detection of TSP1 level using the enzyme-linked immunosorbent assay (ELISA)

To measure the level of TSP1 in culture medium and cell lysate, the Asserachrom Thrombospondin ELISA kit (Diagnostica Stago, France) was used which employed a quantitative sandwich enzyme immunoassay technique. A micro ELISA plate is coated with anti-thrombospondin antibodies. Standard thrombospondin (calibrator) or tests sample are introduced into the plate. Thrombospondin binds by one of its antigenic determinants. Anti-thrombospondin peroxidase is then added and is capted by the other free antigenic determinants. The bound enzyme peroxidase is revealed by its activity on the substrate ortho-phenylenediamine/hydrogen peroxide. After the colour development, the reaction is stopped by addition of sulphuric acid and the absorbance is measured at 492 nm. The observed optical density is directly proportional to the thrombospondin concentration.

All reagents were prepared at room temperature. The thrombospondin standard solution was diluted by the dilution buffer to a volume of 1 ml in appropriately labelled test tubes to give concentrations of 100, 50, 20, 10 and 5 ng/ml. Two hundred microlitres of calibrator or diluted samples were incubated in microtitre plates precoated with thrombospondin antibody for 2

h. After the microplate was washed, 200 µl of anti-thrombospondin peroxidase was added and incubated for 2 h. Wells were washed to remove unbound conjugate and were incubated with ortho-phenylenediamine/hydrogen peroxide substrate for 5 minutes. After adding 50 µl of stop solution (3 M H_2SO_4) into each well and left at room temperature for 10 minutes, the plate was analysed on a spectrophotometric plate reader (Titertek Uniskan II) at 492 nm within 30 minutes. The standard curves were constructed from the mean absorbance values plotted against the corresponding concentration of thrombospondin in ng/ml.

2.12 Proliferation measurement by MTT assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide] (Sigma Biosciences) is a tetrazolium salt which is reduced by mitochondrial dehydrogenase in viable cells to form a dark blue crystal of formazan product. It was made as a stock solution of 5 mg/ml and stored at -20°C. To perform a correlation of optical density of MTT study versus cell density, the following experiment was performed. Cells at a variety of densities from $5x10^3$ to $2x10^5$ were suspended in a volume of 100 µl of growth medium per well uniformly seeded into each well of 96-well microplates (Costar), and incubated at 37°C for 24 h. Twenty microlitres of MTT dissolved in PBS at a concentration of 5 mg/ml were added in each well, and the plates incubated at 37°C for 4 h. After the incubation period, the medium was removed from each well and 200 µl of dimethylsulfoxide were added to

dissolve the formazan crystals. After 5 minutes, 200 µl of the formazan-dissolved solution was transferred into a new 96-well microplate, and then the absorbance was measured by a multiple scanning spectrometer reader (Titertek Uniskan II) using a test wave length of 550 nm.

To compare the growth rate between transfected SK-Hep-1 cells and the parental cells, $5x10^3$ cells were seeded into each well of 24-well plates. Seven plates were set up for the assay of 7 days and this moment was considered to be day '0' of the assay. The MTT assay described above was performed every day for 7 days.

2.13 Thymidine incorporation assay

This assay is based on the fact that radiolabelled thymidine ([^{3}H]-TdR) is incorporated into the newly synthesised DNA of cells which are in the synthetic (S) phase of the cell cycle. After the period of labelling the cells are washed to remove thymidine which has not been transported into the cells. Cells are then lysed and washed so that thymidine which has entered the cells but has not been incorporated is eliminated. The remaining radioactivity which is incorporated into the DNA is then collected onto filters in a cell harvester and is counted by scintillation counting.

Cells in suspension were plated out in a 96-well plate (Falcon) at $1x10^4$ cells per well in 200 µl of RPMI-1640 and 10% FCS. Cells were allowed to grow to complete confluency for 48 h. Then the medium was removed and

replaced with RPMI-1640 and 0.1% FCS. Cells were kept in this medium for 48 h. After that the medium was removed and cells were rinsed once in RPMI-1640. Cells were labelled with [^{3}H]-Thymidine ([^{3}H]-TdR) in the concentration of 1 μCi/well. The period of labelling was extended to 24 h to ensure that all the cells in S-phase of the cell cycle were captured during labelling with [^{3}H]-TdR. At the end of the labelling period, the [^{3}H]-TdR-containing medium was removed and cells were washed once with PBS without Ca^{2+}/Mg^{2+}. After cells were dislodged by using trypsin-EDTA (Gibco-BRL, Life Technologies), they were collected by flushing and aspirating simultaneously several volumes (2-3 ml per well) of distilled water through each well using a semiautomatic cell harvester, Skatron (SkatronAS, Norway). The cells were collected onto 'Filtermats' filter sheets (SkatronAS, Norway). The manifold of the harvester scores and partially cuts 1-cm diameter circles in the filter and the contents of each well were collected onto such circular pieces of filter. Filter sheets were dried and the precut filter circles were pushed into plastic vials (Sterilin, UK). To each vial, 1 ml of 'EcoLume' scintillation fluid (ICN Biomedicals) was added and the radioactivity counted using a Beckman LS600Se Scintillation counter. Triplicates were done for each sample.

2.14 *In vitro* Matrigel invasion assay

The *in vitro* invasion assay is a method to measure the migratory activity of cells. The most widely used technique employs the modified Boyden chamber method. Cells are placed in the upper compartment of a microwell

system which is separated from the lower well by a membrane through which the cells migrate. Microchemotaxis Transwell (Costar) cell culture inserts, 12 mm diameter, with 12 μm pore diameter polycarbonate filters and Matrigel (Collaborative Biomedical Products, Becton Dickson) were used in this study. Matrigel is stored at -20°C and thawed at 4°C at the time of use. Filters were coated with 100 μl, 1:2 dilution of Matrigel basement membrane matrix in serum-free RPMI-1640 by a thin gel method to make a layer 1 mm thick. The Matrigel was solidified in a 37°C incubator for 1 h. Five hundred microlitres of cell suspension containing $1x10^5$ cells were seeded onto the upper chambers and the lower chambers contained 1 ml of RPMI-1640 with 10% FCS (Figure 5). Following 72 h incubation at 37°C and 5% CO_2, the suspension was removed and the Matrigel was wiped gently with cotton wool. To quantitate the number of cells that pass through the Matrigel and attach to the lower surface of the filter, the cells on the filters were fixed in 100% methanol (30 seconds) before staining with haematoxylin (2 minutes).

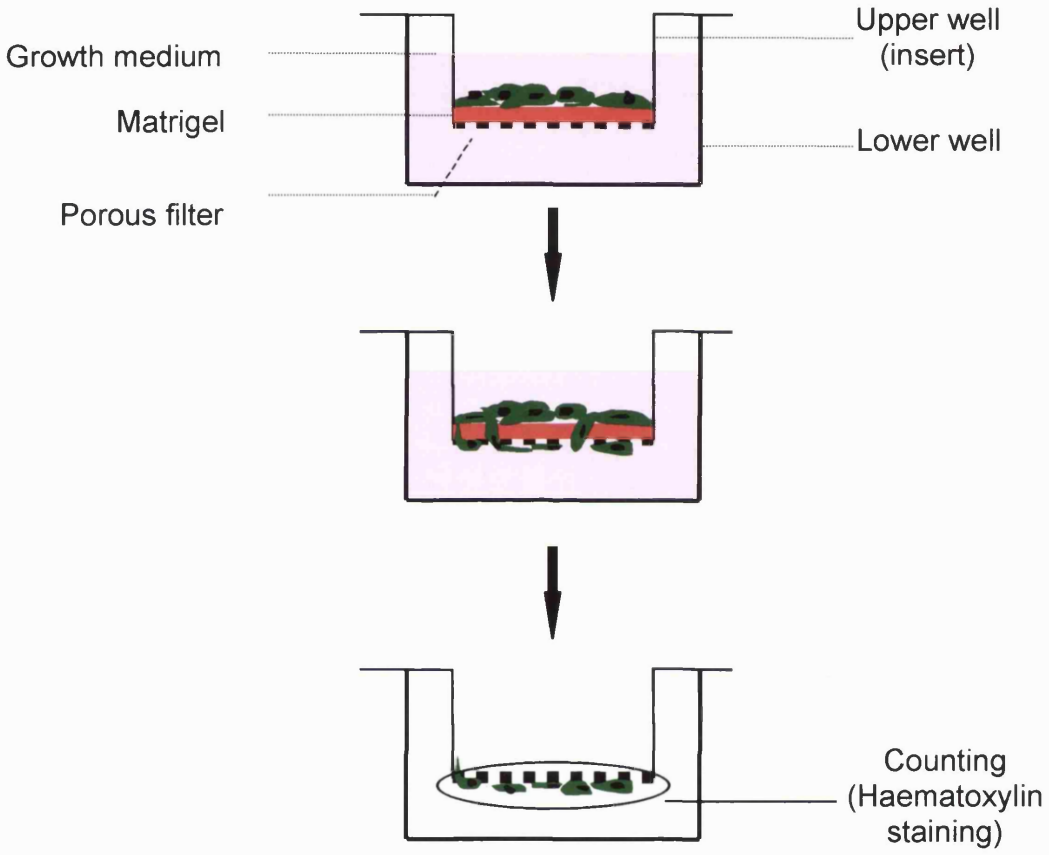


Figure 5. Diagram of *in vitro* Matrigel invasion assay

The filters were removed from the upper chamber support with a sterile scalpel and dehydrated by taking through graded alcohols *ie.* 70%, 90% and 100% methanol. Finally they were cleared by placing in xylene. Each filter was mounted on glass slides by Pertex synthetic mounting medium (Histolab AB, Sweden). Cells which had migrated through Matrigel and attached to the lower surface of the filter were counted under light microscope (Olympus, Japan) at X400 magnification. Five microscopic fields were counted per filter and average number of cells determined.

2.15 Northern blot analysis

2.15.1 RNA isolation from Cell Monolayers

All solutions were autoclaved and, except those containing Tris buffer, were prepared in diethylpyrocarbonate (DEPC)-treated ddH_2O. DEPC is a strong but not absolute inhibitor of RNase. DEPC-treated water was prepared by treating ddH_2O with 0.02% DEPC and autoclaving.

$2x10^7$ cells were dislodged by trypsinisation and collected in RPMI-1640 with 10% FCS. After washing once in PBS, cells were pelleted by centrifugation and either stored at -70°C or used immediately for RNA extraction. Each cell pellet was lysed in 400 µl ice-cold RNA lysis buffer (150 mM NaCl, 10 mM Tris.Cl pH 7.4, 0.5% Nonidet P-40 (NP-40), 1 mM $MgCl_2$) for 5 minutes on ice. Samples were microcentrifuged at a low temperature (1-4°C) for 10 minutes. The supernatant, devoid of unlysed cell nuclei, was transferred into microcentrifuge tubes containing 200 µl of Tris-

buffered phenol (pH 7-8) and 50 µl of 10% SDS. Each sample was vortexed and centrifuged for 5 minutes at room temperature. The upper aqueous phase was added to a microcentrifuge tube containing 200 µl of Tris-buffered phenol (pH 7-8), vortexed and centrifuged as above. The upper phase was mixed with 40 µl of 2 M Na acetate (pH 5.2) and 1 ml of cold absolute ethanol and left at -20°C for few hours or longer for the RNA to precipitate. Precipitated RNA was pelleted by centrifugation at 4°C, washed in ice-cold 70% ethanol, dried under vacuum and resuspended in autoclaved DEPC-treated water.

The concentration of RNA was quantified by measuring its absorbance in solution at wavelength of 260 nm in an UV spectrophotometer. The reading at wavelength of 260 nm allows calculation of the RNA concentration. An optical density (OD) of 1 corresponds to approximately 40 µg/ml. The ratio between the reading at 260 nm and 280 nm (OD260:OD280 ratio) provides the estimate of the purity of RNA. When this was greater than 1.6 the RNA was considered sufficiently pure for Northern blot analysis.

2.15.2 RNA electrophoresis and Northern transfer

Electrophoresis of RNA was carried out after denaturation with glyoxal. Glyoxal denatures RNA by binding covalently to guanine residues and so preventing the formation of guanine-cytosine base pairs. Ten micrograms of RNA samples were mixed with 1.6 µl 0.2 M $NaPO_4$ (pH 7.0), 5.2 µl deionised glyoxal and adjusted to a final volume of 32 µl with sterile distilled water. After incubation at 50°C for 1 h, the reactions were chilled on ice and

8 µl of loading buffer (50% glycerol, 10 mM $NaPO_4$ pH 7.0, 0.05% bromophenol blue) were added. A 0.24 to 9.5 Kb RNA ladder (Gibco-BRL, Life Technologies) was prepared in the same manner as the sample RNA.

Electrophoresis was performed using a horizontal submerged gel apparatus supplied by Flowgen and 1.2% agarose gel in 10 mM $NaPO_4$ (pH 7.0) without ethidium bromide at 70-100 mA. The ethidium bromide intercalation of nucleic acid, which is the basis of visualisation, is believed to alter the mobility of the RNA through the gel, and therefore fractionation under these circumstances may not be accurate. Moreover, saturation of the nucleic acid with the dye appears to reduce the efficiency of transfer. During electrophoresis there was constant recirculation of the running buffer to prevent dissociation of glyoxal from the RNA. Glyoxylated RNA was transferred immediately after electrophoresis to a Hybond-N nylon membrane by a capillary transfer method. In this method, RNA fragments were carried from the gel in the flow of liquid and deposited on the surface of the solid support. The liquid is drawn through the gel by capillary action that is established and maintained by a stack of dry, absorbent paper towels. Using this capillary transfer method, 10X SSC solution (1.5 M NaCl, 1.65 M NaCitrate) was drawn from a reservoir by capillary action through 3MM filter paper, the gel which placed in an inverted position, nylon membrane and through a stack of paper towels above that (Figure 6). A weight (500 gram) applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

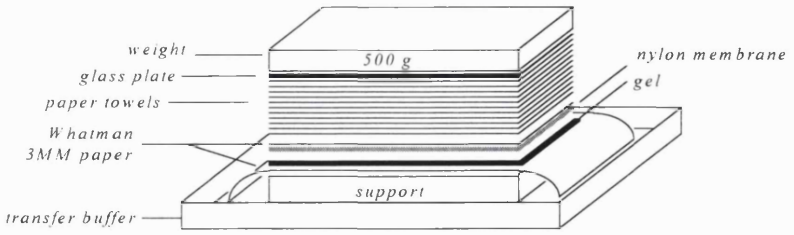


Figure 6. Typical apparatus for the capillary transfer of nucleic acids from an agarose gel to a nylon membrane

The gel was surrounded by Saran wrap to prevent liquid from flowing directly from the reservoir to paper towels. The rate of transfer of the RNA depends on the size of the RNA fragments and the concentration of agarose in the gel.

The transfer of RNA was allowed to proceed for 18 h. To prevent loss during subsequent hybridisation and washing steps, the nylon membrane must be treated to immobilise the nucleic acid after transfer is complete. The membrane was exposed to low doses of ultraviolet irradiation (254 nm). A small fraction of the bases in the RNA was thus covalently cross-linked with the positively charged amine groups on the surface of the membrane. Immobilisation of nucleic acids by ultraviolet irradiation can greatly enhance the hybridisation signal. The membrane was placed between two pieces of 3MM paper and baked at 80°C for 2 h in order to remove the glyoxal. If the membrane was not used immediately in hybridisation experiments, it should be stored at +4°C or wrapped loosely in aluminum foil and stored under

vacuum at room temperature. The RNA marker track was cut from the filter and fixed in 1 M acetic acid for 10 minutes. Then it was stained in 0.4 M sodium acetate, 0.4 M acetic acid, 0.2% (w/v) methylene blue for 30 minutes at room temperature. Excess stain was removed by washing in distilled water to reveal the stained RNA.

2.15.3 TSP1 cDNA probe preparation and transformation

TSP1 cDNA probe was kindly supplied by Dr Luisa Iruela-Arispe, Beth Israel Deaconess Medical Center-Research North, Harvard Medical School, Boston, USA. TSP1 cDNA probe (1.3 Kb) is cloned in the *EcoRI* site of pGem-2 plasmid vector (3.0 Kb). The preparation and transformation of TSP1 probe was performed as described in section 2.5.2-2.5.4.

2.15.4 Radiolabelling of DNA probe

DNA was labelled by the random priming method which generated very high specific activity (typically > 10^9 dpm/μg) DNA probes labelled with ^{32}P. This method utilises the ability of either *E. coli* DNA polymerase I (Klenow fragment) or other DNA-dependent DNA polymerases to synthesise a new DNA strand complementary to a denatured template, utilising a free 3′-hydroxyl group to initiate the reaction. This is provided by the presense in the reaction of short oligodeoxynucleotides (commonly hexamers or nonamers) which anneal non-specifically to single-stranded DNA and thereby prime DNA synthesis. In such reactions, the amount of newly synthesised DNA can often exceed the amount of input DNA, suggesting

that the same region of DNA template can be copied more than once by DNA synthesis initiated from different primers.

TSP1 DNA probe was labelled using the DNA-labelling kit (-dCTP) "Ready To Go" (Pharmacia Biotech) which utilises the procedure with random oligodeoxyribonucleotides, primarily nonamers. Briefly, DNA was denatured by boiling for 3 minutes and placed on ice for 2 minutes. Then 1 µl (20-50 ng) of denatured DNA was mixed with 20 µl of reconstituted reaction mix [dATP, dGTP, dTTP, FPLC*pure* Klenow Fragment (4-8 units), random oligodeoxyribonucleotides, primarily nonamers], 24 µl of dH_2O and 5 µl of [α-^{32}P]-dCTP. Labelling reactions were carried out at 37°C for 5-45 minutes. Labelled DNA was separated from unincorporated nucleotides by Sephacryl S-300 HR resin filtration in Microspin S-300 HR columns (Pharmacia Biotech).

2.15.5 Hybridisation

Optimal conditions of hybridisation are those which allow strong specific binding of the probe to the RNA and minimal non-specific binding to the membrane. Hybridisation conditions are optimised by varying the solvent and temperature used, volume of solvent and length of hybridisation, degree and method of agitation, agents to block the non-specific attachment, concentration of the radiolabelled probe, and stringency of washing following the hybridisation.

The probe becomes irreversibly bound if the membrane is allowed to dry. Therefore, every effort should be made to ensure that the membrane remains wet at all stages during hybridisation, washing and autoradiography. Membrane was pre-hybridised with 15 ml of pre-warmed Rapid-hyb buffer (Amersham) containing 50 μg/ml denatured salmon sperm DNA (Boehringer Mannheim, USA) in a Hybaid hybridisation oven for 30 minutes. This step was performed to block the non-specific attachment of the probe to the surface of the membrane. The radiolabelled probe DNA was denatured by boiling at 95-100°C for 2-5 minutes and was immediately chilled on ice to avoid reannealing just before its addition to the hybridisation mixture. Hybridisation was carried out for 2 h at 65°C. The membrane was then washed by 20 ml of 2X SSC, 0.1% (w/v) SDS and incubated for 20 minutes at 65°C with continuous shaking. The membrane was further washed for an equal period of time with the fresh 1X SSC, 0.1% (w/v) SDS and 0.5X SSC, 0.1% (w/v) SDS solutions respectively. The amount of radioactivity on the membrane was monitored with a Mini-Instruments series 900 mini-monitor.

The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene, 1.2 Kb-cDNA, was used in a hybridisation on the same membrane to control the equivalent RNA loading of each sample.

2.15.6 Autoradiography

The membrane was kept moist and exposed to X-ray film. [^{32}P]-autoradiography was performed at -70°C using intensifying screens to

enhance the signal by converting the β-emission into photons (light) which are also detected by the X-ray film. The response of films to low light intensity is increased when exposures are carried out at low temperature since the stability of the latent image formed in exposed silver hyalide grains is improved at temperatures in the range of -70°C to -80°C. Film was developed and the radiolabelled probe was stripped from the nylon membrane by boiling in 0.5% SDS for 10 minutes. The nylon membrane can be stored for reprobing by wrapping in Saran Wrap at -20°C.

2.16 *In vivo* experiments

The animals used for the *in vivo* experiments were MFI nude mouse and Sprague Dawley rats supplied by CBU unit, Royal Free Hospital. All procedures were carried out according to Home Office regulations and were covered by the Home Office Licence No. PPL 70/4517. These procedures were carried out at the CBU unit, Royal Free Hospital. Cell suspensions were prepared by trypsinisation of adherent cell lines from tissue culture flasks and washing with sterile PBS. Cells were counted and resuspended in the appropriate volume of cold-sterile PBS. The details of the *in vivo* protocol are described in chapter 7.

CHAPTER III

P53 ANALYSIS IN HEPATOCELLULAR CARCINOMAS

III. P53 ANALYSIS IN HEPATOCELLULAR CARCINOMAS

3.1 Tissue specimens

The majority of specimens used in this study are formalin-fixed, paraffin-embedded tissues of cirrhotic explant livers from patients undergoing elective orthotopic liver transplantation (OLT) for end-stage chronic liver disease at the Royal Free Hospital. Most of them were described in a recently published series (Table 4) (Bhattacharya *et al*, 1997). Briefly, the livers were cut ("breadloafed") into coronal slices 10 mm thick and were closely examined for unusual nodules. If any unusual nodules were found, tissue samples were taken from these nodules, preserved in formalin, embedded in paraffin and processed using standard techniques. Five-micron sections and ten-micron sections were serially cut from each block onto glass slides by microtome. The five-micron sections were stained with Haematoxylin and Eosin (H&E) for histological confirmation and for the immunohistochemical studies whereas the ten-micron sections were used for AP-PCR studies. Each lesion was classified on morphological grounds as either an HCC, a borderline lesion, or a regenerative nodule (RN), using the diagnostic criteria outlined by Ferrell and co-workers (1993).

3.2 Immunoreactivity in tumour specimens

Formalin-fixed, paraffin-embedded sections of either HCCs or RNs were studied for p53 expression with immunohistochemistry. None of them showed nuclear immunoreactivity with the DO-7 antibody. The p53 protein was detected in the nuclei of the positive control (colonic adenocarcinoma).

Patient	Cause of cirrhosis	Number of HCC nodules	Number of Regenerative nodules
1	Alcohol	7	1
2	Alcohol	15	1
3	Alcohol	7	2
4	Hepatitis C	0	6
5	Hepatitis C	6	8
6	Alcohol	7	4
7	Hepatitis C	1	1
8	Hepatitis C	0	4
9	Hepatitis B	0	2
10	Hepatitis C	0	1
11	Alcohol	0	1
	Total	**43**	**31**

Table 4. Number of nodules studied and aetiology of cirrhosis

3.3 p53 amplification and direct sequencing

The principle of DNA sequencing is the generation of a set of single-stranded polynucleotides, which differ in length by a single deoxynucleotide at the 3′ end. Four separate reactions are set up to generate batches of polynucleotides which terminate at the 3′ end with either A, T, G or C. The products should represent all possible fragments which can be derived from the DNA sequence of interest. The fragments can then be separated according to length by electrophoresis through a denaturing polyacrylamide gel. The A, T, G and C reaction products are usually resolved in adjacent lanes of a sequencing gel, allowing the DNA sequence to be read from the ladder of bands produced. Detection of the fragments is facilitated by incorporation of a radiolabel in the reaction mixture *ie.* the bands could be visualised by autoradiography. Every genomic DNA sample was successfully amplified by specific primers of exons 5-9. However, when analysed by direct sequencing analysis, there were no point mutations nor rearrangement of nucleotides even at the hotspot (codon 249) on exon 7.

3.4 Discussion

The practice of orthotopic liver transplantation (OLT) provides an opportunity to study small asymptomatic HCCs, as previously undetected incidental HCCs are not infrequently found in cirrhotic livers at the time of OLT and more often on subsequent histopathological examination of the livers (Ferrell *et al*, 1992). Using the diagnostic criteria outlined by Ferrell and co-workers in this study, a study at the Royal Free Hospital previously revealed 45 additional cancers over and above those detected at routine

assessment (17 cancers) (Bhattacharya *et al*, 1997). Routine histological assessment of explant livers underestimates the number of HCCs. The morphological appearance of most of the HCCs was in keeping with an expansile rather than an infiltrative growth. The frequent absence of a dominant lesion, the occurrence of lesions in both lobes of liver, the absence of vascular invasion, and at times the marked morphological heterogeneity among cancers within the same liver suggested that these lesions were synchronous primary tumours rather than intrahepatic metastases. Nevertheless, the molecular biological analysis of these tumours, as presented in this work, is able to provide the relationship between tumour nodules.

DO-7 antibody labels the p53 protein in cells. Because of the low intracellular concentration of p53 and its short half-life, the normal or 'wildtype' p53 protein usually cannot be detected by immunohistochemical methods. Mutations in the p53 gene can usually be demonstrated by immunohistochemical staining of p53 protein because of the metabolic stabilisation and prolongation of the half-life of the mutant protein in the nucleus. However, the diagnostic usefulness of p53 mutations is limited, because the mutation is a relatively late event in the multistep carcinogenesis and hence may not be detected in early HCC. The tissue samples in this study, which demonstrated the negative immunoreactivity are small HCC nodules with the size less than 2 cm in diameter. The p53 protein was detected significantly more frequently in large HCCs (more than 5 cm) than in small HCCs, in poorly differentiated than in well-differentiated HCCs, and in invasive rather than in non-invasive HCC (Hsu *et al*, 1993).

Tanaka and colleagues (1993) reported genetic heterogeneity of the p53 gene in 3 of 9 cases of HCC, which had p53 genetic alterations and comprised two different histological grade of differentiation within each tumour. P53 may not be important for the development of HCC as suggested by Hosono and co-workers (1991), who failed to detect p53 mutations in HCCs from Taiwan and China. Therefore, p53 mutation analysis is not a suitable technique to analyse the clonality of HCCs.

The lack of p53 mutations in this study is possibly related to the aetiological factors. P53 mutations have been found in a large percentage (65%) of HCCs in association with certain aetiologic factors such as aflatoxin (AFB1) (Ozturk *et al*, 1991). The mutations were frequent in non-malignant human liver tissues from high AFB1 exposure areas, which suggested that p53 mutations might be an early event in hepatocarcinogenesis (Aguilar *et al*, 1994). On the other hand, in British and Northern European populations with a low aflatoxin exposure, the frequency of p53 mutations in HCCs is reported to be 9% and 15% respectively (Collier *et al*, 1994; Kress *et al*, 1992), and they tend to occur late in HCCs. A recent study in British patients reported infrequent p53 mutations in HCC patients related to hepatitis viruses and alcohol (Vautier *et al*, 1999). Interestingly, a high frequency (71%) was demonstrated in haemochromatotic HCC patients. The contribution of this genetic alteration to hepatocarcinogenesis in European patients with common aetiology of underlying liver disease seems of little importance.

CHAPTER IV

ESTABLISHMENT AND OPTIMISATION OF AP-PCR

IV. ESTABLISHMENT AND OPTIMISATION OF AP-PCR

4.1 Comparison of different primers

The initial aim of this work was to establish the AP-PCR technique in the laboratory so that it could be used to display the DNA fingerprints in HCC samples. Band patterns varied dramatically depending on the choice of arbitrarily primer used in the PCR step. Panels of 10-mer (10 nucleotides) arbitrary primers (Genosys, USA) were tested to assess their ability to generate high quality band patterns in AP-PCR. All of these primers have a GC content of 50%. The sequences of the arbitrarily primers tested are listed in Table 5.

DNA fingerprint patterns using 10-mer arbitrary primers are shown in Figure 7. The patterns were different with each primer used. The amplification using such short primers occurred at an annealing temperature of 30-35°C throughout the thermo-cycling reaction.

	Sequence of 10-mer arbitrary primers (5′ to 3′)
A	GTGCAATGAG
B	CAATGCGTCT
C	AGGATACGTG
D	TCCCTTTAGC
E	CGGATAACTG
F	AGGTTCTAGC
G	TCCGACGTAT
H	GGAAGACAAC
I	AGAAGCGATG
J	CCATTTACGC
K	AAATCGGAGC
L	GTCCATAGCA
M	TACACTAGCG
N	CATAGCCCTT
O	CTACTAGGGT
P	AGTGAATGCG
Q	ACGATTCCTG
R	TTTACGGTGG
S	ATGGTGTAGC
T	AATCACACCC

Table 5. The sequence of 10-mer arbitrary primers tested in AP-PCR

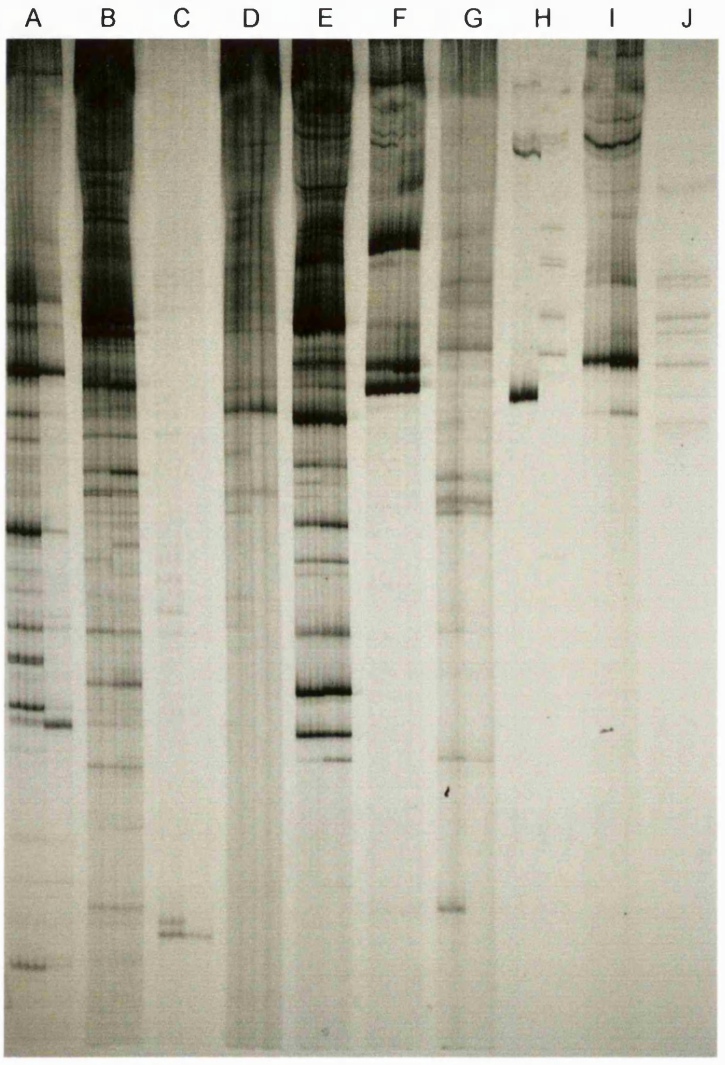


Figure 7. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by various 10-mer arbitrary primers (from table 5) showing different fingerprint patterns.

Another six arbitrary primers with 18-24 nucleotides were selected to study the fingerprint pattern generated because the longer primers can allow more forms of annealing where mismatches occur in the centre of the primer sequence. Therefore, the fingerprint is enhanced with extra bands with longer primers and potentially greater discriminatory ability. The sequences of these arbitrary primers are listed in Table 6.

Arbitrarily Primer	Sequence (5′ to 3′)
AR3	GCGAATTCATGTACGTCAGG
JS1	GATAGCCAGCACAAAGAGAGCTAA
JS2	CGACCGTGTTTTGCAAACAGATGT
SP6long	GGCCGTCGACATTTAGGTGACCAC
T7long	CCGCTAATACGACTCACTATAGGG
ZF3	CCCCACCGGAGAGAAACC

Table 6. The sequence of 18-24-mer arbitrary primers tested in AP-PCR

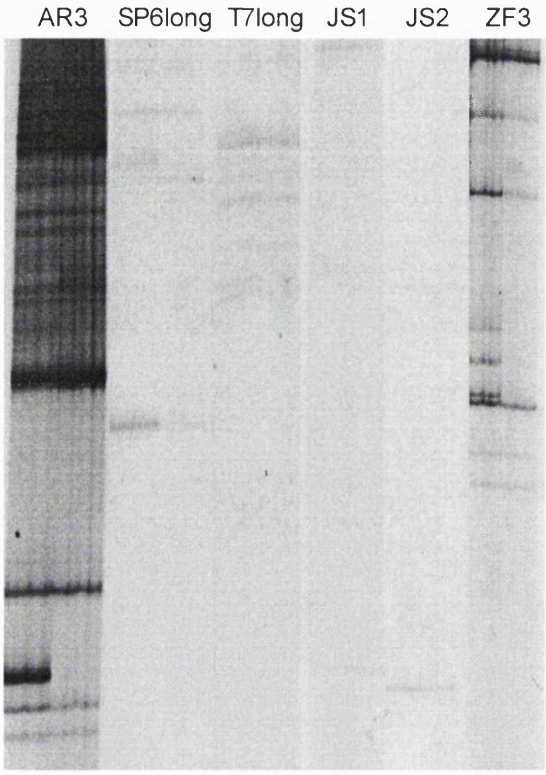


Figure 7b. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by six 18-24-mer arbitrary primers (from table 6) showing different fingerprint patterns. The arbitrary primers are indicated at the top.

Poor band patterns were obtained using arbitrary primers JS1, JS2, SP6long and T7long. Only a few bands were generated using these primers, compared to up to fifty bands of sizes ranging from less than 100 to 2000 bp which were reproducibly amplified with the AR3 and ZF3 primers. Therefore, we chose the AR3 and ZF3 primers that are already in extensive use by other groups to study the DNA fingerprint patterns of HCC samples. (Figure 7b)

The parameters that affected the production of reproducible AP-PCR fingerprints were investigated. The number, reproducibility and intensity of bands in a fingerprint should be a function of several parameters, including the concentration of template, magnesium, potassium, and the level of pH.

4.2 Template concentration

Genomic DNA was varied through six different concentrations in the range 50 ng to 400 ng. Figure 8 shows the effect of varying template concentration on AP-PCR patterns. When run on a high resolution polyacrylamide gel, the pattern generated by AP-PCR with either the AR3 or ZF3 primer is very consistent over a wide range of template concentrations.

4.3 Magnesium concentration

Magnesium (Mg^{2+}) concentration appeared to be important for the pattern obtained. Figure 9 shows the effect of varying Mg^{2+} concentration on AP-PCR patterns. Seven different concentrations in the range 1.5 mM to 7.5 mM were used. When run on a polyacrylamide gel, the pattern generated is very

consistent for Mg^{2+} concentrations between 3.5 to 7.5 mM. Below this level, the AP-PCR is more sporadic. 3.5 mM was selected as the optimal concentration of Mg^{2+} to enhance the stability of primer/template interactions.

4.4 Potassium concentration

Potassium (K^+) concentration also appeared to affect the fingerprint pattern. Figure 10 shows the effect of varying K^+ concentration on AP-PCR patterns. Four different concentrations were studied, 250, 500, 750 and 1000 mM. The pattern generated is very consistent for K^+ concentrations between 750 to 1000 mM. Below this level, the AP-PCR is more sporadic. 750 mM was selected as the optimal concentration of K^+ to enhance the stability of primer/template interactions.

4.5 The level of pH

pH is another important factor in the amplification process. The effect of varying pH on AP-PCR patterns is shown in Figure 11. Seven different pHs between 7.5-10.0 were tested. When run on a polyacrylamide gel, the pattern generated is very consistent for a pH between 7.5-9.2. pH 9.2 was selected as the optimal pH to enhance the stability of primer/template interactions.

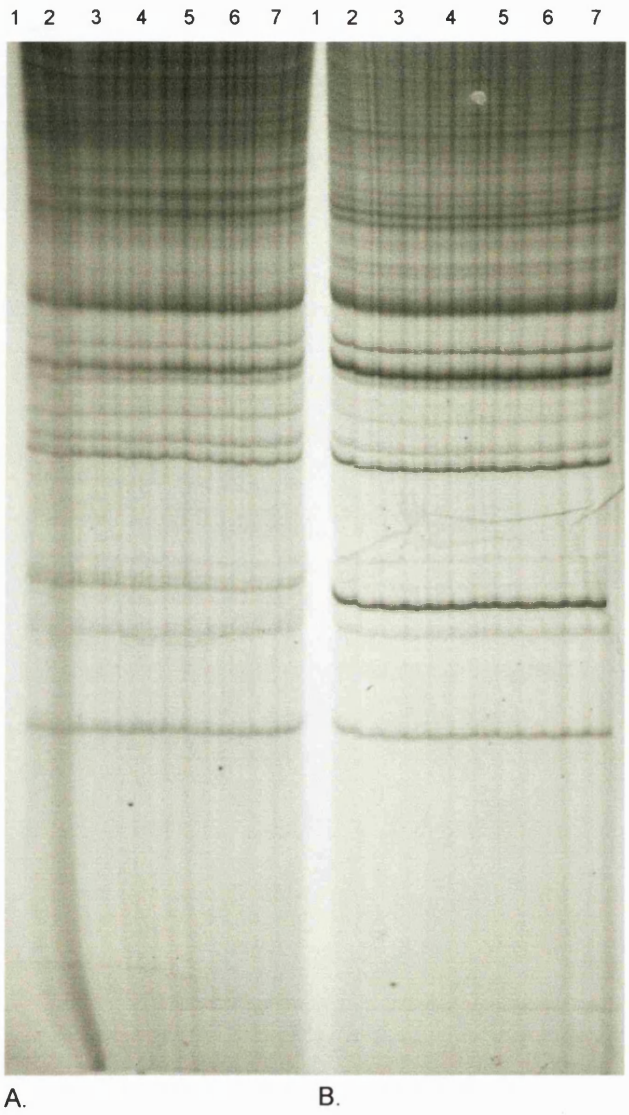


Figure 8. The pattern of AP-PCR products with various template DNA concentrations. AP-PCR was performed using the standard protocol (methods) except the template DNA (A & B) concentration was varied in the range 0 ng to 400 ng. (1, 2, 3, 4, 5, 6 & 7 represent 0, 50, 100, 150, 200, 300 & 400 ng, respectively)

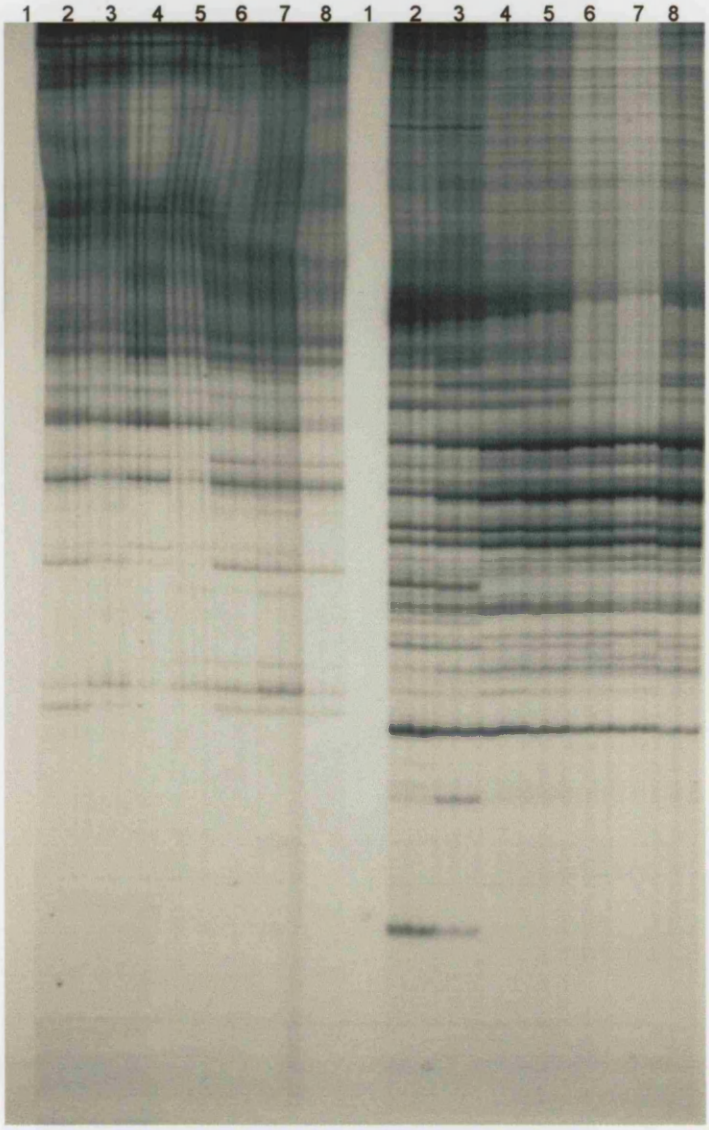


Figure 9. Effect of Mg^{2+} concentration on AP-PCR amplification. AP-PCR was performed using 200 ng of DNA (A & B), and the standard protocol (methods) except the Mg^{2+} concentrations were varied from 0 to 7.5 mM. The Mg^{2+} concentrations were stated on top of each lane. (1, 2, 3, 4, 5, 6, 7 & 8 represent 0, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5 & 7.5 mM, respectively)

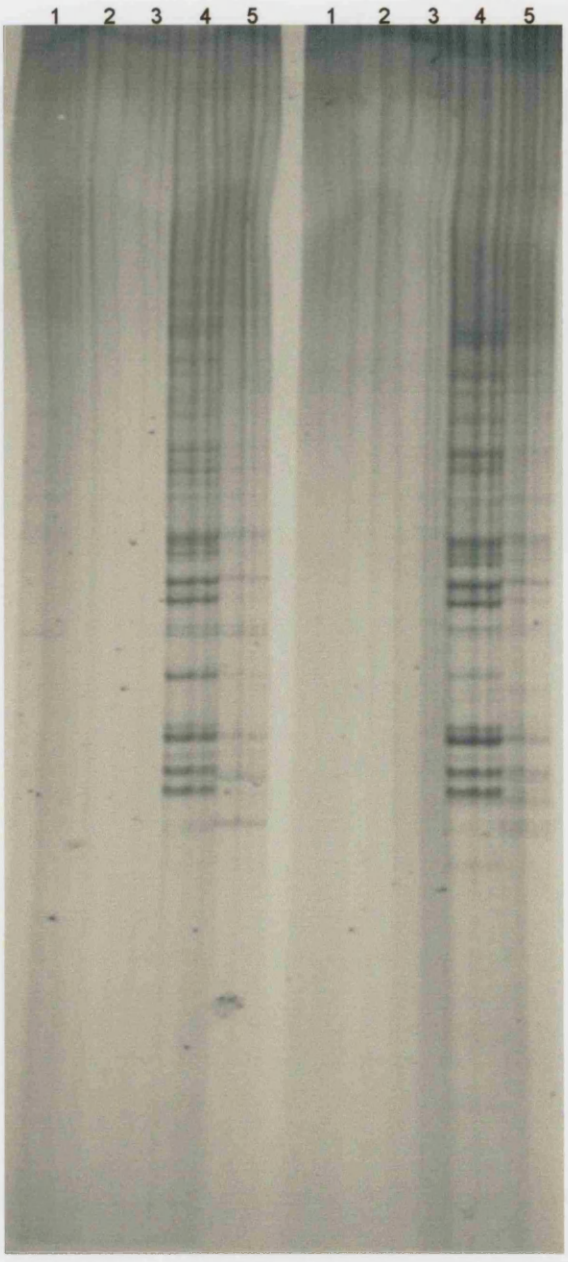


Figure 10. Effect of K^+ concentration on AP-PCR amplification. AP-PCR was performed using 200 ng of genomic DNA (A & B) and the standard protocol (methods) except the K^+ concentrations were varied from 0 to 1000 mM. The K^+ concentrations were stated on top of each lane. (1, 2, 3, 4 & 5 represent 0, 250, 500, 750 & 1000 mM, respectively)

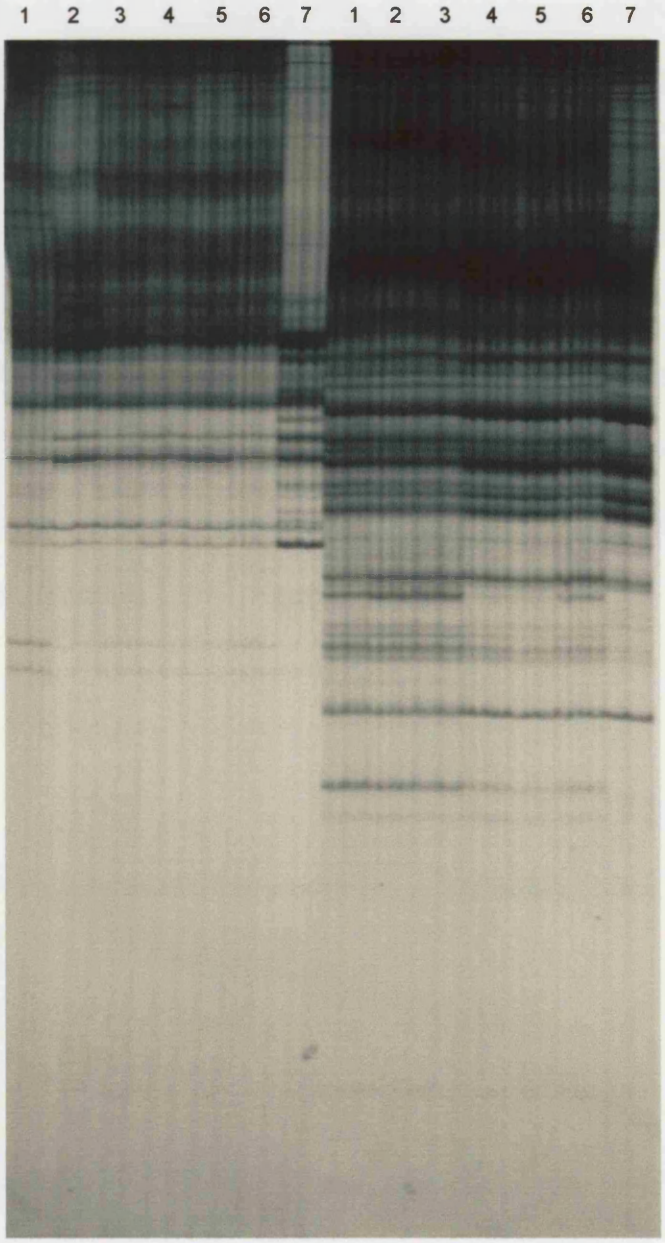


Figure 11. Effect of pH on AP-PCR amplification. AP-PCR was performed using 200 ng of DNA (A & B) and the standard protocol (methods) except the pH were varied in the range of 7.5 to 10.0. (1, 2, 3, 4, 5, 6 & 7 represent pH 7.5, 8.0, 8.2, 8.5, 8.8, 9.2 & 10.0, respectively)

To investigate the combination of parameters that affected the reproducibility of AP-PCR fingerprints, the Opti-Prime PCR Optimization kit (Stratagene) to look for the optimised condition for the AR3 and ZF3 primer was utilised. This kit provides 12 different buffers and 50X master mix. The component in each buffer (1-12) is shown in Table 7.

The fingerprint patterns were consistently generated by the AR3 and ZF3 primers with buffer 11 and buffer 12 respectively. Once the technique had been established and consistent results obtained, the optimised conditions to analyse the clonality of HCC samples were used.

Opti-Prime 10X Buffer	100 mM Tris.Cl, pH	Magnesium (mM)	Potassium (mM)
1	8.3	15	250
2	8.3	15	750
3	8.3	35	250
4	8.3	35	750
5	8.8	15	250
6	8.8	15	750
7	8.8	35	250
8	8.8	35	750
9	9.2	15	250
10	9.2	15	750
11	9.2	35	250
12	9.2	35	750

Table 7. The Opti-Prime PCR Optimization kit with buffers 1-12 (Stratagene)

4.6 Discussion

Amplification polymorphism analyses directed by arbitrary primers has been developed with a variety of nomenclatures. Williams *et al* (1990) invented the RAPD procedure. An arbitrary primer of 10 nucleotides produced amplification products after temperature cycling, which routinely resolved on agarose gels and visualised by ethidium bromide. This technique provides a simple, rapid method of scanning a genome and uses low-cost instrumentation. The DAF and MAAP which utilise the shortest primers, compared to other fingerprint techniques, down to 5 nucleotides in length were developed by Caetano-Anolles and co-workers (1991). The optimal length was found to be 8 nucleotides, a length that does not produce efficient amplification with RAPD. DAF products are routinely separated by thin polyacrylamide gels, backed onto plastic Gel-Bond film. This gel-plastic support is stained by an improved silver-staining method, which detects DNA at about 1 pg. Resultant gels are air-dried and kept for permanent record and evaluation. AP-PCR uses primers of lengths comparable with those used in standard amplification reactions, *ie.* 18-24 nucleotides (Welsh & McClelland, 1990).

The technique of AP-PCR uses primers chosen without regard to the sequence of the genome to be fingerprinted. Thus, AP-PCR requires no prior knowledge of the molecular biology of the DNA samples to be investigated. The large number of bands amplified with a single arbitrary primer generates complex fingerprints that reflect differences between different DNA templates. Each primer gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms between

nodules. AP-PCR generates fewer bands with more unique patterns than does conventional fingerprinting (Preus *et al*, 1993), thereby increasing the ability to discriminate between each clone. Because of the arbitrary nature of the priming events, the *in vitro* amplified sequences represent an arbitrarily chosen small representative sample of the template DNA. Therefore, there is no bias regarding the chromosomal location. Furthermore, and in contrast to the restriction fragment length polymorphism approach, the AP-PCR method permits the direct cloning of the *in vitro* amplified DNA sequences.

AP-PCR amplification depends on the use of one primer and subsequent gel electrophoresis. The major advantage of polyacrylamide gel electrophoresis, compared to agarose gel electrophoresis, is the distinction of many bands at high resolution. Most reports of the AP-PCR used either ^{32}P or ^{35}S detection of the fingerprints. In this study, we used ^{33}P as a tracer because it provides clear resolution of the bands in the gel. When compared with ^{35}S which also allows clear resolution, ^{33}P has a higher energy (^{33}P has the property of being a soft β-particle emitter which has 2-3 times the energy of ^{35}S). The ^{33}P-labelled compound may be more stable at high temperature than those with ^{35}S, and it requires shorter autoradiographic exposure periods than ^{35}S (Tokuyama & Takeda, 1995). Moreover, with a shorter half-life than ^{35}S, it has important safety and radioactive waste storage/disposal implications also. Although a novel technique of non-radioactive AP-PCR, *eg.* silver staining, has been

developed to avoid the hazard to laboratory personnel and the environment, it is not as sensitive as radioactive methods.

The oligonucleotide primer and nucleic acid templates used in PCR can vary in purity and GC content. While the PCR process is a robust reaction, certain primer-template combinations result in either nonspecific amplification products or low yields of the PCR products. By modifying the components of a amplification reaction, it is possible to improve the yield and specificity of the PCR products. As no specific guidelines exist for which buffer conditions to use for the various types of DNA primer-template systems, it is often advantageous to test a range of PCR buffers and additives and thereby determine the optimal buffer for each individual PCR template and primer set. Optimising PCR-base technology is a laborious task as many components can be altered in PCR reactions and not all processes and mechanisms are fully understood. The number, reproducibility and intensity of bands in a fingerprint should be a function of several parameters, including template concentrations, the concentration of salts, primer length and primer sequence. Reproducibility of AP-PCR fingerprints is not strongly affected by the template DNA concentration. Buffer components, such as magnesium, have been shown to influence AP-PCR reactions more than in those standard PCR-based techniques. The Opti-Prime PCR optimization kit is convenient to use for optimising the various components at the same time. It includes 12 Opti-Prime 10X buffers that vary in pH between 8.3 and 9.2, in magnesium chloride concentration between 15 and 35 mM and in potassium chloride concentration between 250 and 750 mM.

One attraction of AP-PCR is that primers can be designed arbitrarily without prior knowledge of the template sequence required. At the low annealing temperature in the first few cycles, a degree of mismatch would be expected. Annealing of the 3′ end is essential for polymerisation. Short and long primers would be expected to anneal perfectly or with a degree of mismatch at the 5′ end. However, longer primers can yield more forms of annealing where mismatches occur in the centre of the primer sequence. Therefore, it is responsible for extra bands with longer primers and potentially greater discriminatory ability. By using a single, random primer and two cycles of low-stringency PCR followed by many cycles of high stringency PCR, we were able to produce a discrete and reproducible set of products characteristic of genomes. At a sufficiently low temperature, primers are expected to anneal to many sequences with a variety of mismatches. Some of these will be within a few hundred base pairs of each other and on opposite strands. Sequences between these positions will be PCR amplifiable. The extent to which sequences amplify will depend on the efficiency of priming of primer annealing sites and the efficiency of extension. At early cycles, those that prime most efficiently will predominate. At later times, those that amplify most efficiently will predominate.

CHAPTER V

CLONAL ANALYSIS OF HEPATOCELLULAR CARCINOMAS

V. CLONAL ANALYSIS OF HEPATOCELLULAR CARCINOMAS

Hepatocellular carcinoma (HCC) arising in cirrhosis is frequently multifocal. Whether HCC in human liver develops from a single clone or from multiple parallel clones is an unresolved question. Information on the clonal origin of tumours will influence management strategies for prevention of recurrence after operation. Of the multiple tumour nodules present in many patients, it has not been established whether the smaller lesions represent intrahepatic metastases or *de novo* cancers. We therefore assessed the degree of genomic heterogeneity in synchronous HCCs using AP-PCR. If two template genomic DNA sequences are different, their arbitrarily primed PCR products display different fingerprinting patterns.

5.1 Polymorphic genomic fingerprints in synchronous HCCs

To compare the effect of tissue fixation and processing, normal noncirrhotic liver tissue was divided into two pieces, the first was snap-frozen in liquid nitrogen while the other was fixed in formalin, embedded in paraffin and processed using standard techniques. A 5-μm section was serially cut from each block onto glass slides and stained with H&E. Tissues from both slides were microdissected from different depths through the block (to test the effects of different lengths of exposure to formalin fixation) and separately collected in microfuge tubes. DNA extraction was performed as described (chapter II, page 67). Tissue samples listed in table 4 (11 patients) plus another 11 excised HCC specimens from 2 patients, representing a total of 54 HCC nodules and 31 regenerative nodules (RNs), were used to analysed

the genomic fingerprint patterns. The mean size of HCCs and RNs analysed were 7.69±5.56 mm and 7.87±3.39 mm in diameter respectively. Normal gallbladder tissues from each patient were used to generate the fingerprint pattern of their normal constitutional DNA for comparison.

Identical fingerprinting patterns in AP-PCR reactions of DNA extracted from paired frozen and formalin-fixed tissues from a normal noncirrhotic liver were generated (Figure 12). Using AP-PCR with each of the two arbitrary primers (AR3 and ZF3), specific and reproducible DNA fingerprints were generated, which were highly polymorphic between different nodules. Each PCR reaction was performed in duplicate with equal aliquots of sample template DNA, and the fingerprint of these paired reactions was identical, confirming the reproducibility of the technique.* Similar results were observed in the tissues dissected from different depths. Genomic homogeneity was detected in the two different sectors (whether closely adjacent or widely separated) of normal tissues of each patient, confirming the specificity of the technique. Nevertheless, DNA fingerprinting of RNs and HCCs was different from those of normal gallbladder tissues in the same patient. Marked genomic heterogeneity was noted amongst the nodules studied (Figures 13 & 14). No two nodules (either RNs or HCCs) had identical electrophoretic patterns. Contrary to expectation, even "satellite" nodules in close proximity within the same segment of the liver were found to have distinct genomic patterns (Figure 15).

*However, different in the intensity of the bands between frozen and formalin-fixed tissue were observed.

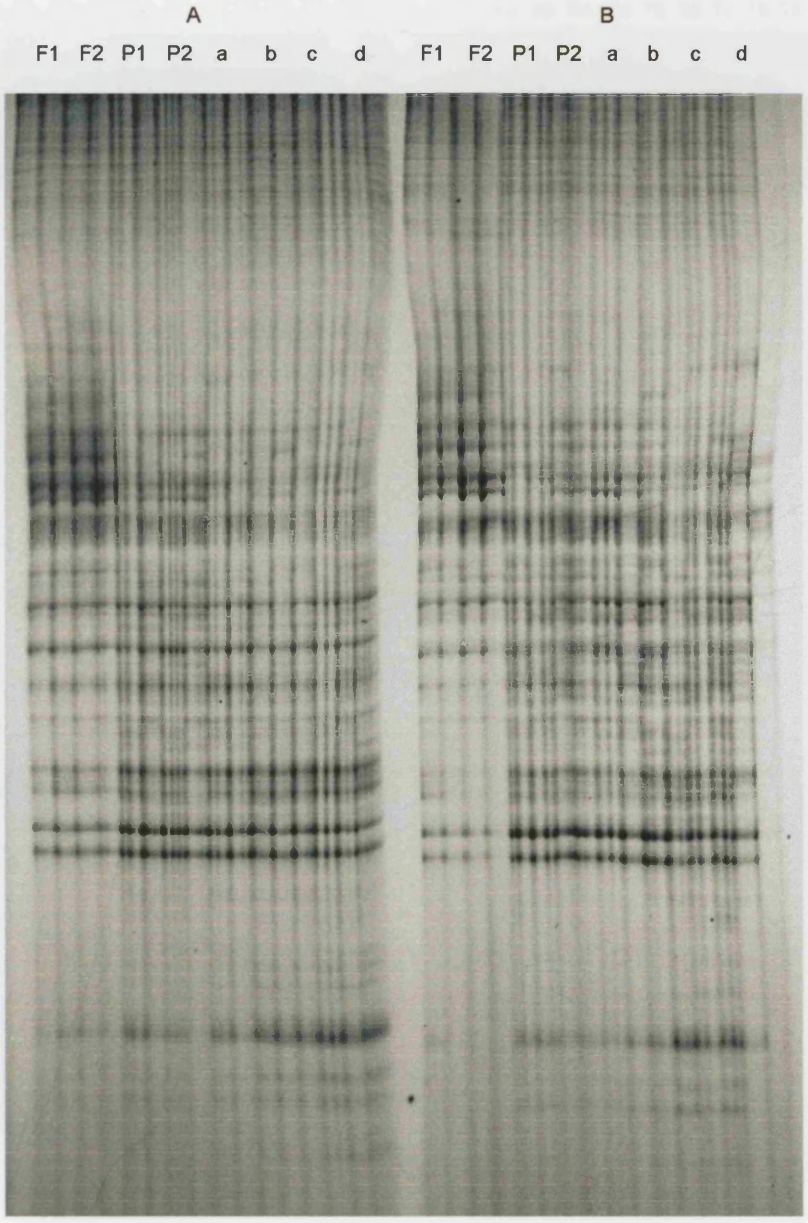


Figure 12. AP-PCR fingerprint patterns of fresh frozen and formalin-fixed, paraffin wax embedded blocks of two segment (A and B) of non-cirrhotic liver from a single patient. Identical profiles were observed on amplification of DNA from microdissected samples at different levels of the frozen blocks (F1 and F2) and paraffin wax blocks (P1 and P2). Similar results were obtained on amplification of various concentrations (a: 50 ng; b: 100 ng; c: 200 ng; d: 400 ng) of template DNA from each of the paraffin wax blocks.

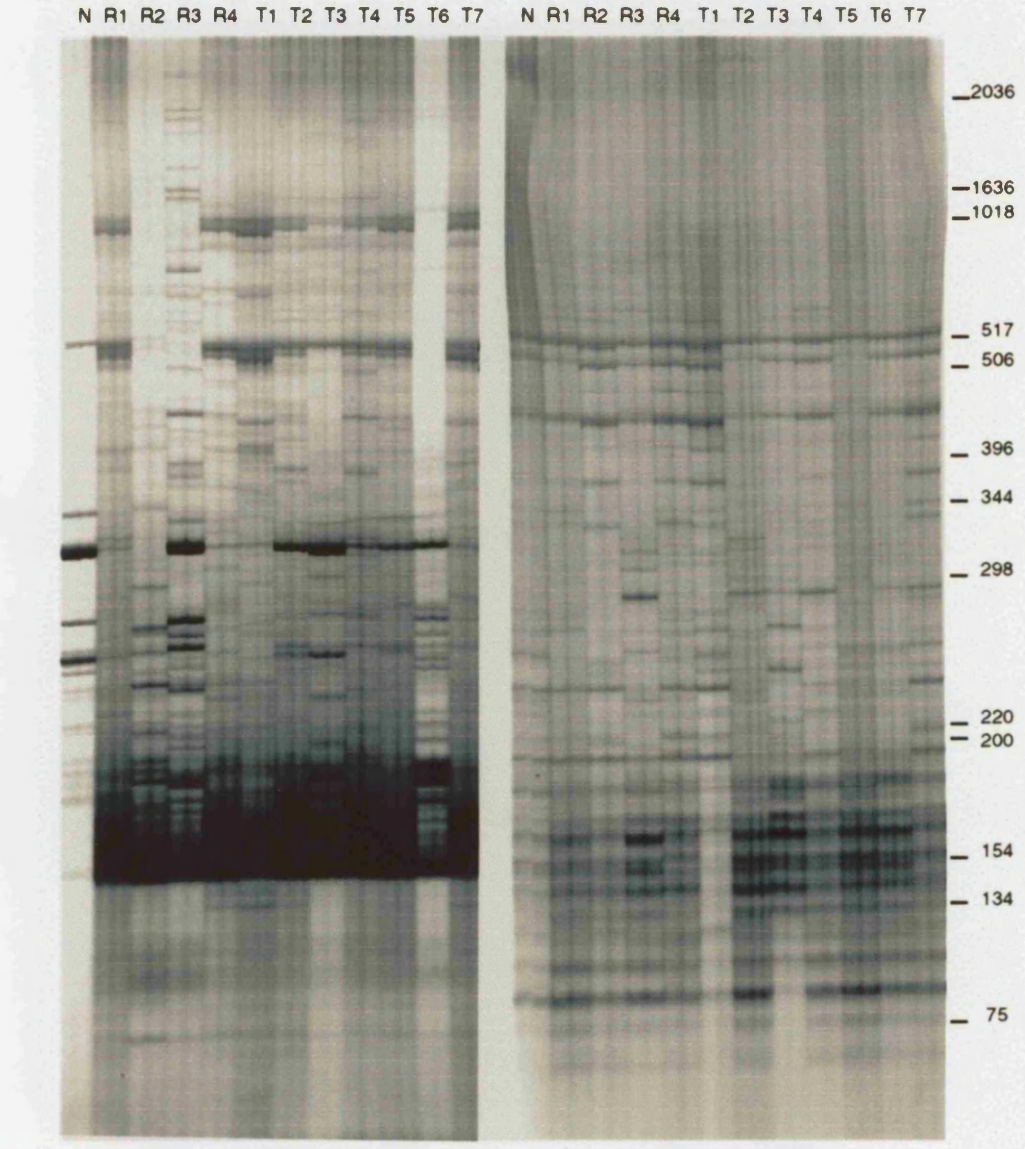


Figure 13. Typical genomic AP-PCR fingerprinting of synchronous hepatocellular carcinoma (HCC) and regenerative nodules (RNs) in the cirrhotic liver of one patient. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by arbitrary primers, AR3 (A) and ZF3 (B), showed high polymorphism between different nodules. N, R1-R4 and T1-T7 represent paired PCR reaction samples from non-tumour tissue, four different RNs and seven different HCC nodules, respectively. Size of bands (in base pairs) is indicated on the right.

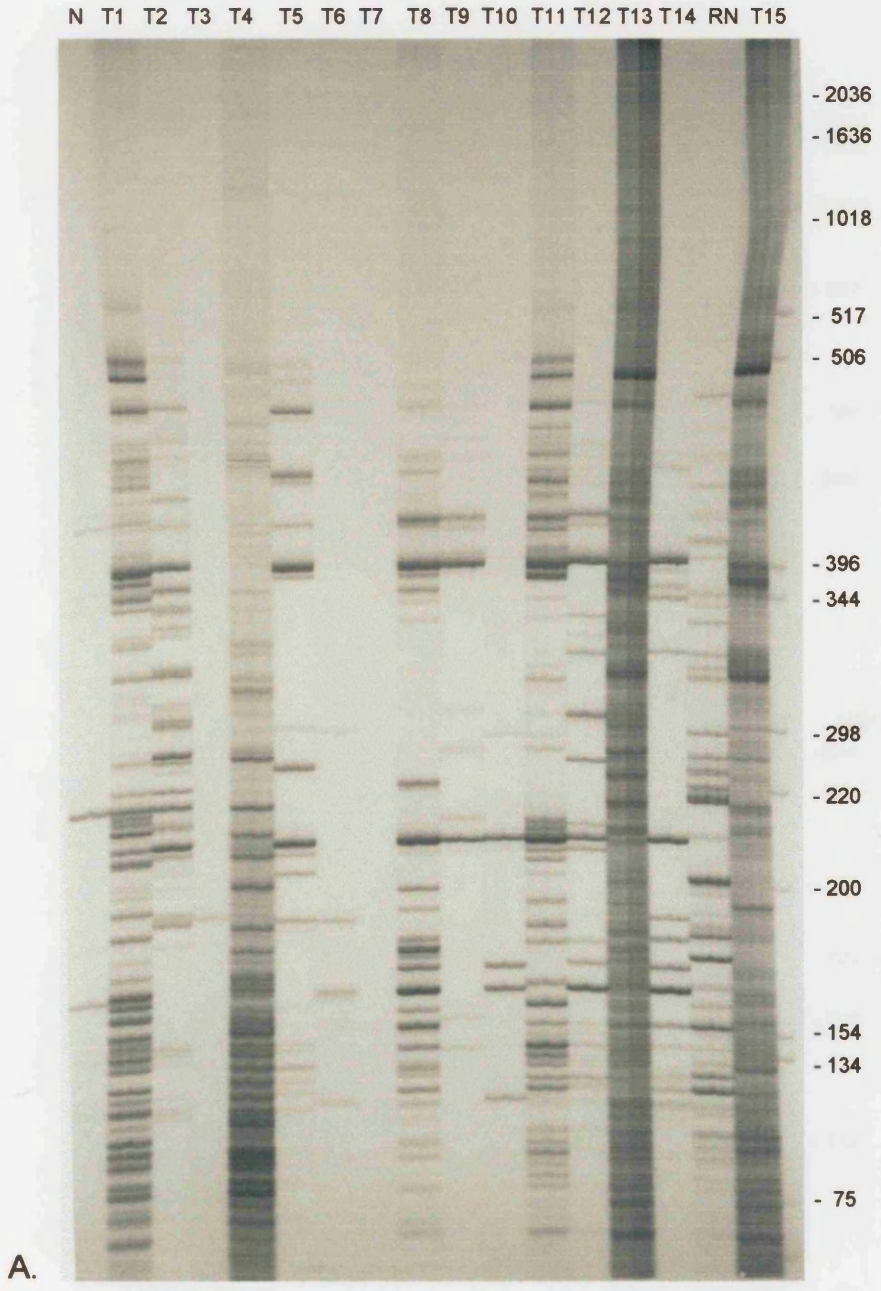
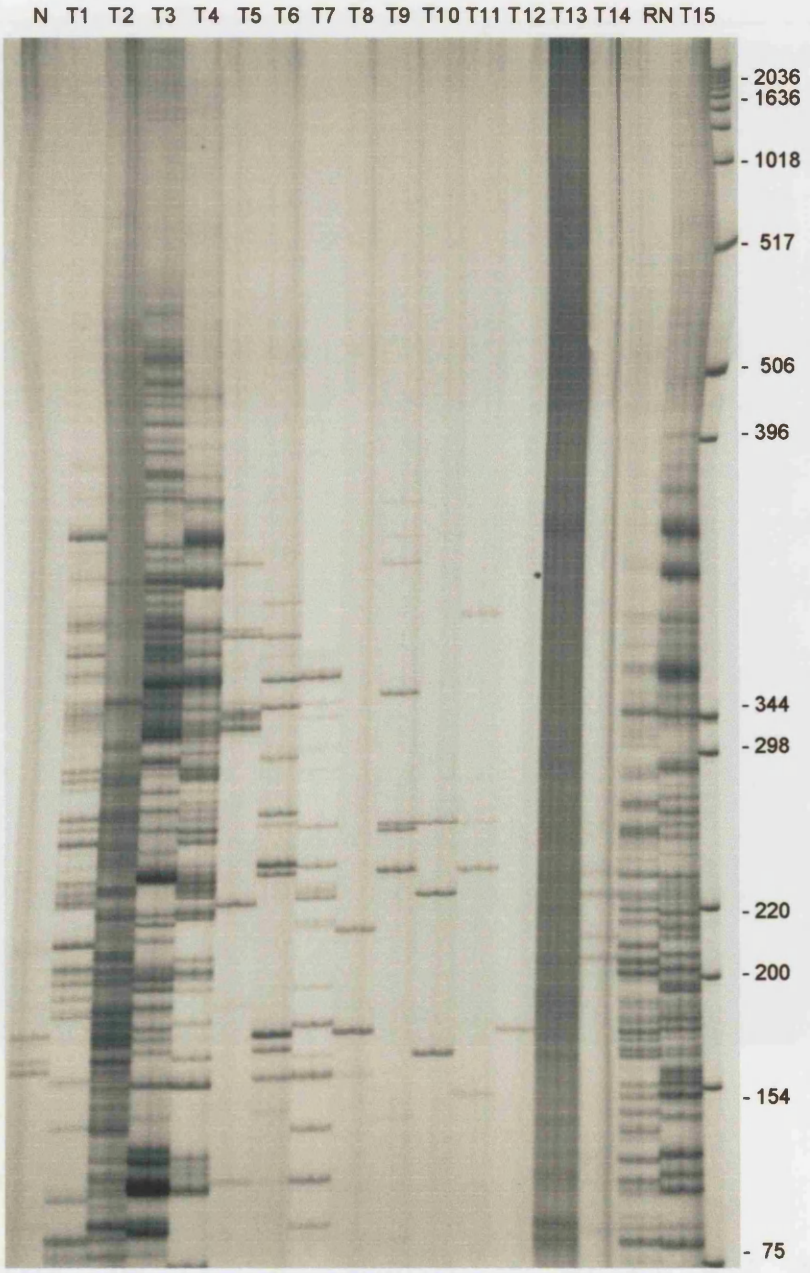


Figure 14. Typical genomic AP-PCR fingerprinting of synchronous hepatocellular carcinoma (HCC) and regenerative nodules (RNs) in the cirrhotic liver of patient Mc. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by arbitrary primers, AR3 (A) and ZF3 (B), showed highl polymorphism between different nodules. N, RN and T1-T15 represent paired PCR reaction samples from non-tumour tissue, RN and fifteen different HCC nodules, respectively. Size of bands (in base pairs) is indicated on the right.

B.


N T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 RN T15
2036
1636
1018
517
506
396
344
298
220
200
154
75

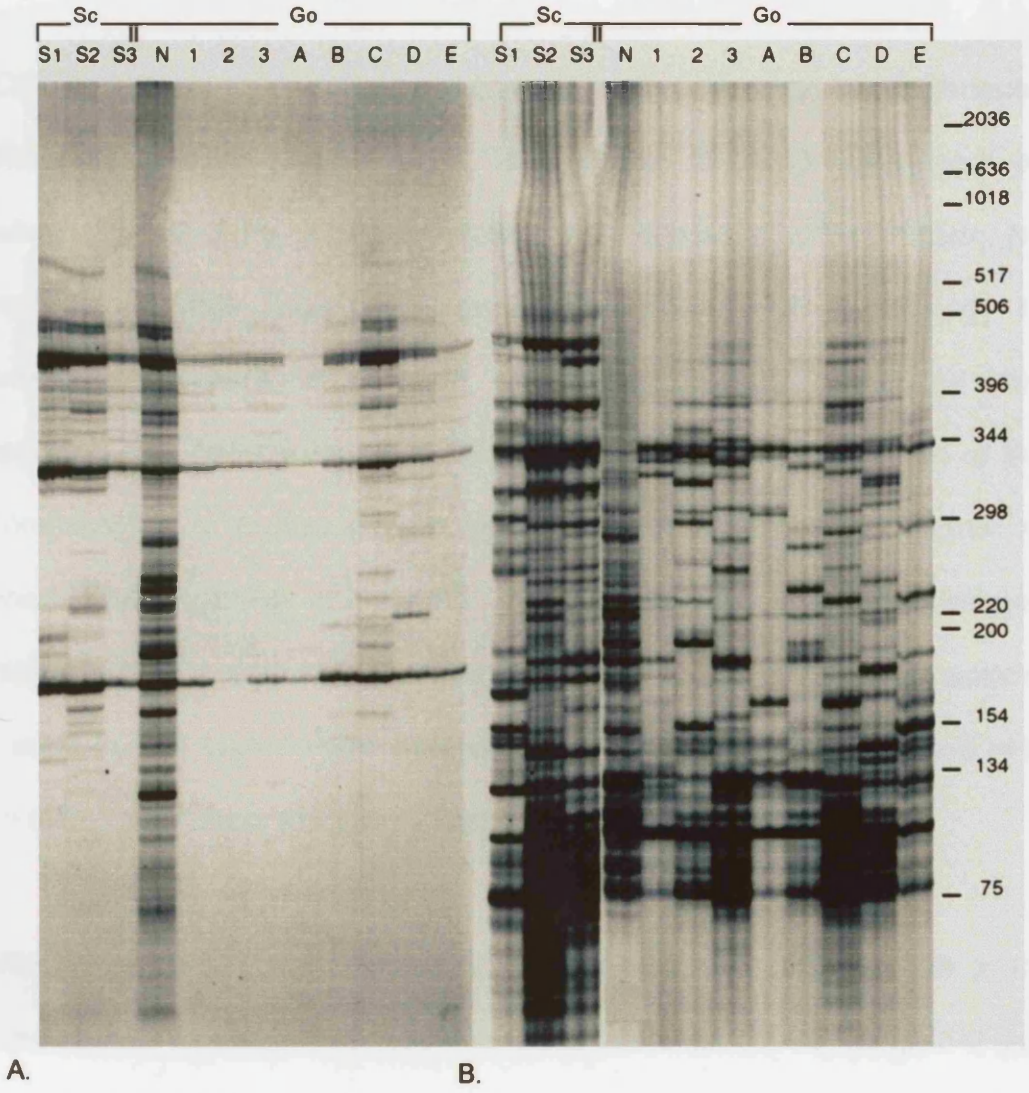


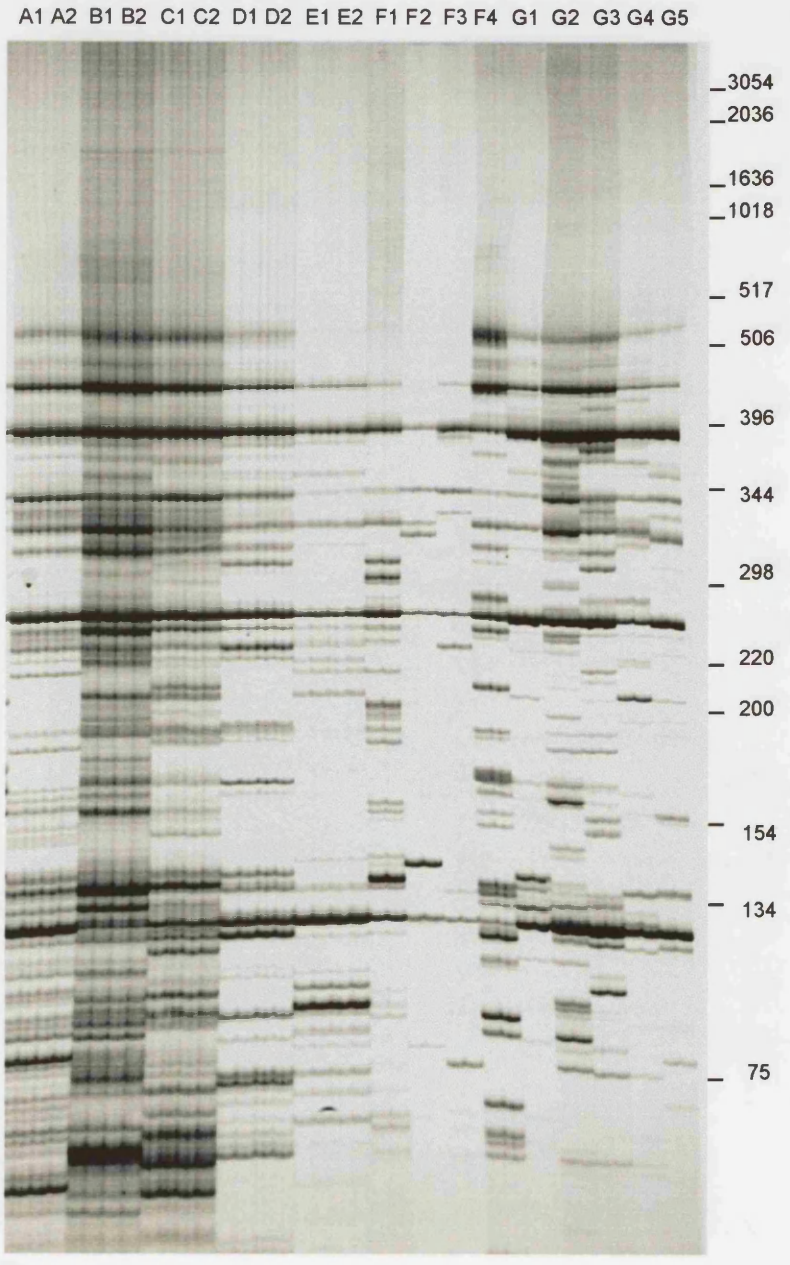
Figure 15. AP-PCR analysis of primary and satellite hepatocellular carcinoma (HCC) nodules. Genomic DNA from each sample was amplified with the AR3 (A) and ZF3 (B) arbitrary primers. S1, S2 and S3 represent the three different sectors in the same primary HCC nodule of patient Sc. N, 1-3 and A-E represent non-tumour tissue, three different sectors of primary and five satellite HCC lesions of patient Go, respectively. Size of bands (in base pairs) is indicated on the right.

5.2 Intratumour genomic fingerprints

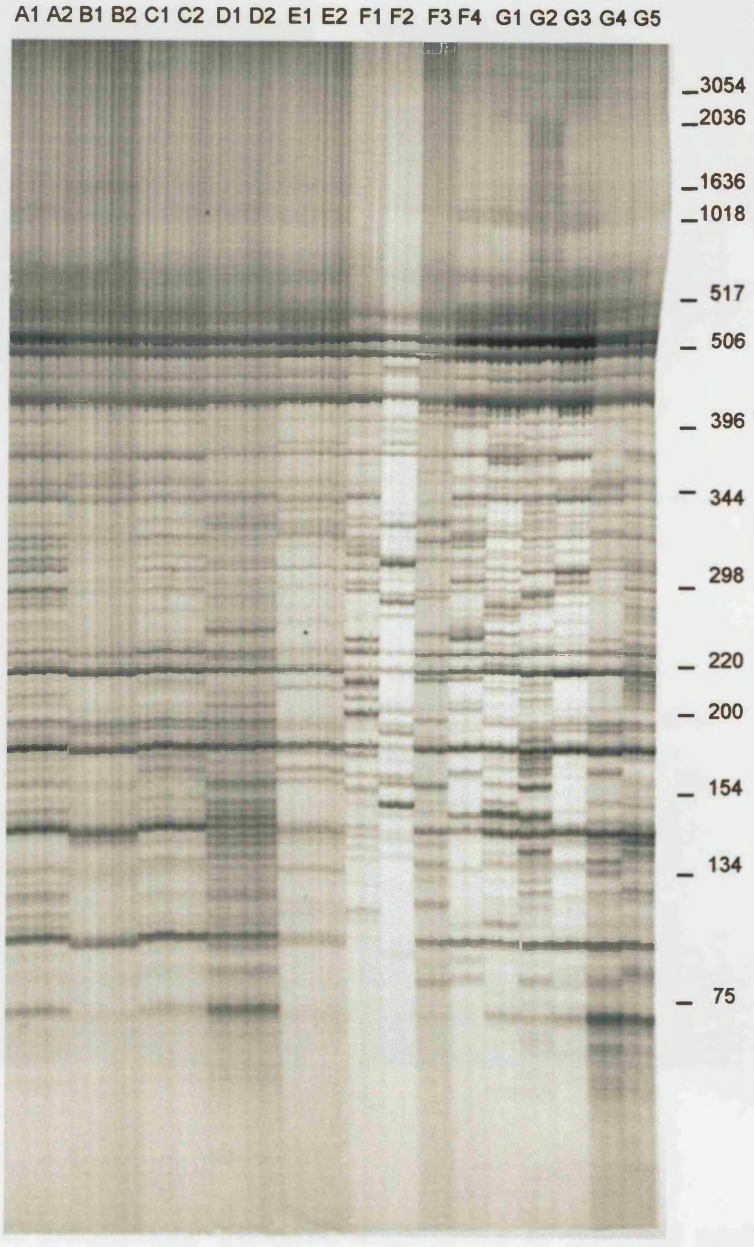
HCCs often contain subpopulations of cells showing heterogeneous differentiation within each tumour. The majority of HCCs first appear as well-differentiated cancers and proliferate with gradual dedifferentiation. No previous attempts have been made to use AP-PCR technology to investigate evidence of mutation and clonal diversity associated with progression in neoplasms, and which may relate to the acquisition of the characteristics of malignancy. In this study, we assessed the degree of genomic heterogeneity of intra-HCC nodules using the AP-PCR technique. Thirty-one HCCs were sampled from 8 cirrhotic livers. Two or more sectors of each nodule were needle-microdissected separately and amplified with two different arbitrary primers in appropriate conditions.

Different genomic patterns were noted between each different RN in the same patient. In every HCC less than 6 mm diameter (N=18, range 3-6 mm, mean diameter 4.7 mm), all sectors of each of these lesions had an identical DNA fingerprint, characteristic for the individual tumour nodule and distinctly different to the fingerprint generated from the normal tissue DNA from the same patient (Figures 16 & 17). All HCC greater than 6 mm diameter (N=13, range 7-30 mm, mean diameter 15.4 mm) showed distinctly different DNA fingerprints in each sector sampled ($P<0.05$, compared to size less than 6 mm diameter) (Figure 18). When synchronous HCCs were present, no tumour had the same DNA fingerprint as any other.

Figure 16. AP-PCR analysis of intratumour genomic DNA fingerprints of hepatocellular carcinoma (HCC) nodules. Autoradiograms were prepared from polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by AP-PCR. Genomic DNA from different sectors of individual nodules from one patient was amplified with the AR3 (A) and ZF3 (B) arbitrary primers. A1-A2, B1-B2, C1-C2, D1-D2 and E1-E2 represent two sectors of different HCC nodules less than 6 mm in diameter. F1-F4 and G1-G5 represent four and five sectors in the HCC nodules which were 10 and 20 mm in diameter, respectively. The size of bands (in base pairs) is indicated on the right.


A1 A2 B1 B2 C1 C2 D1 D2 E1 E2 F1 F2 F3 F4 G1 G2 G3 G4 G5
3054
2036
1636
1018
517
506
396
344
298
220
200
154
134
75

A.


A1 A2 B1 B2 C1 C2 D1 D2 E1 E2 F1 F2 F3 F4 G1 G2 G3 G4 G5
3054
2036
1636
1018
517
506
396
344
298
220
200
154
134
75

B.

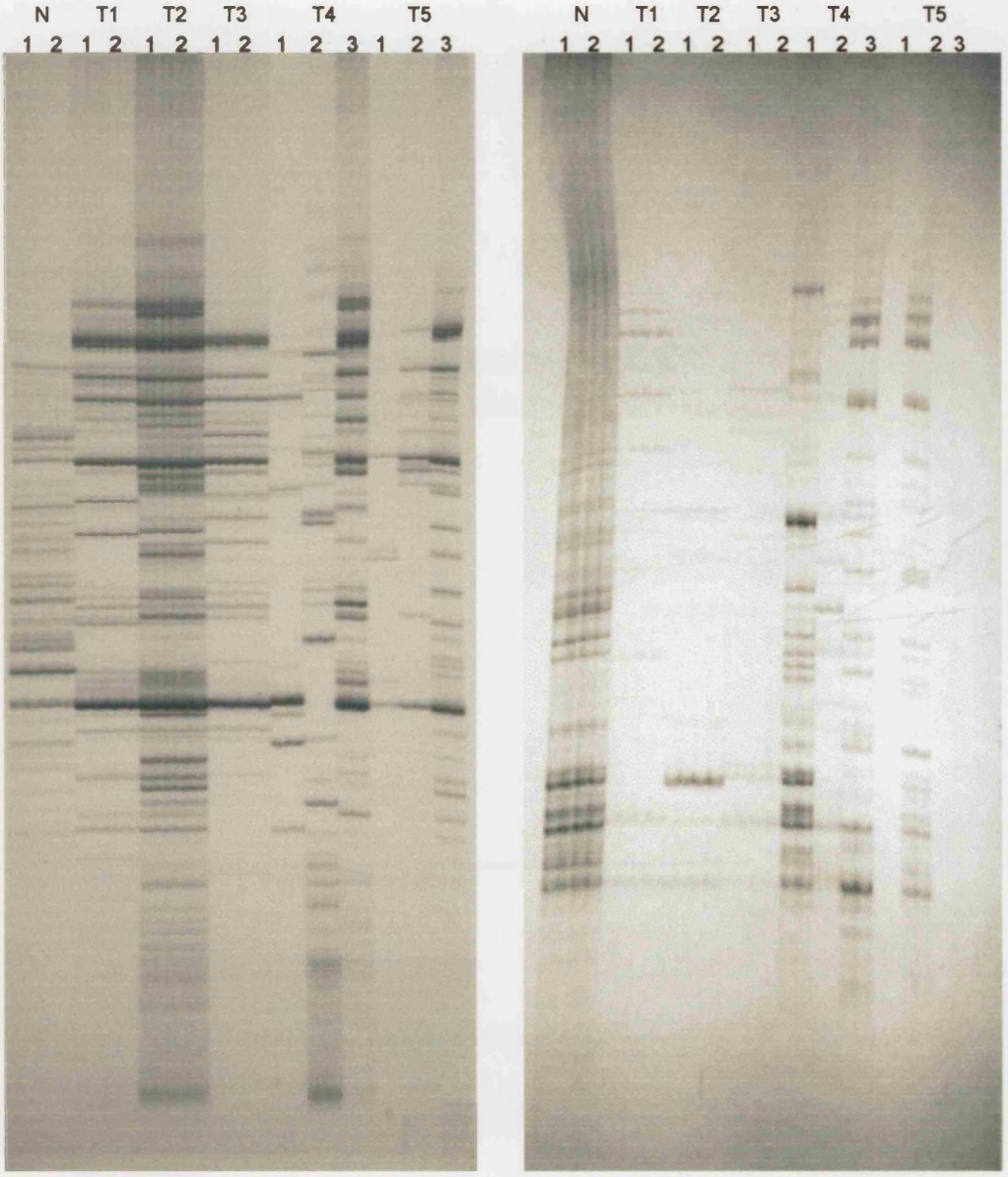


Figure 17. Intratumour genomic fingerprints of different sectors in the same nodules from one patient. Autoradiograms of polyacrylamide gel electrophoresis of DNA fragments amplified by AP-PCR using arbitrary primers, AR3 (A) and ZF3 (B). In every hepatocellular carcinoma (HCC) nodules less than 6 mm diameter (T1, T2 & T3), all sectors of each of these lesions had the same DNA fingerprint whereas all HCC nodules greater than 6 mm diameter (T4 & T5) showed distinct DNA fingerprints in each sector sampled. N, non-tumour tissue; T1-T5, HCC nodules; number of sectors is indicated in the second line at the top.

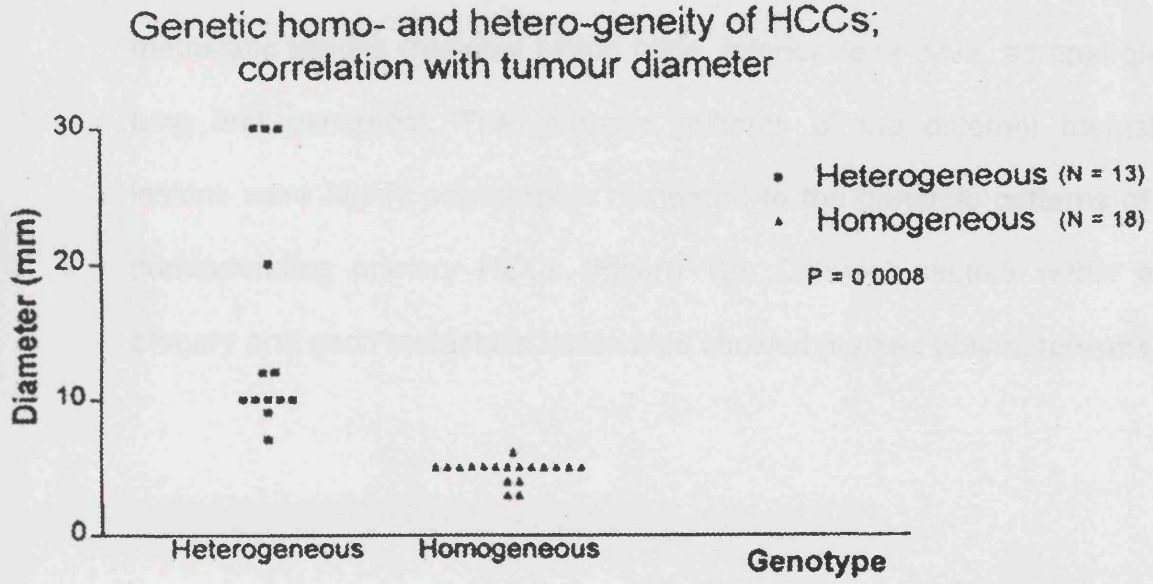


Figure 18. Genetic homo- and hetero-geneity of HCCs; correlation with tumour diameter. *(Homogeneous vs Heterogeneous, t test* p=0.0008*)*

5.3 Genomic heterogeneity in primary and metastatic HCCs

HCC is an aggressive cancer that metastasises frequently. Little is currently known about the molecular relationship between primary HCCs and metastatic lesions. We used the AP-PCR technique to screen the genome DNA for rearrangements and deletions in five primary HCCs and their metastatic lesions (regional lymph node, inferior vena cava, adrenal gland, lung and pancreas). The genomic patterns of the different metastatic lesions were highly polymorphic compared to the genomic patterns of the corresponding primary HCCs (Figure 19). Different sectors within each primary and each metastatic lesion also showed marked polymorphisms.

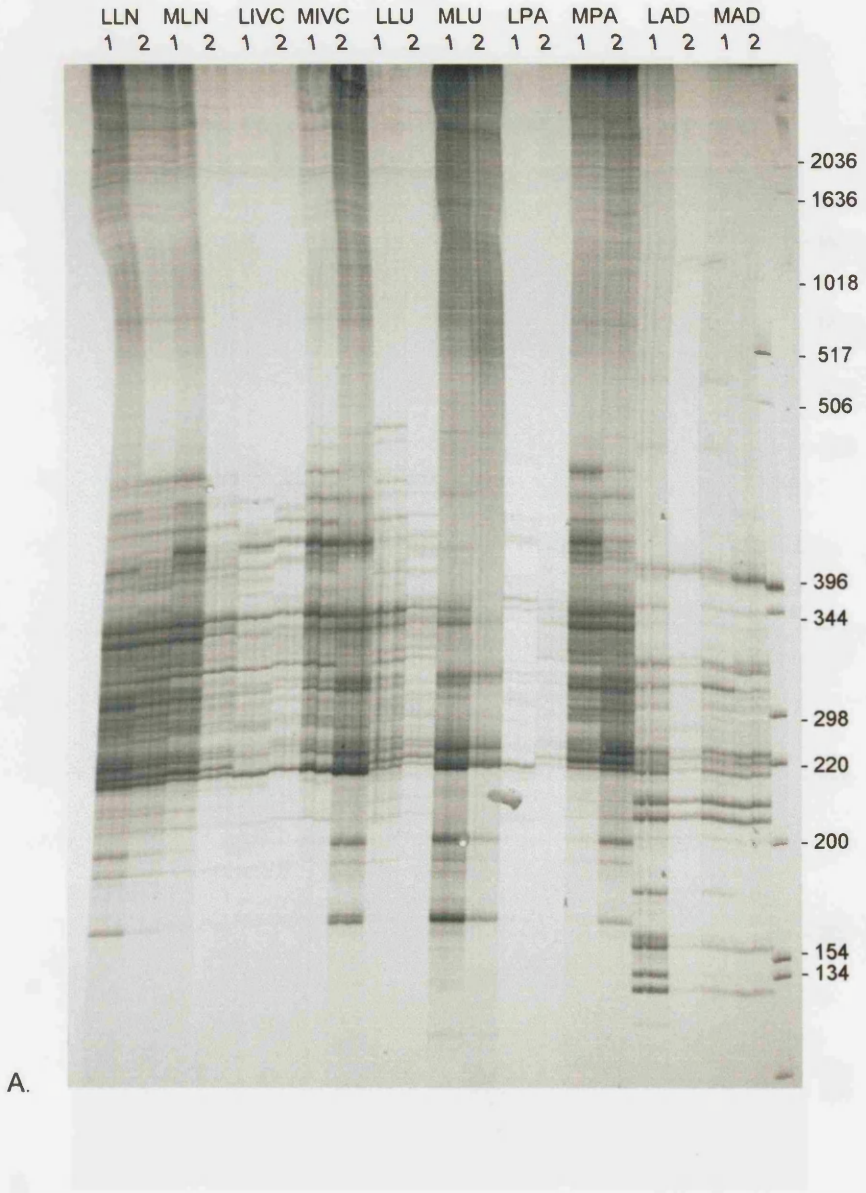
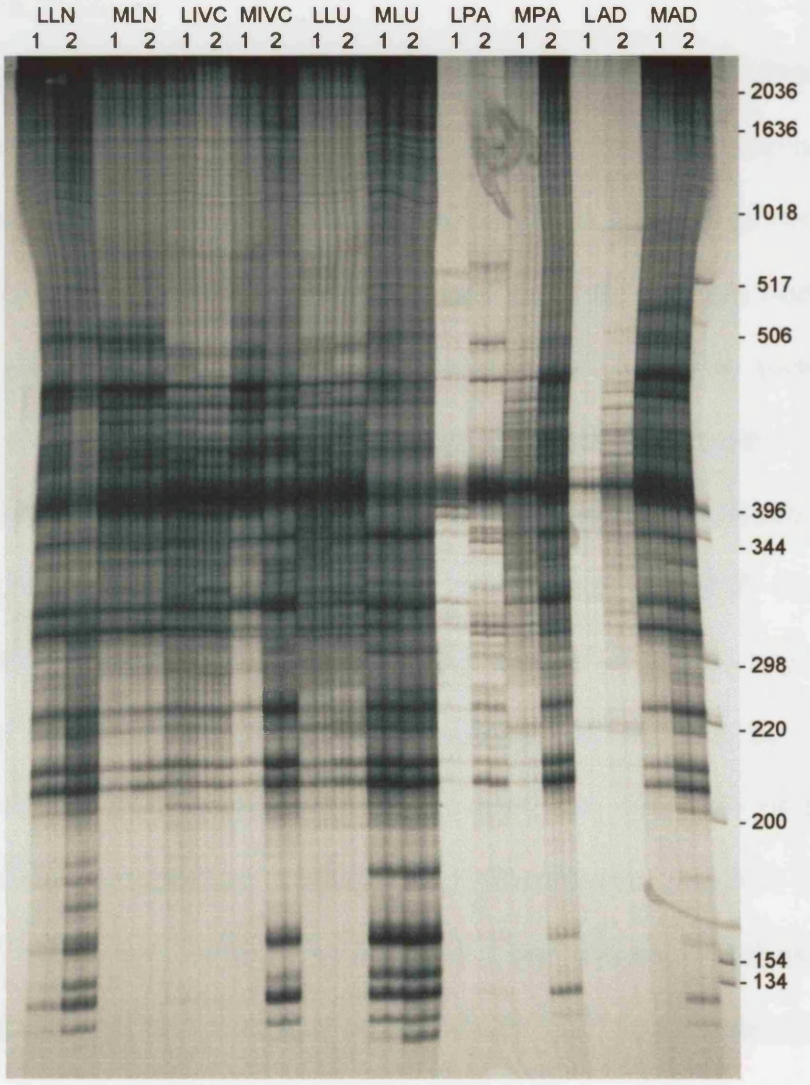


Figure 19. AP-PCR analysis of five primary HCCs (LLN, LIVC, LLU, LPA & LAD) and their metastatic lesions (MLN-regional lymph node, MIVC-inferior vena cava, MLU-lung, MPA-pancreas and MAD-adrenal gland). Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by AP-PCR. Genomic DNA from each sample was amplified with the AR3 (A) and ZF3 (B) primers. Size of bands (in base pairs) is indicated on the right. Each lesion was sampled in two sections (1 and 2 for each case).

B.


LLN 1 2
MLN 1 2
LIVC 1 2
MIVC 1 2
LLU 1 2
MLU 1 2
LPA 1 2
MPA 1 2
LAD 1 2
MAD 1 2
2036
1636
1018
517
506
396
344
298
220
200
154
134

5.4 Discussion

Clonal analysis of HCCs

To study variant or polymorphic sites of DNA target segments, classical DNA fingerprinting is the most commonly used technique employing restriction fragment length polymorphisms by Southern blotting or probes for polymorphic, multiply represented sequences, such as variable number of tandem repeats (VNTRs). Specific PCR primer pairs can be used to identify genes characteristic of a particular species. DNA allelotyping using the highly polymorphic VNTRs has been applied to the detection of polymorphism during malignant transformation (Thein *et al*, 1987) and to study of clonality of tumours (Fey *et al*, 1988). Fey *et al* (1988) reported clonal somatic mutations in 29 of 44 gastrointestinal cancers by DNA allelotyping using three different probes. However, the detection of DNA polymorphisms by established fingerprinting procedures requires prior knowledge of DNA sequence, cloned and characterised probes, or considerable experimental manipulation. AP-PCR has three advantages when compared with classical DNA fingerprinting. First, minor amounts of template DNA are sufficient for analysis (50 ng of genomic DNA for AP-PCR versus 5-10 μg for Southern blotting). Second, somatic mutations detected in tumour fingerprints can by chance directly represent a mutation in a coding sequence, and third, the possibility of reamplification, cloning, and sequencing of polymorphic bands enables the rapid identification of the sequences probably linked to tumour progression.

AP-PCR is a highly specific technique that is able to generate species-specific and individual diagnostic DNA fingerprints (Schlegel *et al*, 1996). This technique has not been previously used to assess genomic relationship between synchronous HCC nodules, but we found it to be a simple and effective approach for analysing the genetic profile. Polymorphisms in genomic fingerprints generated by AP-PCR can demonstrate genetic differences between nodules. Fingerprinting patterns of amplified genomic DNA isolated from paraffin-embedded tissues were shown to be reproducible and interpretable. Therefore, DNA fingerprinting offers a novel approach to determining clonality in tumours and may prove useful for the study of tumour progression.

The determination of clonality of human tumours has important implications for the mechanisms of carcinogenesis. Although various techniques have been developed to assess the clonality in tumours, there are some limitations to these techniques. Using integrated HBV DNA as a marker is applicable only to a portion of patients who have HBV infection. X-linked restriction fragment length polymorphisms are limited to tumours of female patients, while the analysis of DNA content does not detect the cell with a balanced gain or loss of chromosomes, or with genetic rearrangements. Cytogenetic techniques have been used to determine the location of tumour suppressor genes because consistent deletions or inversions of part of a chromosome are indicative of the inactivation of a nearby tumour suppressor genes during neoplastic progression. However, cytogenetic techniques do not detect the entire spectrum of inactivating events during

carcinogenesis, such as microdeletion, chromosomal loss with duplication and homologous recombination with defective chromatid.

Resected liver specimens sometimes show small nodular lesions without definite features of cancer in addition to the main HCC tumour mass. Unfortunately, these lesions are not clearly defined, nor is their nomenclature established. They have been variously referred to as adenomatous hyperplasia, nodular hyperplasia, adenomatous hyperplastic nodules and MRNs. In view of such a confused definition, we used the term regenerative/macroregenerative nodules (RNs/MRNs) according to Ferrell's proposal (Ferrell *et al*, 1993). The data showed that the presence of MRNs was associated with an increased incidence of liver cell dysplasia and HCC, these changes being absent in cirrhotic livers without MRNs (Furuya *et al*, 1988; Hytiroglou *et al*, 1995). It has generally been accepted that MRNs are a possible precancerous lesion. Our findings demonstrated a distinctly different molecular profile among all MRNs examined, whereas previous reports of the incidence of monoclonal MRNs (based on hepatitis B or hepatitis C analysis) have varied from 0.5% to 43% (Aoki & Robinson, 1989; Yasui *et al*, 1992; Aihara *et al*, 1994). DNA fingerprints of MRNs were different from those of HCCs even in the same patient, which suggests that HCC may develop independently of MRN, *ie.* bypass a rather persistent stage of precancerous nodules. Favouring this view, in a previous study of 17 patients with MRNs, none developed into HCC in 13-51 months of follow up; indeed, the nodules disappeared in four cases. HCCs developed apart from the MRNs in four of these patients during this period (Kondo *et al*, 1990). There is also an experimental model of mouse hepatocarcinogenesis

characterised by *de novo* occurrence of HCCs without any precancerous nodular formation (Kohen *et al*, 1983).

Once the tumour becomes detectable by radiological examination, it is usually already about 1 cm in diameter, representing around 10^9 cells. In the evolving process of hepatocarcinogenesis, this represents a relatively late stage. The results from this study suggest genomic heterogeneity in synchronous HCCs and as the tumour nodules expand beyond 6 mm in diameter, the single initiating clone evolves into multiple distinct derivative clones which can be recognised by their DNA fingerprints. On histopathological observations, the small HCCs tend to be composed of homogeneous well-differentiated cancer cells, while more undifferentiated areas appeared as the lesions increased in size. In one study, cancer nodules less than 1 cm in diameter consisted of a uniform distribution of well-differentiated cancerous tissues, whereas approximately 40% of cancer nodules of 1.1-3.0 cm in diameter consisted of more than two cancerous populations of varying levels of dedifferentiation (Kenmochi *et al*, 1987). Although most HCCs were once believed to be monoclonal in origin (Kondo *et al*, 1989; Aihara *et al*, 1994), recent studies have shown that a certain proportion of them are clearly of multiclonal development (Sakamoto *et al*, 1989; Aoki & Robinson, 1989; Hsu *et al*, 1991; Sheu *et al*, 1993). Heterogeneity of DNA content has also been described in multiple synchronous HCCs (Hui *et al*, 1997). The multifocal nature of HCC in cirrhotic patients explains the high rate of intrahepatic recurrence after resection. Belghiti *et al* (1991) reported 60% of patients had intrahepatic recurrence within 3 years after solitary HCC resection. Recurrence was

adjacent to the resection margin in only 9% of cases. In addition, 76% of deaths long after resection of a small HCC (less than 2 cm in diameter) were attributed to cancer recurrence (Nagashima *et al*, 1996). Moreover, by studying of genomic tumour alterations, a different clonal origin between the initial and the recurrent tumour was demonstrated (Chen *et al*, 1989; Nagasue *et al*, 1992)

In normal hepatogenesis, a common stem cell known as the oval cell is bipotential in its ability to replicate into hepatocytes or bile ductular cells (Thorgeirsson, 1996). The oval cells become involved when extensive replication occurs. The parenchymal hepatocytes are responsible for postnatal replication in the normal liver and do not migrate from periportal to pericentral regions as previously suggested. However, the cellular origin of HCC has been controversial. A recent model of retroviral KRAS oncogene transfer into cultured oval cells resulted in HCC and cholangiocarcinoma (CCC) after implantation into animals which were treated with a low dose of the carcinogen diethylnitrosamine (DEN) to induce the additional mutations (Ponder, 1996). *In vitro* transfer of an oncogene into cultured hepatocytes only resulted in HCC in animals but transfer of an oncogene into hepatocytes *in vivo* resulted in both HCC and CCC. Moreover, no cancers were observed in animals treated with oncogene alone or in DEN-treated animals transduced with a control retroviral vector alone supporting the multistep hepatocarcinogenesis theory. Within the background of an anomalous hepatic parenchyma containing numerous potential carcinogenetic cells, multiple HCCs can arise independently from different foci in the liver if a sufficient number of genetic mutations accumulate. The

genetic heterogeneity in each HCC may be the result of (in)activation of specific gene loci in the neoplasm, as alternative consequences to the continued presence of carcinogen. As a result of genetic instability in the expanding tumour population, mutant cells are produced. Most of these variants are eliminated because of metabolic disadvantage or immunological destruction. Occasionally one has an additional selective advantage with respect to the original tumour cells as well as normal cells, and this mutant becomes the precursor of a new predominant subpopulation. Yano *et al* (1993) established two distinct human HCC cell lines from a single nodule showing a three-layered structure with a different histological grade in each layer. Chromosomal abnormalities and p53 point mutations at codon 242 were identical, suggesting that the two cell lines were related in origin and that subsequent clonal dedifferentiation had occurred. Moreover, three established HCC cell lines derived from the same HCC (Hep-3B 14, Hep-3B 217 and Hep-3B F1) were shown to have differences in the pattern of HBV DNA integration, in the number of viral copies per cell and in their ability to synthesise HbsAg (Twist *et al*, 1981). Hence, each HCC nodule may require individual specific therapy, and even this may be prevented by the emergence of a genetically variant subclone resistant to the treatment. Such genomic heterogeneity in synchronous HCCs may explain the poor response to treatment. The high recurrence rate is still a major problem after curative resection of HCC, even for small tumours. If the smaller tumours are *de novo* lesions rather than metastases - as these data suggest - then current concepts regarding liver resection as a curative treatment modality for HCC may require reassessment.

Genomic heterogeneity in primary and metastatic HCCs

Metastasis is the most dangerous outcome and life-threatening aspect of tumour progression. The process of metastasis involves a cascade of linked, sequential steps involving multiple host-tumour interactions. They can be facilitated by proteins that stimulate tumour cell attachment to host cellular or extracellular matrix determinants, tumour cell proteolysis of host barriers such as the basement membrane, tumour cell locomotion, and tumour cell colony formation in the target organ for metastasis. Advances in understanding the molecular mechanisms involved in metastasis have lagged behind other developments in the cancer field. Some genetic changes result in an imbalance of growth regulation, leading to uncontrolled proliferation, but do not result in invasion and metastasis. The process may therefore require additional genetic changes. There are reasons to believe that tumour cells acquire the ability to metastasise through genetic variation, and expression of specific genes has been associated with the metastatic phenotype (Fidler & Radinsky, 1990; Liotta *et al*, 1991). A randomised controlled study of postoperative adjuvant chemotherapy in a group of patients who underwent curative resection of HCC demonstrated more frequent extrahepatic recurrences and lower disease-free survival rates than the curative resection-alone group (Lai *et al*, 1998). The data suggested that chemotherapy may stimulate some clonal variants from the surviving subpopulations and permits cells with a higher metastatic capability to proliferate.

Whether neoplasms are unicellular or multicellular in their origin, the process of tumour evolution and progression can rapidly generate biological

diversity (Fidler & Hart, 1982). Dissemination of malignant cells throughout the body and their survival to form secondary growths is a complicated process dependent on both host and tumour properties. Although a primary tumour may release many cells, only a few (<0.01%) survive the interactions with host defence mechanisms to yield distant cancer growths. A considerable body of evidence has accumulated to suggest that the survival of these few cells is not a random event. Rather, it represents the selection of a pre-existent metastatic subpopulation of tumour cells within the parental population. Metastases may have a clonal origin, and different metastases may develop from different progenitor cells. Such a process has profound implications in determining approaches to therapy and to the elucidation of those tumour-cell properties that are responsible for successful metastatic spread.

CHAPTER VI

LASER CAPTURE MICRODISSECTION AND GENOMIC HOMOGENEITY IN FIBROLAMELLAR CARCINOMAS

VI. LASER CAPTURE MICRODISSECTION (LCM) AND GENOMIC HOMOGENEITY IN FIBROLAMELLAR CARCINOMAS (FLCs)

6.1 Laser-assisted microdissection

There have been dramatic advances in our knowledge of the molecular processes involved in human diseases, but it is certain that other molecular and genetic lesions remain to be identified, and there is a pressing need to integrate such information with structural and architectural data derived from conventional morphological approaches. Tissue microdissection is one of the most advanced techniques in molecular pathology. Together with multiplex molecular approaches, it is now feasible to study genetic alterations and isolate genes and proteins in selected pure populations from complex normal and diseased tissues.

6.1.1 Microdissection strategy

The presence of a wide spectrum of cell types in both the epithelial and the stromal components (including reactive fibroblasts, endothelium and a variety of infiltrating leucocytes) of various organs may complicate the analysis of a particular population. To overcome this difficulty, several microdissection techniques have been developed to procure solely the cells of interests in a tissue specimen. The microdissection techniques originally involved manual or micromanipulator guidance of a needle to scrape off the cells of interest under a microscope, and these have been applied with enthusiasm in various systems for some years. They have been used to isolate a few hundred cells from heterogeneous tissue samples. Modified

methods such as the use of an electrolytically sharpened tungsten needle in a bacteriological loop-holder, a hydraulic micromanipulator and starch adhesive gum fractionation have been reported (Going & Lamb, 1996; Moskaluk & Kern, 1997; Turbett *et al*, 1996). However, the manual approach is very time consuming and operator-dependent requiring a great deal of skill and dexterity, and the identity of dissected cells cannot easily be documented or recorded. To avoid contamination by neighbouring tissues, the superfluous parts surrounding selected cells can be irradiated by selective ultraviolet radiation fractionation (SURF) or ablated by ultraviolet-laser from the PALM (3D-positioning and ablation with the laser microscope) microbeam system (Shibata *et al*, 1992; Schütze & Clement-Sengewald, 1994). Then the target cells are scraped or retrieved by using a needle. Though the techniques have been successfully applied to dissect a single cell, the process of cell transfer is based on needle manipulation and further analysis of the adjacent tissues is not possible (Becker *et al*, 1996). Following the development of membrane-mounted media, various methods termed microbeam microdissection of membrane-mounted native tissue (MOMeNT), laser microbeam microdissection (LMM) and laser pressure catapulting (LPC) have been introduced (Böhm *et al*, 1997). A six-micrometer polyester membrane is used to support the tissue with glycerol or mineral oil as an embedding medium. The microbeam causes dissection by local photolysis of the membrane and adjacent tissue section which are then transferred into a microfuge tube by a 30-gauge needle or small forceps. Though the use of an ultraviolet light allows the microbeam to be focused to a small diameter with a delineation accuracy of 2 to 7

micrometers, the adjacent cells may be destroyed along the dissecting track.

6.1.2 Laser capture microdissection

Recently, a laser capture microdissection (LCM) system, a novel membrane-based microdissection technique, has been developed by Liotta *et al* (Emmert-Buck *et al*, 1996). The system has been subsequently commercialised and used in many laboratories. A thermoplastic ethylene vinyl acetate transfer film containing a near infrared absorbing dye attached to a 6-millimetre diameter rigid, flat cap (Figure 20) is placed in contact with a routinely prepared, haematoxylin and eosin stained tissue section. The film over the cells of interest is precisely activated by a near-infrared laser pulse and bonds strongly to the selected cells. Although the laser transiently raises the temperature of the transfer film to 90°C, its energy is absorbed by the film and is poorly absorbed by biological tissue. Nucleic acids and proteins recovered from these cells are therefore not degraded by heat conduction (Goldstein *et al*, 1998). Removal of the cap from the tissue section effectively procures the targeted cells. The identity of the transferred cells attached to the film can then be recorded by image capture. The cap is fitted in a microfuge tube containing lysis buffer and DNA, RNA and proteins can then be extracted for molecular analysis (Figures 21 & 22). Since no coverslip is used in LCM, the reduction in refractive index means that most light passing through the tissue is scattered

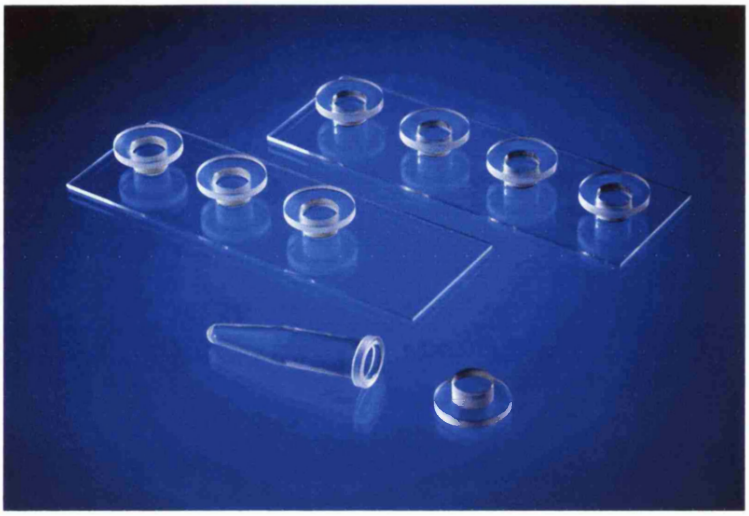


Figure 20. A thermoplastic ethylene vinyl acetate transfer film containing a near infrared absorbing dye attached to a 6-millimetre diameter rigid, flat cap (from http://www.arctur.com/)

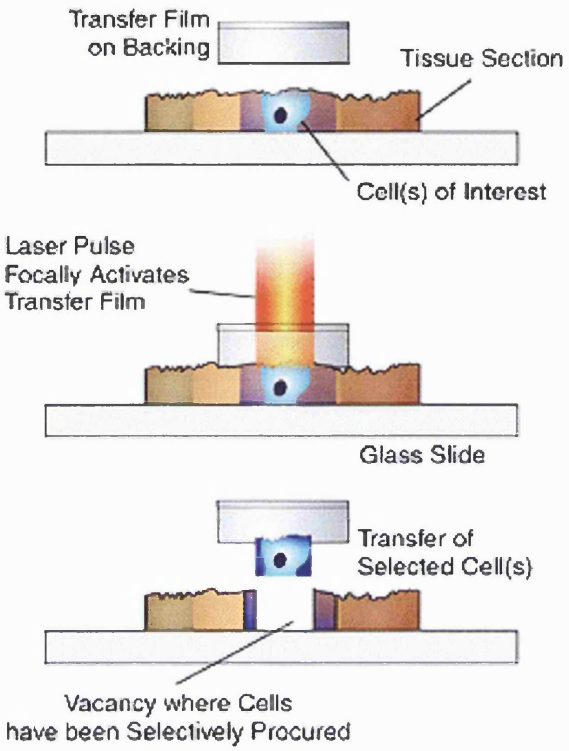


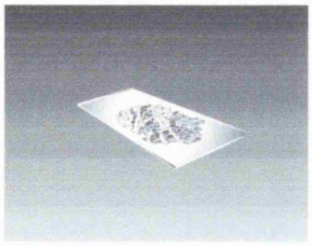
Figure 21. Diagram of the principle of Laser Capture Microdissection

A. The transparent transfer film is applied to the surface of the tissue section mounted on a glass slide. Under the microscope, the diagnostic pathologist or researcher views the tissue through the film and chooses microscopic clusters of cells to study.

B. When the cells of choice are in the centre of the field of view, the operator pushes a button which activates a laser diode integral with the microscope optics. The pulsed laser beam activates a precise spot on the transfer film immediately above the cells of interest. At this precise location the film melts and fuses with the underlying cells of choice.

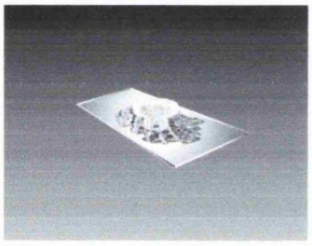
C. When the film is removed, the chosen cell(s) remain stuck to its undersurface, while the rest of the tissue is left behind. The film is then placed directly into the DNA, RNA, or enzyme buffer.

(from http://www.arctur.com/)



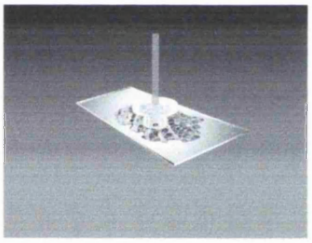
1. Prepare

Follow routine protocols for preparing a tissue or smear on a standard microscope slide.



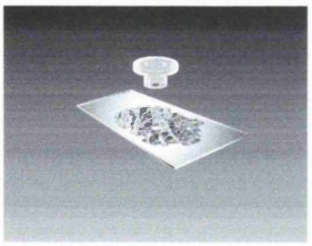
2. Locate

Visualise the sample through the video monitor or the microscope. Use the joystick to locate the cell(s) of interest and position the vial cap film carrier over the cell(s).



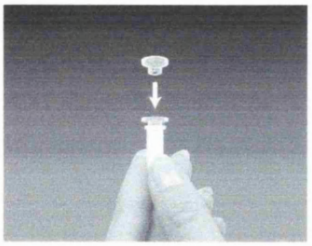
3. Capture

Press a button to pulse the low power infrared laser. The desired cell(s) adhere to the vial cap film carrier.



4. Microdissect

Lift the vial cap film carrier, with the desired cell(s) attached to the film surface. The surrounding tissue remains intact.



5. Analyse

Place the vial cap film carrier directly onto a standard microcentrifuge tube containing the extraction buffer. The cell contents, DNA, RNA or protein are ready for subsequent molecular analysis.

Figure 22. Experimental steps of Laser Capture Microdissection (from http://www.arctur.com/)

which can obscure cellular detail at high magnifications. The isolation of cells from immunohistochemical or molecule-specific fluorescent labelled (fluorescent *in situ* hybridisation) section improves sample imaging and can help in obtaining specific cell populations more precisely (Fend *et al*, 1999).

With the help of Professor NR Lemoine, who recently introduced the LCM system (Arcturus Engineering Inc., Santa Clara, USA) (Figure 23) into the ICRF Molecular Oncology Unit, Hammersmith Hospital, I have performed LCM on formalin-fixed, paraffin-embedded HCC tissues. Under the microscope, the tissue can be viewed through the film, and microscopic clusters of cells chosen to study. When the cells of choice are in the centre of the field of view, pushing a button activates a laser diode integral with the microscope optics. The pulsed laser beam activates a precise spot on the transfer film, which is bonded to the underside of a vial cap. At this precise location the film immediately above the cells of interest melts and fuses with the underlying cells of choice. The cap is lifted off the tissue and placed directly onto a vial for molecular processing. The cells adherent to the film retain their morphological features, and the operator can verify that the correct cells have been procured. Images of the microdissected cells before and after LCM are stored in a computer and serve as a diagnostic record. We found that the LCM technique is simple, fast, requires no moving parts, involves no manual microdissection or manipulations, and enables one-step transfers. The data have shown that from only one tiny sample of selected cells, DNA can be successfully extracted and analysed by the PCR technique (Figures 24 & 25).

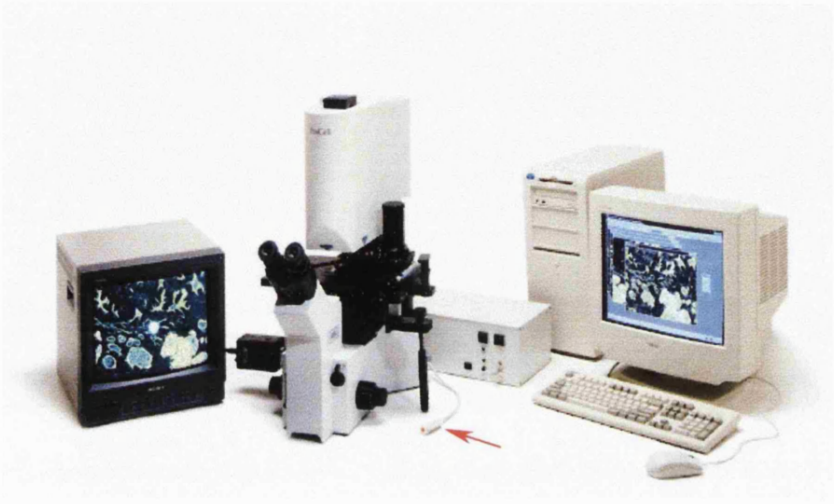


Figure 23. Pixcell instrument for Laser Capture Microdissection

Under the microscope (centre), the tissue can be viewed through the film, and microscopic clusters of cells chosen to study. The cells of interest were also viewed by the monitor (left). When the cells of choice are in the centre of the field of view, pushing a button (red arrow) activates a laser diode integral with the microscope optics. The operator can verify that the correct cells have been procured. Images of the microdissected cells before and after LCM are stored in a computer (right) and serve as a diagnostic record.

(from http://www.arctur.com/)

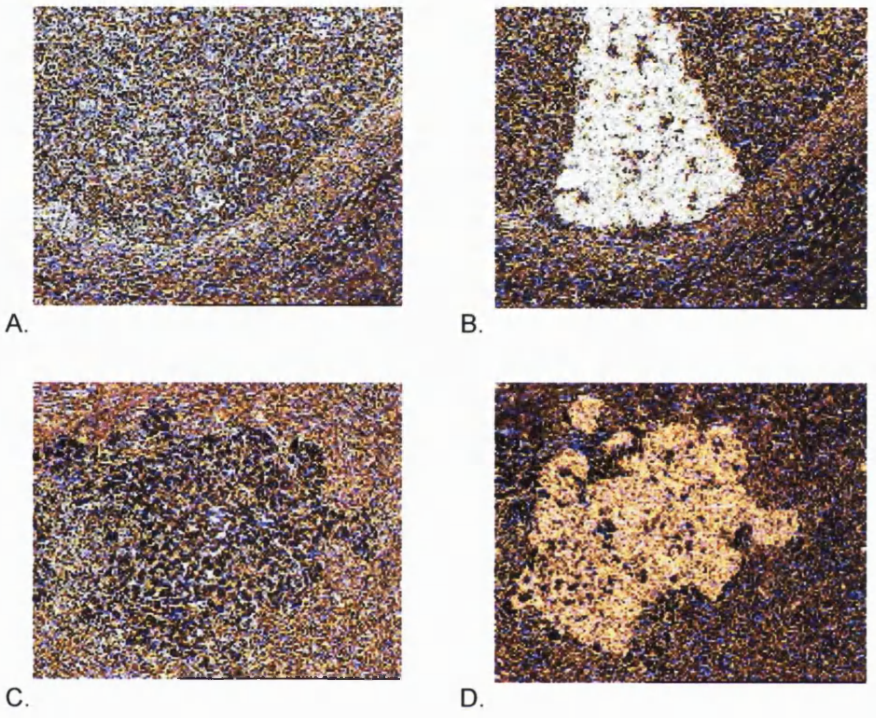


Figure 24. Examples of Laser Capture Microdissection transfer

Two specimens of 5-micron thick paraffin-embedded sections of hepatocellular carcinoma tissues (A & C), stained with Haematoxylin and Eosin. The tissue sections after laser capture with the laser spot size (30 microns) (B & D, respectively).

Figure 25. Laser Capture Microdissection of a HCC tissue

A. Five-micron thick paraffin-embedded of hepatocellular carcinoma tissue, stained with Haematoxylin and Eosin

B. Selected tissue from the section transferred to the film of the cap

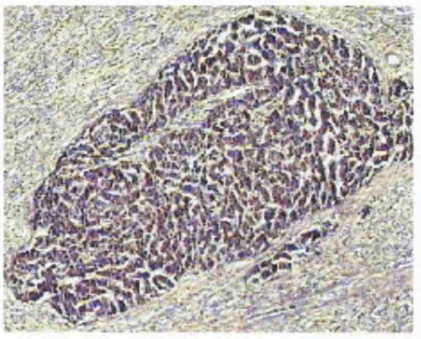
C. The tissue section after laser capture

D. The laser spot size (30 microns)

E. Recovered DNA from single transferred tissue sample (B) was amplified by PCR to generate a 190 base-pair product (GAPDH)
Lane 1: 100 bp DNA ladder
Lane 2: Positive control
Lane 3: Genomic DNA extracted by incubating with proteinase K buffer overnight at 37°C

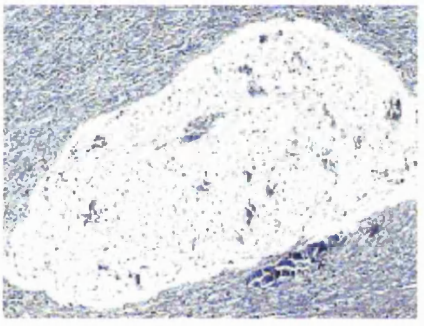
Note: Pixcell Slide Dissection Data
Instrument Serial Number: 0136
Cap Lot Number: 02/03/98-c-1
Sample Thickness: 5.0 microns
Laser Spot Size: 30 microns
Pulse Power: 70 mW
Pulse Width: 50.0 ms
Threshold Voltage: 1.03 V
Total Pulses: 359
Estimated Transfer per Pulse: 90%
Estimated Volume of Tissue Dissected: 1.14e+006 microns^3

A.

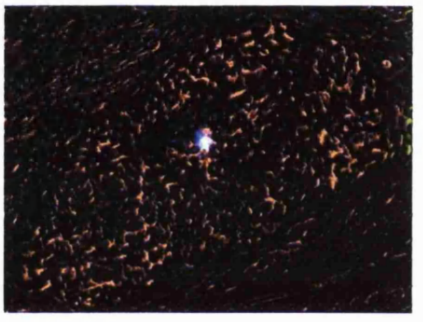


B.

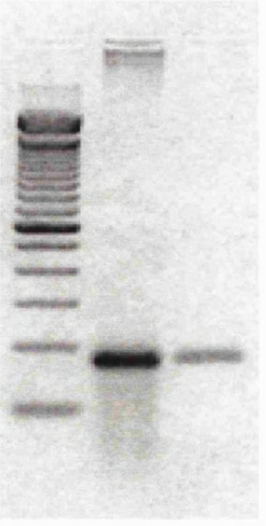
C.



D.



E.



6.1.3 Comparison of material obtained by needle microdissection and laser capture microdissection

Prior to the establishment of the LCM system within our group, all genomic fingerprints of HCC samples were generated from DNA prepared by needle microdissection as described previously. However, we have made a careful comparison of the results obtained from the tissue microdissection procedures (needle microdissection and LCM). Two 10-μm sections were serially cut from four distinct HCC nodules onto glass slides and were stained with H&E. Two sectors of each section were microdissected separately. The first section was needle-microdissected using sterile 27-gauge needles with the assistance of a dissection microscope. The dissected cells of each sector were transferred into a 0.5-ml microfuge tube containing 50 μl of proteinase K buffer and incubated overnight at 37°C. After the incubation period, the buffer is inactivated at 95°C for 10 minutes and the solution was used as a template for PCR.

Both corresponding sectors of the second section were separately microdissected by LCM. The system employs an ethylene vinyl acetate polymer layer coated onto the underside of a rigid, flat vial cap. Under the microscope, the cells of choice can be viewed through the film and the pulsed laser beam activates a spot on the transfer film. At this precise location, the film immediately above the cells of interest melts and fuses with the underlying cells. The cap is lifted off the tissue and placed directly onto a 0.5-ml microfuge tube containing 50 μl of proteinase K buffer. The

tube was inverted and incubated overnight at 37°C. After the incubation period, the tube was centrifuged at 13000 rpm for 5 minutes and the cap was removed. Then the buffer was inactivated at 95°C for 10 minutes and ready to use as a template for PCR.

To examine the quality of the DNA samples from both microdissection techniques, 4 µl of DNA solution were used for amplification with GAPDH primers in a total volume of 50 µl solution containing 50 mM KCl, 10 mM Tris.Cl pH 8.3, 1.5 mM $MgCl_2$, 100 pM primers and 5 units *Taq* polymerase. Templates were denatured for 5 minutes at 95°C and were subject to 35 cycles at 94°C, 1 minute; 55°C, 1 minute and 72°C, 2 minutes. The PCR products were run on 1.5% agarose gel staining with ethidium bromide. Then the fingerprinting of DNA samples was performed by using the AP-PCR technique as described previously. The analyses of sectors of tissue microdissected by needle and laser capture techniques are shown in Figures 26 and 27 respectively. The size of a 27-gauge needle (Figure 26C) is much larger than the laser spot in the LCM system (Figure 27B). The manually microdissected cells can detach from the tip of the needle during transfer whereas the cells obtained by LCM adhere to the film where they retain their morphological features and the operator can verify that the correct cells have been procured (Figures 27C & 27D). DNA prepared from the cells of interest was amplified by AP-PCR technique. Identical DNA fingerprints of each sector dissected by the two microdissection methods are demonstrated in Figure 30.

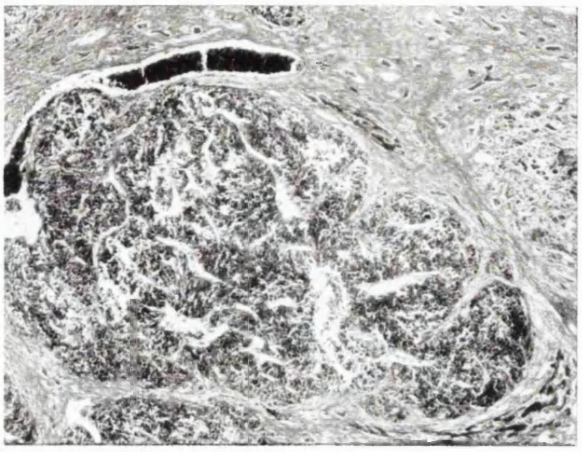
Figure 26. Manual microdissection of a HCC nodule

A. A 10 micron thick paraffin-embedded section of HCC tissue stained with haematoxylin and eosin.

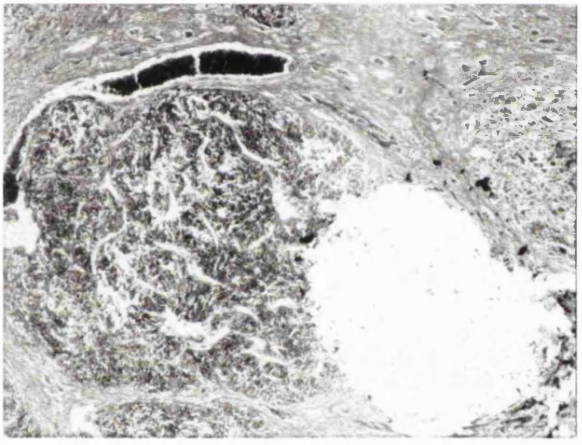
B. A sector of tissue (M1) was needle microdissected and transferred into a microfuge tube.

C. The second sector (M2) was subsequently needle microdissected and transferred into another microfuge tube. The 27-gauge needle tip under microscopic guidance.

A.



B.



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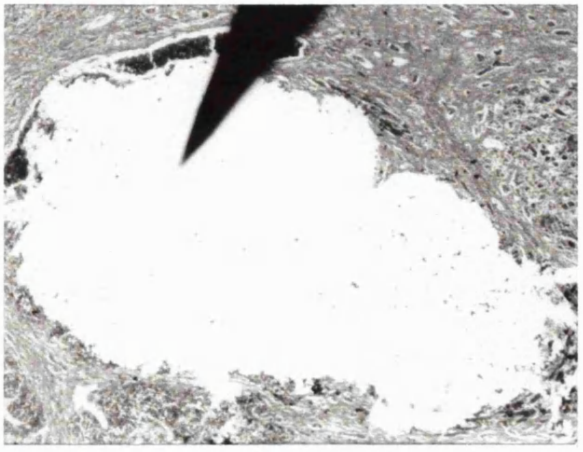


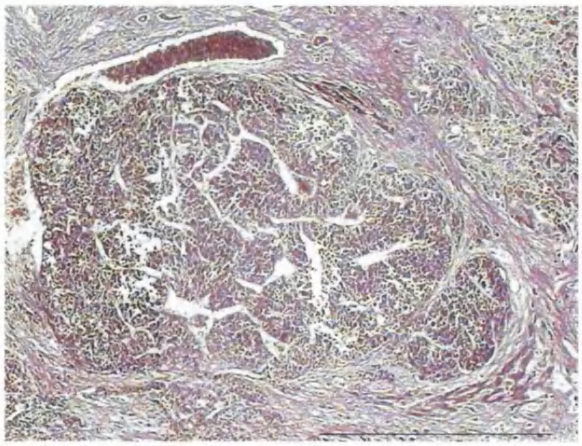
Figure 27. Laser capture microdissection of the same HCC nodule in a serial section of figure 26

A. A 10 micron thick paraffin-embedded section of HCC tissue stained with haematoxylin and eosin.

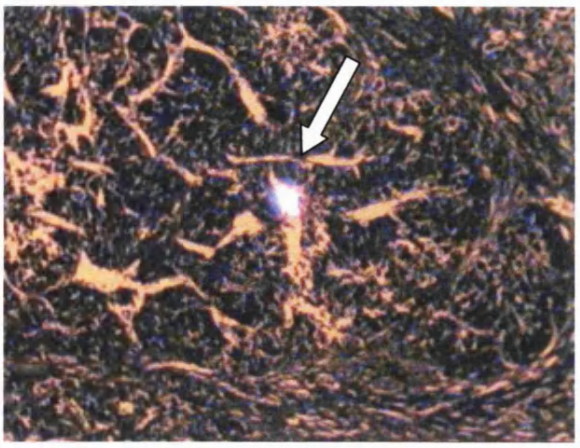
B. The size of the laser spot (30 micron) (*arrow*).

C., D. Selected tissue from the first (L1) and second (L2) sectors transferred to the film of the vial caps.

E., F. The residual tissue section after laser capture.



A.



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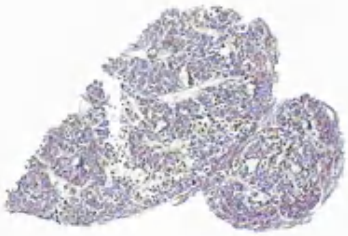

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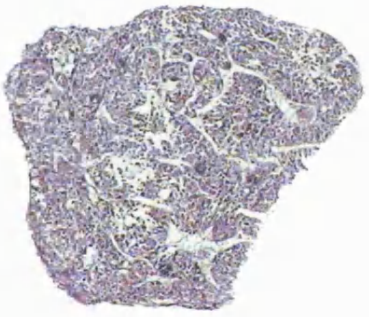

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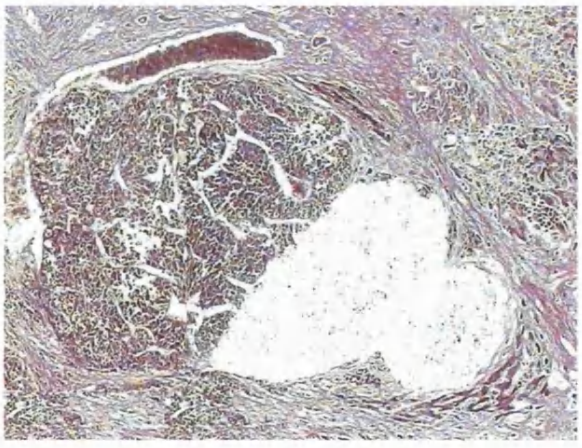

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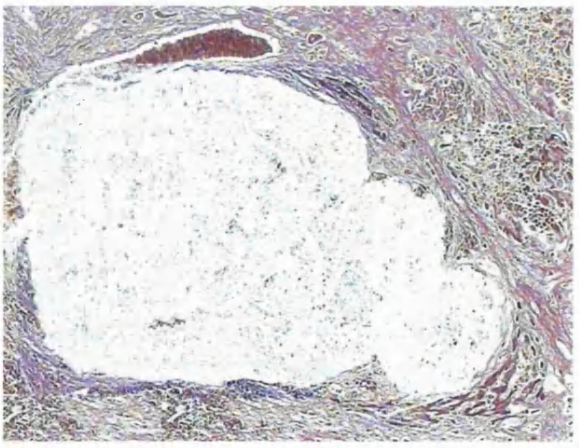

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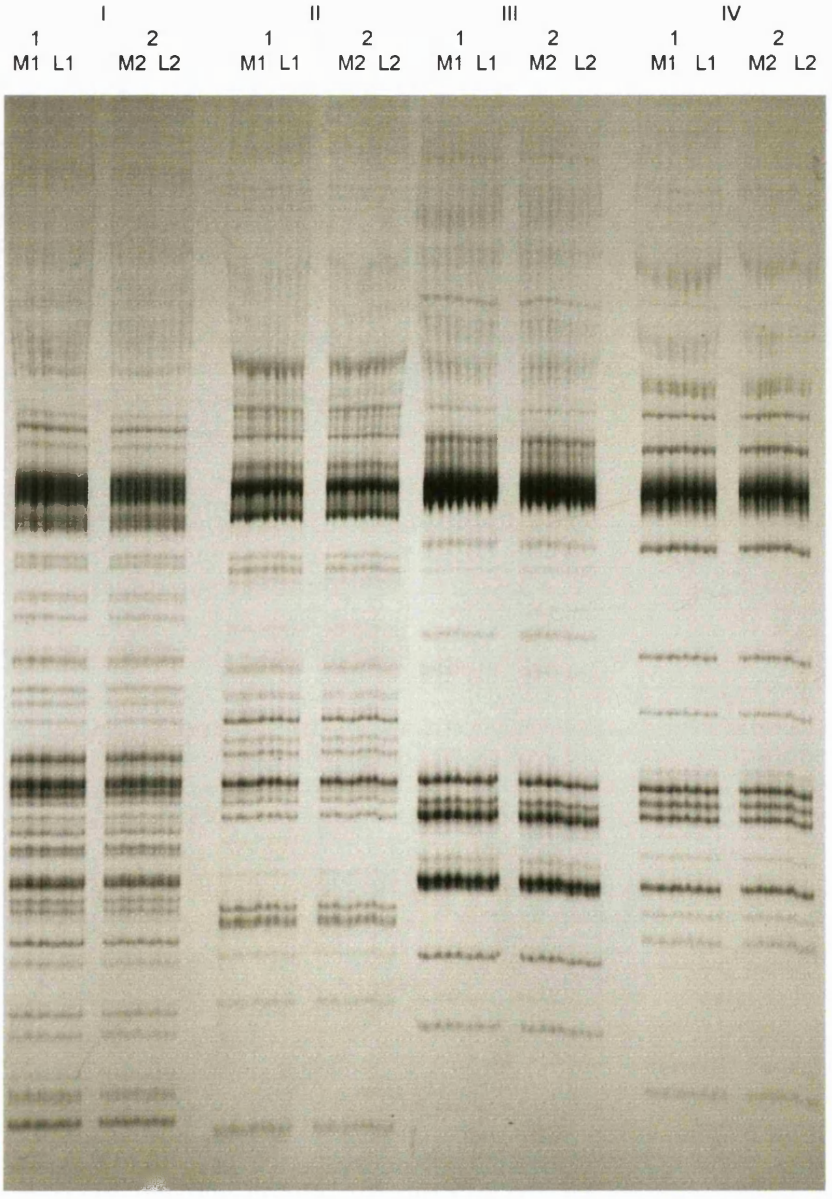


Figure 28. AP-PCR analysis of genomic DNA of manual and laser-captured HCC tissues (nodules I, II, III and IV). Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by AP-PCR. Each sample was amplified in duplicate and run parallel in two lanes. (M – manual microdissection, L – laser capture microdissection, 1 – sector 1, 2 – sector 2)

6.2 Genomic homogeneity in fibrolamellar HCCs

When AP-PCR was performed on the samples of primary and metastatic tumours, we found a striking homogeneity of the genomic fingerprint pattern in a fibrolamellar carcinoma (FLC) and metastatic lesion in the ovary of the same patient. The genomic patterns were therefore studied in a group of FLC patients (Table 8).

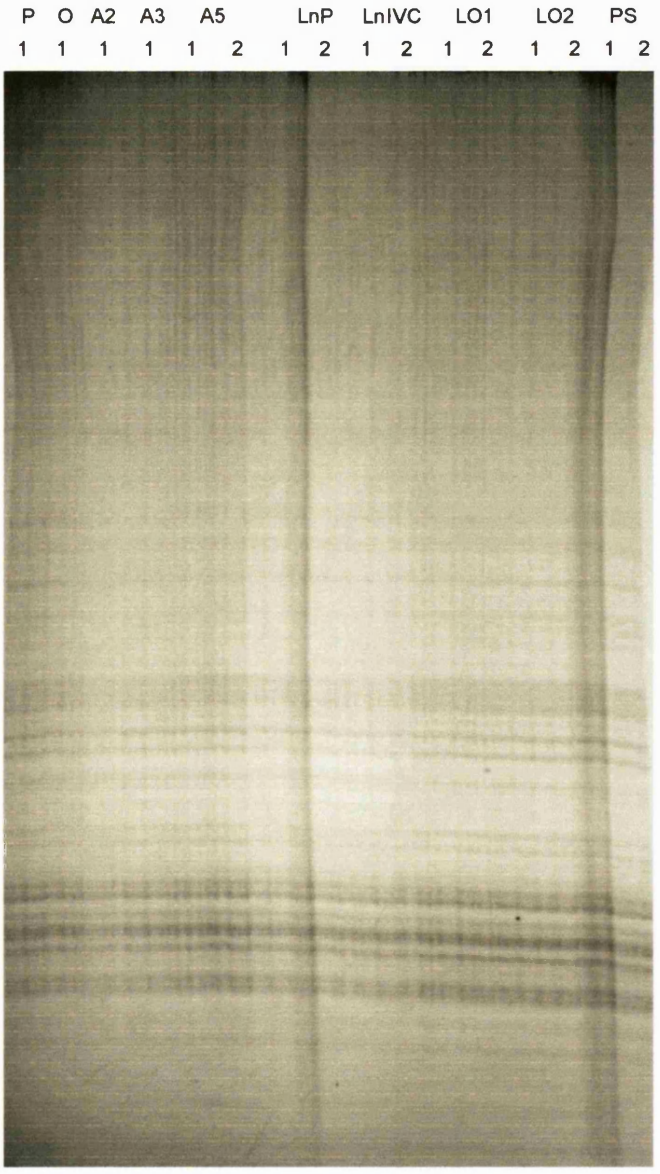
Each tumour was microdissected, and tissue was taken from two sectors of each tumour (for additional assessment of intratumoral genotypic variation). DNA fingerprints of the primary FLCs and all their metastatic lesions were identical in the same patient (Figures 29 & 30). No evidence of intratumour heterogeneity was observed.

Patient	Primary	Metastatic sites	Microdissection	No of samples
1	FLC	Regional lymph nodes, lesser omentum, peritoneum, appendix, ovary	Needle	10
2	FLC	Lungs	Needle	6
3	FLC	Hepatic recurrent lesions	Needle	3
4	FLC	Lymph nodes (regional, hilar, retropancreatic, preaortic, infrarenal, mesenteric)	LCM	12
5	FLC	Lungs, left portal	LCM	9
6	FLC	Retroduodunal lymph nodes	LCM	3
7	FLC	Lymph nodes (pancreatic, hepatic artery, gastric), bowel, chest wall	LCM	7

Table 8. List of fibrolamellar carcinoma samples

Figure 29. AP-PCR analysis of primary fibrolamellar carcinoma (FLC) and their metastatic lesions of patient 1. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by AP-PCR. Genomic DNA from each samples was amplified with the AR3 (A) and ZF3 (B) primers. (P-primary FLC; O-ovary; A2, A3 & A5-appendiceal nodules size 2, 3 & 5 mm respectively; LnP-infrapancreatic lymph node; LnIVC-inferior vena cava lymph node; LO-lesser omentum; PS-peritoneal seeding; number of sectors represents at the top)

B.



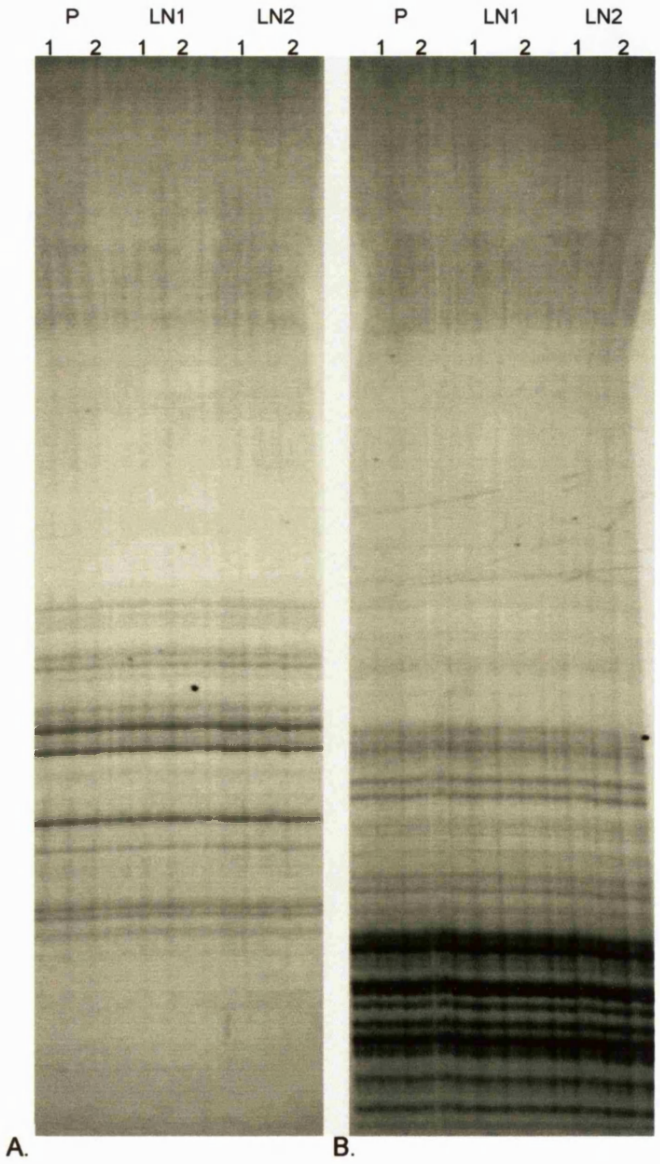


Figure 30. AP-PCR analysis of primary fibrolamellar carcinomas (FLC) and their metastatic lesions from patient 6. Autoradiograms of polyacrylamide gel electrophoresis of ^{33}P-labelled DNA fragments amplified by AP-PCR. Genomic DNA from each samples was amplified with the AR3 (A) and ZF3 (B) primers. (P-primary FLC; LN-lymph nodes metastases; number of sectors represents at the top)

6.3 Discussion

It is obviously an advantage to use microdissected cell samples in molecular analysis because the confounding effect of contaminating cells is eliminated. Precise microdissection of phenotypically similar tissue samples revealed genetic heterogeneity (Macintosh *et al*, 1998). An increase in sensitivity of more than 50% in allelic imbalance analysis was obtained by using microdissected cell populations compared with crushed frozen tumour samples (Giercksky *et al*, 1997). Laser-assisted microdissection assists in the study of molecular pathologies in many applications such as endocrine, neurological, reticuloendothelial and alimentary systems (Fend *et al*, 1999; Suarez-Quian *et al*, 1999; Glasow *et al*, 1998; Luo *et al*, 1999). It has been crucial in enabling selection of minority populations in complex tissues such as the human adrenal gland where cortical and chromaffin tissues are highly intermingled (Glasow *et al*, 1998). Using a modified nucleic acid isolation technique, multiple mRNA and DNA sequences can be identified from a single cell after laser-assisted microdissection (Bernsen *et al*, 1998). In cancer models, laser-assisted microdissection provides the capacity for isolating specific cells including normal, precancerous, malignant and metastatic cells. This will allow us to define the genetic changes associated with functional state, malignant transformation, tumour progression, tumour heterogeneity and clonal progression.

We have demonstrated that AP-PCR is an uncomplicated and effective approach for scanning the genomes of tumour samples to show the evolution of differences. The genetic fingerprinting for this study of HCC nodules was entirely developed by utilising needle-microdissection. By

subsequent introduction of the LCM system into our group, the fingerprinting of much smaller lesions was successfully established. Importantly, the application of LCM with AP-PCR analysis will enhance the detection of clonal cell populations within cancers. Studies of clonality in premalignant lesions and in borderline cases where there is diagnostic doubt between reactive and neoplastic proliferation are also made possible by this approach.

FLC has distinctive histological and clinical features. It occurs predominantly in children and young adults without sex predilection and carries a better prognosis. The indolent growth of FLC is especially obvious when recurrences developed. Even after the diagnosis of tumour recurrence, survival at 1, 3, and 5 years was reported to be 75%, 48% and 28% respectively (Pinna *et al*, 1997). An aggressive surgical approach of recurrent tumour with or without chemotherapy may prolong patient survival. Several studies have attempted to explain the biological behaviour of this disease. Orsatti *et al* (1994) has reported nuclear DNA ploidy patterns in 12 FLCs. All of the tumours showed a nondiploid DNA distribution (six aneuploid and six tetraploid) and the nuclear area of the tumours was significantly larger than that of the surrounding noncancerous livers. The findings suggest that DNA content in FLC is not directly related to the clinical behaviour.

Previous studies demonstrated the positive staining for neuron-specific enolase, the finding of neurosecretory-type granules at the ultrastructural level and the raised serum levels of neurotensin suggesting the

neuroendocrine differentiation of this disease (Collier *et al*, 1984; Payne *et al*, 1986). Neurotensin, an important regulatory peptide in the gut that facilitates translocation of fatty acids from the intestinal lumen, is a useful marker to differentiate FLC from other tumours (Ehrenfried *et al*, 1994). The expression of neurotensin gene is normally localised in the small bowel of the adult in a distinctive temporal- and spatial-specific distribution (Evers *et al*, 1993), but it is more diffuse in the gastrointestinal tract of the fetus. Neurotensin is expressed in the fetal human liver and in FLC, but not in the adult liver and focal nodular hyperplasia (Ehrenfried *et al*, 1994). However, the absence of neurotensin receptor expression emphasises the fact that neurotensin probably does not play a primary role in liver growth.

To date, there is no reported study of the genotypic aspects of this tumour. This is the first report of genomic homogeneity in FLC and metastatic lesions. The absence of clonal evolution which is present in other tumours, particularly HCCs, may explain the distinct behaviour and favourable prognosis in this tumour. These characteristics may reflect the different aetiological factors because there is no recognised association of FLC with viral hepatitis, alcohol, cirrhosis, oral contraceptives or genetic disorders such as Wilson's disease. Surgical treatment is the treatment of choice in this tumour. In advanced cases, these tumours may respond better to chemotherapy than HCCs, though clinical data on such response are scarce.

CHAPTER VII

ROLE OF THROMBOSPONDIN-1 IN HEPATOCELLULAR CARCINOMAS

VII. ROLE OF THROMBOSPONDIN-1 IN HEPATOCELLULAR CARCINOMAS

Recurrence of HCCs is a major problem and is often in areas of the liver distant to the resection site of the primary tumour, suggesting multifocal hepatocarcinogenesis. The concept of tumour dormancy which is often asymptomatic and clinically undetectable followed by rapid growth during relapse might explain this circumstance. The dominant HCCs are capable of releasing angiogenic growth factors that promote their own neovascularisation in excess of angiogenesis inhibitors. The angiogenic inhibitor reaches the vascular bed of a smaller synchronous tumour in greater quantities than the angiogenic stimulator generated by the smaller tumour. In the absence of circulating inhibitor, angiogenesis could reappear in a smaller tumour because of the same excess of angiogenic stimulator in the local vascular bed. Thus, removal or destruction of the dominant lesion alone leads to accelerated growth of the remaining, previously dormant tumours.

Abnormal tumour arterioles are found in HCCs that are between 2 and 3 cm in diameter, and the arterial component of HCC tumour vascular supply becomes dominant compared to the portal venous contribution (Bhattacharya *et al*, 1995). Tumours more than 3 cm in diameter are an exponential growth phase with a positive angiogenic balance, become progressively more dependent on a hepatic arterial supply and are hypervascular. There are not many reports about angiogenic factors in

HCC. The degree of VEGF mRNA expression in HCCs correlated significantly with the density of tumour vasculature in angiograms (Mise *et al*, 1996). An immunohistochemical study by our group on the blood supply of HCCs ranging from 2 to 5 mm in diameter revealed that in the earliest phase (2 mm), there is no sinusoidal capillarisation. As the tumour grows, capillarisation begins centrally, with a leading edge of uncapillarised tumour cells (Bhattacharya *et al*, 1995).

With the evidence of angiogenesis inhibition by human TSP1, treating neoplasms by targeting the HCC cells with this gene might be a novel antiangiogenic therapy. Moreover, further therapy with angiogenesis inhibitors after removal of a dominant tumour could suppress the growth of residual or synchronous tumours and maintain them in a state of dormancy. Therefore, the effect of the TSP1 transfection to a HCC cells was studied both *in vitro* and *in vivo*.

7.1 *In vitro* studies

7.1.1 TSP1 transfection

Thrombospondin-1 cDNA cloned into the *HindIII* and *XbaI* sites of pcDNA1/Neo plasmid vector was transformed successfully into DH5α competent cells. Plasmid DNA was isolated by using a QIAGEN Kit. The plasmid DNA carrying TSP1 gene was confirmed by the diagnostic restriction digest enzymes *HindIII* and *XbaI*. Electrophoresis was carried out

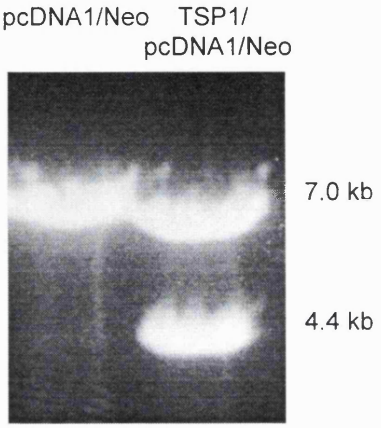
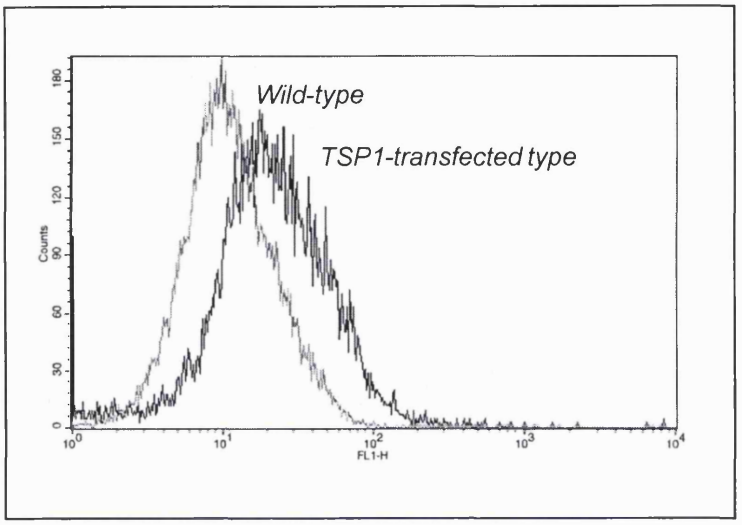


Figure 31. Agarose gel electrophoresis shows the 4.4 kb TSP1 gene inserted in pcDNA1/Neo plasmid vector

in 0.8 % agarose gel containing ethidium bromide and revealed the correct 4.4 kb segment of TSP1 cDNA (Figure 31). The plasmid containing human TSP1 DNA was precipitated with calcium phosphate, and the fine precipitate layered onto the cultured SK-Hep-1 cells. Transfected cells were then cultured under selective conditions using Geneticin sulphate 1% (G-418). The surviving SK-Hep-1 cells were assured of having been successfully transfected with the TSP1 gene because the plasmid vector, pcDNA1/Neo, contained the neomycin-resistant gene which allows the selection of transfected cells in the presence of geneticin, whereas all the wild type cells would be killed by the antibiotic. The stably transfected cells were grown sufficiently after 2-3 weeks of G-418 selection. Transfected cells were checked by FACS and Northern analyses (Figure 32).

A.



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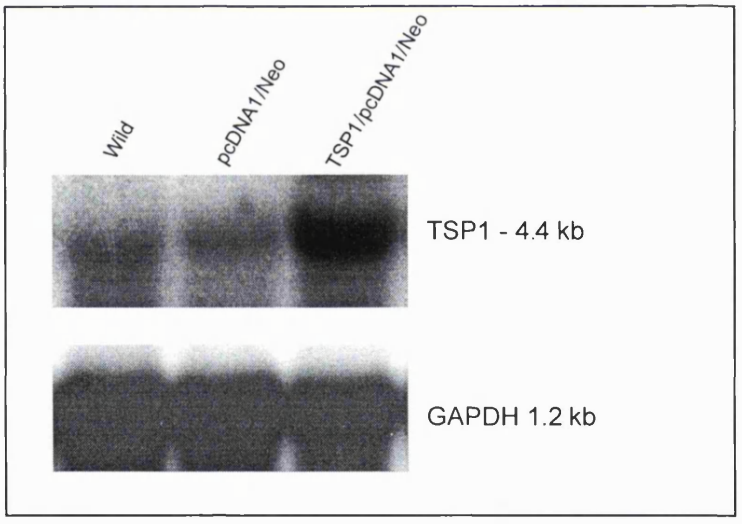

Figure 32. Overexpression of TSP1 in SK-Hep-1 cells transfected with TSP1 cDNA compared to wild type is demonstrated by FACS (A) and Northern blot (B) analyses

7.1.2 TSP1 levels in culture media and cell lysate

The levels of TSP1 produced in culture medium and cell lysate of wild-type and TSP1-transfected-type SK-Hep-1 cells were measured by using the Asserachrom Thrombospondin ELISA kit (Diagnostica Stago, France). The level of TSP1 of each sample was standardised by its protein level. All samples were measured in triplicate and the mean levels are shown (Figure 33). There was no detectable TSP1 level in culture media, RPMI-1640. The TSP1 production in culture media of transfected cells was significantly higher than those of wild-type cells ($p<0.05$). Similarly, cell lysate of transfected cells was also demonstrated in higher level than those of wild-type cells ($p<0.05$).

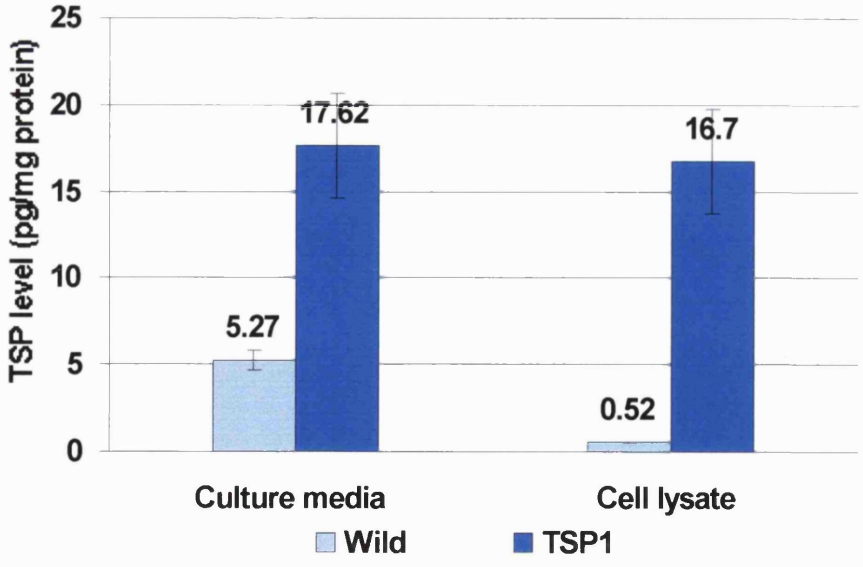

Figure 33. The levels of TSP1 in culture media and cell lysate of wild-type and TSP1-transfected type SK-Hep-1 cells

7.1.3 MTT cell proliferation assay

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by the mitochondrial dehydrogenase enzyme of viable cells. This water insoluble formazan can be solubilised using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye. The MTT assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells. To perform a correlation of optical density of MTT study versus cell density, cell populations from $5x10^3$ to $2x10^5$ were studied in 96-well microplates and the correlation is presented in Figure 34A. The growth curve of TSP1 transfected-type of SK-Hep-1 cells was not significantly different to that of either wild-type or plasmid vector transfected cells during 7-days period (Figure 34B). Each group was studied in quadruplicate and the mean levels are shown.

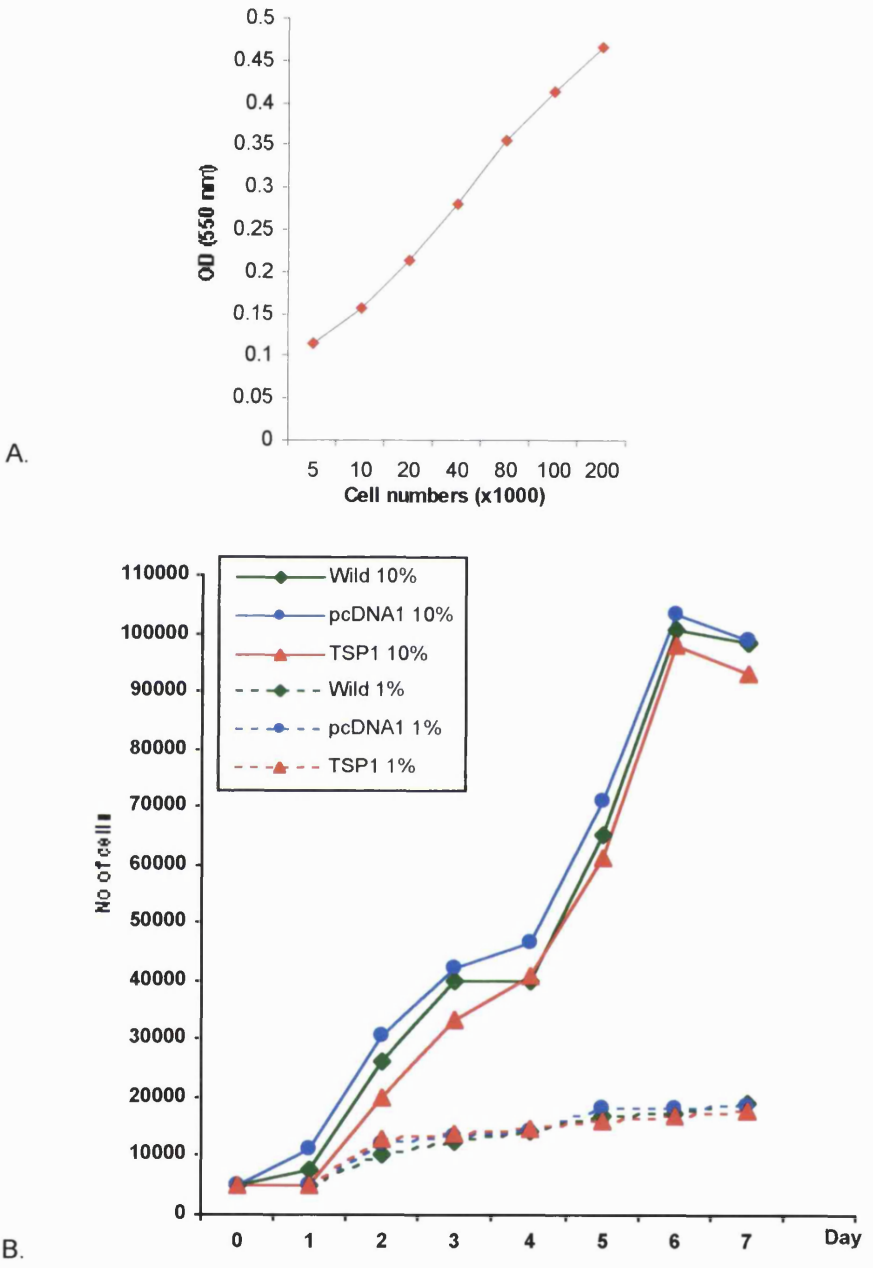

Figure 34. MTT cell proliferation assay of SK-Hep-1 cells with a correlation of optical density versus cell density (A) and the growth rate during 7 days of each cell type (B). Wild, wild-type cells; pcDNA1, plasmid vector pcDNA1-transfected cells, TSP1, thrombospondin 1-transfected cells; 10%, RPMI culture medium with 10% fetal calf serum, 1%, RPMI culture medium with 1% fetal calf serum. Each group was studied in quadruplicate and the mean levels are shown.

7.1.4 Thymidine incorporation assay

The principle of [^{3}H]-thymidine incorporation assay is based on the fact that cells incorporate nucleotide precursors into DNA during the synthetic phase (S phase) of the cell cycle. It relies on the salvage pathway and thymidine kinase by which tritiated thymidine becomes incorporated into DNA, which can then be precipitated and estimated by scintillation counting. Growth arrest is necessary in order to achieve synchrony. Each type of SK-Hep-1 cells was labelled with [^{3}H]-thymidine to a final concentration of 1 μCi/well. Theincorporationof TSP1 transfected cells was 60% lower than that of wild-type cells ($p<0.05$) (Figure 35). The proliferation of plasmid vector transfected cells was no different to that of wild-type cells.

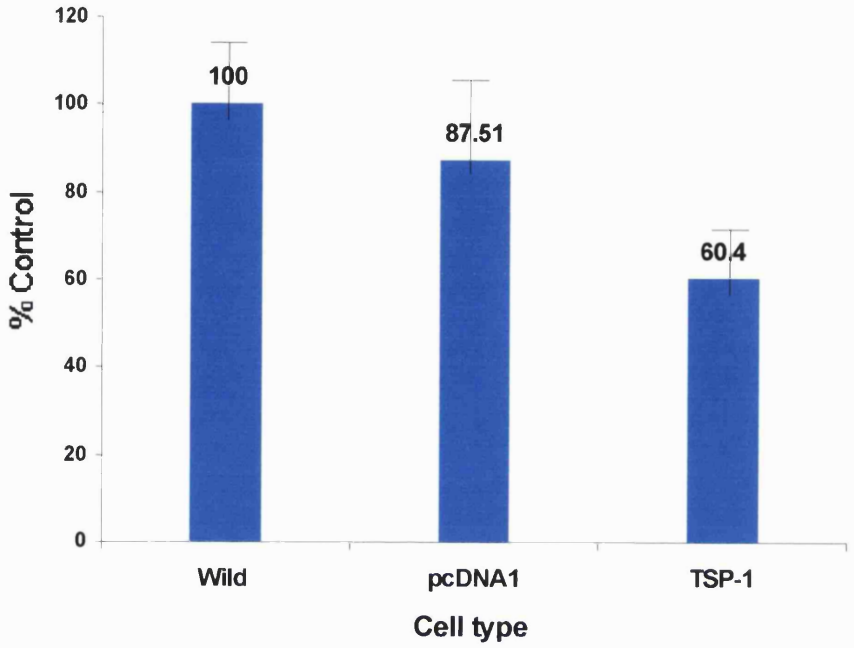

Figure 35. Result of tritiated thymidine incorporation assay of each type of SK-Hep-1 cells. Wild, wild-type cells; pcDNA1, plasmid vector pcDNA1-transfected cells, TSP1, thrombospondin 1-transfected cells. Each group was studied in quadruplicate and the mean levels are shown.

7.1.5 Matrigel invasion assay

Each group of SK-Hep-1 cells (wild-type, plasmid vector transfected, TSP1 transfected) were seeded into the upper chamber of the modified Boyden chamber. The study was performed in triplicate. Cells which had migrated through Matrigel and attached to the lower surface of the filter were counted under light microscope at X400 magnification. Five microscopic fields were counted per filter and average number of cells determined. The results shown in Figure 36 demonstrated that the *in vitro* Matrigel invasion of the TSP1 transfected SK-Hep-1 was no different to that of their parent cells or plasmid vector transfected cells.

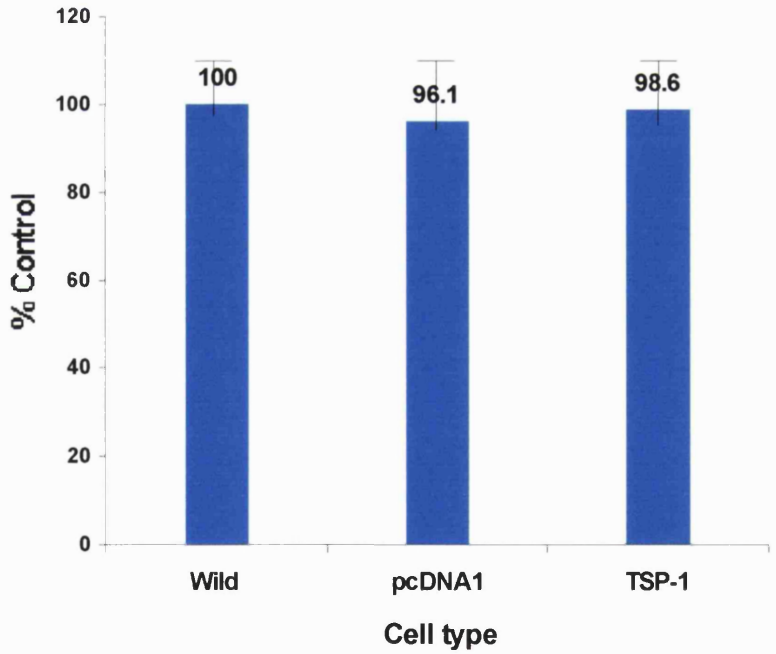

Figure 36. Results of *in vitro* Matrigel invasion. Wild, wild-type cells; pcDNA1, plasmid vector pcDNA1-transfected cells, TSP1, thrombospondin 1-transfected cells. Each group was studied in triplicate and the mean levels are shown.

7.2 *In vivo* study

To study the effect of TSP1 transfection on the tumour growth of SK-Hep-1 *in vivo*, an animal model was utilised. The animals were supplied and the *in vivo* experiments were carried out at the CBU unit, Royal Free Hospital. All procedures were carried out according to home office regulations and were covered by the Home Office Licence No. PPL 70/4517. Tumour volume was determined by external measurement in two dimension according to the published method using equation $V = (L \times W^2) \times 0.5$, when V = volume, L = length, and W = width (Sun *et al*, 1994).

Experiment series I: Direct injection of tumour cell suspension (in PBS) into rat liver parenchyma

Sprague Dawley rats weighing 250 g supplied by CBU unit, Royal Free Hospital were housed in cages and fed a standard laboratory diet and tap water. The strict use of surgical asepsis was performed to prevent infective complications. A million SK-Hep-1 hepatocellular carcinoma cells were prepared by trypsinisation of adherent cells from tissue culture flasks. Cells were washed once with sterile PBS, counted and resuspended in 100 µl cold-sterile PBS. Anaesthesia was induced and maintained by halothane and a 2:1 mixture of nitrous oxide:oxygen. Removal of hair was carried out with electrical clippers and the skin was cleansed with antiseptic solution. Laparotomies were performed through a midline abdominal incision and

bowels were moved to the left to reveal the liver. Rats were divided into study groups as follows:

group A - injected with wild-type cells

group B - injected with plasmid vector transfected cells

group C - injected with TSP1 transfected cells

The cells were injected subcapsular in the right lobe of the liver. After applying pressure for few minutes to stop bleeding at the injected site, the abdominal wall was closed using vicryl 4-0. Rats recovered within minutes after discontinuing the halothane. Analgesia (Tamgesic) was provided postoperatively for the first 24 h. The animals were inspected on a daily basis for any adverse effects. Animals were killed at 45 days by a schedule 1 method. The liver, lung and peritoneal cavity were examined for evidence of tumour.

Results

A total of 18 rats (6 rats of each group) were implanted with wild-type, plasmid vector transfected and TSP1 transfected SK-Hep-1 cells. After 45 days, all the animals were sacrificed. No evidence of tumour was found in liver, lung or peritoneal cavity.

***Experiment series II*: Direct injection of tumour cell suspension (in Matrigel) into rat liver parenchyma**

All procedures were performed as described in experimental series I except the cells were resuspended in Matrigel (Collaborative Biomedical Products, Becton Dickson). Matrigel is stored at -20°C and thawed at 4°C at the time of use. A dilution of 1:2 of Matrigel in serum-free RPMI-1640 was prepared. One hundred microlitres of cell suspension containing $1x10^6$ cells in a 1-ml syringe was carried in ice and was warmed in a 37°C incubator before injection. Rats were divided into study groups as in experimental series I. Animals were killed at 45 days by a schedule 1 method.

Results

Cell suspensions in Matrigel were viscous but not difficult to inject into the liver parenchyma. The cell suspension was also separately grown in a 25-cm^2 culture flask to test the viability of cells. The results showed that cells resuspended in Matrigel survived and grew normally *in vitro*. A total of 9 rats (3 rats of each group) were implanted with wild-type, plasmid vector transfected and TSP1 transfected SK-Hep-1 cells. During inoculation of cell suspension, 3 rats suddenly arrested intraoperatively. The complication was not related to the cell type. The other six rats survived without complication until terminated at 45 days. Unfortunately, no evidence of tumour was found in liver, lung or peritoneal cavity.

***Experiment series III*: Injection of tumour cell suspension (in PBS) into subcutaneous tissues of nude mice**

MFI nude mice (3-weeks old) supplied by CBU unit, Royal Free Hospital were subcutaneously injected in the back using 25-gauge needles. Three million SK-Hep-1 hepatocellular carcinoma cells were prepared from tissue culture flasks and resuspended in 500 μl cold-sterile PBS. Mice were divided into study groups as experimental series I. A successful subcutaneous injection resulted in the formation of a bleb during discharge of the cell suspension. Mice were then left to recover and were assessed for weight loss and other signs of discomfort. The size of tumour was recorded and animals were killed at 45 days by a schedule 1 method.

Results

A total of 18 mice (6 rats of each group) were implant with wild-type, plasmid vector transfected and TSP1 transfected SK-Hep-1 cells. After 45 days, all the animals were sacrificed. The mean tumour volumes in wild-type, plasmid vector transfected and TSP1 transfected cells were 389, 94 and 12 mm^3, respectively.

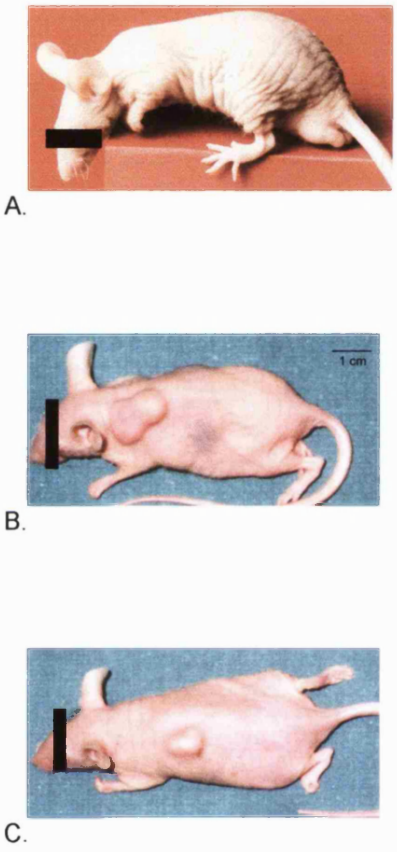

Figure 37. Examples of tumour nodules generated in subcutaneous tissues of nude mice (A). The size of tumour injected by wild-type (B) was similar to that of plasmid vector-transfected cells and was greater than that by TSP1 transfected (C) SK-Hep-1 cells.

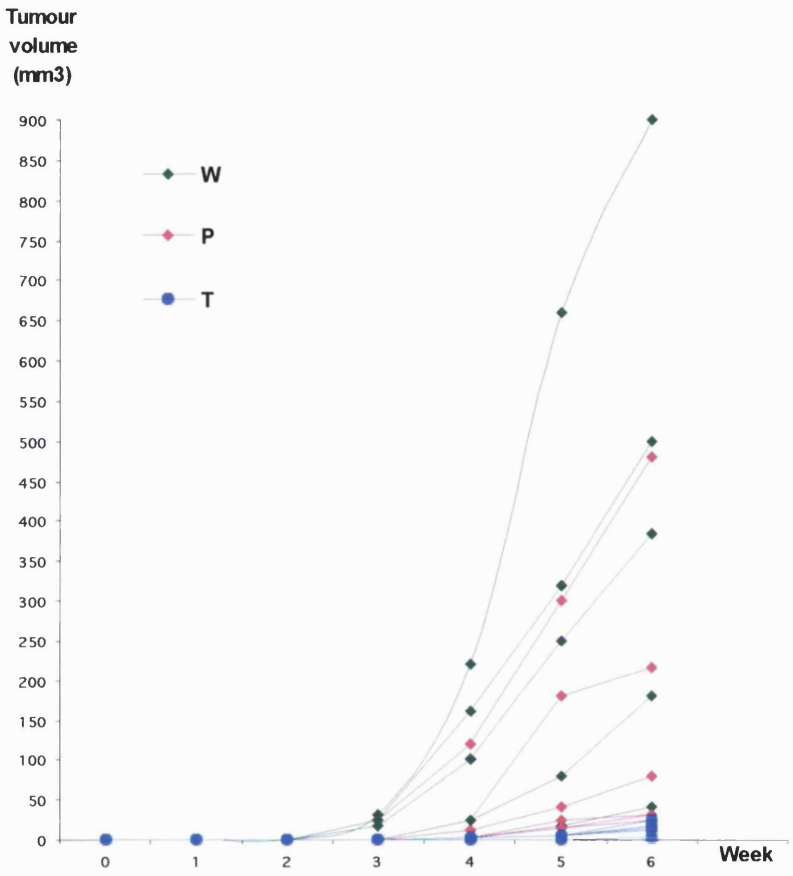

Figure 38. Tumour growth rate in subcutaneous tissues of individual nude mice injected with wild-type (W), plasmid vector transfected (P) and TSP1 transfected (T) SK-Hep-1 cells.

***Experiment series IV*: Implantation of the tumour tissue generated in subcutaneous tissues of nude mice into rat liver parenchyma**

General experimental procedures were similar to those described in experiment series I. On the day of the termination of nude mice, subcutaneous tumour nodules were measured and cut into small cubes about 2 mm^3. Rats were divided into study groups as experimental series I. After depilation and disinfection, the rats underwent a midline abdominal incision. Bowels were removed out to the left to expose the liver. A small superficial incision on the liver was made using the tip of a No.11 surgical blade with the knife at a 15°C angle to the liver surface. A small piece of Gelfoam was inserted into the incision for approximately 1 minute and the incision was compressed by pieces of gauze. When bleeding stopped, the Gelfoam was removed and a tumour fragment was gently placed into the incision. The procedures were performed in both the right and left lobe of the rat liver with small fragments from the same subcutaneous tumour nodule. After the bleeding was assuredly stopped, 20 ml of balanced salt solution was replaced into the peritoneal cavity and the abdominal incision was closed as described. Animals were killed at 45 days by a schedule 1 method.

Results

A total of 18 rats (6 rats for each cell type) were studied. The procedure was uncomplicated. The bleeding from the incision in the liver parenchyma was

simply controlled by Gelfoam and packing for few minutes. There was no intraoperative mortality. One animal of the plasmid vector group died 5 days after the operation. Unfortunately, the animal was not kept for autopsy. The tumour take rate was different between the inoculation site. The tumour take rates in the right lobe of the wild-type, plasmid vector and TSP1 groups were 83%, 67% and 83%, respectively. Whereas that in the left lobe of the wild-type was 50% and 0% in the latter two groups. Figure 40 shows the tumour volume of each group. The mean tumour volumes in the group of wild-type, plasmid vector and TSP1 transfected were 689, 1863 and 87 mm^3, respectively.

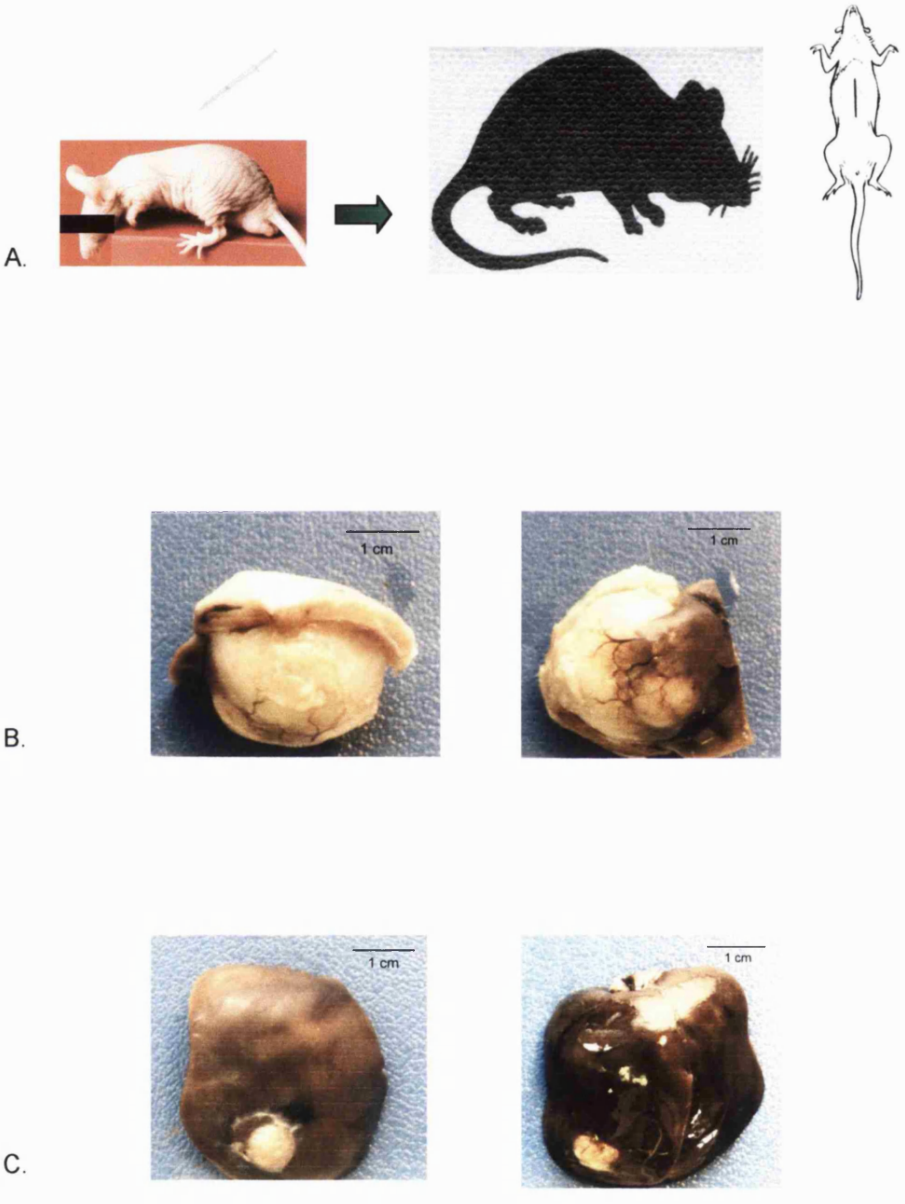

Figure 39. Subcutaneous tumour nodules from nude mice implanted into rat liver parenchyma (A). Samples of tumour in the right lower lobe of rat inoculated by wild-type (B) and TSP1 transfected (C) tumour fragments (pictures are shown in two different views).

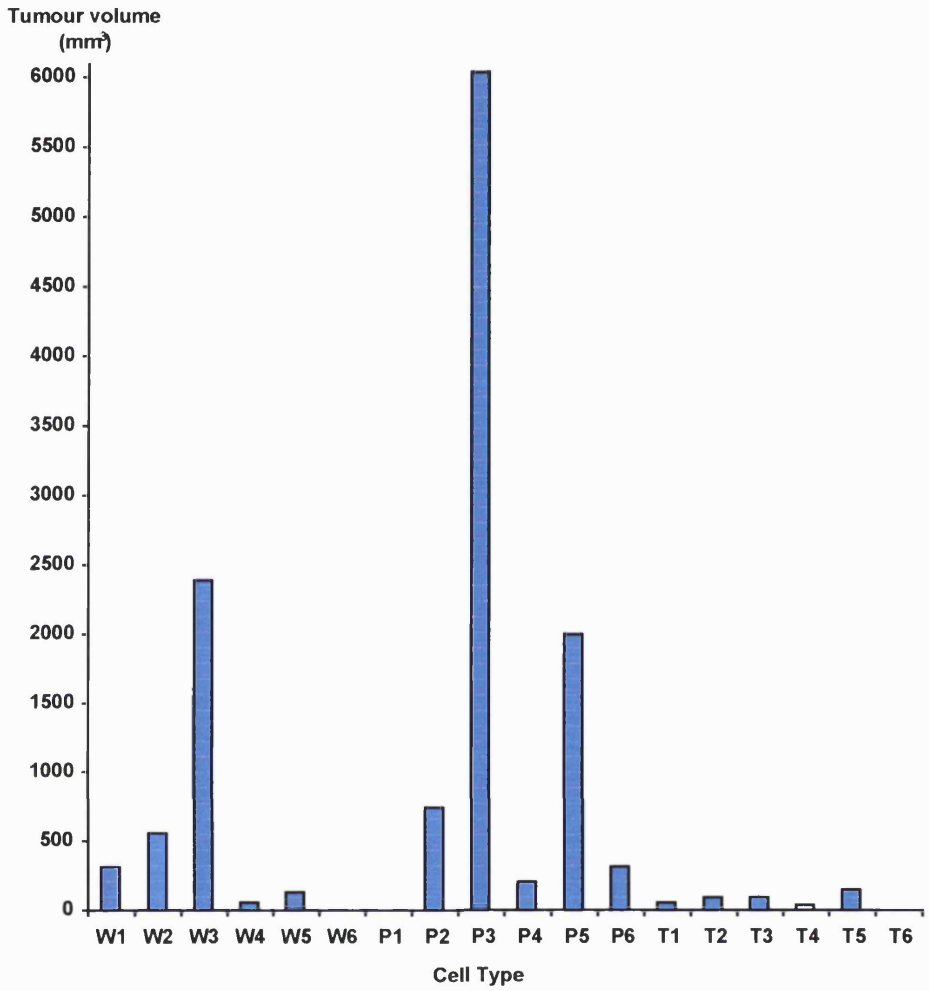

Figure 40. Volumes of tumours in the right lobe of rat liver. (W1-W6, wild-type cells; P1-P6, plasmid vector-transfected cells, T1-T6, thrombospondin-1 transfected cells)

7.3 Discussion

The extracellular matrix is the substance which underlies all epithelia and endothelia, and surrounds all connective tissue cells providing mechanical support and physical strength to tissues, organs and the organism as a whole. It is now quite clear that this matrix exerts profound influences on both the behaviour (*eg.* adherence, spreading and migration) and the pattern of gene expression of the cells in contact with it. Tumour invasion of basement membrane is a crucial step in the complex multistage process which leads to the formation of a metastasis.

Matrigel basement membrane matrix (Becton Dickinson) is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin and nidogen. It also contains TGF-beta, fibroblast growth factor, tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumour. Laminins are a family of large glycoproteins that are distributed ubiquitously in basement membranes. These molecules are multifunctional, performing key roles in development, differentiation and migration through their ability to interact with cells via cell-surface receptors and with other basement membrane components such as type IV collagen, entactin/nidogen and heparan sulphate proteoglycan. Type IV collagen is found exclusively in basement membranes where it provides the major structural support for this matrix. The assembled type IV collagen meshwork provides a scaffold for the assembly of other basement membrane components. In addition, this

meshwork endows the basement membrane with a size-selective filtration property. Entactin/nidogen is a sulphated glycoprotein that is an integral component of basement membranes. It associates specifically with both laminin (in a 1:1 molar ratio) and type IV collagen and is thought to play an important role in linking these two molecules together in the basement membrane.

Direct evidence for the inhibitory effect of TSP1 in cancer cell proliferation and metastasis comes from studies in human breast, human lung and murine melanoma carcinoma cells, where an inverse correlation has been reported between TSP1 mRNA and protein expression and malignant progression (Zabrenetzky *et al*, 1994). The majority of the anti-angiogenic activity of TSP1 resides in the central 70-kD stalk region which alone could block neovascularisation induced by bFGF in the rat cornea *in vivo* and inhibit both migration in a modified Boyden chamber and [3H]thymidine incorporation stimulated by bFGF in cultured capillary endothelial cells (Tolsma *et al*, 1993). Consistent results were obtained by Vogel *et al* (1993) who showed that TSP1 inhibited mitogenesis and migration of endothelial cells and a breast cancer cell line *in vitro.* Somatic cell hybrids between MCF-7 human breast cancer cells and normal immortalised human mammary epithelial cells exhibited a suppressed tumour cell phenotype which was correlated in part with an enhanced expression of TSP1, relative to the MCF-1 tumourigenic cells (Zajchowski *et al*, 1990). Moreover, Taraboletti *et al* (1999) recently reported that TSP1 inhibited endothelial cell proliferation and motility induced by Kaposi's sarcoma cells, and the

angiogenic activity exerted by the transactivating gene product of the human immunodeficiency virus type 1 (HIV-1).

A transfection study in MDA-MB-435S breast cancer cells has demonstrated that tumour cell production of TSP1 exerts an inhibitory effect on tumour progression (Weinstat-Saslow *et al*, 1994). TSP1 overexpression in clones transfected with TSP1 DNA injected into the mammary fat pad of nude mice resulted in a dose-dependent inhibition of primary tumour size and an inhibition of spontaneous pulmonary metastases when compared with controls with low TSP1 expression. Since TSP1 induced programmed cell death in bovine aortic endothelial cells, reduction of angiogenesis in tumours formed by TSP1-transfected MDA-MB-435S cells might result from the induction of apoptosis in endothelial cells during vascularisation of the tumour (Guo *et al*, 1997). Consistent with the data in this study, a recent study demonstrated that transfection-induced overexpression of TSP1 caused tumour suppression in human skin carcinoma cells (Bleuel *et al*, 1999). Coinjection of TSP1 antisense oligonucleotides drastically reduced TSP1 expression and completely restored the tumour phenotype to a well vascularised, progressively solid carcinoma indistinguishable from that induced by the untransfected cells.

A number of studies have underlined two putative different roles of TSP1 in tumour development and progresssion, either proliferative/angiogenic property (Majack *et al*, 1988; Phan *et al*, 1989) or an antiproliferative/antiangiogenic property (Weinstat-Saslow *et al*, 1994; RayChaudhury *et al*, 1994). The results from this study, however, do favour

and support the latter effect of TSP1 in HCC cells. The controversy in the effects of TSP1 may be cell-type specific (Boukerche *et al*, 1995).

Various animal experimental models have chosen an easily accessible region such as the subcutaneous tissue or peritoneal cavity for tumour transplantation and thus have diminished the value of these models in the evaluation of intrahepatic tumour. An orthotopic tumour model is most desirable since the tumour is situated in a topographic location similar to human hepatoma. *In vivo* rat intrahepatic tumour models have been successfully introduced by direct inoculation of rat HCC cells into the portal vein or the spleen (Ikeda *et al*, 1998; Anderson *et al*, 1998). Tumour formed within 12 to 14 days post-inoculation. However, the diffuse colonies of tumour throughout the liver make the estimation of tumour burden very difficult. To attempt to produce a solitary identifiable lesion in liver parenchyma, direct injection of tumour cells into liver parenchyma was performed. This method has been established by using rat HCC cells (McKillop *et al*, 1997). By using human SK-Hep-1 cells in this study, no tumour formation was demonstrated after direct inoculation of the cell suspension. Matrigel, a solubilised tissue basement membrane extract, was reported to enhance the tumour take rate of breast and ovarian cancer cell lines in nude mice (Mullen *et al*, 1996). In this study, Matrigel did not enhance the tumour take rate of SK-Hep-1. Moreover, with direct inoculated into liver parenchyma, the complication of pulmonary embolus causing intraoperative mortality contributed to the unfavourable results of this application. The difficulty in establishing a liver tumour with direct inoculation of human SK-Hep-1 into the liver parenchyma may have been

due to an immunological rejection or failure to localise the cells in particular site.

Nude mice accept tumour xenograft because they lack a thymus, which is the source of T lymphocyte cells. Thus nude mice allow human tumour cells to grow without a mechanism for immunological rejection. In the nude mouse model, TSP1 reduced the tumour growth rate compared with the parent cells. Similar results were demonstrated with the implantation of tumour fragments into rat liver parenchyma.

Tumour angiogenesis has become the focus of intense interest as a potential target for novel cancer therapies. Numerous animal tumour models have been developed to test the anti-angiogenic effect of drugs. Most investigators inject tumour cells subcutaneously into the flank of nude mice and drugs are often administered intravenously. Transfection experiments have become popular to test the effects of overexpressing candidate genes on angiogenesis and tumour growth in animals. Xenografts of the MCF-7 breast carcinoma cell transfected with thymidine phosphorylate gene markedly enhanced tumour growth in nude mice (Moghaddam *et al*, 1995).

A number of human tumour lines can lose their tumourigenic potential as a result of the introduction of a normal tumour suppressor gene or chromosome. Several such transfected-cells simultaneously return to an antiangiogenic phenotype and begin to secrete angio-inhibitory substances.

Reversion to a non-transformed and anti-angiogenic phenotype was observed upon infection of BT549 human breast carcinoma line with a retrovirus encoding wild-type p53. These cells became anti-angiogenic due to the secretion of TSP1 (Volpert *et al*, 1995). A study of the reintroduction of wild-type chromosome 10 to three human glioblastoma cell lines demonstrated that the cells lost their ability to form tumours in nude mice and switched to an antiangiogenic phenotype. This change in angiogenesis was directly due to the increased secretion of TSP1 (Hsu *et al*, 1996). Interestingly, the effect of chromosome 10 on TSP1 production *in vitro* was reflected in patient material. Normal brain and lower grade astrocytomas known to retain chromosome 10 stained strongly for TSP1, but 12 of 13 glioblastomas, the majority of which lose chromosome 10, did not. TSP1 is an effective inhibitor of corneal and inflammatory neovascularisation *in vivo* (Tolsma *et al*, 1993). It can also depress *in vivo* tumourigenicity when it is overexpressed in human breast carcinoma cells (Weinstat-Saslow *et al*, 1994) or in transformed mouse endothelial cells (Sheibani *et al*, 1995)

CHAPTER VIII

CONCLUSIONS AND FUTURE DIRECTIONS

VIII. CONCLUSIONS AND FUTURE DIRECTIONS

Using AP-PCR technique, reproducible and interpretable fingerprinting patterns of amplified genomic DNA isolated from microdissected paraffin-embedded tissues were generated. DNA fingerprinting offers a novel approach to determining clonality in tumours and may prove useful for the study of tumour progression. Polymorphisms in genomic fingerprints generated by AP-PCR can demonstrate genetic differences between nodules. Moreover, unbiased DNA fingerprinting by AP-PCR is a powerful molecular approach for the cytogenetic analysis of solid tumours. It detects both gains of chromosomal regions, reflecting the presence in these regions of cancer genes (*ie.* oncogenes), and losses of genes with negative role in cell growth (*ie.* tumour suppressor genes). Nevertheless, the finding that DNA sequences have undergone heterozygous deletions or gains of extra-copies in tumour relative to normal tissue does not ensure that these sequences are linked to genes playing an active role in oncogenesis due to the high level of random genetic damage in the genome of solid tumours.

Individual neoplasms arise from a single cell of origin and tumour progression results from acquired genetic variability within the original clone, allowing sequential selection of more aggressive subclones. Tumour cells are genetically less stable than normal cells; this instability leads to the development of more mutations in genes which are associated with regulation of the cell cycle and control of DNA repair. The increased genetic instability could produce new subpopulations within the progressing tumour,

leading to clonal heterogeneity. The fingerprints were highly polymorphic amongst HCCs and regenerative nodules. The results from this study suggest that there is genomic heterogeneity in HCCs as they grow beyond 6 mm in diameter. In addition, the genomic fingerprint patterns of the extra-hepatic metastatic lesions were polymorphic compared to those of the corresponding primary HCCs. The single initiating clone evolves into multiple distinct derivative clones which can be recognised by their DNA fingerprints. The multifocal nature of HCC in cirrhotic patients explains the high rate of intrahepatic recurrence after resection. More studies are required on HCC in noncirrhotic livers as well as with borderline or atypical lesions. The information would provide more understanding of hepatocarcinogenesis.

Patients with FLC differ from those with HCC in terms of age at presentation, absence of underlying liver disease and tumour markers. Whether FLC and HCC represent extremes of the spectrum for one disease entity remain unanswered. The cell of origin of FLC remains uncertain. The genomic homogeneity demonstrated in FLC and metastatic lesions may explain the distinct behaviour and favourable prognosis in this tumour. However, despite its slow local growth, FLC presents a high occurrence of initial or late metastatic disease. There is no defined at-risk group to screen other than the young, and thus there is little prospect of improving the prognosis in this way. Further studies are needed to identify the genetic alterations responsible for the apparent dissociation between clinical behaviour and biological characteristics in this tumour.

Laser-assisted microdissection provides the capacity for isolating specific cells which can be defined the genetic changes associated with functional state, malignant transformation, tumour progression, tumour heterogeneity and clonal progression. A new LCM system has been generated to use variable laser-transfer sizes from less than 7.5 to 30 μm. Moreover, a cylinder-based LCM instrument and a novel convex geometry for the ethylene vinyl acetate transfer film have been recently developed (Suarez-Quian *et al*, 1999). The capture zone is 200 times narrower than the original LCM system. Using a smaller beam and briefer pulses (less than 1 ms) limits the melting of the polymer to spots of 6 μm, thus allowing the capture of single cells from different types of tissue sections. The fundamental advantage of this technique is that it offers the investigator the possibility to select, capture and dissect on a single-cell basis. The application of LCM with AP-PCR analysis will enhance the detection of clonal cell populations within cancers.

Cancer has a myriad causes, but many of these may act by damaging DNA. Both dominant and recessive forms of genetic damage have been found in human cancer cells. The damage is likely to play a role in tumourigenesis, and it can therefore form the basis for new approaches to the diagnosis, prognosis and therapy of cancer. Damage to some oncogenes and tumour suppressor genes is found in a wide variety of tumours, whereas damage to others is limited to a more restricted group. Each gene may vary distinctive in its susceptibility to mutagenesis from one tissue to another. However, the malignant phenotype results merely from the cumulative effect of multiple

genetic insults (Fearon & Vogelstein, 1990). Many molecular techniques such as polymerase chain reaction amplification, loss of heterozygosity analysis, *in situ* hybridisation, restriction fragment length polymorphism analysis and X-chromosome inactivation analysis have been applied to determine the genetic alterations in precisely microdissected tissues (Wistuba *et al*, 1999; Radford *et al*, 1995; Wu *et al*, 1999). Other modern technologies for genome-wide analysis, including comparative genomic hybridisation (CGH) and spectral karyotyping, have been utilised to investigate genomic profiles in cancer (James *et al*, 1997; Schrock *et al*, 1996). Genomic changes associated with different stages of cancers have been characterised by using the combination of CGH and microdissection technologies. It was successfully performed in small areas (consisting of 20-100 cells) in paraffin tissue sections and enabled detection of cytogenetic aberrations from clones which are missed when analysing DNA extracted from large cell numbers (Zitzelsberger *et al*, 1998; Weber *et al*, 1998). Interestingly, CGH analysis has been achieved with specimens consisting of 3-40 laser-microdissected cells from routinely processed cervical smears. This might be a useful screening test to identify chromosomal imbalances and early detection of precancerous lesions (Aubele *et al*, 1998). Molecular genetic studies of isolated HCC DNA have been successful and have been useful to detect common regions of chromosomal aberrations. CGH is capable of detecting and mapping relative DNA sequence copy number between genomes which is likely to habour genes that are important for the development or progression of HCCs (Wong *et al*, 1999; Lin *et al*, 1999). Recent studies of CGH in HCCs reported significant correlation of 1q gain and the development of HCC; 4q11-q23 loss and tumours larger than 3 cm

in diameter; 8q gain and HCC in noncirrhotic liver; and 16q and hepatitis B virus carriers. This novel technology has a potential to identify regions that contain candidate oncogenes or tumour suppressor genes responsible for hepatocellular carcinogenesis.

The dismal outcome of this disease is largely due to difficulties in detecting the asymptomatic precursor lesions and early stages of HCC, therefore the majority of HCCs are not amenable to curative therapy at the time they are detected. Attempts to improve the outcome are aimed at earlier detection and prevention. Patients with cirrhosis screened for HCC have an annual conversion rate of about 3% in Western patients and 6% in Japanese patients (Cottone *et al*, 1994; Sato *et al*, 1993). The immunisation programme against HBV in Taiwan reduced the rate of chronic infection in children from 10% in the period of 1981-1986 to less than 1% in the period of 1990-1994, and follow-up revealed a striking reduction in the incidence of HCC (Chang *et al*, 1997).

Changes in gene expression in cells play a pivotal role in a variety of biological processes, including development and differentiation, homeostasis, cell cycle regulation, and angiogenesis. Genetic manipulation to attempt to reduce tumour angiogenesis by introduction of TSP1 gene might be a novel therapeutic approach to HCCs. Up to now, the mechanisms of TSP1 in angiogenesis has not been established, therefore more studies are needed to clarify this subject. The comparison of gene expression in different cell types can provide the important information needed to understand the nature of pathological changes. Many techniques

such as differential display, subtractive hybridisation and microarray assay might be utilised to analyse differential gene expression between cell types or under different conditions.

REFERENCES

References

Adachi, E., Maeda, T., Matsumata, T., Shirabe, K., Kinukawa, N., Sugimachi, K. and Tsuneyoshi, M. (1995) Risk factors for intrahepatic recurrence in human small hepatocellular carcinoma. *Gastroenterology* **108**, 768-775.

Aguilar, F., Harris, C.C., Sun, T., Hollstein, M. and Cerutti, P. (1994) Geographic variation of p53 mutational profile in nonmalignant human liver. *Science* **264**, 1317-1319.

Aihara, T., Noguchi, S., Sasaki, Y., Nakano, H. and Imaoka, S. (1994) Clonal analysis of regenerative nodules in hepatitis C virus-induced liver cirrhosis. *Gastroenterology* **107**, 1805-1811.

Albo, D., Granick, M.S., Jhala, N., Atkinson, B. and Solomon, M.P. (1994) The relationship of angiogenesis to biological activity in human squamous cell carcinomas of the head and neck. *Ann. Plast. Surg.* **32**, 588-594.

Anderson, S.C., Johnson, D.E., Harris, M.P., Engler, H., Hancock, W., Huang, W.M., Wills, K.N., Gregory, R.J., Sutjipto, S., Wen, S.F., Lofgren, S., Shepard, H.M. and Maneval, D.C. (1998) p53 gene therapy in a rat model of hepatocellular carcinoma: intra-arterial delivery of a recombinant adenovirus. *Clin. Cancer Res.* **4**, 1649-1659.

Ang, P.T., Evans, H. and Pazdur, R. (1991) Fibrolamellar hepatocellular carcinoma: therapeutic implications of a ten-year disease free interval. *Am. J. Clin. Oncol.* **14**, 175-178.

Aoki, N. and Robinson, W.S. (1989) State of hepatitis B viral genomes in cirrhotic and hepatocellular carcinoma nodules. *Mol. Biol. Med.* **6**, 395-408.

Aubele, M., Zitzelsberger, H., Schenck, U., Walch, A., Hofler, H. and Werner, M. (1998) Distinct cytogenetic alterations in squamous

intraepithelial lesions of the cervix revealed by laser-assisted microdissection and comparative genomic hybridization. *Cancer* **84**, 375-379.

Austin, H. The role of tobacco use and alcohol consumption in the etiology of hepatocellular carcinoma. In Tabor E, DiBisceglie AM, Purcell RH (eds). Etiology, Pathology, and Treatment of Hepatocellular Carcinoma in North America. Portfolio, The Woodlands, Texas, 1991

Baker, S.J., Preisinger, A.C., Jessup, M., Paraskeva, C., Markowitz, S., Willson, J.K.V., Hamilton, S. and Volgelstein, B. (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.* **50**, 7717-7722.

Beasley, R.P., Hwang, L.Y., Lin, C.C. and Chien, C.S. (1981) Hepatocellular carcinoma and hepatitis B virus: a prospective study of 22707 men in Taiwan. *Lancet* **2**, 1129-1133.

Becker, I., Becker, K.F., Röhrl, M.H., Minkus, G., Schutze, K. and Hofler, H. (1996) Single-cell mutation analysis of tumors from stained histologic slides. *Lab. Invest.* **75**, 801-807.

Belghiti, J., Panis, Y., Farges, O., Benhamou, J.P. and Fekete, F. (1991) Intrahepatic recurrence after resection of hepatocellular carcinoma complicating cirrhosis. *Ann. Surg.* **214**, 114-117.

Berman, M.A., Burnham, J.A. and Sheahan, D.G. (1988) Fibrolamellar carcinoma of the liver: an immunohistochemical study of nineteen cases and a review of the literature. *Hum. Pathol.* **19**, 784-794.

Bernsen, M.R., Dijkman, H.B., de Vries, E., Figdor, C.G., Ruiter, D.J., Adema, G.J. and van Muijen, G.N. (1998) Identification of multiple mRNA and DNA sequences from small tissue samples isolated by laser-assisted microdissection. *Lab. Invest.* **78**, 1267-1273.

Bhattacharya, S., Davidson, B.R. and Dhillon, A.P. (1995) Blood supply of early hepatocellular carcinoma. *Semin. Liv. Dis.* **15**, 390-401.

Bhattacharya, S., Dhillon, A.P., Rees, J., Savage, K., Saada, J., Burroughs, A., Rolles, K. and Davidson, B. (1997) Small hepatocellular carcinomas in cirrhotic explant livers: identification by macroscopic examination and lipiodol localization. *Hepatology* **25**, 613-618.

Bismuth, H., Chiche, L., Adam, R., Castaing, D., Diamond, T. and Dennison, A. (1993) Liver resection versus transplantation for hepatocellular carcinoma in cirrhotic patients. *Ann. Surg.* **218**, 145-151.

Bismuth, H., Chiche, L. and Castaing, D. (1995) Surgical treatment of hepatocellular carcinomas in noncirrhotic liver: experience with 68 liver resections. *World J. Surg.* **19**, 35-41.

Bleuel, K., Popp, S., Fusenig, N.E., Stanbridge, E.F. and Boukamp, P. (1999) Tumor suppression in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization. *Proc. Natl. Acad. Sci. USA* **96**, 2065-2070.

Blum, H.E., Offensperger, W.B., Walter, E., Offensperger, S., Wahl, A., Zeschnigk, C. and Gerok, W. (1987) Hepatocellular carcinoma and hepatitis B virus infection: molecular evidence for monoclonal origin and expansion of malignantly transformed hepatocytes. *J. Cancer Res. Clin. Oncol.* **113**, 466-472.

Böhm, M., Wieland, I., Schütze, K. and Rubben, H. (1997) Microbeam MOMeNT: Non-contact laser microdissection of membrane-mounted native tissue. *Am. J. Pathol.* **151**, 63-67.

Bornstein, P. and Sage, E.H. (1994) Thrombospondins. *Methods Enzymology* **245**, 62-85.

Bosch FX (1997) Global epidemiology of hepatocellular carcinoma. Liver cancer (Okuda K, Tabor E, eds). Churchill Livingstone: New York 13-28

Boukerche, H., Berthiervergnes, O., Tabone, E., Bailly, M., Dore, J.F. and McGregor, J.L. (1995) Thrombospondin modulates melanoma-platelet interactions and melanoma tumor cell growth in vivo. *Br. J. Cancer* **72**, 108-116.

Brechot, C., Pourcel, C., Louise, A., Rain, B. and Tiollais, P. (1980) Presence of integrated hepatitis B virus DNA sequences in cellular DNA in human hepatocellular carcinoma. *Nature* **286**, 533-535.

Bressac, B., Kew, M., Wands, J. and Ozturk, M. (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* **350**, 429-430.

Bruni, R., Argentini, C., D'Ugo, E., Giuseppetti, R., Ciccaglione, A.R. and Rapicetta, M. (1995) Recurrence of woodchuck hepatitis virus integration in the b3n locus in woodchuck hepatocellular carcinoma. *Virology* **214**, 229-234.

Buchmann, A., Bauer-Hofmann, R., Mahr, J., Drinkwater, N.R., Luz, A. and Schwarz, M. (1991) Mutational activation of the c-Ha-ras gene in liver tumors of different rodent strains: correlation with susceptibility to hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA* **88**, 911-915.

Burr, A.W., Hillan, K.J., McLaughlin, K.E., Ferrier, R., Chapman, C., Mathew, J. and Burt, A.D. (1996) Hepatocyte growth factor levels in liver and serum increase during chemical hepatocarcinogenesis. *Hepatology* **24**, 1282-1287.

Bursch, W., Oberhammer, F., Jirtle, R.L., Askari, M., Sedivy, R., Grasl-Kraupp, B., Purchio, A.F. and Schulte-Hermann, R. (1993) Transforming

growth factor-beta 1 as a signal for induction of cell death by apoptosis. *Br. J. Cancer* **67**, 531-536.

Caetano-Anolles, G., Bassam, B.J. and Gresshoff, P.M. (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology (N Y)* **9**, 553-557.

Caetano-Anolles, G., Bassam, B.J. and Gresshoff, P.M. (1992) DNA fingerprinting: MAAPing out a RAPD redefinition? *Bio/Technology* **10**, 937.

Campbell, T.C., Chen, J., Liu, C., Li, J. and Parpia, B. (1990) Non-associated of aflatoxin with primary liver cancer in a cross-sectional ecologic survey in the People's Republic of China. *Cancer Res.* **50**, 6882-6893.

Canfield, A.E., Boot-Handford, R.P. and Schor, A.M. (1990) Thrombospondin gene expression by endothelial cells in culture is modulated by cell proliferation, cell shape and the substratum. *Biochem. J.* **268**, 225-230.

Cariani, E., Dubois, N., Lasserre, C., Briand, P. and Brechot, C. (1991) Insulin-like growth factor II (IGF-II) mRNA expression during hepatocarcinogenesis in transgenic mice. *J. Hepatol.* **13**, 220-226.

Cariani, E., Lasserre, C., Seurin, D., Hamelin, B., Kemeny, F., Franco, D., Czech, M.P., Ullrich, A. and Brechot, C. (1988) Differential expression of insulin-like growth factor II mRNA in human primary liver cancers, benign liver tumors, and liver cirrhosis. *Cancer Res.* **48**, 6844-6849.

Carraway, K.L.III and Cantley, L.C. (1994) A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8.

Chen, T.C., Hsieh, L.L., Ng, K.F., Jeng, L.B. and Chen, M.F. (1998) Absence of APC gene mutation in the mutation cluster region in hepatocellular carcinoma. *Cancer Lett.* **134**,23-28.

Castells, A., Bruix, J., Bru, C., Fuster, J., Vilana, R., Navasa, M., Ayuso, C., Boix, L., Visa, J. and Rodes, J. (1993) Treatment of small hepatocellular carcinoma in cirrhotic patients: a cohort study comparing surgical resection and percutaneous ethanol injection. *Hepatology* **18**, 1121-1126.

Cattoretti, G., Rilke, F., Andreola, S., D'Amato, L. and Delia, D. (1988) P53 expression in breast cancer. *Int. J. Cancer* **41**, 178-183.

Chang, M.H., Chen, C.J., Lai, M.S., Hsu, H.M., Wu, T.C., Kong, M.S., Liang, D.C., Shau, W.Y. and Chen, D.S. (1997) Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. *N. Engl. J. Med.* **336**, 1855-1859.

Chari, R.S., Price, D.T., Sue, S.R., Meyers, W.C. and Jirtle, R.L. (1995) Down-regulation of transforming growth factor beta receptor type I, II and III during liver regeneration. *Am. J. Surg.* **169**, 126-131.

Chen, D.S., Hoyer, B.H., Nelson, J., Purcell, R.H. and Gerin, J.L. (1982) Detection and properties of hepatitis B viral DNA in liver tissues from patients with hepatocellular carcinoma. *Hepatology* **2**, 42s-46s.

Chen, M.F., Hwang, T.L., Jeng, L.B., Wang, C.S., Jan, Y.Y. and Chen, S.C. (1994) Postoperative recurrence of hepatocellular carcinoma: two hundred five consecutive patients who underwent hepatic resection in 15 years. *Arch. Surg.* **129**, 738-742.

Chen, P.J., Chen, D.S., Lai, M.Y., Chang, M.H., Huang, G.T., Yang, P.M., Sheu, J.C., Lee, S.C., Hsu, H.C. and Sung, J.L. (1989) Clonal origin of recurrent hepatocellular carcinomas. *Gastroenterology* **96**, 527-529.

CLIP Group (Cancer of the Liver Italian Programme). (1998) Tamoxifen in treatment of hepatocellular carcinoma: a randomised controlled trial. *Lancet* **352**, 17-20.

de La Coste, A., Romagnolo, B., Billuart, P., Renard, C.A., Buendia, M.A., Soubrane, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A. and Perret, C. (1998) Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA* **95**,8847-8851.

de Souza, A.T., Hankins, G.R., Washington, M.K., Orton, T.C. and Jirtle, R.L. (1995) M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. *Nat. Genet.* **11**,447-449.

Cohick, W.S. and Clemmons, D.R. (1993) The insulin-like growth factors. *Annu. Rev. Physiol.* **55**, 131-153.

Collier, J.D., Guo, K., Mathew, J., May, F.E., Bennett, M.K., Corbett, I.P., Bassendine, M.F. and Burt, A.D. (1992) c-erbB-2 oncogene expression in hepatocellular carcinoma and cholangiocarcinoma. *J. Hepatol.* **14**, 377-380.

Collier, J.D., Carpenter, M., Burt, A.D. and Bassendine, M.F. (1994) Expression of mutant p53 protein in hepatocellular carcinoma. *Gut* **35**, 98-100.

Collier, N.A., Weinbren, K., Bloom, S.R., Lee, Y.C., Hodgson, H.J. and Blumgart, L.H. (1984) Neurotensin secretion by fibrolamellar carcinoma of the liver. *Lancet* **1**, 538-540.

Cottone, M., Turri, M., Caltagirone, M., Parisi, P., Orlando, A., Fiorentino, G., Virdone, R., Fusco, G., Grasso, R., Simonetti, R.G. and Pagliaro, L. (1994) Screening for hepatocellular carcinoma in patients with Child's A cirrhosis: an 8-year prospective study by ultrasound and alphafetoprotein. *J. Hepatol.* **21**, 1029-1034.

Craig, J.R., Peters, R.L., Edmondson, H.A. and Omata, M. (1980) Fibrolamellar carcinoma of the liver: a tumor of adolescents and young adults with distinctive clinico-pathologic features. *Cancer* **46**, 372-379.

Dameron, K.M., Volpert, O.V., Tainsky, M.A. and Bouck, N. (1994) Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* **265**, 1582-1584.

Dejean, A., Bougueleret, L., Grzeschik, K.H. and Tiollais, P. (1986) Hepatitis B virus DNA integration in a sequence homologous to v-erb A and steroid hormone receptor in a hepatocellular carcinoma. *Nature* **322**, 70-72.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. (1987) Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors. *Cancer Res.* **47**, 707-712.

Diamantis, I.D., McGandy, C., Chen, T.J., Liaw, Y.F., Gudat, F. and Bianchi, L. (1994) A new mutational hot-spot in the p53 gene in human hepatocellular carcinoma. *J. Hepatol.* **20**, 553-556.

Dragani, T.A., Manenti, G., Colombo, B.M., Falvella, F.S., Gariboldi, M., Pierotti, M.A. and Della-Porta, G. (1991) Incidence of mutations at codon 61 of the Ha-ras gene in liver tumors of mice genetically susceptible and resistant to hepatocarcinogenesis. *Oncogene* **6**, 333-338.

Ebara, M., Ohto, M., Shinagawa, T., Sugiura, N., Kimura, K., Matsutani, S., Morita, M., Saisho, H., Tsuchiya, Y. and Okuda, K. (1986) Natural history of minute hepatocellular carcinoma smaller than three centimeters complicating cirrhosis. A study in 22 patients. *Gastroenterology* **90**, 289-298.

Ehrenfried, J.A., Zhou, Z., Thompson, J.C. and Evers, B.M. (1994) Expression of the neurotensin gene in fetal human liver and fibrolamellar carcinoma. *Ann. Surg.* **220**, 484-489.

El-Serag, H.B. and Mason, A.C. (1999) Rising incidence of hepatocellular carcinoma in the United States. *N. Engl. J. Med.* **340**, 745-750.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui, R.F., Zhuang, Z., Goldstein, S.R., Weiss, R.A. and Liotta, L.A. (1996) Laser capture microdissection. *Science* **274**, 998-1001.

Esumi, M., Aritaka, T., Arii, M., Suzuki, K., Tanikawa, K., Mizuo, H., Mima, T. and Shikata, T. (1986) Clonal origin of human hepatoma determined by integration of hepatitis B virus DNA. *Cancer Res.* **46**, 5767-5771.

Evers, B.M., Rajaraman, S., Chung, D.H., Townsend, C.M.Jr., Wang, X., Graves, K. and Thompson, J.C. (1993) Differential expression of the neurotensin gene in the developing rat and human gastrointestinal tract. *Am. J. Physiol.* **265**, G482-G490.

Farhi, D.C., Shikes, R.H., Murari, P.J. and Silverberg, S.G. (1983) Hepatocellular carcinoma in young people. *Cancer* **52**, 1516-1525.

Farinati, F., De Maria, N., Fornasiero, A., Salvagnini, M., Fagiuoli, S., Chiaramonte, M. and Naccarato, R. (1992) Prospective controlled trial with antiestrogen drug tamoxifen in patients with unresectable hepatocellular carcinoma. *Dig. Dis. Sci.* **37**, 659-662.

Fausto, N. and Mead, J.E. (1989) Regulation of liver growth: protooncogenes and transforming growth factors. *Lab. Invest.* **60**, 4-13.

Fearon, E.R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767.

Fend, F., Emmert-Buck, M.R., Chuaqui, R., Cole, K., Lee, J., Liotta, L.A. and Raffeld, M. (1999) Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am. J. Pathol.* **154**, 61-66.

Ferrell, L., Wright, T., Lake, J., Roberts, J. and Ascher, N. (1992) Incidence and diagnostic features of macro-regenerative nodules vs small hepatocellular carcinoma in cirrhotic livers. *Hepatology* **16**, 1372-1381.

Ferrell, L.D., Crawford, J.M., Dhillon, A.P., Scheuer, P.J. and Nakanuma, Y. (1993) Proposal for standardized criteria for the diagnosis of benign, borderline, and malignant hepatocellular lesions arising in chronic advanced liver disease. *Am. J. Surg. Pathol.* **17**, 1113-1123.

Fey, M.F., Wells, R.A., Wainscoat, J.S. and Thein, S.L. (1988) Assessment of clonality in gastrointestinal cancer by DNA fingerprinting. *J. Clin. Invest.* **82**, 1532-1537.

Fidler, I.J. and Hart, I.R. (1982) Biological diversity in metastatic neoplasms: origins and implications. *Science* **217**, 998-1003.

Fidler, I.J. and Radinsky, R. (1990) Genetic control of cancer metastasis. *J. Natl. Cancer Inst.* **82**, 166-168.

Folkman, J., Watson, K., Ingber, D. and Hanahan, D. (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339**, 58-61.

Folkman, J. (1994) Angiogenesis and breast cancer. *J. Clin. Oncol.* **12**, 441-443.

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature* **1**, 27-31.

Foster, G.R., Goldin, R.D., Main, J., Murray-Lyon, I., Hargreaves, S. and Thomas, H.C. (1997) Management of chronic hepatitis C: clinical audit of biopsy based management algorithm. *B.M.J.* **315**, 453-458.

Fourel, G., Trepo, C., Bougueleret, L., Henglein, B., Ponzetto, A., Tiollais, P. and Buendia, M.A. (1990) Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumors. *Nature* **347**, 294-298.

Fregene, T.A., Khanuja, P.S., Noto, A.C., Gehani, S.K., Van Egmont, E.M., Luz, D.A. and Pienta, K.J. (1993) Tumor-associated angiogenesis in prostate cancer. *Anticancer Res.* **13**, 2377-2381.

Froesch, E.R., Schmid, C., Schwander, J. and Zapf, J. (1985) Actions of insulin-like growth factors. *Annu. Rev. Physiol.* **47**, 443-467.

Garcia, S., Martini, F., De Micco, C., Andrac, L., Hardwigsen, J., Sappa, P., Lavaut, M.N., Le Treut, Y.P. and Charpin, C. Immunoexpression of E-cadherin and -catenin correlates to survival of patients with hepatocellular carcinomas. *Int. J. Oncol.* **12**,443-447.

Furuya, K., Nakamura, M., Yamamoto, Y., Togei, K. and Otsuka, H. (1988) Macroregenerative nodule of the liver. A clinicopathologic study of 345 autopsy cases of chronic liver disease. *Cancer* **61**, 99-105.

Giercksky, H.E., Thorstensen, L., Qvist, H., Nesland, J.M. and Lothe, R.A. (1997) Comparison of genetic changes in frozen biopsies and microdissected archival material from the same colorectal liver metastases. *Diagn. Mol. Pathol.* **6**, 318-325.

Glasow, A., Haidan, A., Hilbers, U., Breidert, M., Gillespie, J., Scherbaum, W.A., Chrousos, G.P. and Bornstein, S.R. (1998) Expression of Ob receptor in normal human adrenals: differential regulation of adrenocortical and adrenomedullary function by leptin. *J. Clin. Endocr. Met.* **83**, 4459-4466.

Going, J.J. and Lamb, R.F. (1996) Practical histological microdissection for PCR analysis. *J. Pathol.* **179**, 121-124.

Goldstein, S.R., McQueen, P.G. and Bonner, R.F. (1998) Thermal modeling of laser capture microdissection. *Appl. Optics.* **37**, 7378-7391.

Good, D.J., Polverini, P.J., Rastinejad, F., Le Beau, M.M., Lemons, R.S., Frazier, W.A. and Bouck, N.P. (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. USA* **87**, 6624-6628.

Groopman, J.D., Busby, W.J. and Wogan, G.N. (1980) Nuclear distribution of aflatoxin B1 and its interaction with histones in rat liver in vivo. *Cancer Res.* **40**, 4343-4351.

Grossfeld, G.D., Ginsberg, D.A., Stein, J.P., Bochner, B.H., Esrig, D., Groshen, S., Dunn, M., Nichols, P.W., Taylor, C.R., Skinner, D.G. and Cote, R.J. (1997) Thrombospondin-1 expression in bladder cancer: association

with p53 alterations, tumor angiogenesis, and tumor progression. *J. Natl. Cancer Inst.* **89**, 219-227.

Guigui, B., Mavier, P., Lescs, M.C., Pinaudeau, Y., Dhumeaux, D. and Zafrani, S. (1988) Copper and copper-binding protein in liver tumors. *Cancer* **61**, 1155-1158.

Gunduz, N., Fisher, B. and Saffer, E.A. (1979) Effect of surgical removal on the growth and kinetics of residual tumor. *Cancer Res.* **39**, 3861-3865.

Guo, N.H., Krutzsch, H.C., Inman, J.K. and Roberts, D.D. (1997) Thrombospondin 1 and type 1 repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. *Cancer Res.* **57**, 1735-1742.

Hasan, F., Jeffers, L.J., De Medina, M., Reddy, K.R., Parker, T., Schiff, E.R., Houghton, M., Choo, Q.L. and Kuo, G. (1990) Hepatitis C-associated hepatocellular carcinoma. *Hepatology* **12**, 589-591.

Hayashi, K., Kurohiji, T. and Shirouzu, K. (1997) Localization of thrombospondin in hepatocellular carcinoma. *Hepatology* **25**, 569-574.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 Mutations in human cancers. *Science* **253**, 49-53.

Hollstein, M.C., Wild, C.P., Bleicher, F., Chutimataewin, S., Harris, C.C., Srivatanakul, P. and Montesano, R. (1993) p53 mutations and aflatoxin B1 exposure in hepatocellular carcinoma patients from Thailand. *Int. J. Cancer* **53**, 51-55.

Holmgren, L., O'Reilly, M. and Folkman, J. (1995) Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature* **1**, 149-153.

Hosono, S., Lee, C.S., Chou, M.J., Yang, C.S. and Shih, C. (1991) Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines. *Oncogene* **6**, 237-243.

Hsia, C.C., Axiotis, C.A., Di, B.A. and Tabor, E. (1992) Transforming growth factor-alpha in human hepatocellular carcinoma and coexpression with hepatitis B surface antigen in adjacent liver. *Cancer* **70**, 1049-1056.

Hsia, C.C., Di-Bisceglie, A.M., Kleiner, D.E.Jr., Farshid, M. and Tabor, E. (1994) RB tumor suppressor gene expression in hepatocellular carcinomas from patients infected with the hepatitis B virus. *J. Med. Virol.* **44**, 67-73.

Hsieh, D.P. and Atkinson, D.N. (1995) Recent aflatoxin exposure and mutation at codon 249 of the human p53 gene: lack of association. *Food Addict. Contam.* **12**, 421-424.

Hsu, H.C., Chiou, T.J., Chen, J.Y., Lee, C.S., Lee, P.H. and Peng, S.Y. (1991) Clonality and clonal evolution of hepatocellular carcinoma with multiple nodules. *Hepatology* **13**, 923-928.

Hsu, H.C., Tseng, H.J., Lai, P.L., Lee, P.H. and Peng, S.Y. (1993) Expression of p53 gene in 184 unifocal hepatocellular carcinomas: association with tumor growth and invasiveness. *Cancer Res.* **53**, 4691-4694.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J. and Harris, C.C. (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* **350**, 427-428.

Hsu, S.C., Volpert, O.V., Steck, P.A., Mikkelsen, T., Polverini, P.J., Rao, S., Chou, P. and Bouck, N.P. (1996) Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. *Cancer Res.* **56**, 5684-5691.

Hsu, T., Moroy, T., Etiemble, J., Louise, A., Trepo, C., Tiollais, P. and Buendia, M.A. (1988) Activation of c-myc by woodchuck hepatitis virus insertion in hepatocellular carcinoma. *Cell* **55**, 627-635.

Hui, A.M., Kawasaki, S., Imamura, H., Miyagawa, S., Ishii, K., Katsuyama, T. and Makuuch, M. (1997) Heterogeneity of DNA content in multiple synchronous hepatocellular carcinomas. *Br. J. Cancer* **76**, 335-339.

Hytiroglou, P., Theise, N.D., Schwartz, M., Mor, E., Miller, C. and Thung, S.N. (1995) Macroregenerative nodules in a series of adult cirrhotic liver explants: issues of classification and nomenclature. *Hepatology* **21**, 703-708.

Ikeda, K., Saitoh, S., Koida, I., Arase, Y., Tsubota, A., Chayama, K., Kumada, H. and Kawanishi, M. (1993) A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* **18**, 47-53.

Ikeda, Y., Matsumata, T., Takenaka, K., Yamagata, M. and Sugimachi, K. (1998) Effects of doxorubicin and/or cilostazol on cancer cells during liver regeneration after two-thirds hepatectomy in rats. *Oncology* **55**, 354-356.

Imazeki, F., Omata, M., Yokosuka, O. and Okuda, K. (1986) Integration of hepatitis B virus DNA in hepatocellular carcinoma. *Cancer* **58**, 1055-1060.

Inagaki, M., Moustakas, A., Lin, H.Y., Lodish, H.F. and Carr, B.I. (1993) Growth inhibition by transforming growth factor beta (TGF-beta) type I is restored in TGF-beta-resistant hepatoma cells after expression of TGF-beta receptor type II cDNA. *Proc. Natl. Acad. Sci. USA* **90**, 5359-5363.

Innov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D. and Perucho, M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* **363**, 558-561.

Kanai, Y., Ushijima, S., Hui, A.M., Ochiai, A., Tsuda, H., Sakamoto, M. and Hirohashi, S. (1997) The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int. J. Cancer* **71**,355-359.

Inui, Y., Higashiyama, S., Kawata, S., Tamura, S., Miyagawa, J., Taniguchi, N. and Matsuzawa, Y. (1994) Expression of heparin-binding epidermal growth factor in human hepatocellular carcinoma. *Gastroenterology* **107**, 1799-1804.

Isobe, H., Sakai, H., Imari, Y., Ikeda, M., Shiomichi, S. and Nawata, H. (1994) Intratumor ethanol injection therapy for solitary minute hepatocellular carcinoma. A study of 37 patients. *J. Clin. Gastroenterol.* **18**, 122-126.

Jaffe, E.A., Rugiero, J.T., Leung, L.K., Doyle, M.J., McKeown-Longo, P.J. and Moscher, D.F. (1983) Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. *Proc. Natl. Acad. Sci. USA* **80**, 998-1002.

Jaffe, E.A., Ruggiero, J.T. and Falcone, D.J. (1985) Monocytes and macrophages synthesize and secrete thrombospondin. *Blood* **65**, 79-84.

James, L.A., Mitchell, E.L.D., Menasce, L. and Varley, J.M. (1997) Comparative genomic hybridisation of ductal carcinoma in situ of the breast: identification of regions of DNA amplification and deletion in common with invasive breast cancer. *Oncogene* **14**, 1059-1065.

Jeng, J.E. and Tsai, J.F. (1991) Hepatitis C ivrus antibody in hepatocellular carcinoma in Taiwan. *J. Med. Virol.* **34**, 74-77.

Jiang, W.G. and Hiscox, S. (1997) Hepatocyte growth factor/scatter factor, a cytokine playing multiple and converse roles. *Histol. Histopathol.* **12**, 537-555.

Kandel, J., Bossy-Wetzel, E., Radvanyi, F., Klagsbrun, M., Folkman, J. and Hanahan, D. (1991) Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* **66**, 1095-1104.

Kawate, S., Fukusato, T., Ohwada, S., Watanuki, A. and Morishita, Y. (1999) Amplification of c-myc in hepatocellular carcinoma: correlation with clinicopathologic features, proliferative activity and p53 overexpression. *Oncology* **57**,157-163.

Kim, J.R., Kim, S.Y., Kim, M.J. and Kim, J.H. (1998) Alterations of CDKN2 (MTS1/p16INK4A) gene in paraffin-embedded tumor tissues of human stomach, lung, cervix and liver cancers. *Exp. Mol. Med.* **30**,109-114.

Kita, R., Nishida, N., Fukuda, Y., Azechi, H., Matsuoka, Y., Komeda, T., Sando, T., Nakao, K. and Ishizaki, K. (1996) Infrequent alterations of the p16INK4A gene in liver cancer. *Int. J. Cancer* **67**,176-180.

Kao, K.J. and Klein, P.A. (1986) A monoclonal antibody-based enzyme-linked immunosorbent assay for quantitation of plasma thrombospondin. *Am. J. Clin. Pathol.* **86**, 317-323.

Kawahara, N., Ono, M., Taguchi, K.I., Okamoto, M., Shimada, M., Takenaka, K., Hayashi, K., Mosher, D.F., Sugimachi, K., Tsuneyoshi, M. and Kuwano, M. (1998) Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. *Hepatology* **28**, 1512-1517.

Kawakita, N., Seki, S., Sakaguchi, H., Yanai, A., Nakatani, K., Yamada, T., Kitada, T., Sakai, Y., Kuroki, T., Kobayashi, K., *et al* (1994) Immunohistochemical analysis of retinoblastoma gene product (pRB) expression in malignant and non-malignant liver diseases. *Liver* **14**, 295-301.

Kenmochi, K., Sugihara, S. and Kojiro, M. (1987) Relationship of histologic grade of hepatocellular carcinoma (HCC) to tumor size, and demonstration of tumor cells of multiple different grades in a single small HCC. *Liver* **7**, 18-26.

Kohen, H., Pugh, T.D. and Goldfarb, S. (1983) Hepatocarcinogenesis in the mouse: combined morphologic-stereologic studies. *Am. J. Pathol.* **112**, 89-110.

Kohler, M.F., Berchuck, A., Davidoff, A.M., Humphrey, P.A., Dodge, R.K., Iglehart, J.D., Soper, J.T., Clarke-Pearson, D.L., Bast, R.C.Jr. and Marks, J.R. (1992) Overexpression and mutation of p53 in endometrial carcinoma. *Cancer Res.* **52**, 1622-1627.

Kondo, F., Wada, K., Nagato, Y., Nakajima, T., Kondo, Y., Hirooka, N., Ebara, M., Ohto, M. and Okuda, K. (1989) Biopsy diagnosis of well-differentiated hepatocellular carcinoma based on new morphologic criteria. *Hepatology* **9**, 751-755.

Legoix, P., Bluteau, O., Bayer, J., Perret, C., Balabaud, C., Belghiti, J., Franco, D., Thomas, G., Laurent-Puig, P. and Zucman-Rossi, J. (1999) Beta-catenin mutations in hepatocellular carcinoma correlate with a low rate of loss of heterozygosity. *Oncogene* **18**,4044-4046.

Kondo, F., Ebara, M., Sugiura, N., Wada, K., Kita, K., Hiroka, N., Nagato, Y., Kondo, Y., Ohto, M. and Okuda, K. (1990) Histological features and clinical course of large regenerative nodules: evaluation of their precancerous potentiality. *Hepatology* **12**, 592-598.

Kress, S., Jahn, U., Buchmann, A., Bannasch, P. and Schwarz, M. (1992) p53 mutations in human hepatocellular carcinomas from Germany. *Cancer Res.* **52**, 3220-3225.

Kubo, S., Tamori, A., Nishiguchi, S., Kinoshita, H., Hirohashi, K., Kuroki, T., Omura, T. and Otani, S. (1998) Effect of alcohol abuse on polyamine metabolism in hepatocellular carcinoma and noncancerous hepatic tissue. *Surgery* **123**, 205-211.

Lai, E.C.S., Lo, C.M., Fan, S.T., Liu, C.L. and Wong, J. (1998) Postoperative adjuvant chemotherapy after curative resection of hepatocellular carcinoma. *Arch. Surg.* **133**, 183-188.

Lawrence, D.A. (1996) Transforming growth factor-beta: a general review. *Eur. Cytokine Netw.* **7**, 363-374.

Lee, F.I. (1966) Cirrhosis and hepatoma in alcoholics. *Gut* **7**, 77-85.

Lefkowitch, J.H., Muschel, R., Price, J.B., Marboe, C. and Braunhut, S. (1983) Copper and copper-binding protein in fibrolamellar liver cell carcinoma. *Cancer* **51**, 97-100.

Levine, A.J., Momand, J. and Finlay, C.A. (1991) The p53 tumour suppressor gene. *Nature* **351**, 453-456.

Liaw, Y.F., Tai, D.I., Chu, C.M., Lin, D.Y., Sheen, I.S., Chen, T.J. and Pao, C.C. (1986) Early detection of hepatocellular carcinoma in patients with chronic type B hepatitis: a prospective study. *Gastroenterology* **90**, 263-267.

Liu, P., Flejou, J.F., Feldmann, G. and Bernuau, D. (1994) Activation of ras oncogene in livers with cirrhosis. *J. Hepatol.* **21**,1103-1108.

Lien, W.M. and Ackerman, N.B. (1970) The blood supply of experimental liver metastases. II. A microcirculatory study of the normal and tumor vessels of the liver with the use of perfused silicone rubber. *Surgery* **68**, 334-340.

Lin, Y.W., Sheu, J.C., Huang, G.T., Lee, H.S., Chen, C.H., Wang, J.T., Lee, P.H. and Lu, F.J. (1999) Chromosomal abnormality in hepatocellular carcinoma by comparative genomic hybridisation in Taiwan. *Eur. J. Cancer* **35**, 652-658.

Liotta, L.A., Steeg, P.S. and Stetler-Stevenson, W.G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* **64**, 327-336.

Liver Cancer Study Group of Japan (1988) Survey and follow-up study of primary liver cancer in Japan-report 8. *Acta Hepatol.* **29**, 1619-1626.

Liver Cancer Study Group of Japan (1990) Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. *Ann. Surg.* **211**, 277-287.

Lu, S.N., Lin, T.M., Chen, C.J., Chen, J.S., Liaw, Y.F., Chang, W.Y. and Hsu, S.T. (1988) A case-control study of primary hepatocellular carcinoma in Taiwan. *Cancer* **62**, 2051-2055.

Luo, L., Salunga, R.C., Guo, H., Bittner, A., Joy, K.C., Galindo, J.E., Xiao, H., Rogers, K.E., Wan, J.S., Jackson, M.R. and Erlander, M.G. (1999) Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* **5**, 117-122.

Macchiarini, P., Fontanini, G., Hardin, M.J., Squartini, F. and Angeletti, C.A. (1992) Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* **340**, 145-146.

Macintosh, C.A., Stower, M., Reid, N. and Maitland, N.J. (1998) Precise microdissection of human prostate cancers reveals genotypic heterogeneity. *Cancer Res.* **58**, 23-28.

MacSween, R.N.M. (1974) A clinicopathological review of 100 cases of primary malignant tumors of the liver. *J. Clin. Pathol.* **27**, 669-682.

Majack, R.A., Goodman, L.V. and Dixit, V.M. (1988) Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation. *J. Cell Biol.* **106**, 415-422.

Mant, J.W.F. and Vessey, M.P. (1995) Trends in mortality from primary liver cancer in England and Wales 1975-92: influence of oral contraceptives. *Br. J. Cancer* **72**, 800-803.

Martinez-Cerezo, F.J., Tomas, A., Donoso, L., Enriquez, J., Guarner, C., Balanzo, J., Martinez Nogueras, A. and Vilardell, F. (1994) Controlled trial of tamoxifen in patients with advanced hepatocellular carcinoma. *J. Hepatol.* **20**, 702-706.

Massague, J., Cheifetz, Z., Laiho, M., Ralph, D.A., Weis, F.M.B. and Zentella, A. (1992) Transforming growth factor-β. *Cancer Surv.* **12**, 81-103.

Matsumata, T., Kanematsu, T., Takenaka, K., Yoshida, Y., Nishizaki, T. and Sugimachi, K. (1989) Patterns of intrahepatic recurrence after curative resection of hepatocellular carcinoma. *Hepatology* **9**, 457-460.

McKillop, I.H., Schmidt, C.M., Cahill, P.A. and Sitzmann, J.V. (1997) Altered expression of mitogen-activated protein kinases in a rat model of experimental hepatocellular carcinoma. *Hepatology* **26**, 1484-1491.

Mead, J.E. and Fausto, N. (1989) Transforming growth factor alpha may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc. Natl. Acad. Sci. USA* **86**, 1558-1562.

Mise, M., Arii, S., Higashituji, H., Furutani, M., Niwano, M., Harada, T., Ishigami, S., Toda, Y., Nakayama, H., Fukumoto, M., Fujita, J. and Imamura, M. (1996) Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* **23**, 455-464.

Moghaddam, A., Zhang, H.T., Fan, T.P., Hu, D.E., Lees, V.C., Turley, H., Fox, S.B., Gatter, K.C., Harris, A.L. and Bicknell, R. (1995) Thymidine phosphorylase is angiogenic and promotes tumor growth. *Proc. Natl. Acad. Sci. USA* **92**, 998-1002.

Mondazzi, L., Bottelli, R., Brambilla, G., Rampoldi, A., Rezakovic, I., Zavaglia, C., Alberti, A. and Ideo, G. (1994) Transarterial oily chemoembolization for the treatment of hepatocellular carcinoma: a multivariate analysis of prognostic factors. *Hepatology* **19**, 1115-1123.

Morimitsu, Y., Hsia, C.C., Kojiro, M. and Tabor, E. (1995) Nodules of less-differentiated tumor within or adjacent to hepatocellular carcinoma: relative expression of transforming growth factor-α and its receptor in the different areas of tumor. *Hum. Pathol.* **26**, 1126-1132.

Mosher, D.F., Doyle, M.J. and Jaffe, E.A. (1982) Synthesis and secretion of thrombospondin by cultured human endothelial cells. *J. Biol. Chem.* **93**, 343-348.

Moskaluk, C.A. and Kern, S.E. (1997) Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. *Am. J. Pathol.* **150**, 1547-1552.

Motoo, Y., Sawabu, N. and Nakanuma, Y. (1991) Expression of epidermal growth factor and fibroblast growth factor in human hepatocellular carcinoma: an immunohistochemical study. *Liver* **11**, 272-277.

Mullen, P., Ritchie, A., Langdon, S.P. and Miller, W.R. (1996) Effect of matrigel on the tumourigenicity of human breast and ovarian carcinoma cell lines. *Int. J. Cancer* **6**, 816-820.

Murakami, Y., Hayashi, K., Hirohashi, S. and Sekiya, T. (1991) Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinoma. *Cancer Res.* **51**, 5520-5525.

Nagao, T., Inoue, S., Yoshimi, F., Sodeyama, M., Omori, Y., Mizuta, T., Kawano, N. and Morioka, Y. (1990) Postoperative recurrence of hepatocellular carcinoma. *Ann. Surg.* **211**, 28-33.

Nagashima, I., Hamada, C., Naruse, K., Osada, T., Nagao, T., Kawano, N. and Muto, T. (1996) Surgical resection for small hepatocellular carcinoma. *Surgery* **119**, 40-45.

Nagasue, N., Yukaya, H., Hamada, T., Hirose, S., Kanashima, R. and Inokuchi, K. (1984) The natural history of hepatocellular carcinoma. A study of 100 untreated cases. *Cancer* **54**, 1461-1465.

Nagasue, N., Kohno, H., Chang, Y.C., Yamanoi, A., Kimoto, T., Takemoto, Y. and Nakaura, T. (1992) DNA ploidy pattern in synchronous hepatocellular carcinomas. *J. Hepatol.* **16**, 208-214.

Nagaya, T., Nakamura, T., Tokino, T., Tsurimoto, T., Imai, M., Mayumi, T., Kamino, K., Yamamura, K. and Matsubara, K. (1987) The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. *Genes Dev.* **1**, 773-782.

Nakamura, T., Tokino, T., Nagaya, T. and Matsubara, K. (1988) Microdeletion associated with the integration process of hepatitis B virus DNA. *Nucleic Acids Res.* **16**, 4865-4873.

Ogata, N., Kamimura, T. and Asakura, H. (1991) Point mutation, allelic loss and increased methylation of c-Ha-ras gene in human hepatocellular carcinoma. *Hepatology* **13**,31-37.

Nakopoulou, L., Stefanaki, K., Filaktopoulos, D. and Giannopoulou, I. (1994) C-erb-B-2 oncoprotein and epidermal growth factor receptor in human hepatocellular carcinoma: an immunohistochemical study. *Histol. Histopathol.* **9**, 677-682.

Nicolson, G.L. (1987) Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res.* **47**, 1473-1487.

O'Reilly, M., Rosenthal, R., Sage, E.H., Smith, S., Holmgren, L., Moses, M., Shing, Y. and Folkman, J. (1993) The suppression of tumor metastases by a primary tumor. *Surg. Forum* **44**, 474-476.

O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H. and Folkman, J. (1994) Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315-328.

Oda, T., Tsuda, H., Scarpa, A., Sakamoto, M. and Hirohashi, S. (1992) Mutation pattern of the p53 gene as a diagnostic marker for multiple hepatocellular carcinoma. *Cancer Res.* **52**, 3674-3678.

Oda, T., Tsuda, H., Sakamoto, M. and Hirohashi, S. (1994) Different mutations of the p53 gene in nodule-in-nodule hepatocellular carcinoma as a [sic] evidence for multistage progression. *Cancer Lett.* **83**, 197-200.

Okuda, K., Nakashima, T., Sakamoto, K., Ikari, T., Hidaka, H., Kubo, Y., Sakuma, K., Motoike, Y., Okuda, H. and Obata, H. (1982) Hepatocellular carcinoma arising in noncirrhotic and highly cirrhotic livers: a comparative study of histopathology and frequency of hepatitis B markers. *Cancer* **49**, 450-455.

Okuda, K. (1992) Hepatocellular carcinoma: recent progress. *Hepatology* **5**, 948-963.

Orsatti, G., Greenberg, P.D., Rolfes, D.B., Ishak, K.G. and Paronetto, F. (1994) DNA ploidy of fibrolamellar hepatocellular carcinoma by image analysis. *Hum. Pathol.* **25**, 936-939.

Ouchi, K., Matsubara, S., Fukuhara, K., Tominaga, T. and Matsuno, S. (1993) Recurrence of hepatocellular carcinoma in the liver remnant after hepatic resection. *Am. J. Surg.* **166**, 270-273.

Ozturk, M., Bressac, B., Puisieux, A., Kew, M., Volkman, M., Bozcall, S., Mura, J.B., De La Monte, S., Carlson, R., Blum, H. and Takahasi, H. (1991) p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet* **388**, 1356-1359.

Paradinas, F.J., Melia, W.M., Wilkinson, M.L., Portmann, B., Johnson, P.J., Murray-Lyon, I.M. and Williams, R. (1982) High serum vitamin B12 binding capacity as a marker of the fibrolamellar variant of hepatocellular carcinoma. *Br. Med. J.* **285**, 840-842.

Pasquinelli, C., Bhavani, K. and Chisari, F.V. (1992) Multiple oncogenes and tumor suppressor genes are structurally and functionally transgenic mice. *Cancer Res.* **52**, 2823-2829.

Payne, C.M., Nagle, R.B., Paplanus, S.H. and Graham, A.R. (1986) Fibrolamellar carcinoma of liver. A primary malignant oncocytic carcinoid? *Ultrastruct. Pathol.* **10**, 539-552.

Peinado, M.A., Malkhosyan, S., Velazquez, A. and Perucho, M. (1992) Isolation and characterisation of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **89**, 10065-10069.

Phan, S.H., Dillon, R.G., McGarry, B.M. and Dixit, V.M. (1989) Stimulation of fibroblast proliferation by thrombospondin. *Biochem . Biophys. Res. Commun.* **153**, 56-63.

Pinna, A.D., Iwatsuki, S., Lee, R.G., Todo, S., Madariaga, J.R., Marsh, J.W., Casavilla, A., Dvorchik, I., Fung, J.J. and Starzl, T.E. (1997) Treatment of fibrolamellar hepatoma with subtotal hepatectomy or transplantation. *Hepatology* **26**, 877-883.

Ponder, K.P. (1996) Analysis of liver development, regeneration, and carcinogenesis by genetic marking studies. *FASEB J.* **10**, 673-682.

Pratt, D.A., Miller, W.R. and Dawes, J. (1989) Thrombospondin in malignant and non-malignant breast tissue. *Eur. J. Cancer Clin. Oncol.* **25**, 343-350.

Preus, H.R., Haraszthy, V.I., Zambon, J.J. and Genco, R.J. (1993) Differentiation of strains of Actinobacillus actinomycetemcomitans by arbitrarily primed polymerase chain reaction. *J. Clin. Microbiol.* **31**, 2773-2776.

Radford, D.M., Fair, K.L., Phillips, N.J., Ritter, J.H., Steinbrueck, T., Holt, M.S. and Donis-Keller, H. (1995) Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res.* **55**, 3399-3405.

Rastinejad, F., Polverini, P.J. and Bouck, N.P. (1989) Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell* **56**, 345-355.

RayChaudhury, A., Frazier, W.A. and D'Amore, P.A. (1994) Comparison of normal and tumorigenic endothelial cells: differences in thrombospondin production and responses to transforming growth factor-beta. *J. Cell Sci.* **107**, 39-46.

Riestra, S., Rodriguez, M., Delgado, M., Suarez, A., Gonzalez, N., de la Mata, M., Diaz, G., Mino-Fugarolas, G. and Rodrigo, L. (1998) Tamoxifen does not improve survival of patients with advanced hepatocellular carcinoma. *J. Clin. Gastroenterol.* **26**, 200-203.

Ringe, B., Pichlamyr, R., Wittekind, C. and Tusch, G. (1991) Surgical treatment of heptocellular carcinoma: experience with liver resection and transplantation in 198 patients. *World J. Surg.* **15**, 270-285.

Riser, B.L., Varani, J., O'Rourke, K. and Dixit, V.M. (1988) Thrombospondin binding by human squamous carcinoma and melanoma cells: relationship to biological activity. *Exp. Cell Res.* **174**, 319-329.

Rizzi, P.M., Kane, P.A., Ryder, S.D., Ramage, J.K., Gane, E., Tan, K.C., Portmann, B., Karani, J. and Williams, R. (1994) Accuracy of radiology in detection of hepatocellular carcinoma before liver transplantation. *Gastroenterology* **107**, 1425-1429.

Rogler, C.E., Sherman, M., Su, C.Y., Shafritz, D.A., Summers, J., Shows, T.B., Henderson, A. and Kew, M. (1985) Deletion in chromosome 11p associated with a hepatitis B integration site in hepatocellular carcinoma. *Science* **230**, 319-322.

Roninson, I.B., Chin, J.E., Choi, K.G., Gros, P., Housman, D.E., Fojo, A., Shen, D.W., Gottesman, M.W. and Pastan, I. (1986) Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA* **83**, 4538-4542.

Russell, W.E., Coffey, R.J., Ouellette, A.J. and Moses, H.L. (1988) Type beta transforming growth factor reversibly ingibits the early proliferative response to partial hepatectomy in the rat. *Proc. Natl. Acad. Sci. USA* **85**, 5126-5130.

Sage, H., Farin, F.M., Striker, G.E. and Fisher, A.B. (1983) Granular pneumocytes in primary culture secrete several major components of the extracellular matrix. *Biochemistry* **22**, 2148-2155.

Sakamoto, M., Hirohashi, S., Tsuda, H., Sakamoto, Y., Makuuchi, H. and Hosoda, Y. (1989) Multicentric independent development of hepatocellular

carcinoma revealed by analysis of hepatitis B virus integration pattern. *Am. J. Surg. Pathol.* **13**, 1064-1106.

Sambrook, J., Fritsch, E. and Maniatis, T. (eds) (1989) *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press: New York, USA.

Sato, Y., Nakata, K., Kato, Y., Shima, M., Ishii, N., Koji, T., Taketa, K., Endo, Y. and Nagataki, S. (1993) Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. *N. Engl. J. Med.* **328**, 1802-1806.

Saul, S.H., Titelbaum, D.S., Gansler, T.S., Varello, M., Burke, D.R., Atkinson, B.F. and Rosato, E.F. (1987) The fibrolamellar variant of hepatocellular carcinoma: its association with focal nodular hyperplasia. *Cancer* **60**, 3049-3055.

Schlegel, J., Vogt, T., Munkel, K. and Ruschoff, J. (1996) DNA fingerprinting of mammalian cell lines using nonradioactive arbitrarily primed PCR (AP-PCR). *Biotechniques* **20**, 178-180.

Schlesselman, J.J. (1995) Net effect of oral contraceptive use on the risk of cancer in women in the United States. *Obstet. Gynecol.* **85**, 793-801.

Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Bar-Am, I., Soenksen, D., Garini, Y. and Ried, T. (1996) Multicolor spectral karyotyping of human chromosomes. *Science* **273**, 494-497.

Schütze, K. and Clement-Sengewald, A. (1994) Catch and move: cut or fuse. *Nature* **368**, 667-669.

Scorsone, K.A., Zhou, Y.Z., Butel, J.S. and Slagle, B.L. (1992) P53 mutaions cluster at codon 249 in hepatitis B virus-positive hepatocellular carcinomas from China. *Cancer Res.* **52**, 1635-1638.

Segarini, P.R. (1993) TGF-beta receptors: a complicated system of multiple binding proteins. *Biochem. Biophys. Acta* **1155**, 269-275.

Sheibani, N. and Frazier, W.A. (1995) Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis. *Proc. Natl. Acad. Sci. USA* **92**, 6788-6792.

Sheu, J.C., Sung, J.L., Chen, D.S., Yang, P.M., Lai, M.Y., Lee, C.S., Hsu, H.C., Chuang, C.N., Yang, P.C., Wang, T.H., Lin, J.T. and Lee, C.Z. (1985) Growth rate of asymptomatic hepatocellular carcinoma and its clinical implications. *Gastroenterology* **89**, 259-266.

Sheu, J.C., Huang, G.T., Chou, H.C., Lee, P.H., Wang, J.T., Lee, H.S. and Chen, D.S. (1993) Multiple hepatocellular carcinomas at the early stage have different clonality. *Gastroenterology* **105**, 1471-1476.

Shibata, D., Hawes, D., Li, Z.H., Hernandez, A.M., Spruck, C.H. and Nichols, P.W. (1992) Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. *Am. J. Pathol.* **141**, 539-543.

Shiota, G., Okano, J., Kawasaki, H., Kawamoto, T. and Nakamura, T. (1995) Serum hepatocyte growth factor in liver diseases: clinical implications. *Hepatology* **21**, 106-112.

Shirai, Y., Kawata, S., Ito, N., Tamura, S., Takaishi, K., Kiso, S., Tsushima, H. and Matsuzawa, Y. (1992) Elevated levels of plasma transforming growth factor-beta in patients with hepatocellular carcinoma. *Jpn. J. Cancer Res.* **83**, 676-679.

Shiratori, Y., Shiina, S., Imamura, M., Kato, N., Kanai, F., Okudaira, T., Teratani, T., Tohgo, G., Toda, N., Ohashi, M., Ogura, K., Niwa, Y., Kawabe, T. and Omata, M. (1995) Characteristic difference of hepatocellular

carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* **22**, 1027-1033.

Simonetti, R.G., Camma, C., Fiorello, F., Politi, F., D'Amico, G. and Pagliaro, L. (1991) Hepatocellular carcinoma. A worldwide problem and the major risk factors. *Dig. Dis. Sci.* **36**, 962-972.

Soreide, O., Czerniak, A., Bradpiece, H., Bloom, S. and Blumgart, L. (1986) Characteristics of fibrolamellar hepatocellular carcinoma. A study of nine cases and a review of the literature. *Am. J. Surg.* **151**, 518-523.

Srivastava, A., Laidler, P., Davies, R.P., Horgan, K. and Hughes, L.E. (1988) The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. A quantitative histologic study. *Am. J. Pathol.* **133**, 419-423.

Srivatanakul, P., Parkin, D.M., Jiang, Y.Z., Khlat, M., Kao-lan, U.T., Sontipong, S. and Wild, C.P. (1991) The role of infection by Opisthorchis viverrini, hepatitis B virus, and aflatoxin exposure in the etiology of liver cancer in Thailand. A correlation study. *Cancer* **68**, 2411-2417.

Starzl, T.E., Iwatuski, S., Shaw, B.W.Jr., Nalesnik, M.A., Farhi, D.C. and Van Thiel, D.H. (1986) Treatment of fibrolamellar hepatoma with partial or total hepatectomy and transplantation of the liver. *Surg. Gynecol. Obstet.* **162**, 145-148.

Suarez-Quian, C.A., Goldstein, S.R., Pohida, T., Smith, P.D., Peterson, J.I., Wellner, E., Ghany, M. and Bonner, R.F. (1999) Laser capture microdissection of single cells from complex tissues. *BioTechniques* **26**, 328-335.

Sun, L., Wu, G., Willson, J.K., Zborowska, E., Yang, J., Rajkarunanayake, I., Wang, J., Gentry, L.E., Wang, X.F. and Brattain, M.G. (1994) Expression of transforming growth factor beta type II receptor leads to reduced

malignancy in human breast cancer MCF-7 cells. *J. Biol. Chem.* **269**, 26449-26455.

Switalska, H.I., Niewiarowski, S., Tuszynski, G.P., Rucinski, B., Schmaier, A.H., Morinelli, T.A. and Cierniewski, C.S. (1985) Radioimmunoassay of human platelet thrombospondin: different patterns of thrombospondin and beta-thromboglobulin antigen secretion and clearance from the circulation. *J. Lab. Clin. Med.* **106**, 690-700.

Tabor, E. and Kobayashi, K. (1992) Hepatitis C virus, a causative infectious agent of non-A, non-B hepatitis: Prevalence and structure - summary of a conference on hepatitis C virus as a cause of hepatocellular carcinoma. *J. Natl. Cancer Inst.* **84**, 86-90.

Tada, M., Omata, M. and Ohto, M. (1990) Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. *Cancer Res.* **50**, 1121-1124.

Tanaka, S., Toh, Y., Adachi, E., Matsumata, T., Mori, R. and Sugimachi, K. (1993) Tumor progression in hepatocellular carcinoma may be mediated by p53 mutation. *Cancer Res.* **53**, 2884-2887.

Taraboletti, G., Benelli, R., Borsotti, P., Rusnati, M., Presta, M., Giavazzi, R., Ruco, L. and Albini, A. (1999) Thrombospondin-1 inhibits Kaposi's sarcoma (KS) cell and HIV-1 Tat-induced angiogenesis and is poorly expressed in KS lesions. *J. Pathol.* **188**, 76-81.

Taroa, K., Ohkawa, S., Shimizu, A., Harada, M., Nakamura, Y., Ito, Y., Tamai, S., Hoshino, H., Inoue, T. and Kanisawa, M. (1994) Significance of hepatocellular proliferation in the development of hepatocellular carcinoma from anti-hepatitis C virus-positive cirrhotic patients. *Cancer* **73**, 1149-1154.

Teicher, B.A., Williams, J.I., Takeuchi, H., Ara, G., Herbst, R.S. and Buxton, D. (1998) Potential of the aminosterol, squalamine in combination therapy in

the rat 13,762 mammary carcinoma and the murine Lewis lung carcinoma. *Anticancer Res.* **18**, 2567-2573.

Thein, S.L., Jeffreys, A.J., Gooi, H.C., Cotter, F., Flint, J., O'Connor, N.T., Weatherall, D.J. and Wainscoat, J.S. (1987) Detection of somatic changes in human cancer DNA by DNA fingerprint analysis. *Br. J. Cancer* **55**, 353-356.

Thorgeirsson, S.S. (1996) Hepatic stem cells in liver regeneration. *FASEB J.* **10**, 1249-1256.

Tokuyama, Y. and Takeda, J. (1995) Use of 33P-labeled primer increases the sensitivity and specificity of mRNA differential display. *Biotechniques* **18**, 424-425.

Tolsma, S.S., Volpert, O.V., Good, D.J., Frazier, W.A., Polverini, P.J. and Bouck, N. (1993) Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J. Cell Biol.* **122**, 497-511.

Tong, M.J., el-Farra, N.S., Reikes, A.R. and Co, R.L. (1995) Clinical outcomes after transfusion associated hepatitis C virus. *N. Engl. J. Med.* **332**, 1463-1466.

Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T. and Hanada, K. (1995) Epiregulin: A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J. Biol. Chem.* **270**, 7495-7500.

Turbett, G.R., Barnett, T.C., Dillon, E.K. and Sellner, L.N. (1996) Single-tube protocol for the extraction of DNA or RNA from paraffin-embedded tissues using a starch-based adhesive. *Biotechniques* **20**, 846-851.

Tuszynski, G.P., Smith, M., Rothman, V.L., Capuzzi, D.M., Joseph, R.R., Katz, J., Besa, E.C., Treat, J. and Switalska, H.I. (1992) Thrombospondin levels in patients with malignancy. *Thromb. Haemost.* **67**, 607-611.

Twist, M.E., Clark, H.F., Aden, D.P., Knowles, B.B. and Plotkin, S.A. (1981) Integration pattern of hepatitis B virus DNA sequences in human hepatoma cell lines. *J. Virol.* **37**, 239-243.

Ueki, T., Fujimoto, J., Suzuki, T., Yamamoto, H. and Okamoto, E. (1997) Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. *Hepatology* **25**, 862-866.

Ueno, T., Takahashi, K., Matsuguchi, T., Ikejiri, K., Endo, H. and Yamamoto, M. (1988) Reactivation of rat insulin-like growth factor II gene during hepatocarcinogenesis. *Carcinogenesis* **9**, 1779-1783.

Unsal, H., Yakicier, C., Marcais, C., Kew, M., Volkmann, M., Zentgraf, H., Isselbacher, K.J. and Ozturk, M. (1994) Genetic heterogeneity of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **91**, 822-826.

Van Rensburg, S.J., Cook-Mozaffari, P., Van Schalkwyk, D.J., Van der Watt, J.J., Vincent, T.J. and Purchase, I.F. (1985) Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. *Br. J. Cancer* **51**, 713-726.

Vautier, G., Bomford, A.B., Portmann, B.C., Metivier, E., Williams, R. and Ryder, S.D. (1999) p53 mutations in British patients with hepatocellular carcinoma: clustering in genetic hemochromatosis. *Gastroenterology* **117**, 154-160.

Vogel, T., Guo, N.H., Krutzsch, H.C., Blake, D.A., Hartman, J., Mendelovitz, S., Panet, A. and Roberts, D.D. (1993) Modulation of endothelial cell proliferation, adhesion, and motility by recombinant heparin-binding domain

and synthetic peptides from the type I repeats of thrombospondin. *J. Cell Biochem.* **53**, 74-84.

Vogelstein, B., Fearon, E.R., Hamilton, S.R. and Feinberg, A.P. (1985) Use of restriction fragment length polymorphisms to determine the clonal origin of human tumors. *Science* **227**, 642-645.

Volpert, O.V., Stellmach, V. and Bouck, N. (1995) The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. *Breast Cancer Res. Treat.* **36**, 119-126.

Wands, J.R. and Blum, H.E. (1991) Primary hepatocellular carcinoma. *N. Engl. J. Med.* **325**, 729-731.

Wang, J., Chenivesse, X., Heigliein, B. and Brechot, C. (1990) Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. *Nature* **343**, 555-557.

Watanabe, J., Nakashima, O. and Kojiro, M. (1994) Clinicopathologic study on lymph node metastasis of hepatocellular carcinoma: a retrospective study of 660 consecutive autopsy cases. *Jpn. J. Clin. Oncol.* **24**, 37-41.

Weber, R.G., Scheer, M., Born, I.A., Joos, S., Cobbers, J.M., Hofele, C., Reifenberger, G., Zoller, J.E. and Lichter, P. (1998) Recurrent chromosomal imbalances detected in biopsy material from oral premalignant and malignant lesions by combined tissue microdissection, universal DNA amplification, and comparative genomic hybridization. *Am. J. Pathol.* **153**, 295-303.

Weidner, K.M., Sachs, M. and Birchmeier, W. (1993) The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor. *J. Cell Biol.* **121**, 145-154.

Weidner, N., Semple, J.P., Welch, W.R. and Folkman, J. (1991) Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N. Engl. J. Med.* **324**, 1-8.

Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W. and Folkman, J. (1993) Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.* **143**, 401-409.

Weinstat-Saslow, D.L., Zabrenetzky, V.S., VanHoutte, K., Frazier, W.A., Roberts, D.D. and Steeg, P.S. (1994) Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res.* **54**, 6504-6511.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**, 7213-7218.

Welsh, J., Petersen, C. and McClelland, M. (1991) Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acids Res.* **19**, 303-306.

Welsh, J., Pretzman, C., Postic, D., Saint-Girons, I., Baranton, G. and McClelland, M. (1992) Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves Borrelia burgdorferi into three distinct phyletic groups. *Int. J. Systematic Bacteriol.* **42**, 370-373.

Wesley, C.S., Ben, M., Kreitman, M., Haga, N. and Eanes, W.F. (1990) Cloning regions of the Drosophila genome by microdissection of polytene chromosome DNA and PCR with nonspecific primer. *Nucleic Acids Res.* **18**, 599-603.

Wheelock, E.F., Weinhold, K.J. and Levich, J. (1981) The tumor dormant state. *Adv. Cancer Res.* **34**, 107-140.

Yamada, T., De Souza, A.T., Finkelstein, S. and Jirtle, R.L. (1997) Loss of the gene encoding mannose 6-phosphate/insulin-like growth factor II receptor is an early event in liver carcinogenesis. *Proc. Natl. Acad. Sci. USA* **94**,10351-10355.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.

Wistuba, I.I., Miquel, J.F., Gazdar, A.F. and Albores-Saavedra, J. (1999) Gallbladder adenomas have molecular abnormalities different from those present in gallbladder carcinomas. *Hum. Pathol.* **30**, 21-25.

Wong, N., Lai, P., Lee, S.W., Fan, S., Pang, E., Liew, C.T., Sheng, Z., Lau, J.W.Y. and Johnson, P.J. (1999) Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis relationship to disease stage, tumor size, and cirrhosis. *Am. J. Pathol.* **154**, 37-43.

Wong, S.Y., Purdie, A.T. and Han, P. (1992) Thrombospondin and other possible related matrix proteins in malignant and benign breast disease: an immunohistochemical study. *Am. J. Pathol.* **140**, 1473-1482.

Wu, C.D., Wickert, R.S., Williamson, J.E., Sun, N.C., Brynes, R.K. and Chan, W.C. (1999) Using fluorescence-based human androgen receptor gene assay to analyze the clonality of microdissected dendritic cell tumors. *Am. J. Clin. Pathol.* **111**, 105-110.

Yamaguchi, K., Carr, B.I. and Nalesnik, M.A. (1995) Concomitant and isolated expression of TGF-alpha and EGF-R in human hepatoma cells supports the hypothesis of autocrine, paracrine, and endocrine growth of human hepatoma. *J. Surg. Oncol.* **58**, 240-245.

Yamashita, Y., Sendo, S., Hosokawa, T., Tuszynski, G., Kurohiji, T., Beppu, R., Shinohara, T., Kinugasa, T. and Shirakusa, T. (1998) Exogenous thrombospondin stimulates the proliferation of non-thrombospondin-producing cells. *Int. J. Oncol.* **13**, 355-359.

Yamauchi, M., Nakahara, M., Maezawa, Y., Satoh, S., Nishikawa, F., Ohata, M., Mizuhara, Y., Hirakawa, J., Nakajima, H., Fujisawa, K. and Toda, G. (1993) Prevalence of hepatocellular carcinoma in patients with alcoholic cirrhosis and prior exposure to hepatitis C. *Am. J. Gastroenterol.* **88**, 39-43.

Yano, H., Iemura, A., Fukuda, K., Mizoguchi, A., Haramaki, M. and Kojiro, M. (1993) Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. *Hepatology* **18**, 320-327.

Yasui, H., Hino, O., Ohtake, K., Machinami, R. and Kitagawa, T. (1992) Clonal growth of hepatitis B virus-integrated hepatocytes in cirrhotic liver nodules. *Cancer Res.* **52**, 6810-6814.

Yeh, F.S., Yu, M.C., Mo, C.C., Luo, S., Tong, M.J. and Henderson, B.E. (1989) Hepatitis B virus, aflatoxin, and hepatocellular carcinoma in southern Guangxi, China. *Cancer Res.* **49**, 2506-2509.

Yeh, Y.C., Tsai, J.F., Chuang, L.Y., Yeh, H.W., Tsai, J.H., Florine, D.L. and Tam, J.P. (1987) Elevation of transforming growth factor alpha and its relationship to the epidermal growth factor and alpha-fetoprotein levels in patients with hepatocellular carcinoma. *Cancer Res.* **47**, 896-901.

Yokoyama, I., Sheahan, D.G., Carr, B., Kakizoe, S., Selby, R., Tzakis, A.G., Todo, S., Iwatsuki, S. and Starzl, T.E. (1991) Clinicopathologic factors affecting patient survival and tumor recurrence after orthotopic liver transplantation for hepatocellular carcinoma. *Transplant Proc.* **23**, 2194-2196.

Yumoto, Y., Hanafusa, T., Hada, H., Morita, T., Ooguchi, S., Shinji, N., Mitani, T., Hamaya, K., Koide, N. and Tsuji, T. (1995) Loss of heterozygosity and analysis of mutation of p53 in hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* **10**, 179-185.

Zabrenetzky, V., Harris, C.C., Steeg, P.S. and Roberts, D.D. (1994) Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. *Int. J. Cancer* **59**, 191-195.

Zajchowski, D.A., Band, V., Trask, D.K., Kling, D., Connolly, J.L. and Sager, R. (1990) Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 2314-2318.

Zhang, X., Xu, H.J., Murakami, Y., Sachse, R., Yashima, K., Hirohashi, S., Hu, S.X., Benedict, W.F. and Sekiya, T. (1994) Deletions of chromosome 13q, mutations in retinoblastoma 1, and retinoblastoma protein state in human hepatocellular carcinoma. *Cancer Res.* **54**, 4177-4182.

Zitzelsberger, H., Kulka, U., Lehmann, L., Walch, A., Smida, J., Aubele, M., Lorch, T., Hofler, H., Bauchinger, M. and Werner, M. (1998) Genetic heterogeneity in a prostatic carcinoma and associated prostatic intraepithelial neoplasia as demonstrated by combined use of laser-microdissection, degenerate oligonucleotide primed PCR and comparative genomic hybridization. *Virchows Arch.* **433**, 297-304.

APPENDICES

Appendix 1: Solutions and reagents

Luria broth (LB)

10 g enzymatic casein digest (bacto-tryptone)
5 g yeast extract
10 g NaCl
[Dissolve in water to 1 litre and autoclave for 15 minutes at 15 lb/sq]

Luria broth agar

10 g enzymatic casein digest (bacto-tryptone)
5 g yeast extract
5 g NaCl
12 g of bacteriological agar
[Dissolve in water to 1 litre, autoclave and pour 15-20 ml into 90 mm^2 Petri dishes (Sterilin)]

QIAGEN Miniprep and QIAfilter Plasmid Maxi DNA Purification Kit (Qiagen)

Cell Resuspension Buffer (Buffer P1)

100 µg/ml RNase A
10 mM EDTA
50 mM Tris-HCl, pH 8.0

Cell Lysis Buffer (Buffer P2)

200 mM NaOH
1% SDS

Neutralization buffer (Buffer N3 or P3)

3 M potassium acetate, pH 5.5

Column wash buffer (Buffer PE or QC)

1 M NaCl
50 mM MOPS
15% ethanol, pH 7.0

Equilibration buffer (Buffer QBT)

750 mM NaCl
50 mM MOPS
15% ethanol, pH 7.0
0.15% Triton X-100

Elution buffer (Buffer QF)

1.25 M NaCl

50 mM Tris-HCl

15% ethanol, pH 8.5

G-50

4 g medium grade Sephadex G-50 (Pharmacia)

100 ml TES

[Autoclave for 15 minutes at 15 lb/sq]

TES

1X TE (4 ml of 100 x TE)

0.5% SDS (20 ml of 10% SDS)

376 ml of water

[Autoclave for 15 min at 15 lb/sq]

10X TE

121.1 g Tris (hydroxymethyl) aminomethane

3.7 g EDTA

[Dissolve in 800 ml water, adjust to pH 7.5 and to volume of 1 litre]

10X TBE

108 g Tris (hydroxymethyl) aminomethane (Tris base)

55 g Boric acid

9.3 g EDTA

[Dissolve in 1 litre water]

5X reaction buffer for T4 DNA ligase (Life Technologies)

250 mM Tris.Cl, pH 7.6

50 mM $MgCl_2$

5 mM ATP

5 mM DTT

25% (w/v) polyethelene glycol-8000

10X Taq polymerase reaction buffer

200 mM Tris.Cl, pH 8.4

500 mM KCl

20X SSC

175.3 g NaCl

88.2 g Na citrate

[Dissolve in 1 litre water]

0.2 M sodium phosphate buffer (pH 7.0)

8.65 g Na_2HPO_4 (anhydrous)

6.08 g $H_2PO_4.2H_2O$

[Dissolve in water to 1 litre and autoclave for 15 minutes at 15 lb/sq]

10 mM sodium phosphate running buffer (pH 7.0)

25 ml 0.2 M sodium phosphate buffer (pH 7.0)

475 ml deionised water

[Autoclave for 15 minutes at 15 lb/sq]

GNB loading buffer

50% glycerol

10 mM sodium phosphate buffer

0.01% (w/v) bromphenol blue

[Dissolve in water to 100 ml and autoclave for 15 minutes at 15 lb/sq]

RNA ladder filter-staining buffer

0.4 M Na acetate

0.4 M Acetic acid

0.2% (w/v) methylene blue

6X DNA loading buffer

0.25% bromophenol blue

40% (w/v) sucrose in water

Phosphate-buffered saline (PBS)

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

[Dissolve in 800 ml water, adjust to pH 7.4 and to volume of 1 litre]

Restriction endonucleases

The restriction endonucleases common used and the recognition sites.

Enzymes	Recognition sites	Buffer	Suppliers
Xba I	T↓CTAG A A GATC↑T	REact Buffer 2	Life Technologies
Pst I	C TGCA↓G G↑ACGT C	REact Buffer 2	Life Technologies
Xho I	C↓TCGA G G AGCT↑C	REact Buffer 2	Life Technologies
EcoR I	G↓AATT C C TTAA↑G	REact Buffer 3	Life Technologies
BamH I	G↓GATC C C CTAG↑G	REact Buffer 3	Life Technologies
Kpn I	G GTAC↓C C↑CATG G	REact Buffer 4	Life Technologies
Sal I	G↓TCGA C C AGCT↑G	REact Buffer 10	Life Technologies

1X REact Buffer 2

50 mM Tris.Cl
10 mM $MgCl_2$
50 mM NaCl
pH 8.0

1X REact Buffer 3

50 mM Tris.Cl
10 mM $MgCl_2$
100 mM NaCl
pH 8.0

1X REact Buffer 4

20 mM Tris.Cl
5 mM $MgCl_2$
50 mM KCl
pH 7.4

1X REact Buffer 10

100 mM Tris-HCl
10 mM $MgCl_2$
150 mM NaCl
pH 7.6

Appendix 2: Sequence of p53 (exon 4 to exon 9)

EXON 4	TCCCCCTTGCCCGTCCCAAGCAATGGATGATTTGATGCTGTCCCCGGACG ATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGATGAAGCTCCCAGA ATGCCAGAGGCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCTCCTACAC CGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCCC TTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGC ATTCTGGGACAGCCAAGTCTGTGACTTGCACG
EXON 5	TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCC TGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGC GCCATGGCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGC GCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATG
EXON 6	GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTG GAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTA TGAGCCGCCTGAG
EXON 7	GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCC TGCATGGGCGGCATGAACCGGAGGCCCATCTCACCATCATCACACTGGA AGACTCCAG
EXON 8	TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTC CTGGGAGAGACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGG AGCCTCACCACGAGCTGCCCCCAGGGAGCACTAAGCGAG
EXON 9	CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTG GATGGAGAATATTTCACCCTTCAG

Publications related to this work

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Davidson, B.R., Dhillon, A.P., Kakkar, A.K., Williamson, R.C.N. and Lemoine, N.R. (1999) Genomic heterogeneity in synchronous hepatocellular carcinomas. *Gut* **45**, 761-765.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Davidson, B.R., Dhillon, A.P., Kakkar, A.K., Williamson, R.C.N. and Lemoine, N.R. (1999) Evolution of genetic abnormalities in hepatocellular carcinomas demonstrated by DNA fingerprinting. *J. Pathol.* **189**, 344-350.

Sirivatanauksorn, Y., Drury, R., Crnogorac-Jurcevic, T., Sirivatanauksorn, V. and Lemoine, N.R. (1999) Laser-assisted microdissection: applications in molecular pathology. *J. Pathol.* **189**, 150-154.

ABSTRACTS

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Rees, J., Savage, K., Dhillon, A.P., Kakkar, A.K., Lemoine, N.R., Williamson, R.C.N. and Davidson, B.R. (1998) Genomic heterogeneity in synchronous hepatocellular cancers. *Br. J. Surg.* **85**, 712.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Savage, K., Rees, J., Dhillon, A.P., Lemoine, N.R., Williamson, R.C.N. and Davidson, B.R. (1998) Polymorphic genomic fingerprinting of synchronous hepatocellular cancers identified by arbitrarily primed-polymerase chain reaction (AP-PCR). *J. Hepatol.* **28** Suppl, 88.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Davidson, B.R., Lemoine, N.R., Williamson, R.C.N., Savage, K., Rees, J. and Dhillon, A.P. (1998) Clonal evolution in hepatocellular carcinomas: relationship to tumour size. *Hepato-Gastroenterology* **45** Suppl II, LXXXIII.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Davidson, B.R., Savage, A.K., Dhillon, A.P., Kakkar, A.K., Lemoine, N.R. and Williamson, R.C.N. (1998) Genomic homogeneity in fibrolamellar hepatocellular carcinoma and its metastatic lesions. *Hepato-Gastroenterology* **45** Suppl II, LXXXIV.

Sirivatanauksorn, Y., Bhattacharya, S., Sirivatanauksorn, V., Lemoine, N.R., Savage, K., Dhillon, A.P., Williamson, R.C.N. and Davidson, B.R. (1998) Genomic heterogeneity in primary and metastatic hepatocellular carcinoma. *Hepato-Gastroenterology* **45** Suppl II, CXVIII.

Sirivatanauksorn, V., McKie, A.B., **Sirivatanauksorn, Y.** and Lemoine, N.R. (1998) DNA fingerprinting strategies to identify novel genetic events in pancreatic cancer. *Gastroenterology* **114**, A681.

Sirivatanauksorn, Y., Sirivatanauksorn, V., Bhattacharya, S., Lemoine, N.R., Tebbutt, S.J., Kakkar, A.K., Williamson, R.C.N., Dhillon, A.P. and Davidson, B.R. (1998) Clonal evolution in hepatocellular carcinomas. *Br. J. Surg.* **85**, 1577.

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