INVESTIGATION OF NOVEL TUMOR MARKERS BASED ON HYBRIDOMA TECHNOLOGY

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

BY
HİLAL ÇELİKKAYA
AUGUST, 2004

To the hope of cancer patients for recovery…

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

Assoc. Prof. Ayhan Kubar

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

Assist. Prof. Kamil Can Akçalı

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

Assist. Prof. Tamer Yağcı

Approved for the Institute of Engineering and Science.

Prof. Dr. Mehmet Baray
Director of Institute of Engineering and Science

ABSTRACT

Investigation of Novel Tumor Markers
Based On Hybridoma Technology
Hilal Çelikkaya

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Supervisor: Assist. Prof. Tamer Yağcı
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Hybridoma technology is a highly specific technique utilized for the production of monoclonal antibodies. In cancer research, monoclonal antibodies are used as tumor markers for diagnosis of malignant tissue versus benign or normal, for differential diagnosis of tumor type, for pathological grading of tumor biopsy specimens, for detection of the antigens that are up- or down-regulated in tumor cells or in sera of cancer patients, and for detection of primary or metastatic lesions. Additionally, monoclonal antibodies are employed in predicting the course of the disease, in diagnostic approaches for carrying imaging reagents to tumors, and in therapy for targeting cytotoxic reagents to and triggering or blocking cell surface molecules. This study involved generation of 6D5 and 9C11 monoclonal antibodies against apoptosis induced hepatocellular carcinoma cell line HuH-7, followed by characterization experiments. 6D5 antibody recognized 5 different epitopes in a panel of 28 cell lines in Western blotting experiments. In immunohistochemistry studies, 6D5 demonstrated positive staining in cirrhotic and cancerous cells of liver cancer tissue samples. On the other hand, 9C11 antibody recognized a single band in the same panel of 28 cell lines but it was not immunoreactive in immunoperixodase studies of liver cancer tissue samples, under our experimental conditions.

ÖZET

Yeni Tümör Belirleyicilerin
Hibridoma Teknolojisine Dayalı Olarak Araştırılması

Hilal Çelikkaya

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Bölümü

Tez Yöneticisi: Yard. Doç. Tamer Yağcı

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Hibridoma teknolojisi, monoklonal antikor üretimi için yararlanılan oldukça özgün bir tekniktir. Monoklonal antikorlar kanser araştırmalarında; kanserli dokunun benign veya normal dokuya karşı ayırıcı tanısında, tumor tipinin ayırıcı tanısında, tumor biyopsisi örneklerinin evrelerinin belirlenmesinde, tumor hücreleri veya kanserli hasta serumlarında artan veya azalan biçimde düzenlenen antijenlerin saptanmasında ve birincil veya metastazik lezyonların saptanmasında tumor belirleyici olarak kullanılmaktadır. Bunun yanı sıra monoclonal antikorlar; hastalık seyrinin öngörüsünde, görüntüleme ayıraçlarının tumor hücrelerine hedeflenmesi gibi tanısal yaklaşımlarda ve terapide, sitotoksik ayıraçların hücre yüzey moleküllerine yönlendirilmesi ve hücre yüzey moleküllerinin tetiklenmesi veya bloke edilmesi icin kullanılmaktadır. Bu çalışma, apoptoz tetiklenmiş hepatoselüler karsinom hücre hattı HuH-7'a karşı 6D5 ve 9C11 monoklonal antikorlarının üretimini takiben, karakterizasyon deneylerini içermektedir. 6D5 antikoru, Western blotlaması deneylerinde kullanılan 28 hücre hattından oluşan bir panelde 5 farklı epitopu tanımaktadır. Immünohistokimya çalışmalarında 6D5, karaciğer kanseri doku örneklerindeki sirozlu ve kanserli hücreleri pozitif boyamaktadır. Diğer yandan, 9C11 antikoru, 28 hücre hattından oluşan aynı panelde tek bir bant tanımakta ancak kullandığımız deneysel koşullar altındaki immunohistokimya çalışmalarında, karaciğer kanseri doku örneklerinde immunoreaktivite göstermemektedir.

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ABBREVIATIONS

ACF	Aberrant Crypt Foci
AFP	Alpha-Fetoprotein
APS	Ammonium Persulphate
BSA	Bovine Serum Albumin
C	Degree Celsius
CD	Cluster of differentiation
CK	Cytokeratin
cm^2	square centimeter
CYFRA	Cytokeratin 19 Fragments in Serum
dH_2O	Distilled Water
ddH_2O	Doluble Distilled Water
DAB	Diaminobenzidine Tetrahydrochloride
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay

FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FLC	Fibrolamellar Carcinoma
HAT	Hypoxanthine Aminopterin Thymidine
HCC	Hepatocellular Carcinoma
HGPRT	Hypoxanthine Guanine Phosphoribosyl Transferase
HPV	Human Papilloma Virus
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin G
kDa	kilodalton
L	Liter
LOH	Loss of heterozygosity
mA	Miliampere
mAb	Monoclonal antibody
ml	Mmililiter
mm	Milimeter
mM	Milimolar
M-protein	Monoclonal (or Myeloma) Protein
µg	Microgram
µl	Microliter
nm	Nanometer
NSCLC	Non-Small Cell Lung Cancer

PAGE	Polyacrylamide Gel Electrophoresis
Pap	Papanicolaou
PBS	Phosphate Buffered Saline
PSA	Prostate-Specific Antigen
PSMA	Prostate-Specific Membrane Antigen
PVDF	Polyvinyl Difluoride
rpm	Revolutions per Minute
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulfate
TAA	Tumor-Associated Antigen
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β_1	Transforming Growth Factor β1
U	Unit(s)
UV	Ultraviolet
v	Volume
w	Weight
W	Watt

1. INTRODUCTION

1.1. Understanding Cancer

Among the 75 to 100 trillion of an estimated number of cells in the human body, it is not surprising that one or some of our cells may gain an aberrant proliferative activity in the course of a lifetime. Beyond this, any individual may contain hereditary defects leading to abnormal proliferative activity of her/his cells. However, not every aberrant proliferation of cells is referred to as cancer. Excessive division of the cells gives rise to an abnormal mass of tissue, called **tumor** or **neoplasm**. If the cells of the tumor stay clustered together in this tumor mass without spreading to surrounding tissue or farther, the neoplasm is said to be **benign.** On the contrary, **malignant** (cancerous) tumors invade the nearby tissue and metastasize to distant parts of the body (American Cancer Society, 2004; Alberts *et al.*, 2002; DeVita *et al*., 2001; http://www.nci.nih.gov).

1.2. Cancer Incidence and Mortality Rates

In American population, the lifetime risk of a male individual of developing cancer is nearly 1 in 2 and it is nearly 1 in 3 for females. American Cancer Society states that about 1,368,030 new cancer cases are expected to be diagnosed in 2004 in the United States (U.S.) excluding carcinoma in situ of any site except urinary bladder and cancers of basal and squamous cells of skin (**Table 1.1**). The estimated number of basal and squamous cell skin cancers in 2004 is more than one million (American Cancer Society, 2004).

According to National Vital Statistics Reports of leading causes of deaths for 2001 (Arias *et al*., 2001), malignant neoplasms rank in the second order coming after the diseases of heart as being the first cause of deaths during 2000-2001 in the U.S. Malignant neoplasms are still ranking in the second place in the published preliminary data of National Vital Statistics Reports for the year 2002. The

estimation of American Cancer Society for 2004 reaches a consensus on ranking of cancer deaths with those previous reports. As a result, every year, 1 of every 4 people in the U.S. dies of cancer. 563,700 cancer deaths are expected to occur in U.S. in 2004, which means more than 1,500 people dying of cancer per day (American Cancer Society, 2004).

1.3. Cancer Development

It should be noted that anyone can develop cancer. There is a diverse set of genomic abnormalities in cancer cells and those abnormalities affect the phenotype of those cells as well. Loss of differentiation, increased motility leading to invasion and metastasis, deregulation of cell cycle control, self-sufficiency in growth signals, escape from apoptosis, sustained angiogenesis and decreased drug sensitivity are among the significant characteristics of cancer cells. As DeVita *et al*. (2001) underlines, there is a common misconception such as "cancer cells replicate faster than their normal counterparts". However, the growth abnormality of cancer cells is not a result of faster replication but lack of cell cycle control and insensitivity to anti-growth and apoptotic signals. Understanding the interconnections between growth inhibitory and growth stimulatory pathways would reveal the complexity of cell growth control networks.

The progression of cancer from a normal tissue is a multistep process and it takes place between 5 to 20 years. During the years of cancer progression, accumulation of mutations occurs resulting in malignant phenotype and this suggests that genetic instability is an early occurring event during tumor progression. Despite this fact, the exact number of mutations leading to malignancy is not known. Cancer progression is influenced by both inherited factors and somatic genetic changes, which is affected by both intrinsic (hormones, immune conditions, mutations occurring as a result of metabolic activity) and extrinsic factors (tobacco, chemicals, radiation, infectious organisms). About 5% to 10% of cancers are caused by hereditary defects.

Table 1.1: Estimated new cancer cases and deaths by sex for all body sites, U.S., 2004* (taken from American Cancer Society, 2004)

	Estimated New Cases			Estimated Deaths		
	Both Sexes	**Male**	**Female**	**Both Sexes**	**Male**	**Female**
All sites	1,368,030	699,560	668,470	563,700	290,890	272,810
Oral cavity & pharynx	28,260	18,550	9,710	7,230	4,830	2,400
Tongue	7,320	4,860	2,460	1,700	1,100	600
Mouth	10,080	5,410	4,670	1,890	1,070	820
Pharynx	8,250	6,330	1,920	2,070	1,460	610
Other oral cavity	2,610	1,950	660	1,570	1,200	370
Digestive system	255,640	135,410	120,230	134,840	73,240	61,600
Esophagus	14,250	10,860	3,390	13,300	10,250	3,050
Stomach	22,710	13,640	9,070	11,780	6,900	4,880
Small intestine	5,260	2,750	2,510	1,130	610	520
Colon†	106,370	50,400	55,970	56,730	28,320	28,410
Rectum	40,570	23,220	17,350			
Anus, anal canal & anorectum	4,010	1,890	2,120	580	210	370
Liver & intrahepatic bile duct	18,920	12,580	6,340	14,270	9,450	4,820
Gallbladder & other biliary	6,950	2,960	3,990	3,540	1,290	2,250
Pancreas	31,860	15,740	16,120	31,270	15,440	15,830
Other digestive organs	4,740	1,370	3,370	2,240	770	1,470
Respiratory system	186,550	102,730	83,820	165,310	95,460	69,670
Larynx	10,270	8,060	2,210	3,830	3,010	820
Lung & bronchus	173,770	93,110	80,660	160,440	91,930	68,510
Other respiratory organs	2,510	1,560	950	860	520	340
Bones & joints	2,440	1,230	1,210	1,300	720	580
Soft tissue (including heart)	8,680	4,760	3,920	3,660	2,020	1,640
Skin (excluding basal & squamous)	59,350	31,640	27,710	10,250	6,590	3,660
Melanoma	55,100	29,900	25,200	7,910	5,050	2,860
Other nonepithelial	4,910	2,400	2,510	2,340	1,540	800
Breast	217,440	1,450	215,990	40,580	470	40,110
Genital system	323,210	240,660	82,550	59,250	30,530	28,720
Uterine cervix	10,520		10,520	3,900		3,900

Uterine corpus	40,320		40,320	7,090		7,090
Ovary	25,580		25,580	16,090		16,090
Vulva	3,970		3,970	850		850
Vagina & other genital, female	2,160		2,160	790		790
Prostate	230,110	230,110		29,900	29,900	
Testis	8,980	8,980		360	360	
Penis & other genital, male	1,570	1,570		270	270	
Urinary system	98,400	68,290	30,110	25,880	17,060	8,820
Urinary bladder	60,240	44,640	15,600	12,710	8,780	3,930
Kidney & renal pelvis	35,710	22,080	13,630	12,480	7,870	4,610
Ureter & other urinary organs	2,450	1,570	880	690	410	280
Eye & orbit	2,090	1,130	960	180	110	70
Brain & other nervous system	18,400	10,540	7,860	12,690	7,200	5,490
Endocrine system	25,520	6,950	18,570	2,440	1,440	1,300
Thyroid	23,600	5,960	17,640	1,460	620	840
Other endocrine	1,920	990	930	980	520	460
Lymphoma	62,250	33,180	29,070	20,730	11,090	9,640
Hodgkin disease	7,880	4,330	3,550	1,320	700	620
Non-Hodgkin lymphoma	54,370	28,850	25,520	19,410	10,390	9,020
Multiple myeloma	15,270	8,090	7,180	11,070	5,430	5,640
Leukemia	33,440	19,020	14,420	23,300	12,990	10,310
Acute lymphocytic leukemia	3,830	2,110	1,720	1,450	820	630
Chronic lymphocytic leukemia	8,190	5,050	3,140	4,800	2,730	2,070
Acute myeloid leukemia	11,920	6,280	5,640	8,870	4,810	4,060
Chronic myeloid leukemia	4,600	2,700	1,900	1,570	940	630
Other leukemia	4,900	2,880	2,020	6,610	3,690	2,920
Other & unspecified primary sites‡	31,090	15,930	15,160	45,170	22,10	23,160

* Rounded to the nearest 10; excludes basal and squamous cell skin cancers and in situ carcinomas except urinary bladder. Carcinoma in situ of breast accounts for about 59,390 new cases annually, and in situ melanoma accounts for about 40,780 new cases annually.

† Estimated deaths for colon and rectum cancers are combined.

‡ More deaths than cases suggests lack of specificity in recording underlying causes of death on death certificates.

As a result of the fact that cancer progresses in a time-dependent manner including several rate-limiting steps, many types of cancers show an increase of incidence in an age-dependent manner. Most cancer cases affect adults beginning in middle age and about 76% of all cancers are diagnosed at age 55 and older (American Cancer Society, 2004; Qin and Tang, 2002; Vogelstein and Kinzler, 2002; Weber, 2002; DeVita *et al*., 2001; Röcken and Carl-McGrath, 2001; Hanahan and Weinberg 2000; Hunter, 1997; Kinzler and Vogelstein, 1996).

1.4. Cancer Genetics

1.4.1. Tumor Suppressor Genes

There is a big group of genes whose inactivation directly contributes to neoplasmic growth including *TP53, RB1, VHL, WT, PTEN, NF-1, NF-2* and *APC* (**Table 1.2**). They function in growth control of cells and are said to be "gatekeepers" to prevent uncontrolled cell growth and named as tumor suppressors. Tumor suppressor genes are targets of loss-of-function mutations in cancer cells. They function in a diverse set of signaling and growth regulatory networks including cell cycle control, differentiation, cell-cell adhesion, apoptosis and maintenance of genomic integrity. As an example, in patients suffering from familial adenomatous polyposis (FAP), an inherited defect in the *APC* tumor suppressor gene results in development of hundreds of adenomatous polyps. The great number of those polyps guarantees the progression of some of them to malignant neoplasm.

There is also an ever-increasing number of susceptibility genes including *XPB, ATM, MSH2,* and *MLH1* that indirectly suppress neoplasia. Those genes are said to be "caretakers" of the genome and admitted to be a subset of tumor suppressor genes. They locate in DNA repair pathways, not in cell growth regulation or differentiation. While inactivation of a gatekeeper directly contributes to oncogenesis, inactivation of a caretaker contributes to increased mutation rate in the genome. For example, hereditary nonpolyposis colorectal cancer (HNPCC) leads to formation of adenomatous polyps in an individual at about the same rate as the general population

but since those patients are more open to have mutations because of defective mismatch repair, those polyps progress to cancer much more often when compared to general population (Vogelstein and Kinzler, 2002; Kinzler and Vogelstein, 1998; Kinzler and Vogelstein, 1996).

The retinoblastoma gene (*RB1*) is the first tumor suppressor to be cloned and it is found to be mutated in retinoblastoma, small cell lung carcinoma (SCLC) and osteosarcoma. pRb has a role in cell cycle control, DNA replication, differentiation and apoptosis, interacting with more than 100 different cellular proteins. In pRb deficient cells, proliferation rate increases whereas differentiation decreases contributing to malignant phenotype (Classon and Harlow, 2002).

TP53 is the most commonly mutated gene in human cancers with at least 50% of the tumors having abnormal *TP53* gene. Moreover, there are some inactivating mutations of p53 function in some cancers. One of the examples is inactivation of p53 by HPV infection in cervical carcinoma. The reason of importance of *TP53* lies in its multiple involvements in cellular processes. p53 functions in cell cycle control, apoptosis and maintenance of genetic stability. Therefore, loss of p53 activity is extremely dangerous in relation to cancer (Alberts *et al*., 2002; DeVita *et al*., 2001).

1.4.2. Oncogenes

RAS, *MYC*, *EGFR*, *HER2*, *BCL-2*, *CCND-1*, *β-catenin*, *RET*, *SMO*, *MDM2* are some of the oncogenes that are frequently mutated in human cancers (**Table 1.3**). Oncogenes are altered forms of proto-oncogenes that are normal cellular genes whose proteins are highly conserved in evolution. Proto-oncogenic proteins function in networks that regulate cell cycle, apoptosis, cell division and differentiation. As a result, their expression is carefully regulated in different cell types during different stages of cell cycle and embryonic development. In oncogenesis, some of the oncogenes rescue cells from apoptosis and senescence by blocking cell differentiation whereas some of them liberate cells from their growth factor requirements. The activation of proto-oncogenes most frequently occur by

Table 1.2: Some tumor suppressor genes and tumor suppressor proteins (taken from Vogelstein and Kinzler, 2002)

Gene	Associated inherited cancer syndrome	Cancers with somatic mutations	Presumed function of protein
RB1	Familial retinoblastoma	Retinoblastoma, osteosarcoma, SCLC, breast, prostate, bladder, pancreas, esophageal, others	Transcriptional regulator; E2F binding
TP53	Li-Fraumeni syndrome	Approximately 50 % of all cancers	Transcription factor; regulates cell cycle and apoptosis
P16/INK4A	Familial melanoma, familial pancreatic carcinoma	25-30% of many different cancer types (e.g., breast, lung, pancreatic, bladder)	Cyclin-dependent kinase inhibitor (i.e., cdk4 and cdk6)
$P14^{Arf}$ ($p19^{Arf}$)	Familial melanoma?	15% of many different cancer types	Regulates Mdm-2 protein stability and hence p53 stability; alternative reading frame of *p16/INK4A* gene
APC	FAP, Gardner syndrome, Turcot syndrome	Colorectal, desmoid tumors	Regulates levels of β-catenin protein in the cytosol; binding to microtubules
WT-1	WAGR, Denys-Drash syndrome	Wilms tumor	Transcription factor
NF-1	Neurofibromatosis type 1	Melanoma, neuroblastoma	p21ras-GTPase
NF-2	Neurofibromatosis type 2	Schwannoma, meningioma, ependymoma	Juxtamembrane link to cytoskeleton
VHL	Von Hippel-Lindau syndrome	Renal (clear cell type), hemangioblastoma	Regulator of protein stability (e.g., HIFα)
BRCA1	Inherited breast and ovarian cancer	10% of ovarian, rare in breast	DNA repair; complexes with Rad 51 and BRCA2; transcriptional regulation

BRCA2	Inherited breast (both female and male), pancreatic cancer, others?	Rare mutations in pancreatic, others?	DNA repair; complexes with Rad 51 and BRCA1
MEN-1	Multiple endocrine neoplasia type 1	Parathyroid adenoma, pituitary adenoma, endocrine tumors of the pancreas	Not known
PTCH	Gorlin syndrome, hereditary basal cell carcinoma syndrome	Basal cell skin, medulloblastoma	Transmembrane receptor for sonic hedgehog factor; negative regulator of smoothened protein
PTEN/MMAC1	Cowden syndrome, sporadic cases of juvenile polyposis syndrome	Glioma, breast, prostate, follicular thyroid, head and neck squamous	Phosphoinositide 3-phosphatase; protein tyrosine phosphatase
DPC4	Familial juvenile polyposis syndrome	50% of pancreatic, 10-15% of colorectal, rare in others	Transcriptional factor in TGF-β signaling pathway
E-CAD	Familial diffuse-type gastric cancer	Gastric (diffuse type), lobular breast, rare in other types (e.g., ovarian)	Cell-cell adhesion molecule
LKB1/STK1	Peutz-Jeghers syndrome	Rare in colorectal, not known in others	Serine/threonine protein kinase
EXT1	Hereditary multiple exostoses	Not known	Glycosyltransferase; heparan sulfate chain elongation
EXT2	Hereditary multiple exostoses	Not known	Glycosyltransferase; heparan sulfate chain elongation
TSC1	Tuberous sclerosis	Not known	Not known; cytoplasmic vesicle localization
TSC2	Tuberous sclerosis	Not known	Putative GTPase-activating protein for Rap1 and rab5; golgi localization
MSH2, MLH1, PMS1, PMS2, MSH6	Hereditary nonpolyposis colorectal cancer	Colorectal, gastric, endometrial	DNA mismatch repair

Table 1.3: Oncogenes (taken from Vogelstein and Kinzler, 2002)

Oncogene	Neoplasm	Proto-oncogene
Growth factors		
v-sis	Glioma/fibrosarcoma	B-chain PDGF
int 2	Mammary carcinoma	Member of FGF family
KS3	Kaposi sarcoma	Member of FGF family
HST	Stomach carcinoma	Member of FGF family
int-I	Mammary carcinoma	Possible growth factor
Receptors lacking protein kinase activity		
mas	Mammary carcinoma	Angiotensin receptor
Tyrosine kinases: integral membrane proteins, growth factor receptors		
EGFR	Squamous cell carcinoma	Protein kinase (tyr) EGFR
v-fms	Sarcoma	Protein kinase (tyr) CSF-1R
v-kit	Sarcoma	Protein kinase (tyr) stem cell factor R
v-ros	Sarcoma	Protein kinase (tyr)
MET	MNNG-treated human osteocarcinoma cell line	Protein kinase (tyr) HGF/SFR
TRK	Colon carcinoma	Protein kinase (tyr) NGFR
NEU(HER2)	Breast carcinoma , neuroblastoma	Protein kinase (tyr)
RET	Thyroid carcinoma	Protein kinase (tyr) GDNFR
Tyrosine kinases: membrane associated		
SRC	Colon carcinoma	Protein kinase (tyr)
v-yes	Sarcoma	Protein kinase (tyr)
v-fgr	Sarcoma	Protein kinase (tyr)
v-fps	Sarcoma	Protein kinase (tyr)
v-fes	Sarcoma	Protein kinase (tyr)
BCR/ABL	Chronic myelogenous leukemia	Protein kinase (tyr)

Membrane-associated G proteins		
H-RAS	Colon, lung, pancreas carcinoma	GTPase
K-RAS	Acute myelogenous leukemia, thyroid carcinoma, melanoma	GTPase
N-RAS	Neuroblastoma, acute myeloid leukemia, multiple myeloma, melanoma	GTPase
gsp	Thyroid carcinoma	$G_6\alpha$
gip	Ovary, adrenal carcinoma	$G_1\alpha$
GEF family of proteins		
Dbl	Diffuse B-cell lymphoma	GEF for Rho and Cdc42Hs
Ost	Osteosarcomas	GEF for RhoA and Cdc42Hs
Tiam-1	T lymphoma	GEF for Rac and Cdc42Hs
Vay	Hematopoietic cells	GEF for Ras?
Lbc	Myeloid leukemias	GEF for Rho
Serine/threonine kinases: cytoplasmic		
v-mos	Sarcoma	Protein kinase (ser/thr)
v-raf	Sarcoma	Protein kinase (ser/thr)
pim-1	T-cell lymphoma	Protein kinase (ser/thr)
Nuclear protein family		
N-MYC	Neuroblastoma, lung carcinoma	Transcription factor
L-MYC	Lung carcinoma	Transcription factor
v-myb	Myeloblastosis	Transcription factor
v-fos	Osteosarcoma	Transcription factor API
v-jun	Sarcoma	Transcription factor API
v-ski	Carcinoma	Transcription factor
v-rel	Lymphatic leukemia	Mutant NFκB
v-ets	Myeloblastosis	Transcription factor
v-erbA	Erythroblastosis	Mutant thioredoxine receptor

amplification of those genes, by point mutations, by proviral insertion and gene rearrangements.

The complex network of growth inhibitory and growth stimulatory pathways necessitates the interconnection of tumor suppressors and oncogenes on the same pathway for a regular control of cell growth. This fact underlines the importance of such interconnected pathways. Therefore, the acquisition of sequential alterations involving both oncogenes and tumor suppressor genes is required for the progression of the malignant phenotype (Vogelstein and Kinzler, 2002; Hunter, 1997).

1.5. Selected Cancer Types

1.5.1. Bone Cancer (Sarcomas of the Bone)

The most common bone tumor is osteosarcoma and it generally occurs in childhood and adolescence. Osteosarcoma is a high-grade, malignant spindle cell tumor with poor prognosis accounting for 5% of the tumors in childhood. Bones of the knee joint are the most common sites for this cancer. Generally, 80-90% of osteosarcomas arise in the long tubular bones. The most important prognostic feature of the tumor is its resectability because this tumor is highly resistant to radiotherapy. Osteosarcoma is found to be not associated with a specifically recurrent translocation or any other specific chromosomal rearrangements. However, inactivation of *RB1*, *TP53*, *INK2A*, *INK4A* genes, deregulation of *CDK4*, and over-expression of *MDM2, MYC, HER2* and *CCND1* genes are reported (Sandberg and Bridge, 2003; DeVita *et al*., 2001; http://www.cancer.gov/cancertopics/pdq/treatment/osteosarcoma/healthprofessional).

1.5.2. Breast Cancer:

Breast cancer is the most frequent non-skin cancer type among women. It ranks second among cancer deaths in females. Increased risk-associated factors for the disease are a personal or family history of breast cancer (e.g., carrying mutated

BRCA1 and *BRCA2* genes, which are high-penetrance breast cancer susceptibility genes), atypical hyperplasia in the breast, a long menstrual history, obesity, postmenopausal hormone therapy of combination of estrogen and progestin, never having children, and excessive consumption of alcoholic beverages.

Most of the breast cancers are ductal type. Hereditary breast cancer accounts for 5 to 15% of all breast cancer cases. Breast cancer patients who carry an altered gene related to the disease have an increased risk of developing ovarian cancer. Somatic alterations in breast cancer include the genes that are displayed in **Table 1.4** (American Cancer Society, 2004; Vogelstein and Kinzler, 2002, DeVita *et al*., 2001; http://www.cancer.gov/cancertopics/pdq/treatment/breast/patient).

Table 1.4: Somatic alterations in breast cancer (taken from Vogelstein and Kinzler, 2002)

Gene/Region	Modification	Frequency
Growth factors and receptors		
EGFR	Overexpression	20-40%
HER-2/neu	Overexpression	20-40%
FGF1/FGF4	Overexpression	20-30%
TGFα	Overexpression	Not reported
Intracellular signaling molecules		
Ha-ras	Mutation	5-10%
c-src	Overexpression	50-70%
Regulators of cell cycle		
TP53	Mutation/inactivation	30-40%
RB1	Inactivation	20%
Cyclin D	Overexpression	35-45%
TGF-β	Deregulation	Not reported
Adhesion molecules and proteases		
E-cadherin	Reduced/absent	60-70%
P-cadherin	Reduced/absent	30%
Cathepsin D	Overexpression	20-24%
MMPs	Increased expression	20-80%
Other genes		
bcl-2	Overexpression	30-45%
c-myc	Amplification	5-20%
nm23 (*NME1*)	Decreased expression	Not reported

1.5.3. Cervical Cancer

Cancer of uterine cervix risk is closely related to the sexual behavior of the individual. Sexually transmitted infections caused by several strains of human papilloma virus (HPV), sexual activity starting at an early age, having many sexual partners or having sexual partners who have had many sexual partners increase the risk of cervical cancer. Besides, cigarette smokers and overweight patients are at disadvantage in the disease progression. For women aged between 20 and 39 years, cervical cancer is the second leading cause of cancer-related deaths ranking after breast cancer in U.S. However, Pap test is a routine diagnostic tool that can be simply used as a part of pelvic examination for detection of abnormal cells and fortunately, most cervical precancers grow slowly.

In the disease progression with HPV infection, HPV E6 and E7 oncoproteins interact directly with p53 and pRB tumor suppressor proteins, respectively. This leads to degradation of p53 protein by ubiquitin pathway and inactivation pRB protein. c-*myc* and *HER2* gene amplifications are also reported in cervical cancer cases (American Cancer society, 2004; Vogelstein and Kinzler, 2002; DeVita *et al*., 2001).

1.5.4. Colon and Rectum Cancers

In U.S., colon and rectum cancers account for the third most commonly diagnosed cancer both in males and females. In addition, it ranks third among mortality rates for both sexes. This ranking accounts for 10% of deaths caused by cancer. The disease is highly age-dependent with more than 90% of cases diagnosed in individuals older than age of 50. Half of the Western population develops benign colorectal tumors (adenomatous polyps) by age 70, and a fraction of those sporadic tumors progress into cancer (American Cancer society, 2004; Vogelstein and Kinzler, 2002; Kinzler and Vogelstein, 1998).

As mentioned before, tumorigenesis is a process involving activation of oncogenes and inactivation of tumor suppressor genes. In colorectal cancer, the presence of

aberrant crypt foci (ACF) is the earliest premalignant lesion. Dysplastic form of ACF is referred to as adenomatous crypts (microadenoma) and the most frequent reason of this form is loss of heterozygosity on 5q of the adenomatous polyposis coli (APC) gene. Dysplastic ACF are precursors of the adenomatous polyps, which further become colon carcinoma lesions. *TP53* gene mutations appear to be of significant importance during transition from adenoma to high-grade dysplasia. When compared, *TP53* mutation is a late event in contrast to *APC* gene mutation that is the earliest genetic event in colorectal cancer progression (Luebeck and Moolgavkar, 2002). Among the several inherited predispositions to colorectal cancer, the two best characterized are HNPCC and FAP. Notwithstanding, those two account for a small fraction of all cases and most cases still retain a sporadic character. *K-RAS*, *H-RAS*, *N-RAS, CTNNB1* oncogene mutations are frequently observed in colorectal tumors. The most frequently mutated tumor suppressor genes other than *APC* and *TP53* are *DCC*, *SMAD4/DPC4*, *SMAD, TGFBRII, hMSH2, hMSH3, hMSH6, hMLH1 hPMS1, and hMLH1* (Vogelstein and Kinzler, 2002; Grady and Markowitz, 2002; Buda and Pignatelli, 2002; Röcken and Carl-McGrath, 2001; Kinzler and Vogelstein, 1996; http://www.cancer.gov/cancertopics/pdq/treatment/colon/).

1.5.5. Kidney Cancer

Renal cell cancer, also called renal adenocarcinoma or hypernephroma, is a malignant neoplasm found in the lining epithelium of tubules in the kidney. Similar to many other cancer types, renal cell cancer can often be cured if it is diagnosed and treated at a localized stage to the kidney and to the very nearby surrounding tissue. 96% of the renal carcinoma cases are sporadic and there is a strong correlation between cigarette smoking, exposure to asbestos and development of renal carcinoma. *VHL* tumor suppressor gene mutations are of significant importance in renal cell tumorigenesis (DeVita *et al*., 2001; http://www.cancer.gov/cancertopics/pdq/treatment/renalcell/healthprofessional; http://www.cancer.gov/cancertopics/pdq/treatment/renalcell/patient).

1.5.6. Liver Cancer

Among all primary liver malignancies, hepatocellular carcinoma (HCC), which is carcinoma of hepatocytes, accounts for 90% of all cases and most of these cases arise from chronic liver infection by Hepatitis B and Hepatitis C viruses, accompanied by underlying cirrhosis. Hepatocyte necrosis, inflammation, regeneration, and fibrosis are associated with hepatitis and it may proceed to cirrhosis. Quiescent cells start to proliferate following liver necrosis, and chronic hepatitis consists of repetitive cycles of necrosis and regeneration that facilitate cancer development. The pathways that are suggested to be important in HCC development are p53, pRB, TGF-β and APC/β-catenin pathways. In addition, c-myc, c-fos, H-ras, N-ras and insulin-like growth factor gene are suggested to involve in oncogenic activation. Furthermore, there are environmental, metabolic (e.g., hemochromatosis, glycogen storage disease type 1), nutritional (e.g., aflatoxin B_1 uptake, excessive alcohol consumption) and endocrine factors that may contribute to hepatocarcinogenesis.

HCC is a highly malignant disease with poor prognosis and most cases are lately diagnosed. It ranks fifth in frequency in the world and has a male preponderance (2:1; male: female). HCCs are almost always soft tumors with the exception of the fibrolamellar variant (FLC), which is rare. All the same, FLC differs from other HCCs in female preponderance, young mean age of the patients (23 years) and its usual occurrence in the absence of cirrhosis. Serum alpha-fetoprotein is usually normal in FLC contrary to patients with underlying cirrhotic disease who display a progressive increase in alpha-fetoprotein (AFP) and/or in alkaline phosphatase. FLC's importance results from the fact that an increased proportion of fibrolamellar carcinoma patients may be cured if the tumor can be resected. It also generally exhibits a slower clinical course than the more common hepatocellular carcinoma.

Hepatoblastoma is another malignant tumor of liver and it has embryonic origin with differing patterns of differentiation. Hepatoblastoma usually does not spread outside the liver (Nita *et al*., 2002; Röcken and Carl-McGrath, 2001; Dürr and Caselmann, 2000; Habib, 2000; Robinson, 1994; http://www.cancer.gov/cancerinfo/pdq/ treatment/adultprimaryliver/HealthProfessional; http://www.cancer.gov/cancertopics/

pdq/treatment/adult-primary-liver/patient; http://www.vh.org/adult/provider/pathology/LiverPathology/Text/11Epithelial.html).

1.5.7. Myeloma

Myeloma is the systemic neoplasm of B cells, and the most common type is multiple myeloma. In multiple myeloma, cancerous plasma cells are found in the bone marrow. The small tumors made by plasma cells that collect in the bone are called plasmacytomas.

Macroglobulinemia is a type of plasma cell neoplasm in which lymphocytes that make an M-protein build up in the blood. Lymph nodes, liver and spleen may be swollen.

Despite the fact that it is rarely curable, myeloma is a highly treatable disease. Since the cancer arises from a single cell by clonal expansion, too many amount of monoclonal antibody and monoclonal (or myeloma) protein (M-protein) is found in the serum and/or urine of the myeloma patients at the time of diagnosis. Exposure to ionizing radiation is the major environmental cause of myeloma. Other potential environmental risk factors include exposure to nickel, agricultural chemicals, benzene, petroleum products and silicon. More than 40% of all myeloma cases have deletion in *RB1* gene. Additionally, scientists report constitutive phosphorylation of pRB. The high frequency of abnormalities in p16 (75%) and p15 (67%) suggests the importance of pRB regulatory pathway in myeloma development. Furthermore, the most common translocation involving 14q32 results in overexpression of cyclin D (DeVita *et al*., 2001; http://www.cancer.gov/cancertopics/pdq/treatment/myeloma/healthprofessional; http://www.cancer.gov/cancertopics/pdq/treatment/myeloma/patient).

1.5.8. Malignant Melanoma

Melanocytes are the pigment producing cells of the skin from which malignant melanoma arises. Melanoma is the most serious type of all skin cancers. Since it is 10 times more common in whites than in African Americans, it is admitted to be a disease of whites. Melanoma has a quickly spreading character but is highly curable when detected in its early, localized stages. Exposure to UV irradiation is the most influencing environmental factor of the disease. There are some loss of heterozygosity (LOH) regions that are frequently coupled to melanoma formation. Three of those regions contain metastasis suppressor gene *NM23* and tumor suppressor genes *NF1* and *PTEN*. *MLM*, *p16*, *p15*, $p19^{ARF}$ and *CDK4* are the genes whose mutations influence melanoma (American Cancer Society, 2004; Vogelstein and Kinzler, 2002; DeVita *et al*., 2001).

1.5.9. Prostate Cancer

Prostate cancer has the highest incidence among other cancers in men and unfortunately, it is the second leading cause of cancer deaths following lung cancer. Similar to colon and rectum cancers, more than 70% of the individuals diagnosed with this disease are over the age of 65. Histologically, more than 95% of the prostate tumors are adenocarcinomas that arise from acinar cells of the prostate. The genes that are found to be mutated in prostate cancer include *p53*, *PTEN*, *RB1*, *RAS*, *p16*, *Bcl-2*, *E-cadherin* and androgen receptor (American Cancer Society, 2004; Vogelstein and Kinzler, 2002; DeVita *et al*., 2001).

1.6. Hybridoma Technology and Monoclonal Antibody Production

B lymphocytes generate humoral immune response that is polyclonal and therefore, the antibodies present in an individual are heterogeneous. However, one can obtain large amounts of homogeneous antibody reacting with a single epitope (monoclonal) by cloning B lymphocytes. From the time that Koehler and Milstein introduced hybridoma technology based on this fact, scientists have identified a broad range of biological molecules with the help of monoclonal antibodies. Immune stimulatory and regulatory molecules, cellular markers for distinguishing the tissue origin of one cell from another, cellular and tumor markers for characterization of the nature of a tumor biopsy specimen, the antigens that are up- or down-regulated in tumor cells are some of the biomolecules in this range. Additionally, monoclonal antibodies are used in therapeutic approaches to target cytotoxic reagents to and trigger or block cell surface molecules, and in diagnostic approaches to carry imaging reagents to tumors (**Table 1.5**) (White *et al*., 2001; DeVita *et al*., 2001; Goding 1983).

Antibody-secreting hybridoma is the fusion of an indefinitely growing myeloma cell and an immune B lymphoblast expressing a specific antibody gene. Myeloma cells are established to be deficient for hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme. Polyethylene glycol, which is a polywax, is used to facilitate cell fusion because it promotes cell adherence and the exchange of nuclei. HAT medium that contains hypoxanthine, aminopterin and thymidine is used to select successfully fused cells because only hybrid cells can grow in this medium. Homogeneous cell clones that secrete only one specific antibody are obtained by limiting dilution (Abbas and Lichtman, 2003; Little *et al*., 2000).

The culture of a hybridoma cell line can yield 1-10 μg of Ig per ml supernatant. Ascites fluids can produce between 1 and 10 mg of Ig per 1 ml (DeVita *et al*., 2001).

Table 1.5: Clinical applications of monoclonal antibodies (mAbs) in cancer (taken from DeVita *et al*., 2001)

Diagnosis
Screening of body fluids for the presence of circulating TAA*
Nuclear scanning with radiolabeled mAb
Detection of primary or metastatic lesions (intravenous, subcutaneous, or iliopsoas administration of radiolabeled mAb)
The use of radiolabeled mAb and intraoperative γ detecting probe
Immunopathology
Diagnosis of malignant versus benign
Differential diagnosis of tumor type
Subclassification of tumor based on TAA expression
Metastatic potential
Specific favored sites of metastasis
Predicted response (or lack of thereof) to specific therapeutic regimens
Prognosis
Monitoring of disease progression
Screening of body fluids for circulating TAA
Nuclear scanning with radiolabeled mAb to detect or quantify tumor recurrence
Immunopathology for detection of occult metastases
Aspiration cytology
Lymph node or bone marrow biopsy
Cytology of body fluids
Therapy
Direct cytotoxicity of mAb
Complement mediated
Cell mediated
Drug conjugation of mAb (e.g., doxorubicin)
Toxin conjugation of mAb (e.g., ricin)
Radionuclide conjugation of mAb (e.g., α or β emitters)
Ex vivo tumor removal from harvested bone marrow
Inhibition of receptors for growth factors
Administration of antiidiotype mAbs to induce specific active immunity to tumor antigens

* Tumor-associated antigen

1.7. Tumor Markers

Tumor markers are products that are often detected in higher-than-normal amounts in the blood or other body fluids or tissues of some individuals with certain types of cancer. Tumor markers are produced either by the tumor itself (e.g., tumor antigens) or by the body in response to the presence of cancer. Tumor markers can be classified into three categories in terms of the aim of use:

1. **Diagnostic Markers:** They give clues for identifying a disease by its signs, symptoms and laboratory findings.
2. **Prognostic Markers:** They help to predict the course of the disease.
3. **Therapeutic Markers:** They pertain with the treatment of the disease. They are typically of IgG class molecules.

There are cell surface markers (e.g., phenotypic CD antigens of hematopoietic cells), genetic markers (e.g., Philadelphia chromosome), secreted proteins (e.g., paraproteins in the serum), oncofetal proteins (e.g., carcinoembryonic antigen, α-fetoprotein), hormones (e.g. human chorionic gonadotropin), enzymes (e.g., prostate-specific antigen, lactic dehydrogenase, neuron-specific enolase) and cancer antigens (e.g., CA 125, CA 15-3, CA 19-9) whose elevated levels or abnormal expression pattern is used for diagnostic, prognostic or therapeutic purposes (**Table 1.6**). Anyone may notice that a tumor marker can have more than one of the features mentioned in the sentence before (e.g., carcinoembryonic antigen is a cell surface protein and an oncofetal protein) (http://lwwoncology.com; http://cis.nci.nih.gov/fact/5_18.htm). This thesis will focus on protein markers because of the design of the thesis project.

Table 1.6: Some serum and immunohistochemical markers (taken and adapted from http://lwwoncology.com)

Marker	Associated Cancer Type
α-Fetoprotein	Testis, hepatocellular, upper gastrointestinal, germ cell, trophoblastic tumors
α-Lactalbumin	Breast
β_2-microglobulin	Myeloma, lymphoma
CA 15–3	Breast
CA 19–9	Pancreas, transitional cell tumors
CA 125	Ovary, cervix, endometrium, gastrointestinal tract, breast
Calcitonin	Thyroid (medullary carcinoma)
CD antigens	Lymphoma, leukemia, myeloma, endothelial cells
Carcinoembryonic antigen	Colorectal, breast, lung (small cell), gut, pancreas, cervix, urinary tract, medullary thyroid
β_1-Antitrypsin	Hepatocellular
Chromogranin	Neuroendocrine
Collagen type IV	Sarcoma (neurogenic, smooth muscle)
Cytokeratins	Carcinomas, rarely sarcomas
Desmin	Sarcoma (muscle)
Factor VIII	Vascular sarcomas
Glial fibrillary acidic protein	Glial cell tumors, astrocytomas, oligodendrogliomas, ependymomas, schwannomas
Gross cystic disease fluid protein	Breast
HER1	Lung
HER2	Breast, ovary, bladder, pancreas, colon, lung
Human chorionic gonadotropin	Testis, trophoblastic neoplasia, breast
Human placental lactogen	Trophoblastic tumors, germ cell tumors, gastric, lung
Immunoglobulin molecules	Lymphoma, leukemia
Involucrin	Squamous epithelia
Ki-67	All tumor types
Leukocyte common antigen	Lymphoma, leukemia, histiocytic tumors
Muramidase	Histiocytic tumors, myelogenous leukemia
Myelin basic protein	Neurogenic sarcoma
Myoglobin	Sarcoma, corpus uteri
Muscle-specific actin	Sarcoma
Neurofilaments	Neuroendocrine; small cell lung
Neuron-specific enolase	Neuroendocrine; small cell lung, breast
NKI/C3	Melanoma
Pancreatic carcinoma antigen	Pancreas, gut
Prostate-specific antigen	Prostate
Prostate-specific membrane antigen	Prostate
S100 protein	Melanoma, sarcoma, histiocytic tumors, glioma
Vimentin	Sarcoma, renal cell carcinoma, melanoma, lymphoma, leukemia
Thyroglobulin	Thyroid

1.8. Selected Tumor Markers

1.8.1. CA 125

CA 125 is an antigenic determinant on a high molecular weight glycoprotein and one of its two antigenic domains is recognized by the murine monoclonal antibody OC-125 and the other is recognized by the monoclonal antibody M11. Inclusion cysts, metaplasic parts and papillary excrescences found in ovary express CA 125 determinant but it is not expressed in the normal surface epithelium of fetal or adult ovaries. CA 125 is elevated in 85% of epithelial ovarian cancers and 13-21% of squamous carcinoma. In ovarian cancer, preoperative serum level of CA 125 is found to be correlated with tumor stage and histological grade but it is not suggested to be an independent prognostic or diagnostic factor on its own. Elevation of serum CA 125 can also be associated with pancreas, breast, colon, lung malignant neoplasms as well as with some benign neoplasms and physiological states such as pregnancy, and menstruation (Robertson *et al*., 2002; Meyer and Rustin, 2000; http://lwwoncology.com). The major contribution of CA 125 as a tumor marker is said to be its utility in the monitoring of tumor response to chemotherapy (Verheijen *et al*., 1999).

1.8.2. Carcinoembryonic Antigen (CEA)

CEA is an oncofetal serum antigen and it is present in small amounts in adult colon. If an increased serum level of CEA is detected in a pretreatment stage, it implies a negative prognostic significance for the patient. Serum level elevation of CEA is related to many cancer types including colon, rectum, gallbladder, pancreas and stomach. It is frequently used in the management of patients with rectal, colon, gallbladder and stomach cancers but it is suggested that CEA is not useful by itself for screening those cancers and use of CEA alone for monitoring response to treatment is not recommended. This is as a result of lack of tumor type specificity of CEA. Additionally, benign diseases of liver, gastrointestinal tract, lung and cigarette smoking may cause elevation of CEA levels decreasing its tumor specificity

(http://lwwoncology.com; http://www.cancer.gov/cancerinfo/pdq/treatment/rectal/HealthProfessional).

1.8.3. Alpha-Fetoprotein (AFP)

AFP is an oncoprotein, too. This glycoprotein is produced by fetal yolk sac, liver, and upper gastrointestinal tract. Pregnancy and benign liver disease also cause the elevation of AFP. Serum levels of this protein are increased in most of liver tumors and in some of the gastric, pancreatic, colon, and bronchogenic cancers. AFP is accepted to be the best diagnostic serum tumor marker for primary liver cancer. It is suggested that AFP is a reliable factor for monitoring therapeutic response and detecting recurrences in women with endodermal sinus tumors and embryonic carcinomas (Uenishi *et al*., 2003; http://lwwoncology.com).

1.8.4. CD20

CD20 is a transmembrane protein present on almost all of the B cells from the time of their commitment to B cell development until the time that the protein is down-regulated in the differentiated, antibody-secreting plasma cells. Therefore, CD20 is accepted as a pan-B cell antigenic marker. The expression of CD20 is quite heterogeneous both in the tumor sample of an individual and among different lymphoma types.

The first monoclonal antibody to be approved by U.S. Food and Drug Administration (FDA) for the therapy of cancer is rituximab (Rituxan®, 1997). Rituximab is used to target CD20 that is present in many B cells of non-Hodgkin's lymphoma subtypes. It induces apoptosis, antibody-dependent cell cytotoxicity and complement mediated cytotoxicity in these tumors. When used in combination with other chemotherapeutics, it significantly improves disease-free survival rates (Smith, 2003; Ross *et al*., 2003; White *et al*., 2001; Glennie *et al*., 2000; Maloney *et al*., 1994).

1.8.5. Cytokeratins

Cytokeratins are intermediate filaments that build cytoskeleton, being a member of it in all epithelial cells. They are specific markers of epithelial differentiation and although not the same, they are continued to be expressed after malignant transformation. More than 20 cytokeratins have been described in humans. One of the applications of those proteins is their use in differential diagnosis of epithelial tumors and detecting their differentiation status. Cytokeratin fragments can be detected in the serum by monoclonal antibodies because in malignant epithelial cells, activated proteases degrade cytokeratins into fragments that are soluble in the serum. CYFRA 21-1 is an assay developed to measure a soluble fragment of CK 19 in the serum. In non-small cell lung cancer (NSCLC), CYFRA 21-1 is more sensitive than any other established markers. Moreover, it is found to be a useful marker for cervical, esophageal, breast, gastric, and bladder cancers. Uenishi *et al*. (2003) suggest CYFRA 21-1 as a useful diagnostic test for intrahepatic cholangiocarcinoma patients because CK 19 is differentially expressed in bile duct cells of normal liver whereas normal hepatocytes do not express it (Upasani *et al*., 2004; Uenishi *et al*., 2003; Young *et al*., 2002; Kamoi *et al*., 2002; http://lwwoncology.com).

In a study of differential expression of cytokeratin 18 (CK 18) in NSCLC subtypes of adenocarcinoma, squamous cell carcinoma and adenosquamous carcinoma, it was found that CK 18 expression was strongest in adenocarcinoma, weak in adenosquamous carcinoma and undetectable in squamous cell carcinoma concluding that CK18 might be of diagnostic value for those subtypes (Young *et al*., 2002). In another study involving anal carcinoma, the authors concluded that loss of expression of CK 18 and CK 19 is a marker of dedifferentiation in anal carcinoma (Behrendt and Hansman, 2001). Ordonez (2002) underlines that CK 5/6 is among the most useful positive immunohistochemical markers for epitheloid mesothelioma diagnosis.

1.8.6. HER2

HER2 (erbB2) belong to the erbB family of tyrosine kinase receptors (RTKs) -also known as type I receptor tyrosine kinases or EGF receptor family- and it is a relative of HER1. In normal cells, activation of erbB RTKs triggers a broad network of signaling pathways controlling cell growth, differentiation, motility and adhesion. Deregulation of HER2 receptor is observed in many cancers leading to a more aggressive clinical outcome of the disease. In breast cancer, nearly 25% to 30% of patients have overexpression of HER2 caused by amplification of HER2 gene. This amplification is associated with a poor prognosis and chemoresistance of tumor in breast cancer patients. In addition, HER2 has a metastasis-promoting effect promoting the secretion of matrix metalloproteases.

The hypothesis that the blockage of binding of epidermal growth factor (EGF) to its receptor might prevent cell proliferation by inhibiting receptor activation influenced production of anti-EGF receptor monoclonal antibodies. HER2 is the target of the first monoclonal antibody approved by FDA as an erbB receptor inhibitor, trastuzumab (Herceptin®; Genentech, Inc.; South San Francisco, CA). The metastatic breast cancer patients with a profile of HER2 over-expression benefit from therapies involving trastuzumab. HER2-targeted therapies can lead to tumor regression, delay of tumor growth and some symptomatic improvements. There is a consensus among researchers on prognostic value of HER2 amplification/over-expression in node-positive breast cancer patients. In addition, HER2 over-expression has a negative predictive value for the responsiveness of the cancer patients to chemotherapy and endocrine therapy indicating the probability of resistance to those therapies. Apart from this, the approval of trastuzumab provided the use of an immunohistochemical diagnostic test (HercepTest™). HercepTest™ is designed specifically to detect HER2 protein over-expression in routinely processed breast cancer tissues. HercepTest™ is approved by FDA for the measurement of HER2 status when deciding whether patients are eligible for Herceptin® therapy. In Europe, patients are accepted to be eligible for treatment if they demonstrate IHC 3+ HER2 over-expression and in the U.S. patients are eligible if they demonstrate 2+ or 3+ HER2 over-expression. Therefore, HercepTest™ aims a reliable detection of

HER2 over-expression for the success of Herceptin® therapy. (Menard *et al*., 2003; Rowinsky 2003; Ross *et al*., 2003; White *et al*., 2001; Hanahan and Weinberg, 2000; Mendelsohn and Baselga, 2000; Glennie and Johnson, 2000; http://www.dakocytomation.com/dako_facts_1final.pdf).

1.8.7. Prostate-Specific Antigen (PSA)

The measurement of PSA levels in serum is utilized for screening and early detection of prostate cancer. However, PSA is not a highly specific tumor marker. Serum contains two forms of PSA; complexed PSA and free PSA. There are three forms of free PSA and the one that is a proenzyme is associated with cancer of prostate. The ratio of elevated serum level of truncated proenzyme PSA to total serum PSA correlates with an increased risk of prostate cancer (Mikolajczyk *et al*., 2002; Peter *et al*., 2001; Mikolajczyk *et al*., 2000).

1.8.8. Prostate-Specific Membrane Antigen (PSMA)

PSMA is a tissue-specific protein that is accepted to be a very good target for imaging and therapy because of its prostate specificity and its extracellular large domain. Although PSMA is expressed in kidney, proximal small intestine, salivary gland and brain, those tissues have a thousand-fold smaller expression levels than that of prostate. PSMA is up-regulated many folds in prostate cancer and interestingly, it is expressed in neovascularizations of most of the solid tumors but not in neovascularizations of normal tissues (Ghosh and Heston, 2004).

2. AIM OF THE STUDY

Utilization of tumor markers in cancer research and in clinical applications provides benefit of differential diagnosis of cancer versus normal or benign tissue, differential diagnosis of tumor degree, tumor types and subtypes, predicting the prognosis of the disease, and targeted treatment of the tumor versus normal tissue, selectively. Although there are many tumor markers investigated on the basis of monoclonal antibody production, very few markers are specific in terms of cancer and tissue type. This feature restricts the use of tumor markers leading to lack of routinely used informative tumor markers in many cancer types. Therefore, investigation of novel, informative tumor markers is in great demand.

This study aims production of monoclonal antibodies that may be candidates for fulfilling the requirements of an ideal tumor marker. To serve this purpose, two monoclonal antibodies were produced against apoptosis induced hepatocellular carcinoma cell line HuH-7. Next, the monoclonal antibodies were subjected to characterization experiments to explore their immunoreactivity in different cell, tissue and tumor types.

3. MATERIALS AND METHODS

3.1. Production of 6D5 and 9C11 Monoclonal Antibodies

3.1.1. Production of 6D5 and 9C11 Monoclonal Antibody Producing Hybridomas

6D5 and 9C11 producing hybridomas were previously produced by Tamer Yagci. Ten million of cells of apoptosis induced (by UV-C irradiation, 60-120 mJ/cm²) HuH-7 hepatocellular carcinoma cell line was lysed in 2 ml PBS and 0.5 ml of lysate was injected into peritoneal cavity of Balb/c mice. Mice were immunized twice more at two weeks intervals and hybridomas were prepared by spleen cells after the animals were sacrificed. Sp2/0-Ag14 mouse myeloma cell line was used as fusion partner.

Partaking in the project at the time of culturing after the fusion, the antibody producing hybridoma cells were selected by enzyme-linked immunosorbent assay (ELISA). HuH-7 cells were grown in 96-well tissue culture plates in DMEM medium (supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids) in tissue culture incubator that was set to 37 ºC and 5% CO_2. When they reached to desired number, they were fixed with 4% formalin in PBS for 15 minutes in dark at room temperature. Afterwards, they were permeabilized with 0.2% Triton X-100 in 1X PBS for 5 minutes at room temperature. Then, the plates were washed two times with distilled water and dried gently by tapping on a paper towel. 2% BSA in PBS was used for blocking and the cells were incubated with this blocking solution for 1 hour at 37 ºC. Blocking was followed by addition of hybridoma supernatants to the wells that contain HuH-7 cells. The cells were incubated with the supernatants for 1 hour at 37 ºC. Following incubation, wells were washed 2 times with distilled water and secondary antibody (goat anti-mouse IgG-alkaline phosphatase, Sigma) was added as 1:1000 diluted. After an incubation period of 1 hour at 37 ºC, the wells were washed 4 times with distilled water. Next, substrate tablet (pNPP disodium hexahydrate, phosphatase

substrate, Sigma) was dissolved in 20 ml ddH_2O in dark at room temperature and added to each well. At the end of 20-45 minutes of incubation, spectrophotometric measurement was performed at A_{405} at Beckman Biomek ELISA reader. DMEM containing wells were used as negative control. The supernatants were accepted to produce antibody if they had an optic density twice of that of the negative control.

After ELISA assay, the positive hybridoma cells were cloned by limiting dilution. The hybridoma cells were counted and they were plated to 96-well plates with a calculated theoretical amount of 0.5 cell per well. Recloning was done to ensure clonality of the cells.

Clones, producing different antibodies of single cell origin, were screened again by the same ELISA method described before. The antibody producing clones to be selected for further studies were determined from the ones that revealed a measurement result of three times that of the negative control.

Two of the antibody producing clones, namely 6D5 and 9C11, were chosen to be used for further studies.

3.1.2. Culturing 6D5 and 9C11 Hybridoma Cells for Antibody Production

6D5 and 9C11 hybridoma cells were cultured in high glucose containing DMEM medium supplemented with 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM non-essential amino acids. The incubator where the cells were grown was set to 37 ºC and 5% CO_2. The hybridoma supernatants were collected by centrifugation of the cultures at 1500 rpm for 5 minutes at room temperature. They were buffered with 20 mM Tris (pH 8) and 0.02% sodium azide (w/v) was added as preservative to prevent microorganism contamination. The supernatants were stored at -20 ºC if they were not going to be used immediately. New stocks were prepared by freezing 10 million hybridoma cells in 90% FCS and 10% DMSO containing freezing medium.

3.2. Western Blotting With 6D5 and 9C11 Monoclonal Antibodies

3.2.1. Tissue Culture Studies

28 cell lines (**Table 3.1**) were grown as monolayer culture in 150 mm culture dishes in tissue culture facility incubator that was set to 37 ºC and 5% CO_2. The culture media were supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids.

Table 3.1: The cell lines used in Western blotting with 6D5 and 9C11 antibodies

Cell line	Organism	Tissue/Cancer type	Medium
HuH-7	Human	Liver/hepatocellular carcinoma	DMEM
SK-Hep-1	Human	Liver/ascite-adenocarcinoma	DMEM
SNU-449	Human	Liver/hepatocellular carcinoma	RPMI-1640
SNU-387	Human	Liver/hepatocellular carcinoma	RPMI-1640
SNU-398	Human	Liver/hepatocellular carcinoma	RPMI-1640
SNU-475	Human	Liver/hepatocellular carcinoma	RPMI-1640
SNU-182	Human	Liver/hepatocellular carcinoma	RPMI-1640
FOCUS	Human	Liver/hepatocellular carcinoma	DMEM
Mahlavu	Human	Liver/hepatocellular carcinoma	DMEM
Hep3B	Human	Liver/hepatocellular carcinoma	DMEM
Hep3B-TR	Human	Liver/hepatocellular carcinoma	DMEM

HepG2	Human	Liver/hepatocellular carcinoma	DMEM
Hep40	Human	Liver/hepatocellular carcinoma	DMEM
FLC4	Human	Liver/hepatocellular carcinoma	DMEM
CAMA-1	Human	Mammary gland; metastatic site: pleural effusion adenocarcinoma	DMEM
MCF-7	Human	Mammary gland; metastatic site: pleural effusion adenocarcinoma	DMEM
LS411N	Human	Cecum/colorectal carcinoma	DMEM
SW837	Human	Rectum/adenocarcinoma	DMEM
U-2 OS	Human	Bone/osteosarcoma	DMEM
LNCaP	Human	Prostate/from lymph node metastasis	DMEM
HEK293	Human	Fetal kidney/transformed with adenovirus 5 DNA	DMEM
A375	Human	Skin/malignant melanoma	DMEM
HeLa	Human	Cervix/adenocarcinoma	DMEM
MRC-5 (p14)	Human	Fetal lung/normal	DMEM
COS-7	Monkey	Kidney/SV40 transformed	DMEM
IAR 6	Rat	Liver/hepatocellular carcinoma	DMEM
Ankara	Bovine	Lymphoblastoid/transformed with *Theileria annulata* (Ankara)	DMEM
Sp2	Mouse	B lymphocyte/myeloma	RPMI-1640

3.2.1.1 Thawing of Cells

The vial of the cell line was taken from liquid nitrogen and immediately put on ice. Subsequently, the vial was put into water bath at 37 ºC and the frozen cell suspension was thawed. Afterwards, the cells were transferred into a 15 ml sterile tube and 10 ml fresh medium was gently added to the sterile tube. The cells were centrifuged at 1500 rpm for 5 minutes at 4 ºC. The supernatant was discarded. For the anchorage-dependent cells, the pellet was resuspended in 15 ml warm culture medium and the cells were plated into 150 mm culture dishes. Then, the cells were left in the tissue culture incubator overnight and the medium was refreshed the following day. For the anchorage-independent cells, the pellet was resuspended in 5 ml, 37 ºC culture medium and the cells were transferred to 25 cm² flask. The flask was left upright in the tissue culture incubator until subculturing.

3.2.1.2. Subculturing of Cells

The medium of the entire anchorage-dependent cells were refreshed every two or three days. They were splitted at the time when they were 80% confluent. During split, the medium of the dish was aspirated and the cells were washed twice with sterile PBS (pH 7.4). PBS was aspirated and 1.5 ml trypsin/EDTA was added to each 150 mm dish. The cells were incubated in the tissue culture incubator for 5 minutes for detachment from the dish surface. Then, they were plated into appropriate number of new plates according to desired dilution. At the time that anchorage-independent cells reach to the desired number, they were centrifuged at 1500 rpm for 5 minutes at room temperature. Then, the supernatant was aspirated and the cells were resuspended in appropriate amount of fresh medium. When they were subcultured for the first time, they were transferred to a 75 cm² flask from the 25 cm² flask.

3.2.1.3. Freezing of Cells

The anchorage-dependent cells were frozen when they were 60%-70% confluent in 150 mm culture dishes. The cells were washed twice with sterile PBS after aspirating the medium. Then, PBS was removed, trypsin/EDTA was added for detachment and the dish was put into tissue culture incubator to facilitate detachment. The detached cells were collected and transferred to a 15 ml sterile tube. 10 ml fresh medium was added and the cells were centrifuged at 1500 rpm for 5 minutes at 4 ºC. The supernatant was discarded and the cells were resuspended in 1 ml freezing medium containing 20% FCS, 10% DMSO and 70 % medium (DMEM or RPMI-1640). The cells were transferred into a vial and the vial was immediately placed on ice. Then, it was put into -80 ºC. The vial was moved to liquid nitrogen tank in three days. The suspension cells were directly transferred into a 15 ml sterile tube and centrifuged at 1500 rpm for 5 minutes at 4 ºC. The supernatant was discarded and the cells were resuspended in freezing medium containing 90% FCS and 10% DMSO. The next steps were the same of freezing steps of anchorage-dependent cells.

Phosphate-Buffered Saline (PBS):

10X Stock Solution

80 g NaCl
2 g KCl
7.64 g $Na_2HPO_4.2H_2O$
2 g KH_2PO_4

3.2.2. Protein Extraction from Cells

The anchorage-dependent cells in the tissue culture facility were grown to be as confluent as 80%- 90%. Their medium was aspirated and the cells were washed twice with ice-cold PBS. Then, PBS was aspirated and cells were scraped in 1.5 ml

ice-cold PBS. The scraped cells were centrifuged at 1500 rpm for 5 minutes at 4 ºC. After centrifugation, the supernatant was removed and the pellet was either stored at -80 ºC to be used later or lysis was performed. For lysis, freshly prepared NP-40 lysis buffer was used. The pellets were resuspended in NP-40 lysis buffer that was 4 times the volume of the cell pellet. This mixture was incubated on ice for 30 minutes and later, it was centrifuged at 13000 rpm for 30 minutes at 4 ºC. Supernatant was taken into fresh tubes and stored at -80 ºC except a small amount taken for further step of protein quantification.

NP-40 Lysis Buffer:

150mM NaCl
1.0% NP-40
50 mM Tris (pH 8.0)
1 Tablet protease inhibitor (Complete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche)

The final volume was brought to 7 ml with dH_2O.

3.2.3. Bradford Assay for Protein Quantification

The tubes referred in **Table 3.2** were prepared with a BSA stock of 1mg/ml concentration and the tubes in **Table 3.3** were prepared with previously aliquoted total cell extracts. After preparation, BSA and protein samples were measured in spectrophotometer (Beckmann) at 595 nm visible light, no later than fifteen minutes.

A standard linear curve was drawn as XY (scatter) chart type according to A_{595} of BSA standards. The protein concentrations of the samples were calculated according to the equation of this standard linear curve.

Table 3.2: Preparation of BSA samples

Tube numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
BSA stock (µl)	0	2.5	5	7.5	10	12.5	15	20
ddH_2O (µl)	100	97.5	95	92.5	90	87.5	85	80
Bradford working (µl)	900	900	900	900	900	900	900	900

Table 3.3: Preparation of protein samples

Tube numbers	**1**	**2**	**3**	**4**	**5**	**6**
Sample (µl)	0	2	2	2	2	2
ddH_2O (µl)	98	98	98	98	98	98
Bradford working (µl)	900	900	900	900	900	900
Lysis buffer (µl)	2	-	-	-	-	-

(Tube number 1 was blank in both tables.)

<u>Bradford Working Solution</u>:

50% Methanol (v/v)

0.05% (w/v) Coomassie Brilliant Blue R-250

10% (v/v) Acetic acid

40% (v/v) dH_2O

The solution was kept in dark at 4 ºC.

3.2.4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

The glass plates were assembled and the volume of the gel mold was determined according to the manufacturer's instructions (CBS). The appropriate volume of polyacrylamide gel solution of resolving gel was prepared in a disposable plastic tube. In every Western blotting experiment, the gel solution was prepared to contain 10% acrylamide concentration. The effective range of protein separation of a 10% acrylamide containing gel is between 16-68 kDa.

Solution of 10% resolving gel for tris-glycine SDS-PAGE was prepared according to the **Table 3.4**.

Table 3.4: Components of 10% resolving gel for tris-glycine SDS-PAGE

Solution components	**Component volumes (ml)**							
10%	**5**	**10**	**15**	**20**	**25**	**30**	**40**	**50**
ddH_2O	1.9	4	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10	13.3	16.7
1.5 M Tris-HCl (pH 8.8)	1.3	2.5	3.8	5	6.3	7.5	10	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.2	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

10% APS was freshly prepared for every experiment. 10% APS and TEMED were added respectively to the resolving gel solution just before the gel solution was poured. After addition of all of the solution components, the solution was swirled and poured into the gap between the glass plates without any delay. Sufficient space for stacking gel was left and the resolving gel was overlaid by pouring several drops of isobutanol. The gel was left for polymerization at room temperature in upright

position. After polymerization, the overlaying isobutanol was poured off and the top of the gel was washed several times with distilled water. The remaining water drops between the glass plates were dried by the edge of a paper towel.

5% stacking gel solution was prepared according to **Table 3.5**.

Table 3.5: Components of 5% stacking gel for tris-glycine SDS-PAGE

Solution components	**Component volumes (ml)**							
5% gel	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**10**
ddH_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% mix	0.17	0.33	0.5	0.67	0.83	1	1.3	1.7
1 M Tris-HCl (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Freshly prepared 10% APS and TEMED were added as the last components to the stacking gel solution. Afterwards, the mixture was swirled rapidly and the solution was poured on top of the polymerized resolving gel. A comb of desired number of wells was inserted into the stacking gel forthwith, paying attention not to form air bubbles. The gel was left in vertical position at room temperature for polymerization.

During polymerization, the samples to be loaded and the marker protein (Prestained Protein Molecular Weight Marker, Fermentas) were prepared by heat denaturation of the proteins at 100 ºC for 5 minutes in 1X SDS gel loading buffer. Following denaturation, the samples were placed on ice.

After the polymerization of the SDS gel, the comb was removed carefully and the wells were washed with distilled water to remove any unpolymerized acrylamide.

The glass plates containing the gel were mounted to the gel apparatus and tris-glycine electrophoresis buffer was added to the upper and bottom reservoirs. 5 to 10 µg marker protein and 1 to 80 µg of sample proteins were loaded to the wells according to their predetermined order.

The electrophoresis apparatus was connected to power supply. The gel was run at 80 volts until the dye left stacking gel and then, the voltage was increased to 180 volts. The gel was run until the bromophenol blue of the gel loading buffer reached the bottom of the resolving gel. At that moment, the power supply was turned off and the glass plates were removed from the electrophoresis apparatus. The plates were separated from each other on a paper towel with the help of a spatula. The orientation of the gel was marked by cutting top right corner of the gel.

30% Mix (Acrylamide and Bis-acrylamide Solution):

146 g Acrylamide
4 g Bis-acrylamide

Volume was adjusted to 500 ml and the mix was filtered to get rid of air bubbles. It was stored at 4 ºC in dark for maximum 30 days.

1.5 M Tris-HCl (pH 8.8):

54.45 g Tris base
150 ml ddH_2O

The pH was adjusted to 8.8 with 1 M HCl and the final volume was brought to 300 ml with ddH_2O. The solution was stored at 4 ºC.

1 M Tris-HCl (pH 6.8):

12 g Tris base
60 ml ddH_2O

The pH was adjusted to 6.8 with 1 M HCl and the final volume was brought to 100 ml with ddH_2O. The solution was stored at 4 ºC.

10% SDS:

10 g SDS was dissolved in ddH_2O by stirring and brought to 100 ml.

10% APS:

1 ml 10% (w/v) solution was prepared freshly in ddH_2O.

1X SDS Gel Loading Buffer:

3.8 ml distilled water
1 ml 0.5 M Tris-HCl (pH 6.8)
0.8 ml Glycerol
1.6 ml 10% SDS
0.4 ml 0.05% (w/v) Bromophenol blue
0.4 ml 2 β-Mercaptoethanol (added freshly)

2X SDS Gel Loading Buffer:

25 ml 4X Tris-HCl/SDS (pH6.8)
20 ml Glycerol
4 g SDS
2 ml 2 β-Mercaptoethanol (added freshly)

dH_2O was added to 100 ml.

4X Tris-HCl/SDS (pH6.8)

6.05 g Tris base was dissolved in 40 ml dH_2O. pH was adjusted to 6.8 with 1 M HCl. Volume was brought to 100 ml and 0.4 g SDS was added.

5X Tris-Glycine Electrophoresis Buffer:

15 g Tris
72 g Glycine
5 g SDS

Volume was adjusted to 1 L with ddH_2O and the buffer was stored at 4 ºC. 1X buffer was used as working solution.

3.2.5. Transfer of Proteins from SDS-Polyacrylamide Gel to PVDF Membrane

For the transfer of the proteins from the SDS gel to PVDF membrane (Immobilon-P membrane, 0.45 µm, Millipore), four pieces of Whatman 3MM filter paper and a piece of PVDF membrane were cut as the same dimensions of the gel by wearing gloves. The membrane was left in methanol for 20 seconds, washed with distilled water and then soaked into transfer buffer for 15 minutes. The gel was removed from the glass plate and kept in transfer buffer for 10 minutes. After, it was washed with distilled water. Whatman 3MM papers were kept in the transfer buffer for a few minutes.

The transfer sandwich was set on transfer unit as follows:

1. 2 pieces of Whatman 3MM paper were taken out of the transfer buffer and placed on top of the metal transfer surface, which was the anode of the unit. The trapped air bubbles were driven out gently by a roller.
2. The PVDF membrane was placed on top of Whatman 3MM papers aligning it exactly. Air bubbles were squeezed.
3. The SDS gel was taken out of the transfer buffer and placed on the PVDF membrane. The positions of the prestained marker proteins were marked by a needle. Air bubbles were squeezed.

4. 2 pieces of Whatman 3MM paper were taken out of the transfer buffer and placed on top of the SDS gel. The cover was then placed on the unit, which was cathode.

After setting, the transfer unit was connected to power supply and 3.5 mA/cm² current was applied to the sandwich for 45 minutes. At the end of the transfer process, the power supply was turned off and the sandwich was disassembled layer by layer from top-down.

Transfer Buffer:

2.9 g Glycine
5.8 g Tris base
0.37 g SDS
200 ml methanol

The volume was adjusted to 1 L with ddH_2O.

3.2.6. Immunological Detection of Immobilized Proteins (Western Blotting)

Taken from the transfer unit, the membrane was soaked into blocking solution composed of 3% milk powder in 0.1% Tween-20-TBS (TBS-T) solution for one hour at room temperature on a rotating platform. Subsequently, the membrane was incubated with primary antibody, which was either 6D5 or 9C11 hybridoma supernatant, for one hour at room temperature or overnight at 4 ºC on a slowly rotating platform. Following incubation, the membrane was washed with TBS-T for three times, once 15 minutes long and twice 5 minutes long. Next, the membrane was incubated with 1:5000 diluted secondary antibody (goat anti-mouse IgG-peroxidase conjugate, Sigma) for 1 hour at room temperature on a slowly rotating platform. Incubation was followed by four times washing with TBS-T, once for 15 and three

times for 5 minutes. Finally, the membrane was washed with distilled water and became ready for development.

For detection of proteins immobilized on the membrane, ECL detection system (Enhanced Chemiluminescence, Amersham Pharmacia Biotech.) was used according to manufacturer's instructions.

10X Tris-Buffered Saline (TBS):

12.19 g Tris base
87.76 g NaCl

The final volume was brought to 1 L with ddH_2O and the pH was adjusted to 8 with 1 M HCl. 1X solution was used as working buffer.

3.3. Immunoperoxidase Staining of Paraffin-Embedded Liver Cancer Tissue Samples

Three paraffin-embedded liver cancer tissue samples were cut 4 µm thick and placed on slides. Those samples were named as S1, S2 and S3.

Immediately after sectioning, tissue samples were left in 70 ºC oven for 20 minutes for deparaffinization of the tissues. Then, deparaffinization was continued on the bench. The samples were taken into xylene for 5 minutes, 99% ethanol for 5 minutes, 90% ethanol for 5 minutes, 70% ethanol for 5 minutes, 50% ethanol for 5 minutes, and distilled water for 5 minutes, respectively. Next, antigen retrieval step was applied. The samples were placed in pre-warmed citric acid (pH 6) in a glass plate and the glass plate was transferred to a plastic container of warm water. Then, the plastic container was moved into microwave oven that was set to 750 W. The citric acid solution was allowed to boil for 15 minutes and its level was checked against evaporation and plates were refilled whenever necessary. At the end of 15 minutes,

the plastic container was taken out of microwave oven and left at room temperature for cooling. Next, the slides were soaked in distilled water for five minutes and then, washed 3 times (5 minutes for each) with TBS (pH 8). Afterwards, the slides were immerged in 5% Triton X-100 containing 3% hydrogen peroxide for inhibition of endogenous peroxidase activity in the tissues. 20 minutes later, slides were washed 3 times with TBS, each washing step lasting 5 minutes. Then, blocking was performed to prevent binding of the reagents to the unspecific sites in the tissues. 1.5% goat serum in TBS was dropped on each slide covering whole tissue and the slides were incubated for 1 hour at room temperature. 6D5 or 9C11 antibody was used as primary antibody and the slides were incubated with either of them for 1 hour at room temperature or overnight at 4 ºC after blocking. If the antibodies were to be diluted, blocking solution was used. Primary antibody step was followed by 3 times washing with TBS for 5 minutes for each. On the next step, biotinylated link and streptavidin-HRP reagents (LSAB2 System, Dako) were used according to the product information of the supplier. This step was followed by 3 times washing with TBS, 5 minutes each. Then, liquid DAB/Chromogen reagent kit (Dako) was applied to the samples according to the manufacturer's recommendations. Counter staining of the samples was performed with hematoxylene as long as the tissues were apparently stained (30 seconds to a few minutes). Excess of the hematoxylene was washed away with tap water and cover slips were mounted on the slides with glycerol.

Citric Acid (pH 6):

3.8 g citric acid was dissolved in 1.9 L dH_2O and the pH was adjusted to 6 with 2 M NaOH. Final volume was adjusted to 2 L.

4. RESULTS

4.1. Production of 6D5 and 9C11 Monoclonal Antibodies

Balb/c mice was immunized with apoptosis induced (by UV-C irradiation, 60-120 mJ/cm²) hepatocellular carcinoma cell line HuH-7. Hybridomas were prepared from the spleen cells of the sacrificed mice (performed by T. Yagci). Among 22 of monoclonal antibody-producing clones selected, 2 were named as 6D5 and 9C11, and were used for further studies.

4.2. Biochemical Characterization of 6D5 and 9C11 Monoclonal Antibodies

Twenty eight different cell lines (**Table 3.1**) were used to investigate whether 6D5 or 9C11 monoclonal antibody reacted with an epitope present in any of those selected cell lines. Fourteen of the selected cell lines were hepatocellular carcinoma, two of them were breast carcinoma, two were colorectal carcinoma, one was osteosarcoma, one was prostate carcinoma, one was fetal kidney carcinoma, one was malignant melanoma, one was cervical cancer, one was normal fetal lung, one was transformed monkey kidney, one was rat hepatocellular carcinoma, one was transformed bovine lymphoblastoid, and one was mouse myeloma cell line. The cell lines apart from human origin were selected for validation of cross-species reactivity of 6D5 and 9C11 antibodies.

All of the cell lines were grown, as confluent as they should be, in 150 mm dishes. The cells were scraped to obtain cell pellets and their total cell lysates were prepared by using NP-40 lysis buffer. The total cell lysates were loaded to 10% SDS gel. The loaded amounts of the total cell lysates are mentioned in sections 4.2.1 and 4.2.2. Convenient transfer buffers were used to transfer proteins from SDS gels to PVDF membranes. The membranes were subjected to Western blotting either with 6D5 or 9C11 antibody.

4.2.1 Western Blotting With 6D5 Antibody

First, a panel of 9 different HCC cell lines was used in Western blotting with 6D5 antibody. **Figure 4.1** displays banding pattern of those cell lines when 10 µg protein containing total cell lysate was loaded to each well. Hep3B (lane 4), SNU-475 (lane 5), and SK-Hep-1 (lane 7) cell lines revealed a similar banding pattern including an upper band, around 75 kDa, and a lower band just below that one. However, the equal loading control, which was performed with anti-calnexin antibody, revealed that the total amount of protein extract from SK-Hep-1 cell line loaded to the SDS gel was much more less than those of Hep3B and SNU-475. On the contrary, the signal intensity of the bands belonging to SK-Hep-1 cell line was no weaker than those of Hep3B and SNU-475. It seemed that Hep3B and SNU-475 cell proteins were equally loaded to the SDS gel. In Hep3B and SK-Hep-1 cell lines, both of the upper and lower bands seemed to be of equal density when upper was compared to lower one, but the upper band observed in SNU-475 was more intense than the lower band of the same cell line. HuH-7 (lane 1), HepG2 (lane 2), SNU-182 (lane3), Hep40 (lane 8) and SNU-398 (lane 9) cell lines exhibited single banding pattern. However, there were some apparent differences between those cell lines. HuH-7, Hep40 and SNU-398 cell lines expressed a band that is at the same molecular weight with the lower band of Hep3B, SNU-475 and SK-Hep-1 cell lines. Although total amount of loaded HuH-7 protein seemed to be more than those of Hep40 and SNU-398, it had weaker band intensity. On the other hand, HepG2 and SNU-182 cell lines expressed a band that is at the same molecular weight with the upper band of Hep3B, SNU-475 and SK-Hep-1 cell lines. HepG2 seemed to be equally loaded with HuH-7 and it displayed the same band intensity with HuH-7 but, total amount of cell protein of SNU-182 was slightly less loaded than those of HepG2 and Huh-7. In addition, it was slightly weaker in terms of band intensity. Hep3B-TR (lane 6) cell line displayed a completely different banding pattern from the previously mentioned cell lines. The amount of protein loaded from Hep3B-TR total cell lysate seemed definitely more than that of SK-Hep-1 but it was less than those of the rest of the cell lines. On the contrary, Hep3B-TR had a very dense, smear-like banding pattern. The signal intensity of Hep3B-TR was the strongest among those 9 hepatocellular carcinoma cell lines.

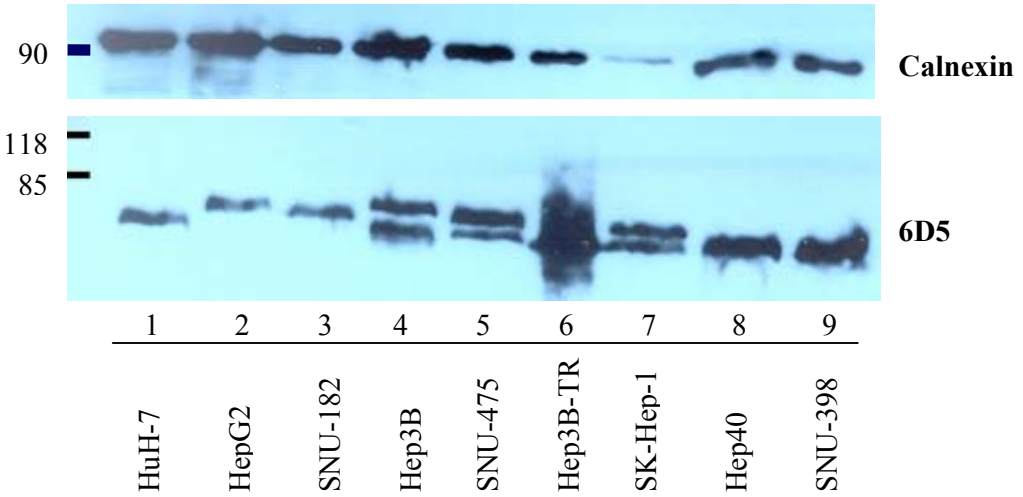


Figure 4.1: 6D5 Western blotting with 9 different HCC cell lines. Expression patterns of Huh-7, HepG2, SNU-182, Hep3B, SNU-475, Hep3B-TR, SK-Hep-1, Hep40 and SNU-398 cell lines are displayed.

Next, 4 different HCC cell lines were subjected to Western blotting. In **Figure 4.2**, the banding pattern of those cell lines are displayed each of which contained 10 µg protein while loading. According to the pattern of equal loading with anti-calnexin antibody, all the total cell proteins were equally loaded. In addition to this, signal intensities of all of the cell lines were equal to each other. However, there were some differences between the banding patterns. FOCUS (lane 1) cell line had two-banding pattern as Hep3B, SNU-475 and SK-Hep-1 cell lines did. Contrarily, band intensities of those upper and lower bands were different. FOCUS cell line was the only one with an upper band weaker than its lower counterpart among the HCC cell lines representing a two-banding pattern. SNU-387 (lane 2) pictured a totally different band with a molecular weight around 40 kDa, which was observed in none of the other cell lines. Each of SNU-449 (lane 3) and Mahlavu (lane 4) cell lines exhibited a single band at the same molecular weight with the lower band of FOCUS cell line.

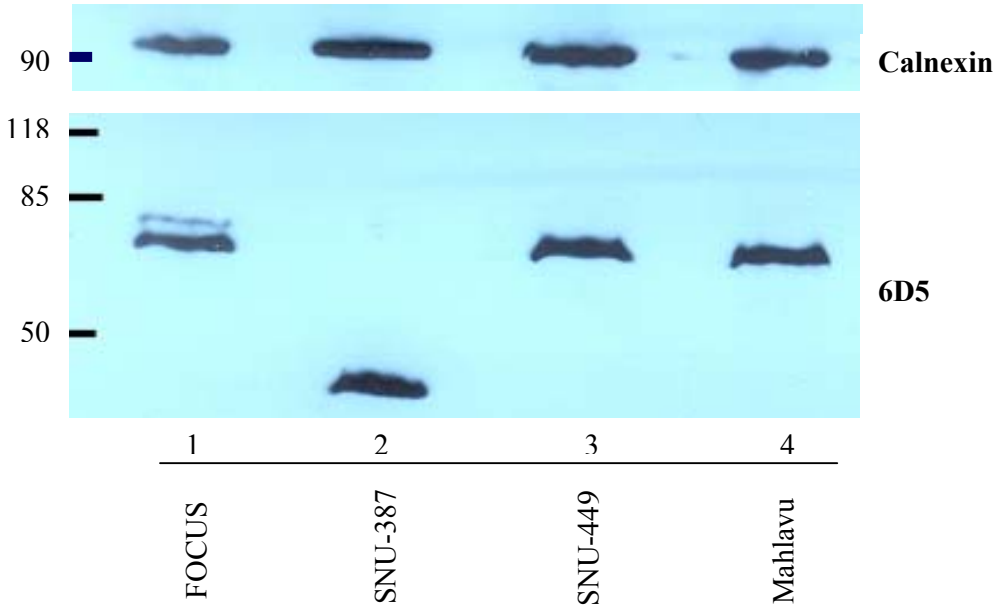


Figure 4.2: 6D5 expression pattern of 4 different HCC cell lines. Western blotting was performed with FOCUS, SNU-387, SNU-449, and Mahlavu cell lines.

Then, Western blotting study was broadened by the addition of different cell lines of liver and non-liver origin to the experiments (**Figure 4.3**). In this panel of study, HuH-7 (lane 1) cell line was used as positive control and SNU-387 (lane 3) was included for its interesting banding pattern that was observed in Figure 4.2. The amount of the loaded protein was increased to 20 µg per well since we wanted to question the presence of any bands other than the ones we previously detected. Equal loading with anti-calnexin antibody revealed that all of the samples were equally loaded. HuH-7 cell line displayed the expected banding pattern confirming the previous experiments. FLC4 (lane 2) cell line did not exhibit any bands although it was an HCC variant. Therefore, it was unique with this property among the human HCC cell lines used in this study. Interestingly, SNU-387 cell line represented a different band than the one it displayed before and this band was at the same molecular weight as the band observed in HuH-7. Moreover, A375 (lane 4) and HeLa (lane 7) cell lines had a banding pattern similar to that of HuH-7. However, it was questionable whether HeLa contained a second band below 50 kDa, whose expression was also observed in U-2 OS (lane 8) and LNCaP (lane 9) cell lines. U-2 OS and LNCaP cell lines revealed the same banding pattern, LNCaP expressing

weaker bands. Although not very clear, HEK293 (lane 5) seemed to display two very near bands, one of which migrated at the same level with band of HuH-7 and the other located slightly lower than this one. MRC-5 (lane 6) cell line had a differential banding pattern with an unusual band just above 50 kDa and one at the same molecular weight with band of HuH-7.

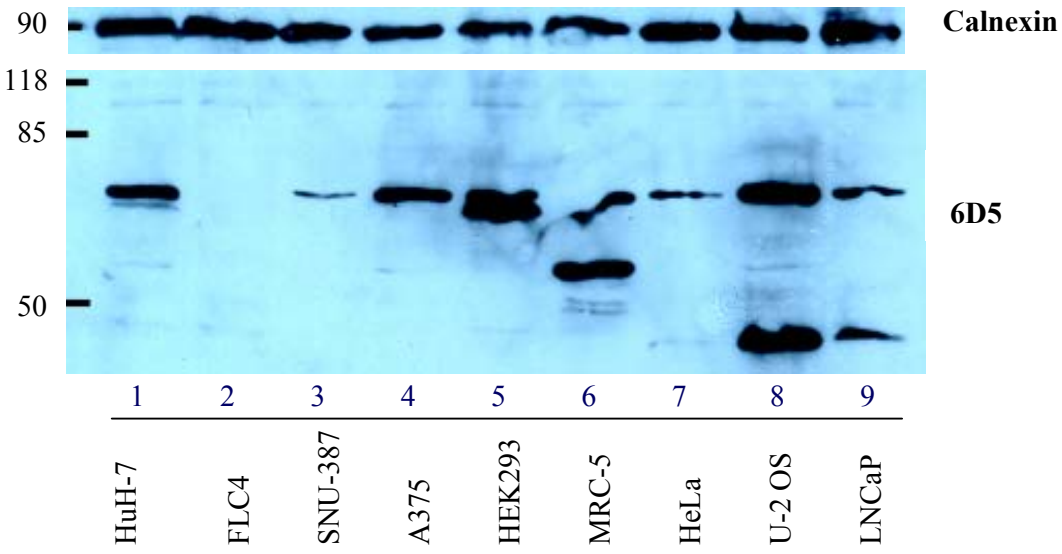


Figure 4.3: 6D5 Western blotting with cell lines from 7 different tissues-20 µg. Banding patterns of HuH-7, FLC4, SNU-387, A375, HEK293, MRC-5, HeLa, U-2 OS, and LNCaP cell lines with 6D5 monoclonal antibody are displayed.

Figure 4.4 involves the same panel of cell lines as Figure 4.3 but this time 50 µg protein was loaded per well since we wanted to clarify the picture of banding pattern. The equal loading was performed with anti-calnexin antibody. The entire cell lines were equally loaded, except LNCaP (lane 9), which was over-loaded. HuH-7 (lane 1) and FLC4 (lane 2) cell lines corroborated their banding pattern. SNU-387 (lane 3) clearly displayed two bands, one of which was previously displayed in Figure 4.2 and the other in Figure 4.3. The lower one of those bands that was below 50 kDa was much denser than the upper one that was around 75 kDa. The probable reason that this denser band was absent but the weaker one was present in Figure 4.3 could be a transfer problem in the membrane. It was observed that A375 (lane 4) cell line

represented two bands, one of which was absent from Figure 4.3. This newly observed band was at the same molecular weight with the upper band of SNU-475, SK-Hep-1 and FOCUS cell lines. The upper band of A375 was expressed weaker than its lower counterpart. HEK293 (lane 5) was far from displaying two bands it used to display in Figure 4.3 concluding that 50 µg of protein load was too much for it for a clear banding pattern. Unlike its profile in Figure 4.3, the banding pattern of MRC-5 (lane 6) cell line was clearly displayed as three equally intense bands. The two upper bands of MRC-5 were at the same molecular weight with the two bands of A375 cell line. With the help of 50 µg of protein load, we figured out that the band around 40 kDa, which we were suspicious about its presence, was expressed in HeLa (lane 7). Both of the upper and lower bands of HeLa represented a profile just the same as that of SNU-387. Moreover, both of the upper bands of those cell lines were weaker when compared to their lower counterparts. It was revealed that 50 µg of protein load was excessive for a clear figure of U-2 OS (lane 8) cell line. As a result of overload, it lost its clear two banding pattern but instead had a smear-like banding. In lane 9, there seems to be a problem in transfer affecting the banding pattern of LNCaP cell line.

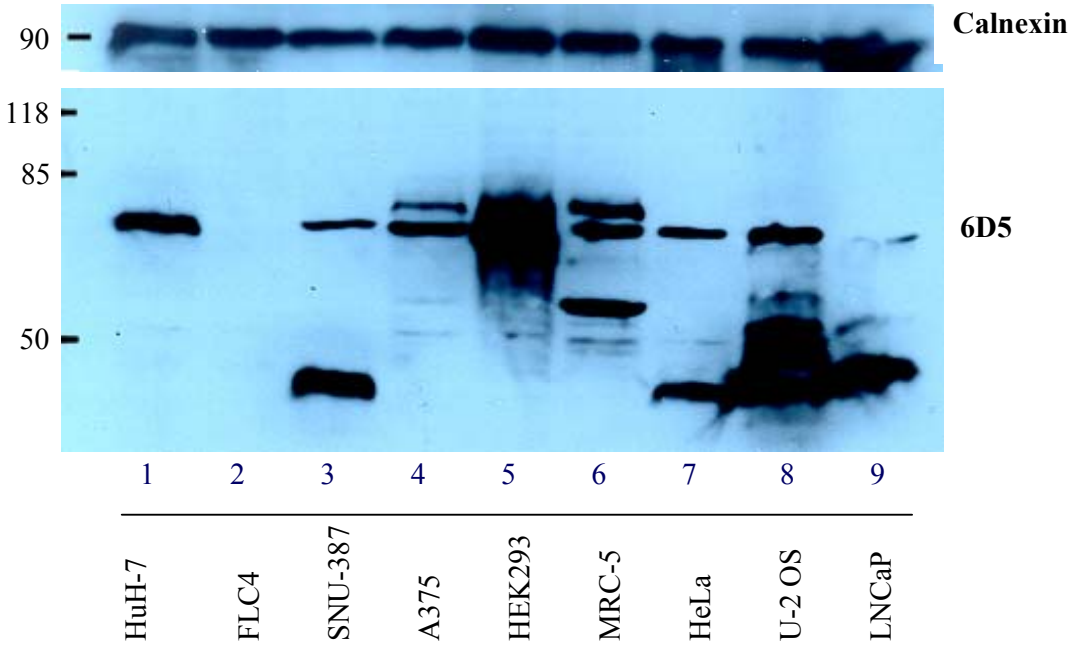


Figure 4.4: 6D5 Western blotting with cell lines from 7 different tissues-50 µg. Banding patterns of HuH-7, FLC4, SNU-387, A375, HEK293, MRC-5, HeLa, U-2 OS, and LNCaP cell lines are displayed.

The cell line panel of **Figure 4.5** is composed of both human and non-human origin cell lines. HuH-7 (lane 1) was included to the panel as positive control. The equal loading was performed with anti-calnexin antibody indicating equal loading of the samples except LNCaP (lane 11), which was slightly overloaded. There were no bands in IAR (rat), Ankara (bovine), COS-7 (monkey) and Sp2 (mouse) cell lines (lane 2, lane 3, lane 4 and lane 5, respectively) indicating that 6D5 was human specific, thus it did not display cross-species reactivity. Furthermore, none of LS411N (lane 6) or CAMA-1 (lane 8) cell lines displayed any bands even though they were of human origin. SW837 (lane 7) exhibited only one band that was at the same molecular weight and equally dense with that of HuH-7. MCF-7 (lane 9) represented only one band that was at the same molecular weight with the lower band of SNU-387, LNCaP, U-2 OS and HeLa cell lines. U-2 OS (lane 10) and LNCaP cell lines displayed the same cloudy banding pattern as in the Figure 4.4 since 50 µg of protein load was excessive for them.

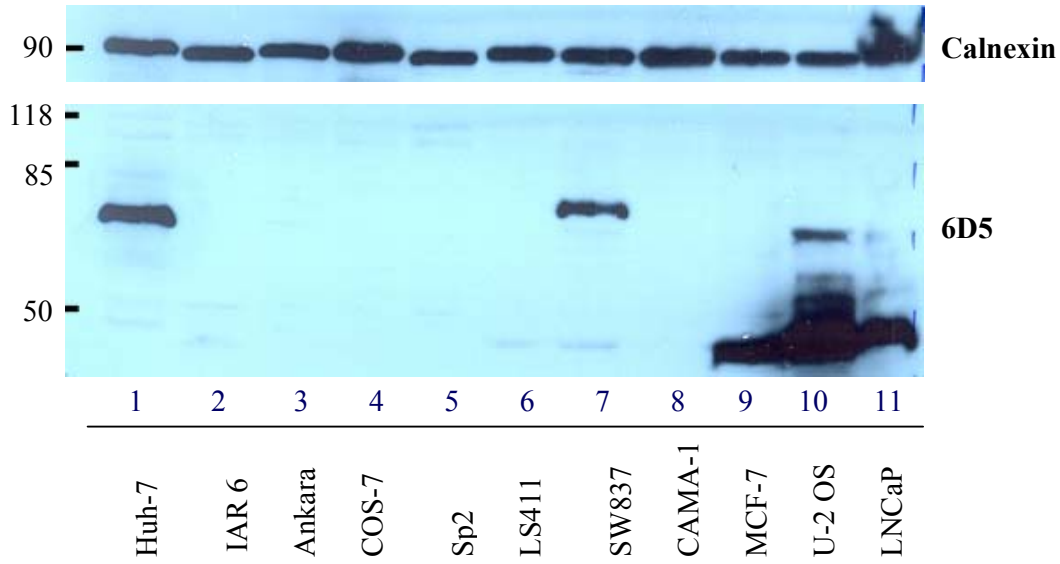


Figure 4.5: 6D5 expression analysis of human and non-human origin cell lines. Western blotting pattern of HuH-7, IAR 6, Ankara, COS-7, Sp2, LS411, SW837, CAMA1, MCF-7, U-2 Os and LNCaP cell lines with 6D5 monoclonal antibody is displayed.

The fact that Hep3B-TR cell line differs from Hep3B being resistant to growth inhibition by transforming growth factor β1 (TGF-β_1) directed us to the idea that it might be interesting to see the differences in the expression pattern of those cell lines. Therefore, we aimed to verify the differential banding pattern of Hep3B and Hep3B-TR, which was demonstrated in Figure 4.1. **Figure 4.6** displays the banding pattern of serially diluted protein loads of Hep3B and Hep3B-TR cell lines. The loaded protein amount was 50 µg for the lanes 1 and 2, 25 µg for the lanes 3 and 4, 10 µg for the lanes 5 and 6, and 5 µg for the lanes 7 and 8. The lanes 5 and 6 revealed the clearest banding patterns with 3 bands displayed by Hep3B and 2 bands displayed by Hep3B-TR. Those banding patterns of Hep3B and Hep3B-TR were totally different from their previous banding patterns. In Figure 4.1, Hep3B displayed two bands with equal densities and Hep3B-TR displayed a smear-like banding pattern without a clear view of the number of its bands. In this figure, one of the three bands of Hep3B, which was around 75 kDa, was more intense than its other two counterparts of equal density. In addition, it was revealed that Hep3B-TR had 2 clear bands at the same molecular weight with two upper bands of Hep3B. The upper band of Hep3B-TR was denser than its lower counterpart and than its counterpart in Hep3B. On the other hand, the lower band of Hep3B-TR was lighter than its counterpart in Hep3B.

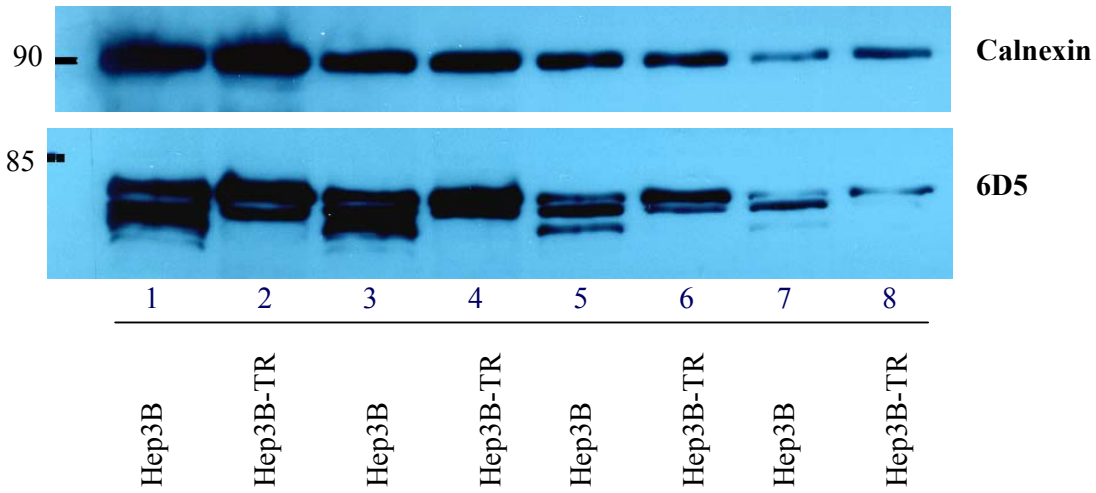


Figure 4.6: Differential expression of Hep3B and Hep3B-TR cell lines with 6D5. Serially diluted Hep3B and Hep3B-TR protein extracts were loaded to the wells.

4.2.2 Western Blotting with 9C11 Antibody

The first of Western blotting experiments with 9C11 was performed with 5 HCC cell lines. **Figure 4.7** displays Western blotting panel of those cell lines using 80 µg total protein extract. The entire cell lines, namely HuH-7 (lane 1), SNU-398 (lane 2), Hep3B (lane 3), SNU-449 (lane 4), and HepG2 (lane 5), exhibited a single banding pattern around 75 kDa. It was revealed that this band was different from the one that was displayed with 6D5 (data not shown). The equal loading control performed with anti-calnexin antibody showed that HuH-7 and Hep3B were equally loaded, SNU-398 was overloaded than those two, and SNU-449 and HepG2 were loaded less than HuH-7 and Hep3B. In spite of containing least amount of protein loads, SNU-449 and HepG2 had clear, strong banding profiles. The other three cell lines had denser bands correlating with their amount of loaded protein.

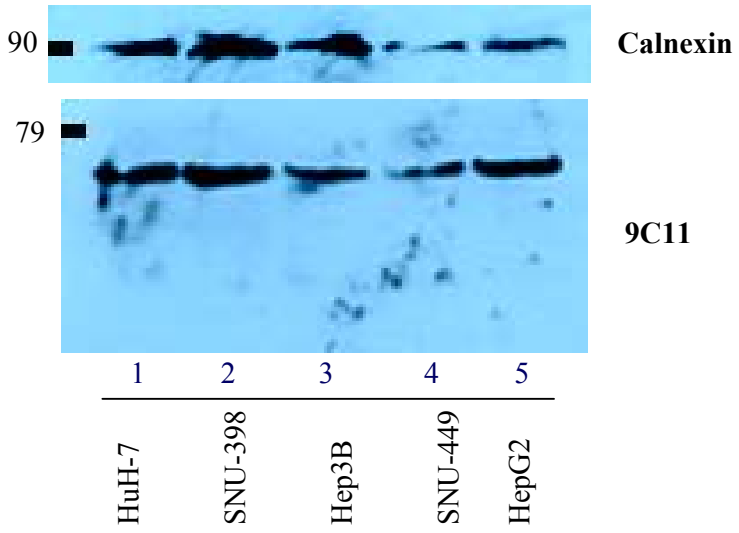


Figure 4.7: 9C11 Western blotting with 5 different HCC cell lines. Expression pattern of HuH-7, SNU-398, Hep3B, SNU-449 and HepG2 cell lines is displayed.

Western blotting experiments with 9C11 antibody was continued with a broader panel of HCC cell lines (**Figure 4.8**). In Figure 4.8, each well contained 50 µg total protein extract of Mahlavu (lane 1), SNU-182 (lane 2), SNU-387 (lane 3), Hep3B-

TR (lane 4), Hep40 (lane 5), FOCUS (lane 6), SK-Hep-1 (lane 7), and SNU-475 (lane 8) cell lines, respectively. The equal loading was performed with anti-GSK-3β antibody. GSK-3β pattern revealed that SNU-182, Hep3B-TR, FOCUS, SK-Hep-1 and SNU-475 cell lines were equally loaded but Mahlavu was a little more loaded, and SNU-387 was slightly less loaded when compared to the equally loaded cell lines. Hep40 was loaded with least amount of protein among entire cell lines. All the cell lines displayed a band around 75 kDa, likewise the expression pattern of the cell lines in Figure 4.7. Considering the equal loading pattern of all cell lines, Hep40 had a strong band contrary to its small amount of protein load when compared to the other cell lines. Likewise, SNU-182 and SNU-387 cell lines displayed dense bands when we considered their protein load. On the other hand, Hep3B-TR, FOCUS, SK-Hep-1 and SNU-475 cell lines had similar band intensities with 9C11 blotting as they did with anti-GSK-3β blotting. Mahlavu had the strongest band in equal loading however; it did not exhibit a band as strong as SNU-182 did.

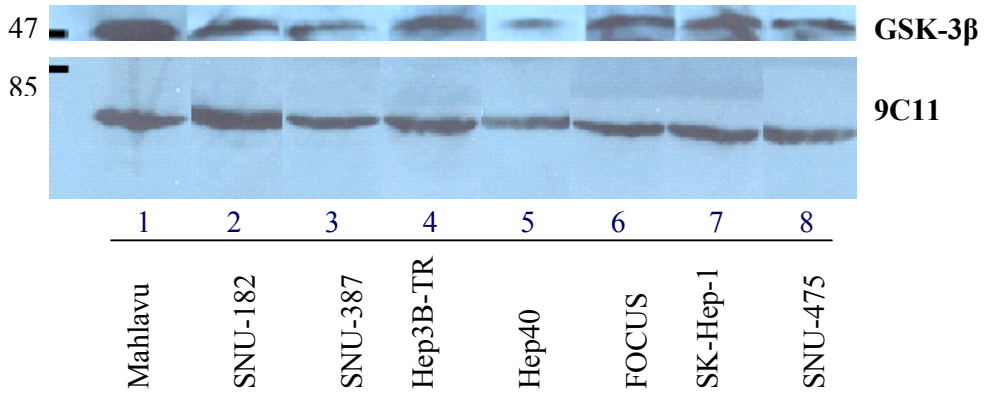


Figure 4.8: 9C11 expression pattern of 8 different HCC cell lines. Western blotting of Mahlavu, SNU-182, SNU-387, Hep3B-TR, Hep40, FOCUS, SK-Hep-1 and SNU-475 cell lines is displayed.

Like that of 6D5 antibody, 9C11 Western blotting panel of studied cell lines was extended to include both liver and non-liver origin cell lines. According to this, **Figure 4.9** displays the banding pattern of HuH-7 (lane 1), FLC4 (lane 2), SNU-387 (lane 3), A375 (lane 4), HEK293 (lane 5), MRC-5 (lane 6), HeLa (lane 7), U-2 OS

(lane 8) and LNCaP (lane 9) cell lines. HuH-7 was included to the panel as positive control and SNU-387 was added to question its previous expression profile in Figure 4.8 because this cell line had an interesting outcome with 6D5 antibody. 50 µg total cell protein was loaded to each well. The equal loading was done with anti-calnexin antibody. Apart from LNCaP cell line, whose protein was slightly overloaded, the protein samples were equally loaded. As seen in the figure, every cell line expressed a band around 75 kDa similar to the ones in Figure 4.7 and Figure 4.8. In this panel of cells, FLC4 was the only one lacking the expression of an epitope reacting with 9C11. SNU-387, A375, HEK293, MRC-5, HeLa, and U-2 OS cell lines, which were equally loaded, had nearly the same band intensities. Contrary to this, LNCaP displayed the weakest banding of all although it contained the most amount of protein in the loading.

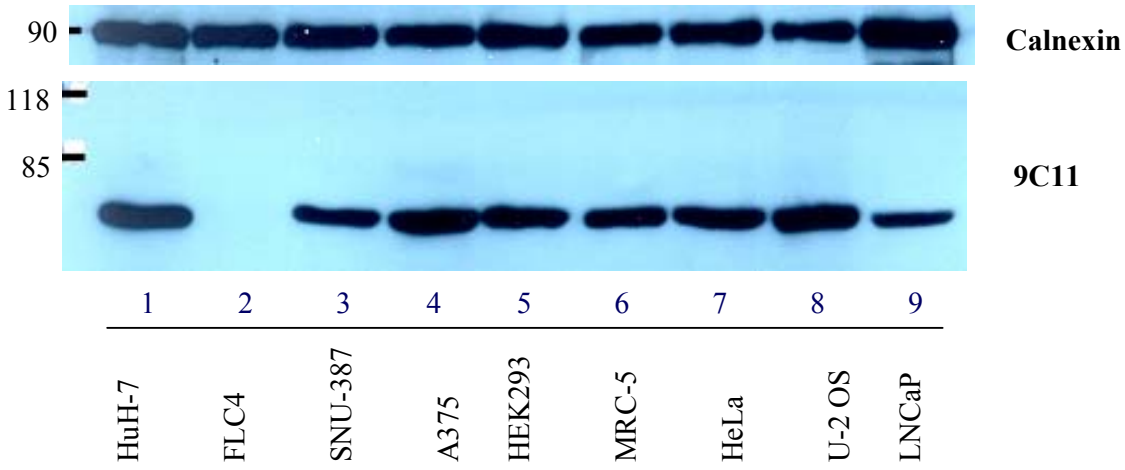


Figure 4.9: 9C11 Western blotting with cell lines from 7 different tissues. Expression profile of HuH-7, FLC4, SNU-387, A375, HEK293, MRC-5, HeLa, U-2 OS and LNCaP cell lines is displayed.

Then, Western blotting of human and non-human origin cell lines was accomplished with 9C11 (**Figure 4.10**). In Figure 4.10, 50 µg total cell protein was loaded to each well and equal loading was performed with anti-calnexin antibody. Calnexin revealed equal band intensities in all cell lines except MCF-7 (lane 9). MCF-7 was slightly less loaded when compared to rest of the cell lines. IAR 6 (lane 2), Ankara

(lane 3), SW837 (lane 7) and MCF-7 were the cell lines that expressed a single band. This single band was at the same molecular weight with that of HuH-7 (lane 1). 9C11 antibody reacted with 2 non-human origin cell lines, IAR 6 (rat) and Ankara (bovine), but did not react with other two, COS-7 (monkey, in lane 4) and Sp2 (mouse, in lane 5). IAR 6 cell line displayed a weak band whereas Ankara had a strong band. LS411N (lane 6) and CAMA-1 (lane 8) were the two human origin cell lines that 9C11 seemed not to react with. SW837 exhibited the strongest band of all and MCF-7 had a weak signal, correlating with its equal loading band intensity, similar to that of IAR 6.

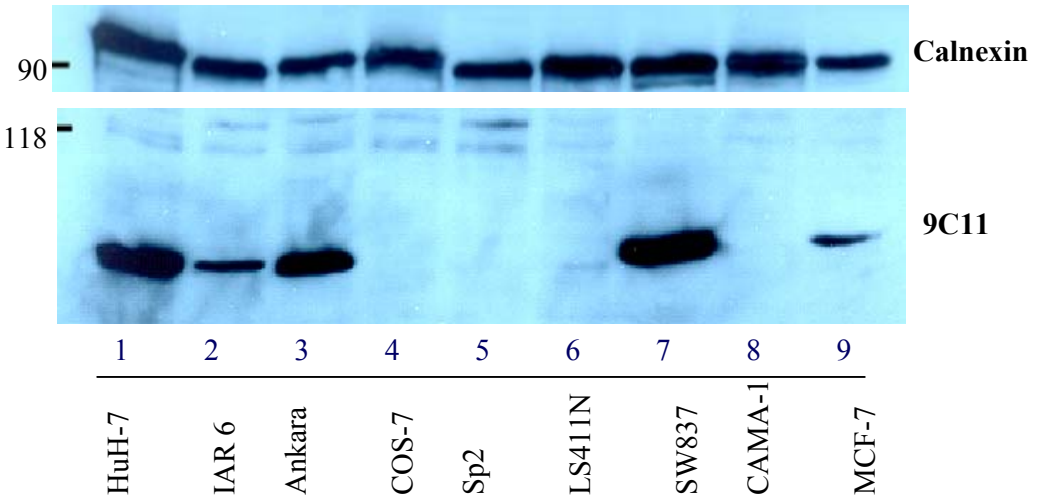


Figure 4.10: 9C11 expression analysis of human and non-human origin cell lines. Western blotting patterns of HuH-7, IAR 6, Ankara, COS-7, Sp2, LS411N, SW837, CAMA-1, and MCF-7 cell lines are displayed.

4.2.3. Banding Patterns of Cell Lines in Western Blotting With 6D5 and 9C11 Antibodies

Table 4.1 is the banding patterns of the cell lines, prepared according to the expression results of 6D5 and 9C11 antibodies in Western blotting. The table also includes information about the studied cell lines.

Table 4.1: Western blotting banding patterns of the studied cell lines

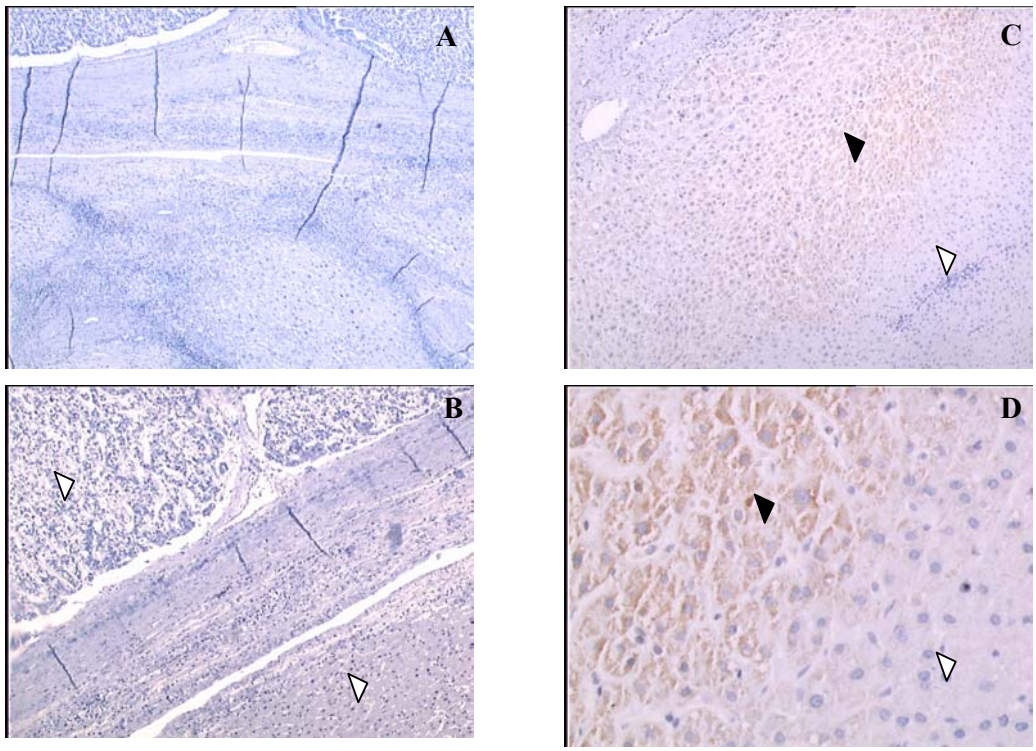
Cell Line	Organism	Tissue/Cancer Type	Morphology	Differentiation Status	Banding Pattern/Intensity	
					6D5	9C11
HuH-7	Human	Liver/hepatocellular carcinoma	Epithelial	Differentiated	b,++	++
SK-Hep-1	Human	Liver/ascite-adenocarcinoma	Epithelial (endothelial origin)	Poorly differentiated	a,+++; b,+++	+
SNU-449	Human	Liver/hepatocellular carcinoma	Epithelial		b,++	++
SNU-387	Human	Liver/hepatocellular carcinoma	Epithelial (pleiomorphic)		b,+; e,++	++
SNU-398	Human	Liver/hepatocellular carcinoma	Epithelial		b,+++	+
SNU-475	Human	Liver/hepatocellular carcinoma	Epithelial		a,++; b,+	+
SNU-182	Human	Liver/hepatocellular carcinoma	Epithelial		a,+	++
FOCUS	Human	Liver/hepatocellular carcinoma	Epithelial		a,+; b,++	+
Mahlavu	Human	Liver	Epithelial	Poorly/moderately diff.	b,++	+
Hep3B	Human	Liver/hepatocellular carcinoma	Epithelial	Well differentiated	a,++; b,++; c++	+
Hep3B-TR	Human	Liver/hepatocellular carcinoma	Epithelial		a,+++; b,++	+
HepG2	Human	Liver/hepatocellular carcinoma	Epithelial	Well differentiated	a,++	++
Hep40	Human	Liver	Epithelial		b, +	+++
FLC4	Human	Liver/hepatocellular carcinoma	Epithelial		Ø	Ø

CAMA-1	Human	Mammary gland; metastatic site: pleural effusion adenocarcinoma	Epithelial	Poorly differentiated	Ø	Ø
MCF-7	Human	Mammary gland; metastatic site: pleural effusion adenocarcinoma	Epithelial	Differentiated	e,++	+
LS411N	Human	Cecum/colorectal carcinoma	Epithelial	Poorly differentiated	Ø	Ø
SW837	Human	Rectum/adenocarcinoma	Epithelial	Poorly differentiated	b,++	+++
U-2 OS	Human	Bone/osteosarcoma	Epithelial	Moderately differentiated	b,++; e,+++; and some unusual banding patterns?	++
LNCaP	Human	Prostate/from lymph node metastasis	Epithelial	Differentiated	b,+; e,++	+
HEK293	Human	Fetal kidney/transformed with adenovirus 5 DNA	Epithelial		a,++; b,++	++
A375	Human	Skin/malignant melanoma	Epithelial		a,+; b,++	++
HeLa	Human	Cervix/adenocarcinoma	Epithelial		b,+; e,++	++
MRC-5 p14	Human	Fetal lung/normal	Fibroblast		a,++; b,++; d,++	++
COS-7	Monkey	Kidney/SV40 transformed	Fibroblast		Ø	Ø
IAR 6	Rat	Liver/hepatocellular carcinoma	Epithelial		Ø	+
Ankara	Bovine	Lymphoblastoid/transformed with Theileria annulata (Ankara)	Lymphoblastoid		Ø	++
SP2	Mouse	B lymphocyte/myeloma	Lymphoblast		Ø	Ø

* **a**, refers to the band slightly above 75 kDa; **b**, refers to the band around 75 kDa, **c**, refers to the band just below 75 kDa; **d**, refers to the band just above 50 kDa; **e**, refers to the band below 50 kDa.

4.3. Immunohistochemistry with 6D5 and 9C11 Antibodies

After Western blotting experiments, we examined in vivo reactivity of 6D5 and 9C11 antibodies in three different paraffin-embedded liver carcinoma tissue samples. Paraffin-embedded carcinoma biopsy samples were cut 4 μ thick and named as S1, S2 and S3. Immunoperoxidase assay was performed as described in Materials and Methods. The pictures of the slides were taken under Zeiss light microscope by Kodak digital photograph machine. The most informative magnifications are demonstrated on the next pages. The white arrows on the images indicate a negative staining and the black arrows indicate a positive staining.



(Black arrows indicate positive staining, white arrows indicate negative staining.)

Figure 4.11: Immunoperoxidase stainings of paraffin-embedded liver carcinoma tissue sample S1. A. Control sample (25X). Immunoperoxidase staining was performed without primary antibody. **B.** Immunoperoxidase staining with 9C11 at 50X. The upper part of the encapsulated liver tissue was composed of cirrhotic cells and the bottom part was composed of tumor cells. 9C11 seemed to stain neither of those pathological areas. **C.** Immunoperoxidase staining of S1 with 6D5 at 50X. 6D5 stained an area that was composed of cirrhotic cells but the tumor cells at the bottom right were not stained. **D.** A larger view (200X) of the positively stained cirrhotic cells. The neighboring tumor cells were not stained.

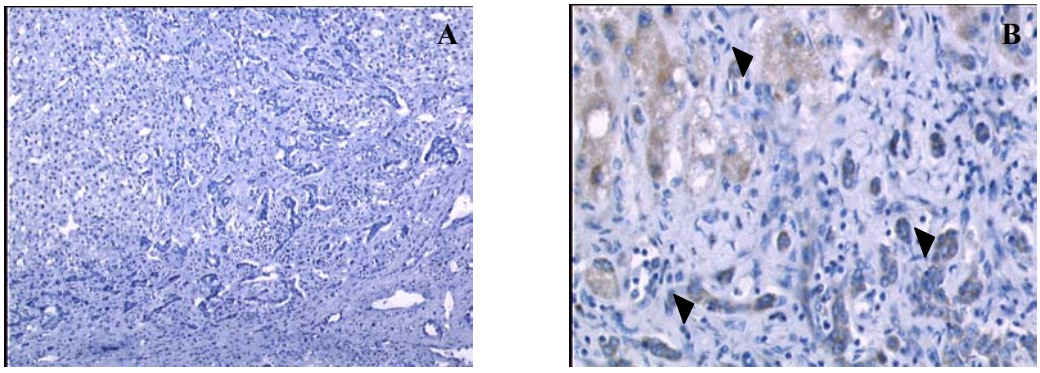


Figure 4.12: Immunoperoxidase stainings of paraffin-embedded liver carcinoma tissue sample S2. Most probably, there is an underlying secondary biliary cirrhosis in S2. Both of the images have 200X magnification. **A.** 9C11 was used as negative control since it did not stain previously studied paraffin-embedded liver tissue samples. In this section, the tissue was crowded with cirrhotic nodules of biliary tract. **B.** Immunoperoxidase staining with 6D5. The cirrhotic nodules of hepatocytes and cirrhotic biliary tract epithelium were both stained.

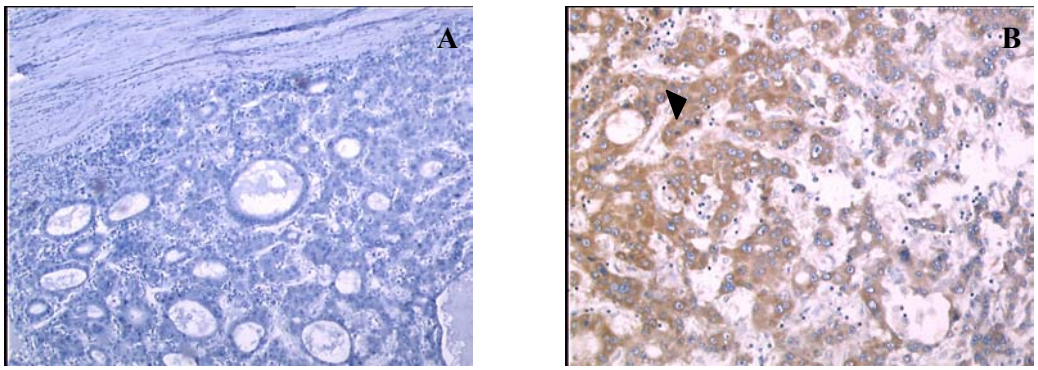


Figure 4.13: Immunoperoxidase stainings of paraffin-embedded liver carcinoma tissue sample S3. A. Staining with 9C11 was used as negative control. Magnification was 50X. **B.** The section in the view was composed of tumor cells stained with 6D5 at 100X.

5. DISCUSSION AND FUTURE PERSPECTIVES

In the multistep progression of cancer, it is not known how many genes are expressed differentially in tumor versus normal cells. In addition to this, it is not clear whether these differential changes are tumor-specific or cell type-specific (Lin and Wei, 1997). Therefore, a better insight into understanding malignancy would reveal the genes and their products that may be useful as prognostic, diagnostic and therapeutic markers.

An ideal tumor marker should be highly specific in terms of cancer and tissue type, should be present in detectable amounts in the patient even in the early stages of the malignancy, and should reflect the tumor grade. However, very few, if any, markers might fulfill those requirements. The most common limitation of the tumor markers is lack of specificity; many of the markers are not tumor-specific but tumor-associated. That means, they are elevated in more than one cancer type and even in some benign or physiological conditions. Besides, the potential role of tumor markers is precluded by the fact that most of them are not limited to the cancerous cell type. Those facts usually restrict the use of tumor markers. The diagnostic, prognostic or therapeutic role of a marker generally results from its differential characteristics in malignant versus benign or malignant versus normal phenotype. (White *et al.*, 2001; http://lwwoncology.com).

6D5 and 9C11 are mouse monoclonal antibodies that are produced against apoptosis induced hepatocellular carcinoma cells. Apparently, they react with different epitopes that are present in a wide panel of cell lines. 6D5 and 9C11 display a differential banding profile in Western blotting in addition to their differential immunoperoxidase staining profiles in tumor tissue sections.

6D5 antibody does not react with protein extracts of COS-7 (monkey), IAR 6 (rat), Sp2 (mouse) and Ankara (bovine) cell lines. As a result, 6D5 seems to react only with human proteins but not with the cell proteins of non-human species. In addition to cell lines mentioned above, 6D5 does not react with FLC4, CAMA-1 and LS411N cell proteins. FLC4 is the only hepatocellular carcinoma cell line that 6D5 does not

react with. The underlying reason may be the fact that FLC4 is a cell line, established by cloning JHH-4 hepatocellular carcinoma cells (Aoki et *al.,* 1998) expressing low levels of AFP. This feature resembles the characteristic of FLC variant of HCC, which is a moderate, highly curable tumor type when detected and resected in the early stages of the tumor development. DeVita *et al*. (2001) describes the distinct appearance and clinical behavior of the fibrolamellar variant as the most important pathologic issue of HCC. Unlike HCC, fibrolamellar carcinoma is not invasive but well circumscribed, more frequent in young women (median age of the patiens is 23 years), highly resectable, naturally occurs in the absence of underlying cirrhosis, and it is AFP and hepatitis B negative. It also generally exhibits a slower clinical course than the more common HCC. Among those features, absence of underlying cirrhosis should be mentioned because in our study, immunoperoxidase staining of liver carcinoma tissue samples displayed positive staining in cirrhotic areas with 6D5 antibody. This finding correlates with the negative banding pattern of FLC4 cell line in Western blotting unlike the other 13 HCC-derived cell lines.

CAMA-1 cell line originates from breast adenocarcinoma just the same as MCF-7 does. However, 6D5 has positive result with MCF-7 in Western blotting. The apparent difference between those cell lines is their differentiation status. CAMA-1 is a poorly differentiated cell line, whereas MCF-7 is a differentiated one. 6D5 may be reacting with a protein that is down-regulated during dedifferentiation of breast tumor cells in the process of cancer progression.

Among the two colorectal carcinoma cell lines, LS411N originates from cecum and SW837 originates from rectum. 6D5 is not immunoreactive to LS411N cell protein but immunoreactive to SW837 cell protein. Both of those colorectal carcinoma cell lines are poorly differentiated therefore, their differential expression pattern in Western blotting seems to result from molecular differences between cecum and rectal carcinomas.

6D5 displays differential positive banding patterns for Hep3B and Hep3B-TR isogenic cell lines. Hep3B-TR cell line differs from Hep3B in terms of being resistant to TGF-β_1 because it lacks TGF-βRII gene. Since TGF-β mediates cell proliferation, growth inhibition and differentiation, absence of TGF- βRII leads to a

cell line that is resistant to the growth-inhibitory effect of TGF-β_1. On the contrary, Hep3B is sensitive to growth inhibition by TGF-β (Inagaki *et al*., 1993). Therefore, the differential expression pattern of those cell lines in Western blotting most probably results from their difference in the presence of TGF-βRII gene.

When we evaluate the banding pattern of the whole cell lines, it is observed that 6D5 has a multiple-banding profile in contrast to single-banding profile of 9C11. Overall, there are five different bands appearing in Western blotting with 6D5 antibody. The probable cause may be the presence of alternatively spliced forms of the protein that 6D5 reacts with. In many cancers, malignant transformation includes aberrant functioning of tumor-specific truncated proteins or deregulation of expression of critical proteins. One of the examples is tumor marker PSMA and its regulation in prostate cancer. PSMA is alternatively spliced to produce at least three variants. One of those alternatively spliced forms is truncated from its transmembrane region. Therefore, the elevated level of this truncated form of PSMA in the serum of an individual is related to prostate cancer development (Ghosh and Heston, 2004).

Among the cell lines that display positive banding pattern; HuH-7, SNU-449, SNU-398, Mahlavu, Hep40, and SW837 cell lines exhibit a single band around 75 kDa. The band intensities of HuH-7, SNU-449, Mahlavu and SW-837 cell lines are equal. SNU-398 has the strongest band intensity, whereas Hep40 has the weakest. SK-Hep-1, SNU-475, FOCUS, Hep3B- TR, HEK293, and A375 cell lines display a double-banding pattern. One of those bands is around 75 kDa and the other is just above this band. The band intensities of those cell lines vary from one another and also between two bands of the same cell line.

SNU-182 and HepG2 cell lines represent a single band just above 75 kDa, HepG2 expressing a denser band.

SNU-387, LNCaP, and HeLa cell lines have a double-banding pattern. U-2 OS may be included to this group but we are not very sure whether it displays some additional bands. The multiple, smear-like banding pattern of U-2 OS may be as a result of the amplification of the gene product that 6D5 is immunoreactive to. The upper band displayed by those cell lines is around 75 kDa whereas the lower band is around 40

kDa. The band intensities of those cell lines are different from each other and the upper and lower bands have different strength of expression.

Hep3B cell line is unique with its banding profile. The lowest of the three bands displayed by Hep3B is observed in none of the rest of the cell lines.

MCF-7 is another cell line whose banding profile is unique. MCF-7 expresses only a single band that is around 40 kDa.

MRC-5 also displays a banding pattern different from the other cell lines. It exhibits 3 bands, the lowest of which is just above 50 kDa. This band is not present in the other cell lines. The differential banding pattern of MRC-5 may depend on either the fibroblastic or normal (mortal) feature of this cell line. In addition, MRC-5 is established from fetal lung, which is worth to consider for evaluation of oncofetal properties of the detected protein. The banding patterns of the cell lines are summarized in **Table 4.1**.

The positive results of Western blotting with 9C11 antibody always display a single-banding pattern. This single band has a molecular weight around 75 kDa. In addition, the band intensities of the positive cell lines are not as diverse as that of 6D5.

9C11 does not react with neither of FLC4, CAMA-1, LS411N, COS-7 or Sp2 cell lines. It is very probable that the reason of this non-immunoreactivity is the same as the reason of non-immunoreactivity of 6D5 with those cell lines. FLC4 is a moderate variant of JHH-4 cell line resembling the FLC variant of HCC with some of its molecular features, CAMA-1 is poorly differentiated when compared to MCF-7, LS411N originates from cecum of colon, probably displaying different molecular pathogenesis from SW837 cell line of rectum adenocarcinoma, and COS-7 and Sp2 cell lines are of non-human origin, which we naturally do not expect to react with 9C11.

Contrary to 6D5, 9C11 reacts with IAR 6 and Ankara cell lines leading to cross-species reactivity. It is surprising that 9C11 reacts with an epitope present in rat and bovine but absent from mouse (Sp2) and monkey (COS-7). Since 9C11 reacts with

rat and bovine cell proteins, it would be expected to react with monkey cell proteins in terms of phylogenic relation. However, 9C11 does not display this expected profile in Western blotting experiments.

Apart from FLC4, CAMA-1, LS411N, COS-7 and Sp2 cell lines, 9C11 reacts with all of the studied cell lines.

Immunoperoxidase experiments performed with paraffin-embedded liver carcinoma tissues reveal a differential staining pattern among 6D5 and 9C11 antibodies. No significant staining is observed with 9C11 concluding that 9C11 cannot recognize its epitope in those paraffin-embedded liver tissue samples in our experimental conditions. One of the reasons may be the limitations of routinely processed paraffin-embedded tissue sections in terms of preserving the antigenic determinants of the molecules (Shi *et al*., 1997). Although our immunoperoxidase experiments involved an antigen retrieval step to increase the sensitivity and to decrease background staining in the samples, masking of the antigens in the paraffin-embedded tissue samples is very common in daily applications of immunohistochemistry.

On the other hand, 6D5 antibody has positive staining in all of the three samples. An interesting outcome is the staining of cirrhotic hepatocytes and cirrhotic cells of bile duct epithelium in addition to staining of some tumor cells. Cirrhosis is one of the most important factors underlying HCC; 90% of the HCC cases develop from cirrhotic liver (Alberts *et al*., 2001). Chronic hepatitis is the major cause of liver cirrhosis leading to inflammation, regeneration, and fibrosis. The cycle of regeneration followed by fibrosis may lead to cirrhosis because fibrosis disrupts normal cell-cell and cell-matrix interactions leading to loss of growth control. During fibrosis, liver cells regenerate in an abnormal pattern, primarily forming nodules that are surrounded by fibrous tissue. Grossly abnormal liver architecture can lead to decreased blood flow to and through the liver. This induces oxidative stress in liver that may result in DNA damage. Besides, the repetitive cycles of inflammation and necrosis contributes to loss of cell growth control (Röcken and Carl-McGrath, 2001; http://cpmcnet.columbia.edu/dept/gi/cirrhosis.html; http://www.cancer.gov/cancerinfo/pdq/treatment/adult-primary-liver/healthprofessional/).

It should be emphasized that 6D5 stains tumor cells not in all cases. Presumably, the underlying reason is related to the molecular differentiation status of the cancerous cells. Additionally, the gender of the patients and gross pathological differences that may be present in the livers, which our biopsy specimens are sampled, may contribute to the molecular differences between the tumor pathology of the individual patients. Those gross pathological differences may involve presence of cirrhosis, presence of fibrosis, underlying genetic diseases (e.g., hemochromatosis, Wilson's disease, hereditary tyrosinemia, type 1 glycogen storage disease), and liver localization of the tumor as primary or secondary site.

As a conclusion, the outcomes of this study are as follows:

- It appears that there is a differential expression of the proteins, which 6D5 and 9C11 reacts with, specific to tissue type of the cell lines.

- None of 6D5 or 9C11 antibodies react with protein extracts of FLC4, CAMA-1, LS411N, COS-7 and Sp2 cell lines.

- FLC4, CAMA-1 and LS411N are the only human cancer cell lines that both 6D5 and 9C11 are not immunoreactive to.

- All but one of the 14 hepatocellular carcinoma cell lines express at least one epitope that is recognized by 6D5 or 9C11 antibody under our experimental conditions. FLC4 is the only liver cancer cell line that does not express any recognizable epitope by 6D5 or 9C11 monoclonal antibody.

- 6D5 does not have cross-species reactivity, whereas 9C11 is immunoreactive to IAR 6 and Ankara cell lines leading to cross-species reactivity.

- Band intensities of Western blotting with 6D5 antibody is diverse than that of 9C11 antibody.

- Differential expression pattern of Hep3B-TR cell line from Hep3B in Western blotting with 6D5 may be as a result of mutation of TGF-βRII gene in Hep3B-TR, leading to resistance of this cell line to growth-inhibitory effect of TGF-β. The difference between the banding patterns of Figure 4.1 and Figure 4.6 most probably results from changing 1X SDS gel loading buffer to 2X SDS gel loading buffer to ensure the separation of smear-like bands from each other and to clarify the banding pattern.

- The differential banding pattern of MRC-5 may be as a result of fibroblastic origin and/or mortal feature of this cell line. In addition, the expression profile of MRC-5 may be different from other cell lines because of its establishment from fetal lung.

- 9C11 does not stain paraffin-embedded liver cancer tissue samples in immunoperoxidase staining contrary to 6D5 antibody. 6D5 stains cancerous hepatocytes as well as cirrhotic hepatocytes and cirrhotic bile duct epithelium.

Further studies with 6D5 and 9C11 antibodies may include Western blotting experiments to compare normal (non-cancerous) cell lines with cancerous ones to find out whether those antibodies recognize differentially expressed epitopes in malignant versus normal cells. Another important point is to question the underlying reason in the differential expression pattern of Hep3B and Hep3B-TR isogenic cell lines in Western blotting experiments with 6D5. Sugano *et al*. (2003) underlines that increased production of TGF-β is characteristic of HCC implying the resistance to autocrine TGF-β. In Hep3B-TR, being resistant to growth-inhibitory effect of TGF-β may be leading to deregulation of several proteins including Smad proteins.

Broadening the tissue sample panel for immunohistochemistry studies may contribute to the expression profile of the epitopes recognized by 6D5 and 9C11 in terms of tissue specificity. Additionally, immunofluorescence studies may reveal the cellular localization of the epitopes.

For the characterization of the proteins recognized by 6D5 and 9C11, protein sequencing is required. With the help of protein sequencing, the banding patterns observed in Western blotting experiments may be elucidated and it would be revealed if those bands represent the alternatively spliced forms of the proteins. Sequencing may also reveal if 6D5 and 9C11 recognize novel proteins or novel epitopes of already investigated proteins. Characterization of the targeted epitopes or proteins may lead us to have an opinion on the function of those molecules. Another contribution of sequencing may be elucidation of value of 6D5 and 9C11 as novel tumor markers. When 6D5 and 9C11 are characterized as novel tumor markers, they can be used in research and/or for diagnostic, prognostic or therapeutic purposes according to the degree of their specificity and informative features.

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