

**CHARACTERIZATION OF NOVEL MONOCLONAL
ANTIBODIES THAT TARGET PROTEINS
DIFFERENTIALLY EXPRESSED IN HEPATOCELLULAR
CARCINOMA: A PROTEOMICS APPROACH**

**A THESIS SUBMITTED TO
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY**

**BY
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JANUARY 2011**

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ABSTRACT

CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES THAT TARGET PROTEINS DIFFERENTIALLY EXPRESSED IN HEPATOCELLULAR CARCINOMA: A PROTEOMICS APPROACH

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Hepatocellular carcinoma (HCC) is the sixth common cancer in the world. Because of the late diagnosis of the disease, survival rates are still poor in the HCC patients. Surveillance strategies have to be developed in populations with high risk groups having premalignant diseases for HCC, such as liver cirrhosis. The usage of serum and histology-based biomarkers assists health professionals to evaluate the patients. Despite of the advances in diagnostic methods, there is still a need to develop novel biomarkers for early detection of HCC. Therefore, we aimed to develop new biomarkers with higher sensitivity and specificity for HCC to improve the surveillance of the patients. Using an apoptotic HCC cell line, HUH7, and SIP1 proteins, we generated novel monoclonal antibodies (mAbs). 6D5, 1C6 and 6E5 hybridoma clones were chosen for characterization studies because of their strong reactivity in cell-ELISA assays. We found differential reactivity pattern for those novel mAbs in a panel of human sections consisting of tumors, benign liver diseases, normal tissues and a variety of cell lines. Using proteomics methods, we identified candidate target proteins for the 6D5 mAb. Better characterization of these target proteins will provide a better understanding of the molecular pathways in the HCC and aid in the research for developing newer therapeutic agents. In conclusion, our candidate biomarker mAbs can be used in the early diagnosis of HCC as well as in drug development studies.

ÖZET

HEPATOSELÜLER KARSİNOMDA FARKLI EKSPRESE EDİLEN HEDEF PROTEİNLERİ TANIYAN MONOKLONAL ANTİKORLARIN KARAKTERİZE EDİLMESİ: PROTEOMİKS YAKLAŞIM

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Hepatoselüler Karsinom (HK) dünyada en çok rastlanan tümörler arasında altıncı sırada yer alır. Bu hastalığın geç teşhis edilmesi nedeniyle HK'li hastaların yaşam süresi daha kısadır. Yaşam süresinin uzatılması yöntemlerinin geliştirilerek özellikle siroz gibi kansere neden olan hastalıkları taşıyan risk grubundaki insanlarda HK'nin erken teşhis edilmesi zorunludur. Serum ve histolojik çalışmalar dayalı biyobelirteçler geliştirilmesi sağlık çalışanlarına hastaların seçilmesinde yardımcı olur. Tanı metotlarındaki gelişmelere rağmen, HK'in erken tanısı için yine de yeni biyobelirteçlerin geliştirilmesine ihtiyaç vardır. Bu nedenle hastaların yaşam süresinin artırılmasına yardımcı olmak için HK'ne güçlü duyarlılıkta ve özellikle yeni biobelirteçleri geliştirmeyi amaçladık. Çalışmamızda apoptotik HK hücre hattı HUH7 ve SIP1 proteinlerini kullanarak monoklonal antikorlar (mAk) ürettik. Hücre-ELISA testlerinde en güçlü reaksiyonu vermeleri nedeniyle 6D5, 1C6 ve 6E5 hibridoma klonlarını, karakterizasyon çalışmaları için seçtik. İnsan dokusu kaynaklı kanser, iyi huylu karaciğer hastalıkları, normal dokular ve hücre hatlarından oluşan panelde antikorlarımızın farklı şekilde reaksiyon gösterdiğini bulduk. Proteomiks metodlar kullanarak 6D5 mAk'nun bağlandığı hedef proteinler için adaylar tespit ettik. Sonuç olarak 6D5 ve SIP1 mAk'larımızın tumor belirteci olarak HK'nin erken tanısında kullanılabileceğini düşünmekteyiz. Ayrıca güncel tedavilere cevabın yetersiz olması nedeniyle, bizim yeni antikorlarımız HK'nin moleküler yolaklarının iyi anlaşılmasında ve yeni tedavi hedefleri geliştirilmesinde yardımcı olabilir.

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ABBREVIATIONS

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ACN	Acetonitrile
AFP	Alpha fetoprotein
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulphate
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cDNA	complementary DNA
CHCA	α -Cyano-4-hydroxycinnamic acid (Matrix)
C-terminus	Carboxy terminus
ddH ₂ O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Ds	Double strand
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ESI	Electrospray ionisation
EST	Expressed sequence tags
FBS	Fetal Bovine Serum
FISH	Fluorescence <i>in situ</i> hybridization
GPC-3	Glypican-3
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase

hTERT	human Telomerase Reverse Transcriptase
IEF	Isoelectric focusing
Ig	Immunoglobulin
IPG	Immobilized pH Gradient
Kan	Kanamycin
Kb	Kilobase
kDa	Kilodalton
LC	Liquid chromatography
LOH	Loss of Heterozygosity
lt	Litre
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption and ionisation
µg	microgram
mg	milligram
min	minute
µl	microliter
ml	milliliter
µm	micrometer
µM	micromolar
mM	millimolar
mRNA	messenger RNA
MS	Mass spectrometry
MS/MS	Tandem MS
MSA	Microsatellite analysis
m/z	mass-to-charge ratio
N-terminal	Aminoterminal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
N-terminus	Amino terminus
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pI	Isoelectric point

pmol	Picomole
PVDF	Polivinilidene flouride
qRT-PCR	Quantitative real time RT-PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT PCR	Reverse transcription PCR
RxFISH	Cross-species color banding
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrilamide gel electrophoresis
sec	Second
SIP1	Smad interacting protein 1
siRNA	small interfering RNA
TBS	Tris buffered saline
TERT	Telomerase reverse transcriptase
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TOF	Time-of-flight
Tris	Tris (Hydroxymethyl)- Methylamine
UV	Ultraviolet
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
w/v	Weight/volume

1 INTRODUCTION

1.1 Liver Anatomy and Histology

The liver is the largest internal organ in the human body receiving blood supply from two major blood vessels. Oxygenated blood comes to the liver via hepatic artery, and the portal vein supplies deoxygenated nutrient-rich blood that provides the most part of the total blood supply of the liver. The Glisson's capsule, a connective tissue, surrounds the liver. This capsule separates the liver into polygonal lobules by connective tissue expansions. The periphery of the polygonal lobule consists of the portal vein, the hepatic artery, bile ducts, lymphatics and nerves. The central vein is located at the center of the lobule. The lobule consists of the parenchymal hepatocytes and nonparenchymal supporting cells. Hepatocytes are the most important cells in the liver and occupy almost 80% of the total volume of the liver and play role in numerous liver functions. On the other hand 6.5% of the liver volume consists of nonparenchymal cells which form 40% of the total liver cells. These cells are localized in the sinusoidal compartment of the tissue. Hepatic sinusoids are covered by three separate cell types. These are sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC) (Fig.1.1) (Bowen, McCaughan et al. 2005). In addition, sinusoidal lumen homes liver-specific natural killer cells. Neighboring nonparenchymal cells release substances that regulate the functions of the hepatocytes in normal and disease conditions. Liver sinusoidal endothelial cells constitute the lining or wall of the hepatic sinusoid (Lautt 1977).

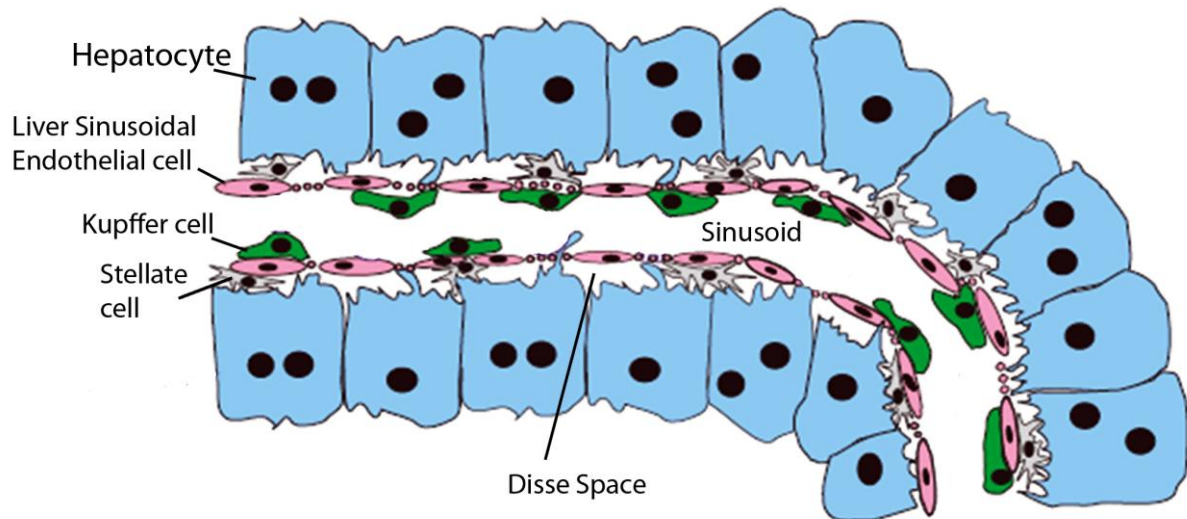


Figure 1.1 Cellular structure of the liver (Bowen, McCaughan et al. 2005).

1.1.1 Functions of Liver

Liver plays a central role in many vital physiological processes as a guard between the digestive system and rest of the body (Vekemans and Braet 2005). The liver functions in energy production and detoxification. It synthesizes lipoproteins, many plasma proteins, especially albumin, clotting factors, as well as cholesterol, glycogen, urea and certain amino acids. Liver changes, transforms and detoxifies many endogenous and exogenous substances. It synthesizes and secretes bile acids (Sasse, Spornitz et al. 1992). In addition it stores lipid soluble vitamins and many minerals. Some other functions of the liver are regulating fat metabolism, protein metabolism, carbohydrate metabolism, besides activation of enzymes important for many metabolic processes (Trauner, Fickert et al. 2001).

1.1.2 Disease Conditions of Liver

Liver has various pathological conditions including, inflammatory (hepatitis), cirrhotic (chronic inflammation progressing into organ failure), carcinomatous (originating from liver or metastatic), or storage (of fats, glycogen, metals) diseases. Chronic alcoholism is a

metabolic perturbant for the liver functions. Hepatocellular carcinoma (HCC) is a primary hepatic neoplasm and the most known type of liver cancer besides intrahepatic bile duct carcinoma, bile duct cystadenomacarcinoma hepatoblastoma, hemangiosarcoma and epitheloid hemangioendothelioma (Anthony 2002; Jemal, Siegel et al. 2010).

1.1.3 Hepatocellular Carcinoma

HCC is described as the malignant tumor of liver originating from liver parenchyma, hepatocytes. It is a primary malignant epithelial neoplasm of the liver. The macroscopic appearance of HCC varies according to the size of tumor and the presence or absence of liver cirrhosis. HCC may arise as a single mass, multinodular, with many tumors scattered through the liver, or as a diffusely growing lesion (Wilkens, Bredt et al. 2000). Histological view of HCC is variable, with various architectural and cytological patterns. Trabecular pattern is the most known architectural variant. Tumor cells of HCCs may demonstrate fatty or clear cell changes. Its histology also shows various forms, like chronic hepatitis and or cirrhosis of dysplastic regions besides adenomatous hyperplasia (Feitelson, Sun et al. 2002; Levy, Renard et al. 2002). Different cytological variants within the same tumor are frequently exhibited in HCCs. The most important differential diagnosis in well-differentiated HCCs is the liver cell adenoma and dysplastic nodules. Moderately differentiated HCCs rarely bring diagnostic problem, whereas poorly differentiated HCCs have to be differentiated from other solid, growing, poorly differentiated carcinomas (Rocken and Carl-McGrath 2001).

1.1.3.1 Pathogenesis of Hepatocellular Carcinoma

The pathogenesis of HCC, being multifactorial, involves environmental, infectious, metabolic, endocrine and nutritional factors that contribute to carcinogenesis. The importance of each factor varies geographically depending on the environmental and socioeconomic influence. High incidence of HCC is detected in African and Far Eastern countries where

hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections are endemic. In these areas, aflatoxin B1, alcoholism, and inherited metabolic diseases, such as hemochromatosis, tyrosinemia, and lipid storage diseases are common etiology for the development of HCC (Wong, Lai et al. 1999; Thorgeirsson and Grisham 2002). Recent studies have brought some understanding for the involvement of HBV in liver malignancies. New oncogenic pathways and tumor suppressor networks were revealed besides chromosomal abnormalities in HCC (Zimonjic, Keck et al. 1999; Levy, Renard et al. 2002).

1.1.3.2 Genomic Changes in Hepatocellular Carcinoma

Cancer develops in the living organism as a result of clonal proliferation of cells acquiring selection for growth as a result of genetic alterations such as mutations in oncogenes and tumor suppressor genes (Buetow, Murray et al. 1989). Identification of genes altered during tumor progression is essential to illuminate the molecular events in carcinogenesis (Yokota 2000). Genetic alterations such as amplification, deletion, translocation and rearrangement in certain chromosomal regions can result in gain or loss of function in genes that modulate cell proliferation, differentiation, motility and survival (Crawley and Furge 2002). Tumor suppressor genes are inactivated somatically, usually via mutations in one allele of the gene and by loss of a region belonging to the second allele. HCCs show variable alterations in the genome, involving rearrangements in DNA, loss of heterozygosity (LOH), amplification in chromosomes, lack of imprinting and many other mutations. Various genes have been proposed in the pathological steps of HCC (Kawamura, Nagai et al. 1999). Chromosomal abnormalities and genetic instability in HCC, such as aneuploidy is a well-known feature of cancers including HCC. Chromosome abnormalities in HCCs are summarized in Table 1.1.

Table 1.1 Review of chromosomal abnormalities in HCCs (http://etd.lib.nsysu.edu.tw/ETD-db/ETD-search/view_etd?URN=etd-0728103-150443).

Sample type	Methods	Position	
		Gain	Loss
HCC cell lines	CGH RxFISH	1, 6p, 7q, 8q, 10p, 17q, 20	4q21, 13, 18q21,Y
HCC cell lines	CGH, FISH	1p34.3-35, 1p33-34.1, 1q21-23, 1q31 -32, 6p11-12, 7p21, 7q11.2, 8q24.1-24.2, 11q11-13, 12q11-13, 12q23, 17q11.2-21, 17q23-24, 20p11.1-q13.2	3p12-14, 3q25, 4p12-14, 4q13-34, 5q21, 6q25-26, 8p11.2-23, 9p12-24, 11q23-24, 13q12-33, 14q12-13, 15q25-26, 18q11.2-22.2, 21q21-22
HCC	CGMA	1q, 6p, 8q, 17q, 5q, 12q, 19p	4q, 8p, 13q, 16q, 17p
HCC	CGH	1q24-25, 8q24, 11q13	17p, 16q, 13q13-14, 4q13-22, 8p, 10q
HCC	CGH	1q, 3q25, 4p15, 6p21-23, 7, 8q, 10q24, 11p14, 12q, 13q31-32, 16p, 17q, 18p 11, 19p10, 20q, Xp21	1p, 4q, 5q13-23, 6q13-23, 8p21-23, 9p, 10q22-23, 11q14-22, 12q14-21, 13q, 14q21-23, 18q12-21, 21q11, Y
HCC	CGH	1q, 8q, 17q, 20q	4q, 8p, 13q, 16q
HCC	MSA	1p, 1q, 2q, 3p, 4q, 6q, 8p, 9p, 13q, 16q, 17p with LOH	
HCC	MSA	1p, 4q, 6q, 8p23, 13q, 16p with LOH	
HCC	MSA	1q, 5q, 6p, 7, 8q	1p, 8p, 17p
HCC	CGH, FISH	1q12-q22, 8q, 20q	16q, 17p, 19p, 4q, 1p, 8p
HCC	MSA	1p33, 1q22-24, 1q25-41, 4q13-23, 4q28, 4q32-qter, 6p24-25, 8p21-23, 8p11-cen, 8q22-24.1, 9p22, 9q31, 10q23.3, 13q14-qter, 16q, 17p with LOH	
HCC	CGH, MSA	1q, 17p, 8q24	17p, 9p21-23, 4q, 16q21-23.3, 13q, 8p21-23, 6q24-27
HCC	MSA, CGH	6q, 8q, 10p, 13q, 17q	4q, 5q, 7q, 9q, 11q, 16q
HCC	MSA	1p, 4q, 8p, 17p, 13q, 14q, 9p, 16p, 3p, 8q, 5q, 10q, 11p, 18, 7q with LOH in biologic indicators of HCC	

HCC: hepatocellular carcinoma, CGMA: comparative genomic microarray analysis, CGH: comparative genomic hybridization, FISH: fluorescence *in situ* hybridization, LOH: Loss of heterozygosity, RxFISH: cross-species color banding, MSA: microsatellite analysis

1.1.4 Serum Markers for Hepatocellular Carcinoma

The prognosis of advanced HCC is poor, however, smaller HCC appropriate for organ transplantation, surgical resection or radio frequency ablation has a better prognosis and longer survival. For this reason detection of HCC at an early stage seriously affects the clinical outcome of these patients (Mor, Kasper et al. 1998). Therefore, a surveillance program using alpha fetoprotein (AFP) and ultrasound in every six months has been recommended by a consensus conference, and is widely practiced (Bruix, Sherman et al. 2001). Using a biomarker is particularly beneficial for the diagnosis of HCC in high risk patients with liver cirrhosis, a premalignant condition (Fattovich, Stroffolini et al. 2004). Surveillance programs seem to be cost-effective but whether they increase survival is still debated. Recently, it has been suggested that surveillance costs are mostly due to HCC treatment rather than the surveillance tests (Patel, Terrault et al. 2005). However, this conclusion depends also on the fact that the diagnostic tests available so far are not beneficial for early detection of HCC, thus affecting the treatment choice, the clinical outcome and the cost-effectiveness. In the case, clinicians have to balance the ethics of an earlier diagnosis while also considering the cost-benefit issues. So far AFP, the only serological marker commonly used in diagnosis may not be a reliable marker mostly because of its poor sensitivity, ranging from 39% to 65% and a specificity ranging from 76% to 97% (Daniele, Bencivenga et al. 2004; Marrero and Lok 2004). AFP seems to be reliable at values over 400 IU/ml; however the percentage of patients with such high levels is very small; this is one of the most important limits of this marker. It is no doubt that ultrasonography is a very powerful technique to identify nodules raising a suspicion of HCC, and technical improvements of diagnostic devices, e.g. use of contrast-enhanced ultrasonography, will further improve the diagnostic accuracy. However, ultrasound has also its major limits, as good results depend on technological advances of the device and

the skill of the operator. Therefore, combination of ultrasound with serological markers such as AFP seems to be the best choice. Serum markers of HCC are summarized in Table 1.2.

Table 1.2 Hepatocellular carcinoma markers (Malaguarnera, Giordano et al. 2010)

HCC marker	Principal use
Alpha-fetoprotein	HCC early diagnosis, monitoring, and recurrence
Lens culinaris agglutinin reactive AFP (AFP-L3%)	HCC early diagnosis and prognosis (vascular invasion and intrahepatic metastasis)
Des- γ -carboxy prothrombin (DCP)	HCC early diagnosis and prognosis (early portal vein invasion and metastasis)
α -l-fucosidase	HCC early diagnosis
Glypican-3	HCC early diagnosis
P-aPKC- ι , E-cadherin, β -catenin	HCC prognosis
Human carbonyl reductase (HCR2)	HCC prognosis
Squamous cell carcinoma antigen (SCCA)	HCC early diagnosis
Serum proteomics	HCC early diagnosis
Golgi protein 73	HCC early diagnosis
Chromogranin A (CgA)	HCC prognosis and possible therapeutic treatment
Vascular endothelial growth factor (VEGF)	HCC prognosis (metastasis development)
Hepatocyte growth factor (HGF)	HCC prognosis and disease recurrence
Transforming growth factor- β (TGF- β)	HCC progression
Serum anti-p53	HCC prognosis (poor differentiation)
Nerve growth factor (NGF)	HCC prognosis and progression
β 2-microglobulin (β 2MG)	HCC progression
Glycylproline dipeptidyl aminopeptidase (GPDA)	HCC early diagnosis

1.2 SIP1 as a Tumor Marker

SMAD interacting protein 1 (SIP1), also known as ZEB2, encoded by ZFH1B, is a member of ZEB family of transcription factors. The protein contains a central homeodomain, CtBP-binding and Smad-interacting domains and two zinc finger clusters each at either end (Remacle, Kraft et al. 1999; Verschueren, Remacle et al. 1999). SIP1 directly binds to bipartite E-boxes on the promoters of different targets by means of its zinc finger domains and mediates transcriptional repression (Verschueren, Remacle et al. 1999). One of these targets is CDH1, the gene encoding for the epithelial adherens junction protein, E-cadherin, whose transcriptional downregulation induces epithelial-to-mesenchymal transition (EMT) in developmental processes and during tumor cell invasion and metastasis (Comijn, Berx et al. 2001). Transcriptional repression is mediated through the association of SIP1 with the corepressor CtBP, however this interaction is dispensable at least for the attenuation of CDH1 transcription (Postigo, Depp et al. 2003; van Grunsven, Michiels et al. 2003). Overexpression of SIP1 in epithelial cells has also been shown to downregulate constituents of cell-cell junctions other than E-cadherin (Vandewalle, Comijn et al. 2005). Although binding of SIP1 to p300 or pCAF was proposed as a mechanism for transactivation and other transcriptional activators associated to SIP1 are yet to be determined, SIP1-mediated up-regulation of EMT and invasion related genes, such as vimentin and matrix metalloproteases, have been reported (Postigo, Depp et al. 2003; Miyoshi, Kitajima et al. 2004; Bindels, Mestdagt et al. 2006).

Despite the overwhelming evidence that SIP1 induces EMT phenotype, its role in tumorigenesis was ill-defined. In fact, SIP1 was originally identified as a binding partner of R-Smads, and shown to be part of the TGF- β pathway, which is frequently involved in carcinogenesis (Verschueren, Remacle et al. 1999). hTERT repression in breast cancer cells was partly mediated by SIP1 in a TGF- β dependent manner (Lin and Elledge 2003). Also, analysis of senescence arrest of clonal HCC cells revealed SIP1 as a mediator of hTERT

repression (Ozturk, Erdal et al. 2006). Impaired G1/S progression was observed upon repression of cyclin D1 by SIP1 (Mejlvang, Kriaievska et al. 2007). SIP1 was also shown to contribute to tumorigenesis in a transgenic mouse model of lymphoma by retroviral tagging (Mikkers, Allen et al. 2002). The differential expression of SIP1 has been described, mostly by RT-PCR, in several human tumors due to the lack of human SIP1-specific antibodies. E-cadherin down-regulation was associated with increase SIP1 expression in intestinal type gastric carcinoma but not in diffuse type gastric carcinoma (Rosivatz, Becker et al. 2002). Elevated SIP1 expression correlated inversely with E-cadherin in advanced stages of pancreatic tumors (Imamichi, Konig et al. 2007). Surprisingly, SIP1 and E-cadherin expression were positively correlated in malignant mesothelioma (Sivertsen, Hadar et al. 2006). In the esophagus, differential expression of SIP1 was observed during keratinocyte differentiation. Only stem cell containing basal cells, but not parabasal cells and keratinocytes expressed SIP1. Consistent with this, SIP1 transcripts were present in all studied esophageal carcinoma cases (Isohata, Aoyagi et al. 2009). High SIP1/E-cadherin ratio correlated with metastatic disease and poor patient survival in breast and ovarian carcinomas (Elloul, Elstrand et al. 2005). Elevated SIP1 transcripts were observed in von Hippel-Lindau-null renal cell carcinomas in a hypoxia-inducible factor 1 alpha (HIF1 α)-dependent manner (Krishnamachary, Zagzag et al. 2006). Immunohistochemical analysis of ovarian tumors revealed a stepwise increase of SIP1 from benign to borderline and to malignant tumors (Yoshida, Horiuchi et al. 2009). In oral squamous cell carcinoma, SIP1 was immunohistochemically detected in a relatively low proportion of tumors and its expression correlated with poor prognosis (Maeda, Chiba et al. 2005). In a previous study, it is found that SIP1 was overexpressed in a series of bladder cancers. Its expression was found to be an independent prognostic factor in bladder cancers and positively stained cases correlated with poor therapeutical outcome (Sayan, Griffiths et al. 2009). With the exception of a few and as

described above, most of the expression studies of SIP1 were done using RT-PCR technique, but SIP1 protein levels have been shown to be tightly regulated by post-transcriptional mechanisms. For instance, Pc2-mediated sumoylation of SIP1 affects the transcriptional regulation of E-cadherin (Long, Zuo et al. 2005). In addition SIP1 also has been identified as a direct target of miR-200 family and miR-205 (Gregory, Bert et al. 2008; Park, Gaur et al. 2008).

1.3 Biomarker Research

A tumor marker is a substance produced by tumor or by host tissue as a response, detectable in biological fluids or tissues and useful to differentiate neoplastic from non-neoplastic disease. These markers are commonly used in diagnosis, staging and prognosis of cancer, and can be useful to localize the tumor burden, besides monitoring therapeutic effectiveness, detecting recurrence or localizing of the tumor, and screening the general population or groups at risk. Tumor markers, also named biomarkers, have been classified as enzymes, isoenzymes, hormones, oncofetal antigens, carbo-hydrate epitopes, oncogene products and genetic alterations. Unfortunately, until now, none of the known biomarkers fit the ideal specificity profile. The most important characteristics for a biomarker are measurement by simple techniques, reliability, reproducibility, minimal invasiveness and low cost. Novel biomarkers are continuously suggested by powerful, high-technology research.

1.3.1 Monoclonal Antibodies

In 1975 the hybridoma technique was introduced (Kohler and Milstein 1975). This strategy utilizes a hybrid cell line to produce an antibody clone with desired binding properties. Hybridomas are obtained by fusion of a selected antibody-producing B cell with an immortal myeloma cell (a cancerous plasma cell). Thereby, an infinite source of antibody molecules is obtained. In many applications, including diagnostics or therapeutic applications, monoclonal

antibodies (mAbs) are preferred (Borrebaeck 2000). This is because of the renewable source and the fact that monoclonal antibodies recognize one single epitope on the target. The detection of the epitope is assumed to give information about the target protein. On the other hand, in applications where multi-epitope recognition is desired more than one monoclonal antibody or a polyclonal serum is required. Monoclonal antibodies are used in many aspects of biomedical research, in diagnosis, and in treatment of diseases, such as infections and cancer. Antibodies are important tools for research and have led to many medical advances.

1.3.2 Analysis of Monoclonal Antibody Targets

1.3.2.1 Tissue Microarrays

Method of using tissue microarrays (TMAs) presents as a technology which allows for the linking of clinical data to the tissues that are combined on one slide. Tissue microarrays have become a tool for tissue-based research since the last decade. In cancer research, depending on the available data attached to the arrayed tissue, many types of arrays from different tissues are commonly manufactured. Prevalence TMAs are suited to estimate the frequency of the occurrence of a particular alteration. Progression arrays include tissues of different stages of disease, and are useful to study the role of a marker protein for tumor initiation, progression, or metastatic growth. Prognosis TMAs include tissues with patient follow-up data. These TMAs are the key components to uncover the clinical impact of molecular markers. In combination with normal tissue arrays representing healthy tissues, prevalence, progression, and prognosis TMAs all allow to a rapid and comprehensive analysis of molecular markers in human cancers. TMAs are also successfully used in many noncancer applications, such as Alzheimer's or inflammatory disease research.

1.3.2.2 Epitope Mapping with Protein Microarrays

Epitope Mapping is a method used for studying the interactions of antibodies with specific regions of protein antigens. Important applications of epitope mapping are found within the area of immunochemistry.

Protein (peptide) microarrays are powerful tools for characterizing antibodies raised against protein antigens. For antibody targets epitope mapping, an array is made from a library of short peptides that span the antigenic protein sequence (Martens, Greiser-Wilke et al. 1995; Frank and Overwin 1996; Reineke, Ivascu et al. 2002). The antigenic determinant recognized by a mAb can then be defined by probing the array with the antibody. An epitope can be defined by constructing an array in which each epitope residue is substituted with other amino acids to assess that residue's contribution to antibody binding and to determine which substitutions affect antibody recognition (mutational analysis). Antibody cross-reactivity can be evaluated with arrays made from large numbers of unrelated synthetic peptides.

Protein microarrays constitute a technology with in situ protein expression directly on the surface of polyvinylidene fluoride (PVDF) membranes. Protein synthesis is performed in an *E. coli* based expression system for recombinant proteins. In this system, a tagged human fusion proteins were expressed in *E. coli*, and after native lysis with lysozyme, crude protein extracts were prepared under non-denaturing conditions in 384-well plate format. The crude bacterial cell extracts were used for incubation overnight with the high density spotted PVDF membranes. The protein microarrays can be used for epitope mapping. The redundant nature of the cDNA expression library represented on protein microarrays allows identification of the epitopic region and determination of possibly shared epitopes in cross-reacting proteins (Bussow, Cahill et al. 1998). Furthermore the protein sequences of the corresponding clones can be used to design different peptides to find the specific epitope recognized by the antibody (Grelle, Kostka et al. 2006).

1.3.2.3 Proteomics Analysis

The “proteome” term is generally attributed to Mark Wilkins, who introduced the term “proteoma” at the Siena Conference in 1994 (Wasinger, Cordwell et al. 1995). Through borrowing the semantics from the ‘genome’ term, it becomes clear that the scope of research is very similar in both cases, although the topics differ. Indeed, it is imperative to analyze the protein content of a cell in order to understand its structure at the molecular level. Analyzing the proteome introduces a more daunting challenge compared to analyzing the genome: apart from spanning a large concentration range, at least 10 orders of magnitude in plasma (States, Omenn et al. 2006), it is dynamic in concentration besides modification state. Indeed, even though cells have the same genome, their proteomes can be different markedly (Collins 2001). Besides, protein sequences are not easily duplicated to large copy-numbers as is the case for nucleic acid sequences through the application of the polymerase chain reaction (PCR). Nowadays the most popular technique to study the proteome is mass spectrometry that relies on separating charged ions by their mass-to-charge ratio (m/z). The general structure of gel based proteome analysis is schematized in Fig.1.2.

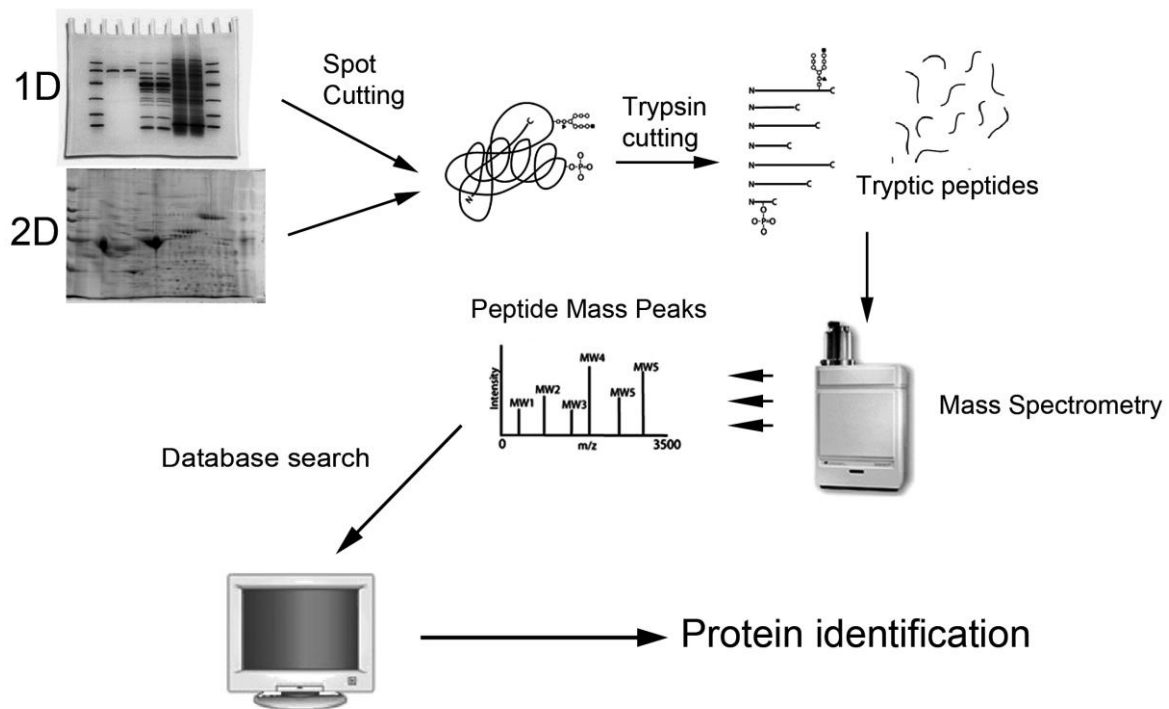


Figure 1.2 Gel based proteome analysis

1.3.2.3.1 Two-dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been in charge of proteomics research for over three decades (O'Farrell 1975; Klose and Kobalz 1995). Its ability in separating complex protein mixtures in two dimensions according to various physicochemical properties was supported by protein identification via mass spectrometry. The separation using 2D-PAGE is based on protein iso-electric point (pI) in the first dimension (isoelectric focusing, IEF) and on apparent molecular weight (SDS-PAGE) in the second dimension. In a typical analysis, a protein mixture is first run on a 2D-PAGE system and after separation the resulting proteome pattern is visualized by staining, e.g. by Coomassie brilliant blue and silver staining (Meyer and Lamberts 1965; Switzer, Merrill et al. 1979). After the visualized protein spots are excised, proteolytic digestion follows either by dissolving the proteins from the gel or, directly inside the gel (in-gel digestion). The

proteolytic enzyme preferred is usually trypsin, which cleaves on the carboxy-terminal (C-terminal) side of arginine or lysine (Olsen, Ong et al. 2004) generating peptides with one of these basic amino acids. This is useful while making the step to mass spectrometry which can only analyze charged ions. Then identification of isolated protein(s) proceeds through a matching of the peptide masses measured by the mass spectrometers to the masses of the in silico produced cleavage products obtained from the entries in a protein sequence database. This comparison is usually too hard to perform manually and specialized software has been generated to allow the automated matching of peptide masses to protein databases (Mann, Hojrup et al. 1993; Pappin, Hojrup et al. 1993; Yates, Speicher et al. 1993; Clauser, Baker et al. 1999; Colinge, Masselot et al. 2003; Geer, Markey et al. 2004). Such algorithms usually need a coverage factor into account when scoring a protein hit. This stems from the assumption that proteins are isolated in pure form after the 2D separation. 2D gel-based methods have proved their usefulness over time

1.3.2.3.2 Mass Spectrometry

Mass spectrometer can be classified into three parts: ion source, m/z analyzer and detector. The detector is usually a specific type of electron multiplier. Due to the high amplification that is typically required, most modern instruments use a type of microchannel plate detector. Additional refinements for peptide/protein sequence analysis include so-called tandem- MS or MS/MS instruments which are capable of more than one round of mass spectrometry. In this technique one mass spectrometer isolates a peptide of a particular m/z , while a second mass spectrometer is used to catalog fragment ions resulting after induced or spontaneous fragmentation. When applied to biomolecular compounds, the ion source of the mass spectrometer can typically take two forms: a Matrix-Assisted Laser Desorption and Ionisation (MALDI) source (Karas and Hillenkamp 1988) or an ElectroSpray Ionisation (ESI) source (Fenn, Mann et al. 1989). In a MALDI source, energy from laser light is converted into

kinetic energy of the irradiated molecules/ions. This light is directed towards a metallic target plate on which the analyte has been crystallized in the presence of so-called matrix molecules. The laser light itself is typically derived from a N₂ laser generating UV light with a wavelength of 337 nm. Some often-used matrix compounds are α -cyano-4-hydroxycinnamic acid for peptide analytes (Beavis and Chait 1990) and sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) for proteins (Beavis and Chait 1989). Crystallization is usually performed in highly organic solvents and in the presence of 0.1% tri-fluoroacetate (TFA). The actual mechanisms leading to desorption and ionisation are subject of debate (Karas 1996; Zhao, Kent et al. 1997; Salih, Masselon et al. 1998; Wong, Lee et al. 1999), yet it is thought to rely on efficient absorption of the laser energy by the matrix molecules, which ultimately convert it into kinetic energy. This theory explains why a high molar excess of matrix molecules is required to obtain efficient desorption of the analyte. Ionisation might, according to one hypothesis, occur in the gas phase by proton transfer between the acidic matrix ions and the basic residues of the analyte (lysine, arginine or histidine). The principle of a MALDI source is depicted according to this theory in Fig 1.3.

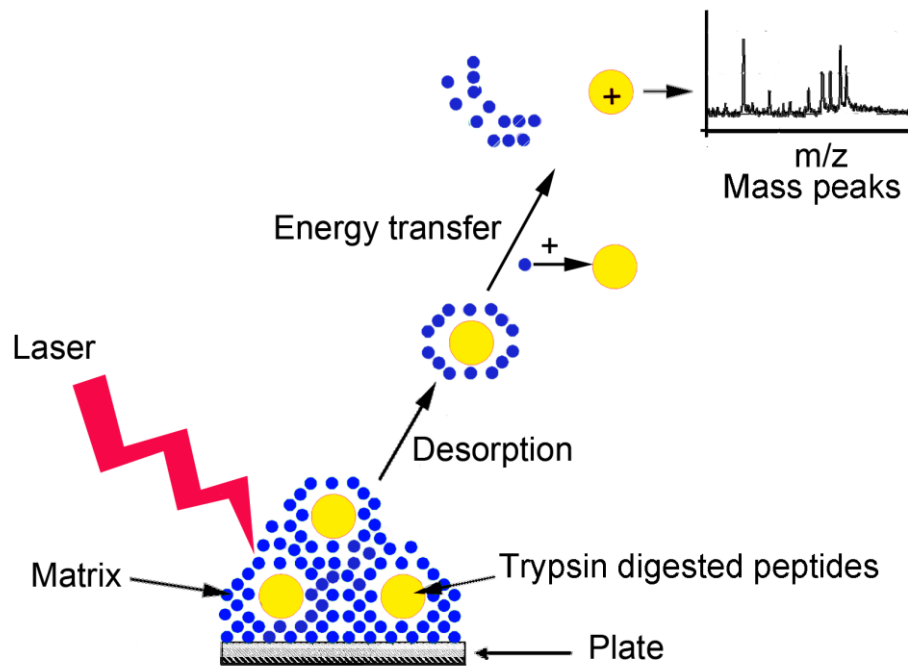


Figure 1.3 MALDI principles for protein analysis

1.3.2.3.3 Identification of Proteins

The process of converting mass spectrometry data into protein lists is managed by two important items: software algorithms that consider establishing mass spectra and, the protein sequence databases that they take as search space. The different types of identification algorithms besides the importance and characteristics of protein sequence databases are available to analyze the mass spectrometry results.

1.3.2.3.4 Protein Sequence Databases

Protein identification is usually based on database search algorithms. Interestingly, even though the sequence database employed represents the most basic source of information in these identifications, its importance is often disregarded. The European Bioinformatics

Institute (EBI) has developed and maintained a number of protein related databases (Table 1.3).

Table 1.3 The list of protein related databases (<http://www.ebi.ac.uk/Databases/protein.html>).

Database	Description
CluSTr	Offers an automatic classification of UniProtKB/Swiss-Prot + UniProtKB/TrEMBL.
CSA	CSA - The Catalytic Site Atlas is a resource of catalytic sites and residues identified in enzymes using structural data.
HPI	Human Proteomics Initiative (HPI) is an initiative, by SIB and the EBI, to annotate all known human sequences according to the quality standards of UniProtKB/Swiss-Prot.
IntEnz	The Integrated relational Enzyme database (IntEnz) will contain enzyme data approved by the Nomenclature Committee. The goal is to create a single relational enzyme database.
InterPro	The InterPro database is an integrated documentation resource for protein families, domains and functional sites.
IPI	International Protein Index contains a number of non-redundant proteome sets of higher eukaryotic organisms constructed from UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, Ensembl and RefSeq.
LGICdb	The Ligand Gated Ion Channel Database.
PANDIT	PANDIT - Protein and Associated Nucleotide Domains with Inferred Trees. PANDIT is a collection of multiple sequence alignments and phylogenetic trees covering common protein domains.
Patentdata Resources	Patent data resources at EBI contain patent abstracts, patent chemical compounds, patent sequences and patent equivalents.
UniProt	The Universal Protein Resource for protein sequences and is the central hub for the collection of functional information on proteins, with accurate, consistent, and rich annotation, the amino acid sequence, protein name or description
UniProt Archive	A non-redundant archive of protein sequences extracted from public databases and contains only protein sequences.
UniProt/UniRef	Features clustering of similar sequences to yield a representative subset of sequences. This produces very fast search times.
UniProtKB-GOA	Provides assignments of proteins in UniProtKB/Swiss-Prot, UniProtKB/TrEMBL and IPI to the Gene Ontology resource.
UniProtKB/Swiss-Prot	An annotated protein sequence database. Part of the UniProtKB.
UniProtKB/TrEMBL	A computer generated protein database enriched with automated classification and annotation. Part of the UniProtKB.
UniProt/UniMES	A repository specifically developed for metagenomic and environmental data.
UniSave	The UniProtKB Sequence/Annotation Version Archive (UniSave) is a repository of UniProtKB/Swiss-Prot and UniProtKB/TrEMBL entry versions.

2 OBJECTIVES AND RATIONALE

Hepatocellular carcinoma is one of the most common cancers with high morbidity and mortality rates and, its incidence is increasing worldwide (O'Brien, Kirk et al. 2004). It is the fifth most frequent cancer in the world and the third most common cause of cancer-related deaths (Parkin 2001). Liver cirrhosis is the most important risk factor for HCC development and will possibly remain so in next decades, due to the high frequency of hepatitis B and C viral infections and excessive alcohol intake (Donato, Tagger et al. 2002; Davila, Morgan et al. 2004). The major contributing phenomenon to this expectancy is the long latency period between infections and the onset of HCC (El-Serag and Mason 2000). For these reasons, patients with liver cirrhosis are periodically surveilled for the diagnosis of HCC at early stages of tumor development. For instance, serum α -fetoprotein levels (AFP) and hepatic ultrasonography are the screening tools of choice (Okazaki, Yoshino et al. 1990) yet, other promising biomarkers, such as des-gamma carboxyprothrombin (Lefrere, Conard et al. 1988), lens culinaris-agglutinin reactive AFP (Kuromatsu, Tanaka et al. 1993), human hepatocyte growth factor-1 (HGF-1) (Wennerberg, Nalesnik et al. 1993) insulin-like growth factor-1 (IGF-1) (Wee and Nilsson 1997) and glypican-3 (Borscheri, Roessner et al. 2001) are currently under intensive investigation. Although useful, these tumor markers have poor sensitivity and specificity for HCC, and accordingly, their use for differential diagnosis of malignant liver cancers are limited (Okazaki, Yoshino et al. 1990; Borscheri, Roessner et al. 2001). Therefore, the development of novel markers for HCC with higher sensitivity and specificity is of great importance for the surveillance of patients with chronic hepatitis and liver cirrhosis, which are at high risk to develop liver tumor. The use of serological markers in patients at the highest risk for developing HCC can thus decrease the cancer-related mortality and reduce medical costs.

We recently generated 3 new monoclonal antibodies (mAb) by using cells of HUH7, a HCC cell line, and recombinant SIP1 proteins as immunogen against novel targets in HCC. To validate their specificity we explored the expression pattern of novel mAbs in human tumor cell lines and in a variety of tissues. In addition to expression profile of these mAbs in tissue sections and cell lines, we aimed to explore the targets (epitopes) of the 6D5 mAb by using proteomics methods. For this reason, the most commonly used proteomic technologies, including protein microarrays, two-dimensional gel electrophoresis and mass spectrometry were applied into this study for the discovery of new diagnostic markers of HCC and targets of pharmaceutical interest to improve patients' prognosis.

3 MATERIALS AND METHODS

3.1 MATERIALS

Immobiline pH-gradient (IPG) DryStrips (pH3-10, length 7 cm), IPG buffer (pH3-10), DryStrip cover fluids, thiourea, urea, CHAPS, DTT, Pharmalyte (pH 3-10), bromophenol blue, Bis, TEMED, Coomassie brilliant blue G-250, protein molecular weight marker, Tris-base, SDS, glycine, horseradish peroxidase-conjugated goat anti-rabbit IgG, horseradish peroxidase-conjugated goat anti-mouse IgG, and the enhanced chemiluminescence (ECL) system were purchased from Amersham Biosciences (Stockholm, Sweden). For immunohistochemistry, secondary antibodies with biotin-avidin complexes and chromogen diaminobenzidine (DAB) were used in universal staining kit purchased from (LabVision, Fremont, CA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI). PVDF membrane and ZipTip C18 columns were obtained from Millipore (Boston, MA). Mercaptoethanol (ME), iodoacetamide (IAA), Acetic Acid, Ammonium Bicarbonate, R-cyano-4-hydroxycinnamic acid (CCA), and HCl were from Sigma-Aldrich (St. Louis, MO). HPLC Acetonitrile (ACN), HPLC Water (Burdick&Jackson), DTT (Calbiochem), TFA (Pierce). All buffers were prepared with Milli-Q water.

3.1.1 Electrophoresis, Autoradiography, Photography and Spectrophotometer

Electrophoresis grade agarose was obtained from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO). Horizontal electrophoresis apparatus were from E-C Apparatus Corporation (Florida, USA). An imaging system, Vilber Lourmat (France), was used to image and analyze. The power supply Power-PAC300 and Power-PAC200, Mini-PROTEAN3 cell

system, programmable IEF unit Protean II IEF cell and Trans-Blot SD semi-dry electrophoretic transfer cell were purchased from Bio-Rad (CA, USA). Univapo 100 ECH vacuum concentrator centrifuge was from UniEquip GmbH (Germany). Immobilon-P transfer (PVDF) membrane was from Millipore (MA, USA) and 3M filter paper was from Whatman International Ltd. (Madison, USA). The films used for autoradiography were from Kodak and the development of the films was performed with Hyperprocessor (Amersham, UK). ECL-Plus, Western Blotting detection reagent was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Beckman Spectrophotometer Du640 was from Beckman Instruments Inc. (CA, USA) and Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer was from Thermo Fisher Scientific (Wilmington, DE).

3.1.2 Tissue Culture Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, trypsin, non-essential amino acids, penicillin/streptomycin mixture and fetal calf serum were obtained from HyClone (South Logan, UT). Tissue culture flasks, petri dishes and cryotubes were purchased from Costar Corp. (Cambridge, England). Geneticin-G418 sulfate was purchased from GibcoBRL (Carlsbad, CA).

3.1.3 Animal Experiments

BALB/c mice were provided by Bilkent University Animal Housing Facility. All animal experiments were performed upon approval by and under the regulations of "Bilkent University Animal Ethical Committee".

3.2 SOLUTIONS AND MEDIA

3.2.1 Tissue Culture Solutions

DMEM/RPMI-1640 growth media: 10% FBS, 1% penicillin/streptomycin, 1% nonessential amino acid were added and stored at 4°C.

Freezing solution: 10% DMSO and 90% FCS were mixed freshly.

Phosphate buffered saline (PBS): Stock solution (10XPBS) was prepared by dissolving 80 g NaCl, 2 g KCl, 17.8 g Na₂HPO₄·2H₂O, and 2.4 g KH₂PO₄ in 1 lt ddH₂O. Working solution (1XPBS) was prepared by dilution of 10XPBS to 1X with ddH₂O. pH of the working solution was adjusted to 7.4.

Geneticin (G418) sulfate: 500 mg/ml solution in ddH₂O was prepared, sterilized by filtration and stored at -20°C (stock solution). 500 µg/ml was used as working solution for stable cell line selection and 250 µg/ml was used as working solution for maintenance of stable cell lines.

Puromycin: 2 mg/ml solution in ddH₂O. Sterilized by filtration and stored at -20°C (stock solution). 2 µg/ml was used as working solution for selection.

3.2.2 Protein Extraction, Quantitation and Western Blotting Solutions

Radio immuno-precipitation assay (RIPA) buffer: 150 mM NaCl, 50mM Tris-HCl pH 8.0, 1% NP-40, 0.1% SDS and 1X protease inhibitor mix (Roche, Basel, Switzerland) were mixed in ddH₂O.

Bradford stock solution: 17.5 mg Coomassie brilliant blue was dissolved in 4.75 ml ethanol and 10 ml phosphoric acid and completed to 25 ml final volume with ddH₂O.

Bradford working solution: 1.5 ml Bradford stock solution was mixed with 0.75 ml 95% ethanol and 1.5 ml phosphoric acid and completed to final volume up to 25 ml with ddH₂O. It was then filtered through whatman paper and prepared freshly.

Acrylamide-bisacrylamide solution: 29 g acrylamide and 1 g bisacrylamide were dissolved in 100 ml ddH₂O and stored in the dark at 4°C (stock solution).

10% Ammonium persulfate (APS): 0.1 g APS was dissolved in 1ml of ddH₂O, prepared freshly.

1.5 M Tris-HCl, pH 8.8: 54.45 g Tris base (18.15 g/100ml) was dissolved in ~150ml ddH₂O. pH was adjusted to 8.8 with 1 N HCl. Final volume was completed to 300 ml with ddH₂O and stored at 4°C.

1 M Tris-HCl, pH 6.8: 12.14 g Tris base was dissolved in ~ 60 ml ddH₂O, pH was adjusted to 6.8 with 1 N HCl. Final volume was completed to 100 ml with ddH₂O and stored at 4°C.

Coomassie brilliant blue solution: 100 mg Coomassie brilliant blue G-250, 50 ml 95% ethanol, and 100 ml 85% phosphoric acid was dissolved and final volume was completed to 1 lt. It was then filtered through whatman paper and stored at 4°C.

5X SDS-gel loading buffer: 5 g SDS, 25 mg bromophenol blue, 15.7 ml 1M Tris pH 6.8, 21.8 ml glycerol (from 87% stock) were mixed and completed to 50 ml with ddH₂O. Before use, β-mercaptoethanol was freshly added to a final concentration of 5% to reach 1% when mixed with protein samples.

10X SDS-gel electrophoresis buffer: Per liter; 30.3 g Tris base, 144.0 g glycine, 10.0 g SDS were added. It was diluted to 1X for working solution and stored up to 1 month at 4°C.

Semi-dry transfer buffer: 48 mM Tris base, 39 mM glycine, 0.037% SDS and 20% methanol were dissolved and final volume was brought to 1 lt.

Wet transfer buffer: 3.03 g Tris and 14.4 g glycine was mixed with 1ml 10% SDS and 20% methanol and completed to final volume of 1 lt. For high molecular weight proteins methanol percentage was decreased by half.

10X Tris buffered saline (TBS): 30 g Tris base, 80 g NaCl and 2 g KCl were dissolved in 1lt of ddH₂O and the pH was adjusted to 8 (stock solution). Diluted to 1X and pH was adjusted to 7.6 with HCl just before use.

TBS-Tween (TBS-T): 0.1% (v/v) Tween-20 was added into 1X TBS solution.

Blocking solution: 5% (w/v) non-fat milk and 0.1% (v/v) Tween-20 were dissolved in 1XTBS (prepared freshly).

3.2.3 Immunofluorescence

Fluorescein isothiocyanate (FITC): anti-mouse IgG (Sigma)

DAPI (4', 6-diamidino-2-phenylindole): 0.1-1 µg/ml DAPI was prepared in ddH₂O (working solution in ddH₂O).

2% paraformaldehyde: 2 g paraformaldehyde dissolved in 100 ml 1X PBS, pH 7.4 and stored in dark, at -20°C.

PBS-TritonX-100 (PBS-T): 0.1% (v/v) Triton X-100 was added into 1X PBS.

Blocking solution: 2% BSA (bovine serum albumin) was prepared in 1X PBS.

3.2.4 Immunohistochemistry Solutions

10 mM Citrate buffer: 2.94 g sodium citrate trisodium salt dihydrate was added to 1lt dH₂O, and pH 6.0 was adjusted with NaOH.

3% Hydrogen Peroxide: 10 ml 30% H₂O₂ was added to 90 ml dH₂O.

3.2.5 2D PAGE Solutions

Rehydration/Sample Buffer: 10 ml of 8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte[®] 3/10 ampholytes, and Bromophenol Blue (trace) were mixed.

Equilibration Buffer I: 20 ml of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% (w/v) DTT were mixed.

Equilibration Buffer II: 20 ml of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), and 20% glycerol were mixed.

Coomassie Brilliant Blue R-250 stain solution: 0.1% Coomassie Blue R-250 in 40% MeOH, 10% acetic acid.

Destain solution: 10% acetic acid, 40% methanol in dH₂O.

30% Glycerol Solution: Sterile 30% (v/v) glycerol. 30 ml glycerol was completed to a final volume of 100 ml by ddH₂O.

Nanopure Water: Sterile nanopure water.

Iodoacetamide: Ultrapure grade of iodoacetamide.

Overlay Agarose: 0.5% low melting point agarose was prepared in a mixture of 25 mM Tris, 192 mM glycine, 0.1% SDS, and a trace of Bromophenol Blue.

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent.

Bio-Lyte[®] 3/10: Ampholytes is a mixture of carrier ampholytes, pH 3 – 10.

3.2.6 In Gel Protein Digestion Solutions

Ammonium Bicarbonate (100mM): 395.3mg ammonium bicarbonate was dissolved and adjusted to a final volume of 50 ml by HPLC water, stored at room temperature.

Ammonium Bicarbonate (100 mM) in 50% Acetonitrile (ACN): 395.3 mg Ammonium Bicarbonate, 25 ml HPLC ACN was brought up to 50 ml by HPLC water, stored at room temperature.

Dithiothreitol (DTT) (10mM): 7.71 mg DTT was dissolved in 5 ml of 100 mM Ammonium Bicarbonate, freshly prepared.

Iodoacetamide (IAA) (50mM): 56 mg iodoacetamide was reconstituted in 6.06 ml ammonium bicarbonate, freshly prepared.

Ammonium Bicarbonate (20mM) in 50% ACN: 10 ml 100 mM Ammonium Bicarbonate, 15 ml HPLC water and 25 ml HPLC ACN, stored at room temperature.

Ammonium Bicarbonate (40mM) in 10% ACN: 20 ml of 100 mM Ammonium Bicarbonate, 5 ml of HPLC ACN and 25 ml HPLC water were mixed and stored at room temperature

Acetic Acid (50mM): 144 μ l Acetic Acid, up to 50 ml HPLC water, stored at room temperature.

Trypsin Solution: 100 μ l 50 mM acetic acid was brought up to 5 ml by 40 mM Ammonium Bicarbonate in 10% ACN, divided into 500 μ l aliquotes and stored at -80°C. Each aliquote can undergo 5 freeze-thaw cycles.

ACN (50%) /TFA (5%): 25 ml HPLC ACN and 2.5 ml TFA were mixed and brought up to 50 ml by HPLC water.

3.2.7 Matrix Preparation for MALDI Sample Spotting

Matrix: 5-10 mg/ml α -Cyano-4-hydroxycinnamic acid (CHCA) was dissolved in ACN: 0.1% aqueous TFA mix, usually at 50:50, vortexed for about 1 min, spinned at 14000 rpm for 1 min to pellet the undissolved matrix, then supernatant was used for sample preparation. After use matrix was discarded.

3.3 METHODS

3.3.1 General Methods

3.3.1.1 Recombinant SIP1 production

Recombinant SIP1 protein was kindly provided by Emre Sayan (Leicester, UK). Briefly the first 360 amino acid part coding region of SIP1 (ZEB2) cDNA was cloned into pET101/D (Invitrogen, Carlsbad, CA) vector with an N-terminal 6-histidine tag. Recombinant protein was expressed in Escherichia coli (BL21) and purified under denaturing conditions using Ni–

NTA resin (Qiagen, Hilden, Germany). Purified protein was then refolded and buffer exchanged to phosphate buffered saline (PBS) using NAP buffer exchange columns (Amersham, Piscataway, NJ). Finally, pure and folded recombinant protein was concentrated (0.5-1 mg/ml) using Centripreps (Millipore, Billerica, MA).

3.3.1.2 Production of SIP1 Monoclonal Antibodies

Recombinant SIP1 protein was injected into the peritoneal cavity of 8 to 10-week-old BALB/c mice at 3 week intervals. During the immunization period, antibody titer of mice sera was evaluated by indirect ELISA. Briefly, ELISA plates were coated by 100 ng of recombinant SIP1 protein in carbonate buffer (pH 9.6). Serially diluted mice sera were assessed for their immunoreactivity with SIP1 protein. Alkaline phosphatase conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was used as secondary antibody. The absorbance of the colorimetric reaction generated upon addition of the substrate para-nitrophenyl-phosphate (Sigma-Aldrich, St. Louis, MO) was measured at 405 nm in an automated plate reader (Biotek Instruments, Winooski, UT). Three days after the final boost, fusion of mouse splenocytes and SP2/0 myeloma cells was performed as previously described (Celikkaya, Ciraci et al. 2007). Hybridoma supernatants were screened by aforementioned indirect ELISA, and hybridomas secreting anti-SIP1 antibodies were subjected to single cell subcloning. Antibody isotype was determined by ImmunoPure Monoclonal Antibody Isotyping Kit (Pierce, Rockford, IL).

3.3.1.3 Production of 6D5 Monoclonal Antibody

For production of mAbs that target HCC, sub-confluent HUH7 cells were harvested by scraping and injected into peritoneal cavity of 6-8 weeks old BALB/c mice. Following two more injections, mice were bled and their sera were assessed for immunoreactivity with HUH7 cells by using cell-ELISA assay. A final boost was given to mice showing highest

immunoreactivity, and after 3 days, these mice were sacrificed, their spleens were harvested, pooled and fused with SP2/0 mouse myeloma cells by using polyethyleneglycol. Cell-ELISA was performed to select positive clones, and hybridomas were sub-cloned by limiting dilution. Isotype of antibodies was determined by using Monoclonal Antibody Isotyping Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

3.3.1.4 Western Blot Analysis

Total cell lysates from cell lines were prepared in NP-40 lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Non-idet P40 (v/v) and a cocktail of EDTA-free protease inhibitors (Roche)]. Protein content was measured by Bradford assay. After protein quantification, protein lysates were aliquoted into fresh tubes and, stored at – 80°C. Equal amounts of cell lysates were solubilized in 5X SDS gel-loading buffer with 5% β -mercaptoethanol (ME), denatured at 100°C for 5 min and incubated on ice for 2 min. After a quick spin, samples were loaded onto SDS-polyacrylamide gel. 10% (15-100 kDa range) resolving gel and 5% stacking gel was used in SDS-PAGE analysis of protein lysates. Mini Protean III (BioRad, Hercules, CA) vertical gel system was set up according to manufacturer's instructions. After electrophoresis at 80 V for 20 min followed by 120 V 53 for 1-2 hr, proteins were transferred onto PVDF transfer membrane with 0.45 μ m pore size (Millipore, Billerica, MA) by using Transblot-Semi Dry (BioRad, Hercules, CA) electroblotting apparatus according to the manufacturer's instructions at 15 V for ~45 min. Membranes were blocked overnight with 5% dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T). Undiluted hybridoma supernatants (6D5, 1C6, 6E5) were used as primary antibody. After washing three times with TBS-T, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody at 1:5000 dilution for 1 hr. The membrane was washed 3 times for 5 min in TBS-T solution at room temperature. After final wash, protein bands were

visualized using ECL Plus chemiluminescent substrate (Amersham). The chemiluminescence emitted was captured on X-ray film within 15 sec to 5 min exposure times.

3.3.1.5 Immunofluorescence Assay

Cells were grown on cover slips in 6 well plates. Phosphate buffered saline (PBS) was used in all washing steps. Cells were fixed in 2% paraformaldehyde in PBS and permeabilized in PBS containing 0.2 % Triton X-100. After blocking with 2% BSA-PBS, cover slips were incubated for 2 hr at room temperature in undiluted 6D5, 1C6 or 6E5 hybridoma supernatant. Fluorescein isothiocyanate (FITC) (Sigma) or Alexa fluor 488-(Invitrogen, Carlsbad, CA) conjugated anti-mouse IgG was used as secondary antibody at 1:200 dilution. Nuclei counterstaining was performed with 4',6-Diamidino-2-phenylindole (DAPI), cover slips were mounted on glass slides and examined under fluorescent microscope (Zeiss GmbH, Germany). Merged images were produced by using AxioVision image processing software (Zeiss GmbH, Germany).

3.3.1.6 Immunohistochemical Staining

For 6D5 and SIP1 immunostaining paraffin blocks containing liver tissues were cut at 4-5 μm thick and tissue sections were mounted on glass slides. For SIP1 immunostaining a total of 123 tissues spotted on three tissue arrays were stained twice by both 1C6 and 6E5 mAbs. Tissue arrays included sections from kidney (22: tumor 18, normal 4), lung (17: tumor 14, normal 3), colon (16: tumor 12, normal 4), uterus (15: tumor 12, normal 3), esophagus (11: tumor 9, normal 2), liver (11: tumor 9, normal 2), breast (11: tumor 9, normal 2), rectum (10: tumor 9, normal 1) and stomach (10: tumor 9, normal 1) tissues. After deparaffinization in xylene and rehydration in graded alcohol series, glass slides were immersed in 10 mM citrate buffer, pH 6.0 and transferred into microwave for 15 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubation of slides in 0.1% H_2O_2 for 30 min. PBS was

used in all washing steps. Tissue sections were incubated for 2 hr with primary antibodies, 6D5 hybridoma supernatant, Ki-67 (DakoCytomation, CA, USA) or AFP (Neomarkers, Fremont, CA) and after washing, universal staining kit (LabVision) was used according to manufacturer recommendations. Aminoethylcarbazol (AEC) or 3,3'-diaminobenzidine (DAB) were used as chromogen, and the slides were counterstained using Mayer's hematoxylin. Cytoplasmic staining of hepatocytes was assessed in entire tissue samples and immunoreactivity was classified into four categories. Positively stained cells were scored as either diffusely positive (D: 50-100 % of the positively stained area), moderately positive (M: 5-50 % of the area), focally positive (F: 1-5% of the area) and negative (N: completely negative). The same scoring was applied for staining with AFP antibody. As for Ki-67 evaluation, samples showing a nuclear staining of more than 10% were assessed as positive, lesser staining intensity was scored as negative. Staining was performed in triplicate for both antibodies. For SIP1 immunoreactivity, the sections were evaluated by light microscopic examination and the intensity of immunostaining in each section was assessed independently by two observers. The intensity of total SIP1 staining on each sample was scored as previously described with a slight modification (Chen, Hsieh et al. 2006). Briefly the staining intensity was graded relatively based on the following scales: 0, 1, 2, and 3 for negative, weak, moderate and strong staining, respectively. An average score was reached as the final score for each tissue with multiple samples. According to the final immunostaining scores, the tissues were classified into four groups: negative group (score 0–0.40), weak staining group (score 0.5–1.4), moderate staining group (scores 1.5–2.4), and strong staining group (scores 2.5–3). (-), (+), (++) and (+++) designations were used for these four groups, respectively. The staining in nuclei and cytoplasm was determined separately on each specimen.

3.3.2 Tissue Culture Techniques

3.3.2.1 Cell Lines

Thirteen hepatoma (HUH7, FOCUS, Mahlavu, Hep40, Hep3B, PLC/PRF/5, SK-Hep1, Snu182, Snu387, Snu398, Snu423, Snu449 and Snu475) and one hepatoblastoma (HepG2) cell lines were cultured as described previously (Celikkaya, Ciraci et al. 2007). For experiments where a defined number of cells were to be seeded, cell counting was performed. Following trypsinization, cells were resuspended in culture medium and counted manually with a hemocytometer.

3.3.2.2 Thawing Cryopreserved Cells

One vial of the frozen cells from the liquid nitrogen tank was taken and immediately put into ice. The vial was left for 1 min on the bench to allow excess nitrogen to evaporate and then placed into 37°C water bath until the external part of the cell solution was thawed (takes approximately 1-2 min). The cells were directly poured into a 15ml sterile tube containing 10ml cold fresh medium. The cells were centrifuged at 1500 rpm at 4°C for 5 min. Supernatant was discarded and the pellet was resuspended in 10ml 37°C culture medium to be plated into 100mm dish. After overnight incubation in a humidified incubator at 37°C supplied with 5% CO₂, culture mediums were refreshed.

3.3.2.3 Growth Conditions of Cell Lines

Focus, Hep40, Hep3B, Hep3B-TR, HepG2, HUH7, Mahlavu, PLC/PRF/5, SK Hep1 cells were cultured in low-glucose DMEM supplemented with 10% FBS, 100U/ml Penicillin-Streptomycin, and 0.1 mM non-essential amino acids (HyClone, Utah, USA). SNU387, SNU398, SNU423, SNU449, SNU475 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin, 0.1 mM non-essential amino

acids (HyClone, Utah, USA). The growth medium was aspirated and the cells were washed once with 1XPBS. Trypsin was added to the flask to remove the monolayer cells from the surface. The fresh medium was added and the suspension was pipetted gently to disperse the cells. The cells were transferred into either fresh petri dishes or fresh flasks using different dilutions (from 1:2 to 1:10) depending on requirements. All media and solutions used for culture were kept at 4°C (except stock solutions) and warmed to 37°C before use.

3.3.2.4 Cryopreservation of Cell Lines

Exponentially growing cells were harvested by trypsinization and neutralized with growth medium. The cells were counted and precipitated at 1500 rpm for 5 min. The pellet was suspended in a freezing solution (10%DMSO, 20%FCS and 70%DMEM for adherent cells) at a concentration of $\sim 4 \times 10^6$ cells/ml. 1ml of this solution was placed into 1ml screw capped cryotubes. The tubes were first frozen at -20°C for 0.5-1 hr and then left at -80°C overnight. The next day, the tubes were transferred into the liquid nitrogen storage tank.

3.3.2.5 Crude Total Protein Extraction

Adherent monolayer cells (both stable and parental cells) were grown to 80% confluency. For the analysis of proteins, cells were collected by trypsinization. Cells were collected by scraping after washing twice with ice-cold PBS to remove any serum residue. Volume of the dry cell pellet was estimated and twice that volume of lysis buffer was pipetted onto the pellet. For nuclear proteins RIPA lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% sodium deoxycholate, 1 % NP-40, 0.1% SDS and 1X Complete Protein Inhibitor mix (Roche Diagnostics, Mannheim, Germany)] was used, and for cytoplasmic and membrane proteins NP-40 lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1X protease inhibitor cocktail (Roche) and 1.0% NP-40] was used. Complete lysis was achieved by pipetting of crude cell lysates several times and by incubating the lysates on ice for 30 min and with continuous

agitation by vortexing at 5 min intervals. Then, the lysate was centrifuged at 10000 rpm for 30 min. Total cell protein was collected as supernatant.

3.3.2.6 Quantification of Proteins

After the cell lysates were prepared, their concentrations were detected by Bradford assay. Briefly, 2 μ l of the samples were diluted with 98 μ l ddH₂O and then 900 μ l of Bradford working solution was added to the samples and mixed well, as described in Table 3.1. Immediately, the protein amounts of the samples were measured at 595 nm versus blank reagent (NP-40 lysis buffer was used as blank). Known concentrations of BSA were prepared according to Table 3.2 as a standard. After reading at 595 nm, samples and standard values were plotted; unknown concentrations were calculated from the standard curve.

Table 3.1 Protein sample preparation for Bradford assay.

	Tube no					
	1	2	3	4	5	6
Sample (μ l)	0	2	2	2	2	2
ddH ₂ O (μ l)	98	98	98	98	98	98
Bradford (μ l)	900	900	900	900	900	900
Lysis buffer (μ l)	2	-	-	-	-	-

Table 3.2 BSA dilutions for standard curve plot.

	Tube no							
	1	2	3	4	5	6	7	8
Sample (μ l)	0	2.5	5	7.5	10	12.5	15	20
ddH ₂ O (μ l)	100	97.5	95	92.5	90	87.5	85	80
Bradford (μ l)	900	900	900	900	900	900	900	900

3.3.2.7 Cell Lines, Tissues Cell Lines, Tissues and siRNA Transfections

Wild-type mouse SIP1 expressing squamous epidermoid carcinoma cell line A431/WTSIP1 with Tet-on doxycycline-inducible SIP1 expression was previously described (Mejlvang, Kriaievska et al. 2007). HCC cell line SK-HEP-1 and colorectal carcinoma cell lines SW480

and SW620 were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU penicillin, 100 µg streptomycin and nonessential amino acids. Multiple Tumor Tissue arrays were purchased from BioChain Institute, Inc. (Hayward, CA). Cells were collected 48 hr after transfection and processed for western blotting.

3.3.2.8 SIP1 Quantitative Real-Time PCR

SIP1 mRNA expression in colon cancer cell lines SW620, SW480 and HCC cell line SK-HEP-1 was determined by quantitative real-time PCR as described previously (Avci, Konu et al. 2008). The expression of SIP1 in cell lines was measured using $\Delta\Delta C_t$ method and normalized to GAPDH gene. The threshold cycle of SIP1 cDNA in SW480 cell line, which showed the lowest expression was set to 1 and relative expression values were plotted as fold changes.

3.3.2.9 6D5 mAb Binding Assay (Epitope Mapping)

A protein microarray (Source BioScience ImaGenes, Berlin, Germany) PVDF membrane (22 x 22 cm) presenting 2 x 27,648 in situ expressed human testis proteins was used for screening of 6D5 mAb targets (epitopes). The protein microarray membrane was placed in a plastic box and rinsed in 70% ethanol at room temperature. Then membrane was washed 2x (~2min) with ddH₂O in order to remove traces of ethanol. After then membrane was rinsed with 25ml TBS-T, and washed by shaking them 3x for 10 min at room temperature. Then membrane was briefly rinsed 2x at room temperature in TBS in order to remove traces of TBS-T. Membrane was soaked for 2 hr at room temperature in blocking solution. After then blocking solution was removed and the 6D5 mAb, as primary antibody, was added into plastic box, agitated overnight at 4°C. Second day, primary antibody was discarded, and membrane was washed 3x 15 min at room temperature in TBS-T. Subsequently membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (Sigma-Aldrich, St. Louis, MO)

secondary antibody at 1:5000 dilution in blocking solution for 1 hr at room temperature. Protein spots were visualized using Super Signal West Dura chemiluminescent substrate (Pierce, Rockford, IL). The chemiluminescence emitted was captured on X-ray film within 15 sec to 5 min exposure times, and films were scanned (Perfection V750 Photo, Epson, Long Beach, CA), then images were merged, and image analysis was performed using an image software (Photoshop CS4, Adobe Systems Inc., San Jose, CA).

3.3.2.10 6D5 Immunoprecipitation with HUH7 Cell Lysate

Protein G beads (100 μ l, 50%) (Pierce) were added to each micro-centrifuge tube. The beads were pelleted at <2000 rpm for 30 seconds and washed in ice cold PBS (1 ml x3) once. 6D5 antibody sera (100 μ l) were combined with HUH7 lysate (30 μ l) in a tube and the mixture was rotated for 1 hr at 4°C. 100 μ l of 50% protein G-agarose slurry were mixed with the mAb and cell lysate mixture, and rotated for 24 hr at 4 °C. Monoclonal antibody culture medium and isotype control antibodies (Invitrogen) were used as control antibodies to evaluate false positivity. The beads were washed with cold PBS (1 ml x3) at 4°C for 30 min under rotation. The agarose beads tubes were centrifuged for 10 sec at 14000 rpm and excess PBS was removed with disposable transfer pipettes. Half of beads were resuspended in gel-loading buffer for one dimensional (1D) electrophoresis. The other half of beads was resuspended in 500 μ l of IEF sample rehydration buffer (Bio-Rad, Hercules, CA) for 2D PAGE running at room temperature for 30 min and gently swirled to maintain the beads in suspension. The supernatant was collected, transferred to a new tube, and centrifuged at 14000 rpm for 2 min.

3.3.2.11 Two-dimensional PAGE and Western Blotting

For 2D PAGE analysis of HUH7 proteins, a modified method was used as described before (Fang, Yi et al. 2006). Extracted HUH7 cell lysates were dissolved in the standard SDS PAGE buffer containing 2% SDS and 5% ME and boiled for 2 min. Solubilized proteins were

precipitated with cold acetone and washed with trichloroacetic acid and ether/ethanol. The final dried protein precipitate was dissolved in the 8 M urea containing IEF sample buffer 1 obtained from Bio-Rad. Adding a small amount of 0.1 M NaOH to the sample buffer to bring the pH to 8 resulted in improved solubilization of proteins. This pH adjustment did not have any noticeable effect on IEF and significantly increased the amount and intensity of protein spots seen in the stained gels. Concentrated supernatant from cell lysate was replaced by a rehydration solution containing urea, CHAPS, DTT, and carrier ampholytes. The tubes were vortexed to ensure that the pellet had dissolved in the rehydration solution and 125 ml of the sample was pipetted into a clean rehydration tray (Bio-Rad). Seven-centimeter precast IPG strips with a pH range of 3–10 (Bio-Rad) were placed gel-side down over each sample, covered with mineral oil, and allowed to sit for approximately 16 hr while they rehydrated. Strips were then transferred to a focusing tray and placed in a programmable IEF unit (Protean II IEF cell, Bio-Rad, CA). IEF was performed at 20°C with a 20-min linear ramp to 250 V, followed by a 2-hr linear ramp to 4000 V, and finally a 10 000 V hr rapid ramp to 4000 V (limit 50 μ A/strip). When focusing was complete, strips were stored immediately at 80°C in sterile tubes. Focused 1D strips were thawed and equilibrated in a buffer containing urea, Tris, SDS, glycerol, DTT and iodoacetamide, with gentle shaking for a total of 20 min. Equilibrated strips were placed on a 7x7 cm 12.5% resolving gel for separation, with a standard-soaked electrode wick with a molecular weight marker (Precision Plus Unstained Standards, Bio-Rad). Glass plates were spaced 1 mm apart and loaded into gel cassette immersed in SDS running buffer. Electrophoresis was carried out in a vertical gel apparatus (Mini-PROTEAN3, Bio-Rad, CA) with the protocol steps as in Table 3.3, and one gel was fixed immediately in a solution containing methanol, acetic acid. Other 2D PAGE gel with separated proteins were transferred to PVDF membranes for western blotting as described before (Eckerskorn, Strupat et al. 1997). Proteins were separated in SDS-PAGE gels identical

to those used for 2D-PAGE. Gels were stained with Coomassie, SYPRO Ruby or silver, and corresponding gel spots with 2D western blots, containing proteins of possible target of 6D5, were cut from the gel for the protein identification by MALDI.

Table 3.3 IPG strips (7 cm) isoelectric focusing.

	Voltage	Time	Volt-Hours	Ramp
Step 1	250	20 min	-----	Linear
Step 2	4,000	2 hr	-----	Linear
Step 3	4,000	-----	10,000 V-hr	Rapid
Total		5 hr	14,000 V-hr	

3.3.2.12 Gel Staining with Coomassie

Coomassie brilliant blue R-250 staining was performed as previously described before (Wilson 1983). After electrophoresis, the gels were stained for 2 hr in Coomassie staining solution with slow shaking, and washed overnight in washing solution containing 10% (v/v) acetic acid and 50% (v/v) methanol. Then staining solution was replaced with destaining 45 solution for 30 min to remove bulk of the excess stain and then the gel was transferred into the destaining solution. Destaining solution was changed twice a day until the background was clear. The gels were rinsed three times with ddH₂O before the image acquisition.

3.3.2.13 Gel Staining with Silver

Silver staining was performed as previously described (Giulian, Moss et al. 1984). The gel was immersed in destain solution for 30 min with gentle shaking. After 30 min Destain was discarded and cross-linking solution was added and again shaken slowly for 30 min. Cross-linking solution was poured off and the gel was washed with ddH₂O several times over 2 hr or

the gel was placed into 2 lt of ddH₂O for overnight storage. The next morning, the gel was washed in fresh ddH₂O for 30 min. The gel was incubated in DTT (dithiothreitol) solution with gentle shaking for 30 min. DTT solution was removed and silver nitrate solution was added and again shaken gently for 30 min. The gel was washed with ddH₂O for two or three times and washed with developing solution for once. Then fresh developing solution was added. Staining had been obtained within 5-10 min. When the desired staining level was reached, development was stopped by replacing the development solution with destain solution. The gels were rinsed three times with ddH₂O before the image acquisition.

3.3.2.14 Gel Staining with SYPRO Ruby

The SYPRO Ruby staining was used as previously described (Berggren, Chernokalskaya et al. 2000). SYPRO Ruby (BioRad) is a commercial ready-to-use solution. Two dimensional gels were fixed using 7% acetic acid and 10% ethanol for 1 hr. Gels were then placed into a tray containing SYPRO Ruby protein gel stain for overnight with continuous gentle agitation. The gels were destained using 7% acetic acid and 10% ethanol for 20 min. Finally, the gels were rinsed three times with ddH₂O before the image acquisition.

3.3.3 Protein Analysis by Mass Spectrometry

Protein spots were excised from the gel before in-gel tryptic digestion (Shevchenko, Wilm et al. 1996) and the resulting peptides were mixed with matrix and spotted on the target plate to analyze by MALDI-TOF mass spectrometry.

3.3.3.1 In-Gel Trypsin Digestion (Coomassie and SYPRO stained gel pieces)

For Coomassie or SYPRO stained gel pieces, in-Gel Trypsin Digestion Procedure was carried out as described before (Gobom, Nordhoff et al. 1999). Target bands or spots excised from 1D or 2D gels were cut into 1 x 1 mm pieces and placed in 1.5 ml tube. Gel pieces were rinsed

with 300 µl HPLC water for 15 min at room temperature. In tubes 300 µl HPLC ACN were added and washed on shaker for 15 min at room temperature. Supernatants were discarded. Gel pieces were washed with 300 µl of 100 mM Ammonium Bicarbonate for 15 min at room temperature on shaker. Supernatants were discarded. 300 µl of 100 mM Ammonium Bicarbonate in 50% ACN were added and washed on shaker for 15 min at room temperature. Supernatants were discarded. 100 µl HPLC ACN was added and washed on shaker for 5 min at room temperature. Supernatants were discarded. Gel pieces were dried in a vacuum concentrator (Univapo 100 ECH, UniEquip GmbH, Germany) for 5 min. 50 µl of 10 mM DTT was added and incubated at 60°C for 1 hr. Supernatants were discarded. 50 µl of 50 mM IAA was added and incubated in dark for 30 min at room temperature. Supernatants were discarded. Gel pieces were washed on shaker with 300 µl of 100 mM Ammonium Bicarbonate for 15 min at room temperature. Supernatants were discarded. Gel pieces were washed on shaker with 300 µl of 20 mM Ammonium Bicarbonate in 50% ACN for 15 min at room temperature. Supernatants were discarded. 100 µl HPLC ACN was added and washed on shaker for 5 min at room temperature. Supernatants were discarded. Gel pieces were dried in a vacuum concentrator centrifuge for 5 min. 20 µl trypsin solution was added and incubated for 1 hr at room temperature. 40 mM Ammonium Bicarbonate in 10% ACN was added to completely cover the gel pieces. Gel pieces were incubated at 37°C overnight. 150 µl HPLC ddH₂O was added on shaker to wash the pieces for 10 min at room temperature. Supernatants were removed and placed in a 0.5 ml tube. The gel pieces were extracted with 50 µl of 50% ACN/5% TFA for 60 min twice at room temperature. Supernatant (extracts) all were collected and pooled and dried in a vacuum concentrator.

3.3.3.2 Matrix Preparation and Samples Spotting on the Target Plate

Three µl of a 10 mg/ml of α -Cyano-4-hydroxycinnamic acid (CHCA) matrix dissolved in 50% ACN and 0.1% TFA solution was mixed with 1 µl of an approximately 10 pmol/µl

sample and 1 µl of this mixture was spotted on the target plate and air-dried by the “dried droplet” technique (Karas and Hillenkamp 1988).

3.3.3.3 MALDI-TOF Mass Spectrometry Analysis

For identification of 6D5 targets, gel spots containing proteins of interest were excised from one or two-dimensional gels stained with either Coomassie or Silver or SYPRO, and submitted to the proteomics laboratories at Ankara University Biotechnology Institute and Marmara University Chemical Engineering Department (Ankara and Istanbul, Turkey) for peptide mass analysis. Samples were analyzed using the MALDI-LR (Waters/ Micromass UK Ltd., Manchester, UK) instrument. After purification/concentration of the tryptic peptides MALDI-TOF peptide mapping was carried out. Spectra were generated using a pulsed nitrogen gas laser (337 nm) in positive linear mode, with a low mass gate of 10000 Da. The accelerating voltage was 15 kV. External calibration was performed using a peptide mixture (ADH, BSA) as molecular weight standards for purposes of mass correction. Three replicated measurements were performed on each sample. TOF-MS spectra were generated from the sum of 100-200 laser pulses and mass determinations were done by finding the peak centroid of a smoothed signal (by Savitzky-Golay algorithm) after background subtraction. Spectra were obtained randomly over the surface of the matrix spot at laser intensity determined by the operator. A S/N ratio of threshold of 10 was used for peak selection. Peak masses and intensities of TOF spectra were detected and collected in the “enhance all” modus and charged ions of peptides were chosen manually for product ion analysis. Product ion spectra (tandem MS) were acquired with collision energies optimized for each peptide to obtain an effective fragmentation pattern with 10% intensity of the residual precursor ion. Data were blasted against the sequence databank using ProteinLynx Global server 2 software (Waters/Micromass UK Ltd., Manchester, UK). Data also were searched by the tandem mass spectrum database matching tool Mascot (<http://www.matrixscience.com>). Positive protein

identifications were assigned on the basis of combined ion scores (calculated by the software) that exceeded the calculated deprecated protein identification threshold.

3.3.4 Statistical Analysis

For comparison of two groups with equal sample numbers, built-in t-test statistics was used in MS Excel (using two-sample with unequal variance, with a two tailed distribution mode).

4 RESULTS

4.1 Expression profile of monoclonal antibodies

4.1.1 SIP1 Monoclonal Antibodies (6E5, 1C6) Expression Profile

Two mAbs, clones 1C6 and 6E5 were obtained by immunizing BALB/c mice with a partial human SIP1 recombinant protein (aa 1-360). The isotypes of antibodies were IgG2a and IgG1, respectively.

4.1.1.1 Monoclonal Antibodies 1C6 and 6E5 Detect Overexpressed SIP1

The specificity of these new SIP1 mAbs were also validated in the inducible cell line system A431 containing mouse WTSIP1. By using both antibodies in immunofluorescence assay, we detected nuclear expression of SIP1 in these cells maintained in the presence of doxycycline for 24 hr (Fig. 4.1). These results showed that the new SIP1 mAbs are specific and able to detect SIP1 in endogenous levels and when overexpressed.

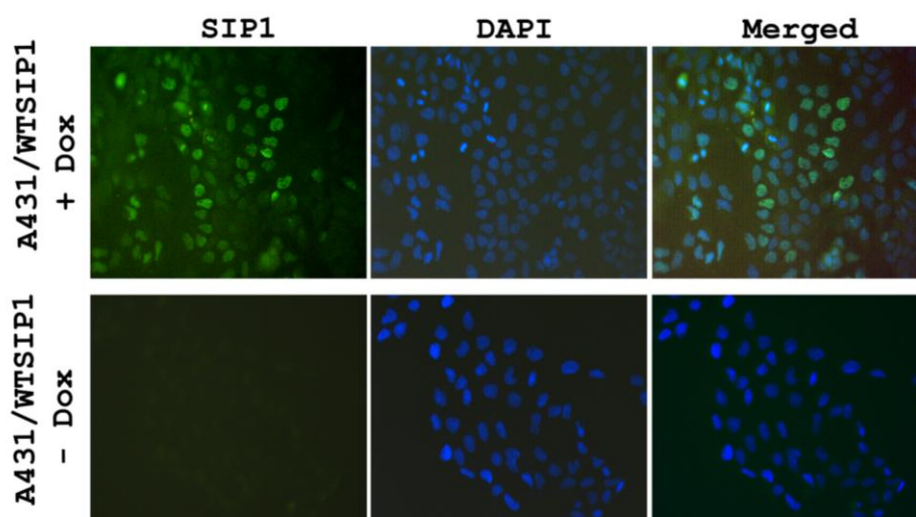


Figure 4.1 SIP1 induced expression detected by 1C6 and 6E5 mAbs. A431/WTsip1 cells maintained in 2 μ g doxycycline for 24 hr and stained with 6E5 mAb displayed nuclear SIP1 expression (first row), whereas no staining was observed in un-induced cells with the same antibody (second row).

4.1.1.2 Cytoplasmic Expression of SIP1 in Tissue

Next, the tissue expression pattern of SIP1 protein was analyzed by staining formalin-fixed and paraffin-embedded tissue arrays with mAbs 1C6 and 6E5. 22 normal and 101 tumor tissues were examined by immunohistochemistry and all samples displayed similar reactivity upon staining by both clones. The SIP1 immunostaining pattern of tissues was summarized in Table 4.1. No immunoreactivity was observed in tissue arrays stained with mouse IgG1 and IgG2a isotype control antibodies.

The majority of tissues displayed cytoplasmic staining of SIP1 and nuclear expression of SIP1 was observed only in 6 cases consisting of one normal and one tumor tissues of rectum and four normal colon samples.

Table 4.1 Immunohistochemistry results of SIP1 expression in human tissues

Tissue (n=123)	SIP1 Expression (Mean staining intensity in positive cases)		
	Cytoplasm	Nucleus	SIP1-positive structure
Kidney			
Tumor (n=18)	+++ (17/18)	-	Tumoral cells
Normal (n=4)	++ (4/4)	-	Proximal-distal tubules
Lung			
Tumor (n=14)	+ (10/14)	-	Tumoral cells
Normal (n=3)	-	-	Surfactant ++(3/3)
Breast			
Tumor (n=9)	+ (5/9)	-	Tumoral cells
Normal (n=2)	-	-	-
Uterus			
Tumor (n=12)	+ (5/12)	-	Tumoral cells
Normal (n=3)	-	-	-
Liver			
Tumor (n=9)	++ (9/9)	-	Tumoral cells
Normal (n=2)	+++ (2/2)	-	Hepatocytes
Stomach			
Tumor (n=9)	+ (4/9)	-	Tumoral cells
Normal (n=1)	+++ (1/1)	-	Glandular cells
Colon			
Tumor (n=12)	+ (4/12)	-	Tumoral cells
Normal (n=4)	++ (4/4)	+ (4/4)	Surface epithelium
Rectum			
Tumor (n=9)	+ (7/9)	++ (1/9)	Tumoral cells
Normal (n=1)	++ (1/1)	++ (1/1)	Surface epithelium
Esophagus			
Tumor (n=9)	+ (5/9)	-	Tumoral cells
Normal (n=2)	++ (2/2)	-	Squamous epithelium

(-): negative, +: weak, ++: moderate, +++: strong staining

4.1.1.3 Differential Expression of SIP1 in Human Tumors

SIP1 is overexpressed in tumors of the kidney, lung, breast and uterus. 1C6 and 6E5 antibodies stained both proximal and distal tubules of kidney, yet the reactivity of the latter was more intense. Compared to the tubular epithelium-restricted expression of SIP1 in normal kidney, SIP1 was extensively expressed in kidney tumors. Out of 18 tumors, 17 clear cell carcinomas displayed strong cytoplasmic staining with both antibodies (Fig. 4.2 A-B), and one transitional cell carcinoma case remained negative. Relative to their normal tissues, which failed to display SIP1 expression, 71% of lung, 56% of breast and 42% of uterus tumors showed cytoplasmic SIP1 positivity, yet with a weak intensity (Fig. 4.2 C-H).

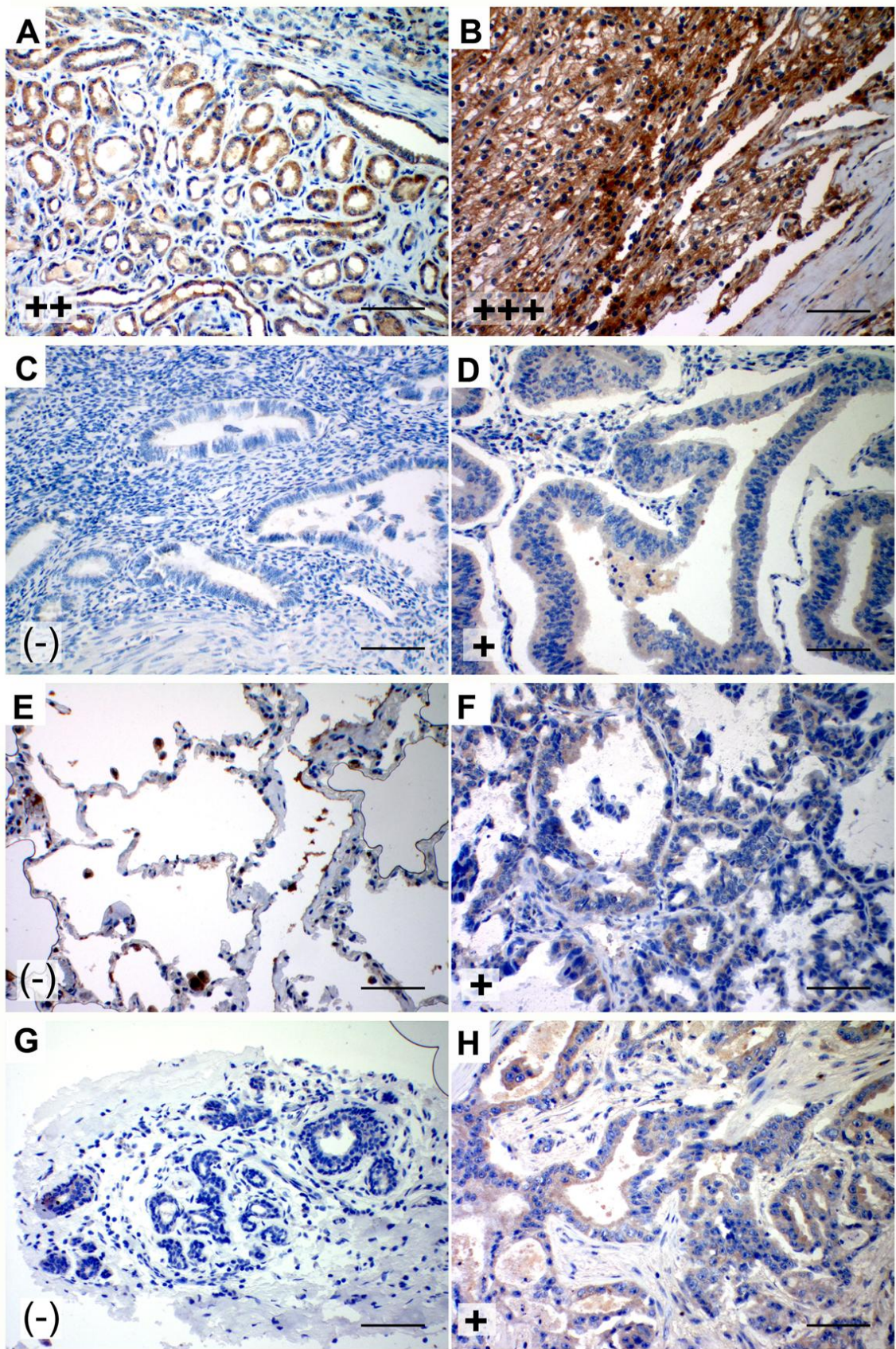


Figure 4.2 Increased expression of SIP1 in kidney, lung, breast and uterus tumors.

Representative photographs show increased SIP1 expression in tumors relative to their normal tissues as detected by immunohistochemistry performed by both antibodies. (A) Distal tubule staining in normal kidney, (B) clear cell renal carcinoma, (C) normal uterus, (D) adenocarcinoma of the uterus, (E) normal lung with non specific surfactant staining, (F) squamous cell carcinoma of the lung, (G) normal breast, (H) breast ductal carcinoma. (-): negative, +: weak, ++: moderate, +++: strong staining intensity. (Scale bars: 50 μ m)

4.1.1.3.1 Downregulated Cytoplasmic SIP1 in Most of the Human Tumors

The cytoplasm of all 9 HCC cases displayed a moderate intensity of SIP1 expression, which could not reach however the strong staining pattern of SIP1 in normal hepatocytes and tumor-adjacent cirrhotic tissues (Fig. 4.3 A, B). Eight of nine stomach adenocarcinomas were weakly positive for SIP1 expression, a pattern far beyond the intense SIP1 staining of glandular cells of normal stomach (Fig. 4.3 C, D). Apical crypt epithelia of 4 normal colon samples displayed cytoplasmic staining with both antibodies with moderate intensity, and a faint nuclear SIP1 expression was also observed in these cells. However, only 4 of 12 colon tumors were weakly positive for cytoplasmic SIP1 (Fig. 4.3 E, F). In tissue arrays, only one normal rectum sample was available, and lumen-facing epithelial cells of this tissue were found to express SIP1 mainly in their nuclei and to a lesser extent in their cytoplasm. Irrespective of its cellular localization, SIP1 expression was of moderate to strong intensity in normal rectum. On the contrary, 78% of rectum tumors were stained, but with weak immunoreactivity (Fig. 4.3 G, H). The dominant cytoplasmic staining pattern of these cancer tissues was accompanied by moderate nuclear staining in only one case, which was the tumor with most advanced stage among others. Tumor cells of 5 squamous cell carcinoma of the esophagus expressed SIP1, yet with a weaker intensity than squamous epithelium of normal esophagus, which was stained with moderate intensity by both antibodies (Fig. 4.3 I, J).

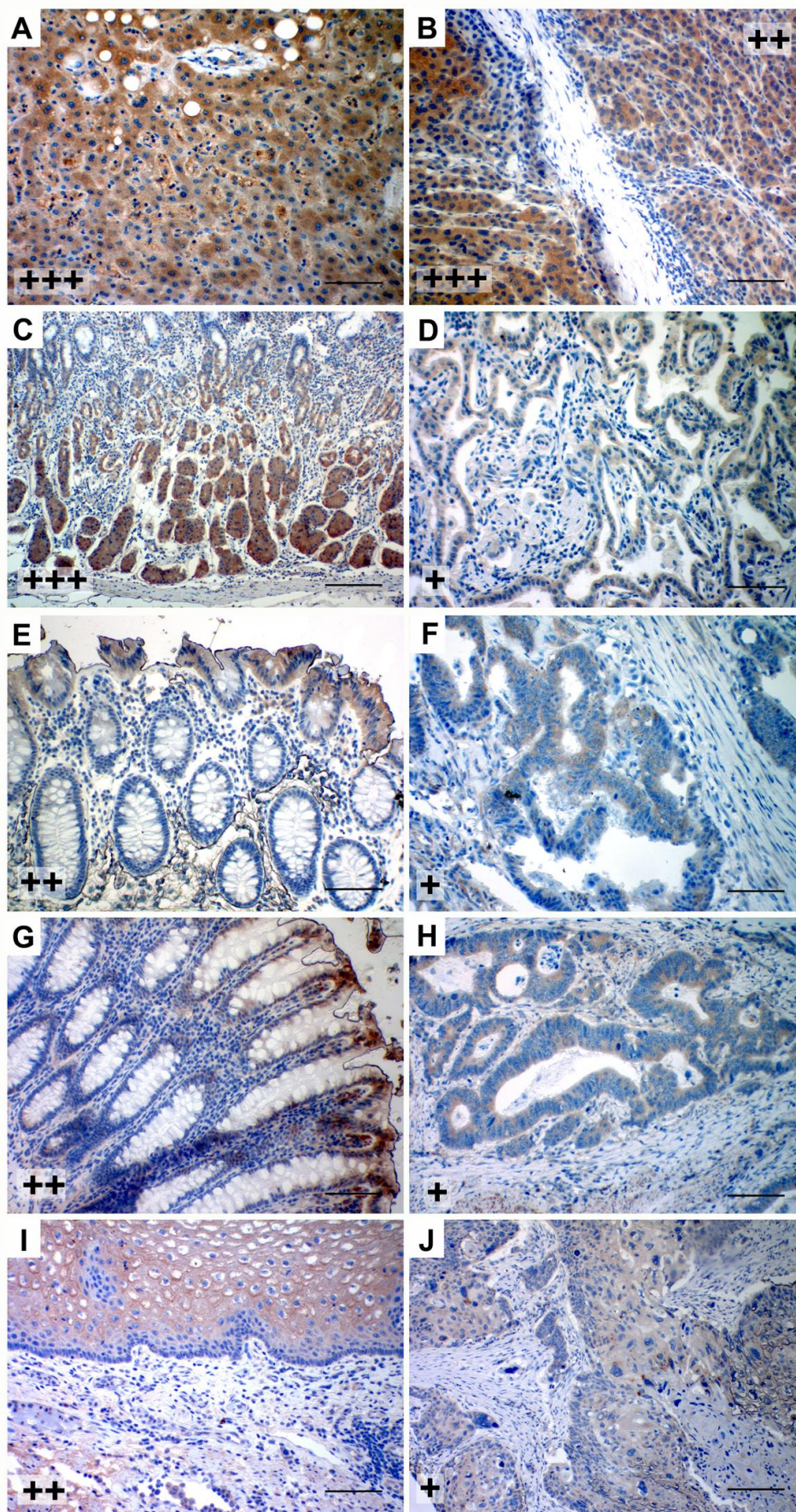


Figure 4.3 Reduced expression of SIP1 in liver, stomach, colon, rectum and esophagus tumors. Representative photographs show decreased SIP1 expression in tumors with respect to their normal tissues. (A) Normal liver, (B-right) hepatocellular carcinoma (HCC) of the liver and (B-left) the adjacent cirrhotic tissue, (C) normal stomach gland cells, (D) adenocarcinoma of the stomach, (E) normal colon surface epithelium, (F) adenocarcinoma of the colon, (G) normal rectum surface epithelium, (H) adenocarcinoma of the rectum, (I) normal esophagus squamous epithelium, (J) squamous cell carcinoma of the esophagus. +: weak, ++: moderate, +++: strong staining intensity (scale bars: 50 μ m).

4.1.1.3.2 Cytoplasmic SIP1 Immunoreactivity in Tumor Cell Lines

Cytoplasmic SIP1 expression in the majority of human carcinomas prompted us to validate this observation in two colon cancer cell lines (SW620 and SW480) and one HCC cell line (SK-HEP-1). Subcellular fractionation was done and the presence of SIP1 protein was analyzed by western blotting. A protein band with strong intensity at the expected size (190 kDa) of SIP1 was observed in the nuclear fraction of the invasive HCC cell line SK-HEP-1 (Fig 4.4 A). Weaker protein bands of the same size also appeared in the cytosolic fraction of SK-HEP-1, and in both cytosolic and nuclear fractions of SW620 and SW480 cells. However, SIP1 expression was barely detectable in total cell lysates. Besides, SIP1 antibody immunoreactive bands at about 120 kDa and lower molecular weights were also observed in the nuclear extracts and total cell lysates of all 3 cell lines (Fig 4.4 A). These results were in accordance with real-time quantitative PCR data in which SK-HEP-1 was the cell line with most abundant SIP1 transcript (Fig. 4.4 B).

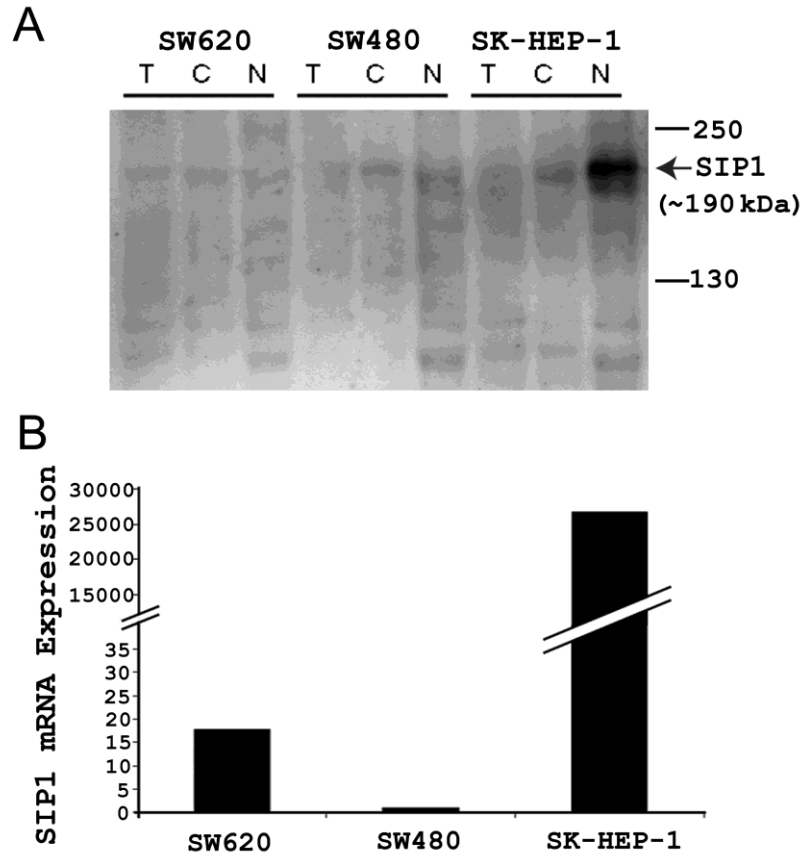


Figure 4.4 Nuclear and cytoplasmic expression of SIP1 protein in cell lines. (A) A strong SIP1 expression at 190 kDa in the nuclear fraction of SK-HEP-1 cells is seen in western blotting experiment with clone 1C6 mAb. The antibody also detects bands with lower molecular weight proteins in nuclear (N) and total cell lysates (T) of all 3 cell lines. SIP1 expression is more abundant in the cytoplasmic (C) but not nuclear extracts of SW480 and SW620 cells. SK-HEP-1 cells express similar pattern for the cytoplasmic SIP1 immunoreactivity. (B) SIP1 transcripts showed the highest SIP1 expression in SK-HEP-1 cell line with qRT-PCR. SIP1 transcript levels in SK-HEP-1 and SW620 cells are represented as fold changes with respect to SW480 reference cell line.

Overall, our results indicated that SIP1 was widely expressed in most normal human tissues that we examined, with moderate to strong intensities, and the overexpression of SIP1 was confined only to a restricted group of human tumors.

4.1.2 6D5 Monoclonal Antibody

4.1.2.1 Generation of 6D5 Monoclonal Antibody

In order to explore proteins differentially expressed in HCC, we immunized mice with HUH7 cells. The heterogeneous characteristics of HUH7 cells make these cells ideal immunogen for the identification of novel markers for liver tumors, as well as the characterization of liver precursor cells involved in hepatocellular carcinogenesis. For instance, we isolated two isogenic clones from this cell line, which kept tumor cell proliferative capacity and underwent senescence in standard culture conditions, respectively (Chu, Ishizawa et al. 2002; Ozturk, Erdal et al. 2006). Out of 127 clones generated by cell fusion, 18 were assessed as positive by cell-ELISA, and supernatants of these clones were evaluated in western blot and immunofluorescence experiments. We selected 6D5 antibody for further studies to investigate its potential as a diagnostic and predictive biomarker. The isotype of clone 6D5 mAb was found to be IgG3 (κ).

4.1.2.2 6D5 Recognizes Differentially Expressed Proteins in HCC Cell Lines

We first analyzed the immunoreactivity of 6D5 antibody with whole cell lysate of HCC cell lines. Positive protein bands were observed in three separate western blot experiments (Fig 4.5 A, B, C). Cell lines derived from undifferentiated and invasive tumors (SNU series, SK-Hep1) seemed to express 6D5 ligands stronger than differentiated cell lines (HUH7, HepG2). However, no other correlation could be established between the protein expression patterns and phenotypic characteristics of cell lines.

Another interesting finding was the differential protein expression in two isogenic cell lines, namely Hep3B and Hep3B-TR (Fig. 4.5 C). These cell lines have been described elsewhere, and Hep3B-TR cells differ from Hep3B cells by their resistance to the growth inhibitory

effect of TGF-beta in cell culture (Wright, Kreikemeier et al. 2007). In the same study, it has also been proven that Hep3B-TR cells are devoid of TGF- β Receptor Type II expression. Proteins were also found to be abundantly expressed in all studied cell lines, since 6D5 detected ligands in western blots performed with total protein content as low as 1 μ g/lane.

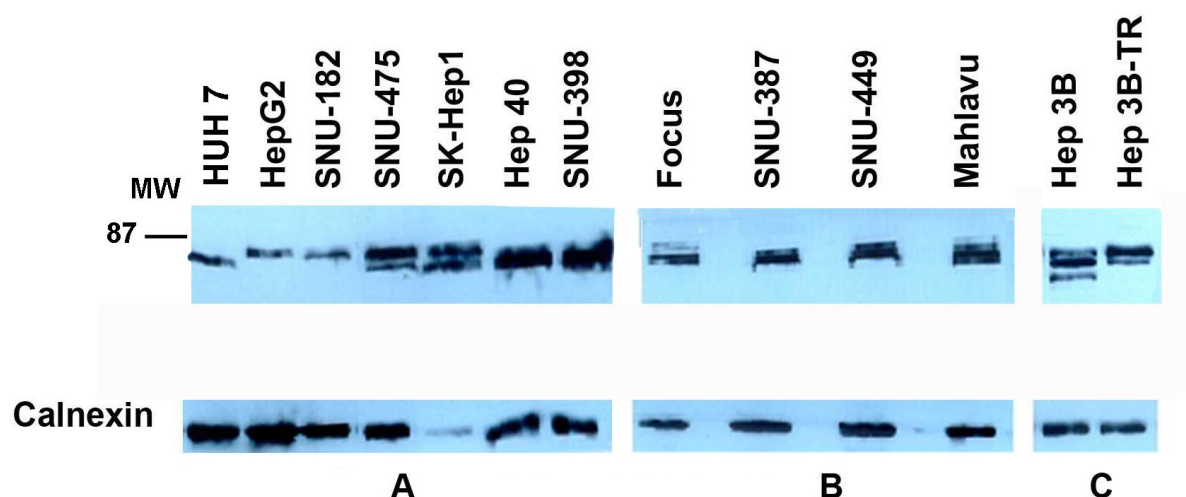


Figure 4.5 6D5 immunoreactivity in HCC cell lines. Target proteins appear to be expressed stronger in poorly differentiated cell lines in three separate western blot experiments.

4.1.2.3 6D5 Stained the Cytoplasm of HCC Cell Lines

Next, we visualized the cellular localization of 6D5 antigens by immunofluorescence microscopy. 6D5 stained the cytoplasm of immunogen HUH7 and other HCC cell lines as well. The strong intensity of the fluorescent signal verified our observation that proteins recognized by this antibody were abundantly expressed in HCC cell lines (Fig. 4.6).

Based on the above *in vitro* data, we planned to explore immunohistological staining pattern of 6D5 antibody in human liver tissue samples to examine whether 6D5 proteins were differentially expressed in distinct clinico-pathological settings.

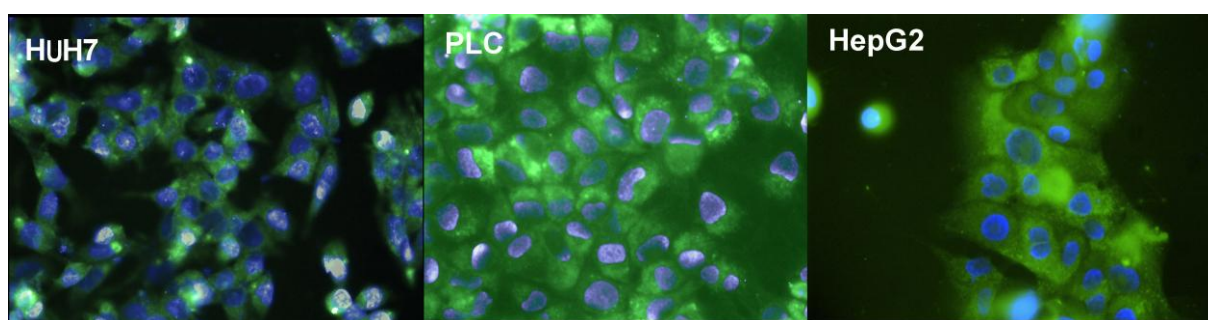


Figure 4.6 Cellular localization of 6D5 mAb targets. Examination of HCC cell lines by immunofluorescence microscopy revealed strong cytoplasmic fluorescent signals.

4.1.2.4 6D5 Antibody Failed to React with Non-Tumoral Liver Tissues

The immunohistological reactivity of 6D5 monoclonal antibody was evaluated in formalin fixed and paraffin embedded human liver tissue samples (Table 4.2).

Table 4.2 Characteristics of human liver tissue samples

Diagnosis	Age $\pm$ SD	Sex	
		Male	Female
HCC (n=14)	66,66 $\pm$ 12,82	7	5
Well differentiated (n=2)			
Moderately differentiated (n=10) ^a			
Poorly differentiated (n=2)			
Cirrhosis (n=46)	52,63 $\pm$ 16,56	9	15
Active Cirrhosis (n=26)			
HBV (n=6)			
HCV (n=3)			
Unknown (n=17) ^b			
Inactive Cirrhosis (n=8)			
HCV (n=2)			
Unknown (n=6)			
Primary Biliary Cirrhosis (n=9)			
Secondary Biliary Cirrhosis (n=3) ^b			
HBV Carrier (n=10)	31,20 $\pm$ 18,94	9	1
Chronic Active Hepatitis B (n=15)	29,66 $\pm$ 06,89	12	3
Mild (n=5)			
Moderate (n=5)			
Severe (n=5)			
Chronic Active Hepatitis C (n=15)	66,66 $\pm$ 12,82	8	7
Mild (n=5)			
Moderate (n=5)			
Severe (n=5)			
Steatohepatitis (n=11)	42,00 $\pm$ 16,76	9	2
Normal (n=19)	33,87 $\pm$ 15,12	16	3

^a: two patient's sex and age are not found in data

^c: one patient's age is not found in data

We examined cytoplasmic staining of hepatocytes in 116 non-tumor cases and found no reactivity in 106 samples (92%) (Fig 4.7).

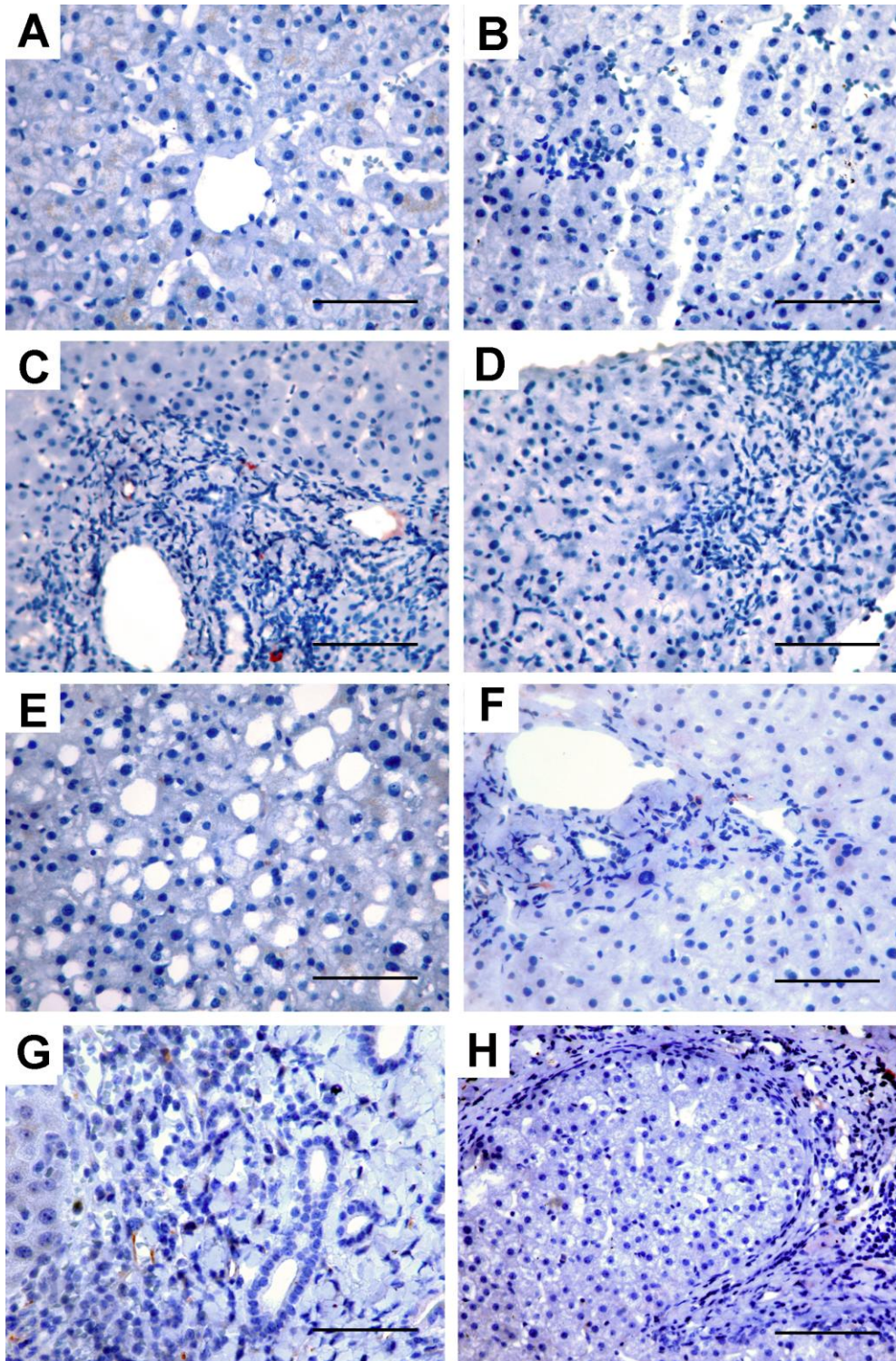


Figure 4.7 No immunoreactivity with 6D5 mAb in non-tumoral liver tissues. Representative images of normal liver (A), HBV carrier (B), chronic active hepatitis C (C), chronic active hepatitis B (D), steatohepatitis (E), secondary biliary cirrhosis (F), primary biliary cirrhosis (G) and reactive changes (H) samples are shown. (Scale bars for all images 50 μ m)

The remaining 10 samples (9%), consisting of 2 normal liver, 2 steatohepatitis, 1 HBV hepatitis, 1 HCV hepatitis and 4 cirrhosis cases, were stained with the antibody. Of these positive samples, only one active cirrhosis case displayed diffuse staining, the remaining 9 samples being stained focally. Interestingly, stained hepatocytes were located at periportal and perivenular areas, suggesting liver tissue reactivity against inflammatory conditions, since these zones are well known as sites of hepatocyte regeneration from precursor cells (Yoon, Choi et al. 2004). We also observed focal staining of portal capillary endothelial cells and portal bile duct epithelial cells in 28 (24%) and 8 (7%) samples, respectively. However, these cells were assessed as reactive because of their focal and restricted staining, as well as their distinct morphology (Fig. 4.8). The staining of aforementioned cell types in non-HCC cases is presented in Table 4.3.

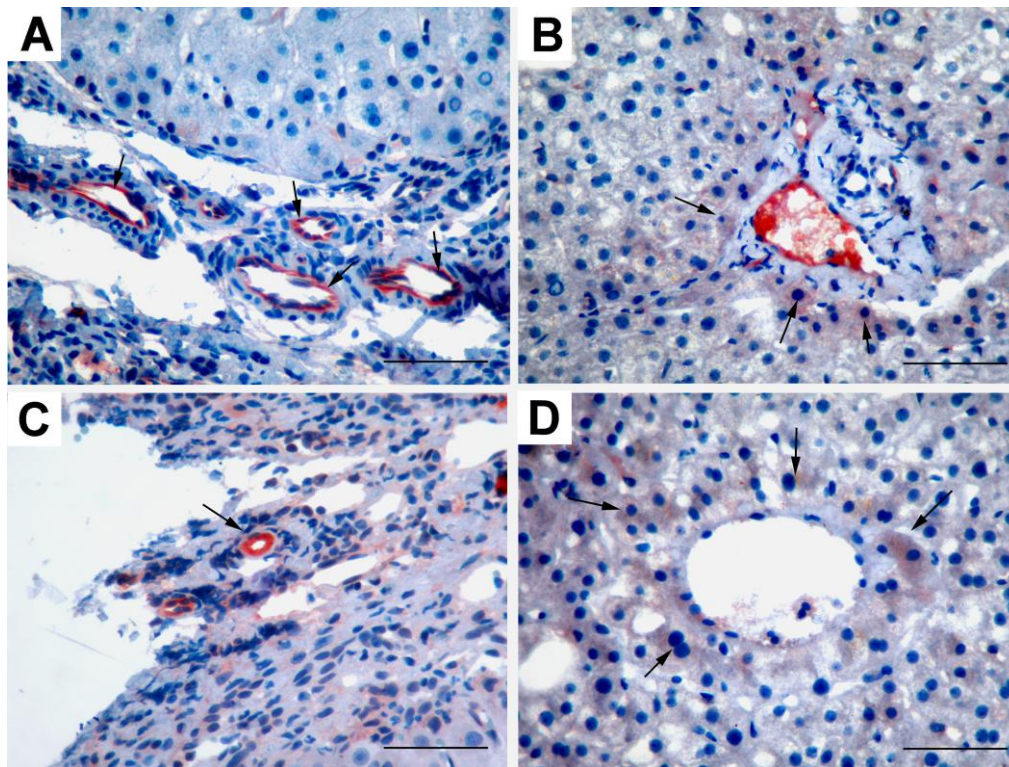


Figure 4.8 Focal staining of non-HCC samples with 6D5 mAb. In some non-neoplastic liver cells, some capillary endothelial cells (A) at portal tract, periportal/perivenular (B), bile duct epithelial cells (C) and lobular hepatocytes (D) showed focal staining (arrows). (Scale bars for all images 50 μ m)

Table 4.3 Staining of Non-HCC cases with 6D5 monoclonal antibody

Diagnosis	# Stained Cases		
	Reactive Endothelial		Reactive Bile Duct
	Hepatocytes	Cells	Epithelial Cells
Normal Liver (n=19)	2	1	1
Steatohepatitis (n=11)	2	4	1
HBV Carriers (n=10)	0	0	0
HBV Hepatitis (n=15)	1	1	0
HCV Hepatitis (n=15)	1	3	1
Cirrhosis (n=46)	4	19	5
Total (116)	10 (9%)	28 (24%)	8 (7%)

4.1.2.5 Diffuse and Strong Staining with 6D5 Monoclonal Antibody in Tumoral and Non-Tumoral Areas of HCC Cases

Next, we continued our immunohistochemical analyses in tumor tissues deriving from 14 HCC patients. 12 of the cases had also clinically diagnosed for cirrhosis. Two well-differentiated, 9 moderately differentiated and 3 poorly differentiated tumors were examined both in tumoral and adjacent non-tumoral areas, and cytoplasmic staining of cells with 6D5 antibody was assessed. Tumor cells were stained with 6D5 antibody in all studied HCC samples, (100%) (Fig. 4.9). Out of 14 tumor samples, diffuse, moderate and focal positivity were observed in 7 (50%), 4 (29%), and 3 (21%) cases, respectively. However, we could not establish any correlation between the differentiation state and staining pattern of tissues because of the small size of our tumor samples. 12 samples also displayed focal staining of endothelial cells (86%), in accordance with tumor neovascularization phenomenon. The high occurrence of reactive endothelial cells in HCC was not surprising, as tumor development is a culmination of tissue inflammation. However, bile duct epithelial cells were absent in all cases due to the loss of the portal tract organization in tumoral zones of HCC samples.

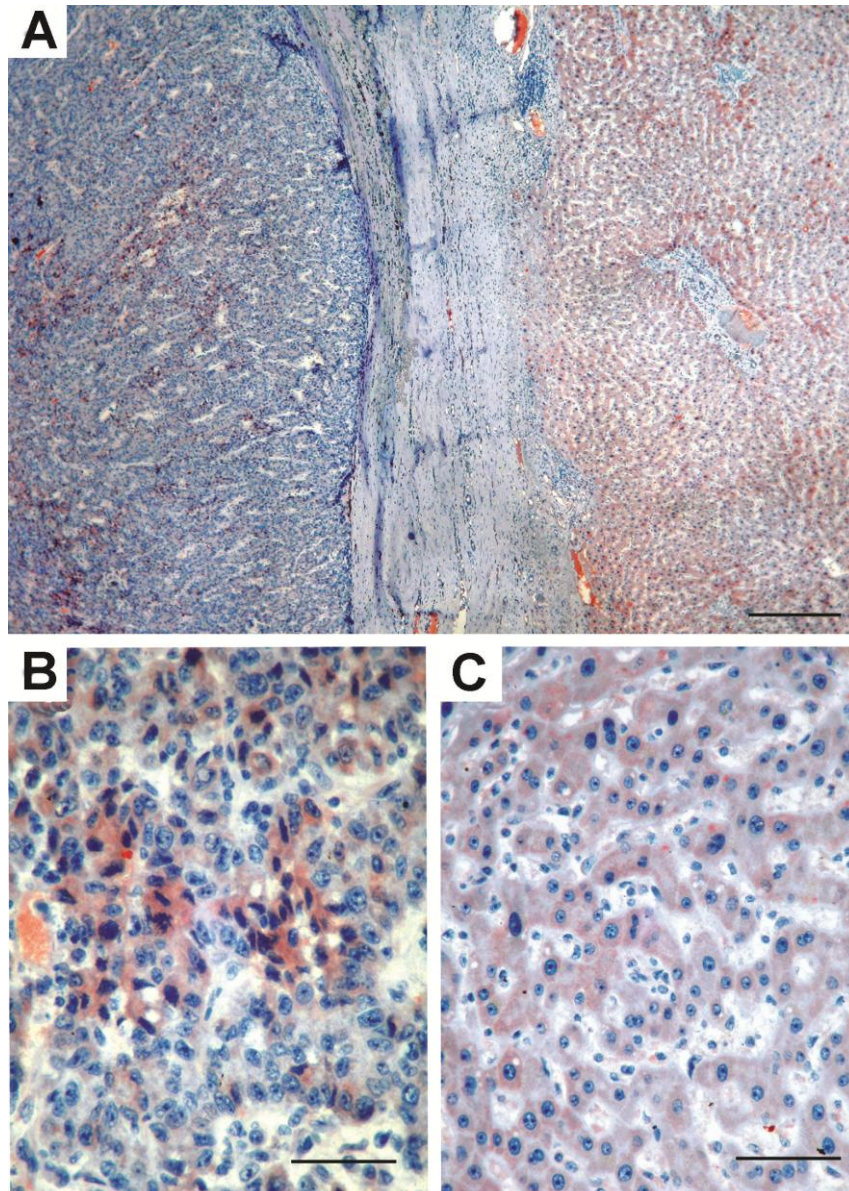


Figure 4.9 Tumor cells stained with 6D5 mAb in HCC samples. Diffuse and strong staining with 6D5 was observed both in tumoral (A-left) and tumor-adjacent tissue areas (A-right) of HCC cases. Higher magnification of tumoral cells (B) and adjacent tissue (C) are seen in detail. (Scale bar: 250 μ m for A and, 50 μ m for B and C)

As for the examination of hepatocytes in non-tumoral areas of HCC samples, we evaluated 13 cases, since 1 moderately differentiated HCC was lacking non-tumor tissue. Unlike the non-reactivity of hepatocytes in samples deriving from normal liver and benign liver diseases, peripheral tissues of HCC cases displayed variable reactivity with 6D5 antibody (10/13;

77%). Most of the cases (8/13) were scored as diffusely positive (62%); moderate and focal positivity was observed for 1 case each (8%) and 3 cases remained completely negative (23%) (Table 4.4).

Table 4.4 Staining scores of HCC cases with 6D5 monoclonal antibody

Case No	Diagnosis	Differentiation	Staining Score				
			Tumor		Non-tumoral area		
			Tumor cells	Endothelial cells	Hepatocytes	Endothelial cells	Bile duct epithelial cells
1	HCC + Cirrhosis	Moderate	F	F	ND	ND	ND
2	HCC + Cirrhosis	Moderate	F	F	F	F	N
3	HCC + Cirrhosis	Moderate	M	F	D	F	F
4	HCC + Cirrhosis	Poor	F	F	N	F	N
5	HCC + Cirrhosis	Moderate	D	F	D	F	N
6	HCC + Cirrhosis	Moderate	D	F	D	F	F
7	HCC + Cirrhosis	Moderate	M	N	D	N	N
8	HCC + Cirrhosis	Moderate	D	F	D	N	N
9	HCC + Cirrhosis	Well	D	F	D	F	F
10	HCC + Cirrhosis	Well	D	F	N	N	N
11	HCC + HCC	Moderate	D	F	D	ND	ND
12	HCC + Cirrhosis	Moderate	M	F	D	N	F
13	HCC + HCC	Poor	M	F	N	ND	ND
14	HCC + Cirrhosis	Poor	D	N	M	N	N

Staining scores expressed as D;diffuse, M:moderate, F: focal, N:negative ND: not determined.

In tumor periphery, the staining of endothelial cells and bile duct epithelial cells could only be evaluated in 11 cases. Endothelial cell reactivity was found in 6 of 11 cases (55%) and was significantly lower than that of tumoral area (86% vs. 55%, $p<0.05$), yet remained higher than that of non-HCC cases (55% vs. 24%, $p<0.05$). However, bile duct epithelial cells were more restrictedly stained (4/11; 36%) than endothelial cells, but 4.5 fold more reactive than ductular epithelia of normal liver and benign liver disease samples (36% vs. 8%, $p<0.05$)

When evaluated along with our non-HCC tissue staining data, these results strongly suggest that 6D5 antibody recognized proteins overexpressed in HCC in a specific manner. Diffuse and strong immunoreactivity was also observed in non-tumoral liver tissue adjacent to tumoral area, suggesting a crosstalk between HCC and tumor microenvironment.

4.1.2.6 6D5 Reactivity Refers to Liver Tumorigenesis not Cellular Proliferation

Next, in order to evaluate the usefulness of our antibody as an HCC specific tissue marker, we compared 6D5 staining pattern with those of AFP and Ki-67 antibodies in a restricted group of patient tissues consisting of 8 cirrhotic and 4 HCC samples. None of the samples reacted with AFP antibody, except one secondary biliary cirrhosis tissue, which stained focally. Variable Ki-67 reactivity (5% - 20%) was observed for all 4 HCC cases (Fig 4.10) and the same secondary biliary cirrhosis sample, which had shown AFP positivity. However, 6D5 diffusely stained 3 HCC and focally stained 1 HCC cases. On the other hand, our antibody reacted in a diffuse manner with one active cirrhosis sample, which failed to be stained with neither of the AFP and Ki-67 antibodies (dysplastic nodule). Moreover, the secondary biliary cirrhosis tissue that was positive both for AFP and Ki-67 (Fig 4.11), remained negative with 6D5 staining (Table 4.5). All together, these data implicate that 6D5 ligand is not a non-specific proliferation marker but rather a protein expressed in early stages of liver tumorigenesis.

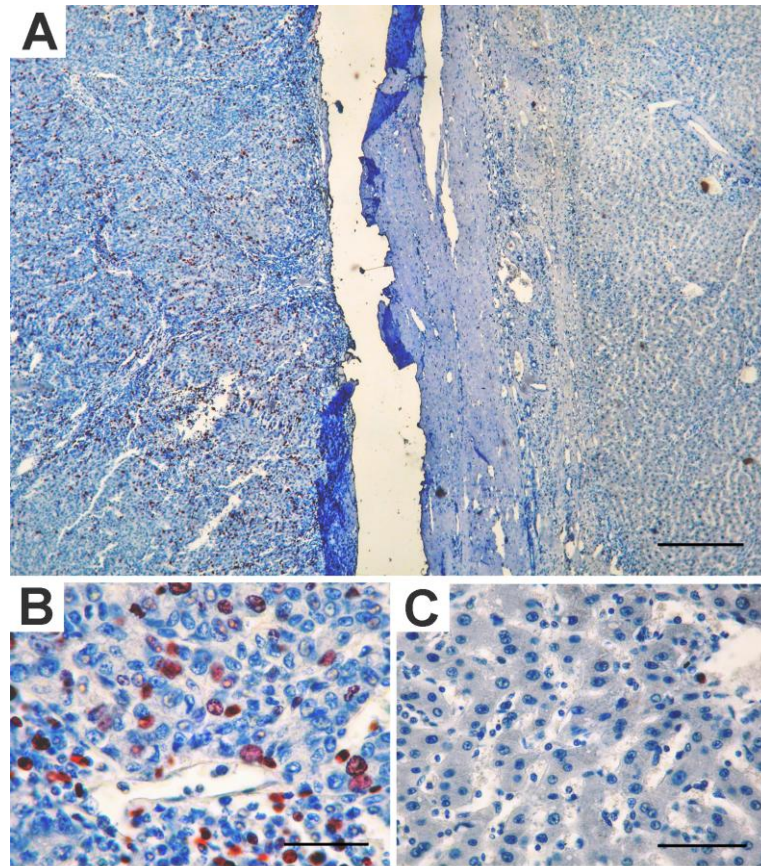


Figure 4.10 Tumor cells and adjacent non tumoral area staining with Ki67 in HCC samples. Ki67 positivity is seen in tumor (A-left) and tumor-adjacent tissue (A-right) showed no immunoreactivity with Ki67 antibody. Higher magnification of tumoral cells (B) and adjacent tissue (C) are seen in detail. (Scale bar; 250 μm for A and 50 μm for B and C)

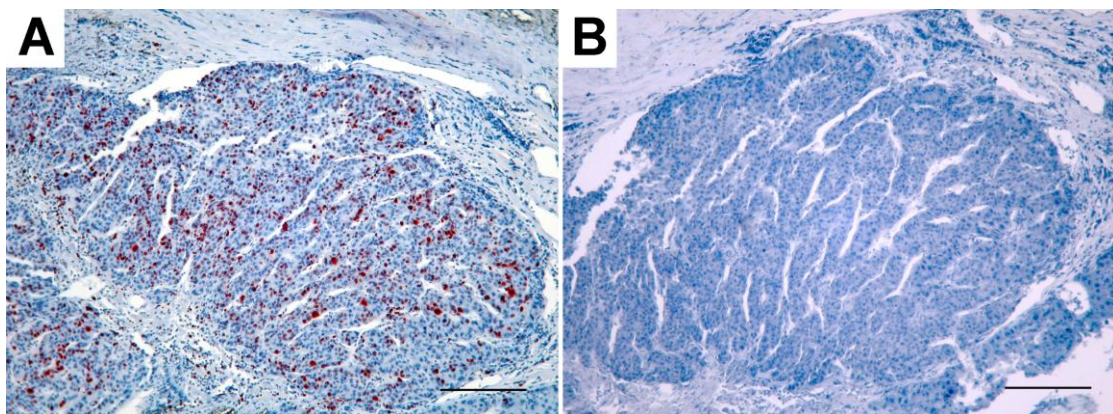


Figure 4.11 Secondary biliary cirrhosis tissue staining with Ki-67 and AFP. Ki67 positivity is seen in nodular structure of the cirrhotic tissue (A), in contrast AFP staining is not seen in the same section (B). (Scale bars; 200 μm)

Table 4.5 Comparison of 6D5 staining with Ki-67 and AFP reactivity

Case No	Diagnosis	Staining					
		Tumor			Non-tumor		
		6D5	Ki-67	AFP	6D5	Ki-67	AFP
1	Macronodular Cirrhosis				N	N	N
2	Macronodular Cirrhosis				N	N	N
3	Active Cirrhosis				D	N	N
4	Active Cirrhosis				N	N	N
5	Inactive Cirrhosis				N	N	N
6	Active Cirrhosis				F	N	N
7	Active Cirrhosis				N	N	N
8	Secondary biliary Cirrhosis				N	P	F
9	HCC + Cirrhosis	D	P	N	D	N	N
10	HCC + Cirrhosis	D	P	N	D	N	N
11	HCC + Cirrhosis	F	P	N	N	N	N
12	HCC	D	N	N	N	N	N

Staining scores expressed as D;diffuse, M:moderate, F: focal, N:negative ND: not determined, P: positive staining for Ki67.

4.1.2.7 Characterization of 6D5 Monoclonal Antibody Target Proteins

4.1.2.7.1 Epitope Mapping

A protein microarray (Imagenes, Germany) containing bacterial clones expressing human testis tissues recombinant His-tagged proteins derived from cDNA libraries was screened with the 6D5 mAb. The probing with 6D5 antibody revealed four big, four middle, and three small size positive spot clones on PVDF membrane (Fig. 4.12). After analyzing spot coordinates, six of them were selected because of their high reactivity. Sequencing of these 6 spot clones by Imagenes revealed cDNA sequences inserted into pQE plasmids (Fig. 4.13). These cDNAs were browsed in BLAST to identify potential peptide sequences and eventually the epitopes of the 6D5 mAb. For each clone, conceptually translated proteins from all six reading frames

were assessed. The lengths of the translated reading frames were calculated, and all amino acid sequences were used to search the combined protein sequence databases for homologous sequences, using the BLAST server at the National Center for Biotechnology Information (NCBI). The raw sequences of clones were read out of the sequencer by Imagenes. One of these sequences is represented in Fig. 4.14. One of them translated a peptide sequence that is shown Fig. 4.15. These six sequences were also aligned in CLC workbench software (www.clcbio.com) to draw a consensus sequence (Fig. 4.16) and the consensus sequence was submitted to NCBI BLAST database. Consensus sequence hit the candidate targets (Table 4.6). Pair-wise deduced amino acid sequence comparison was done by using Clustal W software (Thompson, Higgins et al. 1994). Phylogenetic analysis of our six isolates sequences was performed by using CLC workbench software (www.clcbio.com).

Table 4.6 Candidate 6D5 mAb targets identified by analysis of protein microarray

Acc Name	Protein Description	Function
ZFYVE1	Zinc finger FYVE domain-containing protein 1	Membrane trafficking and cell signaling
TJP2	Tight junction protein 2 (zona occludens 2)	Encodes a zonula occludens-the membrane-associated guanylate kinase
NT5C1B	Cytosolic 5'-nucleotidase 1B isoform 5	Catalyze production of adenosine
RDH14	Retinol dehydrogenase 14 (all-trans/9-cis/11-cis)	Exhibits an oxidoreductive catalytic activity towards retinoids
ZXDA	Zinc finger, X-linked, duplicated A	Encodes one of two duplicated zinc finger genes on chromosome Xp11
QRICH1	Glutamine-rich protein 1	Glutamine-rich putative transcriptional adaptor protein (TIG-1)

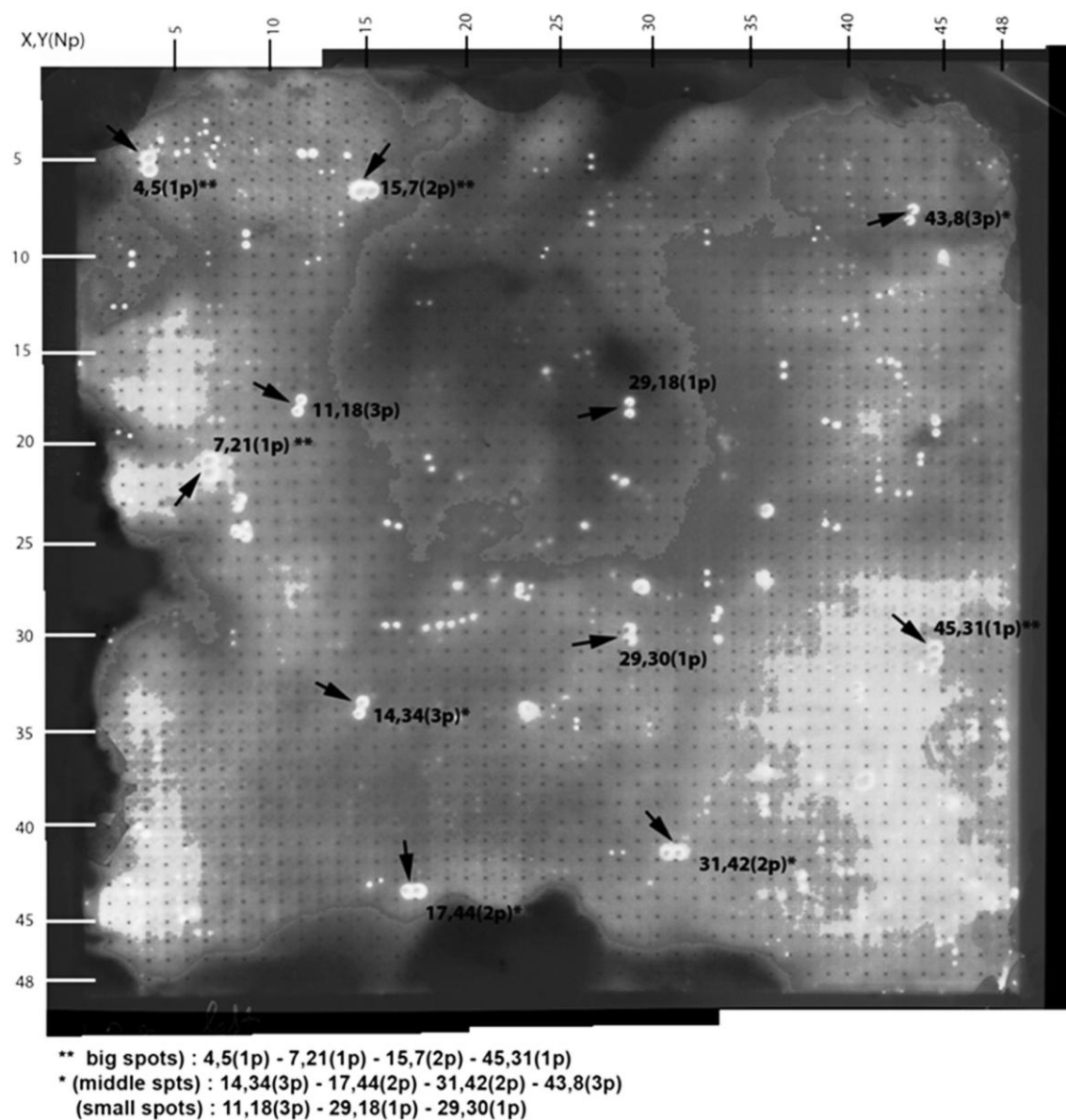


Figure 4.12 Western image of peptide microarray displaying the entire cDNA library expressed proteins. 6D5 mAb probing of the PVDF membrane resulted positive signals (arrows). Coordinates of eleven spots (X,Y) and duplicate pair motifs (p) are seen. A total six clones consisting of four big and two middle size spots (14,34 and 17,44) were selected for sequencing of cDNAs.

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TTCTGCTGCAGCCCTTCCGGGTGCTGATGTGACTGTGGAACACGAGGCCGAAGAGACTGAGGCGTCTCTCACCTTGGTGCCTTGGAGAGG
AGCCAGGATTCGGGTGGCCACATGGCCCTGGGGCCAGGCCCCCTGGCACTGGCCTGTGATGATGGATTCTGAATTGAACATAAAAAAGGGG
ACGAGGTGGGGGGGCTGACCTAACCCAGCACCCCTTTAAAGCAATTCCCTCTGGAGAACAGGGGTT

>C6587DKFzP827I0614-RZPDTypeIII-IV.scf      797      0      797      SCF
ATGAGAGGATCGCATCACCATCACCATCACGGTCGACCCACGCGTCCGCGGGCCAGCGCGGAGCGCCACGCTCGGGTCCGGGGCGGGCTGA
CGCCCGCCGCGCCGCGGGAGGAGGACAAAGGGGTGGGTCTCCGCGGGTCGGCACCCCGCGGCTTGGGCTCGCGGTGAGAGCACTGTCCGGT
GGTGGCCAGGAGGAGTAGGAGCAGGAGCAGAAAGCAGAAAGCGGGTCCGGAGCTGCGCGCTACGCGGGACCTGTGTCCGAATGCCGGTGGC
AGGAGACCGCGGGTTCCACCCCGCGGGGAGCTGTGAGTGGCTCCGCGCCCGAGGCATGGAAGAGCTGATATGGGAACAGTACACTGTGA
CCCTACAAAAGGATTCCAAAAGAGGATTGGAATTGCAGTGTCCGAGGAGCAGAGACAACCCCACTTTGAAATGGAGAAACGTCATTTGTC
ATTTCTGATGTGCTCCCGGTGGGCTGCTGATGGGCTGCTCCAAGAAATGACAGAGTGGTCAATGGCACCCCATGGAGGATGT
GCTTCATTGTTGTCAGTTCAGCAGCTCAGAAAAAGTGGGAAGTTCGTGCTATTGTGGTCAAGAGGCCCGGAAAGTCCAGGTGGCCGCAC
TTCAGGCCAGCCCTCCCTGGATCAGGATGACCGGGCTTTGAGGTGATGGACGAGTTTATGGCAGAAAGTTCCCGAGTGGCTACAGCGAG
AAGAAGCGGGTGAACAGCCATGGGGGCGCAGCCGCACTGGGAAGACAGCCCGGAAAGG

>C6588DKFzP827O1413-RZPDTypeIII-IV.scf      787      0      787      SCF
ATGAGAGGATCGCATCACCATCACCATCACGGTCGACCCACGCGTCCGACGGCCCCACACTACACCGACCGCCATCCACCTCACAGATC
GCCTACACCTCCCTCAGCTCGCCCACTATGGCTCAGCCTTCCCTCCATCTCCCGCCCCAGTTTGACTACTCTGACCATCAGCCCTCAGG
ACCTATTATGGCCATCCGGGCCAGGCTCTGGCTCTACTCGGCTCTCTCTATATGGGGCCCTCGCAGCGGCCCTCTACAGGCCATCT
CTGACCCAGCCCTCAGGGCCCCAGTCCACAGCCCCACACTGGGAGCAGCCAGTATATACGACACTGTCCCGGCCCTAAAGGGGGCCC
TGTGCGCCACACCCCGCCAGCCCTGCCCCCGCGCGATCCGCATGCGAGCTCGATTAGCTGAGCTTGGACTCCTGTGATAGATCCA
GTAATGACCTCAGAACTCCATCTGGATTGTTCAGAACGCTCGGTTGCCCGGGGCTTTTTTATTGGTGAGAATCCAAGCTAGCTTGGCGA
GATTTTCAGGAGCTAAGGAAGCTAAATGGAGAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTG
AGGCATTTCACTCAGTGTGCTCAATGTACTATAACAGACCGTTCACTGGATATTACGGGCTTTTTTAAAGACCGTAAAGAAAAATAGCA
CAAGTTTATCCGGCTTTTATTCACATCTTGCCCGCTGATGAATGCTCC

>C6589DKFzP827E0811-RZPDTypeIII-IV.scf      778      0      778      SCF
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GAGCTGGCCAGCGAGCCTCGGACCCATTTGCTGTGTTGGCCAGCCTGCTGTGCTGCTCAGCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCT
GCTTGGCCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
GGAAGCGGAGAACCTGCCAGCTGATGAGGAAGGCCAACACGGTGGCCTCTGTGCTGCTGGACGTGGCCCTGGGGCTCATGTGCTGCTC
TGGCTCCACGGGAGAAGCCGATCGGGCATCTGGCCGACGCGCTCGTTCCTGTGGCTGACACGTTGGCCGAGGAGCTCCAGCATCTGCTGCA
GTGGCTGATGGGTGCTCCCGCGGGCTCAAGATGAACCGTGCACCTGGAGCAGGTGCTGGGCCGCTTCTTCTCTACACATCCACCTTGGGA
TCAGTACATCCACCTCATGTCCCTTCTGTTGGAGCACATCCTTTGGCAGTGGGCCCTCTCGGCCGCTGGGCTGACGTTGGCCCTGTCC
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GTCTCACTGTGGCGTCTGTTCCGGGAAGAAGTGAACGTC

>C6585DKFzP010-RZPDTypeIII-IV.scf      873      0      873      SCF
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AGGCTGGGGCAGGGCCCTCCACCTACGAGGGCCAGCACAGTCTGGGCAACCAACCGCGCGGGCGGTTTTGCTACAGCCCCACCGCGCGG
GCACCGTCTGCAGCTGCTGCGTGCAGACCCAAAGGCTCCGGTCAGCGCGCGCGCGCTCCCTATAGTGAGTGTATTACGCTTAATTAGCT
GAGCTTGGACTCCTGTTGATACATCCAGTAATGACCTCATACTCCATCTGGATTGTTTCAAGACGCTCGGTTGGCCCGGGCGGTTTTTAT
TGGTGACAAATCAAGCTAGTTTGGCGAGATTTCCGGAGCTAAGGAAGCTAACATGGAGAAAAAATCACTGGATATACACCGCTGATATA
TCCCAATGGCATCGAAAAGAACATCTGAGGCATTTCACTGCTGTTGCTCAATGTACTATAACAGACCGCTCACTGGATATTACGGCCTT
TTTAAAGACCGTAAAGAAAAATAAACACAAGCTTTATCCGCGCTTTATTCACATTTCTGCCCGCTGATGAATGCTATCCGGAATTACGT
AAGGCAATGAAAGAACGCGAGCTGGGGATAAGGGACAGTGTTCACCTTGTACCCGTTTTCTGGAGCAACTGAAACGTTTTCTTCGCC
TGGAGTGGAAACCACGACGATTTCCGGAGTTTCTACCATTTTTTCCCGAGAGGGGGGGTTCGGGGGAAACCTGCCCAATTTCCCAA
AGGGGTTTATGAAAATAAGTTTCTCTCCCCCAATCCAGGGG

```

Figure 4.13 Six clones of cDNAs revealed by sequencing the positive spots. Positive spot coordinates were sent to the company (Imagenes). Target clones were analyzed from the microarray bacteria library. Insert clones were sequenced, and shown in six rows.

```

>DKFZp827J1010-ZPDTypeIII.scf
CAAAAATTTATTTAATAATCGAATCATCCTATACAATACTGCGTATCACGAGAGCCCTTTCTCTCT
CTCCCTCGACACATCATAAAAAATTTATTTGCTTTTCGTTGCAGCGGATAACAATTATAATAGATT
CCATTGTGAGCGGATAACAATTTACACAGAATTCATTAAAGAGGAGAAATTAACCTATGATAGGA
TCG CATCACCATCACCATCAC GGATCCTATTTAGGTGACACTATAGTACATCCTCCACCCACGCG
TCCGCGGTACAGAAAGTTAGGCTGGGGCAGGGCCCTCCACCTACGAGGGGCCAGCACAGTCTGGG
CAACCACACCGGCGGGCCGTTTTGCTCACAGCCCCACCGCGCCGGGCACCGTCTGCAGCTGCTGC
GTGCGAGACCCAAAGGCTCCGGTCAGCGCGCCGGCCGCTCCCTATAGTGAGTCGTATTACGCTTA
ATTAGCTGAGCTTGGACTCCTGTTGATACATCCAGTAATGACCTCATAACTCCATCTGGATTGT
TCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGACAATCCAAGCTAGCTTGGCGAGATT
TTCCGGAGCTAAGGAAGCTAACATGGAGAAAAAAATCACTGGATATACCACCGCTGATATATCCC
AATGGCATCGAAAAGAACATTCTGAGGCATTTTCAGTCTGTTGCTCAATGTACCTATAACCAGACC
GCTCAACTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAACACAAGCTTTATCCGCG
CCTTTATTACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTACGTAAGGCAATGAAAGAA
CGCGAGCTGGGGATAAGGGACAGTGTTACCCCTTGTTACCCCGTTTTCTGGAGCAACTTGAAAC
GTTTCCTTCGCCTGGAGTGGAACACGACGATTTCCGGAGTTTCTCACCATTTTTTCCCCAGAA
GGGGGGGGGTTCCGGGGGAAAACCTGCCCAAATCCCCCAAAGGGGTTTATGAAAATAAGTTTTCCT
CCCCCCCCAATCCCAGGGG

```

Figure 4.14 Representative 6D5 mAb target clone cDNA sequence with insert explanation. The raw sequences of the clone, read out of the sequencer, are seen. Part of the vector sequence is indicated as HisTAG in yellow and clone insert in grey color.

```

>DKFZp827J1010-RZPDTypeIII.scf.frame.1
QKFI**SNHPIQYCVSREPFPLSLDTS*KIYLLSLQRITIIIDSIVSG*QFHTEFIKEEKLTMIG
S HHHHHH GSYLGDTIVHPPPTRPRSQKLGWGRALHLRGPSTVWATTPAGRFAHSPTAPGTVCCSCC
VRDPKAPVSAPAAPYSESYYA * LAELGLLLIHPVMTS*LHLDLFRTLGCRRAFFIGDNPS*LGEI
FRS*GS*HGEKNHWIYHR*YIPMASKRTF*GISVCCSMYL*PDRSTGYYGLFKDRKEK*TQALSA
PLFTFLPA**MLIRNYVRQ*KNASWG*GTVFTLVTPFSWSNLKRFLRLEWKPRRFPEFLTIFSP
GGGFRGKPAQIPQRGL*K*VFLPPQSQGVHPPPTRPRSQKLGWGRALHLRGPSTVWATTPAGRFA
HSPTAPGTVCCSCCVRDPKAPVSAPAAPYSESYYA

```

Figure 4.15 Representative in-frame translation of an insert clone. Reading frame analysis of cDNA sequences was done for possible expressed peptide sequence. In the translated peptide sequence HisTAG is indicated in yellow, first stop is in red color.

T-COFFEE, Version_5.56 Fri Feb 1 01:16:45 WEST 2008
 Cedric Notredame
 CPU TIME:26 sec.
 SCORE=19

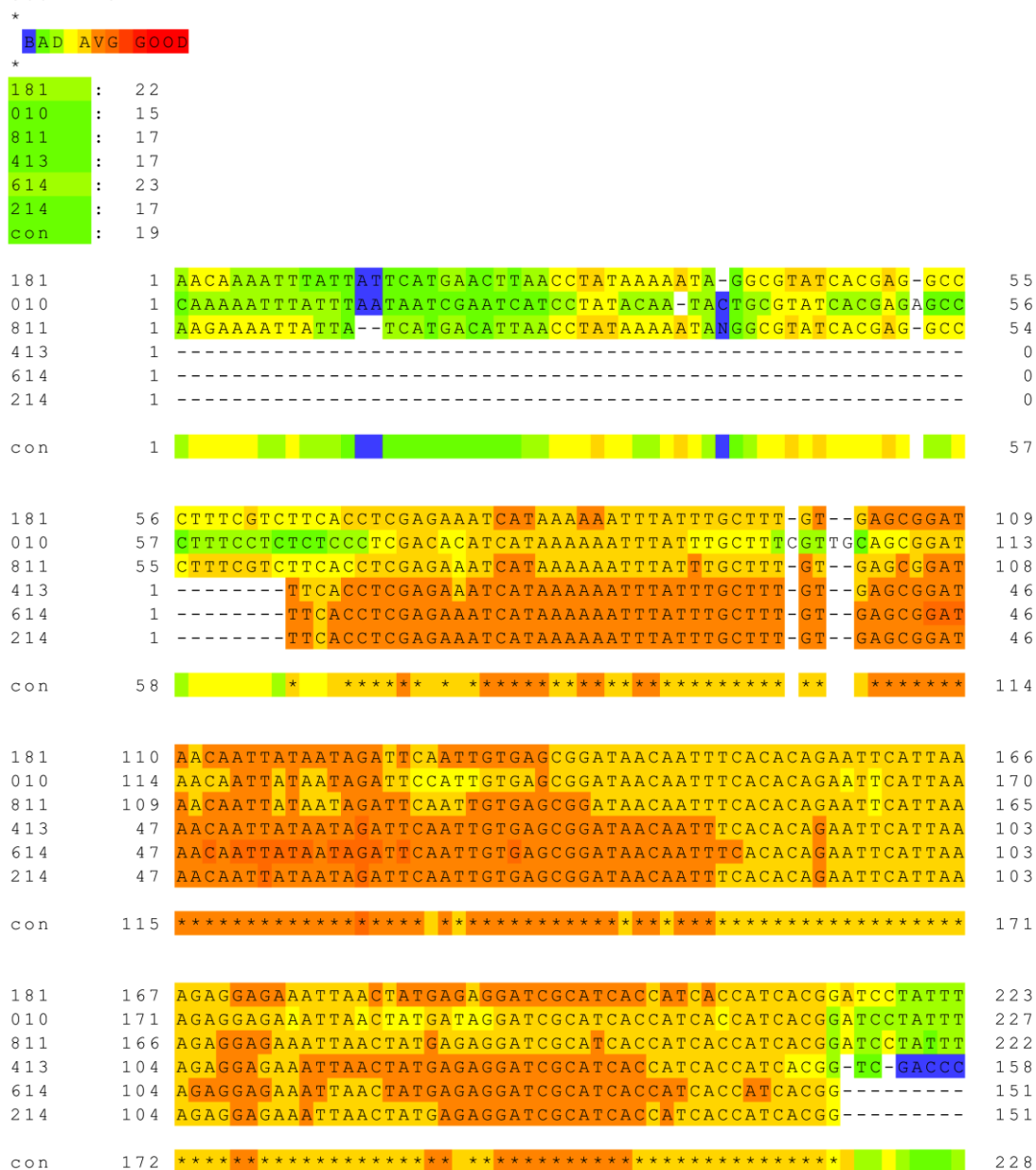


Figure 4.16 Consensus sequences of cDNAs of targets clones. Parts of six clones of cDNA sequences were seen as 181, 010, 811, 413, 614 and 214 (left side). Alignment of these six sequences resulted consensus nucleotides (stars). Good consensus for these sequences were indicated with dark red color.

4.1.2.7.2 2D Electrophoresis

As an alternative approach we performed 2D gel electrophoresis of proteins immunoprecipitated with 6D5 mAb. We also blotted a replica of these gels and probed it with the 6D5 antibody. Proteins detected in 2D gel and western blotting were then compared, and matching protein spots were excised for mass spectrometry analysis (Fig 4.18).

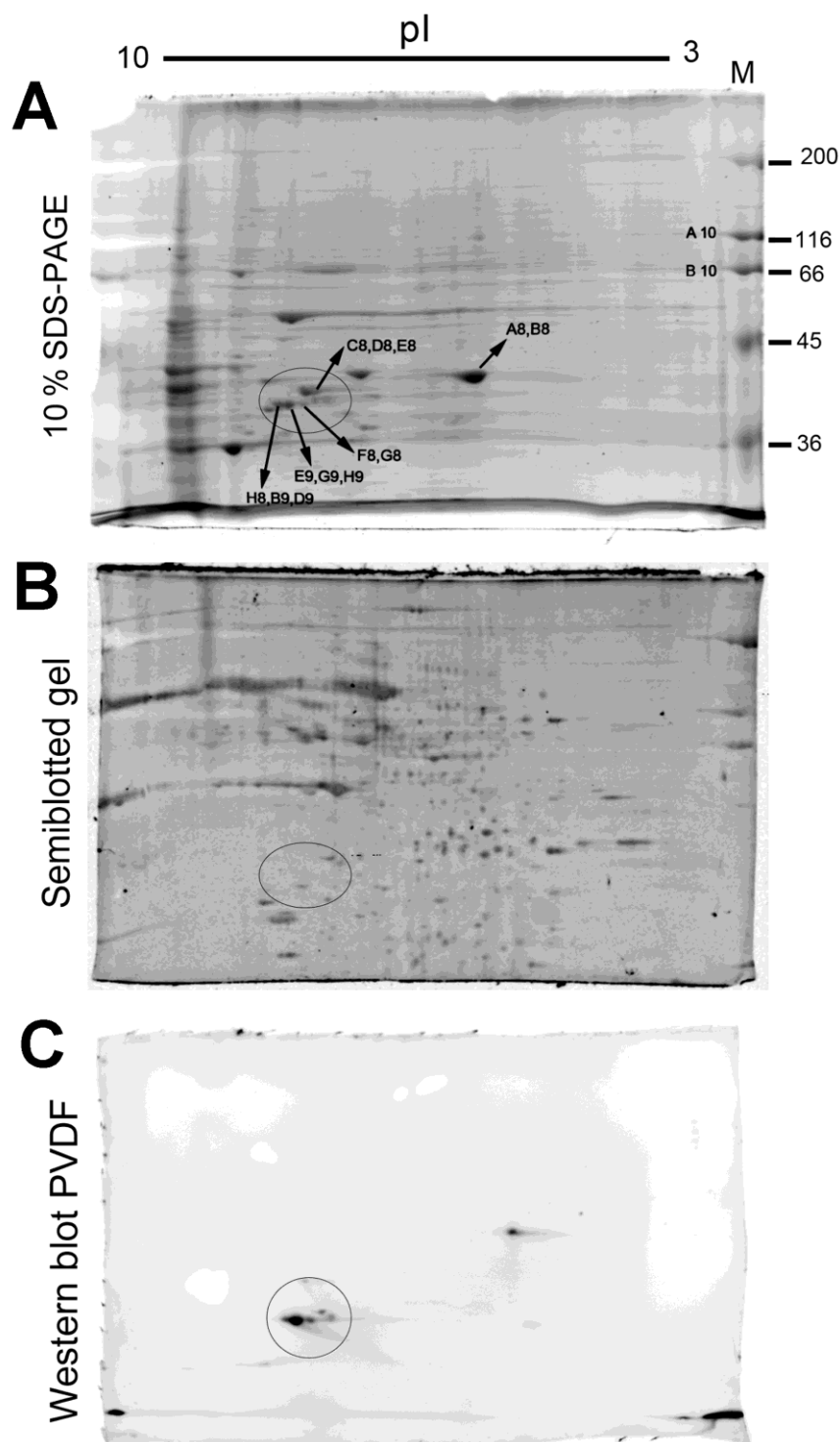


Figure 4.17 2D-PAGE and western analysis of HUH7 proteins. Immuno-precipitated HUH7 total proteins were loaded onto two identical pH 3–10 IPG strips and separated in into two 2D-PAGE. One of the gels was stained with SYPRO (A), the other 2D gel (B) was semi-blotted on the PVDF membrane and then stained with SYPRO. PVDF membrane was probed with 6D5 mAb (C). Possibly target spots of 6D5 mAb is shown as H8, B9, D9, E9, G9 and H9 in 10% SDS-PAGE-SYPRO stained gel. In this gel two of spots from markers (A10, B10) extracted as external control for MALDI-TOF analysis. C8, D8, E8, F8, and G8 spots were cut as controls of the 6D5 mAb targets.

4.1.2.7.3 MALDI Analysis

We extracted and separated the 6D5 immunoreactive protein spots by SDS-PAGE, digested it with trypsin, and subjected it to mass spectroscopic analysis to identify amino acid sequence by peptide mass fingerprinting. We obtained MS spectras from the tryptic peptides. For reliable proteomic identification, we selected high-score peptide sequences with only proteins with a confidence score of more than 99%, and BLASTp comparison of de novo sequences with the NCBI nr mammalian protein database. Data were also blasted against the sequence databank using ProteinLynx Global Server 2 software (Waters/Micromass UK Ltd., Manchester, UK). Data also were searched by the tandem mass spectrum database matching tool Mascot. Redundant proteins or protein isoforms that could not be differentiated from each other on the basis of the MS data were presented as a unique protein group. As a result, 21 unique proteins were identified. The list of proteins identified in this study together with their confidence scores and Entrez gene annotations are shown in Table 4.7.

Table 4.7 Proteins identified by mass spectrometry analysis

Acc Num	Acc Name	Score	Peptide Match Count	Covarage	MW	pI	Protein Description
P79114	MY10_BOVIN	9.2994	17	7.5536	235689	5.9509	Myosin X
09HD67	MY10 HUMAN	8.6434	17	7.7745	237240	5.7697	Myosin X
P28858	DPOL HSVE8	7.7612	13	9.8361	135870	6.2324	DNA polymerase EC2777
Q96AA8	Y555 HUMAN	7.7503	12	13.0864	94875	5.7876	Hypothetical protein KIAA0555
P70589	MY5B RAT	7.6084	27	12.2969	213586	6.5158	Myosin Vb Myosin 5B Myosin heavy chain myr 6
P18709	VTGA2 XENLA	7.4475	19	9.1885	201417	9.2697	Vitellogenin A2 precursor VTGA2 Contains Lipo
P08582	TRFM HUMAN	7.3552	12	14.2276	80190	5.5909	Melanotransferrin precursor Melanoma associated
014139	HRP3 SCHPO	7.2879	14	3.2939	159279	8.3023	Chromodomain helicase hrp3
O9ULV0	MY58 HUMAN	7.1188	20	13.254	146278	8.7874	Myosin Vb Myosin 5B Fragment
090Y53	NPH1 MOUSE	7.0531	8	9.4614	76987	4.966	Mephrocystin
061116	ZF93 MOUSE	6.5529	11	12.093	72965	8.5214	Zinc finger protein 93 Zfp 93
P23116	IF3A MOUSE	6.3745	14	10.2679	161851	6.3393	Eukaryotic translation initiation factor 3 subunit
P39806	SALM DROVI	6.3254	16	11.4836	153648	8.3587	Homeotic protein spalt major
056307	80AL THEMA	6.1873	13	8.5793	127527	5.3948	Beta galactosidase EC32123 Lactase
P14748	REC1 USTMA	6.1778	6	10.3448	56823	6.7592	REC1 protein
P24384	PR22 YEAST	6.1205	17	11.179	129928	7.6741	Pre mRNA splicing factor RNA helicase PRP22
P28561	APCE AGLNE	6.1144	11	11.6384	101090	9.5894	Phycobilisome linker polypeptide Anchor polypeptide
043876	SPS VICFA	6.0933	13	11.5203	118130	5.957	Sucrose phosphate synthase EC24114 UDP glue
P90829	DPOD CAEEL	6.0899	16	12.8585	120752	8.4386	DNA polymerase delta catalytic subunit EC2777
P14605	CYAA SCHPO	6.0367	14	7.4468	190213	5.8032	Adenylate cyclase EC 4 6 1 1 ATP pyrophosphate

5 DISCUSSION AND CONCLUSION

Hepatitis B infections in developing countries and HCV epidemics and excessive alcohol intake in western populations are the major causes of liver cirrhosis (Fattovich 1998). On the other hand, HCC is an end-stage disease which is the major cause of morbidity in cirrhotic patients (Bolondi, Sofia et al. 2001). Hence, patients with chronic liver diseases constitute the population at high risk, which must be screened routinely for the early detection of HCC. AFP has poor sensitivity and specificity for liver cancer and, is far to be a significant detection tool except its use with hepatic ultrasonography in combination (Soresi, Magliarisi et al. 2003). Other promising, non-invasive serum markers have not entered yet into routine clinical practice. Therefore, the need for developing novel HCC markers led us to generate monoclonal antibodies. We expected to find out new antibodies with high specificity and sensitivity for liver cancer, and assessed *in vitro* and *in vivo* reactivity to achieve our goal.

5.1 Expression Pattern of SIP1 Monoclonal Antibody

SIP1 has been reportedly shown to mediate EMT and disease aggressiveness in human tumors (Comijn et al., 2001; Elloul et al., 2005). Several studies indicated increased levels of SIP1 transcripts in association with invasion and metastasis in cancers with advanced stages (Miyoshi, Kitajima et al. 2004; Imamichi, Konig et al. 2007). However, a comprehensive study on SIP1 protein expression in human normal and tumors tissues has not been performed. We produced two mAbs using the N-terminal 360 amino acids of human SIP1 protein as an antigen and assessed their immunoreactivity in cell lines and tissue arrays. Close homology between human and mouse SIP1 proteins allowed us to evaluate the specificity of novel SIP1 antibodies in overexpression studies. Immunofluorescence analysis of A431/WTSIP1 cells with Tet-on doxycycline-inducible wild-type mouse SIP1 expression

revealed nuclear localization of SIP1 in only doxycycline-induced cells (Fig 4.1). This result also suggests that 1C6 and 6E5 recognize epitopes shared in both human and mouse proteins. Endogenous SIP1 expression was analyzed in HCC cell line SK-HEP-1 and colorectal cancer cell lines SW480 and SW620. SK-HEP-1 is a well-known invasive HCC cell line (Lin, Ke et al. 1998); SW480 and SW620 cell lines were established from the primary and metastatic tumors of the same patient, respectively (Leibovitz, Stinson et al. 1976). Consistent with the role of SIP1 in inducing EMT phenotype, we found higher *SIP1* transcript levels in SW620 and SK-HEP-1 cells compared to SW480 cell line (Fig 4.4). SIP1 expression analysis in western blot and qRT-PCR was almost consistent with a basal expression in SW620 and SW480, and an apparent upregulation in SK-HEP-1. Although there was a ~15 fold *SIP1* overexpression in metastatic SW620 cells when compared to SW480, the significance of this difference is questionable given the *SIP1* expression in SK-HEP-1 in thousands scale. Moreover, western blotting revealed protein bands other than the expected 190 kDa size of SIP1 protein. In fact, a previous comprehensive analysis through human and mouse tissues revealed multiple transcripts of SIP1 in both species (Bassez, Camand et al. 2004). Taken together with the immunofluorescence data, these results suggest that SIP1 protein expression is tightly regulated, and may also indicate the existence of alternative SIP1 transcripts. However, at this point, we cannot exclude the possibility of non-specific signal or protein degradation for the aforementioned protein bands with lower molecular weights.

In contrast to nucleus-restricted expression of SIP1 in A431 SIP1 inducible system, most of the analyzed tissues displayed cytoplasmic protein expression. One explanation might be that while cell lines are grown in isolation in culture, tissues are subject to signals from their neighboring cells that may regulate intracellular SIP1 localization. Additionally, cellular stress induced by continuous culture of cell lines may affect the intracellular SIP1 destination. Consistent with our immunohistochemistry findings, a recent report also indicated

cytoplasmic expression of SIP1 in ovarian tumors (Yoshida, Horiuchi et al. 2009). Strong SIP1 expression in normal epithelial cells including hepatocytes, kidney tubules, stomach glandular epithelium and colon surface epithelium suggests the co-existence of E-cadherin and SIP1. Moreover, SIP1 appears to be prevented from translocation into nucleus in these tissues. It is therefore plausible to state that, unlike ZEB1, SIP1 and E-cadherin expression is not necessarily mutually exclusive. Among the analyzed tissues, most of the normal tissues expressed SIP1 from moderate to strong intensity, and we found SIP1 overexpression only in kidney, breast, lung and uterus tumors. On one hand, this differential expression may suggest a protective role for SIP1 against tumorigenesis. In fact, SIP1 was shown to directly repress cyclin D1 (Mejlvang, Kriajevska et al. 2007). Also, induced expression of SIP1 was reported to be partly responsible for hTERT repression in HCC cells (Ozturk, Erdal et al. 2006). On the other hand, SIP1 may be implicated in tumor development irrespective of its role in inducing EMT. In accordance with our results that SIP1 was up-regulated in some tumors, we recently showed that SIP1 protects cancer cells from DNA damage-induced apoptosis (Sayan, Griffiths et al. 2009). Also, SIP1 takes part in the TGF- β pathway and the effects of TGF- β on cells are variable and depend on many factors including cell type and physiological state of tissues (Postigo 2003; Massague 2008).

Given the functional role of SIP1 as a transcriptional repressor, this and aforementioned studies suggest additional levels of regulation on SIP1 activity in tumors in a tissue and/or context-dependent manner. Downstream to TGF- β signaling, ZEB1 and SIP1 regulate transcription of target genes in conjunction with SMADs and CtBPs. ZEB1 and SIP1 were shown to have opposing effects on transcriptional regulation (Postigo 2003). A feedback mechanism was described in which ZEB1 and SIP1 show antagonism by differential recruitment of co-activators and co-repressors to SMAD complexes, respectively (Postigo, Depp et al. 2003). In addition, the expression of ZEB1 and SIP1 was shown to be down-

regulated by micro RNAs (Gregory, Bert et al. 2008). The effects of these post-transcriptional regulation mechanisms on SIP1 protein might be explored by using these novel mAbs in further functional studies.

Herein, we performed a pilot study for the understanding of tissue/tumor specific SIP1 protein expression with the newly developed SIP1 specific antibodies using multi-tissue arrays. We showed that SIP1 protein levels increased only in a restricted group of tumors and most normal tissues displayed SIP1 expression at some extent. We feel to stress a drawback of staining multi-tumor arrays that color reaction has to be stopped when a detectable signal from majority of the samples is observed. Thus, our staining is optimal for high/medium SIP1 expressing tissues and may be sub-optimal for low SIP1 expressing ones. Lower SIP1 expressing tissues may have to be re-tested at their optimal conditions for a better understanding of SIP1 function in tumor development. Our recent paper that we analyzed SIP1 expression by immunohistochemistry and described SIP1 protein overexpression as a marker of poor prognosis in bladder cancers is a good example that SIP1 can be identified as a pro-metastatic protein (Sayan, Griffiths et al. 2009).

To sum up, our observation that SIP1 localized predominantly to the cytoplasm in both tumor and normal tissues suggests the implication of unidentified regulatory mechanisms that prevent translocation of SIP1 into the nucleus. This, in turn, adds another level of complexity to the control of EMT program in tumors progressing towards metastatic state. Therefore, our findings bring novel opportunities to further elaborate the role of SIP1 in tumor development.

5.2 Expression Pattern of 6D5 Monoclonal Antibody

The fact that our 6D5 antibody recognized several bands in western blot analyses with HCC cell lines (Fig 4.5) led us to seek known HCC markers displaying different forms and showing the same pattern as 6D5 in similar studies. The most widely used HCC tumor marker is AFP

which may be translated from multiple RNA transcripts (i.e., 2.2, 1.7, 1.6 and 1.35 kb) resulting in the generation of protein isoforms with molecular weights similar to those of 6D5 ligands (Celikkaya, Ciraci et al. 2007). However, previous studies have clearly shown that AFP expression is restricted to well differentiated cell lines and absent in others. Therefore, according to our western blot experiments, we excluded the possibility that 6D5 antibody is directed against AFP epitopes, since 6D5 ligands are expressed in all HCC cell lines regardless of their differentiation state.

Another protein, which is subjected to intensive investigation, is glypican-3, and results from many studies support its usefulness as a promising marker for HCC (Kandil, Leiman et al. 2007). This protein too, when expressed, gives rise to several isoforms, whose molecular weights approximate to those of protein bands we observed in our western blot analyses. However, in one study, glypican-3 expression has been shown to be absent in SK-Hep1 cell line (Jiang, Man et al. 2006). The strong reactivity of our antibody with cell lysate of SK-Hep1 cells allowed us to exclude this HCC marker as the ligand of 6D5. This conclusion is further supported by studies, which have shown cell surface expression and secretion of glypican-3 from hepatocytes (Grozdanov, Yovchev et al. 2006); however, the staining of human liver tumor samples with 6D5 was strictly cytoplasmic.

In all stained non-HCC cases, the ratio of stained cells to total tissue cell population did not exceed 1%. This was well below our criteria of focal staining, yet we represented this reactivity pattern since the affected cell types (reactive bile duct epithelial cells, periportal hepatocytes, etc.) constitute the origins of precursor cells involved in hepatocyte regeneration in response to liver damage and/or inflammation (Matthews and Yeoh 2005). Moreover, among the stained 45 cases, only 9 displayed moderate staining intensity, the others being stained weakly.

The most striking feature of our immunohistochemistry data in non-HCC cases was the non-reactivity of lobular hepatocytes with 6D5 antibody (Fig 4.7). Therefore, we hypothesized that the staining in other regions of the organ is due to the regenerative capacity of the liver reactive to cellular inflammation and/or damage. The periportal and perivenular staining (Fig 4.8) dominating in HCV hepatitis and steatohepatitis, respectively further supports this idea, since these zones are well known as the primarily affected area of cellular damage in abovementioned liver pathologies (Matthews and Yeoh 2005). Periportal staining in cirrhosis cases was accumulated in the samples deriving from primary and secondary biliary cirrhosis cases. In this group, 6D5 antibody stained periportal hepatocytes in 7 of the 12 samples. Here, our results were consistent with the fact that cellular damage, which is due to auto-antibodies and cholestasis in primary and secondary biliary cirrhosis, respectively, initiates at portal tract. Another interesting finding emerging from our immunohistochemistry analyses was the restricted staining of portal capillaries of endothelial cells (30 of 116 cases) and to a much lesser extent that of bile duct epithelial cells (9 of 116 cases only). We also evaluated both staining as reactive to cellular inflammation and/or damage, since in the majority of samples these two zones were not stained at all.

Unlike the non-reactivity of lobular hepatocytes in non-tumoral samples, the staining of tumor cells in all examined cases strongly implicates the specificity of 6D5 mAb for HCC (Fig 4.9). But, more interestingly, lobular and/or cirrhotic hepatocytes at tumor periphery were stained in the majority of cases, and even with a greater intensity than tumoral cells. This observation is especially striking in that no hepatocyte reactivity was seen in non-tumoral tissues, including our 24 cases of cirrhosis. One possible explanation of this phenomenon might be that hepatocytes in the nodules at tumor periphery were indeed other than cirrhotic cells and most probably dysplastic cells, since none of the cirrhosis cases had displayed reactivity with our antibody. Thus, we suggest that 6D5 target proteins expression occurs at early stages of

tumor development where cirrhotic cells are getting transformed. The staining of reactive endothelial cells and bile duct epithelial cells at tumor periphery was also stronger than that of the same cells in non-tumoral samples (43% vs. 21% and 29% vs. 5%, respectively). We then hypothesized that tumor development, as being a culminating inflammatory event and a damaging process for normal parenchyma, caused this higher reactivity of aforementioned cells with 6D5 antibody.

In conclusion, these results clearly demonstrated that 6D5 monoclonal antibody specifically reacts with liver tumor cells and/or the protein that the antibody recognizes is overexpressed at early stages of liver tumor development.

In our study with 6D5 monoclonal antibody, we have proven that the protein recognized by this reagent showed great specificity and sensitivity for HCC for several reasons. First, the antibody recognized all HCC cases of our study group and did not react with organized lobular hepatocytes in other liver lesions. Second cirrhotic hepatocytes were stained only in tissues deriving from HCC, but not in other cirrhosis cases. Third, besides the staining of endothelial cells lining tumor capillaries, 6D5 also reacted with endothelial cells of liver tissue vasculature in non-tumoral area. But, this observation again, was restricted to HCC cases only. And finally, the staining of bile duct epithelial cells was observed in tumor tissue sections and, such staining pattern was not encountered in other histopathological groups.

5.3 Identification of 6D5 Monoclonal Antibody Targets by Proteomics

Peptide microarrays are increasingly being used to define antibody epitopes. Probing of the 6D5 antibody with protein microarray revealed positive spot clones. Six of them were selected because of their size (Fig 4.12). Sequencing of these 6 spot clones by Imagenes revealed their cDNA sequences (Fig 4.13). Devoted to the polypeptide blast database search, the possible translates of these cDNA sequences did not give the absolute candidate epitopes-

targets for HCC because of low percentage in protein matches. Additionally consensus sequences of our clone's cDNA cannot indicate or hit any real or putative protein target over 99% coverage. The problem can be explained in some way. Antibodies may recognize a linear stretch of amino acids on three dimensional (conformational) structure that is not real sequence of the protein. So this binding might mimic the real binding on peptide sequences. The size of antibody binding site, a continuous epitope, was found to be usually between 5 and 8 amino acids in length. Consequently, the amino acids of the peptides attracting antibody binding site might result with false positivity. This can also be the result of either an accessibility of the key amino acids within the longer sequences or due to the preformation of secondary structures necessary for the antibody binding rather than the length of the epitope itself.

Using 2D PAGE analysis, in this study, 4 spots from the gel were found 6D5 immunoreactive that is confirmed by western analysis. Protein sequencing of 6D5 targets by mass spectrometry showed that the proteins have unusual tandem, non-perfect coverage for the estimation peptide sequence. There are many issues associated with the non-perfect coverage of components of a complex peptide analyte solution containing mixture of proteins. Firstly, all peptides do not ionize with the equal efficiencies that are often mixture dependent with the same peptide ionizing differently depending on the other components of the analyte mixture (Zheng, Ojha et al. 2003). As mass spectrometry continues to improve and as its sensitivity increases, the detection of multiple proteins in individual 2D gel spots is likely to pose less of a problem in the future. Part of this problem can be overcome by increasing the separation of protein spots through the use of multiple narrow range isoelectric focusing strips. Although this is certain to reduce the number of proteins migrating to a particular point on a gel, it also increases the number of 2D gels that need to be produced for coverage of the entire proteome. Using the methodology (Yang, Li et al. 1998), it was possible to assess the validity of using

MALDI-TOF intensity coverage values as a crude measure of protein abundance. This involved the analysis of the remaining analyte samples containing peptides from the fly lines that exhibited an increase in abundance of protein samples.

Secondly, proteomics sequence databases are subject to time instability and changes over time (Batista, del Pozo et al. 2004). Once a sequence has been recorded in a database, it can undergo alterations as well. Certain sequences can be subjected to removal from the database, invalidating the accession number without providing a replacement. This effect is most pronounced for purely theoretical predictions. Change in the prediction algorithm may render a number of previous predictions obsolete and suffers the same fluctuations as its source databases.

The other important problem for data analysis in proteomics is standardization. The use of specialized protocols and very different data processing pipelines add to this problem. In fact, having every single protocol and data processing step documented does not necessarily result in allowing reproduction of the analyses performed in order to validate the results, as specific steps may not be available to other researchers. The Human Proteome Organization (HUPO) (Hanash 2002) has created the Proteomics Standards Initiative (PSI) (Orchard, Hermjakob et al. 2005) to tackle both standardization problems. The heterogeneity of standards will need to be resolved in full before real progress can be made in this matter. This approach has been in place for years for nucleic acid and protein sequence databases and has repeatedly proven to greatly aid researchers worldwide.

In conclusion, in this study, human tissues were used in immunohistochemistry screening of 6D5 target proteins of and SIP1 by using homemade mAbs. It is found that the high-level expression of 6D5 was found to more frequently in human HCCs as compared to normal liver and benign liver lesions. In addition, high-level expression of SIP1 was evaluated to be significantly associated with normal liver tissues. We found that the expression of SIP1 was

semi-quantitatively decreased from normal liver to HCC metastatic carcinoma and the expression of SIP1 in normal tissues was significantly higher than that of corresponding tumor tissues. All these results demonstrated that our data could provide a novel approach in cancer research and after identifying 6D5 target proteins there will be a translation from basic research to clinical applications in the future.

6 FUTURE PERSPECTIVES

It is suggested that hepatitis B-related HCC will decrease with vaccination, while hepatitis C-related HCC will become an increasing problem. Antiviral treatment and chemo-preventive agents will prevent HCC development. Whole-population screening will not be an option, but screening is justified for individuals who can pay for it. There will be more emphasis on the use of tumor markers. The diagnosis of HCC is established serologically (using tumor markers), radiologically, cytologically or histologically, either alone or in combination.

In the protein microarray results, the proteins expressed and purified from the corresponding clones detected in a screening experiment can be used for the further validation and specification of the discovered interactions. For example after the identification of the targets from a novel antibody, the specificity and sensitivity of the interactions can be confirmed by Western immunoblotting and transfectant studies. If the proteins were expressed and purified in a large scale format, specific protein microarrays can be produced. These protein microarrays might be used in routine clinical practice until relevant validation studies have been done in many centers. Ultimately, prediction of the most promising biomarkers requires prospective validation in carefully designed randomized clinical trials using standardized protocols. This will require cooperation across borders and specialties.

Furthermore, we still do not know whether the molecular profile of a tumor changes at the time of disease or even after therapy towards for a more advanced disease. There is little information as to whether primary and metastatic tumors always share the same molecular profile, although there is some evidence for molecular discordance between early and metastatic disease. Therefore our mAbs might be tested in different state of the diseases.

In the field of proteomics, there is a great interest to find binding reagents that specifically interact with the individual protein species. It is recognized that different types of binding

molecules or binders selected from libraries generated by combinatorial design of various alternative scaffolds, are used to obtain target-specific affinity reagents. For any of these binding molecules, there must be a source of target molecules in order to succeed with the generation of target-specific binders. For the herein presented study, we may improve strategies to recovery of the fragments of the target proteins are successfully produced in HCC. Such protein fragments might be then employed to obtain highly target specific (monospecific) antibodies after immunization and careful affinity purification strategies. Monospecific antibody preparations might be obtained to stain TMA in order to localize the target proteins in normal and pathological tissue sections. It is likely that functional proteomics, in the sense of looking for differences in protein abundance in various biological samples, is a useful strategy to find novel drug candidates for diseases. For example, in a publication (Ek, Andreasson et al. 2006), the researchers were guided by information about protein localization, obtained from TMA staining, to point out a number of possible drug targets for an aggressive cancer form.

On the other hand, our candidates are open to test with the molecular techniques such as siRNA or knockdown or knockout of the targets to improve the validity of the proteomics results.

Finally, technical issues are also important in proteomics approaches with mAbs. At present, very few routine clinical laboratories have access to sophisticated molecular techniques, such as qRT-PCR, mutational analysis, FISH, and microarray, although most can do immunohistochemistry. However, standardized, optimized protocols and antibodies need to be applied in order to validate prospective validation; these technologies will also need optimization and standardization before being generally accepted as a valid decision-making tool.

7 REFERENCES

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8 PUBLICATIONS

- 8.1** **Oztas E**, Avci ME, Ozcan A, Sayan AE, Tulchinsky E, Yagci T. Novel monoclonal antibodies detect Smad-interacting protein 1 (SIP1) in the cytoplasm of human cells from multiple tumor tissue arrays. *Exp Mol Pathol*. 2010 Oct;89(2):182-9.
- 8.2** Celikkaya H, Ciraci C, **Oztas E**, Avci ME, Ozturk M, Yagci T. Immunization with UV-induced apoptotic cells generates monoclonal antibodies against proteins differentially expressed in hepatocellular carcinoma cell lines. *Hybridoma (Larchmt)*. 2007 Apr;26(2):55-61.



Novel monoclonal antibodies detect Smad-interacting protein 1 (SIP1) in the cytoplasm of human cells from multiple tumor tissue arrays

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ABSTRACT

Smad-interacting protein 1 (SIP1, also known as ZEB2) represses the transcription of E-cadherin and mediates epithelial–mesenchymal transition in development and tumor metastasis. Due to the lack of human SIP1-specific antibodies, its expression in human tumor tissues has not been studied in detail by immunohistochemistry. Hence, we generated two anti-SIP1 monoclonal antibodies, clones 1C6 and 6E5, with IgG1 and IgG2a isotypes, respectively. The specificity of these antibodies was shown by Western blotting studies using siRNA mediated downregulation of SIP1 and ZEB1 in a human osteosarcoma cell line. In the same context, we also compared them with 5 commercially available SIP1 antibodies. Antibody specificity was further verified in an inducible cell line system by immunofluorescence. By using both antibodies, we evaluated the tissue expression of SIP1 in paraffin-embedded tissue microarrays consisting of 22 normal and 101 tumoral tissues of kidney, colon, stomach, lung, esophagus, uterus, rectum, breast and liver. Interestingly, SIP1 predominantly displayed a cytoplasmic expression, while the nuclear localization of SIP1 was observed in only 6 cases. Strong expression of SIP1 was found in distal tubules of kidney, glandular epithelial cells of stomach and hepatocytes, implicating a co-expression of SIP1 and E-cadherin. Squamous epithelium of the esophagus and surface epithelium of colon and rectum were stained with moderate to weak intensity. Normal uterus, breast and lung tissues remained completely negative. By comparison with their normal tissues, we observed SIP1 overexpression in cancers of the kidney, breast, lung and uterus. However, SIP1 expression was found to be downregulated in tumors from colon, rectum, esophagus, liver and stomach tissues. Finally we did nuclear/cytoplasmic fractionation in 3 carcinoma cell lines and detected SIP1 in both fractions, nucleus being the dominant one. To our best knowledge, this is the first comprehensive immunohistochemical study of the expression of SIP1 in a series of human cancers. Our finding that SIP1 is not exclusively localized to nucleus suggests that the subcellular localization of SIP1 is regulated in normal and tumor tissues. These novel monoclonal antibodies may help elucidate the role of SIP1 in tumor development.

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Introduction

Smad-interacting protein 1 (SIP1, also known as ZEB2) encoded by ZFHX1B is a member of ZEB family of transcription factors. The protein contains a central homeodomain, CtBP-binding and Smad-interacting domains and two zinc finger clusters each at either end (Remacle et al., 1999; Verschuere et al., 1999). SIP1 directly binds to bipartite E-boxes on the promoters of different targets by means of its zinc finger domains and mediates transcriptional repression (Verschuere et al., 1999). One of these targets is CDH1, the gene encoding for the epithelial adherens

junction protein E-cadherin, whose transcriptional downregulation induces epithelial-to-mesenchymal transition (EMT) in developmental processes and during tumor cell invasion and metastasis (Comijn et al., 2001). Transcriptional repression is mediated through the association of SIP1 with the corepressor CtBP, however this interaction is dispensable at least for the attenuation of CDH1 transcription (Postigo et al., 2003; van Grunsven et al., 2003). Overexpression of SIP1 in epithelial cells has also been shown to downregulate constituents of cell–cell junctions other than E-cadherin (Vandewalle et al., 2005). Although binding of SIP1 to p300 or pCAF was proposed as a mechanism for transactivation and other transcriptional activators associated to SIP1 are yet to be determined, SIP1-mediated upregulation of EMT and invasion related genes, such as vimentin and matrix metalloproteases, have been reported (Bindels et al., 2006; Miyoshi et al., 2004; Postigo et al., 2003).

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Despite the overwhelming evidence that SIP1 induces EMT phenotype, its role in tumorigenesis was ill-defined. In fact, SIP1 was originally identified as a binding partner of R-Smads, and shown to be part of the TGF- β pathway, which is frequently involved in carcinogenesis (Verschuere et al., 1999). hTERT repression in breast cancer cells was partly mediated by SIP1 in a TGF- β dependent manner (Lin and Elledge, 2003). Also, analysis of senescence arrest of clonal hepatocellular carcinoma cells revealed SIP1 as a mediator of hTERT repression (Ozturk et al., 2006). Impaired G1/S progression was observed upon repression of cyclin D1 by SIP1 (Mejlvang et al., 2007). SIP1 was also shown to contribute to tumorigenesis in a transgenic mouse model of lymphoma by retroviral tagging (Mikkers et al., 2002). The differential expression of SIP1 has been described, mostly by RT-PCR, in several human tumors due to the lack of human SIP1-specific antibodies. E-cadherin downregulation was associated to increased SIP1 expression in intestinal type gastric carcinoma but not in diffuse type gastric carcinoma (Rosivatz et al., 2002). Elevated SIP1 expression correlated inversely with E-cadherin in advanced stages of pancreatic tumors (Imamichi et al., 2007). Surprisingly, SIP1 and E-cadherin expression were positively correlated in malignant mesothelioma (Sivertsen et al., 2006). In the esophagus, differential expression of SIP1 was observed during keratinocyte differentiation. Only stem cell containing basal cells, but not parabasal cells and keratinocytes expressed SIP1. Consistent with this, SIP1 transcripts were present in all studied esophageal

carcinoma cases (Isohata et al., 2009). High SIP1/E-cadherin ratio correlated with metastatic disease and poor patient survival in breast and ovarian carcinomas (Eloul et al., 2005). Elevated SIP1 transcripts were observed in von Hippel-Lindau-null renal cell carcinomas in a hypoxia-inducible factor 1 alpha (HIF1 α)-dependent manner (Krishnamachary et al., 2006). Immunohistochemical analysis of ovarian tumors revealed a stepwise increase of SIP1 from benign to borderline to malignant tumors (Yoshida et al., 2009). In oral squamous cell carcinoma, SIP1 was immunohistochemically detected in a relatively low proportion of tumors and its expression correlated with poor prognosis (Maeda et al., 2005). In a previous study, we have found that SIP1 was overexpressed in a series of bladder cancers. Its expression was found to be an independent prognostic factor in bladder cancers and positively stained cases correlated with poor therapeutic outcome (Sayan et al., 2009). With the exception of a few and as described above, most of the expression studies of SIP1 were done using RT-PCR technique, but SIP1 protein levels have been shown to be tightly regulated by post-transcriptional mechanisms. For instance, Pc2-mediated sumoylation of SIP1 affects the transcriptional regulation of E-cadherin (Long et al., 2005). SIP1 has been identified as a direct target of miR-200 family and miR-205 (Gregory et al., 2008; Park et al., 2008).

In this study, we generated 2 new monoclonal antibodies (MAb) against the N-terminus of SIP1 protein and validated their specificity by specifically downregulating SIP1 protein, and the other ZFH1

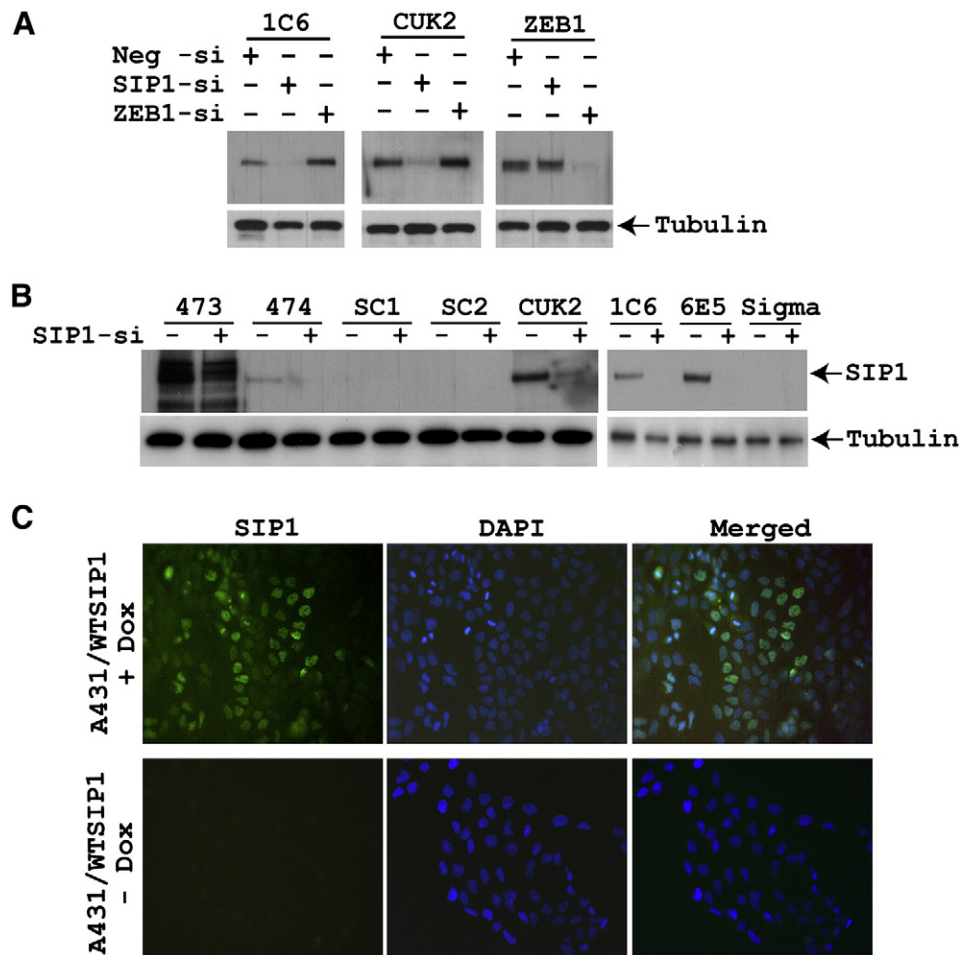


Fig. 1. Endogenous and induced expression of SIP1 is detected by monoclonal antibodies 1C6 and 6E5. (A) HOS2 cells were transfected with control (Neg-si), SIP1 (SIP1-si) and ZEB1 (ZEB1-si) siRNAs. Proteins were extracted 48 h after transfection and western blot was performed with the indicated antibodies. SIP1 specific band was detected with 1C6 and CUK antibodies. Both SIP1 antibodies did not recognize ZEB1 and can detect endogenous SIP1 efficiently. (B) Control siRNA and SIP1 siRNA transfected HOS2 cell lysates were used to compare the new SIP1 MABs with 5 different commercial antibodies. The results from polyclonal antibodies (goat or rabbit anti-SIP1) and monoclonal antibodies (mouse anti-SIP1) are presented in left and right panels, respectively. With the exception of 474, all other commercial antibodies were either weak or non-specific. (C) A431/WTSIP1 cells maintained in 2 μ g doxycycline for 24 h and stained with 6E5 MAB displayed nuclear SIP1 expression (first row), whereas no staining was observed in uninduced cells with the same antibody (second row).

family member protein ZEB1, by siRNA in endogenous levels. Then, by using these antibodies we explored the expression pattern of SIP1 in human tumor cell lines and in a variety of tissues. We detected predominantly cytoplasmic but also nuclear SIP1 staining. Finally, subcellular fractionation of cell lines showed that SIP1 protein can be present in the cytoplasm and nucleus of multiple carcinoma cell lines. To our knowledge, this study is the first description of SIP1 protein expression in a multiple tumor tissue arrays.

Materials and methods

Cell lines, tissues and siRNA transfections

Wild-type mouse SIP1 expressing squamous epidermoid carcinoma cell line A431/WTSIP1 with Tet-on doxycycline-inducible SIP1 expression was previously described (Mejlvang et al., 2007). Osteosarcoma cell line HOS2, hepatocellular carcinoma cell line SK-HEP-1 and colorectal carcinoma cell lines SW480 and SW620 were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU penicillin, 100 µg streptomycin and nonessential amino acids. Multiple Tumor Tissue arrays were purchased from BioChain Institute, Inc. (Hayward, CA). siRNAs targeting ZEB1 (Sayan et al., 2009) and SIP1 (S102364277, Qiagen, Hilden, Germany) were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were collected 48 h after transfection and processed for Western blotting.

Recombinant SIP1 production

The first 360 amino acid part coding region of SIP1 (ZEB2) cDNA was cloned into pET101/D (Invitrogen, Carlsbad, CA) vector with an N-terminal 6-histidine tag. Recombinant protein was expressed in *Escherichia coli* (BL21) and purified under denaturing conditions using Ni-NTA resin (Qiagen, Hilden, Germany). Purified protein then was refolded and buffer exchanged to phosphate buffered saline (PBS) using NAP buffer exchange columns (Amersham, Piscataway, NJ). Finally, pure and folded recombinant protein was concentrated (0.5–1 mg/ml) using Centrprep (Millipore, Billerica, MA).

Production of monoclonal antibodies

Recombinant SIP1 protein was injected into the peritoneal cavity of 8- to 10-week-old BALB/c mice at 3 week intervals. During the immunization period, antibody titer of mice sera was evaluated by indirect ELISA. Briefly, ELISA plates were coated by 100 ng of recombinant SIP1 protein in carbonate buffer (pH: 9.6). Serially diluted mice sera were assessed for their immunoreactivity with SIP1 protein. Alkaline phosphatase conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was used as secondary antibody. Colorimetric reaction generated upon addition of the substrate para-nitrophenyl-phosphate (Sigma-Aldrich, St. Louis, MO) was measured at A₄₀₅ in an automated plate reader (Biotek Instruments, Winooski, UT). Three days after the final boost, fusion of mouse splenocytes and SP2/0 myeloma cells was performed as previously described (Celikkaya et al., 2007). Hybridoma supernatants were screened by aforementioned indirect ELISA, and hybridomas secreting anti-SIP1 antibodies were subjected to single cell subcloning. Antibody isotype was determined by ImmunoPure Monoclonal Antibody Isotyping Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

Western blotting

Total cell lysates from HOS2, SK-HEP-1, SW480 and SW620 cell lines were prepared in NP-40 lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P40 (v/v) and a cocktail of EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany)] or by direct lysis in 2X Laemmli buffer. Nuclear and cytosolic protein

fractions were prepared by NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to manufacturer's instructions. Protein content was measured by Bradford or BCA assay. Equalized lysates were run on 8% SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes by using semi-dry transfer apparatus (Bio-Rad, Hercules, CA). 1C6 and 6E5 hybridoma supernatants were used as primary antibody. Other antibodies used in this study are from Bethyl Labs [473 (A302-473A) and 474 (A302-474A), 1:500; Montgomery, TX], Santa Cruz [SC1 (sc-48789) and SC2 (sc-18392), 1:500; Santa Cruz, CA] and Sigma (WH0009839M1, 1:500; Sigma-Aldrich, St. Louis, MO) for SIP1 immunodetection and Santa Cruz (sc-25388,) for ZEB1 immunodetection. Rabbit polyclonal SIP1 antibody (CUK2) was previously described (Sayan et al., 2009). Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit IgG or anti-goat (Sigma-Aldrich, St. Louis, MO) were used as secondary antibodies at 1:5000 dilution. Protein bands were visualized using Super Signal West Dura or Femto chemiluminescent substrate (Pierce, Rockford, IL).

Quantitative real-time PCR

SIP1 mRNA expression in colon cancer cell lines SW620, SW480 and hepatocellular carcinoma cell line SK-HEP-1 was determined by quantitative real-time PCR as described previously (Avci et al., 2008). The expression of SIP1 in cell lines was measured using $\Delta\Delta C_t$ method and normalized to GAPDH gene. The threshold cycle of SIP1 cDNA in SW480 cell line, which showed the lowest expression was set to 1 and relative expression values were plotted as fold changes.

Immunofluorescence

A431/WTSIP1 cells were grown on cover slips in 6 well plates and induced with doxycycline (2 µg/ml) for 24 h. PBS was used in all washing

Table 1
Immunohistochemistry results of SIP1 expression in human tissues.

Tissue (n = 123)	SIP1 expression		
	Cytoplasm	Nucleus	SIP1-positive structure
Kidney			
Tumor (n = 18)	+++ (17)	—	Tumoral cells
Normal (n = 4)	++ (4)	—	Proximal–distal tubules
Lung			
Tumor (n = 14)	+ (10)	—	Tumoral cells
Normal (n = 3)	— (0)	—	Surfactant ++ (3)
Breast			
Tumor (n = 9)	+ (5)	—	Tumoral cells
Normal (n = 2)	— (0)	—	—
Uterus			
Tumor (n = 12)	+ (5)	—	Tumoral cells
Normal (n = 3)	— (0)	—	—
Liver			
Tumor (n = 9)	++ (9)	—	Tumoral cells
Normal (n = 2)	+++ (2)	—	Hepatocytes
Stomach			
Tumor (n = 9)	+ (4)	—	Tumoral cells
Normal (n = 1)	+++ (1)	—	Glandular cells
Colon			
Tumor (n = 12)	+ (4)	—	Tumoral cells
Normal (n = 4)	++ (4)	+ (4)	Surface epithelium
Rectum			
Tumor (n = 9)	+ (7)	++ (1)	Tumoral cells
Normal (n = 1)	++ (1)	++ (1)	Surface epithelium
Esophagus			
Tumor (n = 9)	+ (5)	—	Tumoral cells
Normal (n = 2)	++ (2)	—	Squamous epithelium

Mean staining intensities expressed as (—): negative, +: weak, ++: moderate, +++: strong. Numbers in parentheses represent positively stained cases.

steps. Cells were fixed in 2% paraformaldehyde in PBS and permeabilized in PBS containing 0.3% triton X-100. After blocking with 2% Bovine Serum Albumin (BSA) in PBS, cells were incubated for 2 h at room temperature with hybridoma supernatants. Alexa fluor 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as secondary antibody at 1:200 dilution. Nuclei counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI); cover slips were mounted on glass slides and examined under fluorescent microscope (Zeiss GmbH, Germany). Merged images were produced by using AxioVision image processing software (Zeiss GmbH, Germany).

Immunohistochemistry

A total of 123 tissues spotted on three tissue arrays were stained twice by both 1C6 and 6E5 MAbs. Tissue arrays included sections from kidney (22: tumor 18, normal 4), lung (17: tumor 14, normal 3), colon (16: tumor 12, normal 4), uterus (15: tumor 12, normal 3), esophagus (11: tumor 9, normal 2), liver (11: tumor 9, normal 2), breast (11: tumor 9, normal 2), rectum (10: tumor 9, normal 1) and stomach (10: tumor 9, normal 1) tissues. Tissue array slides were deparaffinized first at 70 °C and then in xylene. After rehydration in graded alcohol series, glass slides were

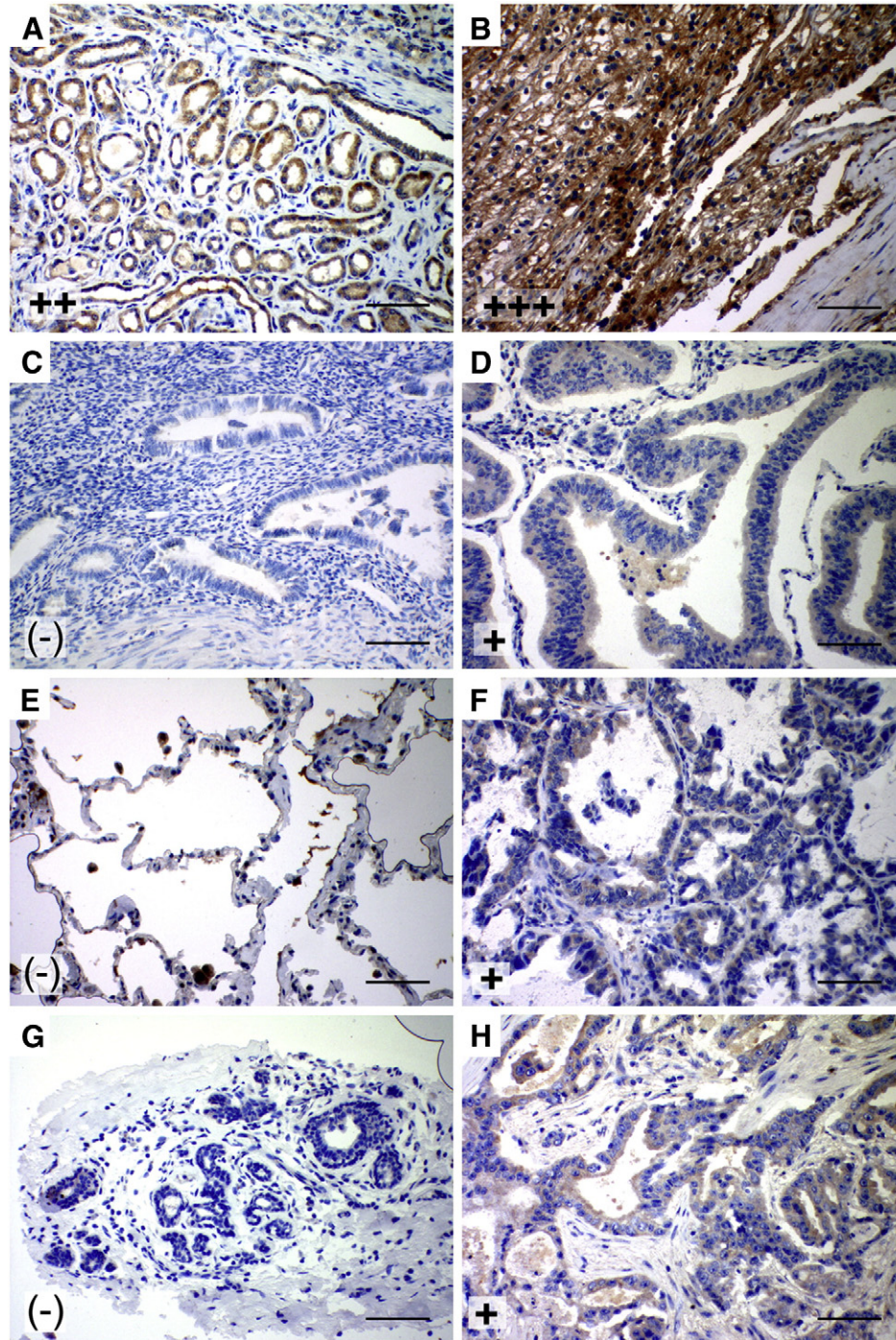
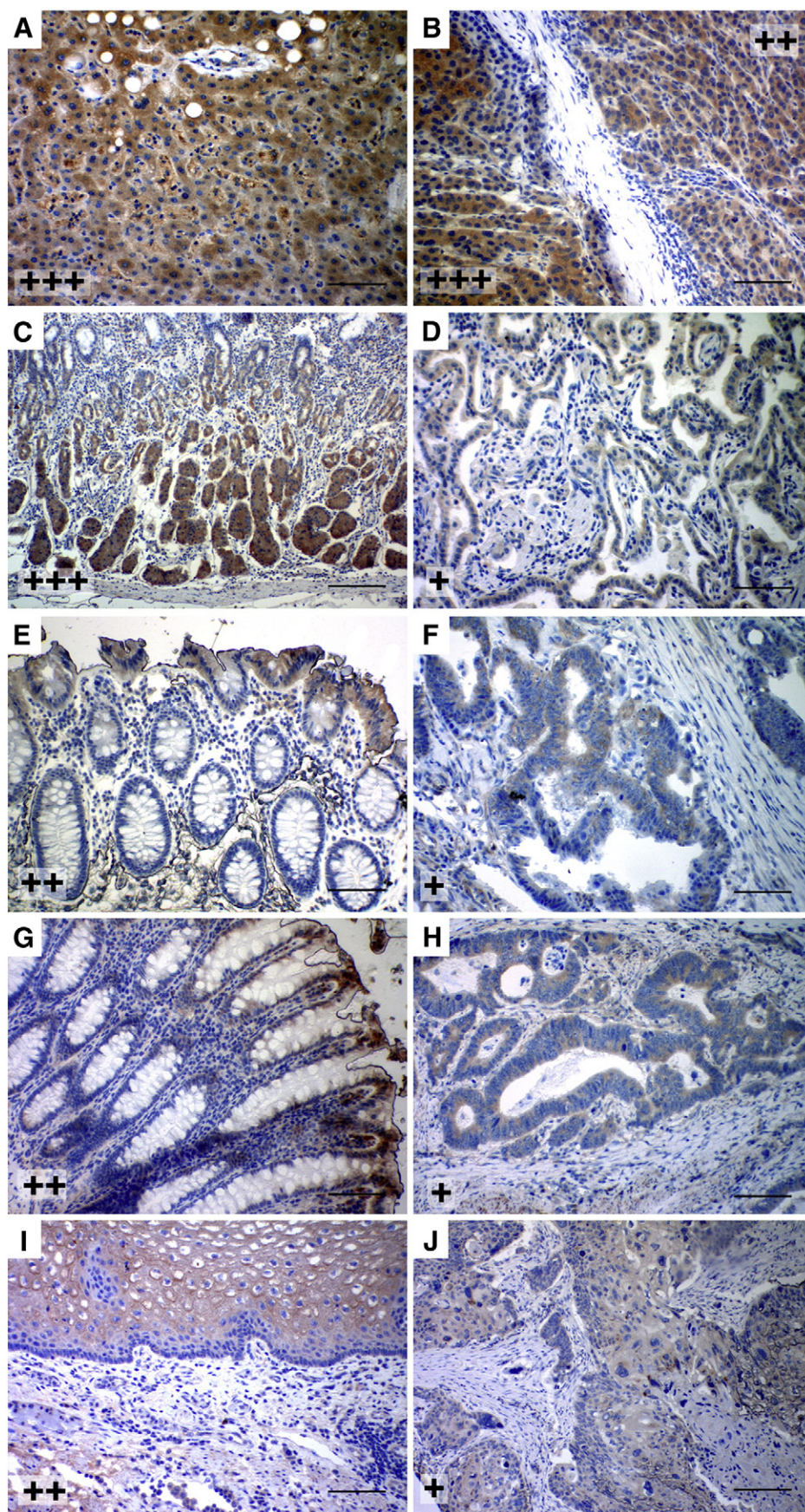


Fig. 2. Increased expression of SIP1 in kidney, lung, breast and uterus tumors. Representative photographs show increased SIP1 expression in tumors relative to their normal tissues as detected by immunohistochemistry performed by both antibodies. (A) Distal tubule staining in normal kidney, (B) clear cell renal carcinoma, (C) normal uterus, (D) adenocarcinoma of the uterus, (E) normal lung with non specific surfactant staining, (F) squamous cell carcinoma of the lung, (G) normal breast, (H) breast ductal carcinoma. (-): negative, +: weak, ++: moderate, +++: strong staining intensity (scale bars: 50 μm).



immersed in 10 mM citrate buffer, pH 6.0 and transferred into microwave oven for 20 min for antigen retrieval. Endogenous peroxidase was blocked by incubation of slides in 3% H₂O₂ for 30 min. PBS was used in all washing steps. Tissue sections were incubated with hybridoma supernatants for 2 h, and after washing, universal staining kit (LabVision, Fremont, CA) was used according to manufacturer's recommendations. 3,3'-diaminobenzidine (DAB) was used as chromogen and the slides were counterstained using Mayer's hematoxylin. Stainings were performed in triplicate for both antibodies. The sections were evaluated by light microscopic examination and the intensity of immunostaining in each section was assessed independently by two observers (E.O and A.O). The intensity of total SIP1 staining on each sample was scored as previously described with a slight modification (Chen et al., 2006). Briefly the staining intensity was graded relatively based on the following scales: 0, 1, 2, and 3 for negative, weak, moderate and strong staining, respectively. An averaged score was reached as the final score for each tissue with multiple samples. According to the final immunostaining scores, the tissues were classified into four groups: negative group (score 0–0.40), weak staining group (score 0.5–1.4), moderate staining group (scores 1.5–2.4), and strong staining group (scores 2.5–3). (–), (+), (++) and (+++) designations were used for these four groups, respectively. The staining in nuclei and cytoplasm was determined separately on each specimen.

Results

Monoclonal antibodies 1C6 and 6E5 detect both endogenous and overexpressed SIP1

Two MAbs, clones 1C6 and 6E5 were obtained by immunizing BALB/c mice with a partial human SIP1 recombinant protein (aa 1–360). The isotypes of antibodies were IgG2a and IgG1, respectively (data not shown). ZEB1 and SIP1 (ZEB2) are members of the zinc finger Homeobox 1 gene family. Among these 2 proteins, there is more than 45% overall protein homology which is much higher (up to 93% identity at C-terminal zinc finger cluster) in the functional domains (Vandewalle et al., 2009). Thus, as a first step, we wanted to assess the specificity of the new antibodies by specifically knocking down ZEB1 and SIP1 proteins in HOS2 osteosarcoma cells. As shown in Fig. 1A, endogenous SIP1 was specifically downregulated by SIP1 siRNA but not by ZEB1 siRNA and detected by 1C6 MAb. The authenticity of 1C6 immunoreactive band (190 kDa) is also confirmed by another SIP1 antibody (CUK2). None of the SIP1 antibodies detected ZEB1 and ZEB1 antibody did not detect SIP1 (Fig. 1A). As a second step, we wanted to test the specificity and strength of several other commercial SIP1 antibodies. We used control siRNA and SIP1 siRNA transfected HOS2 cell lysates to detect endogenous SIP1. With the exception of 474, which recognized endogenous SIP1 weakly, commercial polyclonal SIP1 antibodies were either very weak (SC1, SC2) or non-specific (473) (Fig. 1B, left panel). Also, when compared with another MAb (Sigma), only 1C6 and 6E5, but not the commercial one recognized SIP1 specifically (Fig. 1B, right panel). The specificity of these new SIP1 MAbs were also validated in the inducible cell line system A431 containing mouse WTSIP1. By using both antibodies in immunofluorescence assay, we detected nuclear expression of SIP1 in these cells maintained in the presence of doxycycline for 24 h (Fig. 1C). These results showed that the new SIP1 MAbs are specific and able to detect SIP1 in endogenous levels and when overexpressed.

Tissue expression of SIP1 is predominantly cytoplasmic

Next, the tissue expression pattern of SIP1 protein was analyzed by staining formalin-fixed and paraffin-embedded tissue arrays with MAbs 1C6 and 6E5. 22 normal and 101 tumor tissues were examined by immunohistochemistry and all samples displayed similar reactivity upon staining by both clones. The SIP1 immunostaining pattern of tissues was summarized in Table 1. No immunoreactivity was observed in tissue arrays stained with mouse IgG1 and IgG2a isotype control antibodies (data not shown).

The majority of tissues displayed cytoplasmic staining of SIP1 and nuclear expression of SIP1 was observed only in 6 cases consisting of one normal and one tumor tissues of rectum and four normal colon samples (Table 1).

Differential expression of SIP1 in human tumors

SIP1 is overexpressed in tumors of the kidney, lung, breast and uterus

1C6 and 6E5 antibodies stained both proximal and distal tubules of kidney, yet the reactivity of the latter was more intense. Compared to the tubular epithelium-restricted expression of SIP1 in normal kidney, SIP1 was extensively expressed in kidney tumors. Out of 18 tumors, 17 clear cell carcinomas displayed strong cytoplasmic staining with both antibodies (Fig. 2A–B), and one transitional cell carcinoma case remained negative. Relative to their normal tissues, which failed to display SIP1 expression, 71% of lung, 56% of breast and 42% of uterus tumors showed cytoplasmic SIP1 positivity, yet with a weak intensity (Fig. 2C–H).

Cytoplasmic SIP1 is downregulated in most of the human tumors

The cytoplasm of all 9 hepatocellular carcinoma cases displayed a moderate intensity of SIP1 expression, which could not reach however the strong staining pattern of SIP1 in normal hepatocytes and tumor-adjacent cirrhotic tissues (Fig. 3A, B). Eight of nine stomach adenocarcinomas were weakly positive for SIP1 expression, a pattern far beyond the intense SIP1 staining of glandular cells of normal stomach (Fig. 3C, D). Apical crypt epithelia of 4 normal colon samples displayed cytoplasmic staining with both antibodies with moderate intensity, and a faint nuclear SIP1 expression was also observed in these cells. However, only 4 of 12 colon tumors were weakly positive for cytoplasmic SIP1 (Fig. 3E, F). In tissue arrays, only one normal rectum sample was available, and lumen-facing epithelial cells of this tissue were found to express SIP1 mainly in their nuclei and to a lesser extent in their cytoplasm. Irrespective of its cellular localization, SIP1 expression was of moderate to strong intensity in normal rectum. On the contrary, 78% of rectum tumors were stained, but with weak immunoreactivity (Fig. 3G, H). The dominant cytoplasmic staining pattern of these cancer tissues was accompanied by moderate nuclear staining in only one case, which was the tumor with most advanced stage among others (data not shown). Tumor cells of 5 squamous cell carcinoma of the esophagus expressed SIP1, yet with a weaker intensity than squamous epithelium of normal esophagus, which was stained with moderate intensity by both antibodies (Fig. 3I, J).

Cytoplasmic SIP1 is present in tumor cell lines

Cytoplasmic SIP1 expression in the majority of human carcinomas prompted us to validate this observation in two colon cancer cell lines (SW620 and SW480) and one hepatocellular carcinoma cell line (SK-

Fig. 3. Reduced expression of SIP1 in liver, stomach, colon, rectum and esophagus tumors. Photographs are representative from immunohistochemistry performed by both antibodies and show decreased SIP1 expression in tumors with respect to their normal tissues. (A) Normal liver, (B—right) hepatocellular carcinoma (HCC) of the liver and (B—left) the adjacent cirrhotic tissue, (C) normal stomach gland cells, (D) adenocarcinoma of the stomach, (E) normal colon surface epithelium, (F) adenocarcinoma of the colon, (G) normal rectum surface epithelium, (H) adenocarcinoma of the rectum, (I) normal esophagus squamous epithelium, (J) squamous cell carcinoma of the esophagus. +: weak, ++: moderate, +++: strong staining intensity (scale bars: 50 µm).

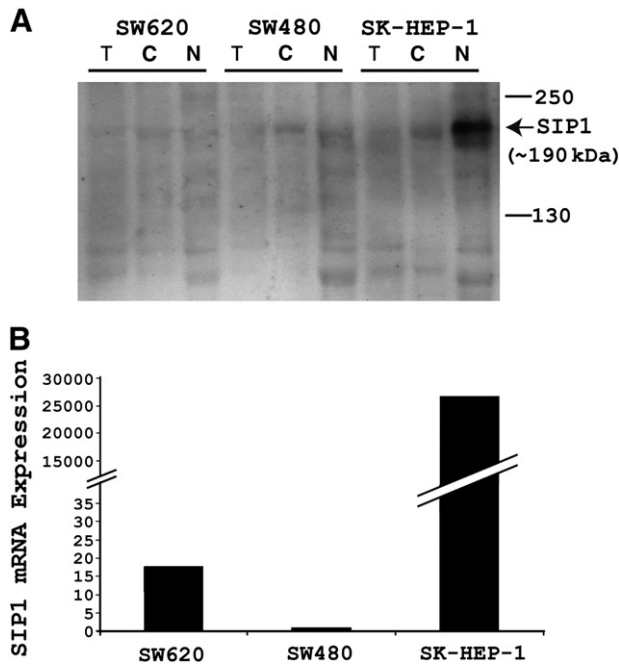


Fig. 4. SIP1 protein is present in nucleus and cytoplasm of carcinoma cell lines. (A) Western blotting performed with clone 1C6 shows strong SIP1 expression at 190 kDa in the nuclear fraction of SK-HEP-1 cells, yet the antibody also detects bands with lower molecular weight proteins in nuclear (N) and total cell lysates (T) of all 3 cell lines. SIP1 expression is more apparent in the cytoplasmic (C) but not nuclear extracts of SW480 and SW620 cells. SK-HEP-1 cells also contain similar levels of cytoplasmic SIP1. (B) Quantification of SIP1 transcripts indicates the highest SIP1 expression in SK-HEP-1 cell line. SIP1 transcript levels in SK-HEP-1 and SW620 cells are represented as fold changes with respect to SW480 reference cell line.

HEP-1). We did subcellular fractionation and analyzed the presence of SIP1 protein by Western blotting. A protein band with strong intensity at the expected size (190 kDa) of SIP1 was observed in the nuclear fraction of the invasive hepatocellular carcinoma cell line SK-HEP-1 (Fig. 4A). Weaker protein bands of the same size also appeared in the cytosolic fraction of SK-HEP-1, and in both cytosolic and nuclear fractions of SW620 and SW480 cells. However, SIP1 expression was barely detectable in total cell lysates. Besides, SIP1 antibody immunoreactive bands at about 120 kDa and lower molecular weights were also observed in the nuclear extracts and total cell lysates of all 3 cell lines (Fig. 4A). These results were in accordance with real-time quantitative PCR data in which SK-HEP-1 was the cell line with most abundant SIP1 transcript (Fig. 4B).

Overall, our results indicated that SIP1 was widely expressed in most normal human tissues that we examined, with moderate to strong intensities, and the overexpression of SIP1 was confined only to a restricted group of human tumors.

Discussion

SIP1 has been reportedly shown to mediate EMT and disease aggressiveness in human tumors (Comijn et al., 2001; Elloul et al., 2005). Several studies indicated increased levels of SIP1 transcripts in association with invasion and metastasis in cancers with advanced stages (Imamichi et al., 2007; Miyoshi et al., 2004). However, a comprehensive study on SIP1 protein expression in human normal and tumors tissues has not been performed. We produced two MAbs using the N-terminal 360 amino acids of human SIP1 protein as an antigen and assessed their immunoreactivity in cell lines and tissue arrays. As an initial study we downregulated SIP1 and the other ZFH1 family member protein, ZEB1, by siRNA in an osteosarcoma derived cell line and compared the strength and specificity of the novel SIP1 antibodies along with 5 different commercial SIP1 antibodies. We found that 1C6 and 6E5 can detect endogenous SIP1,

but not ZEB1, in this system strongly and specifically. We also observed that, with the exception of Bethyl Labs 474 antibody, all other commercial SIP1 antibodies are weak or non-specific. Close homology between human and mouse SIP1 proteins allowed us to evaluate the specificity of novel SIP1 antibodies in overexpression studies. Immunofluorescence analysis of A431/WTSIP1 cells with Tet-on doxycycline-inducible wild-type mouse SIP1 expression revealed nuclear localization of SIP1 in only doxycycline-induced cells. This result also suggests that 1C6 and 6E5 recognize epitopes shared in both human and mouse proteins.

Endogenous SIP1 expression was analyzed in HCC cell line SK-HEP-1 and colorectal cancer cell lines SW480 and SW620. SK-HEP-1 is a well-known invasive hepatocellular carcinoma cell line (Lin et al., 1998); SW480 and SW620 cell lines were established from the primary and metastatic tumors of the same patient, respectively (Leibovitz et al., 1976). Consistent with the role of SIP1 in inducing EMT phenotype, we found higher SIP1 transcript levels in SW620 and SK-HEP-1 cells compared to SW480 cell line. SIP1 expression analysis in western blot and qRT-PCR was almost consistent with a basal expression in SW620 and SW480, and an apparent upregulation in SK-HEP-1. Although there was a ~15 fold SIP1 overexpression in metastatic SW620 cells when compared to SW480, the significance of this difference is questionable given the SIP1 expression in SK-HEP-1 in thousands scale. Moreover, Western blotting revealed protein bands other than the expected 190 kDa size of SIP1 protein. In fact, a comprehensive analysis through human and mouse tissues revealed multiple transcripts of SIP1 in both species (Bassez et al., 2004). Taken together with the immunofluorescence data, these results suggest that SIP1 protein expression is tightly regulated, and may also indicate the existence of alternative SIP1 transcripts. However, at this point, we cannot exclude the possibility of non-specific signal or protein degradation for the aforementioned protein bands with lower molecular weights.

In contrast to nucleus-restricted expression of SIP1 in A431 Sip1 inducible system, most of the analyzed tissues displayed cytoplasmic protein expression. One explanation might be that while cell lines are grown in isolation in culture, tissues are subject to signals from their neighboring cells that may regulate intracellular SIP1 localization. Additionally, cellular stress induced by continuous culture of cell lines may affect the intracellular SIP1 destination. Consistent with our immunohistochemistry findings, a recent report also indicated cytoplasmic expression of SIP1 in ovarian tumors (Yoshida et al., 2009). Strong SIP1 expression in normal epithelial cells including hepatocytes, kidney tubules, stomach glandular epithelium and colon surface epithelium suggests the co-existence of E-cadherin and SIP1. Moreover, SIP1 appears to be prevented from translocating into nucleus in these tissues. It is therefore plausible to state that, unlike ZEB1, SIP1 and E-cadherin expression is not necessarily mutually exclusive. Among the analyzed tissues, most of the normal tissues expressed SIP1 from moderate to strong intensity, and we found SIP1 overexpression only in kidney, breast, lung and uterus tumors. On one hand, this differential expression may suggest a protective role for SIP1 against tumorigenesis. In fact, SIP1 was shown to directly repress cyclin D1 (Mejlvang et al., 2007). Also, induced expression of SIP1 was reported to be partly responsible for hTERT repression in hepatocellular carcinoma cells (Ozturk et al., 2006). On the other hand, SIP1 may be implicated in tumor development irrespective of its role in inducing EMT. In accordance with our results that SIP1 was upregulated in some tumors, we recently showed that SIP1 protects cancer cells from DNA damage-induced apoptosis (Sayan et al., 2009). Also, SIP1 takes part in the TGF- β pathway and the effects of TGF- β on cells are variable and depend on many factors including cell type and physiological state of tissues (Massague, 2008; Postigo, 2003).

Given the functional role of SIP1 as a transcriptional repressor, this and aforementioned studies suggest additional levels of regulation on SIP1 activity in tumors in a tissue and/or context-dependent manner.

Downstream to TGF- β signaling, ZEB1 and SIP1 regulate transcription of target genes in conjunction with SMADs and CtBPs. ZEB1 and SIP1 were shown to have opposing effects on transcriptional regulation (Postigo, 2003). A feedback mechanism was described in which ZEB1 and SIP1 show antagonism by differential recruitment of coactivators and corepressors to SMAD complexes, respectively (Postigo et al., 2003). In addition, the expression of ZEB1 and SIP1 was shown to be down-regulated by micro RNAs (Gregory et al., 2008). The effects of these post-transcriptional regulation mechanisms on SIP1 protein might be explored by using these novel MABs in further functional studies.

Herein, we performed a pilot study for the understanding of tissue/tumor specific SIP1 protein expression with the newly developed SIP1 specific antibodies using multi-tissue arrays. We showed that SIP1 protein levels increased only in a restricted group of tumors and most normal tissues displayed SIP1 expression at some extent. We feel to stress a drawback of staining multi-tumor arrays as the DAB color reaction has to be stopped when a detectable signal from majority of the samples is observed. Thus, our staining is optimal for high/medium SIP1 expressing tissues and may be sub-optimal for low SIP1 expressing ones. Lower SIP1 expressing tissues may have to be re-tested at their optimal conditions for the better understanding of SIP1 function in tumor development. Our recent paper that we analyzed SIP1 expression by immunohistochemistry and described SIP1 protein overexpression as a marker of poor prognosis in bladder cancers is a good example that SIP1 can be identified as a pro-metastatic protein (Sayan et al., 2009).

In conclusion our observation that SIP1 localized predominantly to the cytoplasm in both tumor and normal tissues suggests the implication of unidentified regulatory mechanisms that prevent translocation of SIP1 into the nucleus. This, in turn, adds another level of complexity to the control of EMT program in tumors progressing towards metastatic state. Therefore, our findings bring novel opportunities to further elaborate the role of SIP1 in tumor development.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Immunization with UV-Induced Apoptotic Cells Generates Monoclonal Antibodies Against Proteins Differentially Expressed in Hepatocellular Carcinoma Cell Lines

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ABSTRACT

Early and differential diagnosis of hepatocellular carcinoma (HCC) requires sensitive and specific tissue and serum markers. On the other hand, proteins involved in tumorigenesis are extensively modulated on exposure to apoptotic stimuli, including ultraviolet (UVC) irradiation. Hence, we generated monoclonal antibodies by using UVC-irradiated apoptotic cells of an HCC cell line, HUH7, aiming to explore proteins differentially expressed in tumors and apoptosis. We obtained 18 hybridoma clones recognizing protein targets in apoptotic HUH7 cells, and clone 6D5 was chosen for characterization studies because of its strong reactivity in cell-ELISA assay. Subtype of the antibody was IgG3 (κ). Targets of 6D5 antibody were found to be abundantly expressed in all HCC cell lines except FLC4, which resembles normal hepatocytes. We also observed the secretion of 6D5 ligands by some of the HCC cell lines. Moreover, cellular proteins recognized by the antibody displayed a late upregulation in UVC-induced apoptotic cells. We concluded that 6D5 target proteins are modulated in liver tumorigenesis and apoptotic processes. We therefore propose the validation of our antibody in tissue and serum samples of HCC patients to assess its potential use for the early diagnosis of HCC and to understand the role of 6D5 ligands in liver carcinogenesis.

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most frequently occurring cancers worldwide, and in some regions of Asia, represents the primary cause of death due to cancer.^(1,2) Its incidence continues to increase throughout the world due to hepatitis B virus (HBV) infections in developing countries,⁽³⁾ and hepatitis C virus (HCV) infections⁽⁴⁾ and excessive alcohol intake⁽⁵⁾ in developed western countries and Japan. These liver-damaging agents lead to liver cirrhosis, which, once established, constitutes the major background for HCC development.⁽⁶⁾ Therefore, patients with liver cirrhosis are periodically surveyed for the diagnosis of HCC at early stages of tumor development. Serum α -fetoprotein levels (AFP) and hepatic ultrasonography (HUS) are the screening tools of choice,⁽⁷⁾ yet other promising biomarkers, such as des-gamma carboxypro-

thrombin, lens culinaris-agglutinin reactive AFP, human hepatocyte growth factor-1 (HGF-1), insulin-like growth factor-1 (IGF-1), and glypican-3, are currently under intensive investigation.^(8,9) However, the sensitivity and the specificity of these tumor markers for HCC are variable and most of them are not introduced yet into routine clinical practice.⁽⁹⁾ Therefore, the development of novel markers for HCC with stronger sensitivity and specificity is of great importance for the surveillance of patients with liver cancer risk.

Cancer cells develop various strategies, including resistance to apoptotic stimuli, in order to escape from host immune surveillance mechanisms.⁽¹⁰⁾ They increase the expression of antiapoptotic proteins, while decreasing proapoptotic ones, either by mutations or epigenetic regulations.^(11,12) In order to explore proteins that are modulated in apoptosis and tumorigenesis processes, we produced monoclonal antibodies by using as im-

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munogen UVC-induced apoptotic cells of an HCC cell line, HUH7. The expression of the targets of clone 6D5 was studied in both HCC cell lines and apoptosis-induced HUH7 and Jurkat cells.

MATERIALS AND METHODS

HCC cell lines and antibodies

HCC-derived HUH7, Hep40, Hep3B, Hep3B-TR, PLC/PRF5, FLC4, SK-Hep1, Focus, Mahlavu, SNU182, SNU387, SNU398, SNU449, and SNU475 and hepatoblastoma-derived HepG2 cell lines were analyzed. SNU182, SNU387, SNU398, SNU449, and SNU475 cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) 100 IU penicillin, 100 μ g streptomycin, and nonessential amino acids (RPMI-10). All other cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of FBS, 100 IU penicillin, 100 μ g streptomycin, and nonessential amino acids (DMEM-10). Sp2/0 mouse myeloma cells were grown in high-glucose DMEM-10 and used as fusion partner for hybridoma production. A T-cell leukemia cell line, Jurkat, was grown in RPMI-10. Anticalnexin monoclonal antibody was a kind gift of Dr. Mehmet Ozturk (Bilkent University, Ankara, Turkey). Anti-Fas antibody (clone CH11) was purchased from Upstate Biotechnology (Lake Placid, NY).

Apoptosis induction in HUH7 and Jurkat cells

Two well-established apoptosis-induction methods, UVC irradiation and anti-Fas treatment, were used for HUH7 and Jurkat cells, respectively.⁽¹³⁾ Briefly, HUH7 cells were grown to 80–90% confluency in 10 cm tissue culture plates. Cells were washed three times with phosphate-buffered saline (PBS) and exposed to UVC irradiation (20J/M²) in UV-crosslinker apparatus (Stratagene, La Jolla, CA). After the addition of fresh medium, plates were transferred into CO₂ incubator. Jurkat cells were plated at a density of 5×10^5 /mL and treated for 24 h with 50 ng/mL of anti-Fas antibody. Both treated and untreated HUH7 and Jurkat cells were then collected at desired time points for subsequent apoptosis detection and Western blot assays. Apoptosis was analyzed both by examining morphologic changes under the light microscope and by Annexin V staining (Annexin V-FITC Apoptosis Detection Kit, Sigma-Aldrich, St. Louis, MO) of apoptotic cells, according to the manufacturer's instructions.

Immunization and production of monoclonal antibodies

$5\text{--}10 \times 10^6$ UVC-treated apoptotic HUH7 cells were scraped from culture plates and, after washing with PBS, were injected into the peritoneal cavity of 6- to 8-week-old BALB/c mice (day 0). Three more injections were repeated at 3 week intervals, and immunization efficiency was assessed on days 25 and 45 by cell-ELISA, as described below. Three days after the last injection, immunized mice were sacrificed and their spleen cells were fused with Sp2/0 mouse myeloma cells by standard protocol.⁽¹⁴⁾ Hybrid cells were plated into 96-well tissue culture plates in hybridoma growth medium consisting of high-glucose

DMEM supplemented with 20% FCS, 100 IU penicillin, 100 μ g streptomycin, nonessential amino acids, and hybridoma selection reagents (hypoxanthine, aminopterin, thymidine (HAT); Sigma-Aldrich). Screening of hybrid cells was performed by cell-ELISA and clones with high absorbance values were subjected to subclonings by limited dilution. Isotype of antibodies was determined by ImmunoPure Monoclonal Antibody Isotyping Kit (Pierce, Rockford, IL), according to the manufacturer's instructions.

Cell-ELISA

HUH7 cells were plated in 96-well tissue culture plates at a density of 5×10^4 cells/well, and UVC irradiation was applied when cells reached confluency. After 16 h, medium was aspirated from the wells of culture plates and cells were fixed by 100% ice-cold methanol. Next, fixed cells were incubated at room temperature with supernatant of growing hybridomas for 2 h followed by incubation for 1 h with alkaline phosphatase (AP) conjugated anti-mouse IgG (Sigma-Aldrich) at 1:1000 dilution. Colorimetric reaction was allowed to occur by using paranitrophenylphosphate (pNPP) substrate solution (pNPP tablets, Sigma-Aldrich). Plates were read at A₄₀₅ using a microplate reader (Beckman, Fullerton, CA). Between each step of cell-ELISA, wells were washed gently with PBS to prevent the detachment of fixed cells.

Western blotting

Total cell lysates from 14 HCC and Jurkat cell lines were prepared in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P40 [v/v] and a cocktail of EDTA-free protease inhibitors [Roche Diagnostics, Mannheim, Germany]). Protein content was measured by Bradford assay.⁽¹⁵⁾ Equalized lysates were run on 10% SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes by using semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked overnight with 5% dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T). Undiluted 6D5 hybridoma supernatant was used as primary antibody. After washing three times with TBS-T, horseradish peroxidase (HRP) conjugated anti-mouse IgG (Sigma-Aldrich) was used as secondary antibody at 1:5000 dilution. Protein bands were visualized using ECL Plus chemiluminescent substrate (Amersham, Piscataway, NJ). For equal loading control, membranes were reprobed with anticalnexin antibody.

Immunofluorescence

HUH7, HepG2, and PLC/PRF5 cells were grown on cover slips in 6-well plates. PBS was used in all washing steps. Cells were fixed in 2% paraformaldehyde in PBS and permeabilized in PBS containing 0.2% triton X-100. After blocking with 2% bovine serum albumin (BSA) in PBS, cells were incubated for 2 h at room temperature with undiluted 6D5 hybridoma supernatant. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Sigma-Aldrich) was used as secondary antibody at 1:200 dilution. Nuclei counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI); cover slips were mounted on glass slides and examined under fluorescent microscope (Carl Zeiss, Göttingen, Germany). Merged images were produced by using AxioVision image processing software (Carl Zeiss).

Immunoprecipitation and Western blotting (IP-WB) of secreted proteins

First serum-free culture media of HCC cell lines were incubated for 2 h at 4°C with 100 μ L of 6D5 hybridoma supernatant; then 50 μ L of protein G agarose beads (East Coast Biologicals), pre-cleaned with three PBS washes, were added. The mixture was incubated overnight at 4°C with continuous rotation. Next, beads were precipitated by centrifugation, washed three times with PBS, and antigen-antibody complexes were eluted by resuspending beads with an equal volume of 2X SDS-PAGE sample loading buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and freshly added 200 mM DTT). SDS-PAGE and Western blotting were performed as described above.

RESULTS

Immunization with apoptotic HUH7 cells

To determine the appropriate time for harvesting cells following apoptosis induction, we looked for the presence of apoptotic cells at various times after ultraviolet irradiation. HUH7 cells were grown on cover slips and exposed to UVC at 20 J/M². FITC-labeled Annexin V analyses of UVC-treated and untreated HUH7 cells were performed at 4 h and 24 h post-UVC treatment. Treated cells displayed strong reactivity with FITC-labeled Annexin V at both time points, while very low staining, if any, was observed in unstimulated HUH7 cells (Fig. 1). Therefore, we started immunization of BALB/c mice with cells harvested at 16 h post-UVC treatment, and we repeated injections of apoptotic cells at 3 week intervals. The development of an immune response in mice sera was assessed by cell-ELISA, as described in the above Materials and Methods section (data not shown).

Production of monoclonal antibodies

Following the fusion of spleen cells of immunized mice with Sp2/0 myeloma cells, cell-ELISA assays were performed. This time HUH7 cells were grown to confluency in 96-well tissue culture plates and then exposed to UVC to screen apoptotic cells for their immunoreactivity with antibodies secreted by hybridoma clones. We obtained 18 hybridoma clones recognizing protein targets in apoptotic cells. Out of these, clones 6D5, 6E10, 9C11, and 11G8 displayed higher cell-ELISA absorbance values, with 6D5 showing highest ELISA titer (data not shown). All reactive clones were subcloned two times by limiting dilution, and monoclonal antibody-secreting hybridomas were expanded in culture for supernatant collection and cryopreservation. Characterization studies were then initiated with 6D5 antibody by reasoning that high cell-ELISA titer of this clone might correlate with overexpression of its ligands in both HCC cell lines and cells undergoing apoptosis upon ultraviolet irradiation. The isotype of the antibody was found to be IgG3 (κ).

6D5 recognizes differentially expressed proteins in HCC cell lines

We first analyzed the immunoreactivity of 6D5 antibody with whole cell lysates of HCC cell lines. In three separate Western blot assays, two major proteins with closer molecular weights

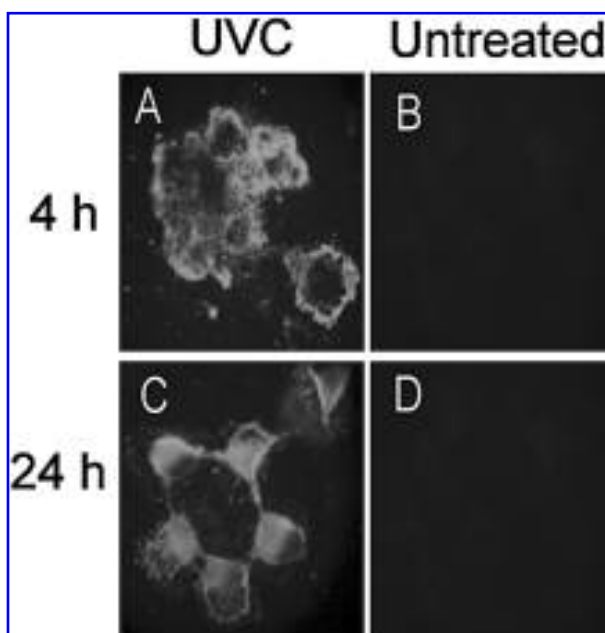


FIG. 1. Annexin V-FITC staining of HUH7 cells after UVC irradiation. Cells were grown on cover slips in 10 cm culture plates and then exposed to 20J/cm² of UVC. Annexin V-FITC staining was performed at 4 h (A) and 24 h (C) post-UVC treatment. (B and D) Untreated controls of A and C, respectively.

of ~80 kDa were found to be expressed either alone or concomitantly in all studied cells (Fig. 2, upper row). Strong band intensity suggested an abundant expression of the proteins in these cell lines. Equal protein loading analysis was performed by reprobing the membrane with anticannexin antibody (Fig. 2, lower row). Lysates of all cells have been shown to contain equal amounts of total protein, except SK-Hep1 cells, which displayed a significant decrease in calnexin protein level. This result repeatedly persisted in independent immunoblots, suggesting a downregulation of this ER chaperone in that cell line. Cell lines deriving from undifferentiated and invasive tumors, including SNU475, SNU398, SNU449, SNU387, SK-Hep1, and Mahlavu, seemed to express 6D5 ligands stronger than did differentiated HUH7 and HepG2 cell lines (Fig. 2, left and middle panels). However, no other correlation could be established between the protein expression patterns and phenotypic characteristics of cell lines. Protein expression was also examined in two isogenic cell lines, Hep3B and Hep3B-TR, and a third protein band with lowest molecular weight was observed only in Hep3B cells (Fig. 2, right panel). These cell lines have been described elsewhere, and Hep3B-TR cells differ from Hep3B cells by their resistance to the growth inhibitory effect of TGF-beta in cell culture.⁽¹⁶⁾ We also compared target protein expression in HUH7 and FLC4 cells and found that the latter was totally devoid of any 6D5 ligands (Fig. 3). The hepatoma cell line FLC4 was established from a well-differentiated HCC and found to possess normal liver functions.^(17,18) We therefore suggest that 6D5 targets are upregulated in HCCs.

6D5 stained the cytoplasm of HCC cell lines

Next we visualized the cellular localization of 6D5 targets by immunofluorescence microscopy in three HCC cell lines.

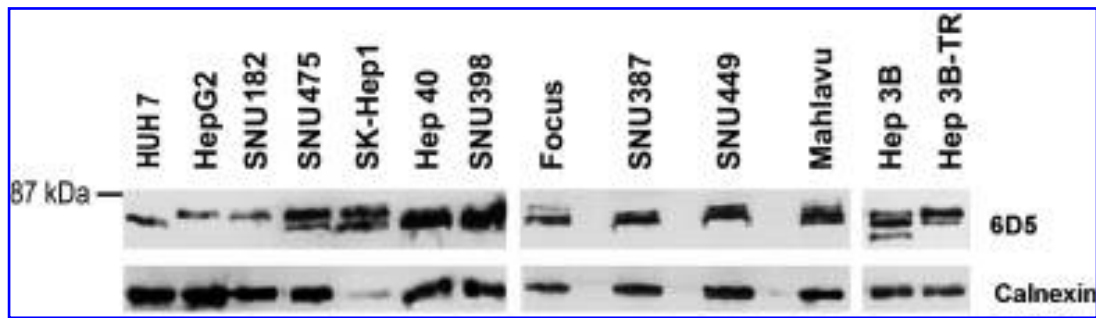


FIG. 2. Expression of 6D5 target proteins in HCC cell lines. Whole cell lysates of HCC cell lines were analyzed in three separate Western blots. Total protein extracts on PVDF membranes were first probed with 6D5 (upper row) and then with anticalnexin (lower row) antibodies. Two major protein bands with molecular weight close to 80 kDa were displayed by all cells alone (HUH7, HepG2, SNU-182, Hep40, SNU-398, SNU-387) or concomitantly (SNU-475, SK-Hep1, Focus, SNU-449, Mahlavu, Hep3B-TR). Only Hep3B cells expressed a third protein band with the lowest molecular weight.

6D5 stained the cytoplasm of HUH7, HepG2, and PLC/PRF5 cells. The strong intensity of the fluorescent signal supported our observation that proteins recognized by this antibody are abundantly expressed in HCC cell lines (Fig. 4).

Secretion of 6D5 targets from HCC cells

Protein secretion was searched first in the supernatant of isogenic cell lines Hep3B and Hep3B-TR. Cells were cultured up to 70–80% confluency and then starved for 48 h before the collection of supernatants. This strategy allowed us to eliminate abundant proteins deriving from fetal calf serum. In the IP-WB experiment, we detected expression of target proteins only in the Hep3B culture medium (Fig. 5). A lower band with sharp intensity and a heavier weak one were observed. However, the third protein band detected in whole cell lysate of Hep3B cells (Fig. 2) was absent in IP-WB (Fig. 5). The differential behavior of Hep3B and Hep3B-TR cell lines in protein secretion also supports our finding that target proteins are differentially expressed in these cells (Fig. 2). We also analyzed the secretion of target proteins from other HCC cell lines, including Focus, HUH7, SNU398, and Mahlavu. Following IP-WB with their

supernatants, we detected faint protein bands in the supernatants of Focus and SNU398 cells, but not in those of Mahlavu and HUH7 cells (data not shown).

Effects of apoptosis induction on the expression of 6D5 targets

Next, we examined the modulation of 6D5 ligands in Jurkat and HUH7 cells treated with anti-Fas antibody and UVC, respectively. Both cells were collected at 24 h post-treatment, and the expression of 6D5 target proteins was visualized by Western blotting. An induction on protein level was observed for UVC-treated HUH7 cells, but unexpectedly neither untreated nor anti-Fas-treated Jurkat cells displayed 6D5 ligand expression (Fig. 6, upper row). The same membrane was then reprobed with 9C11 antibody, and target proteins of this clone were found to be expressed by both cell lines, in both apoptotic and non-apoptotic conditions. We therefore turned to investigate in more detail the modulation of 6D5 proteins in HUH7 cells committed to apoptosis under UVC treatment. We harvested cells at various time points after UVC irradiation and compared the protein expression with that of untreated HUH7 cells. As shown in Figure 7, protein expression was significantly reduced during the initial phases of apoptosis (2, 4, and 8 h after treatment), followed by a recovery at hour 16, with protein levels exceeding those of untreated cells. This recovery was sustained at hour 24 post-UVC treatment, indicating an intermediate-to-late up-regulation of 6D5 ligands in cells undergoing apoptosis.

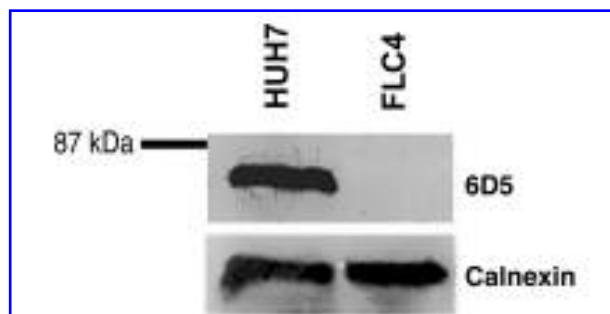


FIG. 3. Lack of target protein expression in FLC4 cell line. Target protein expression of HUH7 cells and FLC4 cells was compared by Western blotting. Immunoblot was first probed with 6D5 antibody. No protein expression was observed in FLC4 cells (upper row). Equal protein loading was controlled by reprobing the membrane with anti-calnexin antibody (lower row).

DISCUSSION

The choice of HUH7 cells as immunogen is based on our previous observations that these cells remain attached to culture plate surfaces even after they commit suicide.⁽¹³⁾ In fact, this property facilitated the collection of cells for immunization with minimal cell loss and allowed us to perform accurate cell-ELISA assays. On the other hand, HUH7 cells consist of heterogeneous cell populations, which make them an ideal source for the identification of novel markers for liver tumors, as well as the characterization of liver precursor cells involved in hepatocellular carcinogenesis.⁽¹⁹⁾

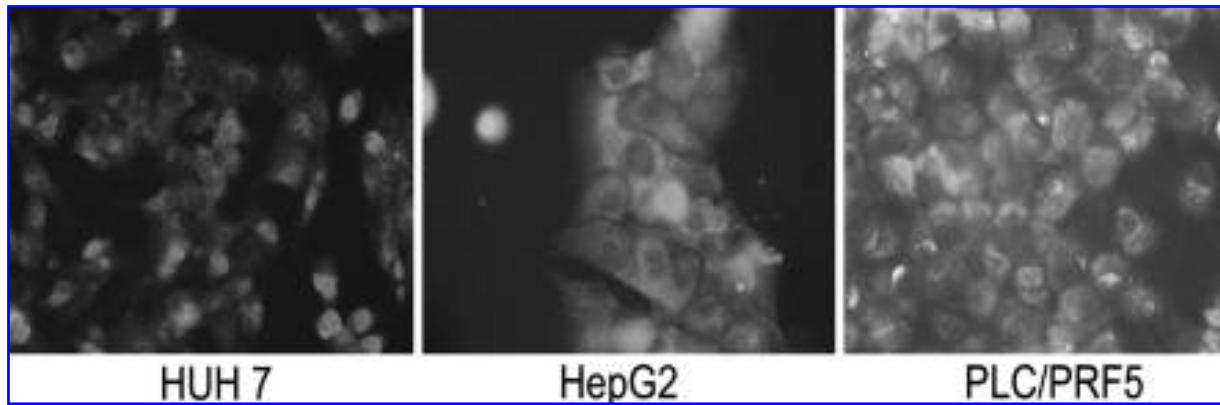


FIG. 4. Immunofluorescence analysis of HCC cell lines with 6D5 antibody. Cells were grown on cover slips and cellular location of target proteins in HUH7, HepG2, and PLC/PRF5 cell lines was evaluated by indirect immunofluorescence using 6D5 antibody. DAPI was used for nuclei counterstaining, and merged images were obtained by using image processing software. All cell lines exhibited strong cytoplasmic staining.

Our Western blot analyses pointed out several important conclusions. First, proteins recognized by 6D5 antibody are abundantly and differentially expressed in HCC cell lines, and this expression appears to increase in poorly differentiated cells. Second, target proteins are differentially expressed in both cell lysates and supernatants of Hep3B and Hep3B-TR isogenic cell lines. The fact that these cells mainly differ in their response to TGF- β suggests a modulation of 6D5 protein expression by this cytokine-mediated pathway. Third, only FLC4 cell line failed to express proteins recognized by our antibody. Well-differentiated FLC4 cells are known to have relatively well-preserved liver cell functions, such as albumin synthesis and enzyme and drug metabolism activities.^(20,21) The absence of target proteins in these cells resembling normal hepatocytes encouraged us to suggest the overexpression of 6D5 ligands in hepatocellular carcinogenesis. Moreover, we also found that Jurkat cells lack

these proteins and hypothesized that 6D5 ligand expression occurs in a tissue-restricted manner. Taken together, we propose that 6D5 target proteins are underexpressed in normal liver, up-regulated in well-differentiated HCC, and attain culmination in poorly differentiated tumors.

The appearance of multiple bands in our Western blots might be due to the expression of isoforms and/or post-translationally modified forms of the 6D5 target protein rather than spontaneous protein degradation, since reprobing the membrane with anti-calnexin antibody revealed single bands in all studied cell lines. This also led us to seek known HCC markers displaying different forms and showing the same pattern as 6D5 ligands in similar studies. The most widely used HCC tumor marker is AFP, which may be translated from multiple RNA transcripts (i.e., 2.2, 1.7, 1.6, and 1.35 kb), resulting in the expression of protein isoforms with molecular weights similar to those of 6D5 target proteins.⁽²²⁾ However, previous studies have clearly shown that AFP expression is restricted to well-differentiated cell lines and is absent in others.^(23,24) Therefore, according to our Western blot experiments showing that 6D5 ligands are expressed in all HCC cell lines regardless of their differentiation state (Fig. 2), we excluded the possibility that 6D5 antibody is directed against AFP epitopes. Another protein, which is subjected to intensive investigation, is glypican-3; results from many studies support its usefulness as a promising marker for HCC.^(8,25) This protein too, when expressed, gives rise to several isoforms, whose molecular weights approximate those of protein bands that we observed in our Western blot analyses. However, glypican-3 is a proteoglycan bound to the cell membrane through GPI anchor, whereas no membrane staining was observed on HCC cells in our immunofluorescence assay with 6D5 antibody (Fig. 4). Another group of proteins that displays differential expression in response to cellular stresses and is overexpressed in many cancers consists of members of heat shock proteins (HSP). Most HSPs reside in the cytoplasm and ER and counteract apoptotic stimuli by preventing misfolding of proteins.⁽²⁶⁾ Two important members, with molecular weights of 70 and 72 kDa, respectively, include Hsp70 and GRP78/BiP and have been recently reported to be upregulated in HCC.⁽²⁷⁾ Their expression was repeatedly shown in Jurkat

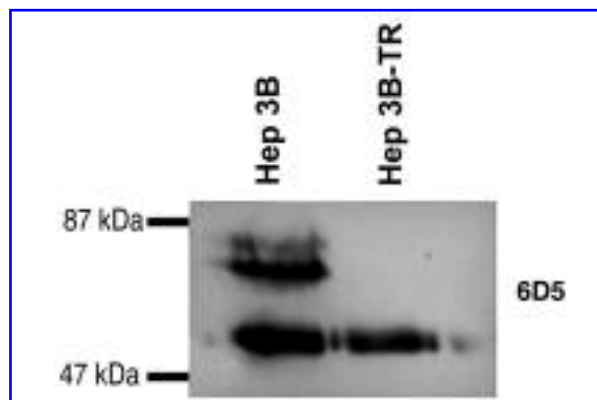


FIG. 5. Secreted forms of 6D5 target proteins. Protein secretion was evaluated in isogenic cell lines Hep3B and Hep3B-TR. Proteins were immunoprecipitated from serum-free supernatants of these cells. Following SDS-PAGE and electroblotting, the membrane was probed with 6D5 antibody. Target protein bands were detected only in the supernatant of Hep3B cells. Lower bands appearing in both supernatants correspond to immunoglobulin heavy chain.

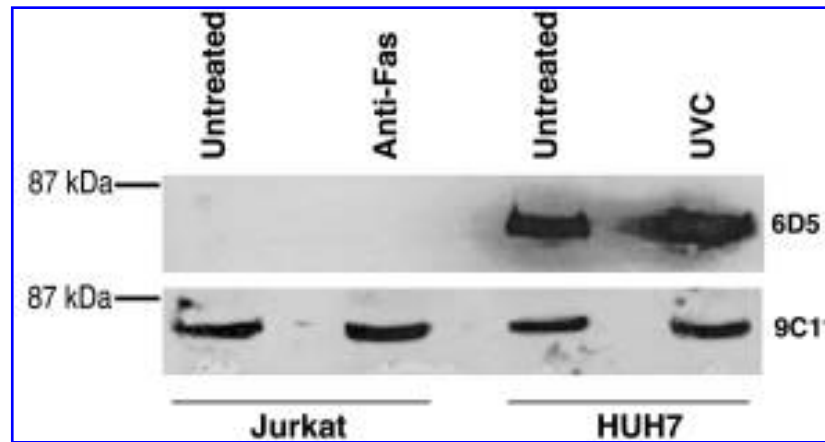


FIG. 6. Induction of apoptosis in HUH7 and Jurkat cells. Apoptosis was induced in HUH7 and Jurkat cells by UVC and anti-Fas antibody treatment, respectively. Cells were collected at 24 h post-treatment and analyzed by Western blotting. An induction on 6D5 target protein expression was observed in HUH7 cells upon UVC treatment. However, no protein expression occurred in anti-Fas treated and untreated Jurkat cells (upper row). The membrane was also probed with 9C11 antibody obtained from the same fusion experiment. This antibody recognized proteins in both cell lines in both control and treated cells (lower row). Protein ligands of 9C11 appear not to be modulated upon apoptosis induction.

cells,^(28,29) which failed to display 6D5 ligands both in apoptotic and nonapoptotic conditions (Fig. 6). We therefore reasoned that these proteins could not be the targets of our monoclonal antibody.

Immunofluorescence analysis with 6D5 antibody revealed perinuclear and granular staining pattern reminiscent of an endoplasmic reticulum location and prompted us to investigate the secretion of proteins recognized by our monoclonal antibody. However, secreted forms of target proteins were observed only in a limited number of HCC cell lines. Nevertheless, we cannot exclude the possibility that the levels of secreted proteins by other cells might be below the detection limit of our IP-WB. Therefore, it would be useful to screen HCC patients' sera with our antibody to validate the tumor marker potential of target proteins.

Tumor cells harbor mechanisms enabling them to bypass apoptotic cell death, which might result from cellular stresses, and attack by immune effector cells and anti-cancer therapeutic agents. Proteins involved in apoptotic processes are subject to differential modulation upon encounter with genotoxic stimuli.⁽³⁰⁾ In our study, we triggered apoptosis by treating HUH7 cells with UVC irradiation and examined the expression of 6D5

target proteins. It has been reportedly shown that protein synthesis is inhibited in cells exposed to UV stress through phosphorylation of the α subunit of eukaryotic translation initiation factor 2.^(31,32) One of the latter studies also reported a sustained suppression of translation for up to 48 h.⁽³¹⁾ This is in sharp contrast with our observation that 6D5 target proteins display an intermediate-to-late upregulation, which culminates at 24 h post-UVC treatment.

The gene expression pattern of cells exposed to genotoxic stresses varies enormously depending on both the experimental conditions and genetic backgrounds of cells.⁽³³⁾ This phenomenon supports our results that 6D5 ligands are subject to modulation in our experimental settings based on UVC irradiation. However, taken together with our data showing abundant expression, as well as secretion of target proteins by HCC cell lines, we can conclude that the ligands of our antibody are some of the molecular players involved in liver tumorigenesis. Target protein identification and examination of the reactivity of this antibody with tissues and sera of HCC patients would be useful for the exploitation of 6D5 in diagnostic and prognostic studies of HCC.

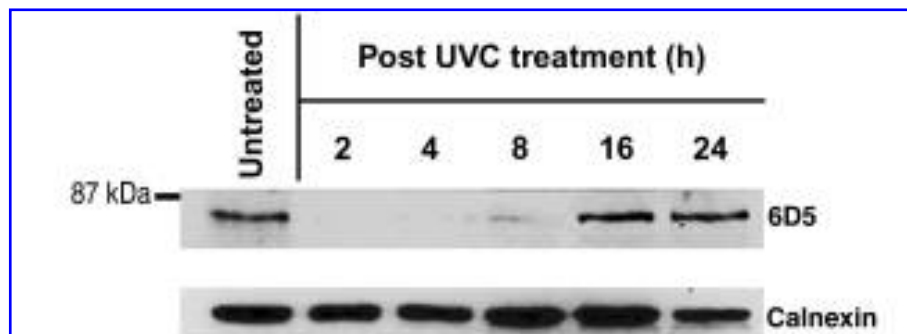


FIG. 7. Intermediate-to-late induction of 6D5 target proteins in apoptotic HUH7 cells. UVC-treated HUH7 cells were collected at given time points and compared with untreated control by Western blotting. Immunoblot was first probed with 6D5 antibody (upper row), and equal protein loading was assessed by reprobing the membrane with anti-calnexin antibody (lower row).

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