Sulfatase 1 and Sulfatase 2 in Hepatocellular Carcinoma: Associated Signaling Pathways, Tumor Phenotypes, and Survival

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The heparin-degrading endosulfatases sulfatase 1 (SULF1) and sulfatase 2 (SULF2) have opposing effects in hepatocarcinogenesis despite structural similarity. Using mRNA expression arrays, we analyzed the correlations of SULF expression with signaling networks in human hepatocellular carcinomas (HCCs) and the associations of SULF expression with tumor phenotype and patient survival. Data from two mRNA microarray analyses of 139 and 36 HCCs and adjacent tissues were used as training and validation sets. Partek and Metacore software were used to identify SULF correlated genes and their associated signaling pathways. Associations between SULF expression, the hepatoblast subtype of HCC, and survival were examined. Both SULF1 and 2 had strong positive correlations with periostin, IQGAP1, TGFB1, and vimentin and inverse correlations with HNF4A and IQGAP2. Genes correlated with both SULFs were highly associated with the cell adhesion, cytoskeletal remodeling, blood coagulation, TGFB, and Wnt/β-catenin and epithelial mesenchymal transition signaling pathways. Genes uniquely correlated with SULF2 were more associated with neoplastic processes than genes uniquely correlated with SULF1. High SULF expression was associated with the hepatoblast subtype of HCC. There was a bimodal effect of SULF1 expression on prognosis, with patients in the lowest or highest tertile having a worse prognosis than those in the middle tertile. SULFs have complex effects on HCC signaling and patient survival. There are functionally similar associations with cell adhesion, ECM remodeling, TGFB, and WNT pathways, but also unique associations of SULF1 and SULF2. The roles and targeting of the SULFs in cancer require further investigation.

INTRODUCTION

Sulfatase 1 (SULF1) and sulfatase 2 (SULF2) are heparin-degrading endosulfatases that act on internal glucosamine 6-O-sulfate modifications within heparan sulfate proteoglycans and modulate heparin-binding growth factor signaling. Emerging evidence suggests that the SULFs play important roles in the pathogenesis of a number of cancers. SULF1 and SULF2 are structurally similar enzymes, but have been shown to have opposite effects on liver cancer cells in vitro and in vivo. SULF1 has a tumor suppressor function through inhibition of receptor tyrosine kinase signaling by desulfation of heparan sulfate proteoglycans that act as coreceptors for heparin binding growth factors and their cognate receptor tyrosine kinases (Lai et al., 2003, 2004b). The tumor suppressor effect of SULF1 has been reported in hepatocellular carcinoma (HCC) (Lai, et al., 2004b,2006), head and neck (Lai et al., 2004a), myeloma (Dai et al., 2005), pancreatic (Li et al., 2005), breast (Narita et al., 2007), and ovarian cancer (Lai et al., 2003). In contrast SULF2 has been shown to have an oncogenic effect through activation of the receptor

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tyrosine kinases and their downstream MAPK and Akt pathways (Lai et al., 2008), and also through activation of the Wnt signaling pathway (Nawroth et al., 2007). The oncogenic role of SULF2 has been demonstrated in HCC (Lai et al., 2008), lung (Lemjabbar-Alaoui et al., 2010), breast (Morimoto-Tomita et al., 2005), and pancreatic cancers (Nawroth et al., 2007). Somewhat paradoxically, emerging evidence suggests that while SULF1 and SULF2 have opposing effects on receptor tyrosine kinase signaling, they both activate Wnt pathway signaling in cancer cells (Nawroth et al., 2007). While SULF1 and SULF2 have highly-conserved N-terminal sulfatase domains, their COOH-terminal regions have divergent structures. The COOH-terminal regions contain charged domains that bind to heparan sulfate chains, and the differences in the heparan sulfate recognition sites in these regions may account for the differences in the functional effects of SULF1 and SULF2. The observed similarities and differences in SULF1 and SULF2 action in cancer are consistent with observations made on their effects in knockout mouse models, which show that although the two enzymes show some functional redundancy, knockouts of the two enzymes have similar but not identical effects on heparan sulfate structure and mouse phenotypes (Lamanna et al., 2006; Lum et al., 2007; Kalus et al., 2009). Given the limited information available on the role of SULF1 and SULF2 in hepatocarcinogenesis, we undertook this study to characterize the disease states and signaling pathways associated with SULF1 and SULF2 in human HCCs examined using high density oligonucleotide mRNA gene expression analysis. Further, we assessed the effects of SULF1 and SULF2 expression on overall patient survival.

MATERIALS AND METHODS

Patient Characteristics of Training and Validation Datasets

Training dataset

Clinical information and microarray data available from a previous analysis were used as the training data set for this study (Lee et al., 2006). Briefly tumor and adjacent benign tissues from 139 HCC patients undergoing surgical resection for HCC were obtained from centers in Asia, Europe, and the United States. The median age of the individuals was 57 and median follow-up was 23.4 months; 73.3% were male and 74 patients died during the follow-up period.

Validation dataset

The 36 patients included in the validation analysis had surgical resection of HCC between December, 2001 and December 2007 at a single institution, Keimyung University Dong-San Medical Center, Daegu, Korea. Gene expression profiling of the HCC tissues and adjacent benign tissues was performed. The study protocol was approved by the Institutional Review Board for the use of human subjects at the Keimyung University School of Medicine, and all participants provided written informed consent.

Microarray Gene Expression Profiling

Training dataset

RNA from HCC and adjacent benign tissue from the 139 HCCs was analyzed at the US National Cancer Institute using the Qiagen Human Array-Ready Oligo Set (version 2.0), which contains 70-mer probes for 21,329 genes. CsCl density-gradient centrifugation was used to isolate total RNAs from frozen liver tissue. Total RNA from 19 normal livers were used as the reference for all microarray experiments. A Cy-5/Cy-3 dye swap strategy was used as described previously (Lee et al., 2006). Expression ratios of each gene (tumor/adjacent benign tissue) were averaged from duplicate experiments. Valid SULF1 and SULF2 expression levels were available in 118 and 139 HCC samples, respectively.

Validation dataset

RNA from HCC and adjacent benign tissue from 36 patients with HCC was analyzed on the Illumina gene expression platform. The quality of total RNA was checked by gel electrophoresis and RNA concentrations were determined using an Ultrospec 3100 pro spectrophotometer (Amersham Bioscience, Buckinghamshire, UK). Biotin-labeled cRNA samples were prepared for hybridization according to the recommended sample labeling procedure: 500 ng of total RNA was used for cDNA synthesis, followed by an amplication/labeling step (in vitro transcription) to synthesize biotin-labeled cRNA using the Illumina® TotalPrep RNA Amplification kit (Ambion, Austin, TX). cRNA concentrations were measured by the RiboGreen method (Quant-iT™ RiboGreen® RNA assay kit; Invitrogen-Molecular Probes, ON, Canada) using a Victor^3 spectrophotometer (PerkinElmer, CT) and cRNA quality was checked on a 1% agarose gel. Labeled, amplified material (1,500

ng per array) was hybridized to Version 3 of the Illumina Human-6 BeadChip (48 K) (Illumina, San Diego, CA). Array signals were developed using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the BeadChip manual. Arrays were scanned with an Illumina BeadArray Reader confocal scanner (BeadStation 500GXDW; Illumina).

Statistical Analyses

Identification of SULF1 and 2 correlated genes

A Pearson correlation comparison was performed comparing gene expression of SULF1 or SULF2 and each of 21,329 genes available in the data set for the 139 patients. Correlation coefficients and two-sided test P values were calculated under the null hypothesis of no correlation. The significance level was set at $\alpha < 0.001$. The step up false discovery rate (FDR) was calculated to adjust for multiple testing. The estimated FDR for the 0.001 cutoff was 0.02 for SULF1 and 0.01 for SULF2. The analyses were performed using Partek software (http://www.partek.com/). Lists of genes that had significant correlation with SULF1 ($P < 0.001$) but not with SULF2 ($P > 0.001$) were defined as SULF1 unique and genes that had significant correlation with SULF2 ($P < 0.001$) but not with SULF1 ($P > 0.001$) were defined as SULF2 unique. Lastly, genes that had significant correlation with both SULF1 ($P < 0.001$) and SULF2 ($P < 0.001$) were defined as SULF1 and SULF2 shared.

MetaCore pathway analysis

MetaCore software (http://www/genego.com/metacore.php) was used to investigate the diseases and pathways associated with the SULF1 unique, SULF2 unique, and SULF1 and SULF2 shared gene sets. Genes with $P < 0.001$ were included in the following analyses using the default Metacore reference database. Diseases and pathways with which the SULF correlated genes were associated more highly than would be expected by chance were identified. The significance of the association with each disease or pathway was estimated by hypergeometric test P value.

Association of SULF1 and SULF2 expression with the hepatoblast phenotype of HCCs

Our previous study of the 139 HCCs identified a subclass of HCCs with a hepatoblast or progenitor cell signature, allowing classification of HCCs into hepatoblast/progenitor cell and mature hepatocyte subtypes (Lee et al., 2006). The association between SULF1 and SULF2 expression and the hepatoblast subtype was examined using Fisher's exact test for trend.

Survival Analysis

Of the 139 individuals, 26 (17 underwent liver transplantation and 9 received palliative treatment) were not included in the survival analysis. Survival of the two groups of patients defined by SULF1 expression below versus above the median, and of the three groups of patients defined by tertiles of SULF1 expression, lower third, middle third, and upper third, were compared. Survival probabilities were estimated using the Kaplan–Meier method and the differences in the overall survival were compared using the log rank test.

Validation

Gene expression profiles of 48,803 probes from 36 individual HCC tissue samples and adjacent benign tissue were obtained. Expression ratios of each gene (tumor/adjacent benign tissues) were used for the validation of (1) pathways and diseases associated with SULF1 unique, SULF1 and SULF2 shared, and SULF2 unique gene sets and (2) the correlation between SULF1 or SULF2 expression and that of individual genes.

Immunohistochemistry and Immunocytochemistry

Immunohistochemical staining was performed as previously described (Lai et al., 2006). Paraffin-embedded blocks of HCC xenografts established from empty vector, SULF1, or SULF2 stably transfected Hep3B cells in nude mice were cut (4 μm) and incubated with polyclonal anti-β-catenin antibody followed by processing in an autostainer (Dako Corporation) using the EnVision+ protocol. For immunocytochemistry, stable Hep3B HCC cell clones transfected with SULF1, SULF2 or vector control were used as previously described (Lai et al., 2004b, 2008). Cells growing in eight-well chamber slides were fixed with 4% formaldehyde. Slides were incubated in anti-β-catenin antibody (mAb, 1:200) for 1 hr at room temperature, followed by incubation with Anti-mouse Alexa 488 (Invitrogen) for 1 hr at room temperature. Slides were mounted with DAPI and observed using a confocal microscope (Zeiss LSM-510) as described (Lai et al., 2004b).

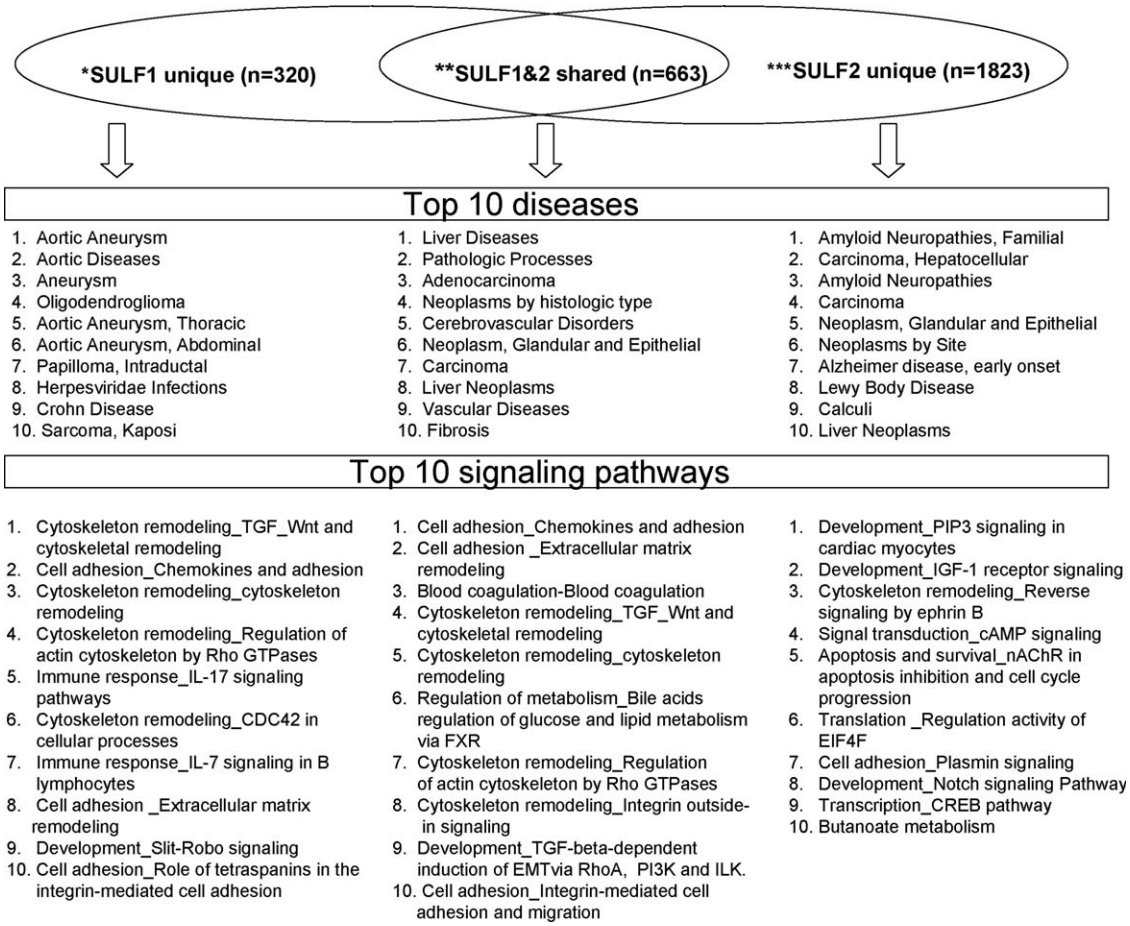

Figure 1. Diseases and pathways associated with SULF correlated genes. Genes included in SULF1 unique, SULF2 unique, SULF1 and SULF2 shared were identified using Partek Software and diseases and pathways associated with individual list of genes were selected using Metacore Software and sorted by ascending order of *P* value. *The SULF1 unique included genes that had significant correlation with SULF1 ($P < 0.001$) but not with SULF2 ($P > 0.001$). **.The SULF1 and SULF2 shared included genes that had significant correlation with SULF1 ($P < 0.001$) and SULF2 ($P < 0.001$). *** The SULF2 unique included genes that had significant correlation with SULF2 ($P < 0.001$) but not with SULF1($P > 0.001$). *P* value for diseases ranges from 5.7E-12 to 7.2E-07 and *P* value for pathways ranges from 1.6E-08 to 1.3E-03 in SULF1 unique gene set. *P* value for disease ranges from 4.0E-23 to 9.9E-20 and *P* value for pathways ranges from 7.6E-14 to 6.7E-06 in SULF1 and SULF2 shared gene set. *P* value for disease ranges from 5.7E-09 to 6.9E-07 and *P* value for pathways ranges from 2.1E-06 to 1.5E-04 in SULF2 unique gene set.

RESULTS

Genes Correlated with SULF mRNA Expression

A total of 983 and 2,486 genes were correlated with SULF1 and SULF2, respectively, while 663 genes were correlated with both SULF1 and SULF2, at a *P* value <0.001 (Fig. 1). The expression of SULF1 and SULF2 were significantly correlated with each other, with a correlation coefficient of 0.41 ($P = 3.8E-06$) (Fig. 2). Table 1 shows the list of the top 40 genes included in the SULF1 and 2 shared gene set, selected by *P* value and ranked by *r* value of correlation with either SULF1 or SULF2 from highest to lowest. Lists of the top 40 genes in the SULF1 unique and SULF2 unique groups are provided as Supporting Information Tables 1 and 2. Periostin (POSTN), which is associated with the epithelial-mesenchymal transition (EMT) and extrahepatic recurrence of HCC, had the highest positive correlation with SULF1. IQGAP1, which encodes a key regulator of cell adhesion and migration, had the third highest positive correlation with SULF2 while IQGAP2 had the second highest inverse correlation with SULF2. HNF4A, the major transcriptional regulator of hepatocellular differentiation, had the highest inverse correlation with SULF2. TGFB1, and multiple additional TGFB regulated genes, most of which have been implicated in the EMT or in cell adhesion, migration, invasion, metastasis or angiogenesis, also had highly significant positive correlations with both SULF1 and SULF2. These genes include CDK2AP1, COL6A3,

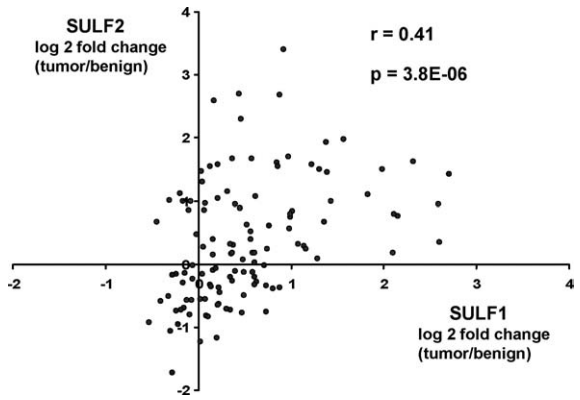

Figure 2. Correlation between SULF1 and SULF2 expression (log 2 tumor/benign expression ratio). The ratios of SULF expressions in tumor versus adjacent benign tissues were expressed as log 2 fold changes and their correlation was demonstrated in a scatter plot.

COL1A2, COL6A2, Lumican (LUM), collagen binding protein 2 (SERPINH1), tissue inhibitor of metalloprotease-1 (TIMP1), tissue inhibitor of metalloprotease-2 (TIMP2), filamin A (FLNA), and vimentin (VIM).

Diseases Associated with SULF Correlated Genes

The top 10 diseases that were most highly associated with SULF1 unique, SULF1 and SULF2 shared, and SULF2 unique gene sets are shown in Figure 1. Five of the 10 disease entities most highly associated with the SULF1 and SULF2 shared gene set were neoplasms and liver neoplasm was the eighth most significant disease. SULF1 unique genes were associated with diseases involving connective tissues and blood vessels, while, in contrast, SULF2 unique genes were still associated with neoplasms, including HCC, neuropathies, and dementia. Table 2 shows the list of known HCC-associated genes whose expression is significantly correlated with SULF1 or SULF2 in HCCs at the $P < 0.0001$ level of significance ($r > 0.5$ or $r < -0.5$ for either SULF1 or SULF2). The gene lists are almost identical and include genes involved in cell adhesion, extracellular matrix (ECM) and cytoskeletal remodeling pathways. Several of the genes, including TGFB1 and VIM, are associated with the EMT, a key phenotypic process in cancer that is associated both with the cancer stem cell phenotype and with tumor invasion and metastasis.

Pathways Associated with SULF Correlated Genes

Next, we explored the molecular signaling pathways associated with SULF1 and SULF2 expression (Fig. 1). The cell adhesion (chemokines, extracellular matrix remodeling, and integrin-related cell adhesion and remodeling), cytoskeleton remodeling (TGFB, Wnt, regulation of actin cytoskeleton by Rho GTPases, and integrin outside-in signaling), blood coagulation, bile acid regulation of glucose and lipid metabolism, and TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK (Supporting Information Fig. 1) were the pathways most highly associated with the SULF1 and SULF2 shared gene sets. Pathways associated with the SULF1 unique gene set included cytoskeleton remodeling (TGFB, Wnt, regulation of actin cytoskeleton by Rho GTPases and CDC42), cell adhesion (chemokines and extracellular matrix remodeling), immune response (IL-17 and IL-7 signaling), and Slit-Robo signaling in development. Several pathways involved in developmental signaling, cell cycle progression, transcriptional, and translational regulation were associated with SULF2 unique genes.

Recent results have suggested that both SULF1 and SULF2 activate Wnt pathway signaling in cancer, including pancreas cancer and HCC (Nawroth et al., 2007; Lai et al., 2010; Lemjabbar-Alaoui et al., 2010). There is also evidence suggesting that both SULF1 and SULF2 are transcriptionally regulated by TGFB (Yue et al., 2008). Given the association of SULF1 and SULF2 expression with the TGFB, Wnt cytoskeletal remodeling pathway, we therefore examined the correlations between SULF1, SULF2, and Wnt and TGFβ pathway molecules. Table 3 shows the list of SULF1 or SULF2 correlated genes that are associated with the cytoskeletal remodeling, TGFB, and Wnt pathways ($P < 0.0001$). Examination of the list of pathway genes significantly correlated with expression of SULFs reveals several cancer-associated genes and pathways, including TGFB1, PLAU, PLAUR, AKT3, and TP53. Cancer phenotypes associated with these genes include the EMT, the cancer stem cell compartment, and cancer cell invasion and metastasis.

We next investigated the effect of SULF1 and SULF2 on β-catenin expression and localization in HCC cells. Figure 3 shows that both SULFs increase expression of cellular β-catenin in Hep3B cells. Control transfection with an empty plasmid vector shows a lower level of β-catenin staining. In experiments examining Hep3B xenografts in nude mice, SULF1 and SULF2 both increased the expression of β-catenin. However,

TABLE 1. List of 40 Genes that had the Highest Positive or Negative Correlation with SULF1 or SULF2 in SULF1 and SULF2 Shared Gene Set

Positive correlation		r		Negative correlation		r	
Symbol	Name	SULF1	SULF2	Symbol	Name	SULF1	SULF2
POSTN	Periostin	0.72	0.48	HNF4A	Hepatocyte nuclear factor 4A	−0.41	−0.65
TIMP2	Tissue inhibitor of metalloproteinase 2	0.52	0.71	IQGAP2	RasGAP-like with IQ motifs2	−0.35	−0.58
IQGAP1	RasGAP-like with IQ motifs1	0.48	0.69	GPAM	Mitochondrial glycerol 3-phosphate acyltransferase	−0.35	−0.56
MRC2	Mannose receptor,C Type 2	0.49	0.67	PECI	D3,D2-enoyl-CoA isomerase	−0.31	−0.56
HK1	Hexokinase 1	0.47	0.66	WBSCR14	MLX interacting protein-like	−0.35	−0.55
CDK2AP1	CDK2-associated protein 1	0.37	0.66	PCYT2	Phosphorylethanolamine transferase	−0.36	−0.54
HRMT1L1	PRMT2 alpha	0.36	0.66	F13B	Coagulation factor XIII, B polypeptide	−0.3	−0.54
COL6A3	Alpha3 (VI) collagen	0.63	0.65	TCEA3	Transcription elongation factor A (SII), 3	−0.39	−0.53
TAX1BP3	Tax1 binding protein 3	0.51	0.65	FLJ20054	DENN/MADD domain containing 1B	−0.32	−0.53
ACTA2	Alpha 2 actin	0.65	0.44	ACF	Asymmetric crying facies	−0.41	−0.52
PMP22	Growth arrest-specific 3	0.60	0.64	PRO2086	Transferrin	−0.36	−0.52
LUM	Lumican	0.64	0.60	IL6R	Interleukin 6 receptor	−0.33	−0.52
RAB31	RAS oncogene family	0.64	0.58	FAM8A1	Autosomal highly conserved protein	−0.3	−0.52
TPM2	Tropomyosin 2 (beta)	0.58	0.64	FASN	Fatty acid synthase	−0.44	−0.51
MGC3047	Matrix-remodeling associated 8	0.58	0.64	BPHL	Biphenyl hydrolase-like	−0.41	−0.51
COL1A2	Alpha 2 Type I collagen	0.64	0.58	N/A	Hypothetical LOC401131	−0.32	−0.51
S100A6	S100 calcium binding protein A6	0.54	0.64	BTN2A3	Butyrophilin, subfamily 2, member A3	−0.31	−0.51
LTBP1	Latent TGFB binding protein 1	0.46	0.64	SC5DL	Sterol-C5-desaturase-like	−0.31	−0.51
CTBP2	C-terminal binding protein2	0.35	0.64	LOC150383	Chromosome 22 ORF 40	−0.47	−0.50
TEAD4	TEA domain family member 4	0.30	0.64	DHCR24	24-dehydrocholesterol reductase	−0.36	−0.50
FSTL1	Follistatin-like 1	0.63	0.63	F5	Coagulation factor V	−0.33	−0.50
MGC4083	Tubulin, beta 6	0.47	0.63	IL12B	Interleukin 12B	−0.41	−0.49
TAGLN	Transgelin	0.63	0.46	N/A	Homo sapiens cDNA: FLJ21782 fis, clone HEP00266	−0.39	−0.49
CHSY1	Chondroitin synthase	0.39	0.63	PECR	Peroxisomal trans-2-enoyl-CoA reductase	−0.36	−0.49
TGFB1	TGF-beta 1 protein	0.33	0.63	MDN1	MDN1, midasin homolog	−0.4	−0.48
GSTP1	Glutathione transferase	0.42	0.62	SSBP1	SSDNA binding protein 1	−0.38	−0.48
SIAT7B	Sialyltransferase 7	0.62	0.41	C21orf42	Non-protein coding RNA 158	−0.37	−0.48
SERPINH1	Collagen-binding protein	0.34	0.62	OPRS1	Sigma non-opioid intracellular receptor 1	−0.37	−0.48
COL3A1	Alpha1 (III) collagen	0.61	0.60	FLJ14146	Chromosome 1 ORF115	−0.34	−0.48
TIMP1	Tissue inhibitor of metalloproteinase 1	0.56	0.61	PKLR	Pyruvate kinase, liver and RBC	−0.33	−0.48
PDE4A	Phosphodiesterase 4A	0.45	0.61	CGREF1	Cell growth regulator with EF-hand domain 1	−0.36	−0.47
MGC15737	TCEA-like protein 3	0.38	0.61	C9orf99	Hydroxysteroid dehydrogenase like 2	−0.34	−0.47
SCGF	Stem cell growth factor	0.49	0.60	POR	P450 oxidoreductase	−0.31	−0.47
C1QR1	CD93 molecule	0.48	0.60	FLJ11164	Radical S-adenosyl methionine domain containing 1	−0.31	−0.47
DNAJC10	Macrothioredoxin	0.47	0.60	DPM3	MPD synthase subunit 3[3]	−0.3	−0.47
FLNA	fllamin A, alpha	0.43	0.60	ACOX2	Acyl-Coenzyme A oxidase 2	−0.37	−0.46
FXYD5	FXYD domain containing ion transport regulator 5	0.49	0.59	N/A	CDNA FLJ42949 fis, clone BRSTN2006583	−0.33	−0.46
SEPW1	Selenoprotein W, 1	0.32	0.59	ZNF533	Zinc finger protein 385B	−0.33	−0.46
VIM	Vimentin	0.49	0.58	TM7SF2	Transmembrane 7 superfamily member 2	−0.32	−0.46
COL6A2	Alpha2 (VI) collagen	0.40	0.58	APOH	Apolipoprotein H	−0.31	−0.46

The SULF1 and SULF2 shared included genes that had significant correlation with SULF1 ($P < 0.001$) and SULF2 ($P < 0.001$). The 40 genes were selected by *P*-value (lower *P* value either for SULF1 or SULF2) and sorted by *r* (higher correlation coefficient either for SULF1 or SULF2). *P*-value ranges from 1.6E-22 to 9.1E-4 for positive correlation and 5.3E-17 to 8.9E-04 for negative correlation.

TABLE 2. List of HCC-Associated Genes Correlated with SULF1 or SULF2 Expression

		r	
Gene symbol	Protein name	SULF1	SULF2
TIMP2	Metalloproteinase inhibitor 2	0.52	0.71
HK1	Hexokinase 1	0.47	0.66
COL6A3	Collagen alpha-3(VI) chain	0.63	0.65
COL1A2	Collagen alpha-2(I) chain	0.64	0.58
S100A6	Protein S100-A6	0.54	0.64
TGFB1	Transforming growth factor beta-1		0.63
GSTP1	Glutathione S-transferase P	0.42	0.62
F2R	Proteinase-activated receptor 1	0.58	0.52
VIM	Vimentin	0.49	0.58
THY1	Thy-1 membrane glycoprotein	0.58	0.45
SPARC	Proliferation inducing protein	0.58	0.42
KRT19	Keratin, type I cytoskeletal 19		0.58
LGALS1	Galectin-1	0.54	0.57
PDGFRA	Alpha-type platelet-derived growth factor receptor		0.57
ETS1	Protein C-ets-1	0.39	0.56
GMNN	Geminin	0.55	0.55
COL4A1	Collagen alpha-1(IV) chain	0.48	0.55
PLAU	Urokinase-type plasminogen activator	0.54	0.50
CXCR4	C-X-C chemokine receptor type 4	0.45	0.53
S100A14	S100 calcium binding protein A14	0.53	0.43
CYR61	Cysteine-rich angiogenic inducer	0.53	0.40
FKBP10	FK506-binding protein 10		0.53
TPM4	Tropomyosin alpha-4 chain	0.47	0.52
BASP1	Brain acid soluble protein 1	0.42	0.51
LAPTM5	Lysosomal protein transmembrane 5	0.51	0.42
JAG1	Jagged 1		0.50
Pro2086	Transferrin		−0.52
ABCC2	Canalicular multispecific organic anion transporter 1		−0.52

SULF1 or SULF2 correlated genes ($P < 0.0001$ and $r > 0.5$ or < -0.5) associated with HCC were generated from Metacore software.

expression of SULF1 and SULF2 in the sulfatase-negative Hep3B cells induce different patterns of β-catenin localization; β-catenin staining was more prominent in the cell membrane in SULF1 transfected cells while cytosolic and nuclear β-catenin staining was predominant in SULF2 transfected cells.

Association Between SULF1 and SULF2 Expression, the Hepatoblast Phenotype, and Patient Survival

Twenty-one (15%) of the 139 HCCs examined had a gene expression pattern consistent with the hepatoblast subtype of HCCs (Lee et al., 2006). Because of the association between SULFs and EMT-associated pathways, and the several hepatoblast/stem/progenitor cell related genes, such as TGFB1, vimentin (VIM), and periostin (POSTN) in the list of genes correlated with SULF1 and SULF2 expression, we examined the association of SULFs with the mature hepatocyte and hepatoblast/progenitor cell phenotypes. Only 5% or less of HCCs within the lower tertiles of SULF1 or SULF2 expression had a gene expression pattern consistent with the hepatoblast phenotype. This proportion increased as SULF expression increased to over 30% of HCCs in the highest tertile of SULF expression (Fig. 4; P trend = 0.0001 for both SULF1 and SULF2). Of note, 5 and 7 of the 10 HCCs with the highest SULF1 and SULF2 expression, respectively, were of the hepatoblast subtype of HCC.

As SULF1 has a tumor suppressor effect in liver cancer cells, while paradoxically high SULF1 expression was associated with the poor prognosis hepatoblast subtype of HCC, we explored the association of SULF1 expression with patient survival. The expression levels of SULF1 and SULF2 were higher in tumor than adjacent benign tissues in more than two thirds and half of the samples, respectively (Figs. 5A and 5B). When we examined the survival of patients with tumor SULF1 expression classified into two groups, survival of patients with SULF1 expression below the median was not different than that of those with SULF1 expression above the median (Fig. 5C). However, when patients

TABLE 3. List of Cytoskeleton Remodeling, Wnt, TGFB Pathway-Associated Genes Correlated with SULF1 or SULF2 Expression

		r	
Gene symbol	Protein name	SULF1	SULF2
ACTA2	Actin, aortic smooth muscle	0.65	0.44
TGFB1	Transforming growth factor beta-1		0.63
LAMB1	Laminin subunit beta-1		0.61
VCL	Vinculin	0.39	0.56
COL4A1	Collagen alpha-1(IV) chain	0.48	0.55
PLAU	Urokinase-type plasminogen activator	0.54	0.50
PLAUR	Urokinase plasminogen activator surface receptor	0.52	0.39
AKT3	RAC-gamma serine/threonine-protein kinase		0.49
ARPC1B	Actin-related protein 2/3 complex subunit 1B	0.48	0.40
FZD1	Frizzled-1		0.46
MSF	Septin 9		0.43
DSTN	Destrin	0.43	
COL4A2	Collagen alpha-2(IV) chain	0.42	0.42
BCAR1	Breast cancer anti-estrogen resistance protein 1		0.42
ACTN1	Actin alpha 1	0.38	0.41
PIK3CD	Phosphoinositide-3-kinase		0.41
CFL1	Cofilin-1		0.41
KRAS2	k-ras p21 protein		0.39
TP53	Cellular tumor antigen p53		0.38
ACTG1	Actin, cytoplasmic 2	0.37	0.37
LIMK1	LIM domain kinase 1	0.37	0.35
LIMK2	LIM domain kinase 2	0.37	
RAC1	Ras-related C3 botulinum toxin substrate 1	0.37	
ARPC2	Actin-related protein 2/3 complex subunit 2		0.36
ARPC3	Actin-related protein 2/3 complex subunit 3		0.35
MAPK13	Mitogen-activated protein kinase 13		0.35
MMP7	Matrilysin	0.32	
RHEB	GTP-binding protein Rheb		−0.32
PLG	Plasminogen		−0.35
ZFYVE9	Novel serine protease	−0.36	
CFL2	Cofilin-2	−0.38	
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha		−0.44

SULF1 or SULF2 correlated genes ($P < 0.0001$) associated with cytoskeleton remodeling, Wnt, TGF beta pathway were generated from Metacore software.

were classified into tertiles by SULF1 expression, high or low SULF1 expression was associated with poor survival whereas patients with intermediate (mid) SULF1 expression had better survival (Fig. 5D). Our previous study has shown that survival of patients with high SULF2 expression is significantly worse than that of patients with low SULF2 expression (Lai et al., 2008).

Validation of Training Set Results Using the Validation Microarray Dataset

For validation of the training set results using the validation data set, we selected six genes for examination; these were POSTIN, the gene with the highest correlation with SULF1 which has been shown to upregulate the expression of EMT marker genes and be associated with extrahepatic recurrence of HCC; HNF4A, a gene with the highest negative correlation with SULF2 which is the major transcriptional driver of liver differentiation; IQGAP1, the oncogenic RAC1 activator which is a key regulator of cell adhesion and migration; IQGAP2, which appears to function as a tumor suppressor, counteracting the effect of IQGAP1; TGFB1, which is a major driver of the EMT and is likely the main inducer of the SULF1 and SULF2 shared gene expression set; and VIM, a well known marker of the EMT. Since the six genes of interest (POSTN, HNF4A, IQGAP1, IQGAP2, TGFB1, and VIM) are known to play important roles in carcinogenesis and their associations with SULF1 and SULF2 were biologically plausible, the correlations between SULF1 or SULF2 and the six genes were examined in a validation HCC dataset (Table 4). Associations between the SULFs and the six genes were confirmed and were

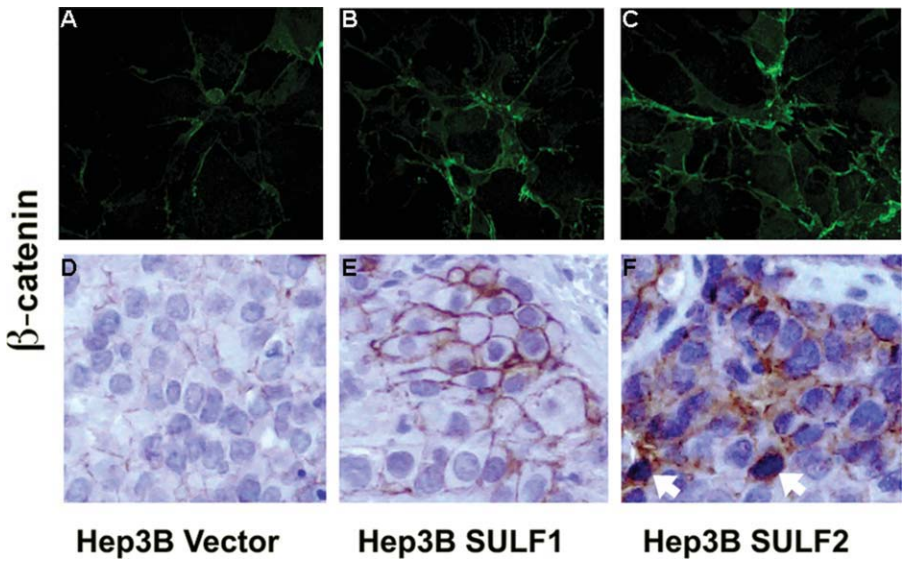

Figure 3. β-catenin expression pattern in HCC cells and HCC xenografts stably expressing SULF1 or SULF2. A, B, and C show immunocytochemical staining of β-catenin by confocal microscopy. (A) Hep3B cells stably transfected with Vector control; (B) Hep3B cells stably transfected with SULF1; (C) Hep3B cells stably transfected with SULF2. D, E, and F show β-catenin immunohistochemical staining of Hep3B-derived xenografts in nude mice. (D) Vector control; (E) SULF1; (F) SULF2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant, except for the association between SULF1 and HNF4A, and SULF2 and both IQGAPs, which showed the expected trends without statistical significance.

Similar to the training dataset, three of the ten most significant diseases associated with the SULF1 and SULF2 shared gene set were malignancies. The SULF1 unique genes were associated with vascular or connective disease (9 out of 10 most significant diseases) while the SULF2 unique gene set had strong associations with neoplasms (6 of the 10 most significant diseases) and connective tissue diseases (Supporting Information Fig. 2).

When considering the broader associations of cell signaling pathways with SULF1 and SULF2 shared gene sets in the validation dataset, cell adhesion (cell–matrix interactions), development (skeletal muscle, cardiac—Wnt/β-catenin, NOTCH, VEGF, IP3 and integrin-mediated, angiogenesis, hedgehog signaling, and ossification and bone remodeling), Wnt signal transduction, muscle contraction, interferon gamma signaling in inflammation, and connective tissue proteolysis were the most significant associated pathways. Similar to the training dataset, cell adhesion (ECM remodeling, chemokines, cell–matrix glycoconjugates, and integrin signaling), cytoskeleton remodeling (TGF and Wnt signaling), development (EDG5 and EDG3, Slit-Robo, and Hedgehog and PTH signaling), and blood coagulation were highly associated with the SULF1 unique

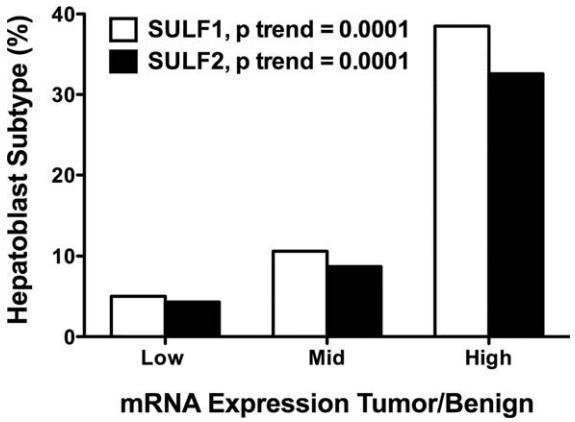

Figure 4. The association of SULF1 and SULF2 with the mature hepatocyte and hepatoblast/progenitor cell phenotypes. Patients were classified into tertiles by SULF1 and SULF2 expression and the association of SULF1 and SULF2 with the mature hepatocyte and hepatoblast/progenitor cell phenotypes were tested by Fisher's exact test for trend.

gene set, while several receptor signaling pathways, including IGF-1, G-protein signaling, and PDGF signaling as well as translational regulation were significant pathways associated with the SULF2 unique gene set (Supporting Information Fig. 2).

DISCUSSSION

In the present study, we show that the expression of the heparan sulfate proteoglycan endosulfatases SULF1 and SULF2 are strongly correlated in HCCs. SULF1 and SULF2

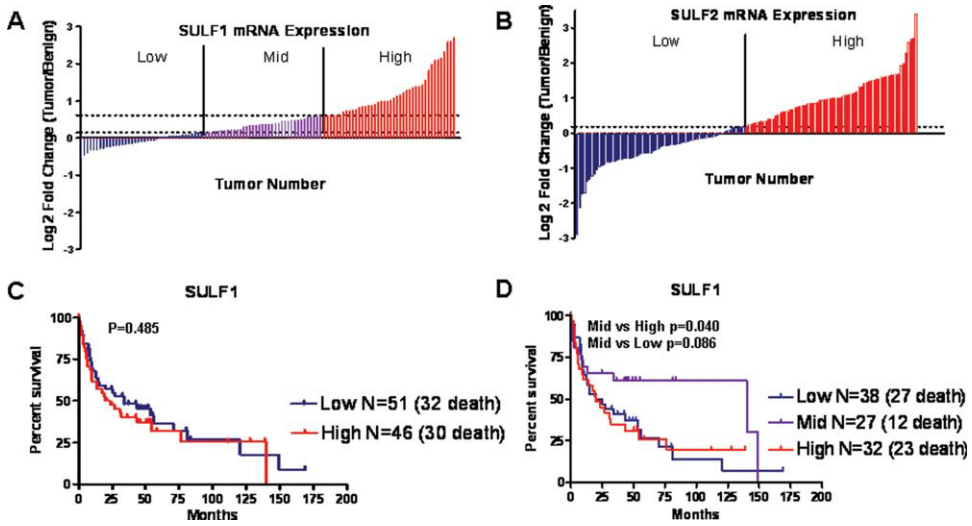

Figure 5. Association between the SULF1 and SULF2 expression and overall survival. (A) The ratios of SULF1 expression in tumor versus adjacent benign tissues were expressed as log 2 fold changes. (B) The ratios of SULF2 expression in tumor versus adjacent benign tissues were expressed as log 2 fold changes. The figure was modified from Lai et al. (2008). (C) Overall survival proportion of individuals with SULF1 expression below the median were compared to those with SULF1 expression above the median by log rank test. (D) Patients were classified into tertiles by SULF1 expression and survival of individuals with medium SULF1 expression and low or high SULF1 expression compared using the log rank test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 4. Correlation Between SULFs and POSTN, HNF4A, IQGAPs, TGFB1, and VIM in the Training and Validation Dataset

	Training dataset				Validation dataset			
	SULF1		SULF2		SULF1		SULF2	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
SULF2	0.41	3.88E-06	1	0	0.43	0.00871	1	0
POSTN	0.72	9.22 E-17	0.48	2.42–07	0.76	9.04 E-08	0.57	3.03 E-04
HNF4A	−0.40	7.91E-06	−0.64	5.27E-17	−0.18	0.27315	−0.48	0.00287
IQGAP1	0.48	3.29E-08	0.68	1.38E-20	0.39	0.01586	0.21	0.21381
IQGAP2	−0.35	8.97E-05	−0.57	8.62E-14	−0.50	0.00152	−0.27	0.10419
TGFB1	0.33	2.57E-04	0.62	1.63E-16	0.37	0.02633	0.34	0.03771
VIM	0.49	1.40E-08	0.58	7.29E-14	0.51	0.00137	0.33	0.04540

correlated genes had strong associations with neoplasms, including HCC, and with cell adhesion, chemokine, extracellular matrix, cytoskeletal remodeling, TGFβ, and WNT pathways. High expression of both SULFs was associated with the hepatoblast phenotype. Patients with intermediate (mid) SULF1 or low SULF2 expression had the best survival.

A significant number of the SULF1 and SULF2 correlated genes were overlapping (Fig. 1). The result is not surprising given the structural and functional similarity between SULF1 and SULF2. Five of ten diseases that were associated most highly with SULF1 and SULF2 shared gene sets were neoplasms, which highlights the roles of the SULFs in carcinogenesis. In contrast to the SULF1 unique gene set, the SULF2 unique gene set was highly associated with neoplastic processes. Similar trends were also observed in the validation data set.

A number of publications have now associated SULF1 and SULF2 with multiple cellular signaling pathways, suggesting that they affect or are associated with a wide range of important cellular functions. Molecules and pathways associated with the SULFs include multiple receptor tyrosine kinases (Lai et al., 2004b, 2008), angiogenesis (Narita et al., 2006; Uchimura et al., 2006; Fujita et al., 2010), Wnt/β-catenin (Nawroth et al., 2007; Lai et al., 2010), hedgehog (Danesin et al., 2006), bone morphogenetic protein (Viviano et al., 2004), TGFB (Yue et al., 2008), and TP53 (Chau et al., 2009). We have shown that SULF1 and SULF2 have opposing effects in liver cancer cell lines, particularly mediated by effects on signaling by receptor tyrosine kinases

such as FGF2, HGF, and HB-EGF (Lai et al., 2003, 2004b, 2008). SULF1 has been shown to inhibit receptor tyrosine kinase signaling, presumably by desulfating heparan sulfate proteoglycan coreceptors that are required for receptor tyrosine kinase signaling while SULF2 activates receptor tyrosine kinase signaling by desulfating RTK-specific sulfate moieties of storage type heparan sulfate proteoglycans, resulting in release of growth factors from extracellular stores and increasing the binding of growth factors to their receptors. In contrast, SULF1 and SULF2 both activate Wnt signaling presumably by desulfating Wnt-specific sulfate moieties of storage type heparan sulfate proteoglycans, resulting in release of Wnt ligands from extracellular stores and increasing the binding of Wnts to their Frizzled receptors (Dhoot et al., 2001; Ai et al., 2003; Nawroth et al., 2007). To further comprehensively examine the cellular signaling programs coordinately or oppositely regulated by SULF1 and SULF2, we examined the gene expression signatures that correlate with SULF1 and SULF2 expression in HCC. We reasoned that this analysis would identify critical pathways involved in SULF1 and SULF2-mediated effects and identify potential target molecules and pathways for further examination in vitro and in vivo. The cell adhesion, blood coagulation and TGFB-Wnt-cytoskeletal remodeling pathways were most significantly associated with SULF expression. This is consistent with studies from our group and others associating the SULFs with the Wnt and TGFB pathways, and confirms a profound association worthy of more detailed exploration. Aberrant activation of the Wnt pathway is common in hepatocarcinogenesis, as was recently shown in a meta-analysis of eight independent gene profiles in which one of three HCC subtypes was characterized by Wnt pathway activation (Hoshida et al., 2009). Recent results from our group have shown that SULF2 activates the oncogenic Wnt signaling pathway (Lai et al., 2010). Here we show that although both SULF1 and SULF2 increase β-catenin expression in HCC cells, SULF1 primarily induces membrane expression, while nuclear staining is prominent in SULF2 transfected cells. These differences in SULF1 and SULF2-mediated localization of β-catenin and their functional consequences remain to be further investigated.

There was a significant association between SULFs and genes and pathways associated with the dedifferentiated phenotype. For example, HNF4A had the strongest inverse correlation with SULF2 expression in the entire set of genes in the training data set. HNF4A plays an important role in the regulation of hepatocyte differentiation and prevention of the EMT. Down-regulation of HNF4A is associated with a dedifferentiated HCC phenotype and tumor progression while up-regulation of HNF4A restores the differentiated phenotype of tumors with less invasive features (Lazarevich et al., 2004; Yin et al., 2008). A recent study has shown that expression of HNF4A prevented tumor progression and metastatic tumor formation in a mouse model (Yin et al., 2008). Interestingly, forced expression of HNF4A dramatically decreased the β-catenin level in HepG2 cells, suggesting a regulatory role of HNF4A on Wnt/β-catenin pathway activation in HCCs (Yin, et al., 2008). Although, there has been no rigorous evaluation of the relationship between SULF2 and HNF4A, SULF2 does have an HNF4A binding site at position 702 in the upstream promoter region. Taken together, these results suggest a regulatory role of HNF4A on SULF2 expression. What is as yet unclear is whether SULF2 is upstream or downstream of the Wnt/β-catenin pathway in HNF4A regulation of the HCC phenotype. Strong correlations were also found between both SULF1 and SULF2 and TGFB1, VIM, and POSTN expression. TGFB1 has both tumor suppressive and oncogenic properties. The tumor suppressor function of TGFB is mainly mediated by growth inhibitory and pro-apoptotic early responses while the oncogenic function is mediated by Wnt pathway activation, which promotes the epithelial to mesenchymal transition (EMT) and tumor cell invasion and metastasis (Benetti et al., 2008; Battaglia et al., 2009; Hoshida et al., 2009; Mishra et al., 2009). Exposure to oncogenic stimuli shifts TGFB signaling from tumor suppression to oncogenesis (Battaglia, et al., 2009; Murata et al., 2009). More recently, a proportion of progenitor cell subtype HCCs has been shown to express a late TGFβ gene expression signature which is associated with progression and a poor outcome in HCCs (Coulouarn et al., 2008).To the best of our knowledge, only one study has investigated the relationship between SULFs and TGFB, showing that TGFB1 up-regulates SULF1 expression in normal human lung fibroblasts and in mice treated with adenovirus encoding active TGFB1 (Yue et al., 2008). Increased SULF2 was also observed in mice treated with adenovirus encoding active TGFB1 (Yue et al., 2008). VIM is a

member of the intermediate filament protein family, is a well known mesenchymal marker that is involved in the epithelial-mesenchymal transition, and regulates cancer invasiveness and metastasis in HCC (Hu et al., 2004). POSTN had the highest positive correlation and tenth highest correlation with SULF1 in the training and validation data sets, respectively. Although the role of POSTN is not well known in HCC, one study has shown that POSTN expression was associated with extrahepatic recurrence of HCC (Iizuka et al., 2006). POSTN is known to interact with multiple cell-surface receptors and pathways to promote cancer cell survival, epithelial-mesenchymal transition (EMT), invasion, and metastasis (Ruan et al., 2009; Hong et al., 2010). Additionally, IQGAP1 and IQGAP2 had the third strongest positive and the second strongest negative correlations with SULF2 in the training data set. The IQGAPs also integrate various signaling pathways such as calcium/calmodulin, BRA, and CDC42 signaling (Ho et al., 1999; Ren et al., 2008). IQGAP1 is known to activate MAPK signaling and the Wnt/β-catenin pathway, enhancing cell migration and proliferation and impairing cell–cell adhesion (Kuroda et al., 1998; Briggs et al., 2002; Mataraza et al., 2003; Roy et al., 2004, 2005; Schmidt et al., 2008; Wang et al., 2008; Zhou et al., 2009). IQGAP2 is a component of a multifunctional scaffolding complex with IQGAP1, β-catenin, and E-cadherin in normal hepatocytes. Loss of IQGAP2 appears to activate the Wnt/β-catenin pathway (Schmidt et al., 2008). There have been no reports of the association between SULF and IQGAP expression. No previous studies have identified the association between SULFs and VIM, POSTN, IQGAPs. Further investigation of the functional association between TGFB1, vimentin, POSTN, IQGAPs, and SULFs in human HCC is clearly needed.

We next analyzed the association between expression of the SULFs and the hepatoblast/progenitor cell phenotype. Lee et al. (2006) previously classified HCCs into two subclasses based on similarity to a fetal hepatoblast/progenitor cell gene expression pattern. Individuals with HCCs that have a hepatoblast gene expression pattern had poor survival compared with those with a mature hepatocyte gene expression profile (Lee et al., 2006). Figure 4 demonstrates the increasing proportion of hepatoblast phenotype HCCs as the expression of SULF1 and SULF2 increases. Recent studies have also shown that Wnt pathway activation is important for maintaining cancer cells with stem cell-like progenitor features (Mishra et al., 2009; Yamashita et al., 2009). The strong association between SULF1 and SULF2 expression, EMT associated genes, the hepatoblast phenotype of HCC, and the Wnt/β-catenin pathway suggest that SULFs may modulate the Wnt/β-catenin pathway and thus promote the EMT process. It remains to be validated with experiments whether SULF1 and SULF2 are directly involved in the EMT process through Wnt pathway activation in human HCCs.

We have been intrigued by the apparently paradoxical effects of SULF1 and SULF2 in HCC cell lines and human HCCs. In established cell lines, SULF1 has a tumor suppressor effect that appears to be mediated by inhibition of heparan sulfate-dependent receptor tyrosine kinase signaling both in vitro and in in vivo mouse xenografts (Lai et al., 2004b, 2006). In contrast, SULF2 has an oncogenic effect on cell lines, both in vitro and in vivo which is at least partly mediated by activation of receptor tyrosine kinase signaling (Lai et al., 2008). Contrary to the demonstrated function of SULF1 as a tumor suppressor in HCC cell lines, expression of SULF1 is mostly higher in HCC than in adjacent benign tissues and approximately a third of HCCs express SULF1 at high levels greater than 1.5× the level in adjacent benign tissue (log 2 of 1.5 = 0.59; upper dotted horizontal line on Fig. 4A). In addition, as shown above, nearly 40% of patients with high tumor SULF1 expression have the hepatoblast phenotype of HCC, which has relatively poor survival (Lee et al., 2006).

We therefore explored the association of SULF1 and SULF2 expression in human HCCs with patient survival. This examination revealed the striking observation that the disparate effects of SULF1 on receptor tyrosine kinase (inhibition) and WNT pathway (promotion) may be reflected in a bimodal effect on patient survival, with low SULF1 abrogating the tumor suppressor effect of SULF1 on receptor tyrosine kinase signaling and leading to worse patient survival and high SULF1 promoting the oncogenic effect of SULF1 via EMT and also resulting in worse patient survival. Patients with mid SULF1 expression therefore have a better survival than those with either high or low SULF1 expression. In contrast to SULF1, SULF2 appears to have a unimodal effect in HCC, causing activation of both the receptor tyrosine kinase and WNT pathways so that elevated SULF2 expression is associated with a worse patient outcome, both in

probability of recurrence and overall survival (Lai et al., 2008).

In summary, SULF1 and SULF2 appear to have complex roles in the regulation of cell signaling in HCCs. There is substantial functional similarity in modulating cell adhesion, ECM remodeling, and WNT pathways. On the other hand, SULF2 appears to be associated with neoplastic processes independent of SULF1. The complete elucidation of the mechanistic bases for these effects could have substantial value in providing the rationale for strategies for targeting SULF1 and SULF2 or their associated molecules and pathways in treatment of HCC. It is perhaps already instructive that the heparan sulfate mimetic agent PI-88, which has been in clinical trials for treatment of HCC, was initially thought to have its primary mechanism of action as a heparanase inhibitor, but has been shown recently to have a profound inhibitory effect on SULF1 and SULF2 activity (Hossain et al., 2010). Newer and perhaps more selective agents currently in development may enhance our ability to target these enzymes in cancer (Dredge et al., 2010).

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