이학박사 학위논문

Down-regulation of *SYK* by promoter CpG island hypermethylation and its potential role in hepatocellular carcinoma

간암에서 *SYK*의 프로모터 CpG island 과메틸화에 의한 발현소실 및 종양억제자로서의 역할에 대한 연구

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서울대학교 대학원
협동과정 종양생물학전공
신 소 현

A thesis of the Degree of Doctor of Philosophy

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The Department of Cancer Biology
Seoul National University
College of Medicine
So-Hyun Shin

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지도교수 강경훈

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신소현

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Down-regulation of *SYK* by promoter CpG island hypermethylation and its potential role in hepatocellular carcinoma

by
So-Hyun Shin

A Thesis Submitted to the Interdisciplinary Graduate Program in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Cancer Biology at the Seoul National University

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Approved by Thesis Committee:

Professor _____ Chairman
Professor _____ Vice chairman
Professor _____
Professor _____
Professor _____

ABSTRACT

The spleen tyrosine kinase (*SYK*) has predominantly been studied in hematopoietic cells in which it is involved in immunoreceptor-mediated signaling. However, *SYK* expression is evidenced in numerous nonhematopoietic cells and its down-regulation has been shown to be involved in tumor formation and progression. Our team has reported that *SYK* promoter methylation identifies a subset of hepatocellular carcinoma (HCC) with poor prognosis but little is known regarding a biological role of SYK in HCC. We found that *SYK* promoter methylation is a common event in HCC and is closely associated with its expression. We established stable HCC cell lines that contain *SYK* gene in inducible expression vector and then compared RNA expression profiles of HCC cell lines with or without induction of *SYK*. Gene ontology analysis revealed that the *SYK*-regulated genes are enriched among genes involved in cell adhesion and cell growth. Indeed, we found that *SYK* increased cell adhesion to fibronectin and decreased cell proliferation. Induced expression of *SYK* decreased cell migration and invasion by coordination with adhesion molecules as well as suppression of Rho-family GTPases. Our findings suggest that

SYK loss is implicated in cell proliferation, migration, and invasion of HCC cells.

Keywords: Spleen tyrosine kinase, DNA mehtylation, Hepatocellular carcinoma, Tumor suppressor gene

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide. In men, it ranks the fifth most common cancer worldwide and the second leading cause of cancer death, with an estimate of more than 520,000 new cases each year (1). The major risk factors associated with the incidence of HCC are well established, such as infection by hepatitis B and hepatitis C viruses, chronic alcoholism and aflatoxin exposure (2). However, the molecular carcinogenesis pathways involving the development and progression of HCC remain largely unclear. Like most solid tumors, it has been believed that the progression of HCC occurs as a consequence of a series of genetic and epigenetic alterations (2). Therefore, it is important to know the genetic/epigenetic changes for understanding the underlying mechanisms that cause the initiation and development of HCC.

CpG islands are DNA sequences (approximately 1 kb) that contain a high density of CpG dinucleotides, and approximately 60% of human genes are known to harbor CpG islands in their promoter sequences (3, 4). DNA methylation is the well-characterized epigenetic alteration and also essential for normal development, X chromosome inactivation and gene imprinting (5). Methylation of CpG islands on promoter regions is related to transcriptional silencing and function as an important tool to inactivate tumor suppressor or tumor-related genes in

cancer cells (6, 7). Hypermethylation of gene promoters has been known to occur in early stage during multistep hepatocellular carcinogenesis (8). In addition to gene inactivation, promoter CpG island hypermethylation has received attention for its potential utility as a biomarker for tumor detection or prediction of prognosis or response of tumor cells to chemotherapeutic agents (9).

The spleen tyrosine kinase (SYK) is a 72 kDa-sized nonreceptor tyrosine kinase that is widely expressed in hematopoietic cells. In these cells, SYK is involved in the signaling, downstream of activated immunoreceptors, that mediates diverse cellular responses including cell proliferation, differenriation, survival and phagocytosis (10, 11). Recently, SYK expression has been evidenced in numerous nonhematopoietic cells and its down-regulation has been shown to be involved in tumor formation and progression (12-15). It has been demonstrated that transfection of *SYK* into a *SYK*-negative cancer cell line dramatically inhibited its cell growth, migration and invasion (12-15). Conversely, knockdown of *SYK* in *SYK*-positive breast cancer cells dramatically increased proliferation and invasion (16). Several researchers have reported that the loss of *SYK* expression correlates with poor survival and tumor metastasis in the patients with breast (17), bladder (18), liver (19), pancreatic (14), or gastrointestinal tract tumor (20). Epigenetic silencing through hypermethylation of critical CpG islands was proposed to be involved in the loss of *SYK* gene expression in these tumors (12-15). Although SYK was shown to affect

cell proliferation, motility and invasion in several types of cancers, its tumor suppressive activity and its molecular mechanism remain to be clarified in HCC.

In hematopoeitic cells, SYK is generally activated by its recruitment on the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of transmembrane immunoreceptors (TCR, BCR, Fcγ and Fcε). However, expression of these receptors is absent in non-hematopoetic cells such as hepatocytes and breast epithelial cells (10). Recently, β1 integrin-mediated activation of SYK has been demonstrated in epithelial cells. Activation of β1 integrin receptors by fibronectin or antibody cross-linking leads to redistribution of SYK from the cytoplasm to the plasma membrane and induction of SYK phosphorylation on tyrosine (21, 22). Furthermore, SYK phosphorylation was prominently increased by activating β1 integrin after plating on a collagen type I matrix in HB2 mammary epithelial and MCF7 breast cancer cell lines (23). These results suggest that integrin receptors and their extracellular matrix ligands might be responsible for SYK activation and signaling in non-immune cells. They also indicate that SYK might be associated with cell adhesion and migration, both critical events for tumor invasion and metastasis.

Aberrant decrease of adhesion to the extracellular matrix is an important characteristic of transformed cells. Focal adhesion of the cell to the matrix is constituted by transmembrane integrins and an

intracellular adhesion molecules such as talin, paxillin, and vinculin (24). In breast cancer cells, SYK inhibits cell motility while promoting the cell adhesion molecules such as vinculin (22), E-cadherin (25) and tensin-2 (26). Focal adhesions are obviously important for regulating the assembly and disassembly of the attached actin polymerization to the plasma membrane (24). Rho family of small GTPases including Rho, Rac and Cdc42 also regulate the assembly of focal adhesion and actin polymerization and transmit the molecular signaling that supports directed cell motility (27, 28). Indeed, several Rho GTPases are up-regulated in many types of human cancer and especially, Rho A shows its high signaling activity as well as high protein level in HCC (29). Furthermore, an inverse correlation was found between stress fibers formed by Rho A signaling pathway and movement (24). Although studies have shown a requirement for SYK in ITAM-dependent actin assembly and SYK's association with actin cytoskeleton or focal adhesion kinase (FAK) in hematopoietic cells (30, 31), the relation between SYK and focal adhesion molecules or Rho GTPases has been poorly understood in non-heamatopoietic cells.

Under a hypothesis that SYK functions as a tumor suppressor in HCC cells, we aimed to investigate the tumor suppressor function of SYK in HCC cells. We found that *SYK* promoter methylation is closely associated with its down-regulation, and that SYK functions as a tumor suppressor to decrease tumor cell proliferation, invasion and migration.

MATERIALS AND METHODS

Cell lines and 5-Aza-dC treatment

Seven different human HCC cell lines (SNU-739, SNU-761, SNU-878, SNU-886, HepG2, Hep3B and Huh7) were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cell lines were seeded at 3×10^5 cells/mL in their respective culture media and were treated with 5 μM 5-aza-2-deoxycytidine (5-Aza-dC, Sigma- Aldrich, St. Louis, MO) for 96 h; media and drugs were replaced every 24 h. As a control, cell lines were mock-treated in parallel with the addition of an equal volume of phosphate buffered saline (PBS) without the drug.

Tet-on inducible expression system

The inducible gene expression system was established using the Tet-on inducible gene expression system (Clontech, Palo Alto, CA) according to the manufacturer's protocols. Target cells (Hep3B and Huh7) were first transfected with pTet-on advanced vector to create a stable cell line. Once a suitable Tet-on advanced cell line is established, the cell lines are then stably transfected with TRE-based vector containing *SYK* gene. Target cells were cultured in DMEM containing 10% tetracycline-free fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. For induction of gene expression, Tet-on inducible cell lines were treated with 1 ug/mL Doxycycline (Dox) for 4

days.

GFP-tagged *SYK* expression vector and transfection

A GFP-tagged, full-length open reading frame clone of human *SYK* and its control vector (pCMV6-AC-GFP) were purchased from Origene (Rockville, MD). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Expression microarray

We conducted expression microarray analysis on the Illumina HumanHT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA) containing 47,231probes according to the manufacturer's protocol. Samples were purified using the RNeasy kit (Qiagen, Valencia, CA). cDNA synthesis and in vitro transcription amplification were followed by hybridization. For the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX), 0.55 μg of total RNA and one round of amplification are required for each sample. Scanning was performed on the Illumina BeadArray Reader. After image scanning, the GenomeStudio software, which is a tool for analyzing gene expression data from scanned microarrays, was utilized to generate data for the genes represented on the array. The gene analysis tool generated output files containing statistics for gene/probe signals and quality control information.

Sodium bisulfite modification and methylation analysis

Sodium bisulfite modification of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research Co., Irvine, CA). For bisulfite genomic sequencing (BGS), The CpG island DNA methylation status was determined by PCR analysis after bisulfited modification and followed by BGS. PCR product was cloned into pGEM-T easy vector (Promega, Medison, WI) and at least 10 individual clones were sequenced. Primer sequences and PCR conditions are shown in Table 1.

RT-PCR

Total RNA was prepared using an RNeasy kit (Qiagen) according to the manufacturer's protocols. A total of 5 μg of RNA was reverse transcribed using oligo-dT and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (RT-qPCR) amplification reactions were performed using SYBR Green PCR master mix (Life Technologiese Applied Biosystems, Foster City, CA). Expression levels of the genes were normalized to expression of GAPDH. Primer sequences and PCR conditions are shown in Table 1.

Western blot

Whole cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were incubated with anti-SYK (4D10), anti-γ tubulin (TU-30), anti-β-actin (C4) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CHK1 (NB100-464) (Novus

biological, Littleton, CO), anti-vinculin (V9131), anti-tensin2 (SAB4200269) (Sigma-Aldrich) and anti-RhoA (2117), B (2098), C (3430) (Cell Signaling Technology Inc., Beverly, MA) at 4℃ overnight. After antibody washing, the blots were reacted with their respective secondary antibodies and were detected using ECL plus reagents (GE Healthcare, Waukesha, WI).

Colony formation assay

Tet-on inducible Huh7 cells were seeded into 60 mm dishes and grown in culture medium containing both geneticin (G418; Sigma-Aldrich) and puromycin (Sigma-Aldrich) for 4 weeks. Antibiotics-resistant colonies were fixed with methanol and stained with a crystal violet solution.

Cell proliferation assay

Cells were seeded at an initial density of 2 x 10^3 cells per well in 96 wells plate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell number.

Cell migration and invasion assay

Cell migration assay and invasion assay were performed using the transwell membrane (Corning, Corning, NY, USA) and matrigel-biocoated invasion chamber (BD biosciences, Bedford, MA, USA), respectively. Cells were trypsinized and resuspended in DMEM at a density of 5x10^4 cells/ml. The cell suspension was added into the

upper chamber consisted of inserts containing 8 μm pore-size membrane. DMEM containg 20% FBS was placed in the lower chamber. After a 48 h incubation at 37℃, cells remained in the upper chamber was removed carefully by cotton swab and the membrane was fixed and stained with 100% methanol and 0.1% crystal violet, respectively.

Immunofluorescence

Cells cultured on glass cover slips were treated sequentially as follows: 3.7% formaldehyde for 10 min at room temperature (fix), 0.1% Triton X-100 in PBS for 10 min at room temperature (permeabilization), 0.1% bovine serum albumin (BSA) in PBS for 30min at room temperature (blocking). Cells were stained with antibodies against F-actin (R415) (Rhodamine-Palloidin, Invitrogen), vinculin, tensin2 (Sigma-Aldrich). Bound primary antibodies were detected using a Texas red-conjugated goat anti-rabbit antibody and a Alexa flour 594-conjugated goat anti-mouse antibody (Invitrogen). Nuclei were counterstained with DAPI. Images were observed and captured using an Olympus confocal microscope.

Cell adhesion assay

96 wells plates were coated with 20 μg/ml fibronectin at room temperature for 1 h. Plates were incubated with 1% BSA in PBS to block nonspecific cell adhesion for 30 min. Thereafter, $5x10^5$ cells were

added to each well for 10, 20, 60 min. Subsequently, non-adherent cells were washed off, the remaining adherent cells were fixed with 96% ethanol and stained with 0.1% crystal violet. Wells were washed extensively with distilled water to remove excess stain and dissolved cells with SDS solution and read absorbance at 570 nm.

Coimmunoprecipitation

Cells were lysed in lysis buffer (150 mM NaCl, 1.0% TritonX-100, 50 mM Tris-Cl (pH 8.0), protease inhibitor and phosphatase inhibitor cocktail). Incubate 300 μg lysate with anti-SYK (Santa Cruz) antibody for a fixed at 4℃ for overnight. Add 20 μl of protein A agarose beads to each lysate and incubate the lysate beads mixture at 4°C under rotary agitation for 6 h. Samples were washed five times with lysis buffer and bound proteins were analyzed by Western blotting with anti- SYK, anti-beta-actin, anti-vinculin, anti-tensin2.

Statistical Analysis

Data are expressed as means ± SD. The Student's *t* test was used to compare the effects of SYK expression on cell proliferation and cell adhesion.

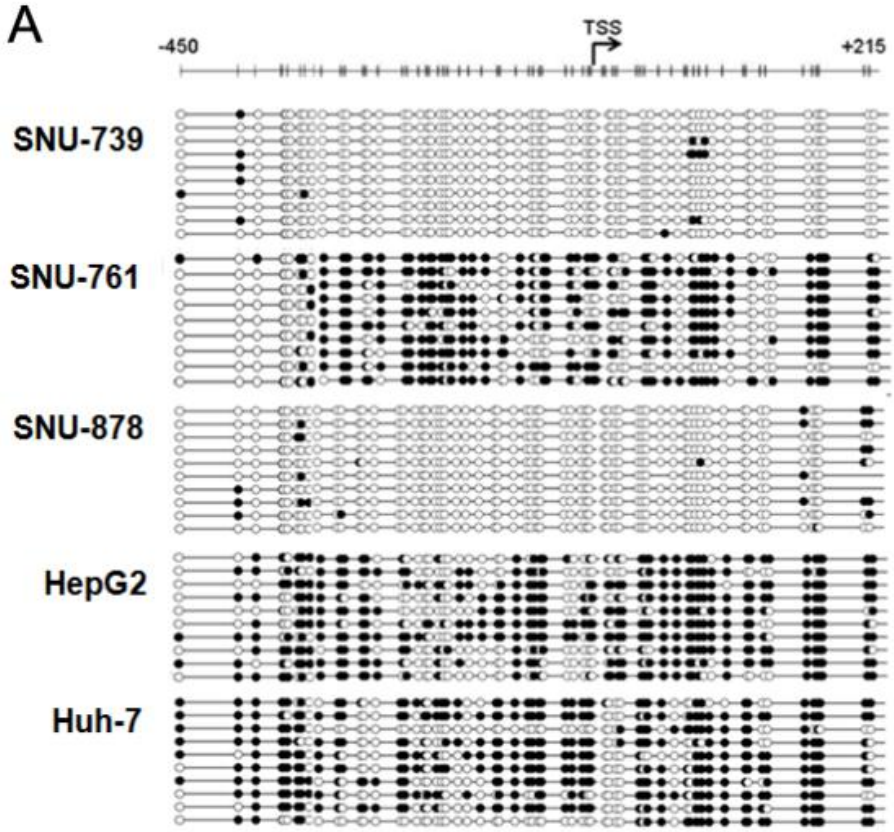
Table 1. Primers for MSP, BGS and RT-PCR

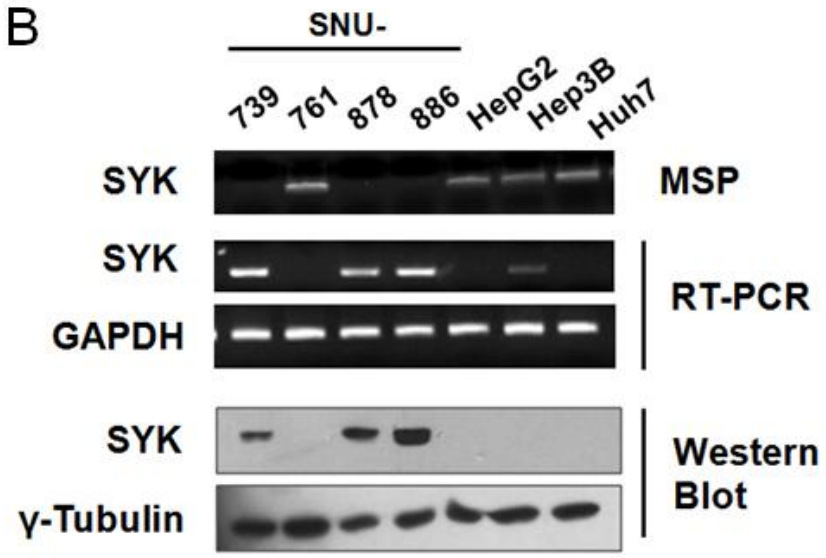
Primer	Gene	Forward (5'-3')	Reverse (5'-3')	Tm (℃)	Product size (bp)
MSP	*SYK*	GTCGGGCGAGGTTCGC	CTACCTACGAAAAACCCTTATCCG	56	104
BGS-1	*SYK*	GATTAAGATATATTTTAGGGAATATG	CACCTATATTTTATTCACATAATTTC	50	664
BGS-2	*SYK*	GGGTAGTTTTATTTTTTTTGTTTG	ACTCTTCCTCATTTTAAACAACTTCC	54	261
BGS-3	*SYK*	GGAAGTTGTTTAAAATGAGGAAGAGT	CCACCTATATTTTATTCACATAATTTCAAC	57	273
RT-PCR	*SYK*	TGTCAAGGATAAGAACATCATAG	CACCACGTCATAGTAGTAATTG	59	508
RT-PCR	*CHK1*	TATGAAGCGTGCCGTAGACT	ATAAACCACCCCTGCCATGA	59	238
RT-PCR	*GAPDH*	CAATGACCCCTTCATTGACC	TGGAAGATGGTGATGGGATT	59	135

RESULTS

Correlation of *SYK* expression with methylation status

To determine whether aberrant promoter hypermethylation of *SYK* was correlated with suppression of *SYK* expression in HCC cells, we performed bisulfate sequencing and methylation-specific PCR. We found that *SYK* was methylated in SNU-761, HepG2, Hep3B and Huh7, which had no detectable *SYK* expression. On the other hand, unmethylated alleles were detected in SNU-739, SNU-878 and SNU-886, all of which had endogenous *SYK* expression (Figure 1A, B). Furthermore, we observed an increase of SYK gene expression after 5-Aza-dC treatment in SNU761 and Huh7 cells (Figure 1C). Therefore, *SYK* promoter hypermethylation is tightly associated with its transcriptional silencing in HCC cell lines.


A
-450
TSS
+215
SNU-739
SNU-761
SNU-878
HepG2
Huh-7


B
SNU-
739
761
878
886
HepG2
Hep3B
Huh7
SYK
MSP
SYK
GAPDH
RT-PCR
SYK
γ-Tubulin
Western Blot

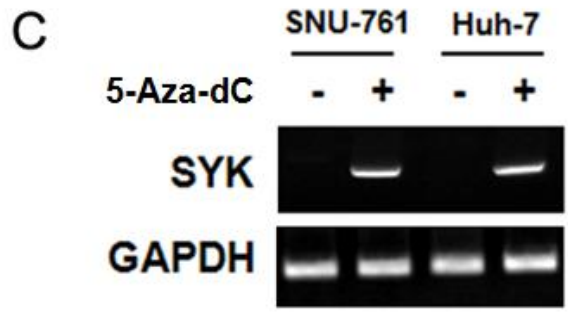

C
SNU-761
Huh-7
5-Aza-dC
-
+
-
+
SYK
GAPDH

Figure 1. *SYK* promoter methylation and expression in HCC cells

(A) Bisulfite sequencing of *SYK* promoter region in 5 HCC cell lines. Vertical lines indicate individual CpG sites. Cloned PCR products were sequenced and each clone is shown as an individual row, representing a single allele of the promoter region. White and black circles denote unmethylated and methylated CpG sites, respectively. (B) Analyses of *SYK* promoter hypermethylation by MSP and *SYK* expression by RT-PCR (*GAPDH* mRNA levels were used as a control) and Western blotting (γ-Tubulin protein levels were used as a control) in 7 HCC cell lines. (C) RT-PCR results of *SYK* gene expression in methylated HCC cell lines SNU-761 and Huh7 following the addition of 5-Aza-dC is shown. Cells were either mock-treated or treated with 5-Aza-dC (5 μM) for 96 h as indicated.

Gene expression profiling

We employed gene expression profiling strategy to identify pathways regulated by SYK-dependent genes in HCC cells. We generated Hep3B and Huh7 cell lines carrying the Tet-on inducible gene expression system in which the expression of *SYK* could be induced by incubation with Dox. We confirmed that *SYK* expression level was dose-dependently induced by treatment with Dox. Also, we confirmed that *SYK* expression level was reduced after Dox was removed (Figure 2). The expression profiles were then analyzed with an Illumina HumanHT-12 v4 Expression BeadChip. Among the 47,231 probes analyzed, 1,673 (3.54%) were found to be significantly regulated (>2-fold) by SYK induction while 175 (0.37%) were found to significantly regulated (>2-fold) by SYK reduction in at least 1 of the two cell lines (Figure 3A and B). Table 2 and 3 summarizes the functions of the genes that were changed in their expression level with SYK induction and reduction in both cell lines, respectively.

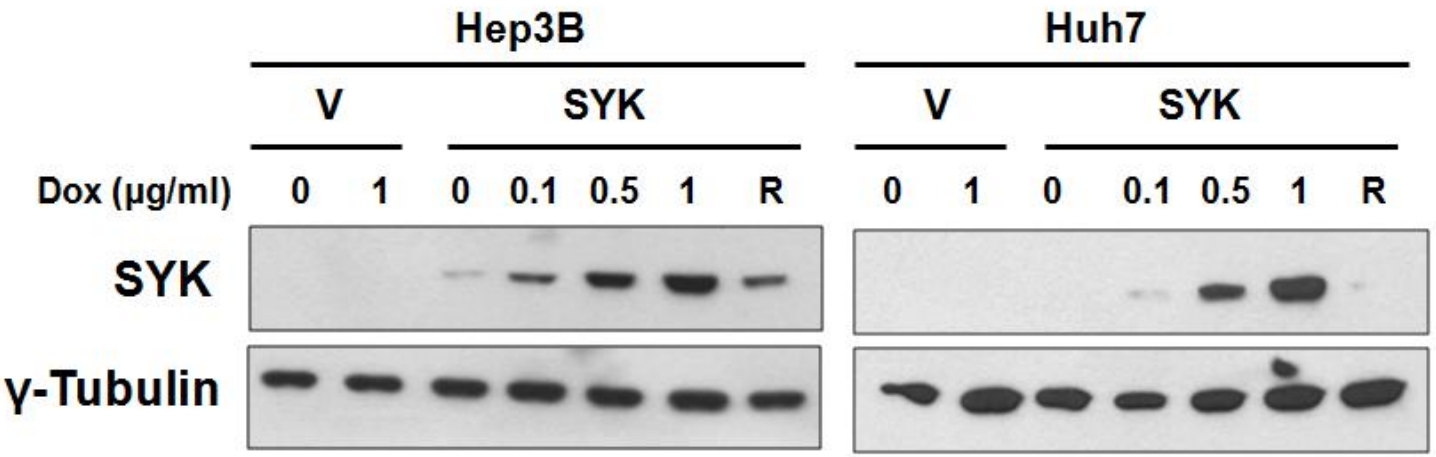
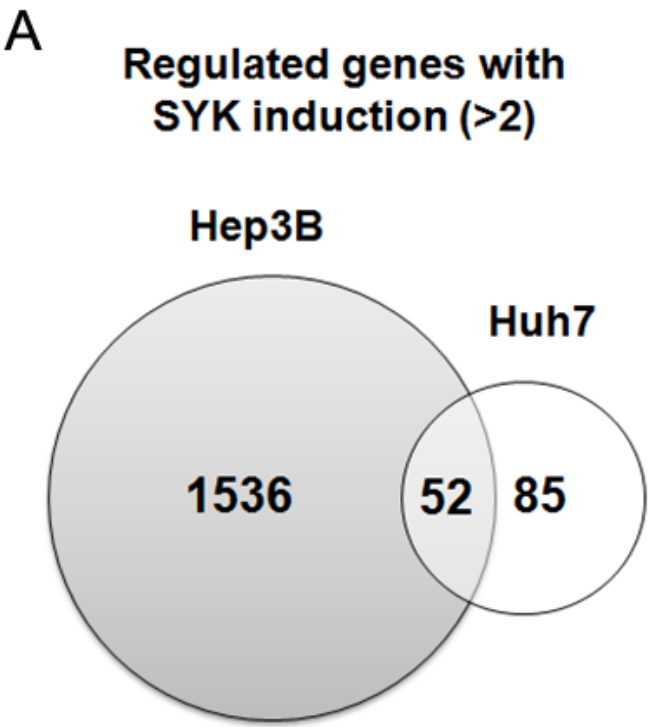

Hep3B
Huh7
V
SYK
V
SYK
Dox (μg/ml)
0 1 0 0.1 0.5 1 R
0 1 0 0.1 0.5 1 R
SYK
γ-Tubulin

Figure 2. Establishment of Tet-on inducible gene expression system

SYK-inducible Hep3B and Huh7 cell lines were generated using a Tet-on inducible gene expression system. The Western blotting results show that the expression level of *SYK* was dose-dependently induced by treatment with Dox (0-1 μg/ml) for 4 days. Also, we confirmed that expression level of *SYK* was reduced after Dox was removed for 4 days (R). V indicates empty vector-transfected cells and SYK indicates SYK expression vector- transfected cells.

A


Regulated genes with
SYK induction (>2)
Hep3B
Huh7
1536
52
85

B

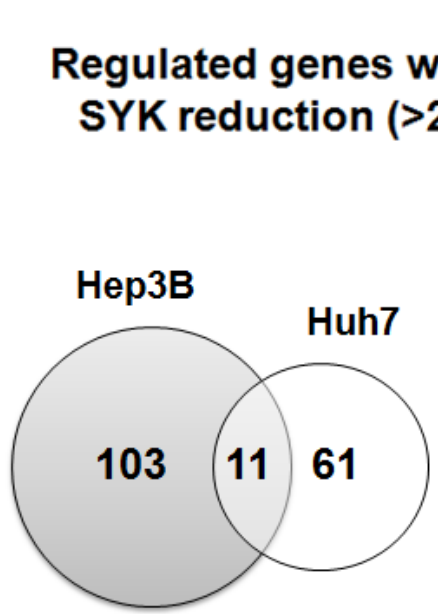

Regulated genes with
SYK reduction (>2)
Hep3B
Huh7
103
11
61

Figure 3. Gene expression profiling

(A) Genes showing greater than 2-fold up-regulation with SYK induction are illustrated in Venn diagram overlapping between Hep3B and Huh7 cells. (B) Genes showing greater than 2-fold down-regulation with SYK reduction are illustrated in Venn diagram overlapping between Hep3B and Huh7 cells.

Table 2. SYK- regulated genes with SYK induction in both Hep3B and Huh7 cells

Symbol	Name	Fold changes (Dox+ vs Dox-)		Process
		Hep3B	Huh7	
CTGF	Connective tissue growth factor	5.53	4.29	Cell-matrix adhesion
TTR	Transthyretin	4.19	3.44	Extracellular matrix organization
JAG1	Jagged 1	3.91	3.63	Notch signaling pathway
CYR61	Cysteine-rich, angiogenic inducer, 61	3.86	3.91	Cell proliferation, Cell adhesion
SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	3.52	2.55	Inflammatory response
CDH6	Cadherin 6, type 2, K-cadherin	3.46	2.65	Cell adhesion
PRSS23	Protease, serine, 23	3.27	2.33	proteolysis
TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	3.22	3.49	Apoptosis
IGDCC3	Immunoglobulin superfamily, DCC subclass, member 3	3.15	2.04	Neuromuscular process controlling balance
OLFML2A	Olfactomedin-like 2A	2.91	2.31	Extracellular matrix organization
SLC4A11	Solute carrier family 4, sodium borate transporter, member 11	2.89	2.28	Borate/sodium ion transport
GLRB	Glycine receptor, beta	2.66	2.06	Ion transport
FLNB	Filamin B, beta	2.56	2.11	Cell differentiation
HES4	Hairy and enhancer of split 4	2.53	2.05	Cell differentiation
KCNJ8	Potassium inwardly-rectifying channel, subfamily J, member 8	2.47	2.16	Defense response to virus

ANGPTL3	Angiopoietin-like 3	2.39	2.86	Cell-matrix adhesion
MATN3	Matrilin 3	2.31	3.06	Extracellular matrix organization
SLC7A7	Solute carrier family 7, member 7	2.27	2.75	Ion transport
SCARA3	Scavenger receptor class A, member 3	2.24	2.53	Response to oxidative stress
KRT17	Keratin 17	2.21	2.06	Keratinization, regulation of cell growth
HS6ST2	Heparan sulfate 6-O-sulfotransferase 2	2.18	2.15	Glycosaminoglycan biosynthetic process
CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3	2.17	2.76	Chondroitin sulfate biosynthetic process
SLC7A5	Solute carrier family 7, member 5	2.14	2.58	Amino acid transport , cell differentiation
DKK3	Dickkopf WNT signaling pathway inhibitor 3	2.13	2.04	Regulation of Wnt signaling pathway
SLC38A4	Solute carrier family 38, member 4	2.13	2.13	Amino acid transport
LUM	Lumican	2.08	2.11	Carbohydrate metabolic process
LCN2	Lipocalin 2	-32.17	-2.92	Apoptosis
SOD2	Superoxide dismutase 2, mitochondrial	-14.28	-2.29	Regulation of cell proliferation
C15orf48	Chromosome 15 open reading frame 48	-10.63	-4.06	Unknown
MMP7	Matrix metallopeptidase 7	-8.63	-2.92	Extracellular matrix disassembly
TNF	Tumor necrosis factor	-6.73	-2.29	Apoptosis
GPX2	Glutathione peroxidase 2	-6.45	-2.45	Response to oxidative stress
RAC2	RAS-related C3 botulinum substrate 2	-6.06	-3.11	Positive regulation of cell proliferation

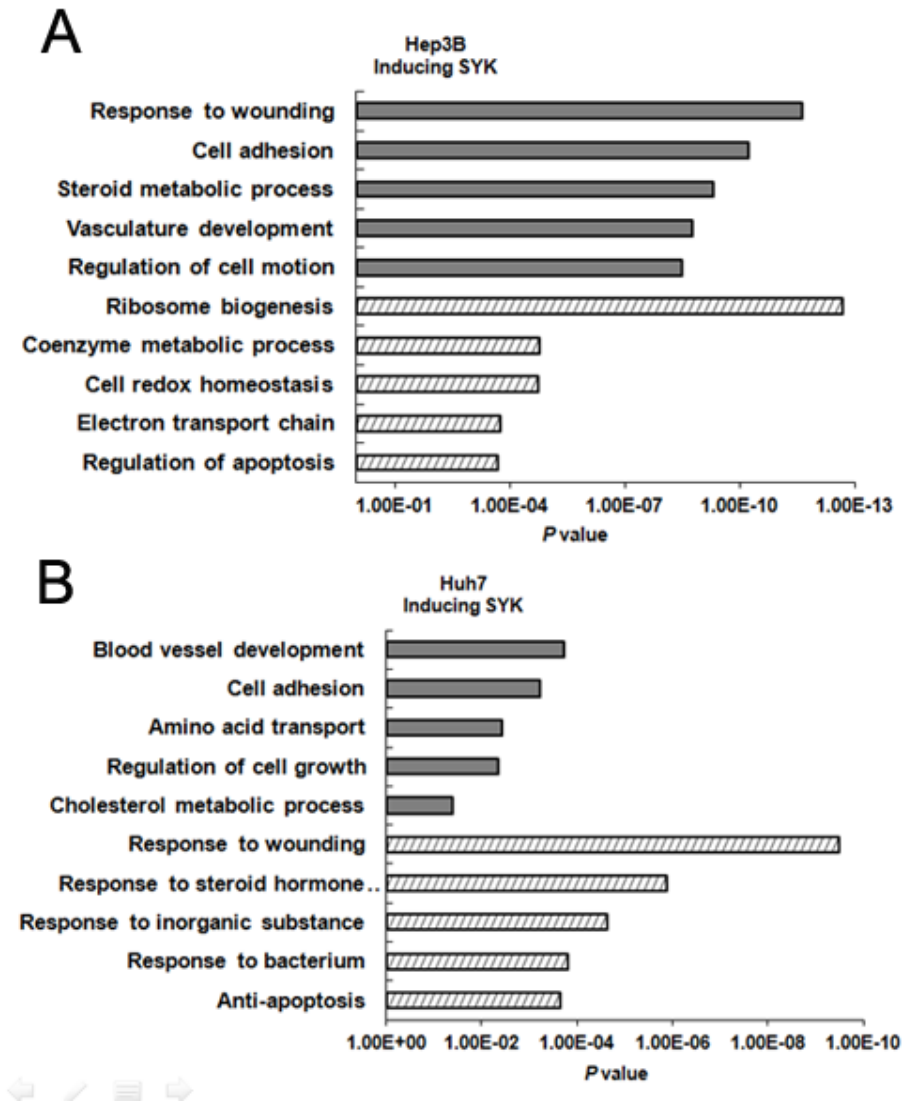
BIRC3	Baculoviral IAP repeat containing 3	-5.96	-2.17	Apoptosis
HOXD1	Homeobox D1	-5.92	-2.3	Embryonic skeletal system development
EHF	Ets homologous factor	-5.23	-2	Cell proliferation, epithelial cell differentiation
CDX2	Caudal type homeobox 2	-3.57	-2.26	Regulation of cell differentiation
LYZ	Lysozyme	-3.26	-2.17	Inflammatory response
EBI3	Epstein-Barr virus induced 3	-3.24	-2.37	Inflammatory response
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	-3	-2.29	Apoptosis
NLF2	C2 calcium-dependent domain containing 4B	-2.99	-3.43	Focal adhesion
LOC440731	Similar to hCG1817424	-2.77	-2.21	Unknown
SERPINA3	Serpin peptidase inhibitor, clade A, member 3	-2.5	-2.07	Inflammatory response
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-2.44	-2.74	Apoptosis
NAMPT	Nicotinamide phosphoribosyltransferase	-2.41	-2.11	Positive regulation of cell proliferation
C2CD4A	C2 calcium-dependent domain containing 4A	-2.36	-2.54	Unknown
TRIM31	Tripartite motif containing 31	-2.14	-2.02	Protein ubiquitination
MT2A	Metallothionein 2A	-2.09	-2.1	Cytokine-mediated signaling pathway
TMPRSS2	Transmembrane protease, serine 2	3.01	-3.11	Proteolysis
FGG	Fibrinogen gamma chain	2.64	-2.11	Blood coagulation
TXNIP	Thioredoxin interacting protein	2.56	-4.23	Cell cycle, regulation of cell proliferation
TPR	Translocated promoter region, nuclear basket protein	2.17	-2.22	Cell division

Table 3. SYK- regulated genes with SYK reduction in both Hep3B and Huh7 cells

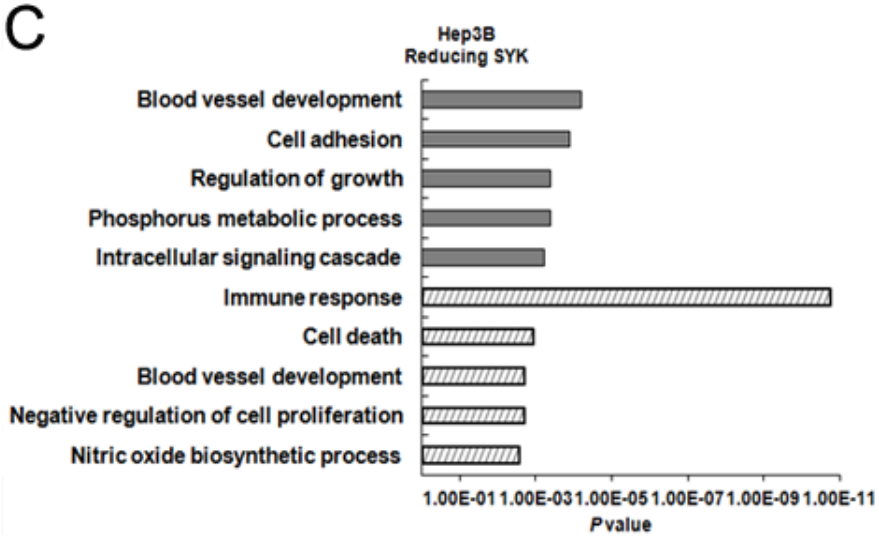
Symbol	Name	Fold changes (Dox- vs Dox+)		Process
		Hep3B	Huh7	
C4BPA	C4BPA complement component 4 binding protein, alpha	2.46	2.04	Immune response
LOC148430	Ribosomal protein S2 pseudogene	2.04	2.15	Unknown
CDKN1A	Cyclin-dependent kinase inhibitor 1A	-2.09	-2.11	Cell cycle arrest
EDN1	Endothelin 1	-2.1	-3.47	Cell growth
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	-2.15	-2.19	Cell proliferation
TAGLN	Transgelin	-2.3	-3.61	Muscle organ development
KRT80	Keratin 80	-2.33	-2.57	Structural molecule activity
CYR61	Cysteine-rich, angiogenic inducer, 61	-2.46	-4.97	Cell proliferation, Cell adhesion
OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	3.28	-2.31	Cell death, cell-cell adhesion
LOC100008588	RNA18S5 RNA, 18S ribosomal 5	2.99	-2.31	Unknown
LOC100008589	RNA, 28S ribosomal 5	2.18	-2.05	Unknown

Gene Ontology Analysis of SYK-dependent genes in HCC cells

To understand the roles of SYK loss during cancer progression in HCC cells, we performed gene ontology analysis of the SYK-dependent genes. Induction of *SYK* expression in Hep3B and Huh7 cells were significantly associated with enrichment of gene set concepts such as cell adhesion, blood vessel development, cell growth and apoptosis. Importantly, when *SYK* expression was tapered in Hep3B and Huh7 cells, many of these gene set concepts were significantly classified (Figure 4A-D).


A
Hep3B
Inducing SYK
Response to wounding
Cell adhesion
Steroid metabolic process
Vasculature development
Regulation of cell motion
Ribosome biogenesis
Coenzyme metabolic process
Cell redox homeostasis
Electron transport chain
Regulation of apoptosis
1.00E-01
1.00E-04
1.00E-07
1.00E-10
1.00E-13
P value
B
Huh7
Inducing SYK
Blood vessel development
Cell adhesion
Amino acid transport
Regulation of cell growth
Cholesterol metabolic process
Response to wounding
Response to steroid hormone..
Response to inorganic substance
Response to bacterium
Anti-apoptosis
1.00E+00
1.00E-02
1.00E-04
1.00E-06
1.00E-08
1.00E-10
P value

C


Hep3B
Reducing SYK
Blood vessel development
Cell adhesion
Regulation of growth
Phosphorus metabolic process
Intracellular signaling cascade
Immune response
Cell death
Blood vessel development
Negative regulation of cell proliferation
Nitric oxide biosynthetic process
1.00E-01 1.00E-03 1.00E-05 1.00E-07 1.00E-09 1.00E-11
Pvalue

D

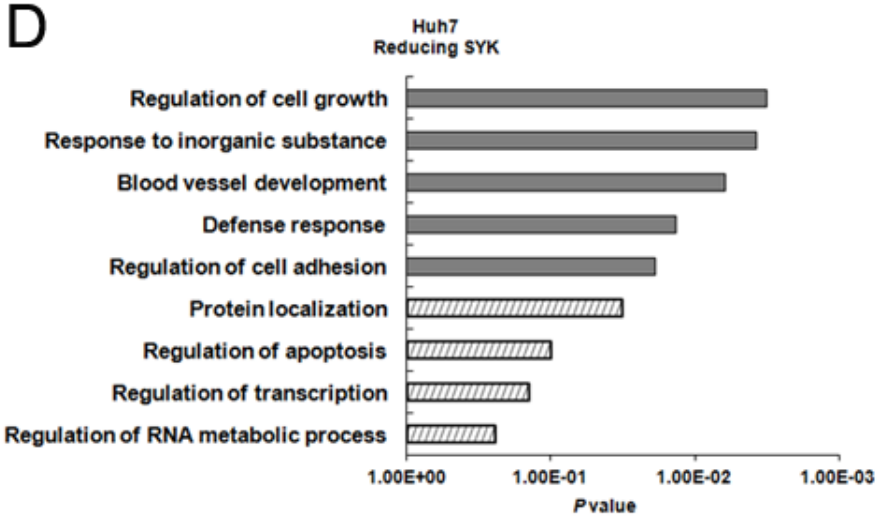

Huh7
Reducing SYK
Regulation of cell growth
Response to inorganic substance
Blood vessel development
Defense response
Regulation of cell adhesion
Protein localization
Regulation of apoptosis
Regulation of transcription
Regulation of RNA metabolic process
1.00E+00
1.00E-01
1.00E-02
1.00E-03
Pvalue

Figure 4. Gene ontology classifications

Gene ontology categories overrepresented in SYK-regulated genes of which expression was increased (black) and decreased (patterned) over 2- fold after Dox treatment in Hep3B (A), Huh7 (B) and over 2-fold after Dox removal in Hep3B (C) and Huh7 (D). The GO term is on the y axis, and the *P* value indicating significance of enrichment on the x axis.

Expression of SYK suppresses cell growth

To investigate the effects of *SYK* expression on cell growth in HCC cells, SYK was induced by Dox treatment in Huh7 cells in which SYK was silenced. The suppressive effect on cancer cell growth was demonstrated by a colony formation assay (Figure 5A). An MTT assay confirmed that cell proliferation was significantly reduced in SYK-induced clones, compared with the non-induced control (Figure 5B). Also, SYK inhibited cellular proliferation in transiently transfected HepG2 cell line, compared with the vector control (Figure 5C). Recently, a report has revealed that checkpoint kinase 1 (CHK1) phosphorylates SYK at Ser295 and promotes its subsequent proteasomal degradation, which negatively regulates SYK function, including suppression of proliferation, suppression of migration, or suppression of invasion (32). We found that *CHK1* was expressed in both Hep3B and Huh7 cells (Figure 6A). To determine the effects of the CHK1 inhibitor GÖ6976, two individual colnes of SYK-inducible Huh7 cells were treated with GÖ6976 and assessed for cell proliferation by MTT assay (Figure 6B). The cells with *SYK* expression and GÖ6976 treatment displayed lower cell proliferation rate than that of the cells with SYK expression alone or treatment of GÖ6976 alone.

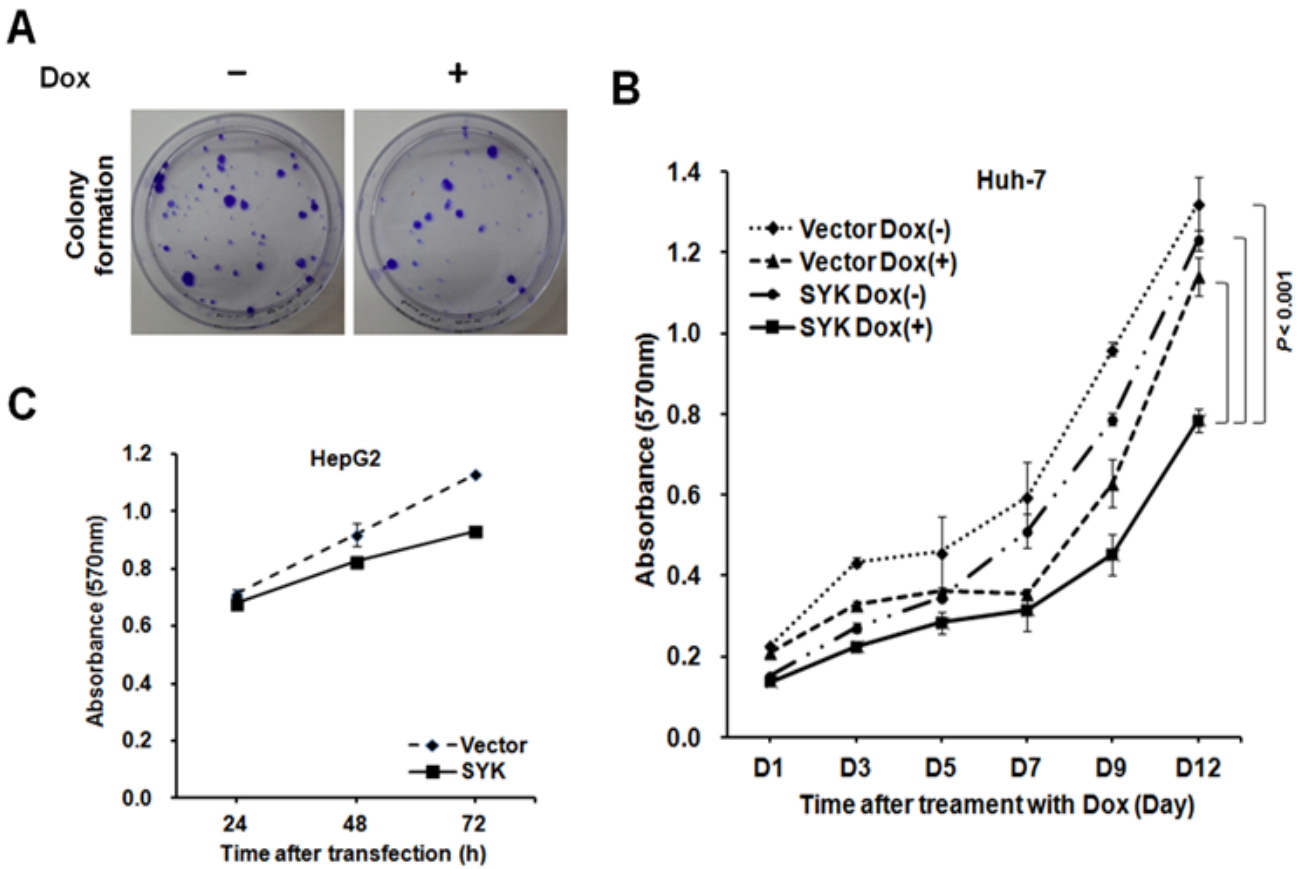

A
Dox
–
+
Colony formation
B
Huh-7
Vector Dox(-)
Vector Dox(+)
SYK Dox(-)
SYK Dox(+)
P< 0.001
Absorbance (570nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D1
D3
D5
D7
D9
D12
Time after treament with Dox (Day)
C
HepG2
Absorbance (570nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vector
SYK
24
48
72
Time after transfection (h)

Figure 5. Suppressive effect of SYK on tumor cell growth.

(A) A colony formation assay demonstrates a reduction in the number of colonies in SYK-induced Huh7 cells compared with those of control cells. (B) Huh7 cells were stably transfected with the indicated plasmid and induction of SYK was controlled by treatment with Dox. The cell proliferative capacity of these cells was measured by MTT assay at the indicated days. Cell growth is expressed as absorbance at a wavelength of 570 nm. Experiments were performed in triplicate, and values indicate means ± s.d. Student's *t* test was performed to compare means, revealing significant differences (***P*<0.001). (C) HepG2 cells were transiently transfected with *SYK* or empty vector. In an MTT assay, cell growth is expressed as absorbance at a wavelength of 570 nm. Experiments were performed in triplicate, and values indicate means ± s.d.

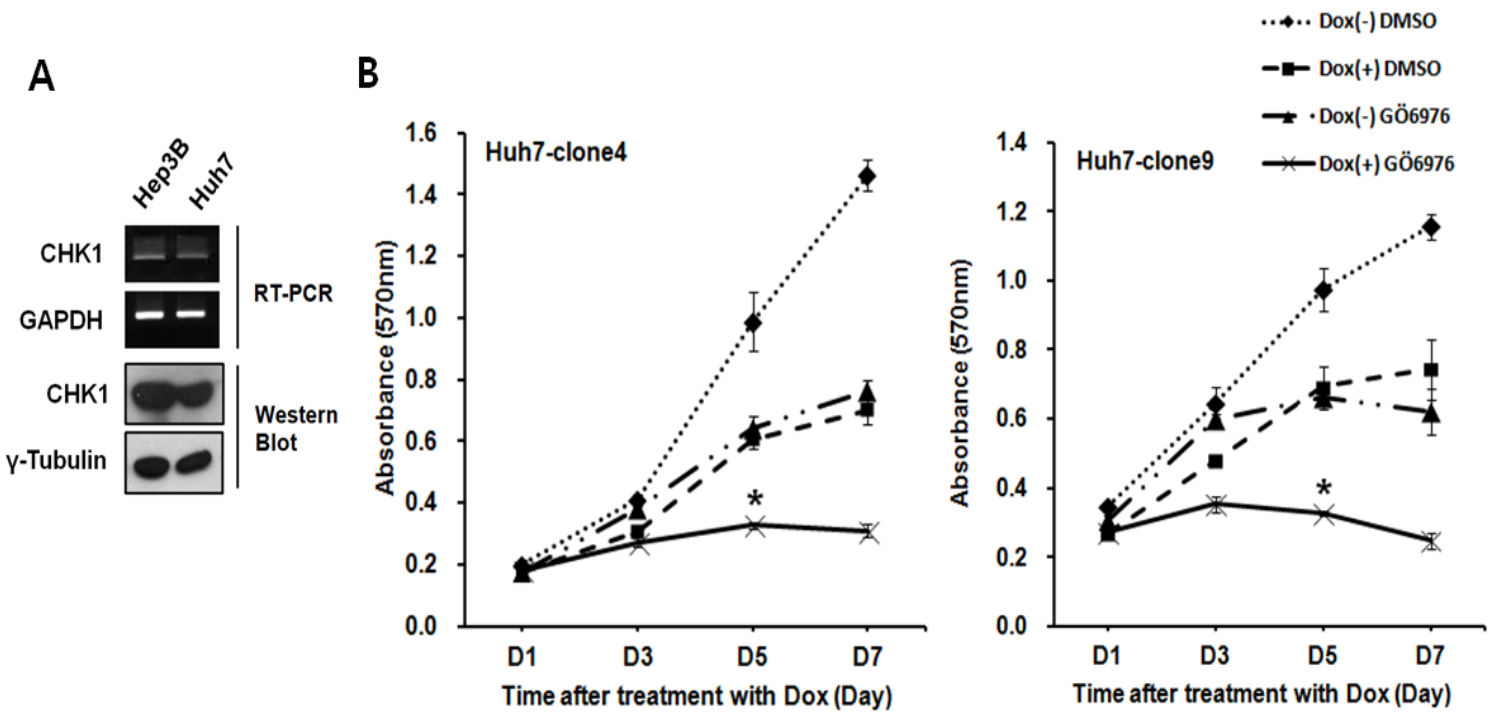

A
Hep3B
Huh7
CHK1
GAPDH
RT-PCR
CHK1
γ-Tubulin
Western Blot
B
Huh7-clone4
Absorbance (570nm)
Time after treatment with Dox (Day)
D1
D3
D5
D7
Huh7-clone9
Dox(-) DMSO
Dox(+) DMSO
Dox(-) GÖ6976
Dox(+) GÖ6976

Figure 6. CHK1 inhibitor suppresses cell proliferation through increasing stability of SYK in Huh7 cells.

(A) Analyses of *CHK1* expression by RT-PCR (*GAPDH* mRNA levels were used as a control) and Western blotting (γ-Tubulin protein levels were used as a control). (B) Two individual clones of Tet-on Huh7 cells were treated with GÖ6976 (100 nM) or DMSO (control) as indicated for days and were subjected to an MTT assay. Cell growth is expressed as absorbance at a wavelength of 570 nm. Experiments were performed in triplicate, and values indicate means ± s.d. (Student's *t* test, **P*<0.05).

Effect of SYK on cell adhesion, migration and invasion

To confirm the role of SYK in cell attachment, migration and invasion, cell attachment, migration and invasion assays were performed. The cells with SYK induction displayed suppression of cell migration (Figure 7A) and invasion (Figure 7B) compared with the cells with no SYK induction. To investigate the effect of SYK in cell adhesion, cell attachment assays were conducted in 96 wells flat bottom microtiter plates coated or uncoated with fibronectin. HCC cells cultured on fibronectin-uncoated plates showed no attachment regardless of *SYK* expression status, whereas HCC cells on coated plates exhibited attachment to the plate and increased attachment with induction of *SYK* expression (Figure 8A). Also, we found that SYK-induced cells increased cell-to-matrix and cell-to-cell adhesion on fibronectin-coated coverslips (Figure 8B). Together, the data indicate that altered capacity of cell-to-matrix and cell-to-cell adhesion is mediated by SYK induction and that SYK might contribute to inhibition of cell migration and invastion.

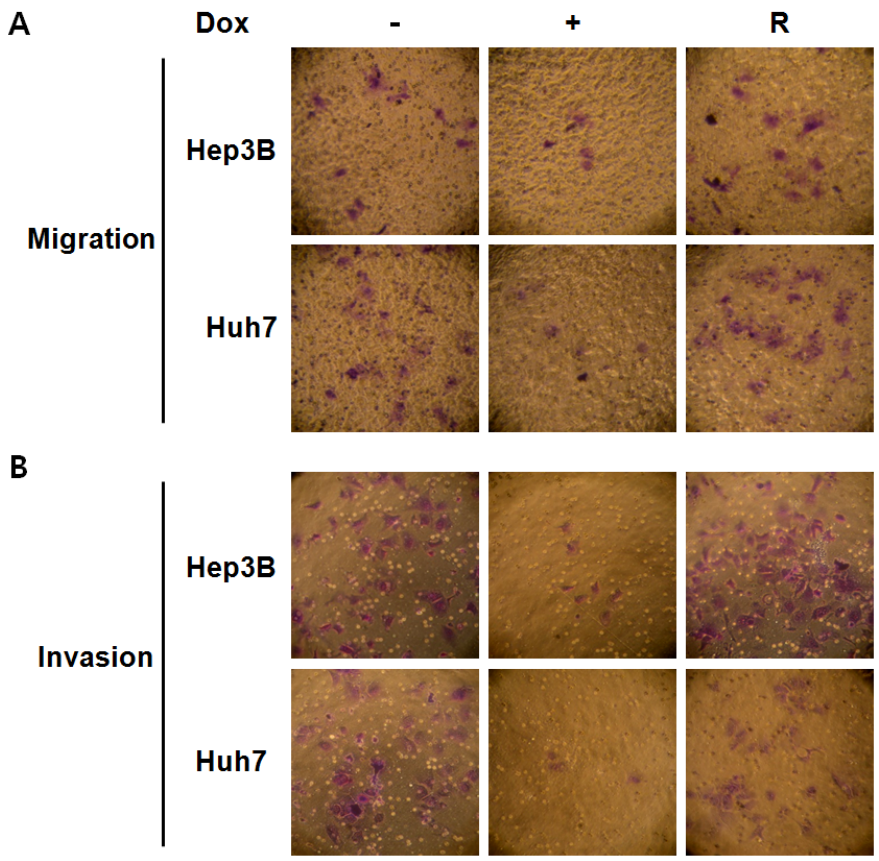


Figure 7. Effect of SYK on cell migration and invasion

(A) Cell migration and (B) invasion assay were performed in SYK-inducible Huh7 cells. Expression of *SYK* decreases cell migration and invasion. -, no treatment of Dox; +, treatment of Dox; R, after removal of Dox.

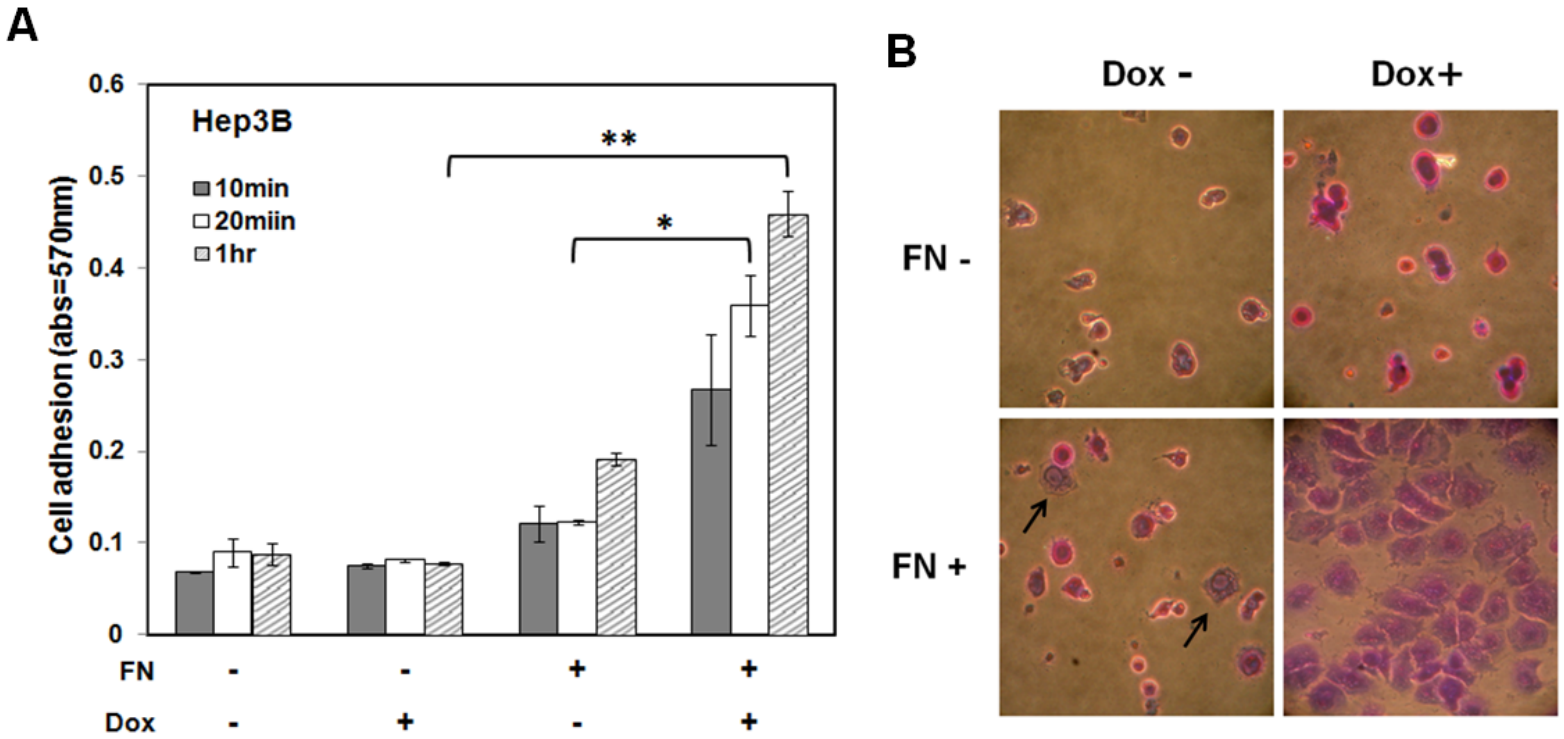

A
Hep3B
10min
20miin
1hr
Cell adhesion (abs=570nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
FN - - + +
Dox - + - +
B
Dox -
Dox+
FN -
FN +

Figure 8. Effect of SYK on adhesion to fibronectin in HCC cells.

(A) SYK-inducible Hep3B cells were tested for adhesion as indicated times to 96 wells plate coated with fibronectin or not. Experiments were performed in triplicate, and values indicate means ± s.d.. **, $P<0.001$; *, $P<0.05$ by Student's *t*-test. (B) *In vitro* morphology of SYK-induced cells following adhesion to fibronectin. Cells were plated on uncoated coverslips (FN-) or fibronectin-coated coverslips (FN+) for 30 min. Subsequently, non-adherent cells were washed off, the remaining adherent cells were fixed with 96% ethanol and stained with 0.1% crystal violet. SYK-induced Huh7 cells showed increased cell-to-matrix adhesion and cell-to-cell adhesion on fibronectin-coated coverslips.

Cellular redistribution of SYK following adhesion to fibronectin

SYK-dependent signaling pathways in epithelial cells were initially activated by participation of β1-integrin receptors. Stimulation of β1-integrin receptors by fibronectin or antibody cross-lilnking has been demonstrated to promote the redistribution of SYK from cytoplasm to plasma membrane localization (21). To identify whether the localization of SYK is changed by stimulating β1-integrin receptors in HCC cells, SYK-tGFP transfected-Hep3B and Huh7 cells were plated on fibronectin-coated surfaces, followed by analysis using confocal microscopy. One hour adhesion to fibronectin induced visible spreading of both Hep3B and Huh7 cells on coverslips. Cells plated on non-coated coverslips (1h) were smaller and more rounded (Figure 9). SYK was distributed in both cytoplasmic and nuclear areas. After adhesion to fibronectin, SYK was redistributed with some localization along the plasma membrane in both cell lines with a difference in nuclear localization: SYK was retained in the nucleus in Huh7 cells but not in Hep3B cells. In contrast, in cells plated on non-coated coverslips kept overnight, SYK was distributed in the cytoplasm without localization along the plasma membrane or to the nucleus (Figure 9). These results indicate that redistribution of SYK was induced by engagement of β1-integrins by fibronectin.

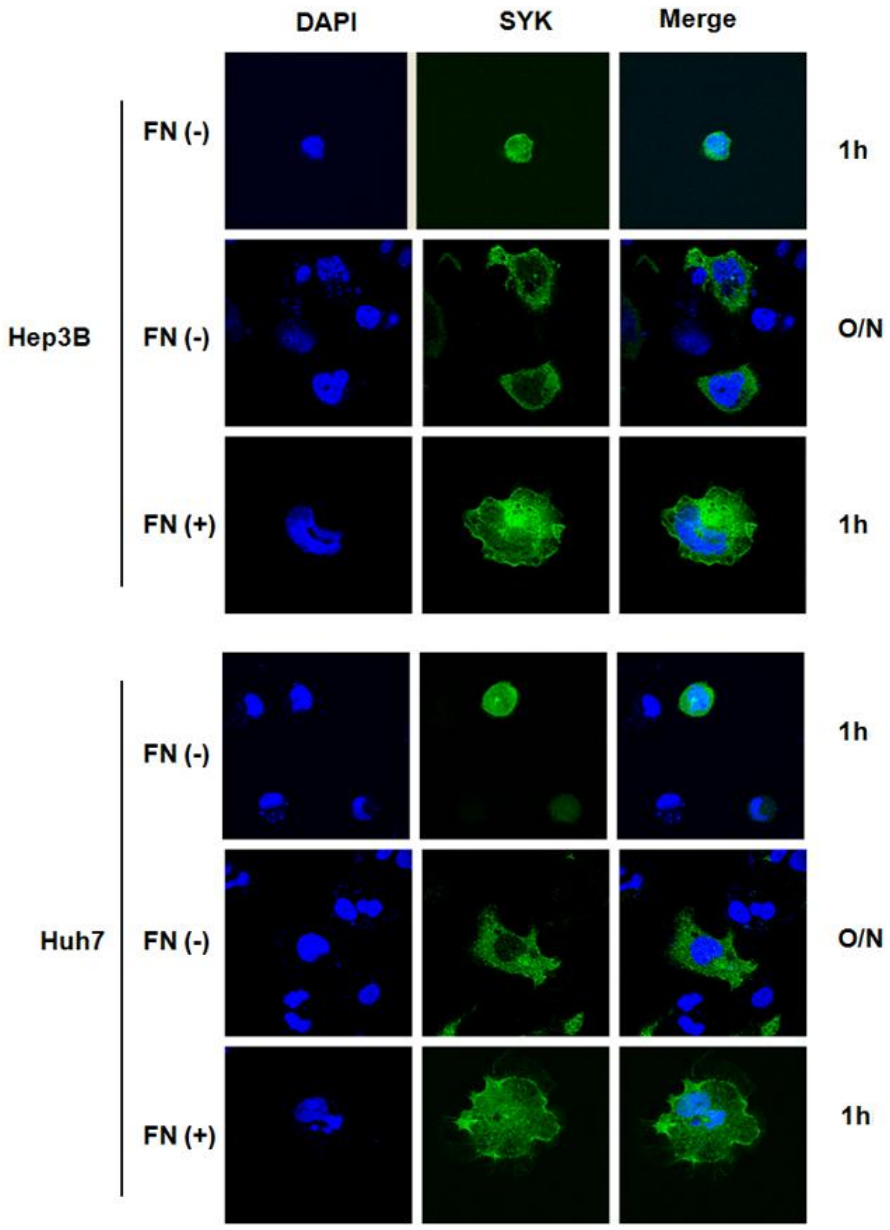
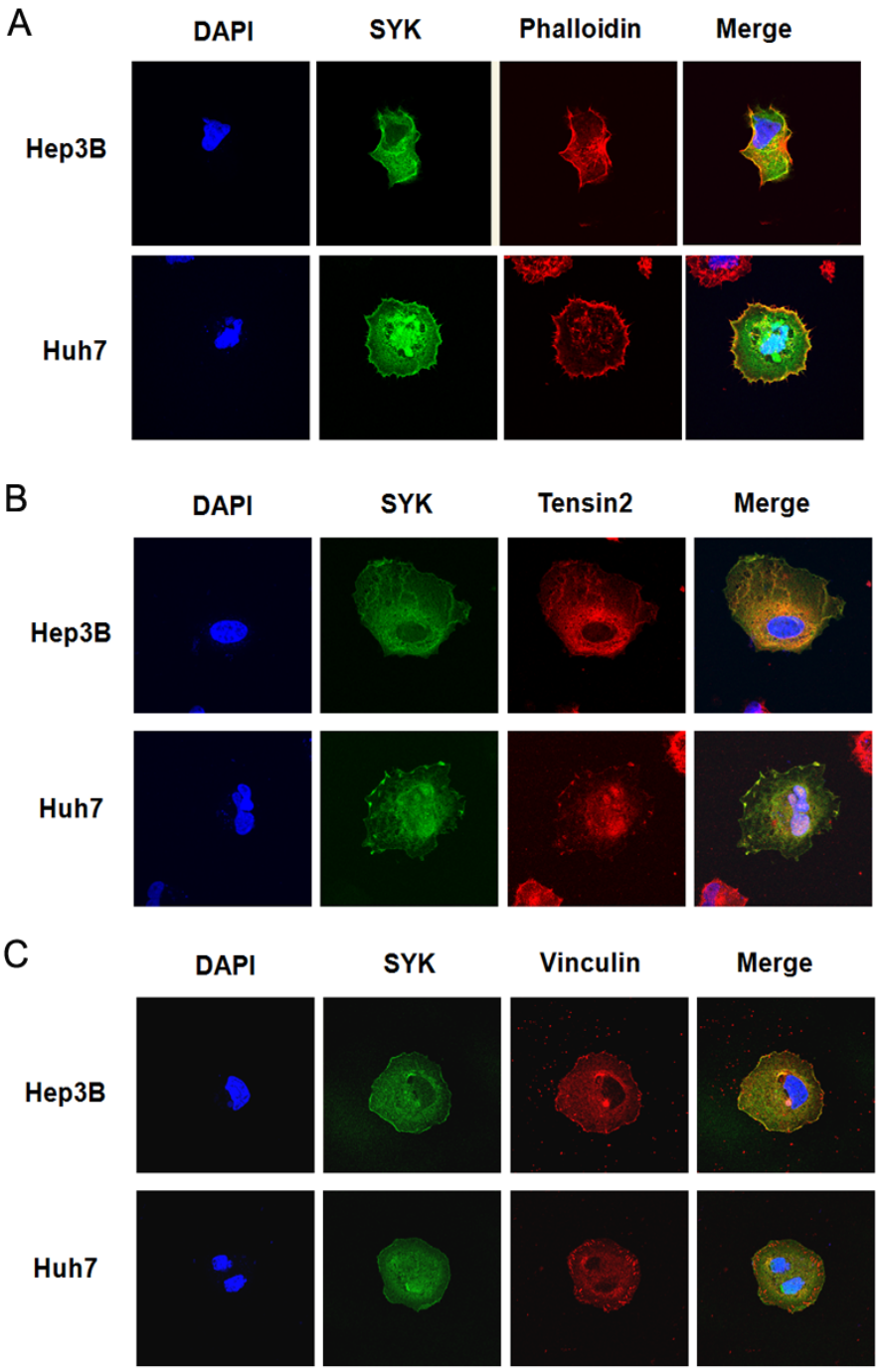

DAPI
SYK
Merge
Hep3B
FN (-)
1h
FN (-)
O/N
FN (+)
1h
Huh7
FN (-)
1h
FN (-)
O/N
FN (+)
1h

Figure 9. Adhesion to fibronectin mediates cellular redistribution of SYK in HCC cells.

Huh7 and Hep3B cells were transiently transfected with tGFP tagged-SYK. Cells were plated on fibronectin- or non- coated glass coverslips for 1h or overnight (O/N), fixed, and counterstained with DAPI nucleic acid stain. Original magnification, x600.

SYK associates with cytoskeleton and adhesion molecules

Focal adhesions are formed at ECM–integrin interactions that bring together cytoskeletal and signaling molecules during the processes of cell adhesion, spreading and migration (28). To explore first a possible association between SYK and microfilaments, we examined the localization of SYK and actin. When expressed in HCC cells, tGFP-tagged SYK appeared co-localized with F-actin stained with fluorescent phalloidin within plasma membrane (Figure 10A). Also, we found a similar distribution of endogenous adhesion molecules, tensin2 and vinculin, stained with their specific antibodies (Figure 10B and C). To look for a physical interaction between SYK and adhesion molecules or F-actin, SYK was immunoprecipitated from lysates of SYK-inducible Hep3B and Huh7 cells and the resulting immune complexes were examined by Western blotting. Both cells showed the interaction between SYK and β-actin or vinculin, but not tensin2 (Figure 10D). To confirm this interaction, SYK also was immunoprecipitated from lysates of SNU-739 cells expressing endogenous SYK and these immune complexes again revealed the interaction with β-actin and vinculin Figure 10D). These data suggest that SYK could localize to focal adhesions with adhesion molecules and affect actin cytoskeletal network in HCC cells.


A
DAPI
SYK
Phalloidin
Merge
Hep3B
Huh7
B
DAPI
SYK
Tensin2
Merge
Hep3B
Huh7
C
DAPI
SYK
Vinculin
Merge
Hep3B
Huh7

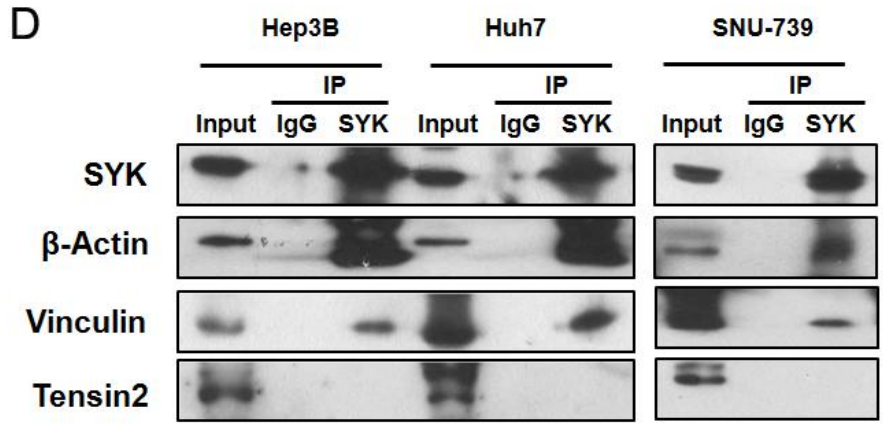


Figure 10. Association of SYK with cytoskeleton and adhesion molecules.

Hep3B and Huh7 cells transiently expressing SYK-tGFP were attached on fibronectin-coated coverslip for 1h, fixed, permeabilized and stained with Rhodamine Phalloidin (A), anti-tensin2 (B) and anti-vinculin (C). Original magnification, x600. (D) Lysates (Input), anti-IgG (IP: IgG) or anti-SYK immune complexes (IP: SYK) prepared from Dox-treated Hep3B, Huh7 (induced-SYK) and SNU-739 (endogenous SYK) cells were analyzed by Western blotting using anti-SYK, anti-beta-actin, anti-vinculin, anti-tensin2 antibodies.

Expression of SYK causes a suppression of Rho-Family GTPases and stress fiber formation

Rho-family GTPases regulate the formation and disassembly of actin cytoskeleton (stress fibers, lamelliopdia and filopodia) and transmit the molecular signaling that supports directed cell motility (28). To investigate the effects of SYK expression on the levels of Rho-family GTPases including RhoA and RhoB, SYK was transiently trasfected in Huh7 cells and compared the levels of Rho-family GTPases with empty vector control. Western blot analysis indicated that expression of SYK resulted in a decrease in the level of RhoA and RhoB (Figure 11A) and stress fiber formation (Figure 11B). These data may imply SYK expression in SYK-silenced HCC cell lines decrease their migration and invasion by suppression of Rho-family GTPases and stress fiber formation.

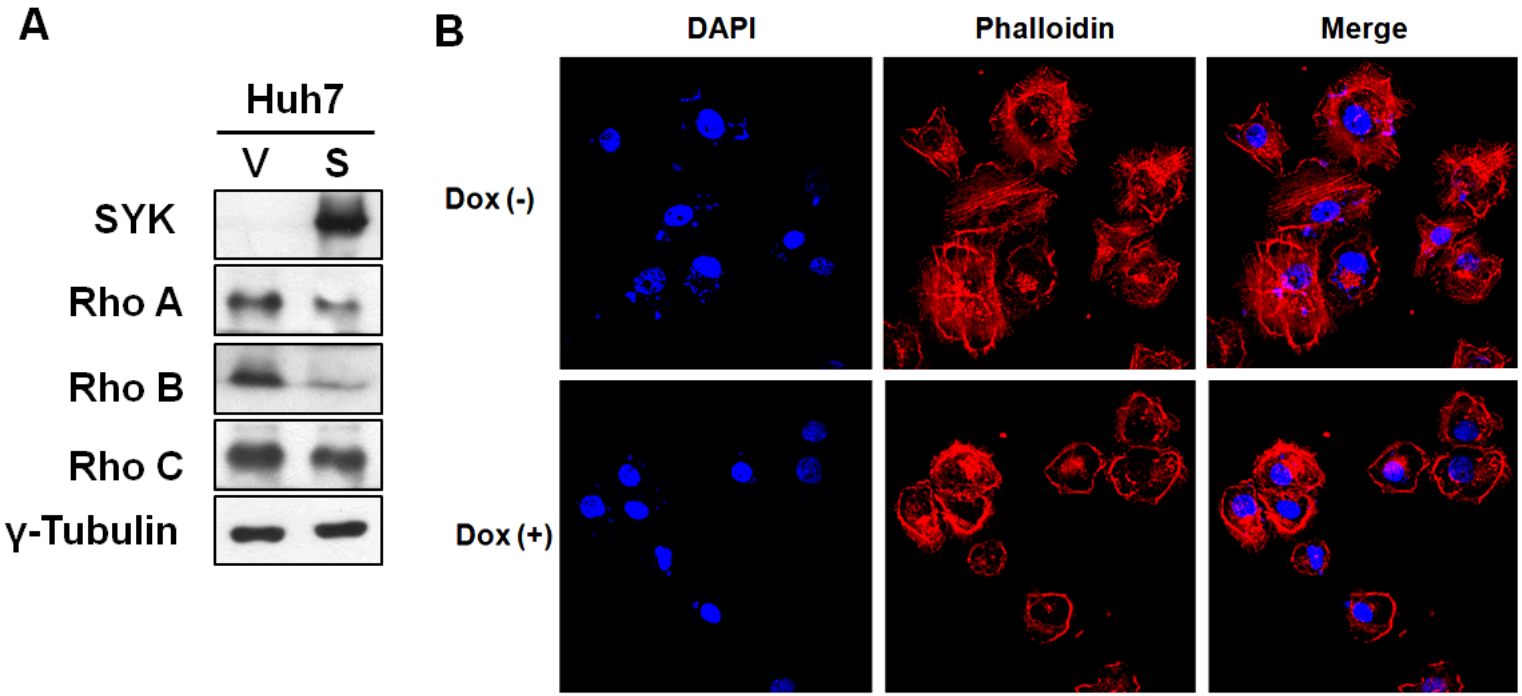

A
Huh7
V
S
SYK
Rho A
Rho B
Rho C
γ-Tubulin
B
DAPI
Phalloidin
Merge
Dox (-)
Dox (+)

Figure 11. Expression of SYK causes a suppression of Rho-family GTPases and stress fiber formation.

(A) SYK-transfected Huh7 or empty vector-transfected Huh7 cells were analyzed by Western blotting using anti-SYK, anti-RhoA, anti-RhoB, anti-RhoC antibodies. γ-Tubulin protein levels were used as a control. V indicates empty vector-transfected cells and S indicates SYK expression vector-transfected cells. (B) Huh7 cells untreated (-) or treated (+) with Doxycycline (Dox) to induce expression of SYK. Cells attached on fibronectin-coated coverslip for 1h, fixed, permeabilized and stained with rhodamine phalloidin.

DISCUSSION

SYK is a putative tumor- and metastasis-suppressor gene recently found to be inactivated through promoter CpG island hypermethylation in several types of cancer (17-20). In the current study, we analyzed the methylation state of *SYK* promoter CpG island locus and its mRNA/protein expression in HCC cell lines, which showed an inverse correlation between promoter CpG island hypermethylation and SYK expression. Treatment of methylated HCC cell lines with a demethylating agent exhibited restored *SYK* expression. Although *SYK* is actively transcribed in hepatocytes, development of aberrant hypermethylation in promoter CpG island loci in association with tumorigenesis leads to transcription silencing of *SYK*. In consideration of previous studies indicating that hypermethylation of *SYK* promoter CpG island locus is closely associated with worse prognosis of HCC patients (19, 33), it could be hypothesized that reduced expression of *SYK* by promoter CpG island hypermethylation may be related to biological aggressiveness of HCC.

In the present study, SYK showed suppression of Huh7 and HepG2 cell growth but not Hep3B cells. The anti-growth activity of SYK was augmented by the treatment of GÖ6976, an inhibitor of CHK1, which stabilizes SYK. Combined effect of SYK induction and CHK1 inhibition was remarkable in terms of suppression of tumor-cell growth compared with SYK induction alone or CHK1 inhibition alone.

Considering that induced SYK shows differential anti-growth activity depending on cell types, it can be speculated that anti-growth activity of SYK operates in a specific context. To identify the mechanisms underlying the anti-growth effect of SYK in Huh7 cells, we performed cell cycle analysis in Huh7 cells with and without induction of SYK expression. However, we did not find a difference in the fraction of cell cycle phases and in specifically sub-G1 fraction (data not shown). This situation is similar to that of Coopman et al's study in which SYK blocked breast tumor cell growth in vitro but no differences in cell proliferation and apoptosis were found between breast cancer cells with and without SYK (12). However, abnormal mitoses with multipolar spindles were found to be significantly increased with SYK expression (12, 34, 35), which suggests involvement of SYK in the regulation of cell proliferation by controlling mechanisms of mitosis and cytokinesis (34). Another mechanism lies in SYK's abilities regulating expression of genes that are involved in cell cycle progression (12). SYK down-regulates the transcription of cell cycle progressive genes such as *CCND1, CCNA1, AKT1* and *FOSL1* (14, 15, 36). On the other hand, reexpression of SYK induces a TP53-dependent accumulation of CDKN1A and a senescence-like growth arrest in melanoma cells (15). However, we did not find significant differences in mRNA levels of *CCND1* and *CDKN1A* between Huh7 cells with and without SYK induction. Our present data show that SYK regulates various genes that are involved in cell proliferation and reexpression of SYK

suppresses cell growth. However, it now remains to identify the exact mechanism for effect of SYK on cell growth in HCC cells.

In contrast with differential anti-growth activity of SYK, increased cell to matrix attachment and decreased cell migration and invasion were shared findings in both Huh7 and Hep3B cells with SYK expression. Induction of SYK expression led to cytoplasmic spreading, while addition of fibronectin exposure prompted cytoplasmic spreading and localization of SYK along cytoplasmic border. SYK was co-localized with actin, vinculin and tensin-2 in cytoplasmic border although immuno-precipitation assay did not prove interaction between SYK and tensin-2. Furthermore, SYK expression led to an increase in CDH1 expression which strengthens cell to cell adhesion (data not shown). Decreased cell motility in association with SYK expression appears to be attributed to increased cell to matrix and cell to cell attachment. Clinical studies have demonstrated that SYK expression is predominantly decreased in invasive and metastatic breast tumors and that decreased expression of SYK is related to an increased risk for distant metastasis (17, 23). In the present study, SYK methylation was closely associated with shortened survival time in HCC patients, which is consistent with results of previous studies demonstrating shortened survival time in HCCs with SYK methylation.

Tumor invasion and metastasis are complicated procedures requiring the ability of cancer cells to interact with endothelial cells and extracellular matrix. Integrins are the major cell surface receptors that

mediated these interactions. Especially, β1-integrins are highly expressed in HCC cell lines including Hep3B and Huh7 cells. Activity and expression of β1-integrins are associated with the invasive ability of HCC cells (37). Several reports have shown that SYK is phosphorylated and activated through β1 integrins signaling in airway epithelial cells and breast cancer cells (21, 22). Our results demonstrated that redistribution of SYK from cytoplasm to plasma membrane induced by stimulation of β1-integrins by fibronectin. Also, the adhesion and spreading of HCC cells occurred rapidly and widely on fibronectin-coated galss coverslips compared to un-coated. Although, more studies will be necessary to fully understand the effects of SYK on cell motility and invasion, we suggest that the ability of SYK to enhance integrin-mediated adhesion and to decrease cell motility may underlie its functions as a metastasis suppressor in HCC.

Cytoskeletal organization is one of the most important mechanisms for cell adhesion and motility. SYK is also involved in the organization of the actin cytoskeleton and associates actin-binding proteins. In platelets, thrombin exposure renders SYK relocated to the actin filament network and associated with FAK (31). Our data show that the expression of *SYK* results in its co-localization with cytoskeletal actin or adhesion molecules such as vinculin and tensin2 at adhesion site. Tensin2 is also known to interact with deleted-in-liver-cancer1 (DLC1), a tumor suppressor and negative regulator of Rho-family GTPases (38, 39). Inhibition of RhoA signaling in HCC cells causes a significant loss

of their in vitro motility and reduces intrahepatic metastasis in injected nude mice (40). Similarly, overexpression of RhoC has a strong correlation with invasion and metastasis of HCC (41). In our results, expression of *SYK* leads to a reduction of motility and decreases the level of RhoA and RhoB. Because the total level of RhoA and RhoB decreases, it suggests that GTP-bound active Rho also decline. Therefore, SYK may participate in both focal adhesion signaling and Rho-dependent signaling.

In conclusion, *SYK* promoter methylation is closely associated with its down-regulation in HCC cells. Findings of the present study support the hypothesis that SYK functions as a tumor suppressor in HCC by demonstrating SYK's anti-proliferative activity and pro-adhesive activity. Restoring *SYK* expression in SYK-silenced HCC cell lines decreased cell growth while increased cell adhesion. Furthermore, expression of *SYK* decreased cell migration and invasion by coordination with adhesion molecules as well as suppression of Rho-family GTPases. Our findings suggest that *SYK* loss is implicated in cell proliferation, migration, and invasion of HCC cells. Further studies are required to define the exact mechanism how SYK regulates cell proliferation of HCC cells.

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국문 초록

간암에서 *SYK*의 프로모터 CpG island 과메틸화에 의한 발현 소실 및 종양억제자로서의 역할에 대한 연구

Spleen tyrosine kinase (SYK)은 면역세포에서 널리 발현이 되고 있다. 면역수용체를 매개로 활성화된 SYK 은 다양한 신호전달에 관여하게 된다. SYK 은 또한 여러 비면역세포에서도 발현되고 있으며, SYK 의 발현 감소로 인해 종양의 형성과 발전에 영향을 준다고 보고되어있다. 본 연구실에서 간암조직에서 SYK 의 과메틸화와 환자의 불량한 예후의 관련성을 보고를 한바 있으나, 간암에서의 SYK 의 생물학적 역할에 대해서는 거의 알려진 것이 없다. 본 연구에서는 SYK 의 메틸화가 간암세포주에서 빈번하게 발생하고, 이것과 유전자 발현 억압과 큰 관련이 있다는 것을 밝혔다. SYK 메틸화에 의해 발현이 억압된 간암세포주 Hep3B 와 Huh7 에 Tet-on inducible expression system 을 적용시킨 stable cell line 을 만들었고, SYK 발현 유도의 유무에 따른 global gene expression 변화를 microarray 를 통해 비교하였다. Gene Ontology 분석을 통하여 SYK 의 유무에 따라 발현이 변화하는 유전자군은 세포성장과 세포부착에 관여하는 유전자가 유의하게 많음을 알 수 있었다. 실제로, SYK 은 간암세포가 Fibroenectin 에 부착하는 능력을 증가시켰고,

세포증식을 감소시킴을 확인하였다. SYK 은 세포부착에 관여하는 단백질들과의 상호작용과 Rho-family GTPase 의 발현감소를 통해 세포의 이동과 침윤을 감소시켰다. 본 연구의 결과로 간암세포에서 SYK 의 발현소실은 세포 증식, 이동, 침윤을 초래할 수 있음을 암시할 수 있다.

주요어 : Spleen tyrosine kinase, DNA 메틸화, 간암, 종양억제유전자

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