Downregulation of spleen tyrosine kinase in hepatocellular carcinoma by promoter CpG island hypermethylation and its potential role in carcinogenesis

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Spleen tyrosine kinase (SYK) has predominantly been studied in hematopoietic cells, where it is involved in immunoreceptor-mediated signaling. However, *SYK* expression has been shown in numerous non-hematopoietic cells, and its downregulation has been shown to be involved in tumor formation and progression. *SYK* methylation has been demonstrated to identify a subset of hepatocellular carcinoma (HCC) cases with poor prognosis, but little is known regarding the biological role of *SYK* in HCC. We found that *SYK* methylation is a common event in HCC, and is inversely associated with its expression. We established stable HCC cell lines with inducible *SYK* expression vectors, and compared the differential RNA expression profiles of HCC cell lines with or without the induction of *SYK*. Gene ontology analysis revealed that the SYK-regulated genes were enriched for genes involved in cell adhesion. Accordingly, we found that the induction of *SYK* expression increased the adhesion of cells to fibronectin and decreased cell migration and invasion, and that cessation of SYK overexpression increased cell migration and invasion. Our findings suggest that SYK is involved in regulating cell to matrix adhesions, and that SYK loss affects the migration, and invasion of HCC cells.
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Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. In men, it ranks the fifth most common cancer worldwide and is the second leading cause of cancer death, with an estimate of >520 000 new cases each year.[1] The major risk factors associated with the incidence of HCC are well established, and include 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]

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase that is widely expressed in hematopoietic cells. Recently, *SYK* expression has been demonstrated in numerous non-hematopoietic cells, and its downregulation has been shown to

be involved in tumor formation and progression.[3–6] The transfection of *SYK* into an SYK-negative cancer cell line dramatically inhibited its cell growth, migration, and invasion.[3–6] Conversely, the knockdown of *SYK* in SYK-positive breast cancer cells increased proliferation and invasion.[7] Several researchers have also reported that the loss of *SYK* expression correlates with poor survival and tumor metastasis in patients with breast,[8] bladder,[9] liver,[10] pancreas,[5] or stomach cancer.[11] Epigenetic silencing through hypermethylation of promoter CpG islands has been proposed to be involved in the loss of *SYK* expression in these tumors.[3–6] Although SYK was shown to affect cell proliferation, motility, and invasion in several types of cancers, in HCC, its tumor suppressive activity and the molecular mechanisms of its effects remain to be clarified.

In hematopoietic cells, SYK is generally activated by its recruitment to the phosphorylated immunoreceptor

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tyrosine-based activation motifs in the cytoplasmic domain of transmembrane immunoreceptors. Conversely, in epithelial cells, $\beta 1$ integrin-mediated activation of SYK has been demonstrated.[12–14] The activation of $\beta 1$ integrin by fibronectin or antibody crosslinking leads to the redistribution of SYK from the cytoplasm to the plasma membrane, and the induction of tyrosine phosphorylation by SYK.[13,15] Focal adhesions are specific types of large protein complexes through which the cytoskeleton of a cell interacts with proteins of the extracellular matrix. In breast cancer cells, SYK inhibits cell motility, while promoting the expression of cell adhesion molecules, such as vinculin,[15] E-cadherin,[16] and tensin2.[17] Although studies have shown that SYK is associated with the actin filament network and focal adhesion kinase (FAK) in hematopoietic cells,[18,19] in non-hematopoietic cells, the relationship between SYK and focal adhesion molecules remains poorly understood.

Under the 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 methylation is closely associated with its downregulation and that SYK functions as a tumor suppressor by decreasing tumor cell proliferation, invasion, and migration.

MATERIALS AND METHODS
Tissue Samples and DNA Preparation
We retrieved 95 cases of archival tissue samples from patients who underwent curative surgery for HCC at the Seoul National University Hospital (Seoul, Republic of Korea) from 2000 to 2001. Approval from the Clinical Research Institute Seoul National University Hospital Institutional Review Board was obtained for the present study. The tumor portions of the tissue slides were identified by microscopic examination, and the DNA was extracted from these regions. The patients' electronic medical records were also reviewed to obtain clinicopathologic information.

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 (Seoul, Korea). The cell lines were seeded at 3×10^5 cells/ml in their respective culture media, and were treated with $5 \mu M$ 5-aza-2′-deoxycytidine (5-aza-dC; Sigma Chemical Co., St Louis, MO, USA) for 96 h. The media and drugs were replaced every 24 h. As a control, cell lines were mock treated in parallel by 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, USA) according to the manufacturer's protocols. The target cells, Hep3B and Huh7, were first transfected with the pTet-On advanced vector, and then with the Tet-responsive element-based vector containing SYK. The target cells were cultured in DMEM containing 10% tetracycline-free fetal bovine serum, 100 units/ml penicillin, and $100 \mu g/ml$ streptomycin. For the induction of gene expression, the Tet-On inducible cell lines were treated with $1 \mu g/ml$ doxycycline (Dox) for 4 days and for the cessation of overexpression, Dox was removed for 7 days.

GFP-Tagged SYK Expression Vector and Transfection
A GFP-tagged, full-length open reading frame clone of human SYK (as transfection-ready DNA) and its control vector (pCMV6-AC-GFP) were purchased from Origene (Rockville, MD, USA). The transfections of the GFP-tagged SYK expression vector into Huh7 and Hep3B cells were performed using Lipofectamine 2000 (Life Technologies Invitrogen, Carlsbad, CA, USA).

Expression Microarray
We conducted expression microarray analysis on the Illumina HumanHT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA, USA) containing 47 231 probes, according to the manufacturer's protocol. Scanning was performed on the Illumina BeadArray Reader. After image scanning, the GenomeStudio software, 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, USA). To determine the methylation status of the SYK promoter CpG island locus, methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were performed. For BGS, the PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and at least 10 individual clones were sequenced. The primer sequences and PCR conditions are shown in Supplementary Table 1. For the semiquantitative methylation analysis of the SYK promoter CpG island locus, MethyLight analysis was conducted as described previously.[10]

RT-PCR
Total RNA was prepared using an RNeasy kit (Qiagen) according to the manufacturer's protocols. A total of $5 \mu g$ of RNA was reverse transcribed using oligo-dT and SuperScript III Reverse Transcriptase (Life Technologies Invitrogen). Quantitative reverse transcription-PCR (RT-qPCR) amplification reactions were performed using SYBR Green PCR master mix (Life Technologies Applied Biosystems, Foster City, CA, USA). The expression levels of the genes were normalized to the expression of GAPDH. The primer sequences and PCR conditions are shown in Supplementary Table S1.

Western Blot

Whole-cell lysates were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The blots were incubated with anti-SYK and anti-gamma tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After the primary antibodies had been washed off, the blots were incubated with their respective secondary antibodies and protein of interest was detected using ECL plus reagents (GE Healthcare, Waukesha, WI, USA).

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. Antibiotic-resistant colonies were fixed with methanol and stained with a crystal violet solution.

Cell Proliferation Assay

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

Cell Migration and Invasion Assay

The cell migration assay and the invasion assay were performed using Transwell membranes (Corning, Corning, NY, USA) and Matrigel-biocoated invasion chambers (BD biosciences, Bedford, MA, USA), respectively. The cells were trypsinized and suspended in DMEM at a density of 1×10^4 cells/ml. The cell suspension was added into the upper chamber of inserts containing 8-μm pore-size membranes. DMEM containing 10% FBS was placed in the lower chamber. After incubation for 24 h at 37 °C, the cells remaining in the upper chamber were removed carefully with a cotton swab, and the membrane was fixed and stained with 100% methanol and 0.05% crystal violet.

Immunofluorescence

The cells were cultured on glass coverslips and were treated sequentially as follows: 3.7% formaldehyde for 15 min at room temperature (fixation), 0.1% Triton X-100 in PBS for 15 min at room temperature (permeabilization), 0.1% bovine serum albumin (BSA) in PBS for 30 min at room temperature (blocking). The cells were stained against F-actin (Rhodamine-Palloidin, Life Technologies Invitrogen) and with antibodies against Vinculin and Tensin2 (Sigma-Aldrich). The bound primary antibodies were detected using a Texas red-conjugated goat anti-rabbit antibody and an Alexa flour 594-conjugated goat anti-mouse antibody (Invitrogen). The nuclei were counterstained with DAPI. The images were observed and captured using an Olympus confocal microscope.

Cell Adhesion Assay

First, 96-well plates were coated with 20 μg/ml fibronectin at room temperature for 1 h. The plates were then incubated with 1% BSA in PBS for 30 min to block non-specific cell adhesion. Thereafter, 5×10^5 cells were added to each well for 10, 20, and 60 min. Subsequently, non-adherent cells were washed off, and the remaining adherent cells were fixed with 96% ethanol and stained with 0.1% crystal violet. The adherent cells were then solubilized with SDS solution, and the absorbance was measured at 570 nm.

Coimmunoprecipitation

The cells were lysed in lysis buffer (150 mM NaCl, 1.0% Triton X-100, 50 mM Tris-Cl (pH 8.0), protease inhibitor and phosphatase inhibitor cocktail). A total of 300 μg lysate was incubated with anti-SYK antibodies (Santa Cruz) at 4 °C for overnight. Then, 20 μl of protein A agarose beads was added to each lysates, and the bead mixture was incubated at 4 °C under rotary agitation for 6 h. The samples were washed five times with lysis buffer, after which the bound proteins were analyzed by western blotting with anti-SYK, anti-beta-actin, anti-vinculin, or anti-tensin2 antibodies.

Statistical Analysis

The data are expressed as the means ± s.d. Student's *t*-test was used to compare the effects of *SYK* expression on cell proliferation and cell adhesion. For survival analysis, the Kaplan–Meier log rank test was conducted to determine the prognostic significance of *SYK* methylation. Survival was measured from the date of resection of HCC to the date of death or the last clinical review before 30 November 2011. The statistical analyses were conducted with the SPSS software (version 15.0).

RESULTS

### *SYK* Methylation is a Prognostic Marker of HCC

From the 99 surgically resected cases of hepatitis B virus-associated HCC which had been studied for the association between *SYK* methylation and poor overall survival,[10] we could follow-up 95 cases in which *SYK* methylation, assessed by the use of the MethyLight assay, was found in 37.9%. The demographic and clinical features of the HCC patients are summarized in Table 1. The Kaplan–Meier log-rank test revealed that *SYK* methylation was significantly associated with a lower overall survival rate in HCC patients (Figure 1). Additionally, multivariate analysis revealed that SYK methylation was an independent prognostic parameter (hazard ratio, 1.720; 95% CI, 1.019–2.904; *P* = 0.037) (Supplementary Table 2).

Correlation of *SYK* Expression with Methylation Status

We performed bisulfite sequencing and MSP and found that *SYK* was methylated in SNU-761, HepG2, Hep3B, and Huh7 cells, which also had no detectable *SYK* expression. On the other hand, *SYK* was not methylated in SNU-739, SNU-878, and SNU-886 cells and non-neoplastic liver tissue samples, all of which had endogenous *SYK* expression (Figures 2a and b). We performed semiquantitative analyses of SYK

Table 1 Patient characteristics and SYK methylation status

	HCC with SYK methylation (n = 36)	HCC without SYK methylation (n = 59)	P-value
Age			
Median (range) (year)	55 (21–76)	53 (32–73)	0.631
Sex			
Male	26 (72.2%)	50 (84.7%)	0.187
Female	10 (27.8%)	9 (15.3%)	
Size			
Median (range) (cm)	6.5 (2.0–14.0)	5.0 (0.5–18.0)	0.533
AJCC stage			
I, II	29 (80.6%)	48 (81.4%)	1.000
III	7 (19.4%)	11 (8.6%)	
AFP			
Average (95% CI) (ng/ml)	31 (5–151 000)	153 (5–70 000)	0.738
Background liver			
Chronic hepatitis	8 (22.2%)	23 (39.0%)	0.116
Cirrhosis	28 (77.8%)	36 (61.0%)	
Gamma GT			0.494
Average (95% CI) (IU/l)	81.4 (44.9–118.0)	95.8 (71.4–120.2)	
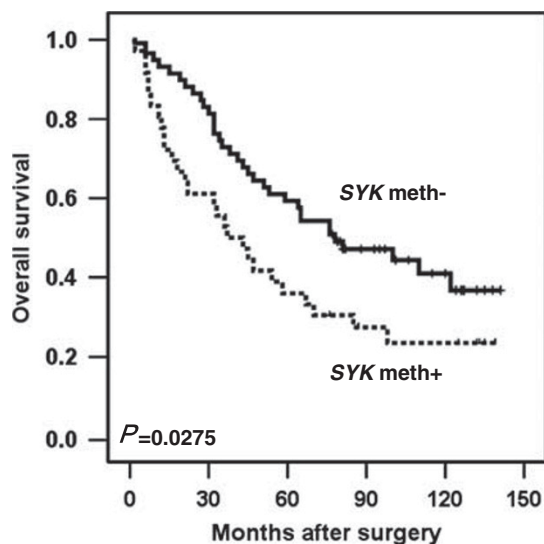
Figure 1 Shorter overall survival of HCC patients with SYK methylation. The overall survival was assessed using the Kaplan–Meier log-rank test.

methylation in five pairs of HCC and adjacent liver tissue sample using MethyLight, which demonstrated no or negligible methylation of SYK promoter CpG island locus in non-neoplastic liver tissue samples in contrast with high level of

SYK methylation in three of five HCC tissue samples (Supplementary Figure 1). Furthermore, we observed an increase in *SYK* expression in SNU-761 and Huh7 cells after 5-aza-dC treatment (Figure 2c). This finding indicates tight association of *SYK* promoter hypermethylation with its transcriptional silencing in HCC cell lines.

Gene Expression Profiling and Ontology Analysis of SYK-Dependent Genes in HCC Cells

We generated Hep3B and Huh7 cell lines with the Tet-On inducible gene expression system, in which the expression of *SYK* could be induced by incubation with Dox (Supplementary Figure 2).

The expression profiles were then analyzed with an Illumina HumanHT-12 v4 Expression BeadChip. Among the 47 231 probes analyzed, 47 genes were found to be significantly regulated ($\geqslant$ 1.5-fold, P-value < 0.05) by SYK induction in both cell lines (Figures 3a and b). Supplementary Table 3 summarizes the functions of the genes showing expression level changes with SYK induction in both cell lines. To understand the roles of SYK loss during cancer progression in HCC cells, we performed a Gene Ontology analysis of the 47 SYK-dependent genes. The induction of *SYK* expression in Hep3B and Huh7 cells was significantly associated with the enrichment of gene set concepts, including cell adhesion, enzyme-linked receptor protein signaling pathway, and protein localization (Figure 3c).

Expression of *SYK* Suppresses Cell Growth

To investigate the effects of *SYK* expression on cell growth in HCC cells, SYK was induced in Huh7 cells by Dox treatment. The suppressive effect on cancer cell growth was demonstrated by a colony formation assay (Figure 4a). An MTT assay confirmed that cell proliferation was significantly reduced in clones with induction of *SYK* expression, compared with the non-induced control clones (Figure 4b). However, SYK did not suppress cellular proliferation in Hep3B cells in either colony formation assay or MTT assay (Supplementary Figure 3a–d). SYK inhibited cellular proliferation in the transiently transfected HepG2 cell line, compared with the vector control (Supplementary Figure 3e). Recently, checkpoint kinase 1 (CHK1) has been demonstrated to phosphorylate SYK at Ser295, which promotes its subsequent proteasomal degradation.[20] To confirm the anti-proliferative activity of SYK, we treated the Tet-On inducible Huh7 cells with the CHK1 inhibitor, GÖ6976 which protected SYK against degradation by CHK1. Two individual clones of Tet-On inducible Huh7 cells were treated with GÖ6976 and assessed for cell proliferation by MTT assay (Supplementary Figure 4). The cells with *SYK* expression and GÖ6976 treatment displayed a lower cell proliferation rate than cells with *SYK* expression and no treatment with GÖ6976.

Effect of SYK on Cell Adhesion, Migration, and Invasion

Cell migration and invasion assays showed that the cells with SYK induction had suppressed cell migration and invasion
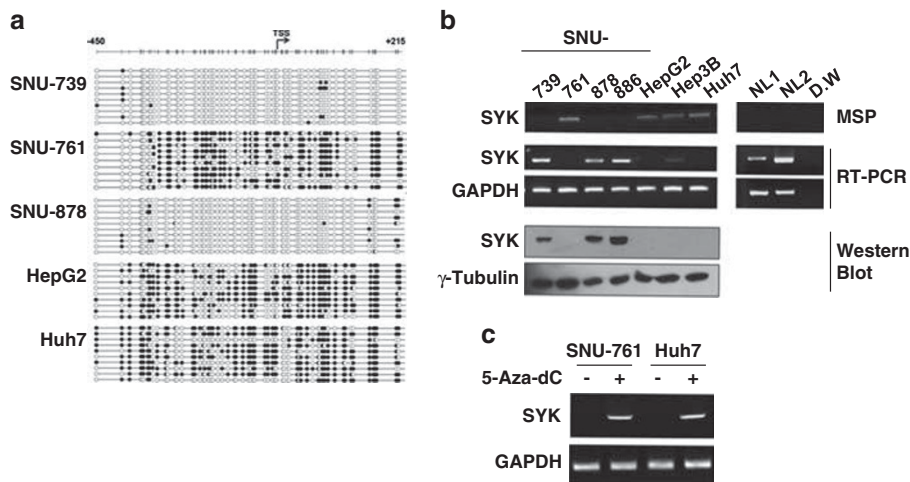
Figure 2 *SYK* promoter methylation and expression in HCC cells. (**a**) Bisulfite sequencing of the SYK promoter region in five HCC cell lines. The vertical lines indicate individual CpG sites. The cloned PCR products were sequenced, and each clone is shown as an individual row representing a single allele of the promoter region. The 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 and western blotting in seven HCC cell lines. (**c**) The RT-PCR results of SYK expression following the addition of 5-Aza-dC in the methylated HCC cell lines, SNU-761 and Huh7, are shown. The cells were either PBS treated or treated with 5-aza-dC (5 μM) for 96 h, as indicated.
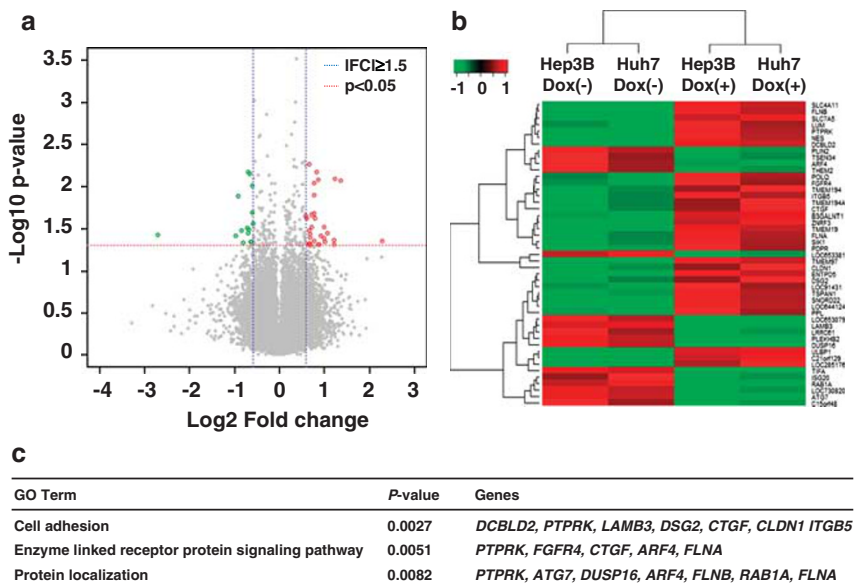

GO Term	*P*-value	Genes
Cell adhesion	0.0027	*DCBLD2, PTPRK, LAMB3, DSG2, CTGF, CLDN1 ITGB5*
Enzyme linked receptor protein signaling pathway	0.0051	*PTPRK, FGFR4, CTGF, ARF4, FLNA*
Protein localization	0.0082	*PTPRK, ATG7, DUSP16, ARF4, FLNB, RAB1A, FLNA*

Figure 3 Gene expression profiling. (**a**) Volcano plots visualize differential gene expression analysis. Red and green dots indicate SYK-regulated genes ($n = 47$) of which expression was >1.5-fold increased and decreased, respectively, after SYK induction in both Hep3B and Huh7 cells and the difference was statistically significant (P-value <0.05, Student's t-test). (**b**) Heat map displays expression changes in a cluster of 47 genes with SYK induction ($-$, no treatment of Dox; $+$, treatment of Dox). Green to red indicates down to up regulation after Dox treatment. (**c**) Top three biological functions of GO term associated with 47 genes regulated by SYK induction.

compared with the cells with no SYK induction (Figures 4c and d). To identify the role of SYK in cell adhesion, cell attachment assays were conducted in 96-well, flat-bottom microtiter plates that were uncoated or coated with fibronectin. Hep3B and Huh7 cells cultured on uncoated plates showed no attachment, regardless of *SYK* expression status. On coated plates, Hep3B and Huh7 cells showed increased attachment to the plate. However, Hep3B cells exhibited further increased attachment with SYK induction, which was not observed in Huh7 cells (Figure 5a).
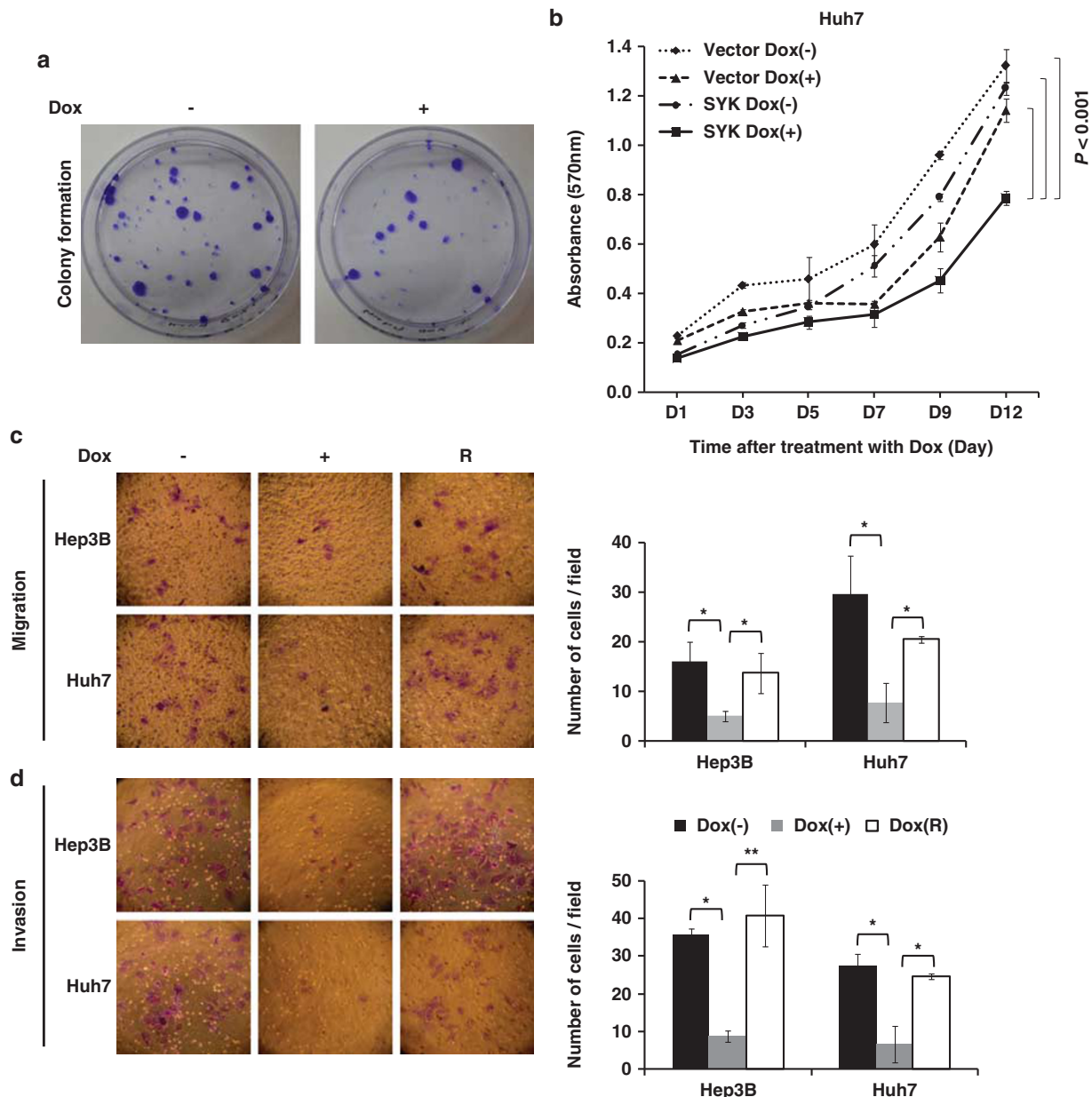
Figure 4 Suppressive effect of SYK on tumor cell growth, migration, and invasion. (**a**) A colony formation assay demonstrating the reduction in the number of colonies in SYK-induced Huh7 cells compared with control cells. (**b**) Huh7 cells were stably transfected with the indicated plasmid, and the induction of SYK was controlled by treatment with Dox. The proliferative capacity of these cells was measured by the MTT assay on the indicated days. Cell growth is expressed as the absorbance at the wavelength of 570 nm. (**c**) Cell migration and (**d**) invasion assays were performed in SYK-inducible Huh7 cells. Representative images (*Left*). Graphs indicate the average number of cells per field (*Right*). The expression of *SYK* decreased cell migration and invasion. −, no treatment of Dox; +, treatment of Dox for 4 days; R, after removal of Dox for 7 days. The experiments were performed in triplicate, and the values indicate means ± s.d. *, $P < 0.05$; **, $P < 0.001$.

Additionally, we found that SYK-induced cells had increased cell-to-matrix and cell-to-cell adhesion on fibronectin-coated coverslips (Figure 5b).

Redistribution of SYK Following Adhesion to Fibronectin
In bronchial epithelial cells, the stimulation of β1-integrin receptors by fibronectin or antibody crosslinking has been demonstrated to promote the redistribution of SYK from the cytoplasm to the plasma membrane.[13] To identify whether the localization of SYK was altered in HCC cells due to the engagement of β1-integrins with fibronectin, SYK-tGFP-transfected Hep3B and Huh7 cells were plated on fibronectin-coated surfaces and subsequently examined by confocal microscopy. After 1 h, the adhesion to fibronectin
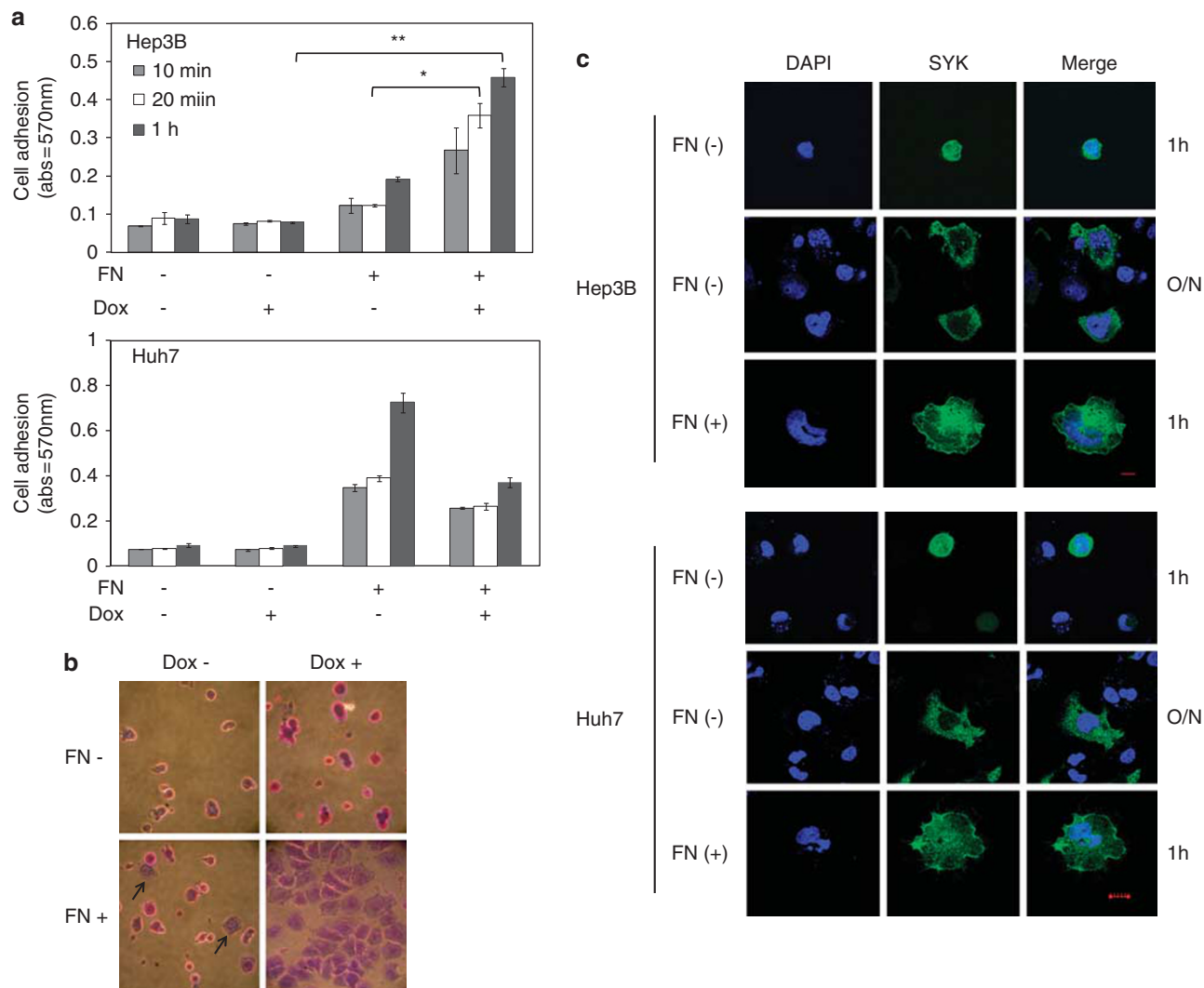
Figure 5 Effect of SYK on the adhesion of HCC cells to fibronectin. (**a**) SYK-inducible Hep3B cells were tested for their adhesion to 96-well plate coated with or without fibronectin at the indicated times. The experiments were performed in triplicate, and the values indicate the means ± s.d. *, $P < 0.05$; **, $P < 0.001$. (**b**) The *in vitro* morphology of SYK-induced cells following adhesion to fibronectin. The cells were plated on uncoated coverslips (FN −) or fibronectin-coated coverslips (FN +) for 30 min. Subsequently, the non-adherent cells were washed off, and the remaining adherent cells were fixed with 96% ethanol and stained with 0.1% crystal violet. SYK-induced cells showed increased cell-to-matrix adhesion and cell-to-cell adhesion on fibronectin-coated coverslips. (**c**) Huh7 and Hep3B cells were transiently transfected with tGFP-tagged SYK. The cells were plated on fibronectin- or un-coated glass coverslips for 1 h or overnight (O/N), fixed, and counterstained with DAPI nuclear acid stain. Scale bar represents 10 μm.

induced visible cytoplasmic spreading of both Hep3B and Huh7 cells on coverslips. However, after 1 h, the cells plated on non-coated coverslips were smaller and more rounded (Figure 5c). SYK was distributed in both the cytoplasmic and the nuclear areas. After adhesion to fibronectin, SYK was redistributed with some localization along the plasma membrane in both cell lines. Additionally, the nuclear localization of SYK was retained in the nucleus in Huh7 cells but not in Hep3B cells (Supplementary Figure 5). In contrast, in cells plated and incubated overnight on non-coated coverslips, SYK was distributed in the cytoplasm, without localization along the plasma membrane or to the nucleus (Figure 5c). These results indicate that the redistribution of SYK was induced by the engagement of β1-integrins by fibronectin. Although SYK phosphorylation on Tyr525/526 has been demonstrated to be also induced by stimulation with fibronectin in airway epithelial cells,[13] we could not find induction of SYK phosphorylation at Tyr525/526 after adhesion of HCC cells to fibronectin (Supplementary Figure 6). Other tyrosine sites might be activated by stimulation with fibronectin in HCC cells.

SYK Colocalizes with Cytoskeleton and Focal Adhesion Molecules

To explore the possible association between SYK and focal adhesion molecules, we first examined the localization of SYK and the endogenous adhesion molecules, tensin2 and vinculin, by immunostaining (Figures 6b and c). Tensin2 and vinculin were found to be colocalized with tGFP-tagged SYK. Additionally, we found that tGFP-tagged SYK also colocalized with F-actin, which was stained with phalloidin conjugates, within the plasma membrane (Figure 6a). Vinculin and actin were detected in anti-SYK immunoprecipitates from fibronectin-engaged, Tet-On inducible Huh7 cells, whereas tensin2 was not detected (Figure 6d). These data suggest that in HCC cells, SYK can localize to focal adhesions with adhesion molecules and affect the actin cytoskeletal network. However, FAK did not coimmunoprecipitate with SYK (data not shown).

DISCUSSION

SYK is a putative tumor- and metastasis-suppressor gene that has been recently found to be inactivated through promoter CpG island hypermethylation in several types of epithelial cell malignancies.[8–11] In the current study, we analyzed the methylation status of the *SYK* promoter CpG island locus and its mRNA/protein expression in HCC cell lines and found an inverse correlation between promoter CpG island hypermethylation and *SYK* expression. The treatment of *SYK*-methylated HCC cell lines with a demethylating agent restored *SYK* expression. Although cancer-associated CpG island hypermethylation tends to occur in inactive genes in normal cells,[21] *SYK* methylation develops despite its active expression in normal hepatocytes.[22] Considering that previous studies and the present study have indicated that *SYK* promoter CpG island hypermethylation is closely associated with poorer prognosis in HCC patients,[10,23] it could be hypothesized that the reduced expression of *SYK* by promoter CpG island hypermethylation may be related to the biological aggressiveness of HCC.

In the present study, SYK suppressed the growth of Huh7 and HepG2 cells, but not Hep3B cells. The anti-growth activity of SYK was augmented by treatment with GÖ6976, an inhibitor of CHK1, which stabilizes SYK. The combined effect of SYK induction and CHK1 inhibition on the suppression of tumor cell growth was remarkable compared with that obtained with SYK induction or CHK1 inhibition alone (Supplementary Figure 4). Considering that induced SYK shows differential anti-growth activity depending on the cell type, it can be speculated that the anti-growth activity of SYK is context specific. 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 the induction of *SYK* expression. However, we did not find a difference in cell-cycle distribution, specifically the sub-G1 fraction (Supplementary Figure 7). This is consistent with the results of a study by Coopman *et al*,[3] in which SYK blocked breast tumor cell growth *in vitro* but no differences in cell
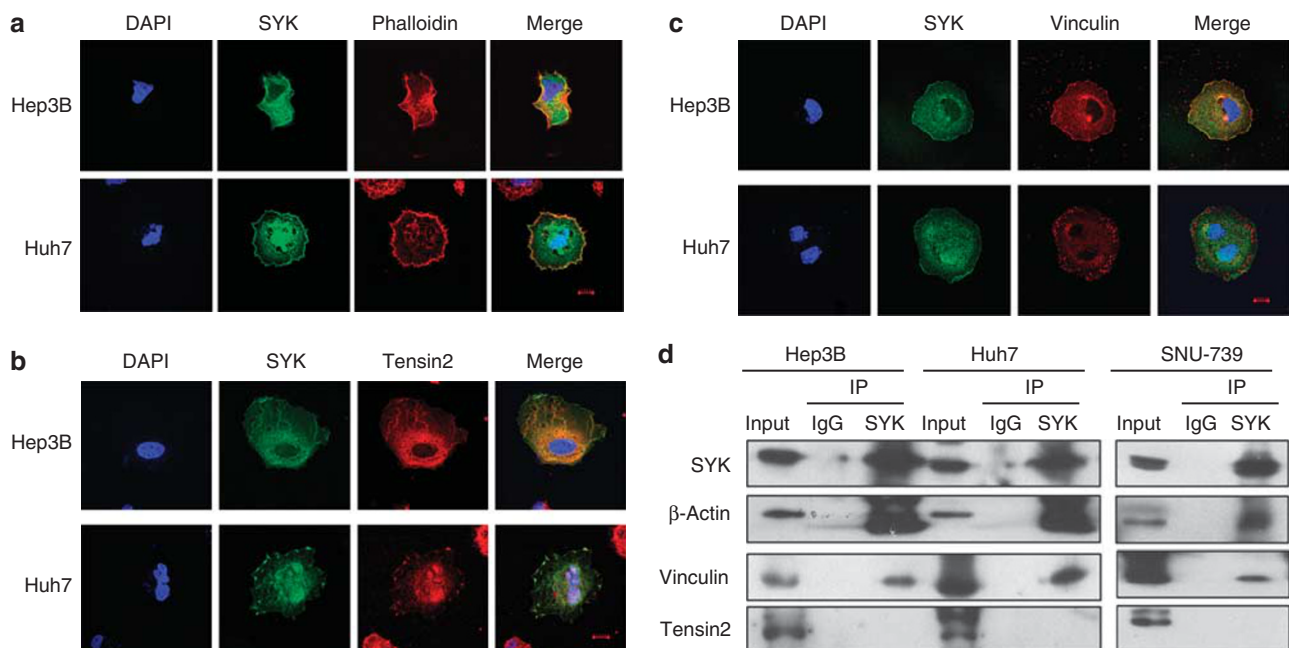

Figure 6 Association of SYK with cytoskeleton and adhesion molecules. Hep3B and Huh7 cells transiently expressing SYK-tGFP were attached on fibronectin-coated coverslip for 1 h, fixed, permeabilized, and stained with Rhodamine Phalloidin (**a**), anti-tensin2 (**b**), and anti-vinculin (**c**). Scale bars represent 10 μm. (**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, and anti-tensin2 antibodies.

proliferation and apoptosis were found between breast cancer cells with and without SYK. However, abnormal mitoses with multipolar spindles were found to be significantly increased with *SYK* expression,[3,24,25] which suggests that SYK regulates cell proliferation by controlling mitosis and cytokinesis.[24] However, we did not find an increase in abnormal mitoses in association with SYK induction. Another mechanism that SYK mediates its effects may be via the regulation of genes that are involved in cell-cycle progression.[14] SYK has been shown to downregulate the transcription of cell-cycle progressive genes, such as *CCND1*, *CCNA1*, *AKT1*, and *FOSL1*.[5,6,26] On the other hand, the re-expression of SYK has been demonstrated to induce a TP53-dependent accumulation of CDKN1A and a senescence-like growth arrest in melanoma cells.[6] However, we did not find significant differences in mRNA levels of *CCND1* and *CDKN1A* in Huh7 cells with and without SYK induction (data not shown). Our present data show that the re-expression of SYK suppresses cell growth. However, the exact mechanism by which SYK affects cell growth in HCC cells remains to be identified.

In contrast with the differential anti-growth activity of SYK, *SYK* expression increased cell to matrix attachment and decreased cell migration and invasion in both Huh7 and Hep3B cells. The induction of *SYK* expression led to cytoplasmic spreading, while the exposure to fibronectin prompted cytoplasmic spreading and localization of SYK along the cytoplasmic border. SYK was colocalized with actin, vinculin, and tensin2 at the cytoplasmic border, although immunoprecipitation assays did not prove an interaction between SYK and tensin2. Furthermore, SYK expression led to an increase in *CDH1* expression, which has been reported to strengthen 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.[8,12] In the present study, *SYK* methylation was closely associated with shortened survival time in HCC patients, which is consistent with the results of previous studies demonstrating shortened survival time in HCC patients with *SYK* methylation.

Tumor invasion and metastasis are complicated procedures that require cancer cells to interact with endothelial cells and the extracellular matrix. Integrins are the major cell surface receptors that mediate these interactions, and activity and expression of β1-integrins are associated with the invasive ability of HCC cells.[27] Several reports have shown that in airway epithelial cells and breast cancer cells, SYK is phosphorylated at several tyrosine residues, including Y342, and activated through β1-integrins signaling.[13,15] β1-integrins are highly expressed in HCC cell lines, including Hep3B and Huh7. Our results demonstrated that the redistribution of SYK from the cytoplasm to the plasma membrane was induced by the engagement of β1-integrins with fibronectin. Additionally, the adhesion and spreading of HCC cells occurred rapidly and widely on fibronectin-coated glass coverslips, compared un-coated glass coverslips. 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 suppressor of metastasis in HCC.

In conclusion, SYK promoter methylation is closely associated with its downregulation in HCC cells. The findings of the present study support the hypothesis that SYK functions as a tumor suppressor in HCC, specifically, by demonstrating the anti-proliferative and pro-adhesive activities of SYK. Restoring SYK expression in SYK-silenced HCC cell lines decreased cell growth, but increased cell adhesion. Furthermore, the expression of SYK decreased cell migration and invasion via interactions with adhesion molecules. Our findings suggest that SYK is involved in cell-to-matrix adhesion, and that SYK loss is implicated in the regulation of cell proliferation, migration, and invasion of HCC cells. Further studies are required to define the exact mechanisms by which SYK regulates the proliferation of HCC cells.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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