Frequent Epigenetic Inactivation of *SYK* Gene in Human Hepatocellular Carcinoma: An Independent Prognostic Marker[*]

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Abstract

Purpose—The aim of present study was to investigate the methylation and expression status of *spleen tyrosine kinase* (*SYK*) in human hepatocellular carcinoma (HCC) and to evaluate this information for its ability to predict disease prognosis. *E-cadherin* and *TIMP-3 methylation* was also analyzed here as control since both were associated with poor prognosis in some types of tumors.

Experimental Design—We analyzed the methylation status of *SYK*, *E-cadherin* and *TIMP-3* in 124 cases of HCC and assessed the correlation of such methylations with clinicopathological parameters and prognosis after tumor resection.

Results—We found that *SYK, E-cadherin,* and *TIMP-3* genes were methylated in 27%, 27%, and 42% of HCC neoplastic tissues, respectively. The loss of *SYK* mRNA or Syk protein expression was highly correlated with *SYK* gene methylation. The patients with methylated *SYK* in neoplastic tissues had a significantly lower overall survival rate after hepatectomy than those with unmethylated *SYK*. No significant difference in overall survival rates, however, was found between groups of patients with methylated and unmethylated *E-cadherin* or *TIMP-3*. Patients with negative Syk protein expression had a significantly lower overall survival rate than those with positive Syk protein expression. Multivariate analyses indicated that factors affecting overall survival were TNM stage, Child-Pugh classification, *SYK* methylation or Syk protein status.

Conclusions—Our results indicate that *SYK* methylation and loss of Syk expression in HCC neoplastic tissues are independent biomarkers of poor patient outcome and that determination of *SYK* methylation or Syk expression status may offer guidance for selecting appropriate treatments.

Keywords

DNA methylation; spleen tyrosine kinase; E-cadherin; TIMP-3; hepatocellular carcinoma; prognosis

Abbreviations

5-aza-dc, 5-aza-2'-deoxycytidine; DNMT, DNA methyltransferase; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; MSP, methylation-specific PCR; RT-PCR, reverse transcriptase-PCR; SYK, spleen tyrosine kinase; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; GGT, gamma-glutamyltransferase; AFP, α-fetoprotein

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death in the world (1). China has one of the highest prevalence of HCC, largely because carriers of chronic hepatitis B account for more than 10% of its population (2). Although the incidence of HCC in the United States is relatively lower, the reported new cases have been increasing steadily (3). The prognosis for patients with HCC is generally poor, even after surgery or chemotherapy. The 5-year overall survival rate is between 35% and 41% after resection of primary tumors (4,5) and between 47% and 61% after liver transplantation (6). Systemic chemotherapy gives a low response rate of only 10% to 20% and has shown no significant benefit with regard to overall survival (7). Given this poor therapeutic efficacy, the development of biomarkers for early detection and accurate prognosis of HCC is crucial for prescribing the most timely and effective treatment.

Although the etiology of HCC remains unclear, chronic infection with hepatitis B or C virus, chemical carcinogens (aflatoxins), and other environmental and host factors have been linked to hepatocarcinogenesis (8,9). In China, most cases of HCC develop from liver cirrhosis with chronic infection of hepatitis B virus and/or chronic exposure to aflatoxin B1. In western countries, however, chronic alcoholism and chronic infection with hepatitis C virus are the major etiological factors. These various factors are believed to induce a spectrum of molecular alterations that contribute to the initiation and progression of HCC, including the genetic and epigenetic inactivation of tumor suppressor genes (8,9). Similar to what has been shown in other tumor types, DNA methylation frequently occurs in HCC, represented by *p16, p15, GSTP, E-cadherin, TIMP-3, APC, SOCS-1, RASSF1A,* and *14-3-3δ* (10–14). The prognostic value of methylation of these genes in HCC was either not systematically studied or was found not important in HCC.

The *spleen tyrosine kinase (SYK)* is a tumor/metastasis suppressor gene recently found to be silenced through DNA methylation in breast cancer (15) and T-lineage acute lymphoblastic leukemia (16). Loss of *SYK* expression has been implicated in increased invasiveness and proliferation of breast tumors (17). Concordantly, over-expression of *SYK* was shown to inhibit the invasiveness, proliferation, and motility of breast cancer cells (17–20). *SYK* was regarded as a novel regulator of metastatic behavior of melanoma cells (21). Decreased *SYK* expression in primary breast tumors was shown to predict shorter survival among cancer patients (22). Given that *SYK* methylation is primarily responsible for the loss of *SYK* expression, aberrant *SYK* promoter hypermethylation may serve as a valuable prognostic marker.

In this study, we correlated epigenetic alterations of *SYK* with clinical and pathological parameters to determine its prognostic value in HCC. Because methylation of *E-cadherin* and *TIMP-3* have been shown to be associated with poor prognosis in gastric and esophageal cancer (23,24), respectively, we also analyzed the *E-cadherin* and *TIMP-3* methylation status in parallel to compare their prognostic value with that of *SYK* methylation.

PATIENTS AND METHODS

Cell Lines

Liver cancer cell lines HepG2 and Hep3B were purchased from the American Type Culture Collection (Manassas, VA) and maintained in recommended culture conditions. Cells were maintained at 37°C in a humidified environment containing 5% CO_2.

Study Population and Tissue Samples

One hundred and twenty-four patients who were consecutively diagnosed with HCC and had undergone hepatectomy from 1998 to 2001 in a single group at the Department of Hepatobiliary Oncology, Sun Yat-sen University Cancer Center were enrolled in the study. Tissue samples, including 124 samples from primary tumors and 34 samples from matched adjacent non-neoplastic liver tissues, were archived in the institution's liver tumor bank and stored at −80° C until use. All non-neoplastic and neoplastic samples were histologically confirmed. Neither chemotherapy nor radiation therapy was given before tumor excision. The tumor stages of HCC were classified according to the tumor-node-metastasis (TNM) criteria (25). The degree of underlying cirrhosis was graded based on the size of gross cirrhotic nodules and histological examination: (1) no cirrhosis: the liver was soft and smooth with no cirrhotic nodules. No pseudolobule formation was found microscopically; (2) mild cirrhosis: the largest nodule on liver surface was <0.4 cm, or cirrhosis was identified by microscopic examination; (3) moderate cirrhosis: the degree of cirrhosis was between mild and severe cirrhosis; (4) severe cirrhosis: the largest cirrhotic nodule on liver surface was >0.8 cm, or the liver was notably deformed and complicated by portal hypertension. The study protocol was approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer Center.

Methylation-specific Polymerase Chain Reaction

A blinded methylation-specific polymerase chain reaction (MSP) analysis was carried out; no clinicopathological or follow-up data were revealed to the bench researchers until the MSP results were finalized. Genomic DNA was isolated from frozen tissue by digestion with proteinase K, followed by standard phenol/chloroform extraction and ethanol precipitation. Sodium bisulfite (Sigma, St. Louis, MO)–induced conversion of genomic DNA was performed as described previously (15). The modified DNA was subjected to a two-step MSP protocol to determine the methylation status of *SYK, E-cadherin,* and *TIMP-3* promoter regions (15, 26,27). Primers were designed to distinguish between bisulfite-sensitive and -resistant modifications of unmethylated and methylated cytosines, respectively. For the first-round MSP, a 30-μl reaction that contained 30 ng of bisulfite-treated DNA was processed in 40 thermal cycles. An aliquot (2 μl) of diluted (1:40) PCR product was subjected to the second-round PCR in another 30-μl reaction. For *SYK* gene, both methylation and unmethylation primers were included in the same reaction. For *E-cadherin* and *TIMP-3*, separate reactions for methylation and unmethylation detection were carried out. The primer sequences, PCR condition, and product sizes for each gene are listed in Table 1. To prepare the positive methylation control, 1 μg of genomic DNA from normal human liver was treated *in vitro* with *Sss*I methyltransferase (NEB, Beverly, MA), yielding completely methylated DNA at all CpG-rich regions. Untreated genomic DNA was used as negative control. For positive (S*ss*I-treated) or negative (S*ss*I-non-treated) controls, 1 μg DNA each was modified by sodium bisulfite. Thirty ng of bisulfite-treated control DNA template underwent nested PCR amplication side-by-side with testing specimens. H_2O was also used as negative control in nested MSP. The PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Reverse Transcriptase-PCR

Reverse transcriptase-PCR (RT-PCR) was performed as described previously (15). Briefly, total RNA was prepared using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using 18-mer oligo(dT) and Superscript II (Invitrogen, Carlsbad, CA). Reactions lacking RT were used to verify the absence of amplification from genomic DNA contamination. The cDNA templates were subjected to PCR amplification. To verify cDNA integrity, β_2-microglobulin expression was analyzed as well. The primer sets were 5'-TGTCAAGGATAAGAACATCATAG-3' (forward) and 5'-CACCACGTCATAGTAGTAATTG-3' (reverse) for *SYK*, and 5'-ACCCCCACTGAAAAAGATGA-3' (forward) and 5'-GCATCTTCAAACCTCCATGAT-3' (reverse) for β_2-microglobulin.

In some experiments, cells were treated for 5 days with a DNA methyltransferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC; Sigma) at a final concentration of 2.0 μM. Cells were then collected for RNA extraction.

Immunohistochemical Assay

Formalin-fixed paraffin-embedded sections of HCC tumors and adjacent non-neoplastic liver tissues were subjected to immunostaining with an antibody against Syk using the rabbit EnVision Plus kit (DakoCytomation, Carpinteria, CA). Briefly, 5-pm thick tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval by boiling in sodium citrate buffer (10 mM, pH 6.0). The sections were incubated at 4°C overnight with Syk primary antibody (1:200 dilution; Cell Signaling, Beverly, MA) and then stained with 3,3'-diaminobenzidine. After visualization of immunoreactivity, the sections were counterstained with hematoxylin, and mounted. The immunostained sections were evaluated without any knowledge of the patients' clinical information and status of MSP and RT-PCR of *SYK*. Normal liver tissues were taken as internal positive controls. The stains were graded as: (*a*) positive when immunoreactivity equivalent to that seen in normal liver cells or immunoreactivity moderately decreased; and (*b*) negative when weak immunoreactivity or no immunoreactivity.

Statistical Analysis

All clinicopathological and follow-up data were collected in a database. Overall survival times were measured from the date of resection of primary tumors to the date of death or of the last follow-up. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. The prognostic factors for survival after hepatectomy were elucidated by univariate and then multivariate analyses. The following variables were analyzed: patient sex, age, Child-Pugh classification, GGT (gamma-glutamyltransferase) level, AFP(α-fetoprotein) level, tumor size, tumor encapsulation status, presence of macro-tumor thrombus in the portal vein, presence of satellite nodules, degree of underlying cirrhosis, TNM stage, expression status of Syk protein and methylation status of *SYK, E-cadherin,* and *TIMP-3* genes in tumor tissues. Significant prognostic factors found by univariate analysis were entered into a multivariate analysis using the Cox proportional-hazards model. The SPSS software package (Version 10.0; SPSS Inc., Chicago, IL) was used for the statistical analyses. $P < 0.05$ was considered to be statistically significant.

RESULTS

Promoter Hypermethylation Leads to *SYK* Silencing in HCC

SYK is expressed in many epithelial cell types. We began our study by analyzing *SYK* expression status in two liver cancer cell lines, HepG2 and Hep3B. RT-PCR showed that Hep3B but not HepG2 cells expressed *SYK* mRNA (Figure 1A). Since DNA methylation is

primarily responsible for *SYK* gene silencing (15), we surmised that the *SYK* gene promoter might be methylated in HepG2 cells. To explore this possibility, we used MSP to measure both methylated and unmethylated *SYK* promoter (15). MSP analyses indicated that *SYK* was methylated in HepG2 but not in Hep3B (Figure 2A), consistent with the *SYK* expression status in these cells. To further substantiate that *SYK* methylation is primarily responsible for the loss of *SYK* expression, we treated HepG2 cells with a DNMT inhibitor, 5-aza-dc, to determine whether demethylation restored *SYK* expression. As shown in Figure 1B, 5-aza-dc reactivated *SYK* expression in the HepG2 line as detected by RT-PCR while not affecting that in Hep3B, suggesting that DNA methylation plays a causal role in the *SYK* loss of expression in HCC.

SYK Is Hypermethylated in Primary HCC

We next examined whether the epigenetic alteration of *SYK* observed in the HCC cell lines could be extrapolated to primary HCCs. All 124 patients included in the present study underwent surgical resection of primary tumors. The pathological diagnosis of all HCC cases was confirmed by histological reviews. We used MSP to evaluate the *SYK* methylation status in the 124 primary HCC tumors, in which 27 (21.8%) and 90 (72.6%) specimens were found to be *SYK* methylated and unmethylated, respectively. The remaining 7 cases (5.6%) showed amplification of both *SYK* methylation and unmethylation (Figure 2B). Coexistence of both methyaltion status in a given tumor could reflect the heterogeneity of HCC, although contamination from normal tissue DNA cannot be ruled out.

To ascertain whether *SYK* methylation leads to gene silencing in primary HCC, we used immunohistochemistry to assess the Syk protein expression in all 124 tumors (Figure 3). Immunohistochemical analyses showed that Syk protein was not expressed in 32 (25.8%) HCC cases; in this group, *SYK* was methylated, methylated/unmethylated, and unmethylated in 24, 5, and 3 cases, respectively. Among the remaining 92 (74.2%) Syk protein-positive cases, *SYK* was methylated, methylated/unmethylated, and unmethylated in 3, 2, and 87 cases, respectively. The correlation between *SYK* methylation and loss of Syk protein expression was highly significant ($P < 0.001$, Spearman test). The 3 cases in which Syk was expressed but methylated may reflect the heterogeneity of HCC; that is, methylation may occur in a subpopulation of neoplastic tissues that is readily detectable by MSP (31). The 3 cases in which Syk was not expressed but unmethylated suggest that there are other mechanisms to suppress SYK expression.

We also measured the *SYK* methylation and expression status in matched normal liver tissues. Among the 124 cases, 34 had samples of matched adjacent pathologically non-neoplastic liver tissues that were used for MSP, RT-PCR and immunohistochemical analyses. *SYK* gene was found methylated, methylated/unmethylated, and unmethylated in 0, 3 and 31 in non-neoplastic specimens, respectively, in comparison with 6, 2 and 26 cases of neoplastic tissues, respectively. If the "methylation/unmethylation" was grouped into the "methylation-positive" category, the percentage of patients with positive methylation were 8.8% (3/34) and 23.5% (8/34), respectively, for neoplastic and non-neoplastic tissues. The difference in percentage of methylation-positive patients was not statistically significant ($P = 0.186$, Fisher test). The statistical significance could be reached if more samples were available. *SYK* methylation in non-neoplastic tissues was also observed in earlier studies that may represent DNA methylation in pre-malignant lesions (28,29). Aging-related gene methylation could also be a contributor (30). The corresponding primary tumors of these 3 cases were found to have unmethylated *SYK*.

The expression of *SYK* mRNA as measured by RT-PCR and Syk protein by immunohistochemistry in the 34 cases was entirely consistent, indicating the SYK expressional control occurs at the transcriptional level. Both *SYK* mRNA and Syk protein were positive in all 34 matching non-neoplastic liver tissues. By contrast, 5 of the 34 primary HCCs expressed

neither *SYK* mRNA nor Syk protein. Among the 29 SYK-positive HCCs, *SYK* was found methylated, methylated/unmethylated, and unmethylated in 1, 2, and 26 specimens, respectively. These numbers were in comparison with 5, 0, and 0 SYK-negative cases, respectively. Using Spearman correlation test, *SYK* methylation and SYK expression was strongly correlated ($P < 0.001$). Collectively, these results indicated that hypermethylation of *SYK* promoter was largely tumor-specific and responsible for the loss of SYK expression in HCC.

Like *SYK*, *E-cadherin* and *TIMP-3* are thought to be tumor/metastasis suppressor genes. DNA methylation could lead to silencing of *E-cadherin* (27,32) and *TIMP-3* (13,29,33) in certain types of tumors, including HCC. Thus we also assessed the methylation status of *E-cadherin* and *TIMP-3* genes in the 124 HCC cases. We found that *E-cadherin* was methylated, methylated/unmethylated, and unmethylated in 21, 9, and 82 cases, respectively (non-informative in 12 cases). *TIMP-3* was methylated, methylated/unmethylated, and unmethylated in 32, 14, and 64 cases, respectively (non-informative in 14 cases) (Figure 4). Gene methylation is believed to be an aberrant alteration that is associated with neoplastic progression; amplification from unmethylation allele is likely contributed by common contaminant of normal tissues. Thus we classified cases with both methylation and unmethylation amplication into methylation-positive group in this study for clinical correlation analyses and prognostic evaluation. When this criteria is adopted, the percentage of patients with positive *SYK* methylation, *E-cadherin* and *TIMP-3* gene became 27.4% (34/124), 26.8% (30/112) and 41.8% (46/110), respectively.

Correlation of Gene Methylation with Clinicopathological Parameters

We next correlated the methylation status of *SYK, E-cadherin,* and *TIMP-3* with 12 clinicopathological parameters, including patient gender, age, Hepatitis B infection status, Child-Pugh classification, GGT and AFP values, tumor size, status of macro tumor thrombus in the portal vein, satellite nodule, tumor capsule, degree of underlying cirrhosis, and TNM stage (Table 2). The patient age ranged from 23 to 76 years, with a median age of 48 years. Fourteen (11.3%) of the patients were women and 110 (88.7%) were men. Hepatitis B surface antigen (HbsAg) was detected in 115 patients (92.7%). Hepatitis C antibody was positive in only 2 cases (1.6%), whose HbsAg was negative. One hundred and five patients (84.7%) had histologically confirmed liver cirrhosis, and the remaining 19 (15.3%) did not. Tumor size ranged from 2 to 21 cm, with a median size of 7.5 cm. After a median follow-up of 2.6 years among 124 patients, 40 patients died of HCC and 8 patients died of other disease. Seventy-six patients were still alive at the time of last follow-up report. The 3- and 5-year overall survival rates were 58.3% and 40.9%, respectively. No significant correlation was observed among of *SYK* methylation and the above clinicopathological parameters. The percentage of patients with positive methylation of *E-cadherin* and *TIMP*-3 were significantly higher among those with Child-Pugh class B than those with Child-Pugh class A. In addition, *E-cadherin* methylation was significantly more frequent in patients with moderate or severe underlying cirrhosis, although the pathophysiological mechanism is not clear.

Prognostic Value of Gene Methylation in HCC

The prognostic value of 11 widely used clinicopathological parameters and the methylation status of *SYK*, *E-cadherin,* and *TIMP-3* were analyzed in the 124 HCC cases. Univariate analyses showed that Child-Pugh B classification, GGT level >100 U/L, the presence of macro tumor thrombus in the portal vein, presence of satellite nodule, presence of severe or moderate cirrhosis, and TNM stage >II predicted relative poor patient survival (Table 3).

We also divided all cases into two groups according to the methylation status of *SYK, E-cadherin,* or *TIMP-3* to determine whether these factors had prognostic value. Patients whose

primary tumors exhibited *SYK* hypermethylation had lower rates of overall survival ($P = 0.0288$, log-rank test) after resection; the 3- and 5-year overall survival rates were 40.6% and 30.5%, respectively, for patients with tumors that showed *SYK* hypermethylation, compared with 66.3% and 56.1%, respectively, for those without *SYK* methylation. The status of *E-cadherin* ($P = 0.8578$) and *TIMP-3* ($P = 0.6725$) methylation did not significantly influence patient survival (Table 3, Figure 5). In addition, prognostic value of the expression status of Syk protein was analyzed. Univariate analyses showed patients whose tumors exhibited negative Syk protein expression had lower rates of overall survival ($P = 0.0405$, log-rank test) after surgical resection; the 3- and 5-year overall survival rates were 40.5% and 30.4%, respectively, for patients with tumors that showed negative expression of Syk protein, compared with 65.7% and 55.6%, respectively, for those with positive expression of Syk protein.

The 6 clinicopathological factors and methylation status of *SYK* (or Syk protein status) found to be prognostic on univariate analysis were entered into a multivariate model to identify independent predictors of overall survival. Cox's multivariate proportional-hazards model indicated that the factors significantly affecting overall survival were Child-Pugh classification ($P = 0.038$), TNM stage ($P = 0.003$), and *SYK* methylation status ($P < 0.001$) (Table 4). When we used the expression status of Syk protein to replace the methylation status of *SYK* in Cox's multivariate model analysis, the factors significantly affecting overall survival were Child-Pugh classification ($P = 0.040$), TNM stage ($P = 0.025$), cirrhosis ($P = 0.048$) and Syk protein expression ($P = 0.007$). These data suggested that *SYK* gene methylation represented a surrogate for loss of SYK gene expression as an independent prognostic marker.

DISCUSSION

In this study, we analyzed methylation of the *SYK, E-cadherin,* and *TIMP-3* genes in 124 cases of HCC and correlated the methylation status with clinical and pathological features to determine whether these markers can predict disease outcomes. The *E-cadherin* and *TIMP-3* tumor suppressor genes have been extensively studied and their suppressor activity has been characterized in several experimental settings (32–35). Our results support these two genes' suppressor roles in HCC by showing methylation of *E-cadherin* and *TIMP-3* in 26.8% and 41.8% of the cases, respectively. *SYK,* however, has been less well characterized. It was initially implicated as a tumor suppressor gene in breast cancer (17). *SYK* promoter methylation leading to gene silencing has been shown in breast cancer (15) and acute lymphoblastic leukemia (16). The loss of *SYK* expression is thought to contribute to tumor progression by promoting tumor invasion, proliferation, and motility. Here we showed that *SYK* hypermethylation was present in 27.4% of the HCCs and was associated with gene silencing. The tight correlation between *SYK* methylation and loss of *SYK* expression, together with the causal role of *SYK* methylation in gene silencing, indicates that epigenetic inactivation of *SYK* contributes to the progression of HCC. In this project, we explored the possibility of using *SYK* methylation as a prognostic marker in comparison to *E-cadherin* and *TIMP-3* gene methylation.

The main focus of this study was to identify accurate biomarkers of prognosis for HCC patients after hepatectomy. Several clinicopathological features and molecular markers, with varied predictive power, have been linked to HCC prognosis. They include clinical indices (tumor size, tumor number and vascular invasion, underlying liver cirrhosis, Child-Pugh classification, and tumor microvessel density) (36–39) and molecular markers (p27 expression and *p53* mutation) (40,41). In this study, the prognostic value of *SYK*, *E-cadherin,* and *TIMP-3* methylation in tumor cells was investigated. Although methylation of *E-cadherin* and *TIMP-3* have been shown to predict a worse prognosis in node-positive diffuse gastric cancer and in esophageal adenocarcinoma, respectively (23,24), we did not find any correlation between either *E-cadherin* or *TIMP-3* methylation and HCC patient survival. In contrast,

methylation of *SYK* in HCC tissues predicted poor overall survival after hepatectomy on univariate analysis. Furthermore, Cox's multivariate proportional-hazard model confirmed that methylation of *SYK* in HCC was an independent and strong predictor of overall survival of these patients. *SYK* methylation appears to be a more powerful biomarker for risk prediction in HCC than other classic clinicopathological features, such as TNM staging and Child-Pugh classification (Table 4). It remains to be seen whether the use of *SYK* methylation as a prognostic tool can be extended to other tumor types, such as breast carcinoma. An earlier study indicated that in breast cancer patients, low *SYK* mRNA expression in tumors predicted short survival time (22). Presuming that the loss of *SYK* expression results from DNA methylation, *SYK* methylation is conceivably suitable for use as a biomarker of breast cancer prognosis.

The association between *SYK* methylation and poor survival rates suggests that *SYK* plays an important role in HCC progression. Since this study included only Chinese patients, it is not known the prognostic value of *SYK* methylation can be extended to HCC cases resulting from other etiological factors. It has been reported that rates of *p16* methylation in HCC vary significantly among different geographic locations (e.g., it is present in 34.4% of cases from China and Egypt but only 12.2% of those from the United States and Europe). Similar geographic variations have been observed for estrogen receptor-α methylation and CpG island methylator phenotype (CIMP) (42). Whether *SYK* methylation has such geographic and ethnic variation and whether *SYK* methylation is associated with certain etiological factors need to be further investigated.

Since CpG island methylation is a reversible epigenetic change, the use of demethylation agents presents a novel therapeutic opportunity (43). Early clinical trials with demethylation compounds, such as 5-azacytidine and 5-aza-dc, have shown disappointing results in solid tumors. Their use in hematological malignancies, however, has yielded promising responses (44,45), despite their high toxicity and chemical instability. The therapeutic outcome could be compromised without knowledge on the methylation status of tumor-related genes; demethylation agents should be effective only for patients with epigenetic inactivation of key tumor suppressor genes. Therefore, sensitive detection and a better understanding of the frequency of gene methylation must be obtained before the use of such demethylation drugs can be optimized. The present study showed that one, two, and all three of the *SYK, E-cadherin,* and *TIMP-3* genes were methylated in 38.7% (48/124), 17.7% (22/124), and 4.8% (6/124) of our HCC cases, respectively. Thus, 61.3% of the HCC patients had at least one of the three genes methylated. They may benefit from the demethylation-based therapy. Furthermore, a new generation of demethylation drugs that are more chemically stable, such as zebularine, could be more effective clinically and may be applicable in solid tumors (46).

In conclusion, the present data demonstrate that the *SYK* gene can be silenced through epigenetic pathway and that positive methylation of *SYK* is an adverse prognostic factor among HCC patients. This information can be used to identify high-risk HCC patients who may benefit from adjuvant or more aggressive therapy after resection of primary tumors. It also justifies further studies of novel demethylating agents in the treatment of HCC.

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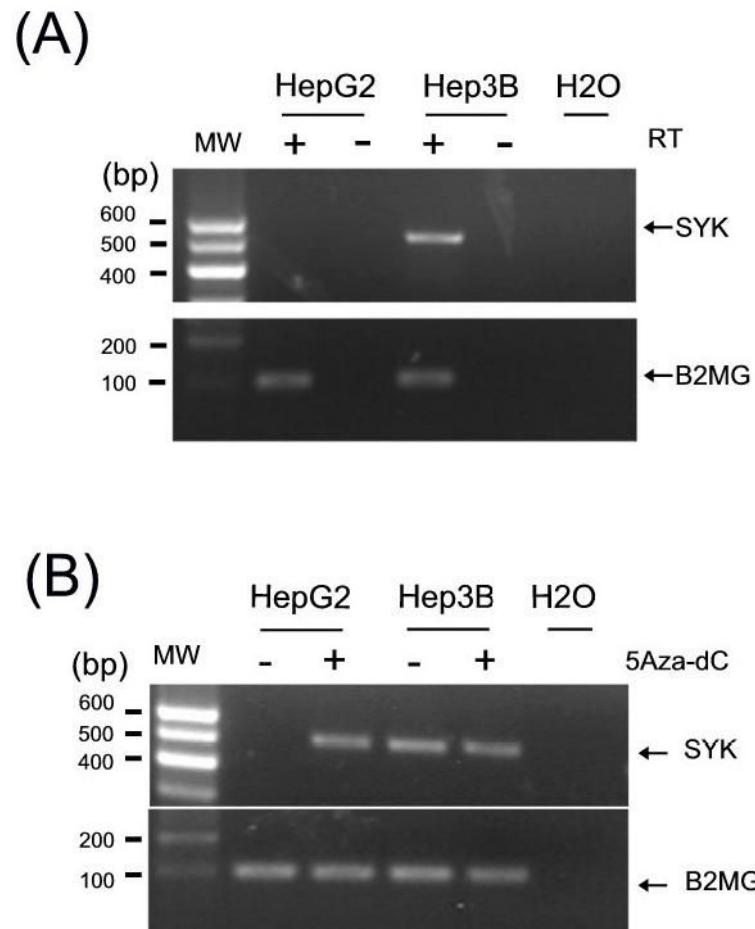
Figure 1.
(**A**) Expression of *SYK* mRNA in HCC cell lines HepG2 and Hep3B. *SYK* mRNA expression was determined by RT-PCR. An RT-negative control (−) was used to rule out false positives resulting from contaminated genomic DNA. mRNA for *β₂-microglobulin* (B2MG) was also analyzed to verify the RNA integrity. A blank control (H_2O) was included in each PCR experiment. PCR products and a molecular weight (MW) marker were run on an agarose gel following by ethidium bromide staining. Bands of 507 bp and 115 bp are expected for *SYK* and *β₂-microglobulin* transcripts, respectively. At least two independent experiments were carried out. (**B**) Restoration of *SYK* mRNA by treatment with DNMT1 inhibitor. HepG2 was treated for 5 days with (+) or without (−) 2.0 μM 5-aza-dC. As a control, Hep3B cells were processed in parallel. Total RNA was harvested and RT-PCR amplified as detailed in (A).
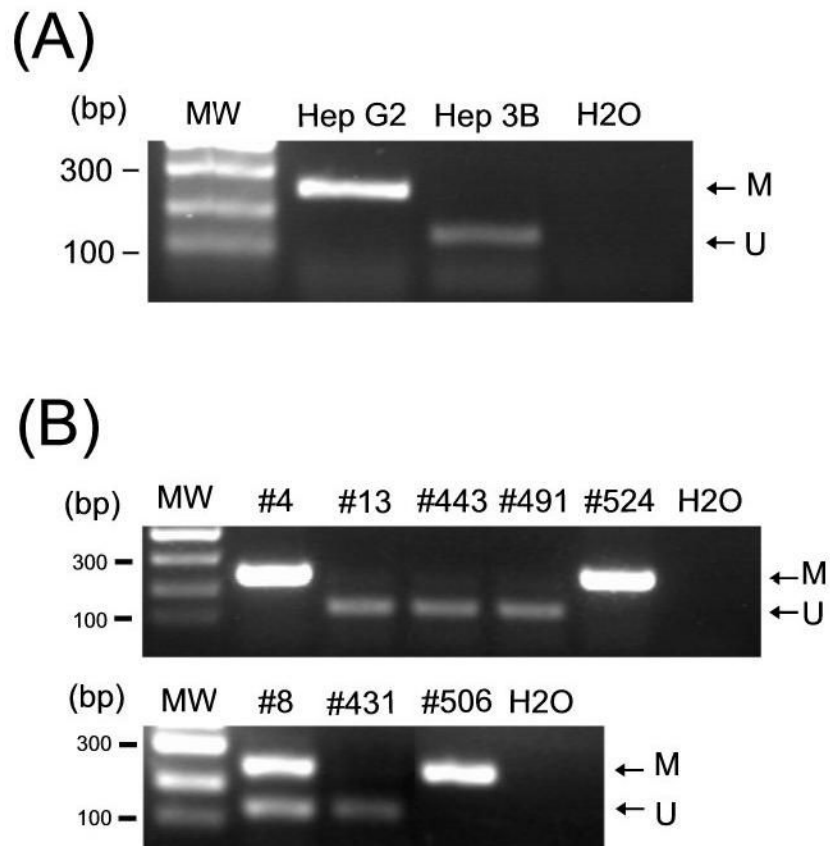
Figure 2.
The *SYK* gene was hypermethylated in HCC cell lines **(A)** and primary tumors **(B)**. DNA was treated with sodium bisulfite followed by the first round of PCR amplification. In the second round of PCR (nested), both methylation-specific and unmethylation-specific primers were used in the same reaction. DNA template-negative control (H$_2$O) was also included. Products of 243 bp and 140 bp were expected for methylated (M) and unmethylated (U) DNA, respectively. The methylation status of three representative methylation-positive (#4, #524 and #506), one intermediate (#8), and four methylation-negative (#13, #443, #491, and #431) cases is shown in **(B).**
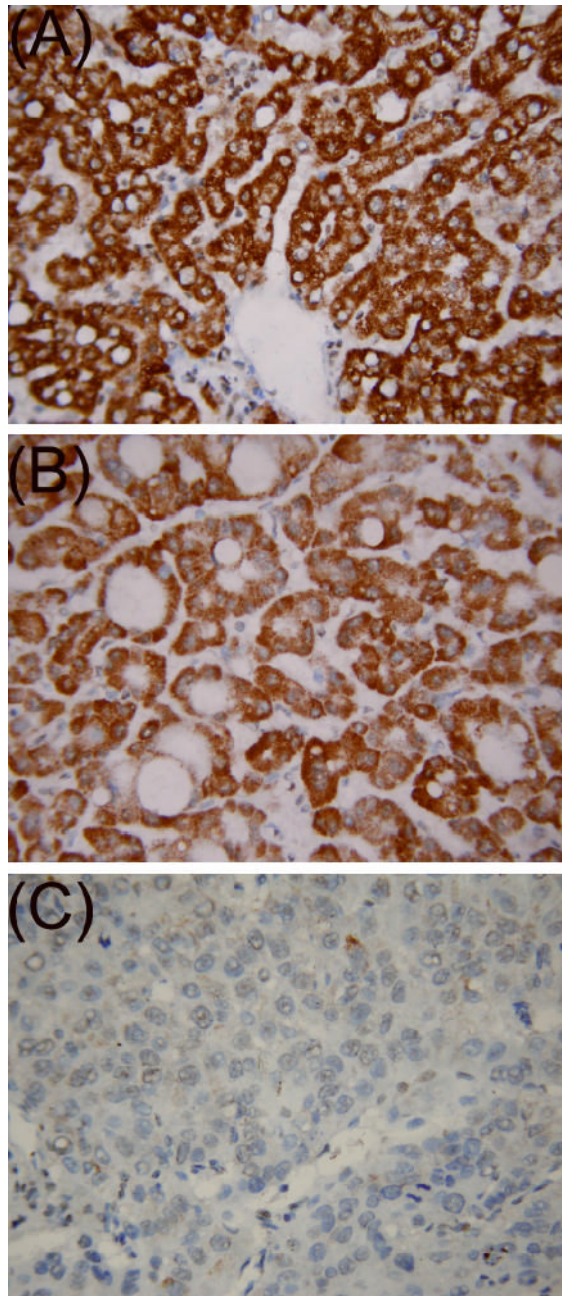
Figure 3.
Immunohistochemical analyses of *SYK* gene product in primary HCC specimens. Staining of representative specimens of normal liver tissue **(A)**, primary HCC with unmethylated *SYK* **(B),** and primary HCC with methylated *SYK* **(C)** is shown.
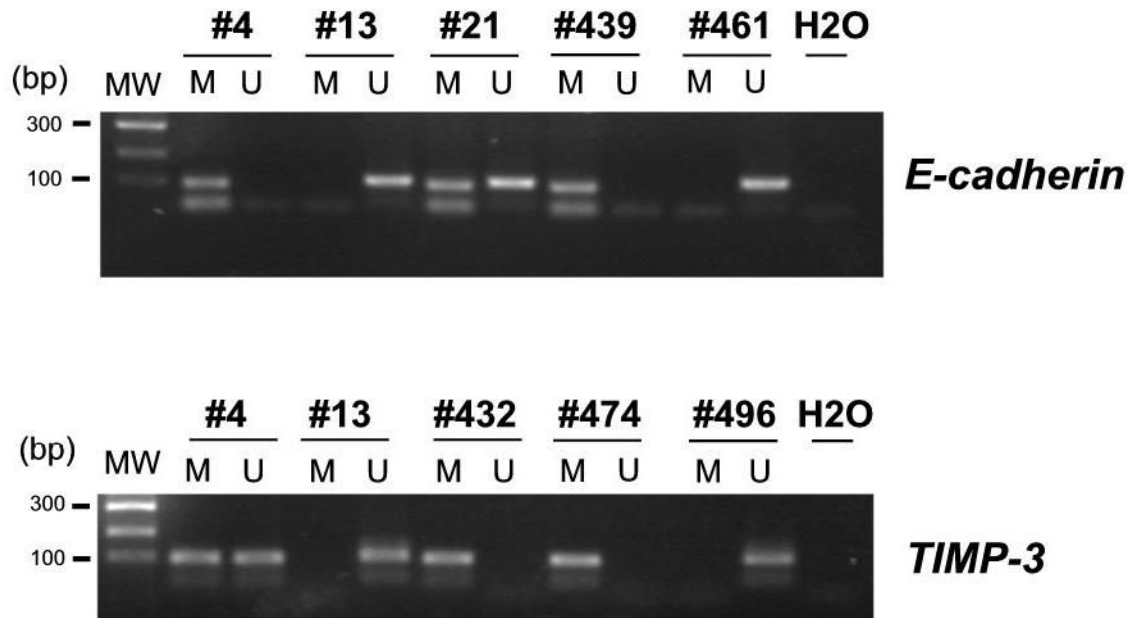
Figure 4.
The *E-cadherin* and *TIMP-3* genes were hypermethylated in primary HCC tumors. A two-step MSP protocol was used to analyze the gene methylation status. DNA was extracted from tissues, treated with sodium bisulfite, and then subjected to first-round PCR amplification. Then, in a nested PCR, methylation-specific or unmethylation-specific primers were used in separate reactions. For the *E-cadherin* gene, products of 112 bp and 120 bp were expected for methylated (M) and unmethylated (U) DNA, respectively. For the *TIMP-3* gene, products of 116 bp and 122 bp were expected for methylated (M) and unmethylated (U) DNA, respectively.
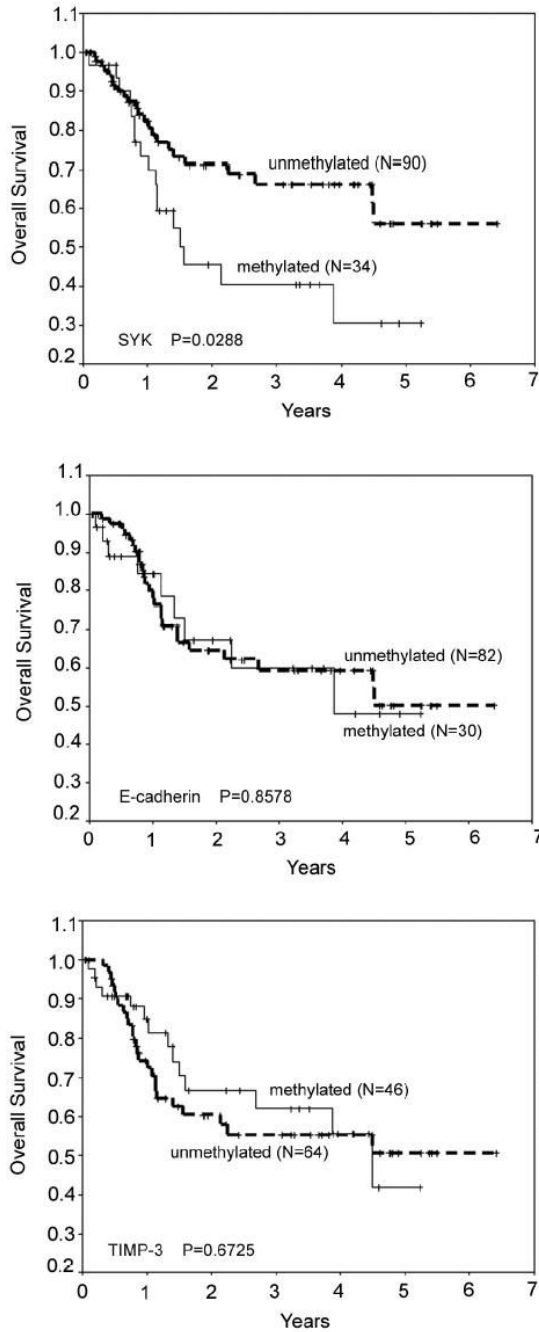
Figure 5.
Overall survival of 124 HCC patients grouped according to methylation status of *SYK* (top panel), *E-cadherin* (middle panel), and *TIMP-3* (bottom panel). Actuarial probabilities were calculated by the Kaplan-Meier method and compared with the log-rank test. After resection of primary tumors, patients with *SYK* hypermethylation in primary tumors had a worse overall survival rate than those without *SYK* methylation ($P = 0.0288$). No significant difference in survival was observed when analyzing the methylation status of *E-cadherin* ($P = 0.8578$) or *TIMP-3* ($P = 0.6725$). Solid lines represent gene methylation and dashed line gene unmethylation.

Table 1

Summary of primer sequence and PCR amplification for methylation analysis

Genes	Sense primer 5' → 3'	Antisense Primer 5' → 3'	Annealing Temp. (°C)	No. cycles	Size (bp)
	External (stage 1)	External (stage 1)			
SYK	TTTAGGGAATATGTTATGTAGTG	CACATAATTTCAAACACTTTTACC	57	40	645
E-cadherin	GTTTAGTTTTGGGGAGGGGGTT	ACTACTACTCCAAAAACCCATAACTAA	49	40	270
TIMP-3	GYGGTATTATTTTTTATAAGGATTTGA	ACCRAATAATATAAACRCTAAACCCC	56	40	185
	Internal methylated (stage 2)	Internal methylated (stage 2)			
SYK	CGATTTCGCGGGTTTCGTTC	AAAACGAACGCAACGCGAAAC	57	24	243
E-cadherin	TGTAGTTACGTATTTATTTTTAGTGGCGTC	CGAATACGATCGAATCGAACCG	54	24	112
TIMP-3	CGTTTCGTTATTTTTTTGTTTTCGGTTTC	CCGAAAACCCCGCCTCG	60	27	116
	Internal un-methylated (stage 2)	Internal un-methylated (stage 2)			
SYK	ATTTTGTGGGTTTGTTTGGTG	ACTTCCTTAACACACCCAAAC	57	24	140
E-Cadherin	TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT	ACACCAAATACAATCAAATCAAAACCAAA	54	24	120
TIMP-3	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	60	27	122

Table 2

Correlation of methylation of *SYK*, *E-cadherin,* and *TIMP-3* genes with clinicopathological features in patients with HCC. Values of statistical significance are shown in bold face type.

Characteristics	No. patients	% of patients showing a methylated gene		
		SYK	***E-cadherin**[*]	***TIMP-3**[#]
Gender				
Female	14	42.9% (6/14)	38.5% (5/13)	53.8% (7/13)
Male	110	25.5% (28/110)	25.3% (25/99)	40.2% (39/97)
P value		0.205	0.329	0.382
Age (years)				
<40	35	25.7% (9/35)	16.1% (5/31)	38.7% (12/31)
40–60	66	25.8% (17/66)	29.0% (18/62)	42.4% (25/59)
>60	23	34.8% (8/23)	36.8% (7/19)	45.0% (9/20)
P value		0.681	0.231	0.899
HbsAg				
Negative	11	18.2% (2/11)	22.2%(2/9)	30.0% (3/10)
Positive	113	28.3% (32/113)	27.2% (28/103)	43% (43/100)
P value		0.726	1.000	0.516
Child-Pugh classification[$]				
A	118	26.3% (31/118)	24.5% (26/106)	39.0% (41/105)
B	6	50.0 (3/6)	66.7% (4/6)	100% (5/5)
P value		0.344	**0.043**	**0.011**
GGT (U/L)				
<50	60	30.0% (18/60)	20.0% (11/55)	38.2% (21/55)
50 – 100	38	28.9% (11/38)	36.1% (13/36)	44.1% (15/34)
>100	26	19.2% (5/26)	28.6% (6/21)	47.6% (10/21)
P value		0.571	0.232	0.718
AFP (µg/L)				
<20	35	22.9% (8/35)	38.7% (12/31)	48.4% (15/31)
20–400	43	27.9% (12/43)	23.1% (9/39)	42.5% (17/40)
>400	46	30.4% (14/46)	21.4% (9/42)	35.9% (14/39)
P value		0.748	0.208	0.571
Tumor size (cm)				
<5	29	17.2% (5/29)	38.5% (10/26)	42.3% (11/26)
>=5	95	30.5% (29/95)	23.3% (20/86)	41.7% (35/84)
P value		0.234	0.137	0.954
Tumor thrombus				
No	103	28.2% (29/103)	25.0% (23/92)	39.3% (35/89)
Yes	21	23.8% (5/21)	35.0% (7/20)	52.4% (11/21)
P value		0.793	0.407	0.329
Satellite nodule				
No	86	31.4% (27/86)	27.8% (22/79)	43.2% (32/74)
Yes	38	18.4% (7/38)	24.2% (8/33)	38.9% (14/36)
P value		0.190	0.817	0.686
Tumor capsule				
Yes	45	26.7% (12/45)	28.6% (12/42)	38.1% (16/42)
No or incomplete	79	27.8% (22/79)	25.7% (18/70)	44.1% (30/68)
P value		0.877	0.826	0.557
Cirrhosis				
No	19	21.1% (4/19)	12.5% (2/16)	38.9% (7/18)
Mild	61	23.0% (14/61)	21.4% (12/56)	36.4% (20/55)
Moderate	40	37.5% (15/40)	36.1% (13/36)	45.5% (15/33)
Severe	4	25.0% (1/4)	75.0% (3/4)	100% (4/4)
P value		0.383	**0.032**	0.09
TNM stage				
I	68	33.8% (23/68)	28.6% (18/63)	43.9% (25/57)
II	11	27.3% (3/11)	20% (2/10)	27.3% (3/11)
III	45	17.8% (8/45)	25.6% (10/39)	42.9% (18/42)
P value		0.173	0.834	0.585

[*] Methylation of *E-cadherin* was non-informative in 12 cases.

[#] Methylation of *TIMP-3* was non-informative in 14 cases.

[$] No patients with Child-Pugh class C were studied.

Table 3

Univariate analyses of overall survival rates among 124 HCC patients

Variable	No. patients	Overall survival rate (%)		
		3-Year	5-Year	
Gender				
Female	14	62.5	62.5	0.9282
Male	110	58.4	48.4	
Age (years)				
<40	35	53.9	40.5	0.1248
40–60	66	66.3	59.7	
>60	23	43.5	34.8	
HbsAg				
Negative	11	88.9	59.3	0.2965
Positive	113	55.7	48.8	
Child-Pugh classification				
A	118	59.6	50.0	**0.0466**
B	6	0.0	0.0	
C	0			
GGT (U/L)				
<50	60	73.6	53.4	**0.0002**
50 – 100	38	56.2	56.2	
>100	26	21.3	0.0	
AFP (µg/L)				
<20	35	76.7	61.4	0.1355
20–400	43	50.9	45.8	
>400	46	50.8	40.6	
Tumor size (cm)				
<5	29	70.4	61.6	0.2934
5–10	72	50.8	40.7	
>10	23	65.7	65.7	
Tumor thrombus				
No	103	63.2	60.2	**0.0033**
Yes	21	33.5	0	
Satellite nodule				
No	86	68.4	65.0	**0.0006**
Yes	38	33.7	11.2	
Tumor capsule				
Yes	45	59.0	53.8	0.5433
No/incomplete	79	58.1	46.4	
Cirrhosis				
No	19	58.7	58.7	**0.0010**
Mild	61	71.5	61.2	
Moderate	40	32.3	16.1	
Severe	4	66.7	0.0	
TNM stage				
I	68	73.4	69.3	**0.0001**
II	11	53.3	53.3	
III	45	36.6	0.0	
SYK protein expression				
Negative	32	40.5	30.4	**0.0405**
Positive	92	65.7	55.6	
SYK gene				
Methylated	34	40.6	30.5	**0.0288**
Unmethylated	90	66.3	56.1	
E-cadherin gene [*]				
Methylated	30	59.9	48.0	0.8578
Unmethylated	82	59.3	50.2	
TIMP-3 gene [#]				
Methylated	46	62.0	41.8	0.6725
Unmethylated	64	55.2	50.6	

[*] Methylation of *E-Cadherin* was non-informative in 12 cases.

[#] Methylation of *TIMP-3* was non-informative in 14 cases.

Table 4

Cox's multivariate analysis of factors contributing to long-term survival in HCC patients

Variable	β	SE[*]	Hazard ratio (95% CI)	*P*value
Child-Pugh classification	1.612	0.776	5.013 (1.094–22.962)	0.038
GGT level	0.422	0.217	1.524 (0.996–2.332)	0.052
Tumor thrombus	−0.451	0.492	0.637 (0.243–1.670)	0.359
Satellite nodule	0.626	0.438	1.870 (0.793–4.413)	0.153
Cirrhosis	0.455	0.265	1.575 (0.936–2.650)	0.087
TNM stage	0.871	0.289	2.389 (1.357–4.207)	0.003
SYK **methylation**	−1.377	0.393	0.252 (0.117–0.545)	<0.001

[*] SE, standard error; CI, confidence interval