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 because 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 clinicopathologic variables 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 tumor-node-metastasis 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.

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 >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 etiologic 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 and overexpressed in HCC. SYK gene expression is controlled by DNA methylation and is postulated to be involved in the oncogenesis of HCC. Therefore, the goal of this study is to determine the methylation status of SYK in HCC and to evaluate the association of SYK methylation with disease outcome.
methylated 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, overexpression 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 pathologic variables 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% CO₂.

**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 nonneoplastic liver tissues, were archived in the liver tumor bank of the institution and stored at −80°C until use. All nonneoplastic and neoplastic samples were histologically confirmed. Neither chemotheraphy 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, as follows, based on the size of gross cirrhotic nodules and histologic examination: (a) No cirrhosis: The liver was soft and smooth with no cirrhotic nodules. No pseudolobule formation was found microscopically. (b) Mild cirrhosis: The largest nodule on liver surface was <0.4 cm, or cirrhosis was identified by microscopic examination. (c) Moderate cirrhosis: The degree of cirrhosis was between mild and severe cirrhosis. (d) 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 PCR.** A blinded methylation-specific PCR (MSP) analysis was carried out; no clinicopathologic 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 done 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 bisulfite-resistant modifications of unmethylated and methylated cytosines, respectively. For the first-round MSP, a 30-μl reaction that contained 30 ng bisulfite-treated DNA was processed in 40 thermal cycles.

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The heterogeneity of HCC, although contamination from tence of both methylation status in a given tumor could reflect SYK both remaining seven cases (5.6%) showed amplification of tumors, in which 27 (21.8%) and 90 (72.6%) specimens were found to be both 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 three 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 (28). The three 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 nonneoplastic 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 nonneoplastic 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 was 8.8% (3 of 34) and 23.5% (8 of 34), respectively, for nonneoplastic and neoplastic tissues. The difference in percentage of methylation-positive patients was not statistically significant (P = 0.186; Fisher’s test). The statistical significance could be reached if more samples were available. SYK methylation in nonneoplastic tissues was also observed in earlier studies that may represent DNA methylation in premalignant lesions (29, 30). Aging-related gene methylation could also be a contributor (31). The corresponding primary tumors of these three 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 expression control occurs at the transcriptional level. Both SYK mRNA and Syk protein were positive in all 34 matching nonneoplastic 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

Table 1. Summary of primer sequence and PCR amplification for methylation analysis

| Genes   | Sense primer 5’→3’ | Antisense primer 5’→3’ | Annealing temperature (°C) | No. cycles | Size (bp) |
|---------|---------------------|------------------------|---------------------------|------------|-----------|
|         | External (stage I)  | External (stage I)     |                           |            |           |
| SYK     | TTAGGAGGATATGATGTAGT | CACATAATTTCCACACTTTACC | 57                        | 40         | 645       |
| E-cadherin | GGTAGTTTTGTGGAGGGTTT | ACTACTCTCGAAAAACCATCTAA | 49                        | 40         | 270       |
| TIMP-3  | TTTGATTTATTTTATAGGATTGA | ACCRAATAATTAACCTTAACCC  | 56                        | 40         | 185       |
|         | Internal methylated (stage II) | Internal methylated (stage II) |                           |            |           |
| SYK     | CGATTGCGGGGTGGTTC | AAAACGACGCCACCCGCAAC  | 57                        | 24         | 243       |
| E-cadherin | TGTAGTTATCTATTTTTTATTGGGTC  | CGAATGCGTCTGAAGAACCGCG  | 54                        | 24         | 112       |
| TIMP-3  | CTTTCTGTTATTTTATTTTGTGTTC  | CGGAAAAACCGGCTCGG       | 60                        | 27         | 116       |
|         | Internal unmethylated (stage II) | Internal unmethylated (stage II) |                           |            |           |
| SYK     | ATTTTTGGGTGGTGGTTTGTTG | ACTCCCTAAACACCCCCAAC  | 57                        | 24         | 140       |
| E-cadherin | TGTTGTGTTATGATTTTTATTTTTGTGTG | ACACCAATACATCAAATCAACCAA | 54                        | 24         | 120       |
| TIMP-3  | CTTTTGGTTGTTATTTTTGTTTTG | CCCCCAAAAACCCCACTCA    | 60                        | 27         | 122       |

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 (Fig. 1A). Because 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 (Fig. 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 DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, to determine whether demethylation restored SYK expression. As shown in Fig. 1B, 5-aza-2′-deoxycytidine 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 pathologic diagnosis of all HCC cases was confirmed by histologic 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 seven cases (5.6%) showed amplification of both SYK methylation and unmethylation (Fig. 2B). Coexistence of both methylation 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 (Fig. 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.
RT-PCR amplified as detailed in (41). Total RNA was harvested and analyzed to verify the RNA integrity. A blank control (H2O) was included in each PCR experiment. PCR products and a molecular weight (MW) marker were run on an agarose gel followed by ethidium bromide staining. Bands of 507 and 115 bp are expected for SYK and j2-microglobulin transcripts, respectively. At least two independent experiments were carried out. B. Restoration of SYK mRNA by treatment with DNA methyltransferase 1 inhibitor. HepG2 was treated for 5 days with (+) or without (−) 2.0 μmol/L 5-aza-2’-deoxycytidine (5Aza-dC). As a control, Hep3B cells were processed in parallel. Total RNA was harvested and RT-PCR amplified as detailed in (A).

methylated 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/malignant cells. The methylation status of SYK, E-cadherin, and TIMP-3 genes was analyzed in the 124 HCC cases. Univariate analyses showed that Child-Pugh B classification, γ-glutamyltransferase level >100 U/L, the presence of macro tumor thrombus in the portal vein, the presence of satellite nodule, the 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 30.4%, 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 six clinicopathologic 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 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 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 pathologic 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 the suppressor roles of these two genes 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

Fig. 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).

Fig. 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 and 120 bp were expected for methylated and unmethylated DNA, respectively. For the TIMP-3 gene, products of 116 and 122 bp were expected for methylated and unmethylated DNA, respectively.
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 compared with 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 clinicopathologic features and molecular markers, with varied predictive power, have been linked to HCC prognosis.

### Table 2. Correlation of methylation of SYK, E-cadherin, and TIMP-3 genes with clinicopathologic features in patients with HCC

| Characteristics                  | No. patients | Percentage 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                                | 0.205        | 0.329              | 0.382        |
| Age (y)                          |              |                    |              |         |
| <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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 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                                | 0.173        | 0.834              | 0.585        |

NOTE: Values of statistical significance are shown in boldface.

Abbreviation: GGT, γ-glutamyltransferase; HbsAg, hepatitis B surface antigen.

*Methylation of E-cadherin was noninformative in 12 cases.

†Methylation of TIMP-3 was noninformative in 14 cases.

‡No patients with Child–Pugh class C were studied.
They include clinical indices (tumor size, tumor number and vascular invasion, underlying liver cirrhosis, Child-Pugh classification, and tumor microvessel density; refs. 36–39) and molecular markers (p27 expression and p53 mutation; refs. 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 multivariate proportional-hazards model confirmed that methylation of SYK in HCC was an independent and strong predictor of overall survival of these patients. SYK

**Table 3. Univariate analyses of overall survival rates among 124 HCC patients**

| Variable                      | No. patients | 3 y Survival (%) | 5 y Survival (%) | P     |
|-------------------------------|--------------|------------------|------------------|-------|
| Gender                        |              |                  |                  |       |
| Female                        | 14           | 62.5             | 62.5             | 0.9282|
| Male                          | 110          | 58.4             | 48.4             |       |
| Age (y)                       |              |                  |                  |       |
| <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 (units/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             | 0.0              |       |
| 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             | 0.0100|
| 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             |       |

Note: Values of statistical significance are shown in boldface. *Methylation of E-cadherin was noninformative in 12 cases. †Methylation of TIMP-3 was noninformative in 14 cases.
methylation seems to be a more powerful biomarker for risk prediction in HCC than other classic clinicopathologic 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. Because this study included only Chinese patients, it is not known whether the prognostic value of SYK methylation can be extended to HCC cases resulting from other etiologic 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 (42). Whether SYK methylation has such geographic and ethnic variation and whether SYK methylation is associated with certain etiologic factors need to be further investigated.

Because 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-2′-deoxycytidine, have shown disappointing results in solid tumors. Their use in hematologic 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 of 124), 17.7% (22 of 124), and 4.8% (6 of 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 show 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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