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Expression of Variant Isoforms of the Tyrosine Kinase SYK Determines the Prognosis of Hepatocellular Carcinoma

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Abstract

The tyrosine kinase SYK has been reported as a novel biomarker for human hepatocellular carcinoma (HCC), but the functional contributions of its two isoforms SYK(L) and SYK(S) are undefined. In this study, we investigated their biologic functions and possible prognostic values in HCC. SYK(L) was downregulated in 38% of human specimens of HCC examined, whereas SYK(S) was detectable in 40% of these specimens but not in normal liver tissue samples without cirrhosis. SYK(S) expression correlated with pathological parameters characteristic of tumor metastasis, including multiple tumors ($P = 0.003$) and vascular invasion ($P = 0.001$). Further, SYK(S) was specifically associated with epithelial-mesenchymal transition (EMT) in HCC specimens. Functional studies showed that SYK(S) promoted tumor growth, suppressed apoptosis and induced EMT through the ERK pathway, countering the opposite effects of SYK(L). Patients with SYK(L+/S-) tumors exhibited longer overall survival and time to recurrence than those with SYK(L-/S-) or SYK(L+/S+) tumors ($P < 0.001$). Taken together, our findings showed that SYK(S) enhances invasion whereas SYK(L) inhibits metastasis in HCC. We suggest that SYK(L) downregulation or SYK(S) elevation are strong predictors of poor survival in HCC patients, indicative of a need for aggressive therapeutic intervention.

Instruction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignances worldwide (1). Treatment of HCC remains highly challenging because of the high incidence of tumor recurrence and metastasis even after surgical resection (2, 3). It is clinically relevant to

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understand the molecular changes associated with HCC recurrence and metastasis and to identify significant biomarkers with which to monitor disease progression.

Spleen tyrosine kinase (SYK) is a non-receptor protein tyrosine kinase expressed in cells of hematopoietic or epithelial origin. A significant drop in full-length SYK [SYK(L)] level was first observed in breast carcinoma (4). Altered SYK expression was later found in gastric cancer, melanoma and oral squamous cell carcinoma (5). Lowered SYK levels have been strongly correlated with an increased risk of metastasis (6, 7). As a result, decreased SYK levels have been proposed as a useful prognostic marker in a few tumor types including breast carcinoma, HCC, oral squamous cell carcinoma and melanoma (8–11). Although most studies have shown a correlation between SYK loss and neoplastic progression, some studies have found that SYK is unregulated in tumors and is required for tumor cell survival, such as retinoblastoma (12), head and neck squamous cell carcinomas (13). The reason for this discrepancy is not completely understood. One explanation is that SYK variants are possibly associated with a different biologic response and an opposite prognostic value from SYK(L).

An alternatively spliced SYK transcript [short form or SYK(S)] that lacks a 69-bp sequence has been reported (14). This in-frame transcript variant creates a SYK isoform that lacks 23 residues within interdomain B (Supplementary Figure 1A). While preserving the major structural domains (two tandem Src homology-2 domains and a kinase domain), SYK(S) does not share the biologic responses elicited by SYK(L) (15). Over-expression of SYK(L) leads to the inhibition of proliferation and invasion, but expression of SYK(S) in SYK-negative cells failed to lead to these activities (14, 16). Coincident with their differing phenotypic responses, SYK(L) and SYK(S) have different subcellular distributions. SYK(L) is present in both the cytoplasm and nucleus, whereas SYK(S) is excluded from the nucleus (14, 17). The interdomain B in SYK(L), which is absent in SYK(S), contains a nuclear localization signal that is required for the nuclear presence of SYK(L) (14, 18). It has been proposed that the transcriptional repressor activity of SYK(L) is required to suppress the expression of oncogenes, accounting for SYK(L) functions. In agreement with that hypothesis, a loss of nuclear SYK was found to be closely associated with poor prognosis in gastric cancer (19). However, the biologic significance of SYK(S) in carcinogenesis and its relationship with SYK(L) are not clear.

We reported earlier that decreased SYK(L) expression resulting from promoter methylation was an adverse prognostic factor among patients with HCC (9), and checkpoint kinase 1 (CHK1) phosphorylated SYK(L) to promote its subsequent proteasomal degradation which induces HCC development (20). It was, however, unclear whether SYK(S) was expressed in HCC. In the present study, we profiled the expression status of both SYK(L) and SYK(S) in HCC and evaluated the prognostic significance and phenotypic effects of SYK(L) and SYK(S).

Materials and Methods

Cell lines and clinical samples

Five HCC cell lines (MHCC-97H, MHCC-97L, BEL-7402, Huh7, SMMC7721) and one human immortalized liver cell line (LO2) were obtained from Liver Cancer Institute of Fudan University, Shanghai, China. Two HCC cell lines (PLC/PRF/5 and Hep-3B) and one human colon carcinoma cell line (HCT116) were purchased from the American Type Culture Collection (Manassas, VA). Cell lines were cultured as previously reported (20). All cell lines used in this study were regularly authenticated by morphologic observation and tested for absence of mycoplasma contamination (MycAlert, Lonza). HCC tissue samples and matched adjacent non-neoplastic tissue samples were obtained from 152 consecutive

patients who underwent curative liver resection from January 2003 to March 2004 at Sun Yat-sen University Cancer Center. The clinical typing of tumors was determined according to the Barcelona Clinic Liver Cancer (BCLC) staging systems (21). None of the patients received anticancer treatment before hepatectomy. Thirty specimens of normal liver tissue without cirrhosis (specimens of focal nodular hyperplasia or liver hemangioma) were also included in the study. The study endpoints included overall survival (OS) and time to recurrence (TTR) (22). Clinical samples were collected from these patients after obtaining informed consent according to an established protocol approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer Center. Additional clinical sample information is presented in Supplementary Materials and Methods.

SYK(L) and SYK(S) stable cell lines

Two HCC cell lines, SMMC7721, with no detectable SYK(L) and SYK(S) expression, and MHCC-97H, with high SYK(L) and SYK(S) expression, were used to generate stable cells by retroviral transduction. Recombinant retrovirus expressing the vector pLNCX2 alone or the vector with SYK(L) or SYK(S) cDNA was generated using a retroviral packaging system from Clontech (Mountain View, CA). Pooled cells expressing SYK(L), SYK(S), or vector alone were selected with 750 μ g/ml of G418 (Calbiochem, Darmstadt, Germany).

Gene expression analyses—Reverse transcriptase–polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR), immunoblotting and immunohistochemistry (IHC) are detailed in Supplementary Materials and Methods.

Cell proliferation, colony formation, and apoptosis assay—Reagents and protocols for these assays are described in Supplementary Materials and Methods.

RNA Interference (RNAi)—The protocol is described in Supplementary Materials and Methods.

Cell fractionation and immunofluorescence assay—Refer to the Supplementary Materials and Methods for a detailed description of experiment protocols.

Matrigel invasion assay—*In vitro* Matrigel invasion assays were performed as described previously (14) using Transwell inserts (8- μ m pore size; Costar) that had been coated with 150 μ g Matrigel (BD Biosciences; Franklin Lakes, NJ). Twenty thousand cells were plated into the upper chamber of each insert and incubated for 48 h at 37°C. Cells that invaded through the Matrigel were stained using a Hoechst 33342 (Beyotime, Jiangsu, China) and quantified.

Matrix metalloproteinase-2 (MMP2) activity assay—Details are described in Supplementary Materials and Methods.

Animal studies

Both subcutaneous and orthotopic models were used for animal studies. The subcutaneous model was performed as described previously (20). For the orthotopic model, 2×10^6 MHCC-97H cells stably expressing SYK(L) or SYK(S) were mixed in 50 μ l of DMEM and Matrigel (1:1). With a microsyringe, the cell suspension was then injected into the left hepatic lobe of 4-week-old nude mice. Transplanted cells were allowed to grow for up to 7 weeks, when mice were sacrificed. The livers and lungs were dissected, fixed and paraffin-embedded. The mice were housed and handled according to protocols approved by the Use Committee for Animal Care of Sun Yat-sen University.

Statistical analyses

Survival curves were constructed using Kaplan-Meier method and analyzed by the log-rank test. Significant prognostic factors found by univariate analysis were entered into a multivariate analysis using the Cox proportional hazards model. The Fisher's test was used to analyze the association of SYK(L)/SYK(S) expression with various clinicopathologic characteristics. Student's *t* test or the Mann-Whitney *U* test was employed to compare the values between subgroups. Data were expressed as mean $\pm$ standard deviation (SD). All analyses were performed using SPSS software (version 16.0; Chicago, IL).

Results

SYK(L) and SYK(S) expression in HCC

Immunoblotting and qRT-PCR were used to examine the expression of SYK(L) and SYK(S) in an immortalized normal liver cell line LO2 and seven human HCC cell lines; the colorectal cancer cell line HCT116 was used as a positive control. Five HCC cell lines (BEL-7402, Hep-3B, Huh7, PLC/PRF/5 and SMMC7721) had low or no metastatic potential. By contrast, MHCC-97H and MHCC-97L cell lines were highly metastatic, with MHCC-97H cells being the most metastatic among all lines used in this study (23). Immunoblotting showed that SYK(L) was expressed in LO2 cells and six HCC cell lines (BEL-7402, Hep-3B, Huh7, MHCC-97H, MHCC-97L and PLC/PRF/5). SYK(S) protein was detected in MHCC-97H and MHCC-97L cells, with a higher SYK(S) level in MHCC-97H cells (Figure 1A). Neither SYK(L) nor SYK(S) was detectable in SMMC7721 cells. qRT-PCR analyses showed that SYK(L) and SYK(S) transcript levels were mostly consistent with their respective protein status (Figure 1B), indicating that SYK expression can be reliably determined by qRT-PCR.

qRT-PCR was then used to determine the level of SYK(L) and SYK(S) in 152 pairs of HCCs (tumor and corresponding non-tumor tissue) and 30 normal liver tissue samples without cirrhosis (N). SYK(L) expression in HCC was found to be significantly down-regulated compared to that in either non-tumor or N. By contrast, SYK(S) expression was higher in HCC than that in non-tumor or N (both $P < 0.05$; Figure 1C and D). As outlined earlier, a decrease in SYK(L) expression by 8 fold relative to the average level of SYK(L) mRNA in 30 normal liver tissue specimens was defined as negative SYK(L). Likewise, an increase in SYK(S) mRNA level by 2 fold was the cutoff for positive SYK(S). By these criteria, 38.2% (58/152) of HCCs were SYK(L)-negative, and 40.1% (61/152) of HCC cases were SYK(S)-positive. By contrast, SYK(S) was rarely found in non-tumor (1.3%, 2/152) and not in N (0%, 0/30), indicating that SYK(S) expression is tumor-specific in HCC. Moreover, the RT-PCR data show that SYK(S) was only expressed in HCC, and only in conjunction with SYK(L) (Supplementary Figure 1C).

SYK(S) possesses oncogenic activities

Our earlier studies showed tumor suppressor function of SYK(L) in HCC.(20) To verify the activity of SYK(L) in HCC and, more importantly, to explore the biologic consequences of SYK(S) expression, we compared the growth of cultured SMMC7721 cells in response to ectopic expression of SYK(L) or SYK(S). SMMC7721 parental cells had neither SYK(L) nor SYK(S) at detectable level. Stable expression of SYK(L) prohibited the proliferation of SMMC7721 cells. The growth-suppressing response to SYK(L) contrasted with a modest but consistent growth-promoting effect of SYK(S) (Figure 2A and B). SYK(L) and SYK(S) were also compared with respect to their effect on *in vivo* xenograft establishment. Ectopic expression of SYK(L) in SMMC7721 cells resulted in less proficient tumor growth in athymic mice than parental cells. In contrast, SYK(S) accelerated xenograft growth (Figure 2C), reaffirming a growth-stimulatory role of SYK(S).

The subcellular distribution of SYK(L) and SYK(S) in HCC cells was evaluated. Both cell fractionation and immunofluorescence studies showed that, in agreement with studies of breast cancer, SYK(S) was distributed exclusively in cytoplasm, whereas SYK(L) was localized in both nuclear and cytoplasm (Supplementary Figure 2 A–C). The distribution of SYK(L) in both nucleus and cytoplasm was confirmed in HCC specimens by immunohistochemical staining (Supplementary Figure 2D). These results suggested that differentiated subcellular distribution of SYK(L) and SYK(S) may account for their opposing biologic responses.

The potential effect of SYK(L) and SYK(S) on apoptosis was then explored. Mitogen removal by serum deprivation effectively induced apoptosis. SMMC721-SYK(L) stable cells were found to be more susceptible to serum starvation than parental cells as measured by annexin-V staining. The increase in apoptotic incidence resulting from SYK(L) was supported by elevated poly(ADP ribose) polymerase (PARP) cleavage. Expression of SYK(S), however, led to a significant decrease in apoptosis that was accompanied by reduced PARP cleavage (Figure 2D), suggesting that SYK(S) is a pro-survival factor capable of overcoming the lack of mitogen stimuli. The opposing activity of SYK(L) and SYK(S) was recapitulated in their effect on cisplatin-induced cell death (Figure 2E). These results suggested a differential role of SYK(L) and SYK(S) in cell response to apoptotic signals, which might be partly responsible for the contrasting effect of SYK(L) and SYK(S) on cell growth. The opposing effect of SYK(L) and SYK(S) in HCC cells was supported by our RNAi experiments. Suppression of SYK(L) expression resulted in accelerated cell proliferation, while inhibited SYK(S) expression was accompanied by a slower growth (Supplementary Figure 3). The growth suppression effect of SYK(L) was associated with lowered levels of p-ERK1/2 and p-Jnk in MHCC-97H or SMMC7721 cells. Expression of SYK(S) resulted in a marked increase in p-Erk1/2 level in SMMC7721. This response is, however, less robust in MHCC-97H cells. P-Akt levels were not affected by either SYK(L) or SYK(S). The effect of SYK(L) and SYK(S) on p-ERK1/2 was also recapitulated in our xenograft specimens (Supplementary Figure 4). These results suggest a differential effect of SYK(L) and SYK(S) on MAPK/ERK activity that may be responsible for their opposing effects on cancer cell growth and invasion.

Correlation of SYK(L) and SYK(S) with clinicopathological variables

To verify the functions of SYK(L) and SYK(S) identified in the experimental setting, we correlated SYK(L) or SYK(S) status in 152 HCC specimens with 10 widely recognized clinicopathologic parameters. Our analyses showed that positive SYK(L) was associated with better tumor differentiation and the absence of intrahepatic multiple nodules, thereby predicting a favorable clinical outcome. By contrast, SYK(S) expression was strongly correlated with poor tumor differentiation, the presence of intrahepatic multiple nodules, absent or incomplete tumor capsules, vascular invasion, and advanced BCLC stage (Table 1). Notably, both negative SYK(L) and positive SYK(S) were found to be associated with multiple nodules and poor differentiation, suggesting that SYK(L) and SYK(S) possess contrasting functional activities. It was also recognized that SYK-associated features were related to metastasis. Notably, the correlation of intrahepatic satellite nodules, absent or incomplete tumor capsules and vascular invasion with SYK(S) positivity suggested a role for SYK(S) in increased invasion and metastasis of HCC.

Opposing effect of SYK(L) and SYK(S) on invasion and metastasis

We then used an experimental metastasis model to evaluate how SYK(L) and SYK(S) affect HCC invasion and metastasis. SMMC7721 and MHCC-97H cells were retrovirally infected with SYK(L) or SYK(S) cDNA (Figure 2A and 3A). Compared with parental cells, MHCC-97H or SMMC7721 cells expressing SYK(L) exhibited markedly decreased

Matrigel invasion. By contrast, expression of SYK(S) significantly promoted cell invasiveness *in vitro* (Figure 3B), suggesting that SYK(L) and SYK(S) have opposite effects on cell invasion. These data were supported by our RNAi experiments. Inhibition of SYK(L) and SYK(S) expression was accompanied by increased and reduced invasiveness of HCC cells, respectively (Supplementary Figure 2). For an *In vivo* metastasis model, the HCC cell line with a high metastatic potency, MHCC-97H, was chosen. Cells stably expressing SYK(L) or SYK(S) were inoculated into the liver of athymic mice. Metastatic nodules in the lung were then measured. We found that expression of SYK(L) resulted in a significant decrease in the number of metastatic foci in lung. By contrast, SYK(S) expression led to elevated lung metastasis (Figure 3C), suggesting that SYK(L) and SYK(S) impose opposite regulatory effects on HCC metastasis.

Secretion of MMPs by cancer cells is a key mechanism for accomplishing invasion and metastasis. We explored how expression of MMP2 and MMP9 (and their respective cognate partner TIMP2 and TIMP1) was affected by SYK(L) and SYK(S) in SMCC7721 and MHCC-97H cells. Our semi-quantitative RT-PCR analyses showed that, in both cell types, SYK(L) expression lowered MMP2 and TIMP2 mRNA levels but had no discernible effect on MMP9 and TIMP1 expression (Figure 3D). SYK(S), however, led to increased expression of all four MMP family genes examined. In both cell lines, the effect of SYK on MMP2 expression was accompanied by changes in the secretion of bioactive MMP2. SYK(L)-expressing stable cells were found to release a lower quantity of active MMP2 to surrounding media than control cells. By contrast, expression of SYK(S) was associated with increased secretion of active MMP2 (Figure 3E).

SYK(S), but not SYK(L), is associated with epithelial-mesenchymal transition (EMT)

To explore the relationship of SYK(L)/(S) and EMT, we performed IHC staining to assess E-cadherin and vimentin levels in 152 HCC specimens. We found that SYK(S) expression inversely correlated ($P = 0.002$) with the reduction of E-cadherin, but positively correlated with vimentin expression ($P = 0.016$). In contrast, there was no significant association between SYK(L) and E-cadherin ($P = 0.869$) or vimentin ($P = 0.732$) (Figure 4A and B). We therefore evaluated the protein levels of several EMT markers in 5 HCC cell lines. We confirmed that a higher level of SYK(S) accompanied with elevated levels of mesenchymal markers vimentin, fibronectin, N-cadherin and Twist, and a reduced level of epithelial protein E-cadherin in high-metastasis cells (Figure 4C). The suppression of SYK(S) resulted in a higher E-cadherin level, accompanied with vimentin decrease, but did not significantly influence the other EMT markers. This effect was confirmed by SYK(S) overexpression (Figure 4D). The SYK(L) had no detectable effect on EMT markers (data not shown). Moreover, the effect of SYK(S) on E-cadherin and vimentin was also observed in the xenograft specimens (Figure 4E and F). Taken together, these results indicated that HCC cells overexpressing SYK(S) undergo EMT to achieve higher invasiveness and metastasis.

ERK activation is critical for SYK(S)-induced EMT

Next, we sought to determine the signaling mechanisms involved in SYK(S)-mediated EMT. Increasing evidence suggests that activated MAPK/ERK and PI3K/Akt pathways activate EMT in HCC (24–29). To investigate whether SYK(S)-mediated EMT occurs through activation of ERK and/or Akt pathways, we used inhibitors of ERK1/2 (U0126) and Akt (LY294002) in SYK(S)-overexpressing Huh7 cells. We found that either U0126 or LY294002 restored SYK(S)-induced expression of epithelial marker E-cadherin and inhibited the mesenchymal markers vimentin and N-cadherin (Figure 5A). This effect was confirmed by the suppression of ERK1/2 or Akt by siRNA (Figure 5B). In addition, our data showed that SYK(S) increased levels of phosphorylated ERK1/2 (p-ERK1/2), but not phosphorylated Akt (p-Akt) in HCC cells (Figure 5C&D; Supplementary Figure 4). In

contrast, SYK(L) decreased levels of p-ERK1/2. We found no correlation between SYK(L) and EMT markers (E-cadherin and vimentin) among 152 HCC specimens (Figure 4A), and SYK(L) did not affect EMT markers in HCC cells (Figure 5C&D). Taken together, these data suggest that SYK(S) promotes EMT, at least in part, through the up-regulation of ERK signaling pathways.

Prognostic value of SYK(L) and SYK(S)

The prognostic value of the several clinicopathological variables and SYK expression status was analyzed. Univariate analyses showed that portal hypertension, GGT level, tumor size, tumor number, vascular invasion, intraoperative blood loss, perioperative blood transfusion, resection margin, tumor differentiation, and BCLC stage are associated with TTR and OS of HCC patients (Supplementary Table 2). We divided all cases into two groups based on SYK(L) or SYK(S) status to evaluate their impact on TTR and OS. Patients with SYK(L)-positive HCC had significantly longer 3- and 5-year TTR and OS than those with SYK(L)-negative HCC ($P = 0.037$ and 0.013 , respectively; log-rank test; Figure 6A and B). In contrast to SYK(L), positive SYK(S) expression predicted a worse disease outcome. Patient with SYK(S)-positive HCC had significantly shorter TTR and OS than those with SYK(S)-negative tumors (both $P < .001$; Figure 6C and D, Supplementary Table 2). The tumor recurrences after surgical resection were divided into early recurrences (≤ 24 months, commonly regarded as a true metastasis disseminated from primary tumors) and late recurrences (> 24 months, likely to be *de novo* carcinogenesis) (30, 31). Among patients with SYK(L)-negative tumors, the incidence of early recurrence was markedly higher than those with SYK(L)-positive HCC (77.6% vs. 56.4%; $P = 0.009$, chi-square test). By contrast, no difference in late recurrence was found between these two groups (26.8% vs. 30.8%; $P = 1.00$). SYK(S)-positive cases showed a higher rate of both early recurrence (80.3% vs. 53.8%; $P = 0.001$) and late recurrence (66.7% vs. 16.7%; $P = 0.002$) than SYK(S)-negative patients.

We divided all cases into three groups based on SYK status: L+/S-, L-/S-, and L+/S+. SYK(L+/S-) was associated with better histological differentiation (I/II). By contrast, SYK(L-/S-) or SYK(L+/S+) expression was associated with poor differentiation (III/IV). We compared TTR and OS among the three groups and found that the 5-year tumor recurrence rate of SYK(L+/S-) patients (32.4%) was significantly lower than that of SYK(L-/S-) or SYK(L+/S+) patients (84.5% and 91.2%, respectively; $P < 0.001$). The 5-year OS rate of SYK(L+/S-) patients (86.9%) was significantly higher than that of SYK(L-/S-) or SYK(L+/S+) patients (24.1% and 16.9%, respectively; $P < 0.001$; Figure 6 E and F, Supplementary Figure 5).

All clinicopathologic factors that were found to be prognostic by the univariate analyses, except those involved in BCLC stage system (tumor size, tumor number and vascular invasion), were entered into a multivariate model to identify independent predictors of TTR and OS. Our analysis showed that AST level, BCLC stage, and SYK status were independent factors that affected TTR. We also found that portal hypertension, tumor differentiation, BCLC stage, and SYK(L)/(S) expression were independent predictors of OS among HCC patients (Table 2). Among all parameters, loss of SYK(L) and presence of SYK(S) were the two most powerful independent predictors of TTR and OS.

Discussion

Tumor recurrence and metastasis remain major obstacles to the long-term survival of patients with HCC. Early intervention with aggressive systemic treatment offers a significant survival benefit. A better understanding of molecular events governing the pathogenesis of HCC metastasis is critically needed. The identification of biological markers

for aggressive therapy to impede disease progression is highly desirable for the improvement of clinical outcome. Here, we report that SYK(S) expression is a frequent alteration in HCC that is associated with poor prognosis resulting from increased tumor invasion and metastasis.

In agreement with the results obtained in breast cancer (32–34), we found in the present study that SYK(L) suppressed the proliferation and invasion of HCC cells. Lowered SYK(L) expression presumably promotes tumor progression by stimulating cell growth and metastasis. Indeed, down-regulation of SYK(L) has been linked to metastasis in multiple cancer types, including oral (10), pancreatic (7), and breast cancer (35). However, SYK(L) was reported to promote tumor malignancy in ovarian cancer (15). We speculate that SYK(L) may have functions that differ depending on tumor type; there is precedent for this concept for other cellular proteins (36–38). Our present study showed decreased SYK(L) expression in 38% of HCC specimens. In addition to SYK(L) down-regulation, the SYK(S) variant was found in 40% of HCC cases but was virtually absent in matched non-tumor samples (or normal liver tissues without cirrhosis). Contrary to SYK(L), SYK(S) expression led to increased growth but compromised apoptosis of HCC cells. More importantly, our *in vitro* and *in vivo* functional studies showed that SYK(S) contributed to tumor invasion and metastasis, which likely accounted for shorter TTR and OS among patients with SYK(S)-positive tumors. We found that SYK(S) expression regulate the levels of E-cadherin and vimentin, both of which have been reported as important EMT markers involving in HCC metastasis (28, 39). Moreover, we found that ERK activation was critical for SYK(S)-induced EMT. How deletion of 23 amino acid residues is able to convert the full-length SYK to a protein with completely opposite phenotypes is not understood. One possible explanation is that aberrantly expressed SYK(S) interferes with normal SYK(L) signaling, which would predict that SYK(S)-associated phenotypes rely on the presence of SYK(L). However, expression of SYK(S) by itself in SYK-negative cells, such as SMMC7721, is sufficient to stimulate cell growth and invasion. These results suggest that the proposed SYK(S) oncogenic signaling is rather SYK(L)-independent. Nevertheless, SYK(S)-inducible responses appear to be similar to those mediated by SYK(L) down-regulation, suggesting that SYK(L) and SYK(S) may interact with common signaling molecules to elicit downstream effects, albeit in opposite directions. Taken together, our data indicate that about three-quarters of HCC cases may be attributed to deregulated SYK signaling via two mechanisms: SYK promoter hypermethylation that lowers SYK(L) expression or alternatively splicing that creates SYK(S) variant.

Tumor-specific SYK(S) expression raises the possibility of using SYK(S) as a biomarker in HCC, which we believe would offer considerable therapeutic benefits. First, L+/S+ tumors are more frequently found in HCC patients with the presence of intrahepatic multiple nodules, with absent or incomplete tumor capsules, with vascular invasion, or with poor tumor differentiation, or with advanced BCLC stage, all of which are associated with increased invasiveness of malignancy and metastasis (Table 1). The elevated metastatic potential associated with abnormal SYK(S) expression is also supported by evidence in HCC cell lines. MHCC-97H cells with a high metastatic potency, had a higher level of SYK(S) than MHCC-97L cells (Figure 1). Most importantly, patients with SYK(S)-positive tumors had an increased risk of recurrence, which led to a reduction in postoperative OS. Hence, SYK(S) expression appears to be a more powerful independent prognostic marker of TTR and OS in HCC than other clinicopathologic variables, including SYK(L) expression. Although not all recurrent tumors express SYK(S), our findings indicate that SYK(S) status can predict very poor prognosis regardless of tumor size, the presence of multiple nodules or vascular invasion. Collectively, our results suggest that SYK(S) could be a new predictor of HCC prognosis as associated with tumor invasion and metastasis. Second, qualitative detection of SYK(S) is more desirable in a clinical setting than quantitative analysis [such as

SYK(L) level], which is often subjective. Inclusion of SYK(S) in an immunohistochemical testing panel would be a feasible approach to provide diagnostic and prognostic evaluations of HCC. Because high-titer antibodies that are uniquely reactive to SYK(S) are not available, we used qRT-PCR in the present study to evaluate the expression of two SYK isoforms. Preparation of antibodies against SYK(S) is expected to be challenging; to prevent cross-reactivity to SYK(L), only a restricted peptide sequence comprising the joined flanking residues can be used as an immunogen. An alternative approach to differentiate SYK variants is to evaluate cytoplasmic and nuclear SYK immunostaining by a pan-SYK antibody. This method appears to be feasible in gastric cancer, in which the 5-year survival rate was found to be significantly lower among patients with negative SYK expression than in those with nuclear SYK expression (19). Whether SYK cytoplasmic and nuclear distribution offers prognostic value in HCC needs to be further investigated.

In conclusion, SYK(L) and SYK(S) expression are strong indicators of outcome after primary tumor resection in HCC patients. SYK(L)/(S) status may be used to identify high-risk patients who may benefit from timely aggressive adjuvant therapy after primary tumor resection. As an adverse prognostic factor associated with metastatic phenotypes of HCC, SYK(S) can be used as a diagnostic marker for early metastasis after curative resection of primary HCC. These efforts to provide personalized therapy are expected to improve the overall clinical management of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of SYK(L) and SYK(S) in HCC

(A) Measurement of SYK(L) and SYK(S) protein level in HCC cell lines by immunoblotting. GAPDH was used to control equivalent loading. HCT116 cells with both SYK(L) and SYK(S) expression were used as a control. (B) The relative expression of SYK(L) and SYK(S) in HCC cell lines was detected by qRT-PCR; GAPDH was used an internal control. The bars represent the mean $\pm$ SD of 3 independent experiments. (C&D) Boxplots of the relative expression of SYK(L) (C) and SYK(S) (D) mRNA levels in normal liver tissue without cirrhosis, non-tumor tissues, and their matched HCC counterparts. Data are presented as medians (line), 25th and 75th percentiles (box), and maximum and minimum percentiles (bars). P values were corrected by using the Bonferroni method.

Figure 2. SYK(L) and SYK(S) exhibit opposing activities

(A) Inhibition of cell growth by SYK(L) and stimulation of cell growth by SYK(S). SMMC7721 cells stably expressing SYK(L), SYK(S), or control vector (immunoblotting as shown in insert) were grown in dishes for 1 to 5 days (mean $\pm$ SD; $*P < 0.05$). (B) Colony formation of SMMC7721 cell lines stably expressing SYK(L) or SYK(S). (C) Opposing effects of SYK(L) and SYK(S) on *in vivo* tumor growth. Xenografts in nude mice were established by subcutaneous injection of SMMC7721 cells stably expressing SYK(L) or SYK(S). The tumor volumes were measured and recorded every three days, and tumor growth curves were created for each group (mean $\pm$ SD; $n = 6$; $*P < 0.05$). Four weeks later,

mice were euthanized and tumors were weighed. The mean value is indicated by a solid line. Bonferroni-correction was used to determine statistical significance. **(D&E)** Opposing apoptotic responses to SYK(L) and SYK(S). SMMC7721 cell lines used in A were serum-starved for 24 h (D) or treated with 20 μ M cisplatin for 36 h (E). Cells that underwent apoptosis were captured by annexin-V staining followed by flow cytometry analysis. The percentage of apoptotic cells, including early and late ones, was calculated and is summarized in the bar chart. The values represent the mean $\pm$ SD of 3 independent experiments. After the treatments, the levels of PARP were also detected by Western blot analysis. β -actin was used as a loading control.

Figure 3. Effects of SYK(L) and SYK(S) on tumor invasion and metastasis

(A) Expression of SYK(L) and SYK(S) in MHCC-97H stable cells as assessed by immunoblotting. (B) MHCC-97H and SMMC7721 cells stably expressing SYK(L) or SYK(S) were subjected to *in vitro* Matrigel invasion assay. Cells that invaded through the Matrigel were stained with Hoechst 33342 and counted (mean $\pm$ SD; $n = 3$; Student's *t*-test). Left panel, a representative microscopy image of invaded cells in three independent experiments. (C) MHCC-97H stable cells were injected orthotopically into the livers of nude mice. Seven weeks after injection, mice were euthanized and their lungs were harvested for evaluation of metastatic foci. Top: Macroscopic metastatic nodules on the surface of lung (arrowheads) were quantified. Statistical analysis was performed using a Mann-Whitney *U*-test. Bottom: Sections of lung tissues were H&E-stained to analyze microscopic metastatic nodules (shown within blue outlines). (D) The stable cell lines made from both SMMC7721

and MHCC-97H were subjected to RT-PCR using primers specific for the indicated genes. β_2 -microglobulin (β_2 -MG), internal control. **(E)** SMMC7721 and MHCC-97H cells stably expressing SYK(L) or SYK(S) were cultured in serum-free media. Activity of MMP2 in conditioned media was quantified (mean $\pm$ SD; n = 3; Student's *t*-test).

Figure 4. SYK(S), but not SYK(L), promotes tumor invasion by inducing EMT

(A) E-cadherin expression patterns (low or high staining) were analyzed in 152 HCC patients with negative vs positive SYK(L)/(S) mRNA expression using the two-sided Fisher exact test. A significant negative correlation ($P = 0.002$) between SYK(S) and E-cadherin was shown in the right panel, but not SYK(L) and E-cadherin ($P = 0.869$; Left). (B) Representative immunostained images of E-cadherin and Vimentin protein expression in the serial sections from the same HCC tissues with SYK(S) mRNA negative or positive expression, respectively. Scale bars, 100 μ m. (C) Immunoblotting of SYK and EMT markers showed in five HCC cell lines with different metastasis potentials, which MHCC-97H have the relatively highest ability. (D) Over-expression SYK(S) expression dramatically suppressed the E-cadherin with the increase of vimentin level. Consistently,

knocking down SYK(S) by siRNA could recover E-cadherin expression, and suppress vimentin level. The other EMT markers were not obviously influenced by the change of SYK(S). (E) Expression of SYK(S) in SMMC7721 stable cells detected by immunoblotting. HCT116 cells with both SYK(L) and SYK(S) expression were used as a control. (F) IHC measurement of levels in E-cadherin and vimentin levels in xenograft tumors (Figure 2C). Sections of xenograft tissues were also H&E-stained.

Figure 5. ERK activation is critical for the SYK(S)-induced EMT

(A, B) SYK(S)-overexpressing Huh7 cells were treated with U0126 (20 μ M) and LY294002 (20 μ M) for 12 h (A), or with ERK1/2 and Akt siRNA for 48 h (B), and subjected to western blot analysis with indicated antibodies. (C) Immunoblotting analysis of the activation of ERK1/2, Akt, and EMT markers (E-cadherin, N-cadherin and Vimentin) in Huh7 cells overexpressing either SYK(L), empty vector or SYK(S). (D) These proteins (C) were also examined by immunoblotting after SYK depletion by siRNA in Huh7 cells [with only SYK(L) protein expression], and SMMC7721-SYK(S) stable cells, respectively. GAPDH was used as a loading control.

Figure 6. Time to recurrence (TTR) and overall survival (OS) curves based on SYK(L) and/or SYK(S) status

TTR and OS curves were generated based on the SYK(L) (A&B) or SYK(S) expression (C&D), or SYK(L)/(S) co-expression (E&F) status from 152 HCC samples, respectively. Actuarial probabilities were calculated by the Kaplan-Meier method and compared with the log-rank test in HCC patients after curative resection.

Table 1

Correlation of SYK(L) and SYK(S) mRNA expression with clinicopathologic features in 152 HCC patients

Features	Total	% of patients showing positive mRNA	
		SYK(L)	SYK(S)
Gender			
Female	17	64.7% (11/17)	35.3% (6/17)
Male	135	61.5% (83/135)	40.7% (55/135)
<i>P</i> -value		1.000	.795
Age, y			
50	81	64.2% (52/81)	45.7% (37/81)
> 50	71	59.2% (42/71)	33.8% (24/71)
<i>P</i> -value		.616	.184
HBsAg			
Negative	13	61.5% (8/13)	46.2% (6/13)
Positive	139	61.9% (86/139)	39.6% (55/139)
<i>P</i> -value		1.000	.769
AFP, µg/L			
< 20	37	64.9% (24/37)	32.4% (12/37)
20 – 400	50	52.0% (26/50)	48.0% (24/50)
> 400	65	67.7% (44/65)	38.5% (25/65)
<i>P</i> -value		.208	.320
Tumor size, cm			
< 2	7	71.4% (5/7)	14.3% (1/7)
2 – 3	13	69.2% (9/13)	53.8% (7/13)
> 3	132	60.6% (80/132)	40.2% (53/132)
<i>P</i> -value		.719	.227
Tumor number			
Single	97	69.1% (67/97)	30.9% (30/97)
Multiple	55	49.1% (27/55)	56.4% (31/55)
<i>P</i> -value		.023	.003
Tumor capsule			
No/incomplete	110	60.9% (67/110)	45.5% (50/110)
Yes	42	64.3% (27/42)	26.2% (11/42)
<i>P</i> -value		.850	.041
Vascular invasion			
No	117	59.0% (69/117)	32.5% (38/117)
Yes	35	71.4% (25/35)	65.7% (23/35)
<i>P</i> -value		.235	.001
Tumor differentiation			
I/II	82	69.5% (57/82)	31.7% (26/82)
III/IV	70	52.9% (37/70)	50.0% (35/70)
<i>P</i> -value		.045	.031

Features	Total	<u>% of patients showing positive mRNA</u>	
		SYK(L)	SYK(S)
Tumor stage (BCLC ^a)			
0/A	20	70.0% (14/20)	40.0% (8/20)
B	105	57.1% (60/105)	33.3% (35/105)
C	27	74.1% (20/27)	66.7% (18/27)
<i>P</i> -value		.196	.007

NOTE. Fisher's exact test. Statistical significance ($P < .05$) is shown in bold.

^aBarcelona Clinic Liver Cancer.