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

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Abstract

The spleen tyrosine kinase (SYK) has been reported as a novel biomarker for human hepatocellular carcinoma, 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 hepatocellular carcinoma. SYK(L) was downregulated in 38% of human specimens of hepatocellular carcinoma 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 pathologic 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 hepatocellular carcinoma specimens. Functional studies showed that SYK(S) promoted tumor growth, suppressed apoptosis, and induced EMT through the extracelluar signal–regulated kinase pathway, counteracting 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 hepatocellular carcinoma. We suggest that SYK(L) downregulation or SYK(S) elevation are strong predictors of poor survival in patients with hepatocellular carcinoma, indicative of a need for aggressive therapeutic intervention.

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Instruction

Hepatocellular carcinoma is one of the most prevalent malignances worldwide (1). Treatment of hepatocellular carcinoma remains highly challenging because of the high incidence of tumor recurrence and metastasis even after surgical resection (2, 3). It is clinically relevant to understand the molecular changes associated with hepatocellular carcinoma recurrence and metastasis and to identify significant biomarkers with which to monitor disease progression.

Spleen tyrosine kinase (SYK) is a nonreceptor 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, hepatocellular carcinoma, 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 Fig. S1A). Although 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). Overexpression 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 hepatocellular carcinoma (9), and checkpoint kinase 1 phosphorylated SYK(L) to promote its subsequent proteasomal degradation, which induces hepatocellular carcinoma development (20). It was, however, unclear whether SYK(S) was expressed in hepatocellular carcinoma. In the present study, we profiled the expression status of both SYK(L) and SYK(S) in hepatocellular carcinoma and evaluated the prognostic significance and phenotypic effects of SYK(L) and SYK(S).

Materials and Methods

Cell lines and clinical samples

Five hepatocellular carcinoma cell lines (MHCC-97H, MHCC-97L, BEL-7402, Huh7, and SMMC7721) and one human immortalized liver cell line (LO2) were obtained from Liver Cancer Institute of Fudan University (Shanghai, China). Two hepatocellular carcinoma cell lines (PLC/PRF/5 and Hep-3B) and one human colon carcinoma cell line (HCT116) were purchased from the American Type Culture Collection. 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 (MycoAlert; Lonza). Hepatocellular carcinoma tissue samples and matched adjacent nonneoplastic 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 (Guangzhou, China). 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; ref. 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 hepatocellular carcinoma 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. Pooled cells expressing SYK(L), SYK(S), or vector alone were selected with 750 μg/mL of G418 (Calbiochem).

Gene expression analyses

Reverse transcriptase PCR (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

The protocol is described in Supplementary Materials and Methods.

Cell fractionation and immunofluorescence 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 μL of Dulbecco’s Modified Eagle Medium (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 (Guangzhou, China).

Statistical analyses

Survival curves were constructed using the 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 test was used to analyze the association of SYK(L)/SYK(S) expression with various clinicopathologic characteristics. The Student t test or the Mann–Whitney U test was
used to compare the values between subgroups. Data were expressed as mean ± SD. All analyses were performed using the SPSS software (version 16.0).

Results

**SYK(L) and SYK(S) expression in hepatocellular carcinoma**

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 hepatocellular carcinoma cell lines; the colorectal cancer cell line HCT116 was used as a positive control. Five hepatocellular carcinoma 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 hepatocellular carcinoma 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 (Fig. 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 (Fig. 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 hepatocellular carcinomas (tumor and corresponding nontumor tissue) and 30 normal liver tissue samples without cirrhosis (N). SYK(L) expression in hepatocellular carcinoma was found to be significantly downregulated compared with that in either nontumor or N. By contrast, SYK(S) expression was higher in hepatocellular carcinoma than that in nontumor or N (both P < 0.05; Fig. 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 of 152) of hepatocellular carcinomas were SYK(L)-negative, and 40.1% (61 of
SYK(L) possesses oncogenic activities

Our earlier studies showed tumor suppressor function of SYK(L) in hepatocellular carcinoma (20). To verify the activity of SYK(L) in hepatocellular carcinoma 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) (Fig. 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-proliferative tumor growth in athymic mice than parental cells. In contrast, SYK(S) accelerated xenograft growth (Fig. 2C), reaffirming a growth-stimulatory role of SYK(S).

The subcellular distribution of SYK(L) and SYK(S) in hepatocellular carcinoma 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 Fig. S2A–S2C). The distribution of SYK(L) in both nucleus and cytoplasm was confirmed in hepatocellular carcinoma specimens by immunohistochemical staining (Supplementary Fig. S2D). 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 our RNA interference (RNAi) experiments. Suppression of SYK(L) expression resulted in accelerated cell proliferation, whereas inhibited SYK(S) expression was accompanied by a slower growth (Supplementary Fig. S3). 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. Phosphorylated Akt (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 Fig. S4). These results suggest a differential effect of SYK(L) and SYK(S) on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) activity that may be responsible for their opposing effects on cancer cell growth and invasion.

Correlation of SYK(L) and SYK(S) with clinicopathologic 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 hepatocellular carcinoma 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 hepatocellular carcinoma.

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 hepatocellular carcinoma invasion and metastasis. SMMC7721 and MHCC-97H cells were retrovirally infected with SYK(L) or SYK(S) cDNA (Figs. 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 (Fig. 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 hepatocellular carcinoma cells, respectively (Supplementary Fig. S2). For an in vivo metastasis model, the hepatocellular carcinoma 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.
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 inset) were grown in dishes for 1 to 5 days (mean ± 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 3 days, and tumor growth curves were created for each group (mean ± 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. The Bonferroni correction was used to determine statistical significance. D and E, opposing apoptotic responses to SYK(L) and SYK(S). SMMC7721 cell lines used in A were serum-starved for 24 hours (D) or treated with 20 μmol/L cisplatin for 36 hours (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. Values, mean ± SD of three independent experiments. After the treatments, the levels of PARP were also detected by Western blot analysis. β-Actin was used as a loading control.
Table 1. Correlation of SYK(L) and SYK(S) mRNA expression with clinicopathologic features in 152 patients with hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Features</th>
<th>Total</th>
<th>SYK(L) %</th>
<th>SYK(S) %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>64.7%</td>
<td>35.3%</td>
</tr>
<tr>
<td>Male</td>
<td>135</td>
<td>61.5%</td>
<td>40.7%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>1.000</td>
<td>0.795</td>
<td></td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>81</td>
<td>64.2%</td>
<td>45.7%</td>
</tr>
<tr>
<td>&gt;50</td>
<td>71</td>
<td>59.2%</td>
<td>33.8%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.616</td>
<td>0.184</td>
<td></td>
</tr>
<tr>
<td><strong>Hepatitis B surface antigen (HBsAg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>61.5%</td>
<td>46.2%</td>
</tr>
<tr>
<td>Positive</td>
<td>139</td>
<td>61.9%</td>
<td>39.6%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>1.000</td>
<td>0.769</td>
<td></td>
</tr>
<tr>
<td><strong>AFP, µg/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>37</td>
<td>64.9%</td>
<td>32.4%</td>
</tr>
<tr>
<td>20–400</td>
<td>50</td>
<td>52.0%</td>
<td>48.0%</td>
</tr>
<tr>
<td>&gt;400</td>
<td>65</td>
<td>67.7%</td>
<td>38.5%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.208</td>
<td>0.320</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor size, cm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>7</td>
<td>71.4%</td>
<td>14.3%</td>
</tr>
<tr>
<td>2–3</td>
<td>13</td>
<td>69.2%</td>
<td>53.8%</td>
</tr>
<tr>
<td>&gt;3</td>
<td>132</td>
<td>60.6%</td>
<td>40.2%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.719</td>
<td>0.227</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor number</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>97</td>
<td>69.1%</td>
<td>30.9%</td>
</tr>
<tr>
<td>Multiple</td>
<td>55</td>
<td>49.1%</td>
<td>56.4%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.023</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor capsule</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/incomplete</td>
<td>110</td>
<td>60.9%</td>
<td>45.5%</td>
</tr>
<tr>
<td>Yes</td>
<td>42</td>
<td>64.3%</td>
<td>26.2%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.850</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td><strong>Vascular invasion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>117</td>
<td>59.0%</td>
<td>32.5%</td>
</tr>
<tr>
<td>Yes</td>
<td>35</td>
<td>71.4%</td>
<td>65.7%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.235</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor differentiation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II/III</td>
<td>82</td>
<td>69.5%</td>
<td>31.7%</td>
</tr>
<tr>
<td>III/IV</td>
<td>70</td>
<td>52.9%</td>
<td>50.0%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.045</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor stage (BCLC)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O/A</td>
<td>20</td>
<td>70.0%</td>
<td>40.0%</td>
</tr>
<tr>
<td>B</td>
<td>105</td>
<td>57.1%</td>
<td>33.3%</td>
</tr>
<tr>
<td>C</td>
<td>27</td>
<td>74.1%</td>
<td>66.7%</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.196</td>
<td>0.007</td>
<td></td>
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NOTE. Fisher exact test. Statistical significance (P < 0.05) is shown in bold.

(Fig. 3C), suggesting that SYK(L) and SYK(S) impose opposite regulatory effects on hepatocellular carcinoma metastasis.

Secretion of matrix metalloproteinase (MMP) by cancer cells is a key mechanism for accomplishing invasion and metastasis. We explored how expression of MMP2 and MMP9 [and their respective cognate tissue inhibitor of matrix metalloproteinase (TIMP2) and TIMP1] was affected by SYK(L) and SYK(S) in SMCC7721 and MHCC-97H cells. Our semiquantitative 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 (Fig. 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 (Fig. 3E).

**SYK(S), but not SYK(L), is associated with epithelial–mesenchymal transition**

To explore the relationship of SYK(L)/(S) and epithelial–mesenchymal transition (EMT), we performed IHC staining to assess E-cadherin and vimentin levels in 152 hepatocellular carcinoma 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 = 0.732; Fig. 4A and B). We therefore evaluated the protein levels of several EMT markers in 5 hepatocellular carcinoma 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 (Fig. 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 (Fig. 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 (Fig. 4E and F). Taken together, these results indicated that hepatocellular carcinoma 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 phosphoinositide 3-kinase/Akt pathways activate EMT in hepatocellular carcinoma (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 (Fig. 5A). This effect was...
confirmed by the suppression of ERK1/2 or Akt by siRNA (Fig. 5B). In addition, our data showed that SYK(S) increased levels of phosphorylated ERK1/2 (p-ERK1/2), but not p-Akt in hepatocellular carcinoma cells (Fig. 5C and D; Supplementary Fig. S4). 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 hepatocellular carcinoma specimens (Fig. 4A), and SYK(L) did not affect EMT markers in...
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 patients with hepatocellular carcinoma with negative versus 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 is shown on the right, 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 hepatocellular carcinoma tissues with SYK(S) mRNA negative or positive expression, respectively. Scale bars, 100 μm. C, immunoblotting of SYK and EMT markers is shown in five hepatocellular carcinoma cell lines with different metastasis potentials, which MHCC-97H have the relatively highest ability. D, overexpression 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 (Fig. 2C). Sections of xenograft tissues were also H&E-stained.
and 5-year TTR and OS than those with positive hepatocellular carcinoma had significantly longer 3- and 5-year TTR and OS than those with SYK(S)-negative tumors (both P < 0.001; Fig. 6C and D, Supplementary Table S2). 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; refs. 30, 31). Among patients with SYK(L)-negative tumors, the incidence of early recurrence was markedly higher than those with SYK(L)-positive hepatocellular carcinoma (77.6% vs. 56.4%; P = 0.009, χ² 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 histologic 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; Fig. 6E and F, Supplementary Fig. S5).

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 aspartate aminotransferase 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 patients with hepatocellular carcinoma (Supplementary Table S3). 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 hepatocellular carcinoma. Early intervention with aggressive systemic treatment offers a significant survival benefit. A better understanding of molecular events governing the pathogenesis of hepatocellular carcinoma metastasis is critically needed. The identification of biologic 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 hepatocellular carcinoma 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 hepatocellular carcinoma cells.
Lowered SYK(L) expression presumably promotes tumor progression by stimulating cell growth and metastasis. Indeed, downregulation 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 hepatocellular carcinoma specimens. In addition to SYK(L) downregulation, the SYK(S) variant was found in 40% of hepatocellular carcinoma cases but was virtually absent in matched nontumor samples (or normal liver tissues without cirrhosis). Contrary to SYK(L), SYK(S) expression led to increased growth but compromised apoptosis of hepatocellular carcinoma 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 hepatocellular carcinoma 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 seem to be similar to those mediated by SYK(L) downregulation, 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 hepatocellular carcinoma 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.

Figure 6. TTR and OS curves based on SYK(L) and/or SYK(S) status. TTR and OS curves were generated based on the SYK(L) (A and B) or SYK(S) expression (C and D) or SYK(L)/SYK(S) coexpression (E and F) status from 152 hepatocellular carcinoma samples, respectively. Actuarial probabilities were calculated by the Kaplan–Meier method and compared with the log-rank test in patients with hepatocellular carcinoma after curative resection.
in hepatocellular carcinoma cell lines. MHCC-97H cells with a high metastatic potency, had a higher level of STK(S) than MHCC-97L cells (Fig. 1). Most importantly, patients with STK(S)-positive tumors had an increased risk of recurrence, which led to a reduction in postoperative OS. Hence, STK(S) expression seems to be a more powerful independent prognostic marker of TTR and OS in hepatocellular carcinoma than other clinicopathologic variables, including STK(L) expression. Although not all recurrent tumors express STK(S), our findings indicate that STK(S) status can predict very poor prognosis regardless of tumor size, the presence of multiple nodules, or vascular invasion. Collectively, our results suggest that STK(S) could be a new predictor of hepatocellular carcinoma prognosis as associated with tumor invasion and metastasis. Second, qualitative detection of STK(S) is more desirable in a clinical setting than quantitative analysis [such as STK(L) level], which is often subjective. Inclusion of SYK(S) in an immuno-histochemical testing panel would be a feasible approach to provide diagnostic and prognostic evaluations of hepatocellular carcinoma. 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 STK(S), only a restricted peptide sequence comprising the joined flanking residues can be used as an immunogen. An alternative approach to differentiate STK variants is to evaluate cytoplasmic and nuclear SYK immunostaining by a pan-SYK antibody. This method seems 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 hepatocellular carcinoma needs to be further investigated.

In conclusion, STK(L) and STK(S) expressions are strong indicators of outcome after primary tumor resection in patients with hepatocellular carcinoma. STK(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 hepatocellular carcinoma, SYK(S) can be used as a diagnostic marker for early metastasis after curative resection of primary hepatocellular carcinoma. These efforts to provide personalized therapy are expected to improve the overall clinical management of hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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