Expression of Variant SYK Isoforms Determines Prognosis of hepatocellular carcinoma

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Supplementary Figure legends

**Supplementary Figure 1.**  (A) Domain structure of SYK(L) and SYK(S). Sequence and cross-species homology of 23 amino acid residues deleted in SYK(S) are shown.  (B) Relative position of PCR primers unique for SYK(L) or SYK(S).

**Supplementary Figure 2.** Differential subcellular distribution of SYK(L) and SYK(S) in HCC.  (A, B) Huh7 [SYK(L) positive], MHCC-97H [positive for both SYK(L) and SYK(S)], and SMMC7721 stable cells overexpressing SYK(L) or SYK(S) cDNA were subjected to subcellular fractionation. The cytoplasmic (C) and nuclear (N) fractions were then processed together with whole cell lysate for immunoblotting. PARP and α-tubulin were used as nuclear and cytoplasmic markers, respectively. Immunoblotting shows that SYK(S) is localized exclusively in the cytoplasm while SYK(L) in both nuclear and cytoplasm.  (C) Analyses of subcellular localization of SYK(L) or SYK(S) by immunofluorescence. SYK(L) or SYK(S) ectopically expressed in SMMC7721 cells were immunostained with anti-SYK antibody (N-19) that recognizes both SYK(L) and SYK(S). Cell nuclei were stained with DAPI.  (D) IHC detection of SYK(L) in normal liver tissues with cirrhosis, non-tumor liver tissues and HCC specimens. An SYK(L)-specific antibody, anti-SYK-23, was used for immunostaining. Scale bar = 50 μm.

**Supplementary Figure 3.** Effects of suppressed expression of SYK(L) or SYK(S) on cell proliferation and invasion in Huh7 [SYK(L)-positive] (A), MHCC-97H [both SYK(L) and SYK(S)-positive] (B), and SMMC7721 cells ectopically expressing SYK(L) (C) or SYK(S) (D). Cells were transfected with SYK siRNA target pools or scramble sequence (Con). SYK expression in these cells was verified by immunoblotting (left panels). Transfected cells were then subjected to cell proliferation analyses (middle panels) and cell mobility assay (right panels). *P < .05 vs. control, Student’s t-test.
**Supplementary Figure 4.** Suppression of MAPK/Erk signaling by SYK(L), but not SYK(S), in HCC cell lines and xenografts. (A) Cultured MHCC-97H and SMMC7721 cells overexpressing SYK(L) or SYK(S) were subjected to immunoblotting to measure the level of ERK1/2, p38, Jnk, Akt (and their phosphorylated form). The relative quantification of p-ERK1/2 or p-Jnk was also shown. (B) IHC measurement of p-ERK1/2 levels in xenograft tumors established from SMMC7721 stable cell lines expressing SYK(L), SYK(S), or vector control. Scale bar = 50 μm.

**Supplementary Figure 5.** Time to recurrence (TTR) and overall survival (OS) curves based on SYK(L) and SYK(S) co-expression. After resection of primary tumors, patients bearing tumors with SYK(L+/S-) status had longer TTR (A) and OS (B) than those bearing ones with SYK(L-/S-) or SYK(L+/S+). Actuarial probabilities were calculated by the Kaplan-Meier method and compared with the log-rank test.
Supplementary Materials and Methods

**HCC patients**

The inclusion and exclusion for all patients in this study were: (a) distinctive pathologic diagnosis of HCC; (b) no prior anticancer treatment; (c) underwent primary and curative resection of all tumor nodules or cancerous thrombus with the cut surface being free of cancer by histologic examination; (d) availability of qualified tissues; and (e) availability of complete clinicopathologic and follow-up data.

The 152 enrolled patients aged from 23 to 77 with a median of 50. Seventeen (11.2%) of these patients were female. Hepatitis B infection as measured by hepatitis B surface antigen (HBsAg) was detected in 139 (91.4%) patients. Hepatitis C antibody was positive in 4 (2.6%) patients, whose HBsAg was negative. One hundred and thirty-four patients (88.2%) had histologically confirmed liver cirrhosis. Tumor size ranged from 1.2 to 20.0 cm with a median of 7.0 cm. Additional details on clinicopathologic characteristics are provided in Table 1.

The diagnosis of HCC was confirmed by histologic reviews, and the tumor cell differentiation was evaluated using Edmondson-Steiner criteria. The presence of preoperative portal hypertension was evaluated retrospectively. Direct measurement of venous pressure was not performed routinely in our series. Thus portal hypertension was indirectly inferred by: (i) esophageal varices detectable at endoscopy; or (ii) splenomegaly (major diameter, > 12 cm) with a platelet count < 100,000/mm³. None of the patients of the present study group had undergone portosystemic shunts before or at the time of hepatic resection. No case was treated as an emergency.

The last follow-up was in May 31, 2012. The follow-up time ranged from 2 to 88 months (median 47 months). Among all 152 cases, 113 (74.3%) patients further developed metastasis, including 92 intrahepatic and 36 extrahepatic metastases (18 lung, 7 bone, 5 lymph nodes, 3 peritoneum, 2 adrenal cortex, and 1 spleen). None of these patients had significant comorbidities that could condition life-expectancy. Overall survival (OS) was defined as the interval from curative surgery to the date of
death or the date of last contact if the patient was still alive. Time to recurrence (TTR) was defined as the interval from surgery to the date when tumor recurrence was diagnosed. The intrahepatic tumor recurrence or distant metastasis detected by imaging diagnosis after tumor resection was designated as recurrence. The 1-, 3-, and 5-year tumor recurrence rates were 46.1%, 71.1% and 74.5%, respectively. The 1-, 3-, and 5-year OS rates were 72.9%, 41.3%, and 37.1%, respectively. Fifty-nine patients (38.8%) were still alive at the time of last follow-up report.

**RNA extraction, reverse transcription (RT), and quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from HCC cell lines and tissues with Trizol reagent kit (Invitrogen). After treatment with DNase I (TaKaRa, Dalian, China), 2 μg total RNA was used for cDNA synthesis with random hexamers and Superscript III (Invitrogen). The cDNA templates were subjected to PCR amplification. To verify cDNA integrity, β2-microglobulin (β2-MG) expression was also analyzed.

The mRNA levels of SYK(L), SYK(S) and GAPDH was evaluated by qRT-PCR using SYBR Green dye (Invitrogen) and an ABI 7900HT Sequence Detector System (Applied Biosystems, Carlsbad, CA). The cycling parameters were 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s for 45 cycles, followed by a melting curve analysis. The threshold cycle (C_T) values of the housekeeping genes (GAPDH) were determined for normalization purposes, and the ΔC_T between the mean of housekeeping genes values and target genes values was calculated. For normal liver tissues without cirrhosis (N), HCC and matched adjacent non-cancerous liver tissues (NT) samples, the fold change of target gene is given by 2^{-ΔΔC_T}(ΔΔC_T = ΔC_T^{HCC/NT} - ΔC_T^{N}). The average level of SYK(L) in HCCs (0.6279) was roughly 8 times lower than that in Ns (5.0260). The average level of SYK(S) in HCCs (1.6217) was about twice as much as that in Ns (0.8105). Thus, we selected 8 and 2 fold as cutoffs for SYK(L) and SYK(S) positivity, respectively. All samples used in this study were subjected to these cutoff parameters. Values herein are the mean of triplicate in 3 independent experiments. Primer sequence and location are shown in Supplementary Table 1 and Supplementary Fig. S1B.
**RNA interference**

Pre-confluent HCC cells were transfected with SYK ON-TARGET plus SMART pool (L-003176; Thermo Fisher Scientific, Lafayette, CO), and ERK1/2 and Akt siRNA (Cell Signaling Technology; Beverly, MA) using the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) following manufacturers’ instructions. The SYK RNAi sequences were expected to inhibit the expression of both SYK(L) and SYK(S). Scramble RNAi sequences were chosen as controls. Twenty-four hours after transfection, cells were pooled for cell proliferation and Matrigel invasion assays.

**Immunoblotting and Reagents**

Cells were harvested with lysis buffer (Cell Signaling Technology; Beverly, MA) containing protease inhibitor cocktail (Roche). Equivalent amounts (40 μg) of proteins were separated by SDS-PAGE under reducing condition and blotted onto polyvinylidene difluoride membranes (PVDF; Millipore). Primary antibody (1:5,000-1,000) was used for immuno-detection that was afforded by horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) and enhanced chemiluminescence reagents (Pierce, Rockford, IL). The primary antibodies were purchased from Santa Cruz Biotechnology [SYK (N-19; rabbit polyclonal), vimentin (V9; mouse monoclonal), Fibronectin (16E5, mouse monoclonal) and GAPDH (mouse monoclonal)], Sigma-aldrich [β-actin (mouse monoclonal) and Flag (mouse monoclonal)], BD Biosciences [E-cadherin (mouse monoclonal)], Abcam [Twist, (rabbit polyclonal)], Cell Signaling Technologies [PARP, α-tubulin, phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, phospho-p38 (Thr180/Tyr182), phospho-Jnk (Thr183/ Tyr185), phospho -Akt (Ser473) and pan-Akt, Snail, all rabbit monoclonal; N-cadherin, rabbit polyclonal]. ERK1/2 inhibitor U0126 and PI3K/Akt inhibitor LY294002 were purchased from Calbiochem (San Diego, CA).

**Immunohistochemistry (IHC) staining**
Formalin-fixed and paraffin-embedded tissue samples were cut in 4-μm sections and mounted on polylysine-coated slides. Sections were then deparaffinized in xylene and rehydrated with alcohols. After samples were treated with 3% hydrogen peroxide in methanol for 15 min to exhaust endogenous peroxidase activity, slides were boiled in 0.01 M sodium citrate buffer (pH 6.0) using a microwave oven to retrieve antigens. After 60 min of preincubation with 10% normal goat serum to block nonspecific staining, samples were incubated overnight in a humidified container at 4°C with a primary antibody, either anti-E-cadherin (1:100), anti-vimentin (1:100), anti-p-Erk1/2 (1:300), or anti-SYK-23 (1:100), in a humidified container at 4°C. Anti-SYK-23 is an SYK(L)-specific antibody generated by immunizing rabbits with conjugated peptide \( ^{283}TWSAGGIISRIKSYSFPKPGHRK^{305} \). The tissue slides were treated with a non-biotin horseradish peroxidase detection system according to the manufacturer’s instructions (Gene Tech, Shanghai, China).

The results of IHC were evaluated by two different pathologists who specialize in liver cancer. The intensity of staining for E-cadherin, vimentin and SYK(L) on the scale of 0-3 according to the percentage of positive tumor (0, < 5% positive cells; 1, 5-20%; 2, 20-50%; and 3, > 50%) were recorded. E-cadherin, vimentin and SYK(L) expression were classified as high-level when the score reached 1.5; if the score ≤ 1.5, the case was classified as low-expressing.

**Cell fractionation**

The extraction and isolation of nuclear and cytoplasmic protein were carried out by using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). Approximately \( 1 \times 10^7 \) cells (parental or stable lines) were collected by centrifugation. The cell pellet was resuspended in cytoplasmic protein extraction reagent A, followed by incubation on ice for 15 min. The cytoplasmic protein extraction agent B was then added. After vortexing and incubation on ice, the cell suspension was spinned at 14,000 \( \times \) g for 5 min at 4°C. The supernatant was collected as the cytosolic fraction. The remaining pellet was resuspended in nuclear protein extraction reagent. After intermittent vigorous vortexing, the suspension was subjected to centrifugation at
12,000 xg at 4°C for 10 min. The supernatant was collected as the nuclear fraction. The cytoplasmic and nuclear fractions were analyzed by immunoblotting.

**Immunofluorescence**

SMMC7721 stable cell lines expressing SYK(L) or SYK(S) were plated and attached onto culture slides (Costar, Cambridge, MA) in 24-well template. Twenty-four hours later, adherent cells were rinsed with phosphate buffered saline (PBS) and fixed in ice-cold methanol-acetone for 10 min at room temperature. The cells were then treated for 30 min with 10% BSA in PBS, followed by incubation with primary SYK antibody (N-19) for 2 h at room temperature. After washes with PBS for three times, the slides were incubated for 1 h in the dark with secondary goat anti-rabbit antibodies (Invitrogen). Then the slides were stained with 4-, 6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) for 5 min to visualize cell nuclei. Immunostaining signals were captured by using an Olympus confocal imaging system (Olympus FV100).

**Cell proliferation assay**

Cell proliferation was measured with a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan). Two thousand cells suspended in 100 μl of culture medium were seeded in each well of a 96-well plate. At indicated time points, 10 μl CCK-8 reagents were added into the media. Cells were subsequently incubated for 2 h before measurement of absorbance at 450 nm. Three independent experiments were performed.

**Colony formation assay**

SMMC7721-SYK(L), SYK(S) and Vector stable cells were plated in 6-well plates in triplicate at 300 single cells/well, and cultured with complete medium for 10 days. After most of the single cells had expanded to > 50-cell colonies, cells were fixed in methanol and stained with crystal violet. After washing out the dye, the plates were photographed. To quantify the colonies objectively, Quantity One software was used.
and colonies that were larger than the averaging parameter of 3 or 1 and the minimum signal intensity of 1.0 were counted.

**Apoptosis assay**

SMMC-7721 cells stably expressing SYK(L) or SYK(S) were plated overnight to attach to dishes. Cells were then incubated either for 24 h in serum-free medium or for 36 h in complete medium containing 20 μM cisplatin. Both adherent and floating cells were then pooled and washed twice with ice-cold 1× PBS. Annexin-V labeling of apoptotic cells was carried out by using an annexin V-FITC Apoptosis Detection Kit I (BD Biosciences; Franklin Lakes, NJ) following the manufacturer's protocol. The FITC and propidium iodide (PI) signals from 12,000 individual cells were analyzed for each sample by FACS flow cytometry (Becton Dickinson; San Jose, CA). Cells that were annexin-V(+)/PI(-) and annexin-V(+)/PI(+) were considered early and late apoptotic cells, respectively.

**Matrix metalloproteinase-2 (MMP2) activity assay**

To measure the active MMP2 proteins that were secreted into the medium, cells were plated at a density of 6×10^5 cell/well in a 6-well dish. After cells attached to the plate overnight, they were incubated with serum-free medium for 24 h before the conditioned medium was collected and cleared by filtration. The conditioned media were then subjected to MMP2 activity assay using a kit from GE Healthcare Biosciences (Piscataway, NJ).