Signal Transducers and Activators of Transcription 5b Activation Enhances Hepatocellular Carcinoma Aggressiveness through Induction of Epithelial-Mesenchymal Transition

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Abstract

Poor prognosis of hepatocellular carcinoma (HCC) is associated with a high potential of vascular invasion and metastasis. Epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion process. Recently, signal transducers and activators of transcription 5 (STAT5) has been linked to tumor progression by EMT induction. However, the precise roles of STAT5 genes (STAT5a and STAT5b) in human epithelial cancers have not been elucidated clearly. The aim of this study is to analyze the roles of STAT5 isoforms in HCC progression using HCC clinical samples. We showed that activation of STAT5b, but not STAT5a, was found in HCC clinical samples and its expression was significantly associated with younger age (P = 0.037), advanced tumor stages (P = 0.003), venous infiltration (P = 0.016), microsatellite formation (P = 0.024), multiple tumor nodules (P = 0.02), and poor patient survival. To specifically investigate the mechanism underlying constitutive activation of STAT5b in HCC, EGFP-HBX was introduced into Huh-7 cells. STAT5b activation in HCC is at least partially mediated by HBX activation. Ectopic STAT5b transfection conferred increased HCC cell motility and invasiveness by induction of EMT changes. In conclusion, STAT5b activation enhanced HCC aggressiveness by induction of EMT, which was possibly mediated by HBX activation. STAT5b could serve as a novel molecular target for HCC treatment. (Cancer Res 2006; 66(20): 9948-56)

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and second most common cancer in Hong Kong (1, 2). Poor prognosis of HCC is associated with a high potential of vascular invasion, metastasis, and recurrence even after curative surgical resection (3). HCC invasiveness is the ability of tumor cells to invade the capsule and portal vein (4, 5). Epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion process whereby epithelial cell layers lose polarity together with cell-cell contacts and then undergo a dramatic remodeling of the cytoskeleton (6). The loss of E-cadherin expression is a hallmark of EMT (6). E-cadherin plays a central role in cell-cell adhesion junctions in maintenance of cell polarity and environment (7, 8). Loss of E-cadherin expression is commonly associated with tumor invasiveness, metastasis, and poor prognosis, including HCC (7). Understanding the molecular mechanism of EMT could allow the development of novel therapies targeting at HCC invasion.

Signal transducers and activators of transcription (STAT) signaling is well known to be activated by cytokines and growth factor receptors and is involved in a wide variety of cellular processes, including differentiation, survival, or cell growth (9, 10). Seven STAT genes have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Among these STAT genes, constitutive activation of STAT1, STAT3, and STAT5 has been shown in a variety of cancer types (11–13). Each STAT gene shows different functions. STAT1 functions as a tumor suppressor gene, whereas STAT3 and STAT5 have shown to play a role in development and tumor progression. Recently, STAT5 has been shown to play a key role in tumor progression through induction of proliferation and inhibition of apoptosis. STAT5 activation has been shown primarily in hematopoietic malignancies, which are associated with Bcl-Abl fusion protein (14). Later, STAT5 was correlated with aggressiveness of solid tumors, such as prostate cancer (13). Thus far, a variety of cytokines and growth factors, including epidermal growth factor, have been shown to stimulate STAT5 activation (15). STAT5 is a latent cytoplasmic protein, which is composed of two highly homologous isoforms, STAT5a and STAT5b (16). Like other STAT members, STAT5 becomes activated by phosphorylation of a specific tyrosine residue, after which phosphorylated STAT proteins dimerize and translocate to nucleus to regulate gene transcription (17, 18). STAT5a and STAT5b are derived from closely linked genes on chromosome 11 with exhibition of 93% amino acid identity. Although STAT5a and STAT5b share high homology at the amino acid level, their expression patterns vary in different cancer types. Some reports have shown that STAT5a is implicated in breast cancer (19), whereas STAT5b is correlated with prostate cancer progression (20). The above intriguing data raised our interest in examining the significance of STAT5 activation in HCC.

Although STAT5 was reported to be activated in various cancer types, its downstream modulating pathway is not clear. Among those limited reports, STAT5 activation was found to turn on gene expression of antiapoptotic proteins, such as Bcl-xL, which contributes to tumor progression (21). Apart from its antiapoptotic role, recent evidence has suggested the involvement of STAT5 in EMT, although the underlying mechanism is far from understood (22, 23). Given the significant role of EMT in HCC invasion, a positive role of STAT5 activation in HCC cancer-promoting HCC invasiveness by down-regulation of E-cadherin expression was...
hypothesized. Thus far, there have been no data on the role of STAT5 in HCC. In addition, a distinct role of STAT5 genes (STAT5a and STAT5b) in human epithelial cancers has not been elucidated yet. In this study, STAT5a/STAT5b expression, nuclear localization, and tyrosine phosphorylation status were evaluated in a large series of 50 primary HCC tissues through combination of immunohistochemistry, immunoprecipitation, and Western blot. Our result first showed STAT5b, but not STAT5a, activation in HCC clinical samples, and its expression was significantly correlated with aggressive tumor behaviors. In addition, STAT5b activation was correlated with poor clinical outcome. STAT5b activation was much prominent in hepatitis B virus (HBV)-related HCC when compared with non-hepatitis B and non-hepatitis C type HCC. Our in vitro result showed that STAT5b activation in HCC was at last in part mediated by HBX activation. Consistent with the clinical significance of STAT5b in HCC clinical samples, ectopic STAT5b transfection conferred increased HCC cell motility and invasion by induction of EMT changes. This line of evidence provides not only the prognostic significance of STAT5b activation in HCC but also the novel regulatory mechanism of STAT5b activation, leading to HCC invasiveness.

Materials and Methods

Patient samples. Liver tumor and adjacent nontumor tissues were obtained with informed consent from 50 patients who underwent hepatectomy for HCC from 1999 to 2001 in the Department of Surgery, The University of Hong Kong, Queen Mary Hospital (Pokfulam, Hong Kong). These 50 samples were prospectively collected and randomly selected to do this study. There were 42 males and 8 females. The median age of the patients at diagnosis was 52.5 years (range, 16-73 years). Forty patients had solitary tumors, whereas the other 10 patients had multifocal tumor nodules detected grossly by preoperative imaging analysis or intraoperative ultrasonography. Thirty-two patients had underlying cirrhosis. All patients had a potentially curative resection with tumor-free margin macroscopically and microscopically.

Immunostaining. Formalin-fixed and paraffin-embedded sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven [12 minutes in sodium citrate buffer (pH 6)], the endogenous peroxidase was inhibited by 0.3% H2O2 for 30 minutes, and the sections were incubated with 10% normal goat serum for 30 minutes. Rabbit polyclonal anti-STAT5a (1:700; Santa Cruz Biotechnology, Santa Cruz, CA), anti-E-cadherin (1:100; Zymed Laboratories, Inc., South San Francisco, CA), and rabbit serum against HBX antigen (from Dr. M.A. Feitelson, Fox Chase Cancer Center, Philadelphia, PA) were applied overnight in a moist chamber at 4 °C. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA) was applied. Briefly, biotinylated goat anti-rabbit immunoglobulin, goat anti-mouse, and avidin-biotin peroxidase complex were applied for 30 minutes each, with 15-minute washes in PBS. The reaction was finally developed by DAKO Liquid 3,3′-Diaminobenzidine Substrate-Chromogen System (DAKO). Nuclear STAT5a and STAT5b expressions were regarded as positive staining, whereas absence of nuclear staining and presence of cytoplasmic expression were regarded as negative staining. The staining was evaluated by two independent observers (K.M. and K.N.).

Plasmids and reagents. pCI-STAT5b was a gift from Dr. Warren Leonard (Laboratory of Molecular Immunology, NIH, Bethesda, MD; ref. 24), and E-cadherin promoter was a gift from Prof. S.W. Tsao (Department of Anatomy, The University of Hong Kong). EGFP-HBX was constructed by inserting HBX gene (subtype ayw, accession no. U95551; 1376-1840) containing flanked EcoRI and SalI restriction sites into pEGFP-C2 vector (Clontech, Palo Alto, CA). Rabbit serum against HBX antigen was a gift from Dr. M.A. Feitelson.

Cell lines. Two metastatic HCC cell lines, MHCC-97L and MHCC-97H (low and high metastatic potential, respectively, from Liver Cancer Institute, Fudan University, Shanghai, China; ref. 25), and nonmetastatic cell lines Huh-7 (a gift from Dr. H. Nakabayashi, Hokkaido University School of Medicine, Sapporo, Japan; ref. 26), PLC (Japanese Cancer Research Bank, Tokyo, Japan), and HepG2 and Hep3B (American Type Culture Collection, Manassas, VA) were maintained in DMEM with high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 mg/ml penicillin G, and 50 μg/ml streptomycin (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO2.

Cell transfection. The cells were cotransfected with 2 μg of plasmid DNA of either pCI-STAT5b or pCI and neomycin using Fugene 6 according to the manufacturer's protocol (Boehringer Mannheim GmbH, Mannheim, Germany). After 48 hours, the medium was replaced with fresh DMEM with geneticin (G418) at 0.7 mg/ml. After 2 weeks of clonal selection, all the clones were grown in the presence of G418 at 0.4 mg/ml to ensure stable transfection. Isolated clones were expanded to 25 cm2 flasks. All the transfected cells used in this experiment were in early passages (passages 4-8). For transient transfection of EGFP-HBX, the procedure was the same as above except no selection by G418.

Luciferase promoter assay. Huh-7 cells (5 × 104 per well) were plated into 24-well culture plates and allowed to grow for 24 hours. E-cadherin promoter (provided by Prof. S.W. Tsao) and pRL-CMV-Luc were cotransfected with either pCI-STAT5b or pCI into the cells using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN). The cells were lysed 48 hours after transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was measured at 48 hours after transfection, and the reading was then normalized with the Renilla luciferase activity, which served as internal control for transfection efficiency. Each experiment was done at least thrice in duplicate wells, and each data point represented the mean and SD. The percentage decrease in luciferase activity of E-cadherin promoter was calculated relative to that of the vector controls. The mean percentage decrease in luciferase activity was presented as the final results, and the SD of the mean was used as error bars.

E-cadherin immunofluorescence staining. Cells were plated onto chamber slides in DMEM at ~70% confluence for 24 hours. They were fixed in ice-cold acetone and methanol (1:1), washed with PBS, and then stained with mouse anti-E-cadherin (1:100; Zymed Laboratories) at 4°C. After washing, the cells were applied with goat anti-mouse FITC or anti-rabbit tetramethyl rhodamine isothiocyanate–conjugated secondary antibody for 30 minutes at room temperature and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 1 minute at room temperature. The cells were examined under a fluorescence microscope.

Wound-healing and invasion assays. Cell migration was assessed by measuring the movement of cells into a scraped, acellular area created by 200 μl pipette tube (time 0), and the speed of wound closure was monitored after 24 hours. Invasion assays were done with 24-well BioCoat Matrigel Invasion Chambers (Becton, Dickinson and Company, Franklin Lakes, NJ) using 5 × 104 cells in serum-free DMEM and plated onto either control or Matrigel-coated filters. Conditioned medium from PLC or PLC-Twist cells was placed in the lower chambers as chemoattractants. After 22 hours in culture, the cells were removed from the upper surface of the filter by scraping with a cotton swab. The cells that invaded through the Matrigel and were adherent to the bottom of the membrane were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid, and its absorbance at 395 nm was determined. Each experiment was done in triplicate, and the mean values ± SE were presented.

Immunoprecipitation. Whole-cell protein extracts were incubated with 1 μg anti-phosphotyrosine monoclonal antibody (PY20; Transduction Laboratories, Inc., Lexington, KY) for 1 hour at 4°C followed by immunoprecipitation by protein A/G for 1 hour. After washing thrice with radiomunoprecipitation assay buffer, precipitated proteins were centrifuged and boiled with 2× sample buffer. STAT5 activation was determined by immunoblotting with either STAT5a or STAT5b antibody (Santa Cruz Biotechnology).

Western blotting. The cells were lysed, and protein extraction was done. The samples were separated in 10% SDS-polyacrylamide gel and
electrophoretically transferred to polyvinylidene difluoride membrane (Amersham, Buckinghamshire, United Kingdom). The membrane was blotted with 10% nonfat milk, washed, and then probed with STAT5a, STAT5b, α-actin, and vimentin (Santa Cruz Biotechnology) and E-cadherin (Zymed Laboratories). After washing, the membrane was incubated with horseradish peroxidase–conjugated anti-mouse or rabbit or goat antibody (Amersham) and then visualized by enhanced chemiluminescence plus according to the manufacturer's protocol.

Statistical analysis. Continuous data were expressed as median and range and compared between groups using the Mann-Whitney U test. Pearson test was used for bivariate correlation comparison. Significance was defined as P < 0.05. Categorical variables were compared using the χ² test (or Fisher's exact test where appropriate). All statistical analyses were done using a statistical software (Statistical Package for the Social Sciences version 9.0 for Windows, SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant. Disease-free survival and overall survival were analyzed by Kaplan-Meier method, and the results were compared by log-rank test. Multivariate analysis was done using the Cox proportional hazards model to identify independent prognostic factors.

Results

Increased STAT5b, but not STAT5a, activation in HCC clinical samples. To determine STAT5 activation in HCC, we evaluated the intracellular protein expression of STAT5a and STAT5b in 50 HCC clinical samples by immunostaining. Strong
expression of STAT5a was observed in nontumor liver (cirrhotic liver and chronic hepatitis), whereas absent to weak expression of STAT5b was observed (Fig. 1A). In 50 cases of primary HCC, nuclear STAT5a and STAT5b expression could be detected in 23 of 50 (46%) and 34 of 50 (68%), respectively (Fig. 1A). STAT5a and STAT5b protein expression in primary HCC and their matched nontumor counterparts was summarized in Table 1. To further confirm the relative contribution of STAT5a and STAT5b to HCC, we did immunoblotting studies using antibody specific for each STAT5 isoform. These experiments showed down-regulation of STAT5a protein in HCC tumor when compared with nontumor liver (Fig. 1B). On the contrary, STAT5b protein was found to be up-regulated in HCC tumor (Fig. 1B). To determine STAT5a/STAT5b phosphorylation levels, immunoprecipitation was done on the same set of HCC clinical cases. Consistently, this experiment showed a 3.3-fold increase in STAT5b expression in HCC tumor when compared with the nontumor counterpart. However, a 1.7-fold decrease in STAT5a expression was found in HCC tumor.

Clinical significance of STAT5b activation in HCC. By immunostaining of STAT5b on 50 cases of clinical HCC samples, 34 of 50 cases (68%) showed positive nuclear staining. The correlation between STAT5b activation with clinicopathologic features was summarized in Table 2. These 34 cases with STAT5b activation were significantly associated with younger age ($P = 0.037$), advanced tumor stages ($P = 0.003$), venous infiltration ($P = 0.016$), microsatellite formation ($P = 0.024$), number of tumor nodules ($P = 0.02$), and HBV infection ($P = 0.037$). We then analyzed the disease-free survival and overall survival by comparing the HCC patients with or without positive STAT5b expression in tumor cells. Assessed by Kaplan-Meier analysis (Fig. 2), patients with STAT5b overexpression had a shorter disease-free survival than patients who had no STAT5b

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<th>STAT5b protein expression (n)</th>
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*Fisher’s exact test.
**$\chi^2$ test.
*Significant difference.
Total number <50 due to missing data.
overexpression \((P = 0.019)\). Consistently, patients with STAT5b overexpression had a shorter overall survival than those who did not show STAT5b overexpression \((P = 0.045)\). STAT5b expression, tumor-node-metastasis (TNM) stage, venous infiltration, and tumor size were compared by Cox regression analysis of these factors with forward stepwise procedure. Among the above factors, only STAT5b and TNM stage were independent indicators of poor prognosis. The relative risks for the disease-free survival for TNM stage and STAT5b expression were 2.6 \([95\% \text{ confidence interval (CI)}, 1.3-4.5; P = 0.002]\) and 0.45 \([95\% \text{ CI}, 0.3-1.6; P = 0.031]\), respectively.

STAT5b activation in HBV-related HCC. A previous report has shown that Janus-activated kinase (JAK)-STAT pathway was induced by HBX transactivation \((27, 28)\). To assess the possible correlation of STAT5b activation with HBV infection in HCC clinical samples, we further did immunostaining of STAT5b on 25 cases of non-hepatitis B or non-hepatitis C–related HCC and compared with the results obtained in HBV-related HCC. Our result showed that STAT5b expression was found in 72\% \((32 \text{ of } 44)\) in HBV-related HCC. On the contrary, overexpression of STAT5b was found in 36\% \((9 \text{ of } 25)\) of non-hepatitis B or non-hepatitis C–related HCC. The above result showed that STAT5b overexpression was significantly correlated with HBV infection \((P = 0.003; r = 0.395; r^2 = 0.156; \text{Fig. } 3)\). The tumor staging was similar between the patients with HBV and those without HBV/HCV. To further characterize the role of HBX in the activation of STAT5b, an \textit{in vitro} experiment was done by transfecting the HBX-expressing construct into Huh-7 cells. As shown in \text{Fig. } 3B, a remarkable increase in both STAT5b and STAT5b phosphorylation levels was seen on transfection of EGFP-HBX. The above result suggested that STAT5b activation was at least in part mediated by HBX activation.

Ectopic STAT5b up-regulation led to increased invasion ability. The above clinical data suggested the role of STAT5b in

![Figure 2. Correlation of STAT5b with disease-free survival and overall survival. A and B, Kaplan-Meier curves for disease-free survival and overall survival analysis according to STAT5b expression in tumor tissue. The mean disease-free survival and overall survival of HCC patients with STAT5b expression in their tumors were 24.15 and 39.49 months compared with 47.6 and 56.59 months in patients without STAT5b expression, respectively.](image)

![Figure 3. Correlation of STAT5b with HBX in HCC. A, correlation between STAT5b with HBX was shown in consecutive sections of HCCs. Top, case 385 showed both strong immunostaining of cytoplasmic HBX and nuclear STAT5b expression; bottom, in case 656 (non-hepatitis B or non-hepatitis C–related HCC), it showed negative staining for both STAT5b and HBX. B, direct correlation between HBX and STAT5b was evaluated by transient transfection of EGFP-HBX into Huh-7 cells. After transient transfection, increased HBX expression was found by increased detection of green fluorescent protein (GFP). Accompanied with HBX transfection, increased STAT5b and STAT5b-p expressions were detected by Western blot.](image)
dissemination of tumors. To further confirm this correlation, we used six HCC cell lines with various metastatic potential and evaluated STAT5b and STAT5b-p levels by Western blot. Consistently, both STAT5b and STAT5b-p protein levels were found to be elevated in metastatic HCC cell lines (MHCC-97L and MHCC-97H) when compared with primary nonmetastatic ones (HepG2, PLC, Hep3B, and Huh-7; Fig. 4A). We then investigated the effect of STAT5b activation on HCC cell invasion in vitro by ectopic transfection of STAT5b cDNA into Huh-7 cell, which showed the lowest expression of STAT5b in the Western blot. After transfection and subsequent G418 selection, four different clones and a pool of Huh-7-STAT5b transfectants were isolated. Among these clones, clone 1 showed the highest expression of STAT5b, whereas clone 4 showed the lowest (Fig. 4B). Next, we studied the invasion and migration ability of Huh-7 after ectopic STAT5b transfection. As shown in Fig. 4C, by Matrigel invasion assay, an approximately 105% to 235% increase in cell invasion was observed in various STAT5b-transfected Huh-7 cell lines when compared with empty vector control. This result showed that STAT5b increased the invasive ability in this HCC cell line. The cell migration was also investigated by wound-healing assay. As shown in Fig. 4D, the migration rate of Huh-7-STAT5b (clone 1) was much higher than the vector control. These results indicated that STAT5b activation might lead to increase the invasive and migratory abilities of HCC cell lines.

Ectopic introduction of STAT5b led to EMT activation through E-cadherin repression. Previous data suggested the novel role of STAT5b in HCC cell invasiveness in vivo. EMT is a key event in tumor invasion whereby epithelial cell layers lose cell-cell contacts. Given that loss of E-cadherin is the major event in EMT, we postulated that STAT5b activation might lead to EMT changes through repression of E-cadherin. To test this hypothesis, we evaluated E-cadherin expression in various Huh-7-STAT5b transfectants by Western blot. As shown in Fig. 5A, a decrease was shown in the epithelial marker E-cadherin in various Huh-7-STAT5b transfectants. On the contrary, gain of the fibroblast marker vimentin was found on STAT5b activation. Such EMT changes resulted in a morphologic change of Huh-7-STAT5b (clone 1). STAT5b overexpression resulted in morphologic changes from tightly packed colonies to scattered growth structure (Fig. 5B). E-cadherin expression was also evaluated by immunofluorescence staining as the membrane localization indicated its biological function. For PLC-empty vector transfectant, E-cadherin was mainly localized at the cell membrane, whereas E-cadherin was mainly detected in the cytoplasm of Huh-7-STAT5b transfectant (clone 1; Fig. 5C). To further examine whether STAT5b inactivated E-cadherin transcriptionally, we then cotransfected the pCI or pCI-STAT5b together with a luciferase reporter harboring E-cadherin promoter and generated transient transfectants. We found that E-cadherin promoter activity was decreased by ~ 4.1-fold in STAT5b transfectants when compared with the vector control (Fig. 5C). To examine the negative correlation between STAT5b and E-cadherin in HCC clinical tumor cases, we further did E-cadherin immunostaining on the same set of 50 cases of HCC tumor samples. Loss of E-cadherin expression was found in 28 of 50 HCC cases. Thirty-three patients had positive STAT5b expression. It was found that STAT5b was negatively correlated with E-cadherin expression ($P = 0.006; r = -0.384; r^2 = 0.147$; Fig. 5D).

Discussion

In the present work, we first showed STAT5b but not STAT5a activation in HCC clinical samples. STAT5b activation was associated with HCC aggressive behavior and poor clinical outcome. It was also an independent predictor of HCC poor prognosis in addition to TNM staging. In addition, we also showed that STAT5b activation was at least partly induced by HBX transactivation. Consistent with the HCC clinical correlation of STAT5b, ectopic introduction of STAT5b led to increased invasiveness of HCC cells by induction of EMT changes. Our data not only provided the mechanistic insight on the role of STAT5b in HCC invasiveness but also added STAT5b to the class of EMT inducers.

STAT5 activation has been previously reported in hematopoietic malignancies and prostate and breast cancers (13, 14, 19). For prostate cancer, STAT5 activation was associated with a high
histologic grade (20). Although constituent activation of STAT5 was reported in cancers, they have not distinguished activation of STAT5a from STAT5b, resulting in failure to determine the key functional role of each STAT5 isoform. Studies of knockout mice revealed the functional difference between these two closely linked STAT proteins. STAT5a-deficient mice showed defective mammary gland development and lactogenesis (29), whereas STAT5b exhibited the functions of growth hormone action (30). Consistent with the above findings, different isoforms have distinct roles in cancer. STAT5a was found to play an important role in breast cancer progression (31). In contrast, STAT5b activation contributes to carcinogenesis of head and neck cancer (32). Due to such contrasting data, it is of great importance to determine not only STAT5 activation but also the distinct role of each STAT5 isoform in HCC. From our protein expression study of STAT5a and STAT5b in HCC clinical samples, STAT5a protein was down-regulated in HCC tumor tissues when compared with the corresponding nontumor counterparts. On the contrary, STAT5b protein was found up-regulated in tumor cells. The Western blot and immunoquantitation results also supported the above observation. In addition, STAT5b activation was significantly correlated with the aggressive behavior of liver cancer cells. From the available data on the differential expressions of each STAT5 isoform in cancers, it seems that expression of each isoform is cancer type specific. STAT5 activation alone has not been reported to be an essential event for cellular transformation. However, the aggressiveness of STAT5b has been shown by accelerating v-Src-induced cell motility and cell growth in NIH-3T3 cells (33). These studies suggested that STAT5b may potentiate the aggressive behavior of cancers, which is consistent with our finding on the clinical significance of STAT5b. By Kaplan-Meier analysis, STAT5b activation was significantly associated with shorter disease-free survival and overall survival. From this result, STAT5b activation can serve as a good marker for poor prognosis of HCC patients.

Figure 5. Effect of ectopic STAT5b expression on EMT changes. A, we examined the expression of STAT5b, E-cadherin, and vimentin in various STAT5b-Huh-7 transfecteds and Huh-7-pCI by Western blot. Decrease in E-cadherin and gain of vimentin expression in various Huh-7-STAT5b transfecteds. B, STAT5b induced morphologic changes from tightly packed colonies to scattered growth structure. C, by immunofluorescence staining, we found that E-cadherin was mainly detected in the cytoplasm of Huh-7-STAT5b transfected (clone 1), indicating function inactivation. Red arrows, membrane staining of E-cadherin in Huh-7-pCI. The cells were counterstained with DAPI. To further examine whether STAT5b inactivated E-cadherin transcriptionally, we did promoter assay. Decreased promoter activity was found in Huh-7-STAT5b transfected when compared with the vector control. D, correlation between STAT5b with E-cadherin was shown in consecutive sections of HCCs. Case 135 showed no immunostaining of E-cadherin but was strong for STAT5b (top), whereas case 168 showed strong E-cadherin expression but with no STAT5b expression (bottom). It was found that STAT5b was negatively correlated with E-cadherin expression ($P = 0.007; r = -0.384; r^2 = 0.147$).
The mechanism for STAT5 activation in human cancers is not clear and may probably depend on the specific cell type and activating stimuli in the tumor microenvironment. In prostate cancer, STAT5 can be activated by prolactin in prostate epithelium (20). In our HCC clinical specimens, most of them are HBV related. In addition, one of the STAT family members, STAT3, was previously reported to be activated by HBX activation (28). Taken together, it raised the possibility that STAT5b was activated by HBV infection. Figure 3A showed 36% STAT5b positive in 25 cases of non-hepatitis B or non-hepatitis C–related HCC clinical samples, which is in great contrast with the results obtained in HBV-related HCC tumors. By statistical analysis, STAT5b activation was significantly associated with HBV infection in our patients’ samples. To confirm the direct interaction of HBX in STAT5b activation, we transiently transfected EGF-P–HBX, one of the well-defined proteins produced by HBV, into Huh-7 cells and examined the activation status of STAT5b by Western blot. Our results showed that both STAT5b expression level and its phosphorylation form were elevated. A previous report showed that JAK1-STAT pathway was activated by HBX transactivation (27). However, it remains unclear of which STAT5 isoform gets activated. Our data suggested that STAT5b activation was at least partly mediated by HBX activation. However, our current data did not provide a detailed mechanism for interaction of HBX and STAT5b and thus it needs further investigation.

Invasiveness is a key step that leads to metastasis resulting in poor prognosis (3). Therefore, it is of great value to study the molecular mechanism of HCC invasiveness. Our data showed that both STAT5b expression and phosphorylation status was elevated in metastatic cell lines when compared with nonmetastatic primary ones. The above results strongly suggested the role of STAT5b in HCC invasiveness. The direct effect of STAT5b on HCC aggressiveness was confirmed by stable transfection of STAT5b cDNA into Huh-7 cell, which showed the lowest STAT5b expression. Consistently, ectopic STAT5b introduction led to increased invasion ability as evidenced by wound-healing and invasion assay.

Recently, increasing evidence has shown that EMT, a process first identified in embryogenesis, (34), mediates tumor progression, including local invasion, spreading through the circulation and metastasis. Several developmental genes that induce EMT have been shown to act as E-cadherin repressors. The first of these is the Zinc finger protein Snail, a DNA-binding factor that recognizes E-box motifs in target promoters, such as E-cadherin (35). Therefore, we hypothesized that STAT5b induced EMT-mediated invasion through E-cadherin repression. As loss of E-cadherin expression is the hallmark of EMT, we examined whether STAT5b directly induced EMT changes by stable ectopic transfection of STAT5b cDNA into Huh-7 cell. This experiment showed that ectopic STAT5b conferred morphologic changes from epithelial to fibroblastic appearance, which was accompanied with a gain of mesenchymal marker vimentin and loss of epithelial markers, such as E-cadherin. In addition, these changes were accompanied with increased translocation of E-cadherin from membrane to cytoplasm, indicating function inactivation.

To further examine whether STAT5b inhibited transcription from E-cadherin promoter via E-boxes, which were also targeted by Snail, we did the promoter assay. The result (Fig. 5D) showed that STAT5b repressed E-cadherin expression transcriptionally through suppression of E-cadherin promoter. However, it remains to be determined whether the interaction of STAT5b with these E-boxes is direct or through other mediators. However, our result is in contrast with the previous report showing an inhibitory effect of STAT5 in breast cancer cell invasiveness by up-regulation of E-cadherin (23). STAT5 was also found to be overexpressed and associated significantly with good prognosis of breast cancer (19). In view of this contrasting result, we postulated that prognostic indication of STAT5b is cancer type specific.

In conclusion, we showed for the first time that STAT5b was correlated with HCC aggressive behavior and poor clinical outcome. STAT5b activation was at least partially mediated by HBX activation. STAT5b induced HCC invasiveness through EMT. Our findings not only provide a molecular basis for the role of STAT5b in HCC but also suggest a novel therapeutic target for the treatment of HCC.

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Signal Transducers and Activators of Transcription 5b Activation Enhances Hepatocellular Carcinoma Aggressiveness through Induction of Epithelial-Mesenchymal Transition
