Identification of c-Yes expression in the nuclei of hepatocellular carcinoma cells: Involvement in the early stages of hepatocarcinogenesis

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Abstract. It is thought that the subcellular distribution of Src-family tyrosine kinases, including c-Yes binding to the cellular membrane, is membranous and/or cytoplasmic. c-Yes protein tyrosine kinase is known to be related to malignant transformation. However, the expression patterns of c-Yes in hepatocellular carcinoma (HCC) remains unknown. In the present study, we report that c-Yes is expressed not only in the membrane and cytoplasm, but also in the nuclei of cancer cells in some human HCC tissues and in a human HCC cell line. We examined the expression and localization of c-Yes in human HCC cell lines (HLE, HLF, PLC/PRF/5 and Hep 3B) by Western blotting and immunohistochemical analyses; we also examined the expression of c-Yes by immunohistochemistry and Western blotting in the tissues of various liver diseases, including 39 samples from HCC patients. We used an antibody array to detect proteins that bind to nuclear c-Yes in PLC/PRF/5 cell line. c-Yes was found to be expressed in the membranes and cytoplasm of HLE, HLF and Hep 3B HCC cells; it was also detected in the nuclei in addition to the membranes and cytoplasm of PLC/PRF/5 HCC cells. HCC with nuclear c-Yes was detected in 5 of 39 cases (13.0%), and nuclear c-Yes expression was not detected in normal, chronic hepatitis or cirrhotic livers. All HCCs with nuclear c-Yes expression were well-differentiated, small tumors at the early stages. In the PLC/PRF/5 cell line, the nuclear localization of c-Yes with cyclin-dependent kinase 1 was confirmed by a protein antibody array. In conclusion, nuclear c-Yes expression was found in cancer cells at the early stages of hepatocarcinogenesis, suggesting that nucleus-located c-Yes may be a useful marker to detect early-stage HCC.

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Introduction

A large number of cellular protein tyrosine kinase (PTK) genes have been cloned and sequenced. They may be classified into two major groups: growth factor receptor PTKs, and retroviral PTKs or their cellular homologues (1). The primary representatives of the latter are non-receptor-linked and membrane-associated (2). There are at least 9 known Src-related tyrosine kinases: c-Src, c-Yes, c-Lck, c-Fyn, c-Hck, c-Lyn, c-Blk, c-Fgr and c-Yrk proto-oncogene products (3). The cellular oncogene c-Yes, a member of the Src family, encodes a 62-kDa, cytoplasmic and membrane-associated PTK (4). c-Yes expression and its kinase activity are known to increase in colorectal cancer (5,6), melanoma (7,8), hepatocellular carcinoma (HCC) (9) and metastatic liver cancer (10).

It is thought that Src-family tyrosine kinases including c-Yes bind to the cellular membrane; their subcellular distribution is membranous and/or cytoplasmic. However, we report here that c-Yes is expressed not only in the membrane and cytoplasm, but also in the nuclei of cancer cells in a subset of HCC cells. In addition, nuclear colocalization of c-Yes with cyclin-dependent kinase 1 (Cdk1) was confirmed by a protein antibody array. To the best of our knowledge, this report is the first study demonstrating that the expression of c-Yes was detected in the nuclei of HCC cells. Furthermore, all HCCs with nuclear c-Yes expression were well-differentiated, small tumors at the early stages.

Materials and methods

Tissue samples. Tissue samples were obtained by surgical resection or liver biopsy from 39 patients with HCC (31 males, 8 females; mean age, 65.6±11.9 years; range, 31-89

Table I. Clinical parameters of HCC patients with and without nuclear c-Yes expression.

	Expression of c-Yes in nuclei			
	(-)	(+)	Total	Test
	34	5	39	
Age (years)				
Mean ± SD	65.9±11.7	63.2±14.1	65.6±11.9	p=0.7365
Med (Min-Max)	68.5 (31-89)	67 (40-77)	68 (31-89)	Wilcoxon
Sex				
Men	28	3	31	p=0.2677
Female	6	2	8	Fisher
HBs Ag (+) or HCV (+)				
HBs Ag (+)	6	1	7	p=1.0000
HCV (+)	23	4	27	Fisher
Non B-non C	5	0	5	
CH or LC				
CH	9	1	10	p=1.0000
LC	25	4	29	Fisher
Differentiation of HCC				
Poor	9	0	9	p=0.0005[a]
Moderate	21	0	21	Mantel-Haenszel
Well	4	5	9	
Stage				
I	1	3	4	p=0.0027[a]
II	6	1	7	Mantel-Haenszel
III	8	1	9	
IV A	15	0	15	
IV B	4	0	4	
Diameter of tumor (mm)				
Mean ± SD	49.9±30.0	20.6±7.3	46.1±29.3	p=0.0216[a]
Med (Min-Max)	40 (15-100)	20 (11-30)	40 (11-100)	Wilcoxon
Portal thrombus				
(-)	27	5	32	p=0.5628
(+)	7	0	7	Fisher
AFP (ng/ml)				
Mean ± SD	38656±154614	38.9±46.8	33705±144676	p=0.1104
Med (Min-Max)	203 (1-874000)	21 (3.5-117)	117 (1-874000)	Wilcoxon
PIVKA-II (mAU/ml)				
Mean ± SD	4995±17817	21±11.5	4529±16997	p=0.0560
Med (Min-Max)	417 (10-95700)	20 (10-33)	356.5 (10-95700)	Wilcoxon
AFP-L3 (%)				
Mean ± SD	33.5±31.0	7.5±7.1	29.4±29.9	p=0.2325
Med (Min-Max)	27.5 (0-84.9)	6.6 (0-16.9)	16.9 (0-84.9)	Wilcoxon

[a]Significantly different between nuclear c-Yes-positive and -negative groups.

years). Of the 39 patients, 27 were positive for hepatitis C virus (HCV)-RNA and 7 were positive for hepatitis B surface antigen (HBs Ag), and the remaining 5 were negative for both (Table I). The histological grade and the stage of HCC were determined according to the criteria of the International Working Party (11) and the International Union against Cancer (12). The fibrotic stage of the liver was evaluated according to the classification by Desmet *et al* (13).

Additional tissues were obtained by liver biopsy from 58 patients (41 males and 17 females; mean age, 57.8±9.4 years; range, 40-76 years) with chronic hepatitis (CH) including cirrhosis. Fifty-three patients with CH were positive for HCV-RNA and 4 patients were positive for HBs Ag. Fifty-eight CH cases included 12 at fibrosis stage 1 (F1), 8 at F2, 9 at F3 and 29 at F4. Five normal liver samples were obtained from patients with liver metastases of colon cancer (4 males and 1 female; mean age, 60.2±4.1 years; range, 56-67 years) during surgery. All serum samples of these patients were negative for both HCV and HBs Ag.

Cell culture. HLE, HLF, Hep 3B and PLC/PRF/5 cells, a kind gift of the Health Science Research Resource Bank (Osaka, Japan), were used as human HCC cell lines. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco), 100 μg/ml penicillin and 100 μg/ml streptomycin at 37˚C under 5% CO_2 in air.

Chemicals and antibodies. Avidin-biotin-peroxidase complex (ABC) kits were purchased from Funakoshi Chemical Co. (Tokyo, Japan). All other chemicals were purchased from Sigma Chemical Co. or Wako Pure Chemical Co. (Tokyo, Japan), unless otherwise mentioned. Two anti-c-Yes monoclonal antibodies used in this study were purchased from Santa Cruz Biotechnology (clone F7; Santa Cruz, CA, USA) and Transduction Laboratories (clone 1; Tokyo, Japan). The optimal dilutions of antibodies used for Western blotting in the present study were as follows: anti-c-Yes antibody (clone 1), 1:1000; anti-horseradish peroxidase (HRP)-anti-mouse IgG, 1:2000. The optimal dilution of anti-c-Yes antibody (clone F7) for immunohistochemistry was 1:500.

Immunohistology for human liver tissues. We prepared 2-μm sections from formalin-fixed, paraffin-embedded tissue blocks. Sections of the HCC tissue specimens were immunohistologically stained using the ABC method, as described in our previous study (14-16). Briefly, to detect c-Yes, sections were placed in 10 mM citrate buffer (pH 6.0) and processed at 500 W at 95˚C for 10 min in a microwave oven. Sections were deparaffinized in xylene, dehydrated in a graded series of alcohol solutions, and then mixed with a solution containing 0.5% hydrogen peroxide to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were processed for immunostaining. Primary incubation was performed overnight at 24˚C with the monoclonal antibody (clone F7) against c-Yes. Immunoreactivity products were visualized using diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. The specificity of immunostaining was examined using non-immune mouse IgG as a negative control for primary antibodies.

Immunohistology of HCC cell lines. Immunohistological staining of c-Yes in human HCC cells was performed by means of the ABC method as described in our previous report (16). Briefly, HCC cells were cultured on tissue culture chamber slides (Nunc, Naperville, IL, USA). The cells were rinsed three times with ice-cold PBS, air-dried at room temperature, fixed with acetone for 1 min and washed three times with PBS at room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. The cells were washed with PBS, immunostained, and then incubated overnight at 24˚C with monoclonal antibody (clone 1) against c-Yes. Immunoreactivity products were visualized using diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin.

Gel electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) was performed following the technique described by Laemmli (17), and Western blotting was performed as described by Towbin *et al* (18), using the optimal dilution of primary antibodies and HRP-linked secondary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Japan Co., Tokyo, Japan) on a radiograph film. The exposure time for Western blotting was 30 sec at room temperature for all samples.

Preparation of subcellular fractionations from liver tissues and human HCC cells. In order to examine the distribution of c-Yes in the liver tissues and HCC cell lines, we thawed and homogenized the samples with 2 volumes of lysis buffer containing 0.25 M sucrose, 0.1 M $MgCl_2$, 10 mM Tris-HCl at pH 7.4, 1 mM of each of the protease inhibitors phenylmethylsulphonylfluoride, pepstatin and aprotinin, and 50 mM leupeptin. The homogenate was centrifuged at 8,000 x g for 30 min to remove unbroken tissues and nuclei. The supernatant was then centrifuged at 100,000 x g for 60 min in a Beckman Airfuge to separate the membranous and cytoplasmic fractions. The particulate and supernatant were the membranous fraction and cytoplasmic fraction, respectively. The membranous fraction was washed with a lysis buffer, and then resuspended in a solubilization buffer (2.3% SDS, 62.5 mM Tris-HCl at pH 6.6, 10 mM ethylaminediamine-N, N, N', N'-tetra acid disodium salt tetrahydrate and protease inhibitors). Equivalent amounts of protein from the membranous and cytoplasmic fractions were used for SDS-PAGE and Western blotting.

The extraction of the nuclear protein was performed following the method described in our previous report (19). All steps were carried out at 4˚C. Liver tissue and cell line samples were homogenized in 5 volumes of 50 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose, 1 mM ethylene glycol-bis (ß-aminoethyl ether)-N, N, N', N'-tetraacetic acid, 3 mM benzamidine, 0.1 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 25 mM KCl and 5 mM $MgCl_2$. The homogenate was centrifuged at 600 x g for 10 min. The pellets were collected and homogenized in 2 volumes of 2.2 M sucrose containing 3.3 mM $CaCl_2$, 3 mM bezamidine, 0.1 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin. After centrifugation at 4,000 x g for 60 min, the pellet was collected in the same buffer and subjected to SDS-PAGE and Western blot analyses.

Protein assay. The protein concentration was determined according to the Bradford's dye-binding assay (20).

Antibody array to screen proteins binding to nuclear c-Yes. To detect proteins binding to nuclear c-Yes, we used an antibody array (Hypromatrix, Inc., Worcester, MA, USA) in

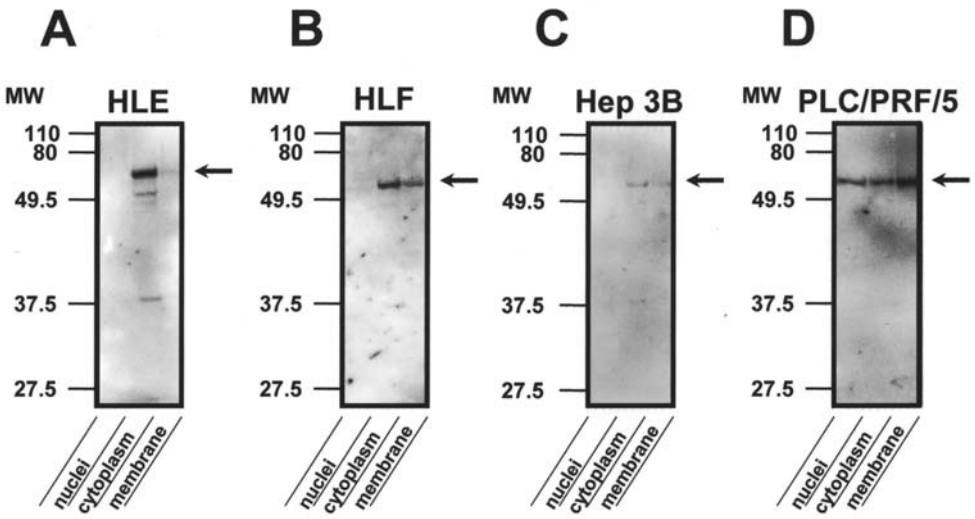

Figure 1. c-Yes expression in the nuclear, cytoplasmic and membranous fractions of various HCC cell lines. A, B, C and D represent Western blot analyses of c-Yes in the HLE, HLF, Hep 3B and PLC/PRF/5 cell lines, respectively. Note that the expression of c-Yes in PLC/PRF/5 cells was detected not only in the cytoplasmic and membranous fractions, but also in the nuclear fraction. Arrows indicate bands corresponding to c-Yes.

which 400 antibodies against signal transduction molecules are immunobilized at each predetermined position on the nitrocellulose membrane. The antibody array membranes were incubated for 1 h at room temperature with 5% dry milk in Tris-buffered saline Tween-20 (TBST) consisting of 150 mM NaCl, 25 mM Tris-HCl, 0.05% Tween-20 (pH 7.5), and then incubated with nuclear lysates from nuclear c-Yes positive PLC/PRF/5 and nuclear c-Yes negative Hep 3B cells for 2 h. After incubation, the membranes were washed with TBST three times for 10 min each time and incubated for 2 h with HRP-conjugated anti-c-Yes monoclonal antibody. Interactive proteins for nuclear c-Yes in PLC/PRF/5 cells were visualized using diaminobenzidine as the color substrate.

Statistical analysis. Data are expressed as means ± SD. The significance of differences between observations was determined according to the Wilcoxon, Fisher and Mantel-Haenszel tests. Statistical significance was set at $p<0.05$.

Results

Western blotting of c-Yes in nuclear, cytoplasmic and membranous fractions of the HCC cell lines, HLE, HLF, Hep 3B and PLC/PRF/5. The expression of c-Yes in HLE (Fig. 1A), HLF (Fig. 1B) and Hep 3B (Fig. 1C) cells was detected in cytoplasmic and membranous fractions, but was not found in nuclear fractions (Fig. 1, arrows); it was detected in the cytoplasmic, membranous and nuclear fractions in the PLC/PRF/5 cell line (Fig. 1D).

Immunohistochemistry of c-Yes in the HCC cell lines, HLE, HLF, Hep 3B and PLC/PRF/5. The expression of c-Yes in HLE (Fig. 2A), HLF (Fig. 2B) and Hep 3B (Fig. 2C) cells was localized in both the cytoplasm (Fig. 2A-C, arrows) and membranes (Fig. 2A-C, arrowheads), while its expression in PLC/PRF/5 cells was also identified in the nuclei (Fig. 2D, arrows) as well as in the cytoplasm and membrane. The localization of c-Yes in HLE, HLF, Hep 3B and PLC/PRF/5 cells based on immunohistochemical analysis was consistent with the results obtained by Western blotting (Fig. 1).

Immunohistochemistry of c-Yes in human normal, CH and cirrhotic livers. Immunohistological studies of c-Yes showed no staining in hepatocytes of normal (Fig. 3A) or CH (Fig. 3B) liver samples. In contrast, c-Yes expression in cirrhotic livers was detected in the cytoplasm and membranes of hepatocytes along the limiting plates and at the periphery of regenerating nodules (Fig. 3C, arrow). However, the expression of c-Yes was not detected in the nuclei of hepatocytes in cirrhotic livers. No nuclear c-Yes expression was detected in any normal, CH or cirrhotic liver samples analyzed in the present study.

c-Yes expression in malignant liver tissues. Immunohistological examinations showed that the expression of c-Yes in human HCC was divided into two staining patterns: the cytoplasmic and nuclear staining patterns. As shown in Fig. 4A, HCC with cytoplasmic c-Yes staining pattern had weak c-Yes expression in the membranes of some cancer cells (arrow heads), but did not have nuclear c-Yes expression. As shown in Fig. 4B, HCC with nuclear c-Yes staining pattern had weak c-Yes expression in the membranes and cytoplasm of cancer cells. In total, the cytoplasmic staining pattern was detected in 34 (87%) of 39 HCC cases and the nuclear staining pattern was detected in the remaining 5 (13%) cases.

Western blotting of c-Yes in the membranous, cytoplasmic and nuclear fractions of human HCC tissues. Fig. 5 shows the Western blotting results of HCC samples corresponding to Fig. 4, respectively. In HCC tissues without nuclear c-Yes expression, c-Yes expression was detected in the membranous and cytoplasmic fractions, but not in the nuclear fraction (Fig. 5A), while in those with nuclear c-Yes expression, it was found in the nuclear fraction of cancer cells as well as in the membranous and cytoplasmic fractions (Fig. 5B). These data are consistent with the immunohistochemical results (Fig. 4).

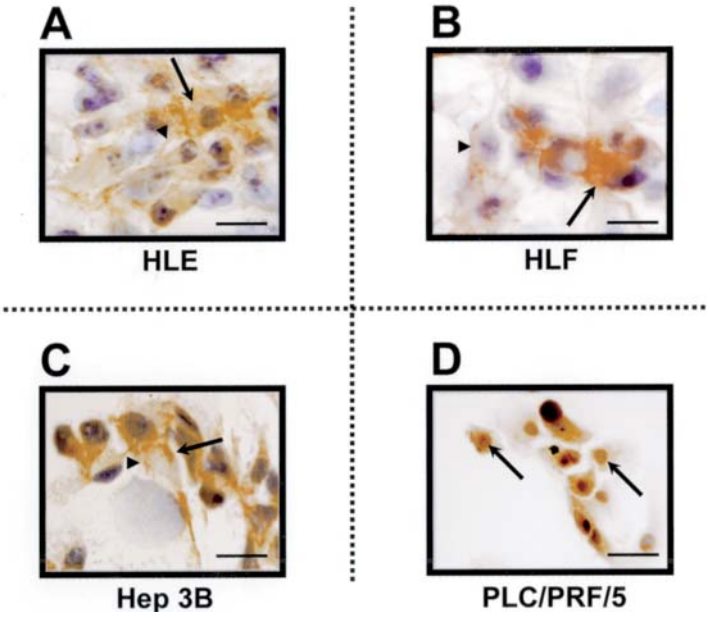

Figure 2. Immunohistological localization of c-Yes in HCC cell lines. A, B, C and D represent immunohistochemical analyses of c-Yes in the HLE, HLF, Hep 3B and PLC/PRF/5 cell lines, respectively. The expression of c-Yes in HLE, HLF and Hep 3B cells was localized in both the cytoplasm (A-C, arrows) and membranes (A-C, arrow heads), while its expression in PLC/PRF/5 cells was also detected in the nuclei (D, arrows) as well as in the cytoplasm and membranes. All cell lines were counterstained with Mayer's hematoxylin. Bars, 20 μm.

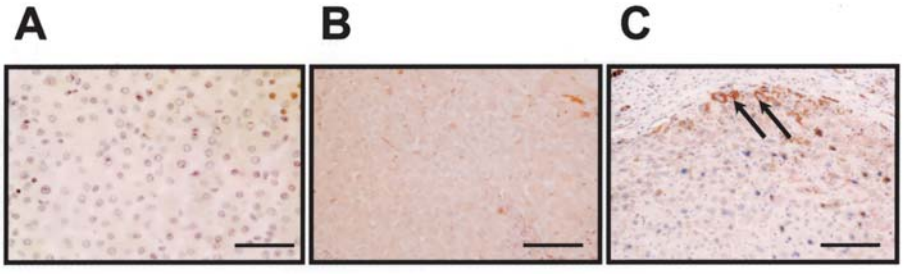

Figure 3. Immunohistochemistry of c-Yes in normal, CH and cirrhotic livers. A-C show sections reacting with a c-Yes antibody in normal, CH and cirrhotic samples, respectively. No c-Yes expression was detected in normal (A) or CH (B) samples, however, in the cirrhotic samples (C) it was immuno-stained in some hepatocytes along the limiting plates and at the periphery of the regenerating nodules (arrows). Note that no expression of c-Yes was detected in the hepatocellular nuclei of normal, CH or cirrhotic samples. All sections were counterstained with Mayer's hematoxylin. Bars, 50 μm.

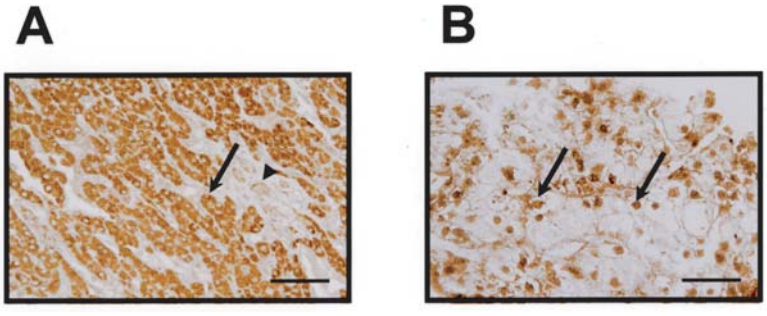

Figure 4. Immunohistochemistry of c-Yes in human HCC. A and B show moderate and well-differentiated clear cell HCC, respectively. (A) Shows the cytoplasmic (arrow), membranous staining pattern (arrow head) of c-Yes, while (B) shows the nuclear staining pattern (arrows) as well. In the present study, HCC without nuclear c-Yes was detected in 34 (87%) of 39 cases, and HCC with nuclear c-Yes was identified in the remaining 5 (13%). All sections were counterstained with Mayer's hematoxylin. Bars, 50 μm.

Relationship between HCC with nuclear c-Yes and clinical parameters. As shown in Table I, HCC with nuclear c-Yes expression was detected in 5 (13%) of 39 HCC cases. HCC cases with nuclear c-Yes expression were detected at a

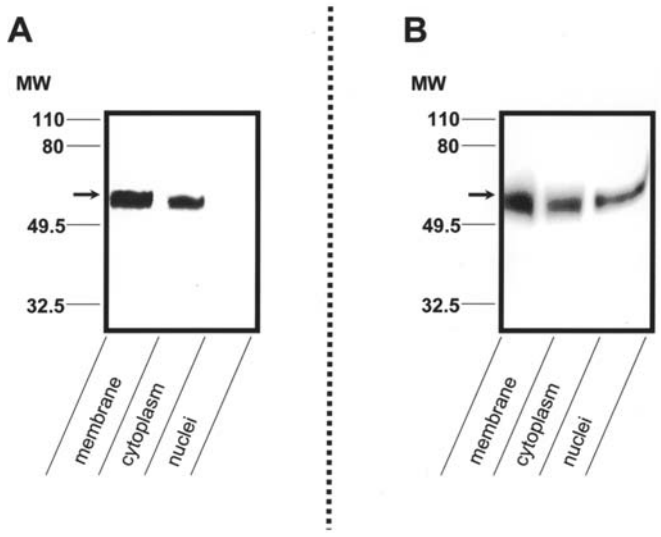

Figure 5. Western blotting of c-Yes in the membranous, cytoplasmic and nuclear fractions of human HCC. (A) and (B) correspond to (A) and (B) in Fig. 4, respectively. As shown in (A), c-Yes expression (arrow) was detected in the membranous and cytoplasmic fractions, but not in the nuclear fraction of HCC. On the other hand, (B) shows that c-Yes expression (arrow) was found not only in the membranous and cytoplasmic fractions, but also in the nuclear fraction of HCC.

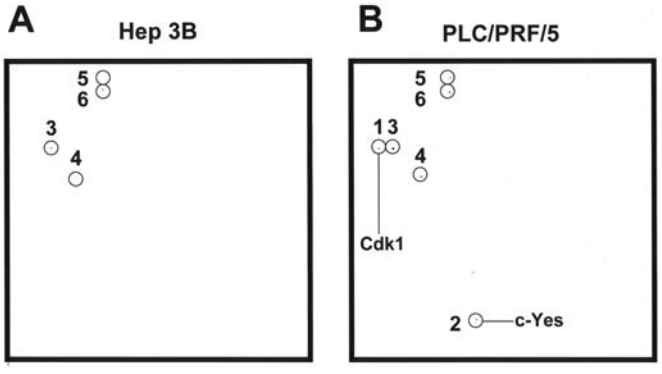

Figure 6. Comprehensive analysis using a protein antibody array to screen proteins binding to nuclear c-Yes. Representative result of the protein antibody array in Hep 3B cells without nuclear c-Yes expression is shown in (A). Representative result of the protein antibody array in PLC/PRF/5 cells with nuclear c-Yes expression is shown in (B). Spots 3-6 are non-specific. Spot 1 was identified as Cdk1, and Spot 2 as c-Yes.

significant rate in well-differentiated, small tumors at early stage HCC. There were no significant differences in the relationships between nuclear c-Yes expression and sex, age, hepatitis viral infection, background liver tissues, portal thrombus or tumor markers.

Detection of nuclear c-Yes-interacting proteins. In order to identify nuclear c-Yes-interacting proteins in PLC/PRF/5, we used a protein antibody array. This examination demonstrated the binding of nuclear c-Yes to Cdk1 (Fig. 6B, spot 1). As shown in Fig. 6, because spots 3-6 in PLC/PRF/5 were also detected in Hep 3B without nuclear c-Yes expression, they were regarded as non-specific spots. Spot 2 was identified as c-Yes.

Discussion

Src-family tyrosine kinases are proto-oncogene products and non-receptor type tyrosine kinases that bind to the cellular membrane; their subcellular distribution is membranous and/or cytoplasmic. In the present study, we found the nuclear expression of c-Yes in a subset of human HCC tissues and in a human HCC cell line. In addition, all HCCs with nuclear c-Yes expression were well-differentiated, small tumors at the early stages. To the best of our knowledge, the present report is the first study showing the identification of nuclear c-Yes in cancer cells and discussing the relationship between nuclear c-Yes expression and the clinicopathological data of HCC.

Among Src-family tyrosine kinases, subcellular localization of c-Lyn has been detected in the nuclei as well as in membranes and cytoplasm in HL-60 myeloid leukemia (21) and HeLa cells (22). Moreover, another Src-family kinase, c-Src, has been shown to be localized in the nuclei of calcium-induced differentiating keratinocytes (23). However, prior to the present study, no subcellular localization of c-Yes had been examined in various liver diseases, including HCC. It was shown in the present study that nuclear c-Yes expression was not detected in hepatocytes of normal, CH or cirrhotic livers but was detected clearly in a subset of HCC tissues. Furthermore, all HCCs with nuclear c-Yes expression were well-differentiated, small tumors at the early stages, suggesting that nuclear c-Yes may play a role in the early stages of hepatocarcinogenesis. Furthermore, nuclear c-Yes expression could be a useful marker to detect early-stage HCC.

Although the present study demonstrated the presence of nuclear c-Yes in cancer cells in a subset of HCC samples, the mechanism for the existence of nuclear c-Yes in the cancer cells remains unclear. c-Yes is known to play an essential role in the signal transduction pathways involved in the regulation of cell differentiation (24-27). Therefore, to investigate its role in differentiation in HCC, we analyzed the relationship between the expression of nuclear c-Yes and the histopathological grade of HCC. Our results demonstrated that all cases with nuclear c-Yes expression were well-differentiated HCC, suggesting that the expression of nuclear c-Yes may play an important role in maintaining differentiation and in preventing the appearance of less differentiated HCC.

In the present study, we found evidence that nuclear c-Yes binds to Cdk1, however, the function of this phenomenon remains unclear. The binding of another Src-family tyrosine kinase, nuclear c-Lyn, to Cdk1 has also been reported (21,22), supporting the present data. It is possible that the association of c-Yes with Cdk1 in the nuclei may contribute to malignant transformation in a subset of HCC cases.

In conclusion, c-Yes expression in the nuclei of cancer cells was found at the early stages of hepatocarcinogenesis and well-differentiated HCC, suggesting that such nucleus-located c-Yes could be a useful marker in detecting early stage HCC. Additionally, HCC may be a novel model for studying the function of nuclear c-Yes in malignant transformation. The suppression of nuclear c-Yes may offer a novel strategy in prohibiting the development of HCC. Further studies are necessary to investigate such possibilities.

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