Aberrant expression of serine/threonine kinase Pim-3 in hepatocellular carcinoma development and its role in the proliferation of human hepatoma cell lines

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Most cases of human hepatocellular carcinoma develop after persistent infection with human hepatitis B virus or hepatitis C virus, and host responses are presumed to have major roles in this process. To recapitulate this process, we have developed the mouse model of hepatocellular carcinoma using hepatitis B virus surface antigen transgenic mice. To identify the genes associated with hepatocarcinogenesis in this model, we compared the gene expression patterns between pre-malignant lesions surrounded by hepatocellular carcinoma tissues and control liver tissues by using a fluorescent differential display analysis. Among the genes that were expressed differentially in the pre-malignant lesions, we focused on Pim-3, a member of a proto-oncogene Pim family, because its contribution to hepatocarcinogenesis remains unknown. Moreover, the unavailability of the nucleotide sequence of full-length human Pim-3 cDNA prompted us to clone it from the cDNA library constructed from a human hepatoma cell line, HepG2. The obtained 2,392 bp human Pim-3 cDNA encodes a predicted open reading frame consisting of 326 amino acids. Pim-3 mRNA was selectively expressed in human hepatoma cell lines, but not in normal liver tissues. Moreover, Pim-3 protein was detected in human hepatocellular carcinoma tissues and cell lines but not in normal hepatocytes. Furthermore, cell proliferation was attenuated and apoptosis was enhanced in human hepatoma cell lines by normal hepatocytes. Furthermore, cell proliferation was attenuated in human hepatocellular carcinoma tissues and cell lines but not in normal liver tissues. We also provided evidence to suggest the involvement of Pim-3 in the proliferation of human hepatoma cell lines.

Material and methods

Experimental animals

HBsAg transgenic mouse lineage 107-5D (official designation Tg[Alb-1,HBV]Bri66; inbred B10D2, H-2d) was provided by Dr. F.V. Chisari (The Scripps Research Institute, La Jolla, CA). Lineage 107-5D contains the entire HBV envelope-coding region (subtype ayw) under the transcriptional control of the mouse albumin promoter, and expresses the HBV small, middle and large envelope proteins in their hepatocytes. They display no evidence of liver disease during their lifetime unless they receive the adoptive transfer of HBsAg-specific cytotoxic T lymphocytes, due to their immunological tolerance to the HBs transgene at the T cell level.

Chronic hepatitis-related liver disease model was generated as described previously. Briefly, after male HBsAg transgenic mice were thymectomized and irradiated (900 cGy), their hematopoietic system was reconstituted with the bone marrow cells from syngeneic non-transgenic B10D2 (H-2d) mice. At 1 week after the bone marrow transplantation, the animals received 10⁵ splenocytes from syngeneic non-transgenic B10D2 (H-2d) mice that were infected intraperitoneally with a recombinant vaccinia virus expressing HBsAg 3 wk before the splenocyte transfer. At 12–15 months after the lymphocyte transfer, multiple HCC foci developed in mice. Non-tumor and tumor portions were demarcated macroscopically and were removed separately. A pathologist without a prior knowl-

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FDD, fluorescent differential display; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCV, hepatitis C virus; HBs, HBV surface; HBsAg, HBV surface antigen; HBsTg, HBs transgenic mice; HCC, hepatocellular carcinoma; IL, interleukin; PBS (−), phosphate buffered saline; PCR, polymerase chain reaction; Rnai, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, short interfering RNA; STAT, signal transducers and activators of transcription; VCP, valosine-containing protein.

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edge on the experimental procedures confirmed the presence of hepatocytes with atypical configurations but not malignant cells in this non-tumor portion. Non-tumor portions were designated as pre-malignant lesions in the following experiments. Liver tissues were also obtained from untreated or HBsTg mice transplanted with tolerant splenocytes as a control.

Fluorescent differential display
Total RNAs extracted from liver tissues were subjected to FDD according to the method described by Ito et al. Briefly, total RNAs were isolated with RNA-Beet (Tel-Test, Inc., Friendswoods, TX), followed by the treatment with RNase-free DNase (Takara Shuzo, Kyoto, Japan). The purified total RNAs (2.5 μg) were reverse-transcribed with SuperScript II reverse transcriptase (Invtrogen, Carlsbad, CA) and fluorescein-labeled anchor primer, GTiA, GTiC or GTiG. The resultant cDNA equivalent to 50 ng of RNA was subjected to polymerase chain reaction (PCR) with 0.5 μM anchor primer, 0.5 μM arbitrary primer (10 mer kit A: Operon Biotechnology, Huntsville, AL), 50 μM each dNTP, 1 unit of Gene Taq DNA polymerase (Nippon Gene, Toyama, Japan), and 1 U of Taq DNA polymerase (Takara Shuzo). PCR products were separated with 6% polyacrylamide-8 M urea gel and analyzed by employing Vistra Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA). The bands of interest were excised from the gel and cloned into pSTBlue-1 Vector (Novagen, San Diego, CA). The inserted cDNA was sequenced with CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, CA) and analyzed with the BLAST program to search the GenBank database.

Cell culture
Human hepatoma cell lines (HepG2, Hep3B, HLE, HLF, HuH7 and SK-Hep1) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA) at 37 °C in a humidified atmosphere with 5% CO2 in the air.12

Semi-quantitative RT-PCR
Total RNAs were isolated with RNA-Beet, followed by the treatment with RNase-free DNase (Takara Shuzo), and a semi-quantitative RT-PCR analysis was carried out as described previously.13 The cDNA was amplified using the sets of the primers that quantitatively synthesized by employing Vistra Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA). The bands of interest were excised from the gel and cloned into pSTBlue-1 Vector (Novagen, San Diego, CA). The inserted cDNA was sequenced with CEQ 2000 DNA Analysis System (Beckman Coulter).

Northern blot analysis
Human Pim-3 mRNA expression was analyzed by using Human 12-Lane MTN Blot (Clontech, Palo Alto, CA). In vitro transcribed digoxigenin-labeled probes were hybridized overnight at appropriate temperatures (70°C for Pim-3 and 68°C for GAPDH). After being washed sequentially each for 15 min in 2× and 0.5× SSC buffer containing 0.1% sodium dodecyl sulfate at room temperature and at 68°C, respectively, the hybridized probes were detected by the DIG detection kit (Boehringer Mannheim Biochemicals, Mannheim, Germany), according to the manufacturer’s instructions.

Preparation of anti-Pim-3 polyclonal antibodies
Anti-Pim-3 antibodies were prepared by Asahi Techno Glass Co. (Tokyo, Japan). Briefly, chickens were immunized with keyhole limpet hemocyanin-conjugated Pim-3 peptide, CGPG-GVHDLPVKILQPAAKAD, which corresponds to the amino acid residues between 13–32 in human Pim-3 and is conserved in murine Pim-3, and their egg yolks were harvested before and after the immunization. IgY proteins were purified with EGGstract IgY Purification System (Promega, Madison, WI) according to the manufacturer’s instructions. They were affinity-purified with Pim-3 peptide conjugated NHS-activated HP (Amersham Biosciences, Tokyo, Japan). Purified antibodies were quantified by measuring the absorbance at 280 nm.

Immunohistochemical analysis
Human liver specimenst were surgically obtained from the patients with their informed consent. Mouse liver tissues were obtained from HBsTg mouse at the indicated time intervals after splenocyte transfer. Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol (70–100%). After incubation with 0.3% hydrogen peroxide in 10 mM phosphate buffer, pH 7.4, containing 150 mM NaN3 (PBS [−]), sections were incubated sequentially with 3% normal rabbit serum (DAKO, Kyoto, Japan) and 2% BSA in PBS (−) and with Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA). Slides were treated subsequently with 10 μg/ml anti-Pim-3 IgY or pre-immunized IgY at 4°C overnight, followed by the incubation with 2.5 μg/ml biotin-conjugated rabbit anti-chicken IgY antibodies (Promega) at room temperature for 30 min. The immune complexes were visualized by using the Vectastain Elite ABC kit (Vector Laboratories) and Vectastain DAB substrate kit (Vector Laboratories) according to the manufacturer’s instructions. The slides were counterstained with hematoxyl (DAKO), mounted, and observed under a microscope (BX-50; Olympus, Tokyo, Japan).

Immunocytochemical analysis of HuH7 cells
Cells were cultured on Lab-Tec chamber slides (Nalge Nunc, Roskilde, Denmark). They were fixed with 4% paraformaldehyde in PBS (−) and permeated in methanol. Then, they were blocked by incubation with 3% normal rabbit serum and 2% BSA in PBS (−) at room temperature for 30 min, with Avidin-Biotin blocking kit. They were treated subsequently with 20 μg/ml affinity-purified anti-Pim-3 IgY or pre-immunized IgY at 4°C overnight, with 2.5 μg/ml biotin-labeled rabbit anti-chicken IgY at room temperature for 30 min. The signals were amplified and visualized by the Vectastain Elite ABC kit and Vectastain DAB substrate kit according to the manufacturer’s instructions. The slides were counterstained with methyl green (DAKO), mounted, and observed under a microscope (BX-50; Olympus).

RNA interference
Short interfering RNA (siRNA) was synthesized with Silencer siRNA Construction Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. By employing siRNA Target Finder and Design Tool (Ambion), siRNA duplexes were designed to target AA(N3)UU sequences in the open reading frame of mRNA encoding Pim-3. The selected siRNA target sequence (5′-
The target sequence (GCACGUGGUGAAGGAGCGG) was further subjected to BLAST searches against other human genome sequences to ensure its target specificity. We identified 2 distinct cDNAs, which exhibit identity with the target sequence at 16 of 19 nucleotides. We could not, however, detect any specific bands corresponding to these cDNAs in HuH7 and Hep3B cell lines.

**TABLE I – IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN PRE-MALIGNANT LESION OF HBsTg MICE**

| Classification                  | Description                                                                 | Accession number |
|---------------------------------|-----------------------------------------------------------------------------|-----------------|
| Upregulated                     | Y box protein 3                                                             | AK029441        |
| Transcription factors           | Complement component 3                                                      | BC043338        |
| Immune system proteins          | Ceruloplasmin                                                               | NM_00775        |
| Xenobiotic metabolism           | Metallothionein II                                                          | AK002567        |
| Oncogenes                       | 24p3 (lipocalin 2)                                                          | X85627          |
| Metabolism enzymes              | Aldehyde dehydrogenase family 1, subfamily A1                               | BC044729        |
|                                 | Hemoglobin α, adult chain 1                                                 | NM_008218       |
|                                 | Phosphoenolpyruvate carboxy-kinase-1                                        | NM_011044       |
|                                 | Succinate dehydrogenase complex, subunit A flavoprotein                    | BC031849        |
| Growth factors, cytokines, and | Growth differentiation factor 15 (macrophage inhibiting compound-I)        | NM_011819       |
| chemokines                      | Insulin-like growth factor binding protein 1                                | NM_008341       |
| Non-receptor protein kinases     | Serine/threonine kinase pim-3                                               | NM_145478       |
| Not classified                   | BetaKlotho                                                                  | AF178429        |
|                                 | Serine (or cysteine) proteinase inhibitor, clade A, member 6                | NM_007618       |
| Putative proteins               | Hypothetical Esterase/acyetylhydrolase structure containing protein         | NM_026347       |
|                                 | Putative el protein                                                         | AK090127        |
| Est                              | Similar to bile acid Coenzyme A: amino acid N-acyltransferase               | NM_145368       |
|                                 | Hemoglobin, adult major chain                                               | NM_008218       |
|                                 | Stearoyl-Coenzyme A desaturase 1                                            | BC007474        |
|                                 | Isocitrate dehydrogenase 1 (NADP+), soluble                                 | AK087063        |
| Not classified                   | Endogenous retrovirus 3' LTR                                                | K02892          |
| Metabolism enzymes              | Glutathione S-transferase                                                   | BC009805        |
|                                 | Group 1 major urinary protein                                               | X03208          |
| Extracellular transport/carrier | Major urinary protein 1                                                     | BC012221        |
| proteins                        | Major urinary protein 2                                                     | BC012259        |
|                                 | Major urinary protein 3                                                     | XM_135398       |
|                                 | Major urinary protein 11 and 8                                              | AK011413        |
|                                 | Preimplantation protein 2                                                   | AK028563        |
|                                 | Ubiquitin-associated protein 1                                              | NM_023305       |
| mitochondria                    | Cytochrome oxidase A1                                                       | V00711          |
| Putative proteins               | Archerase                                                                    | AY071852        |
| Mitochondrial gene              | Di3 protein homolog                                                         | AK052991        |
|                                 | Glutatione S-transferase                                                   | BC009805        |
|                                 | Group 1 major urinary protein                                               | X03208          |
|                                 | Major urinary protein 1                                                     | BC012221        |
|                                 | Major urinary protein 2                                                     | BC012259        |
|                                 | Major urinary protein 3                                                     | XM_135398       |
|                                 | Major urinary protein 11 and 8                                              | AK011413        |
|                                 | Preimplantation protein 2                                                   | AK028563        |
|                                 | Ubiquitin-associated protein 1                                              | NM_023305       |
|                                 | Endogenous retrovirus 3' LTR                                                | K02892          |
|                                 | Glutatione S-transferase                                                   | BC009805        |
|                                 | Group 1 major urinary protein                                               | X03208          |
|                                 | Major urinary protein 1                                                     | BC012221        |
|                                 | Major urinary protein 2                                                     | BC012259        |
|                                 | Major urinary protein 3                                                     | XM_135398       |
|                                 | Major urinary protein 11 and 8                                              | AK011413        |
|                                 | Preimplantation protein 2                                                   | AK028563        |
|                                 | Ubiquitin-associated protein 1                                              | NM_023305       |
|                                 | Endogenous retrovirus 3' LTR                                                | K02892          |
|                                 | Glutatione S-transferase                                                   | BC009805        |
|                                 | Group 1 major urinary protein                                               | X03208          |
|                                 | Major urinary protein 1                                                     | BC012221        |
|                                 | Major urinary protein 2                                                     | BC012259        |
|                                 | Major urinary protein 3                                                     | XM_135398       |
|                                 | Major urinary protein 11 and 8                                              | AK011413        |
|                                 | Preimplantation protein 2                                                   | AK028563        |
|                                 | Ubiquitin-associated protein 1                                              | NM_023305       |

**FIGURE 1** – Semi-quantitative RT-PCR analysis for proto-oncogene Pim family mRNA expression in HBsTg mice. (a) Total RNAs were extracted from HBsTg ice before (N), 9 (whole liver, 9), 15 months (pre-malignant lesions, 15) after splenocyte transfer, or transgenic splenocytes transfer (C). (b) Total RNAs were extracted from pre-malignant (P) or malignant (M) tissues of HBV transgenic mice 15 months after splenocyte transfer. Representative results from 3 independent experiments are shown in the upper panels. The ratios of the PCR product for Pim-3 to GAPDH were determined, and relative intensities were calculated to assume the ratio of untreated mice as 1.0. Means and SD were calculated and are shown in the lower panels. Statistical significance was evaluated using ANOVA test, and \( p < 0.05 \) was accepted as statistically significant. *\( p < 0.05 \) compared to N.
lines by RT-PCR analysis (our unpublished data), further indicating the specificity of the used target sequence. Scramble siRNA (5′-GGCGCUUUGAGGACUCG-3′ designed by B-Bridge International, Inc.) was used as a negative control. Each siRNA duplex (final concentration 50 nM) was mixed with 12.5 μl and 12 μl of Lipofectamine 2000 (Invitrogen) for HuH7 and Hep3B, respectively. The mixtures were added into 2.5 ml of Opti-MEM (Invitrogen) and allowed to stand at room temperature for 20 min. The final mixture was then added directly into the semi-confluent cells in 6-cm culture dishes, which were washed with serum-free DMEM beforehand. The following day, 2.5 ml of DMEM plus 20% FBS medium was added to adjust the FBS concentration to 10%. At the indicated time intervals, cells were harvested for further analyses.

Semi-quantitative RT-PCR analysis of siRNA transfectant

Hep3B and HuH7 cells were harvested at 2 and 4 days after the transfection, respectively. Total RNAs were extracted and a semi-quantitative RT-PCR analysis was carried out as described above. The cDNA was amplified using the sets of the primers that specifically amplify Pim-3 (sense 5′-ATGCTGCTCTCCAAGTTCCGCTCCAGCACC-3′, antisense 5′-TCCTGTGCCGGCTCGGGTCGCTCCAGCACC-3′) and GAPDH.

Cell proliferation assay

Cells were trypsinized at 2 days after the transfection, and 5 × 10³ cells were plated to each well of 96-well plate. This time point was designated as Day 0. The cell viability was determined every day using WST-1 reagent (an MTT analog from Boehringer-Mannheim Biochemicals) according to the manufacturer’s instructions. The ratios to Day 0 were calculated.

Cell-cycle analysis by a flow cytometry

HuH7 cells were harvested at 4 days after the transfection and fixed with graded concentrations of ethanol on ice. They were incubated with 50 μg/ml propidium iodide and 1 μg/ml of RNase A for 30 min at room temperature and quenched by adding EDTA to a final concentration of 10 μM. The filtered cells were analyzed using a FACSCalibur (Becton Dickinson, Bedford, MA). The distribution in each cell-cycle phase was determined by using Cell Quest analysis software (Becton Dickinson).

Chromatin condensation analysis by Hoechst 33258

HuH7 and Hep3B cells were harvested at 4 days after the transfection and stained with Hoechst 33258 to detect the cells with condensed nuclei under a fluorescence microscope (BX-50; Olympus).

Results

Identification of the genes differentially expressed in pre-malignant liver tissue

We compared the gene expression patterns between pre-malignant lesions and normal liver tissues by employing a FDD method. The determination of the nucleotide sequence of the resultant bands identified 24 and 19 distinct genes among the upregulated and downregulated bands in pre-malignant lesions, respectively (Table I). Among these genes, Pim-3 expression has not been reported in normal hepatocytes. We focused on Pim-3, a member of proto-oncogene Pim family including Pim-1 and Pim-2. A semi-quantitative RT-PCR analysis confirmed that Pim-3 mRNA expression was significantly enhanced in the pre-malignant tissues.

![Figure 2](image-url)

**Figure 2** – Immunohistochemical analysis of Pim-3 in HBsTg mice. HBsTg mice liver tissues before (a) and 15 months after splenocyte transfer (b,d,e: non-tumor portions; c,f: tumor portions) were immunostained by anti-Pim-3 IgY as described in Material and Methods. Representative results are shown here. (b) Pre-malignant lesion is indicated with arrowheads. (c) Tumor portion is indicated with arrowheads. (d) Positively-stained regenerated proliferating bile ductule. The arrow indicates regenerated proliferating bile ductule. (e,f) Positively-stained hepatocytes at a higher magnification of the square in (b) and (c). Original magnification = (a–c) ×100; (d–f) ×400. Scale bars = 50 μm.
and to a lesser degree, in HCC tissues, compared to control (Fig. 1). In contrast, specific Pim-1 and Pim-2 transcripts were barely detected under these conditions (Fig. 1). These results indicate that Pim-3 mRNA expression is enhanced during HCC development in this model.

We further localized Pim-3 protein immunohistochemically in liver tissues obtained from HBsTg mouse after splenocyte transfer. We failed to detect Pim-3 protein in unmanipulated mice (Fig. 2a) or 9 months after splenocyte transfer, when hepatocytes with atypical nuclear configuration were not detected (data not shown). On the contrary, Pim-3 protein was weakly detected in the cytoplasm of hepatocytes with atypical nuclear configurations in pre-malignant lesion (Fig. 2b,e) and highly differentiated neoplastic hepatocytes in the tumor portion (Fig. 2c,f). Moreover, Pim-3

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FIGURE 3 – (a) Structure of human Pim-3 cDNA. The nucleotide and predicted amino acid sequences of human Pim-3 are shown. The nucleotide sequence is numbered. The predicted amino acid sequence is shown in a single-letter code below the nucleotide sequence. The AT-rich motifs are indicated in boxes and highlights. The region used as the probe for Northern blot analysis is underlined. (b,c) Amino acid alignment of Pim family proteins. The amino acid sequences of human, rat, and mouse Pim-3s (b) or other members of human Pim family kinases (c) were aligned using DNASIS-Mac version 3.0 software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). The residues identical to human Pim-3 are highlighted.
protein was detected in regenerated proliferating bile ductules (Fig. 2d, arrow), which are assumed to be the proliferation of hepatic stem cells after chronic liver injuries (i.e., infection, tumors). These results may indicate that Pim-3 protein expression was aberrantly enhanced in liver during the course of hepatocarcinogenesis in this model.

Because the full length human Pim-3 cDNA nucleotide sequence has not been determined yet, we initially cloned and determined the nucleotide sequence of human Pim-3 cDNA by screening cDNA library constructed from a human hepatoma cell line, HepG2. Three positive clones were obtained after 2 rounds of screening, and these 3 distinct cDNA clones contained the same insertion consisting of 2,392 bp. The 5'-untranslated region is 82.3% G and C, whereas the 3'-untranslated region contains 5 copies of the ATTTA motif and 8 copies of TATT motif (Fig. 3a). This sequence exhibits an identity with a partial human Pim-3 cDNA sequence predicted from EST database (data not shown). Its open reading frame encodes the protein consisting of 326 amino acids with a calculated molecular weight of 35,861 (Fig. 3a).

Moreover, the amino acid sequence of the predicted open reading frame, shares a high degree of identity with the mouse16 and rat Pim-3 (KID-1)8 proteins (95.0%; Fig. 3b). Based on these results, we judged this clone as human Pim-3 cDNA. Human Pim-3 protein showed a high sequence identity with the quail qPim17 (73.9%) and Xenopus Pim (Pim-1)18 (68.7%) at the amino acid level (data not shown). Moreover, human Pim-3 protein shows a high sequence identity with human Pim-119 (57.1%) and Pim-220 (44.0%) at the amino acid level (Fig. 3c). Northern blotting analysis detected 2.4-kb mRNA in various organs including heart, skeletal muscle, brain, spleen, kidney, placenta, lung and peripheral blood leukocytes (Fig. 4). In contrast, no specific band was detected in colon, thymus, liver and small intestine under the present experimental conditions (Fig. 4).

**FIGURE 4** – Human Pim-3 mRNA expression in human normal tissues. Northern blot analysis was carried out as described in Material and Methods. Representative results are shown. sk, muscle, skeletal muscle; PBL, peripheral blood leukocytes. GAPDH mRNA expression was analyzed in parallel to evaluate the amount of mRNA loaded in each lane.

**FIGURE 5** – Immunohistochemical analysis of Pim-3 in HCC tissues. Human normal liver tissue (a) or HCC tissues (b–f) were immunostained by anti-Pim-3 IgY as described in Material and Methods. Representative results are shown. (b) Precancerous lesions. The lesions surrounded with arrowheads and arrows are precancerous lesions and HCC lesions, respectively. (c) Precancerous lesion in the square of (b) is shown at a higher magnification. Arrows indicate regenerated proliferating bile ductules. (d,e) Positively-stained lesion in the square of (b) is shown at a higher magnification. Arrows indicate regenerated proliferating bile ductules. Original magnification: (a,b,d) 100; (c,e,f) ×400. Scale bars = 50 μm.
**Pim-3 is expressed aberrantly in human HCC**

Immunohistochemical analysis failed to detect Pim-3 protein in normal liver tissues (Fig. 5a), consistent with the Northern blotting analysis. On the contrary, Pim-3 protein was weakly but diffusely detected in most of large regenerative nodules and adenomatous hyperplasia, lesions with precancerous potential, which were located adjacent to HCC areas (19 of 27 cases; Fig. 5b,c). Moreover, a substantial proportion of HCC cells were immunostained with anti-Pim-3 IgY (6 of 27 cases; Fig. 5d,e) but not the pre-immunized IgY (data not shown). Furthermore, Pim-3 protein was observed markedly in regenerating proliferating bile ductules (27 of 27 cases; Fig. 5c,f, arrows). Because the staining patterns were similar to that observed in HBsTg mouse model (Fig. 2), these results would indicate that Pim-3 protein expression was aberrantly enhanced in precancerous lesion, also in humans, and a portion of HCC cells.

**Constitutive Pim-3 expression in human hepatoma cell lines**

Immunohistochemical analysis indicated that Pim-3 protein expression was aberrantly enhanced not only in precancerous lesion but also in a portion of HCC cells in human HCC tissues (Fig. 5). This finding prompted us to examine Pim-3 expression in human hepatoma cell lines, by RT-PCR. To exclude the possibility that contaminated genomic DNA gave rise to the generation of the amplified bands, we used total RNA samples that were treated with DNase. Under the present condition, Pim-3 transcript was detected in all hepatoma cell lines, whereas no specific band was detected in the normal liver tissue (Fig. 6a), consistent with the Northern blotting analysis. The exclusion of reverse transcriptase failed to give rise to any bands, further indicating the specificities of RT-PCR (Fig. 6a, non-RT). Moreover, an immunocytochemical analysis detected immunoreactive Pim-3 proteins in HuH7 cell line, when incubated with anti-Pim-3 antibodies (Fig. 6b[a]) but neither pre-immunized IgY (Fig. 6b[b]) nor anti-Pim-3 adsorbed with the relevant peptide (Fig. 6b[c]). Immunoreactive Pim-3 proteins were similarly detected in all 6 human hepatoma cell lines, consistent with RT-PCR analysis (data not shown). Collectively, these results would indicate that Pim-3 was constitutively expressed in human hepatoma cell lines.

**RNAi ablation of Pim-3 induces cell death to hepatoma cell lines**

Because Pim-1 and Pim-2 were required to induce cell-cycle progression21,22 and anti-apoptotic effects,21–25 we examined the role of Pim-3 in cell proliferation by ablating endogenous Pim-3 mRNA expression in HuH7 and Hep3B cell lines with RNAi. Endogenous Pim-3 mRNA level was decreased after the transfection with specific Pim-3 siRNA but not Scramble siRNA (Fig. 7a). Under these conditions, transfection with Pim-3 siRNA significantly retarded cell proliferation, compared to Scramble siRNA-transfected and the control cells (Fig. 7b,c). These results sug-
siRNA transfectants exhibited a higher ratio of sub-G1 populations with reduced G1 and G2/M populations, compared to Scramble siRNA transfectants and control cells (Fig. 8b). Furthermore, the proportion of cells with condensed nuclei was significantly higher in both HuH7 and Hep3B cells transfected with Pim-3 siRNA, than those transfected with Scramble siRNA (Fig. 8c). These observations would indicate that the ablation of Pim-3 might induce apoptosis in these hepatoma cell lines.

Discussion

Transcriptome analysis has been applied widely to elucidate molecular mechanisms of various types of diseases and can provide many important clues, particularly for understanding the molecular pathogenesis of oncogenesis, where the expression of many genes changes simultaneously.26,27 Several independent groups carried out transcriptomical studies on human HCC.28–33 In most studies, however, the gene expression pattern was compared between tumor and non-tumor portions obtained from the same patients.28–33 Because these non-tumor portions exhibit usually hepatocyte dysplasia, this type of analysis may fail to detect the changes in gene expression that have already existed at the stage of hepatocyte dysplasia. To circumvent these pitfalls, we compared gene expression patterns among pre-malignant lesions and normal tissues by using FDD. We observed that various genes were selectively changed in pre-malignant lesions. Moreover, a semi-quantitative RT-PCR analysis did not detect any significant differences in the expression of several of these genes between malignant and pre-malignant lesions (our unpublished data), supporting our assumption that the changes in gene expression which have already existed at the stage of hepatocyte dysplasia, might be undetected in the preceding studies.

Among the genes identified in our study, we focused on Pim-3. Pim-3 was originally identified as depolarization-induced gene KID-1 in PC12 cell line, a rat pheochromocytoma cell line.8 Subsequently, several independent groups observed a selective expression of its mRNA in neuronal system,16,34,35 but not the liver. By using human Pim-3 cDNA as a probe, we detected Pim-3 mRNA in several organs such as the brain and spleen but not the liver. On the contrary, Pim-3 mRNA expression was detected in all human hepatoma cell lines that we examined. Moreover, immunohistochemical analysis detected immunoreactive Pim-3 protein in precancerous lesions and a portion of HCC cells. Furthermore, Pim-3 protein was also detected in regenerating bile ductules, which are assumed to be the proliferation of hepatic stem cells after chronic liver injury (i.e., infection, tumors).14

Deneen et al.36 provided evidence on the crucial involvement of Pim-3 in EWS/ETS-mediated malignant transformation of mouse NIH 3T3 cells. They demonstrated that Pim-3 was a common transcriptional target of EWS/ETS. EWS/ETS fusion proteins retain an intact ETS DNA-binding domain and can bind to a binding sequence in the target genes through this domain.37 Pim-3 gene transcription may be regulated not only by EWS/ETS fusion proteins but also other Ets family proteins. Several independent groups reported that Ets-1, one of the Ets family proteins, was expressed in human HCC tissues.38,39 Ito et al.38 demonstrated that Ets-1 expression was markedly enhanced in non-cancerous lesions adjacent to HCC lesions and suggested that Ets-1 had a crucial role in hepatocarcinogenesis and HCC progression during their early phases. In line with these observations, we also observed that another transcription factor with an ETS-domain, polyomavirus enhancer A binding protein-3, was expressed selectively in HCC and induced constitutive gene expression of a pro-angiogenic factor, interleukin (IL)-8, in HCC.40 It is tempting to speculate that a transcription factor(s) with an ETS-domain, may induce ectopic Pim-3 gene expression in liver, during the course of hepatocarcinogenesis.

Several lines of evidence demonstrated that the gene expression of Pim-1 and Pim-2 could be regulated by IL-6-gp130-mediated signal transducers and activators of transcription (STAT) family
protein, STAT3.41,42 STAT3 signals can advance cell-cycles and prevent apoptosis by inducing Pim-1 and c-Myc in lymphomagenesis.42 In the liver, IL-6-deficient mice exhibited an impaired liver regeneration after a partial hepatectomy.43 Several lines of evidence have shown that Bcl-xL expression is upregulated by IL-6 gp130-mediated STAT3 and prevents hepatocyte apoptosis,44,45 and the constitutive activation of STATs observed during oncogenesis can cause a permanent alteration in the genetic program.46,47 These observations suggest that STAT3 signals could regulate hepatocyte regeneration during the course of HBV-induced hepatocarcinogenesis. We observed that Pim-3 gene ablation by RNAi attenuated proliferation rates and caused cell death in hepatoma cell lines. Thus, if Pim-3 was also regulated by STAT3, these observations suggest that Pim-3 would be involved in STAT3-mediated prevention of apoptosis or cell-cycle progression.

Pim-1 and Pim-2 are also known as proto-oncogene to be involved in lymphomagenesis.48,49 Because Pim-1 and Pim-2 can induce and anti-apoptotic effects,21–25 Pim-3 may be involved in cell-cycle regulation or anti-apoptosis. We also observed that gene ablation of Pim-3 caused cell death to human hepatoma cell lines, later than 3 days after the transfection. These results suggest that Pim-3 can regulate cell-cycle or apoptosis process indirectly by phosphorylating a molecule(s) upstream in these processes. Although Pim-1 can phosphorylate several molecules such as Cdc25A,49 a G1/S cell-cycle regulator, Pim-3 could not interact with Cdc25A.36 Pim-1 can also phosphorylate valosine containing protein (VCP)/p97,41,42 a mammalian homolog of Saccaromyces cerevisiae Cdc48p. Pim-1 can upregulate further the expression of an anti-apoptotic molecule, Bcl-2 and Bcl-xL, by augmenting the expression of VCP.42,44 In HCC tissues and human hepatoma cell lines, evidence is accumulating to indicate that Bcl-xL is constitutively expressed and is a major executor to prevent apoptosis.39,50,51 Moreover, VCP was also detected in human HCC tissues.53 If VCP could be phosphorylated by Pim-3 as well as Pim-1, Pim-3 may exert an anti-apoptosis effect by augmenting indirectly the expression of an anti-apoptotic molecule, similarly as Pim-1. Moreover, if the contents of target molecules may differ between HuH7 and Hep3B cell lines, these may account for different patterns of the effects of Pim-3 gene ablation on the proliferation of these cell lines.

The kinase activity of Pim-3 was crucially involved in EWS/ETS-mediated malignant transformation of mouse NIH 3T3 cells.36 Our present observations suggest that Pim-3 can regulate anti-apoptosis process or cell-cycle progression by modulating molecules involved in these processes. Accumulating evidence indicated that Pim-3 can auto-phosphorylate itself,16,17 but it still remains elusive on physiological substrates of Pim-3. The identification of a substrate(s) may shed novel light on Pim-3-mediated regulatory mechanisms of apoptosis or cell-cycle progression.

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