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Loss of runt-related transcription factor 3 expression leads hepatocellular carcinoma cells to escape apoptosis

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Abstract

Background: Runt-related transcription factor 3 (RUNX3) is known as a tumor suppressor gene for gastric cancer and other cancers, this gene may be involved in the development of hepatocellular carcinoma (HCC).

Methods: RUNX3 expression was analyzed by immunoblot and immunohistochemistry in HCC cells and tissues, respectively. Hep3B cells, lacking endogenous RUNX3, were introduced with RUNX3 constructs. Cell proliferation was measured using the MTT assay and apoptosis was evaluated using DAPI staining. Apoptosis signaling was assessed by immunoblot analysis.

Results: RUNX3 protein expression was frequently inactivated in the HCC cell lines (91%) and tissues (90%). RUNX3 expression inhibited 90 ± 8% of cell growth at 72 h in serum starved Hep3B cells. Forty-eight hour serum starvation-induced apoptosis and the percentage of apoptotic cells reached 31 ± 4% and 4 ± 1% in RUNX3-expressing Hep3B and control cells, respectively. Apoptotic activity was increased by Bim expression and caspase-3 and caspase-9 activation.

Conclusion: RUNX3 expression enhanced serum starvation-induced apoptosis in HCC cell lines. RUNX3 is deleted or weakly expressed in HCC, which leads to tumorigenesis by escaping apoptosis.

Background

Hepatocellular carcinoma (HCC)[1] is the sixth most common cancer and responsible for more than half a million deaths worldwide each year [1-3]. Although most HCC cases occur in East Asia and Middle and West Africa, its incidence in some developed countries is increasing [1,4]. In most cases, HCC is fatal because of an incomplete understanding of the pathogenic mechanisms and inadequacies of early detection [1,5].

The activation of proto-oncogenes plays a major role in the development of HCC [1,6-8], and a number of tumor suppressor genes may be associated with the development and progression of HCC [1,9-12]. Although several cancer-related genes are altered in HCC, the frequency of alterations for each individual gene is relatively low. In HCC, the alteration of tumor suppressor genes seems to be more important than that of oncogenes. Established genetic events include the loss of an allele, mutation, or promoter methylation [13-16]. A higher loss of heterozygosity (LOH) frequency was detected at several loci on chromosomes 8p23, 4q22-24, 4q35, 17p13, 16q23-24, 6q27, 1p36, and 9p12-14, suggesting the presence of important tumor suppressor genes at these loci [17]. However, there is little understanding of the several key pathways and the genes involved in these pathways.

Runt-related transcription factor 3 (RUNX3), located on chromosome 1p36, is correlated with tumorigenesis and gastric cancer progression [18,19]. RUNX3 acts as

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an apoptotic factor, downstream of transforming growth factor-β (TGF-β), and as a cell differentiation mediator in intestinal metaplasia of gastric mucosa [19-21]. In gastric cancer cell lines, RUNX3-induced apoptosis depends on Bim expression [22]. RUNX3 protein expression is decreased about 45-60% in human gastric cancer [21] and has been detected in some human malignancies such as those of the colon, lung, pancreas, and bile duct [23-26]. RUNX3 gene expression decreased in 30-80% of HCCs due to LOH and methylation of its promoter [27,28]. The loss or decrease of RUNX3 expression in HCC tissue has been recently reported [29], but the precise function of RUNX3 in HCC needs to be elucidated.

Methods

Cell lines and cell culture

The HCC cell lines HepG2, Hep3B, PLC/PRF/5 (PLC), and SK-Hepl were obtained from the American Type Culture Collection (Manassas, VA), and the Huhl, Huh7, JHH1, JHH2, JHH4, HLE, and HLF cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). Normal human hepatocytes were obtained from Sanko Junyaku Co. Ltd. (Tokyo, Japan). JHH2 and normal human hepatocytes were cultured in William's medium E (Invitrogen, Carlsbad, CA). Other cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen). Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin solution (Sigma). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Quiescence was carried out under restricted serum conditions with 0.1% dialyzed FBS for the indicated time periods.

RNA preparation and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells using Trizol™ reagent (Invitrogen). Reverse transcription was performed using random primers and ReverTra Ace™ (Toyobo, Osaka, Japan) reverse transcriptase (RT). Ps-CA and Ps-CB, previously published primer set for RUNX3, were utilized [21]. For each polymerase chain reaction (PCR), 20 μl (total volume) of reaction mixture contained 0.1 μg template DNA, 4 pmol each of the forward and reverse primers, 2 μl deoxynucleoside triphosphates (200 mM each), 1 U pfu Turbo™ DNA polymerase (Stratagene, La Jolla, CA), and 2 μl of 10× pfu reaction buffer. PCR amplification was conducted on an iCycler™ (Bio-Rad, Hercules, CA) with the following cycle conditions: cycle 1, 95°C for 2 min; cycles 2-30, 95°C for 30 s, 58°C for 30 s, and 72°C for 120 s, with a final elongation step of 72°C for 10 min.

Immunoblot analysis

Cells were plated onto 6-well tissue culture plastic dishes and grown to confluence. After cultivating the cells under the indicated conditions, they were washed twice with cold phosphate-buffered saline (PBS) and lysed in 150 μl of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate [SDS], 1% bromophenol blue, 10% β-mercaptoethanol). The samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P™ polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA), which were blocked using Tris-buffered saline with Tween-20 (TBS-T) (Sigma) containing 5% bovine serum albumin for 1 h. The membranes were incubated with antibodies against RUNX3 (R3-G54; Abcam, Cambridge, MA), poly-histidine (His) (Roche Diagnostics, Basel, Switzerland), Bax, Bcl-2, Bim, cleaved caspase-3 and -9 (Cell Signaling Technology, Beverley, MA), and β-actin (Sigma) overnight at 4°C. We washed the membranes three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies before developing them using an ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ) by enhanced chemiluminescence.

HCC tissue and immunohistochemistry

Thirty-one patients including 24 men with age ranging from 18 to 71 years (average age, 58 years) and 7 women with age ranging from 59 to 67 years (average age, 63 years) at the time of hepatic resection were included in this study. HCC tissues along with adjacent liver tissues were used for analysis. As per the institutional guidelines, we obtained informed consent from all donors of liver tissue samples, and the study was approved by the Research Ethics Committee of Okayama University.

Immunohistochemistry was performed on formalin-fixed paraffin sections that were dewaxed and dehydrated. After rehydration, endogenous peroxidase activity was blocked for 30 min in a methanol solution containing 0.3% hydrogen peroxide. After antigen retrieval in citrate buffer, the sections were blocked overnight at 4°C. The sections were probed with rabbit polyclonal antibody (ab49117; Abcam) followed by biotinylated anti-rabbit secondary antibody (Dako Japan, Tokyo, Japan). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with hematoxylin, after which the sections were dehydrated in alcohol and xylene, and mounted for observation. The sections were scored on a four-tier scale; 0, negative; 1, weak signal; 2, intermediate signal; and 3, strong signal [30]. All sections were scored independently by two observers (Y. K. and K. N.) without prior knowledge. All discrepancies in scoring were reviewed and a consensus was reached.

RUNX3 cloning and transfection

We obtained human RUNX3 cDNA by PCR-based cloning from normal human hepatocytes (Sanko Junyaku). Briefly, cDNA was amplified by PCR using sense (5'-TATGCGTATTCCCGTAGA) and antisense (5'-CTCGAGGCGGCCGCTCAATGGTGATGGTGAT-GATGACCGGTACGGTAGGGCCGCCACAC; including the six-His tag) oligonucleotide primers with Pfu Turbo™ Hotstart DNA polymerase (Stratagene) and cloned into the PCR II TA cloning vector (Invitrogen). The size of the PCR product was ~1.2 kb. After confirmation by sequencing, RUNX3 cDNA was subcloned into pCEP4 (Stratagene), downstream from a cytomegalovirus promoter. The poly-His tag was replaced with green fluorescent protein (GFP) cDNA from pEGFP-C1 (Clontech, Palo Alto, CA). The human RUNX3 and/or chloramphenicol acetyltransferase (CAT) (control) constructs were transfected into Hep3B cells using FuGENE™6 transfection reagent (Roche), as per the manufacturer's instruction. Cells were selected in complete medium containing 250 μg/ml of hygromycin (Roche). Polyclonal lines consisting of more than 20 colonies were established. At least two independent stably transfected lines were established for each construct.

Transient RUNX3 expression was also conducted using FuGENE™6 in Hep3B, Huh7, HLE, and HLF cells. After transfection, the cells were cultured under serum starved condition for the indicated periods, if needed, and utilized for the following experiments.

MTT assay

Cell proliferative activity was assessed with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded at 2,000 cells/well in 96-well tissue culture plastic dishes and quiesced for 6 h with 0.1% dialyzed FBS. After 24-120 h of quiescence, the cells were cultured for the indicated periods with or without 10% FBS. At the end of the treatment, 10 μl of MTT (5 mg/ml in PBS) was added to each well, and the wells were incubated for an additional 2 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 200 μl of dimethyl sulfoxide (Sigma). The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm with a microplate reader (Bio-Rad).

DAPI staining

Cells were plated at 50% confluence on glass chamber slides (Labtek II, Nalgen Nunc, Roskide, Denmark) and quiesced for 6 h with a media containing 0.1% dialyzed FBS. Then, they were treated with 10% FBS, 100 μM caspase inhibitor (caspase inhibitor IV, Calbiochem, Gibbstown, NJ), 1 nM transforming growth factor-α (TGF-α) (Peprotech Inc. Rocky Hill, NJ), 1 nM epidermal growth factor (EGF) (Peprotech), and/or 5 ng/ml platelet derived growth factor (PDGF)-BB (Peprotech). Chromosomal DNA was stained with 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, treated cells were washed with PBS and stained with DAPI working solution (1 μg/ml in PBS) for 2 min. The percentage of cells with condensed chromatin and/or fragmented nuclei was established in 300-500 DAPI-stained cells examined under a fluorescence microscope (IX-70, Olympus, Tokyo, Japan).

Flow cytometry analysis

Annexin V and propidium iodide (PI) staining was performed using an annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) to measure apoptosis. Cells were cultured in 10-cm tissue culture plates and quiesced for 6 h with a media containing 0.1% dialyzed FBS. Cells were cultured in medium with or without 10% FBS for 24 h. Then, they were washed twice with PBS, collected, and re-suspended in 85 μl of 1× annexin V-FITC binding buffer. Five microliters of annexin V-FITC conjugate and 10 ml of PI buffer were added, and the cells were incubated at room temperature for 15 min in the dark. After adding 400 μl of 1× annexin V-FITC binding buffer, cells were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ).

Gene silencing of Bim with small interfering RNA

RUNX3-expressing Hep3B cells were transfected with either scrambled negative control small interfering RNA (siRNA) or Bim siRNA (Applied Biosystems, Foster City, CA). siRNAs were transfected into cells using RNAi-Fect™ transfection reagent (Qiagen, Hilden, Germany). Cells were incubated with scrambled negative control siRNA or Bim siRNA for 24 h before 48 h of serum starvation. The MTT assay and DAPI staining for detecting apoptosis were performed as described above.

Results

Loss of RUNX3 expression in HCC cell lines and human HCC tissues

A decreased level or absence of RUNX3 mRNA expression was observed in 10 of 11 HCC cell lines (Figure 1A). RUNX3 mRNA was undetectable in eight cell lines (HepG2, Hep3B, Huh1, Huh7, JHH1, JHH2, JHH4, and HLE). In HLF and SK-Hep1 cells, RUNX3 mRNA was significantly underexpressed (Figure 1A). Normal human hepatocytes expressed RUNX3 mRNA. Sequence analysis was performed in HLF, PLC, and SK-Hep1 cells, and no mutation was detected. In accordance with the mRNA analysis, RUNX3 protein expression was

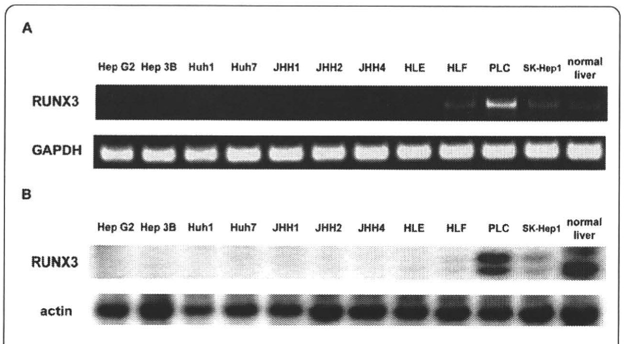

Figure 1 RUNX3 mRNA (A) and protein (B) expression in HCC cell lines. (A) RUNX3 and GAPDH mRNA expression levels were determined by RT-PCR. Shown here are representative gels from three independent experiments. (B) RUNX3 and α-actin protein expression was analyzed by immunoblot using the anti-RUNX3 antibody. Shown here are representative blots from more than three independent experiments.

undetectable in the HepG2, Hep3B, JHH1, JHH2, JHH4, HLE, and HLF cell lines, while the RUNX3 protein was expressed in HLF, PLC, and SK-Hep1 cells (Figure 1B). The RUNX3 protein was significantly underexpressed in HLF and SK-Hep1 cells.

RUNX3 protein expression in human HCC tissue was compared to that in the corresponding tumor-free resection margins using immunohistochemical analysis (Figure 2). Twenty eight (~90%) of these pairs showed a negative or weak signal for RUNX3 expression in HCC tissue, but showed RUNX3 protein expression in tumor-free resection margins (Table 1). In the remaining three pairs, a weak RUNX3 expression signal was detected in the tumor-free resection margins; thus, no negative RUNX3 signal was detected in the tumor-free resection margins.

Ectopic RUNX3 protein expression in Hep3B cells

To assess whether RUNX3 protein expression affected cell survival in the HCC cell lines, a RUNX3 construct was introduced into RUNX3-negative Hep3B cells (Figure 3A). Overall, the clones were expressed at similar

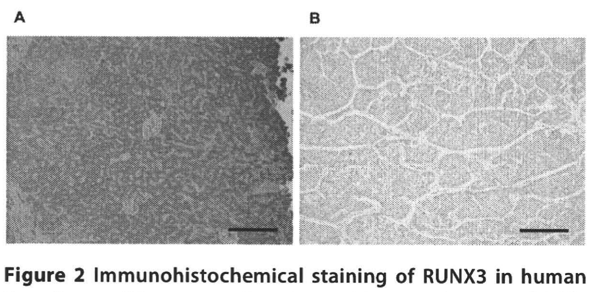

Figure 2 Immunohistochemical staining of RUNX3 in human liver tissues. (A) nontumor liver tissue with a protein score of 3 (B) HCC tissue with a protein score of 0. Bar = 100 μm.

Table 1 RUNX3 expression in HCC samples (n = 31) and the corresponding tumor-free resection margins

RUNX3 protein expression score	HCC samples (n = 31)	Tumor-free sections (n = 31)
0 (negative signal)	13 (41.9%)	0
1 (weak signal)	15 (48.4%)	3 (9.7%)
2 (intermediate signal)	3 (9.7%)	16 (51.6%)
3 (strong signal)	0	12 (38.7%)

levels in all cells, as determined by immunocytochemical analysis (data not shown). RUNX3-expressing Hep3B cells grew slightly slower than normal Hep3B cells in the presence of FBS.

RUNX3 expression inhibited cell growth under serum starvation

RUNX3 has been reported to induce apoptosis in a gastric cancer cell study [21]. The MTT assay was performed to determine whether RUNX3 expression influenced cell growth. RUNX3-expressing Hep3B cells grew slightly slower than CAT-transfected Hep3B cells in the presence of FBS, whereas the growth of RUNX3-expressing Hep3B cells was markedly suppressed in the absence of FBS; growth inhibition could be observed as early as 24 h, and reached 70 ± 12% and 90 ± 8% at 48 and 72 h, respectively (Figure 3B). The inhibition levels were over 4 times than those found in the condition with 10% FBS. This effect was confirmed with GFP-tagged RUNX3-expressing Hep3B cells (70 ± 11% growth inhibition at 72 h).

RUNX3 expression induced apoptosis under serum starvation

The effect of RUNX3 expression on cell survival and the cell cycle with and without FBS was assessed to investigate whether the elicited growth suppression in RUNX3-expressing cells under serum starved conditions was due to an increase in cell death or due to cell cycle inhibition, or both. DAPI staining demonstrated that serum starvation induced apoptosis in RUNX3-expressing Hep3B cells (31 ± 4%) but not in CAT-transfected Hep3B cells (4 ± 1%) in the absence of FBS (Figure 3C). Flow cytometry analysis with annexin V antibody was also performed. RUNX3-expressing Hep3B cells showed a significant increase in a pre-apoptosis population (Annexin V+ PI-) after 24 h of serum starvation compared with CAT-transfected Hep3B cells (Figure 3D).

RUNX3-induced apoptosis through the Bim-caspase pathway

Because a RUNX3-induced apoptotic pathway has been described previously, the effect of altering RUNX3 expression was investigated. Bim protein expression was

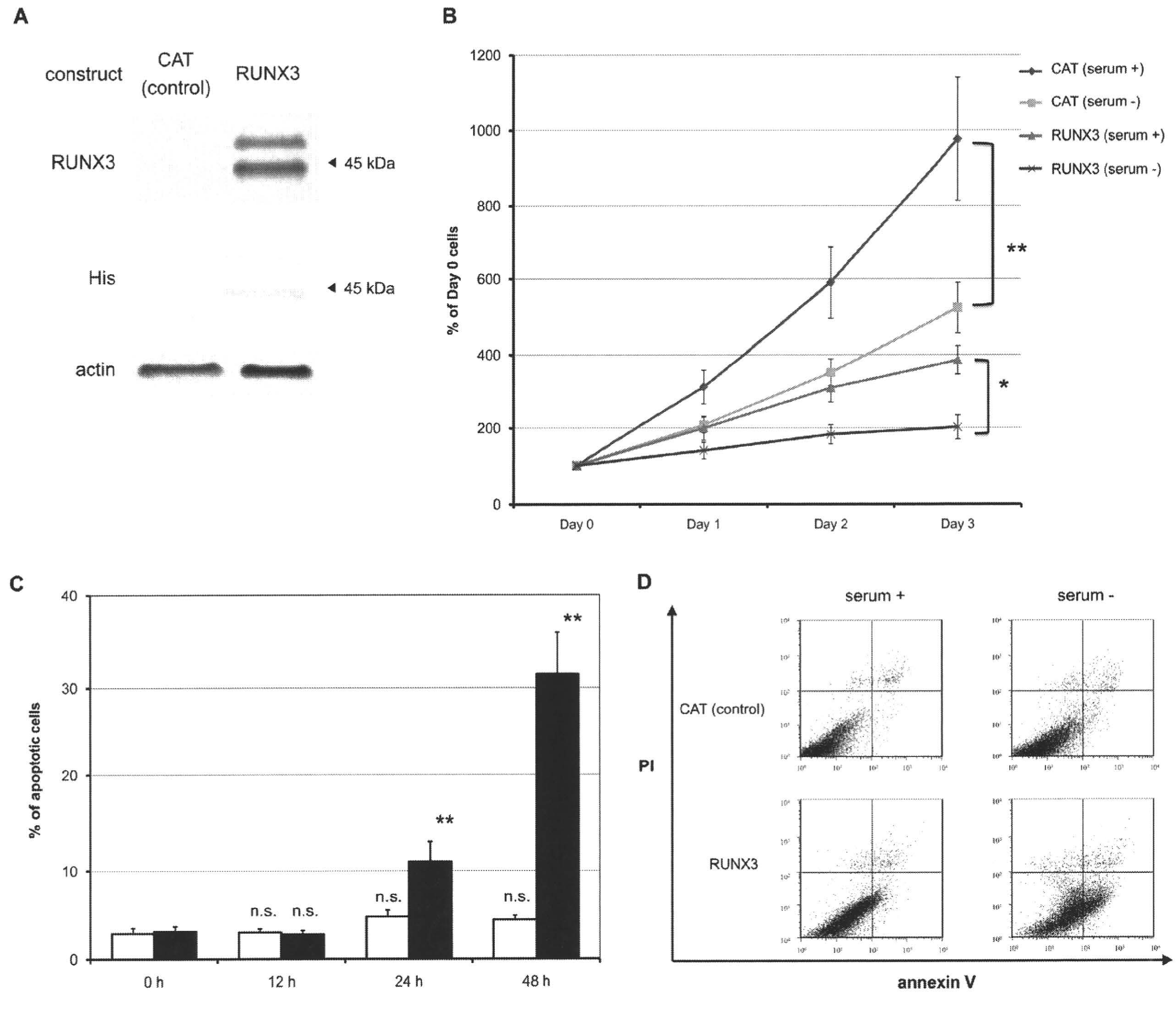

Figure 3 RUNX3 expression in Hep3B cells. CAT (control) and RUNX3 constructs were introduced into Hep3B cells. Polyclonal stably expressing cell lines were selected in the presence of hygromycin. (A) Immunoblot analysis RUNX3 protein expression was analyzed by immunoblot using anti-RUNX3 and anti-His antibodies. Shown here are representative blots from more than three independent experiments. (B) Effect of RUNX3 expression on cell growth Cell proliferative activities were measured by the MTT assay. All results are expressed as ratios to cell number at day 0. Data represent the mean ± S.E. of more than three independent experiments, each with four replicates. *, $P < 0.05$; **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test. (C) Apoptosis determined by DAPI staining Cells were cultured in medium without FBS for the indicated periods. The cells were stained with DAPI, and the percentage of apoptotic cells was examined by fluorescence microscopy (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. n.s., not significant; **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test. (D) Flow cytometry analysis Cells were cultured in medium with or without 10% FBS for 24 h. They were harvested and washed in PBS without Ca^{2+}/Mg^{2+}, and stained using the Mebcyto™ apoptosis detection kit. The cells were analyzed with a Becton Dickinson FACS Calibur flow cytometer. Shown here are representative plots from more than three independent experiments.

enhanced by serum starvation in RUNX3-expressing Hep3B cells but not in control cells (Figure 4A). Activated apoptosis executors, caspase-9 and -3, were found in serum starved RUNX3-expressing Hep3B cells. Expression of the Bim attenuators, Bax and Bcl-2, was not affected by serum starvation. These results imply that Bim plays a major role in serum starvation-induced apoptosis in RUNX3-expressing cells.

Serum starvation-induced apoptosis was abrogated by an apoptosis inhibitor (Figure 4B). Various growth factors were employed to determine whether serum starvation-induced apoptosis was caused by the absence of a growth factor-induced survival signal. As a result, TGF-α, EGF, and PDGF abrogated serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells (Figure 4B).

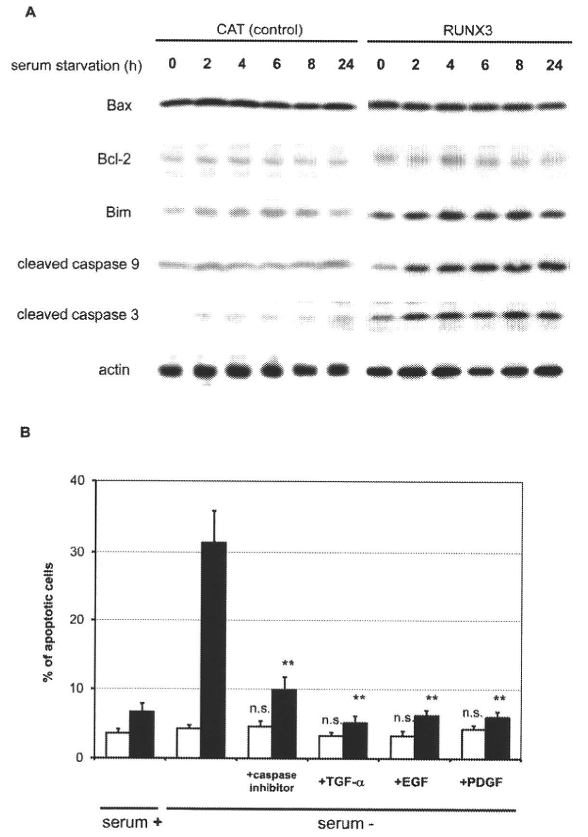

Figure 4 Effect of RUNX3 on apoptosis-related signaling molecules. (A) Immunoblot analysis Equal amounts of cell lysates were size-fractionated with 10% SDS-PAGE and immunoblotted with antibodies against Bax, Bcl-2, Bim, cleaved caspase-9, cleaved caspase-3, and actin. Shown here are representative blots from at least three independent experiments. (B) Apoptosis determined by DAPI staining Cells were cultured in media with or without 10% FBS, caspase inhibitor, and/or TGF-α for 48 h. Cells were stained with DAPI, and the percentage of apoptotic cells was determined under a fluorescent microscope (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. n.s., not significant; $P > 0.05$; **, $P < 0.01$ (vs. data with no serum); Student's *t*-test.

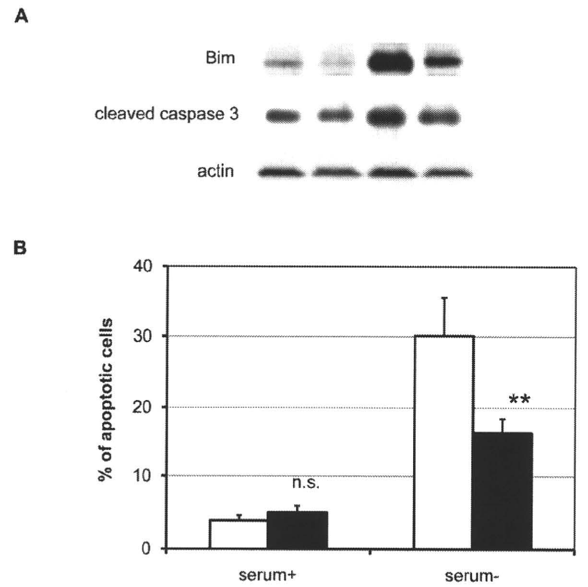

Figure 5 Effect of Bim siRNA gene silencing on cell growth inhibition and apoptosis. After treatment with Bim siRNA duplexes, an immunoblot analysis (A) and DAPI apoptosis detection assay (B) were performed. Shown here are representative blots from more than three independent experiments. All results are expressed as ratios to control siRNA-treated cells (control siRNA, white bars; Bim siRNA, black bars). Data are mean ± S.E. from at least three independent experiments, each performed in triplicate. Statistical analysis was performed with the Student's *t*-test. n.s., not significant; **, $P < 0.01$ (vs. control siRNA-treated cells).

siRNA against Bim reduced serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells

siRNA against Bim was used to knockdown Bim expression in Hep3B cells (Figure 5A). The expression level of cleaved caspase-3, decreased in Bim siRNA-treated cells (Figure 5A). Bim siRNA inhibited serum starvation-induced apoptosis by 46 ± 7% in RUNX3-expressing Hep3B cells (Figure 5B).

Transient ectopic RUNX3 expression in various HCC cell lines

RUNX3 was transiently expressed in various HCC cell lines, including Hep3B, Huh7, HLE, and HLF, introducing a RUNX3 construct-induced RUNX3 protein expression (Figure 6A). Transient RUNX3-expressing cells also showed growth inhibition after 48 h of serum starvation; the inhibition was 50 ± 10%, 46 ± 11%, 60 ± 8%, and 52 ± 9% in Hep3B, Huh7, HLE, and HLF cells, respectively. The RUNX3-expressing HCC cell lines demonstrated enhanced serum starvation-induced apoptosis; the percentage of apoptotic cells determined by DAPI staining was 21 ± 2%, 25 ± 2%, 19 ± 1%, and 20 ± 2% in Hep3B, Huh7, HLE, and HLF cells, respectively (Figure 6B). Serum starvation-induced Bim expression and caspase-3 cleavage were also confirmed in RUNX3-expressing Hep3B, Huh7, HLE, and HLF cells (Figure 6C).

Discussion

The results of the present study demonstrated that RUNX3 is a tumor suppressor gene for HCC. A significant down-regulation of RUNX3 was observed in a high percentage of human HCC cell lines (91%) and tissues (90%) (Figures 1, 2, and Table 1). RUNX3 has been described as a gastric cancer tumor suppressor [21]. In many cancer types, deletion of the RUNX3 locus and reduction of its expression by promoter hypermethylation has been

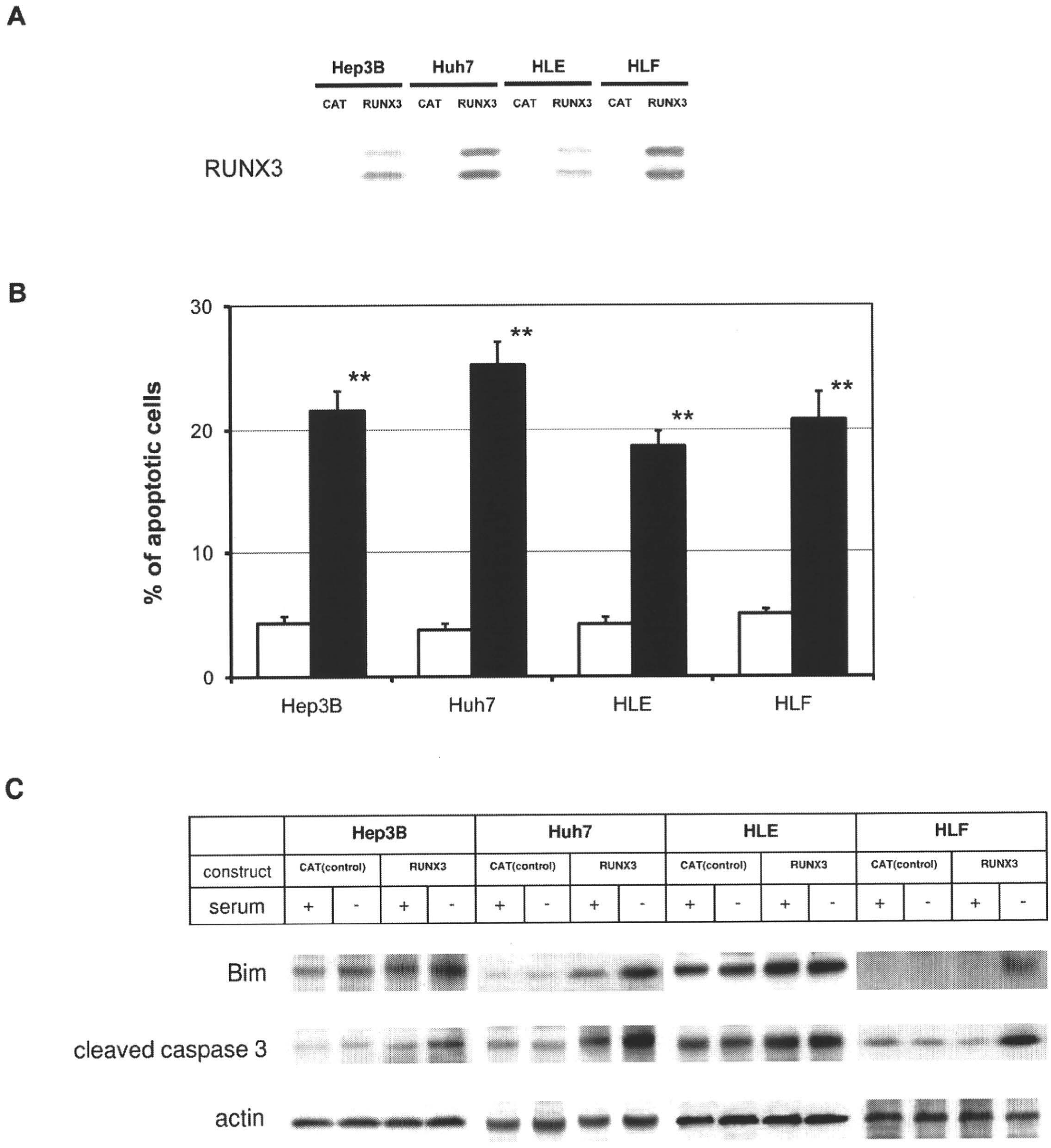

Figure 6 Effect of transient ectopic RUNX3 expression. CAT (control) and RUNX3 constructs were introduced into HCC cell lines. After a 48-h incubation period, an immunoblot analysis for RUNX3 expression (A), a DAPI apoptosis detection assay (B), and an immunoblot analysis for Bim and caspase (C) were performed. Shown here are representative blots from more than three independent experiments. All results are expressed as ratios to control CAT-transfected cells (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test.

reported [23-26]. However, little is known about the role of RUNX3 in HCC tumor suppression. We hypothesized that loss of RUNX3 expression contributes the development of HCC by escaping apoptosis. The results of the present study provide clear evidence that RUNX3 elicits serum starvation-induced apoptosis in HCC cells by activating the Bim-caspase pathway.

Stable expression of RUNX3 protein was established in Hep3B cells (Figure 3A), and they showed apoptosis under serum starved conditions (Figure 3B). This effect was reproducible in the Hep3B, Huh7, HLE, and HLF HCC cell lines transiently expressing RUNX3. The inhibition of cell growth in transient RUNX3-expressing cells was generally lower than that in stable RUNX3-

expressing Hep3B cells, probably due to low transfection efficiency.

Serum starvation-induced apoptosis is caused by caspase activation in ectopic RUNX3-expressing Hep3B cells (Figures 3C and 3D). To explore the signaling molecule responsible for apoptosis, Bim protein expression was induced in serum starved RUNX3-expressing Hep3B cells (Figure 4A). This is the first report demonstrating that RUNX3 enhances Bim expression under serum starved conditions in HCC cells, which appears to be consistent with the important role of Bim in previous studies on other types of cells. Bim expression was induced by the cooperation of RUNX3 and TGF-β in a study of gastric epithelial cells [21,31]. Bim protein also plays an important role in cell death [32]. Bim induces sequential activation of caspase-9 and -3 [32]. The potency of Bim as a cell death inducer is attenuated by Bax and Bcl-2 subfamily proteins [33]. The expression of Bax and Bcl was not affected by RUNX3 expression (Figure 4A). The expression of Bad (data not shown), a Bcl-2 antagonist known as a serum starvation-induced apoptosis initiator [34], increased with serum starvation but was not attenuated by RUNX3 expression (Figure 4A). Bim siRNA was used to evaluate whether Bim expression regulates serum starvation-induced apoptosis in RUNX3-expressing cells. As a result, Bim siRNA successfully knocked down Bim expression in RUNX3-expressing Hep3B cells (Figure 5A). Knockdown of Bim expression abrogated serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells (Figure 5B). Consequently, RUNX3 expression enhanced serum starvation-induced apoptosis through the Bim-caspase pathway in Hep3B cells. This effect was reproducible in the Huh7, HLE, and HLF HCC cell lines transiently expressing RUNX3 (Figure 6).

Serum starvation triggered apoptosis in RUNX3-expressing HCC cells. As this leads to the question of how serum prevents apoptosis in RUNX3-expressing cells, RUNX3-expressing Hep3B cells were treated with TGF-α, EGF, or PDGF (Figure 4C). These growth factors reduced apoptosis in RUNX3-expressing Hep3B cells by activating the PI3/Akt signaling pathway (data not shown), which is consistent with a previous report [34].

RUNX3 induces apoptosis in the presence of TGF-β [21]. In a study of gastric epithelial cells, RUNX3 enhanced Bim expression during TGF-β-induced apoptosis [21,31]. In a study of a gastric and esophageal cancer cell lines, RUNX3 expression made cancer cells sensitive to TGF-β-induced apoptosis [21,35-38]. These reports suggest that TGF-β is required for RUNX3-related apoptosis. In the present study, ectopic RUNX3 expression enhanced serum starvation-induced apoptosis in the absence of TGF-β. This discrepancy may be explained by the autocrine action of TGF-β in Hep3B cells, which have an intact TGF-β signaling pathway [39]. Furthermore, some HCC cell lines, including Hep3B, produce TGF-β [40]. Further study is required to establish whether TGF-β is involved in the enhanced apoptosis of HCC.

It has been reported that p53, Rb, p16, phosphatase, and tensin homolog (PTEN) are altered in HCC. The p53 gene is the most extensively studied gene of solid tumors. Alteration of this gene occurs at a relative low frequency (28-42%) in HCC compared to other solid tumors [11,17,41,42]. The Rb gene is another well-studied tumor suppressor gene in HCC and other solid tumors. Rb mutations are found in only 15% of HCCs [42]. The LOH of chromosome 13q, where Rb gene is located, is more frequent in HCC (25-48%) [43,44]. The p16 gene, also known as the cyclin-dependent kinase inhibitor 2A gene, regulates the Rb pathway and is found in 64% of HCCs [9]. PTEN negatively regulates the PI3K/Akt signaling pathway, which is involved in the regulation of cell survival [45]. Alteration of PTEN was found in ~40% of HCCs [10]. The frequency of alteration of each individual gene was relatively low, while RUNX3 expression was frequently down-regulated in both human HCC cell lines (91%) and tissues (90%).

Alterations in some tumor suppressor genes are due to LOH in HCC [17]. Similar to other tumor suppressor genes, some of the alterations in RUNX3 are due to the LOH of chromosome 1p36, where RUNX3 is located. Perhaps another mechanism for RUNX3 down-regulation is hypermethylation of the RUNX3 promoter region [13-16]. In a previous report, 30-40% of HCCs showed LOH of the RUNX3 gene and 40-80% showed promoter hypermethylation [28]. In agreement with these reports, RUNX3 down-regulation was detected in ~90% of HCC tissue specimens.

Conclusions

RUNX3 expression elicits serum starvation-induced apoptosis in HCC cells via the Bim-caspase pathway. Because RUNX3 expression is generally suppressed in HCC cell lines and tissues, loss of RUNX3 expression leads to tumorigenesis by escaping apoptosis.

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Authors' contributions
HS conceived the design and drafted the manuscript. YN performed experiments. NT, ST, SN, MU, MM, MI and AT helped performing experiments for YN. SN, YK, KN, KK, HH, JT, HO and TY contributed for the collection of HCC tissues. YN performed immunohistochemical study. KY provides financial supports and participates in the discussion of the results. All authors read and approved the final manusctipt.

Competing interests
The authors declare that they have no competing interests.

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治療の新たな取り組み　臓器別集学的治療—EBM に基づいて—

肝細胞癌の最新治療

池田健次

Current therapy of hepatocellular carcinoma

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Abstract

Multimodality treatment is applied according to varied stages of hepatocellular carcinoma (HCC), and to varied status of liver function. Surgical resection is regarded as the most radical way of therapy for an early stage of HCC (single large tumor, or small tumor of 3 cm or less with 3 nodules or less). Among percutaneous local ablation therapies, radiofrequency ablation is the most effective from the viewpoint of local recurrence and survival rate. Transcatheter arterial chemoembolization prolongs the survival period of those patients with an intermediate stage of HCC (tumors of more than 3 cm and/or 4 nodules or more). Sufficient evidence of the efficacy of chemotherapy is still lacking for advanced stages of HCC with or without portal vein invasion. Although sorafenib is the first molecular targeted medicine approved for the advanced HCCs, its usefulness remains unknown in Japanese patients.

Key words: hepatocellular carcinoma, local ablation, surgery, transcatheter arterial chemoembolization (TACE), radiofrequency ablation

はじめに

肝癌治療に関する先進国である我が国では，C型肝炎の新規感染の激減を受けて，肝癌死亡が横ばいから減少傾向を示し始めている．しかし，実際の臨床の場ではいまだ肝癌治療のニーズは衰えず，肝癌治療・肝癌予防の努力が続けられている．

本稿では各種肝癌の治療に関する治療法について，肝細胞癌の治療アルゴリズムとして総論を述べたのち，外科切除，局所治療，経動脈的治療，抗癌剤治療の順にエビデンスとしての観点から最新の知見を示す．

1. 肝細胞癌の治療法選択の基準

肝細胞癌の病態に応じた治療法の選択基準として幕内班の'肝細胞癌治療アルゴリズム'(図1)が推奨されている[1]．この肝癌治療に関するアルゴリズムは，肝障害度・腫瘍数・腫瘍径の3因子を基に設定されており，種々の治療法選択基準の中で最も単純化されている．

肝障害度AまたはBの症例においては，①腫瘍が単発ならば腫瘍径にかかわらず肝切除が推奨される(ただし，肝障害度Bの症例で腫瘍径

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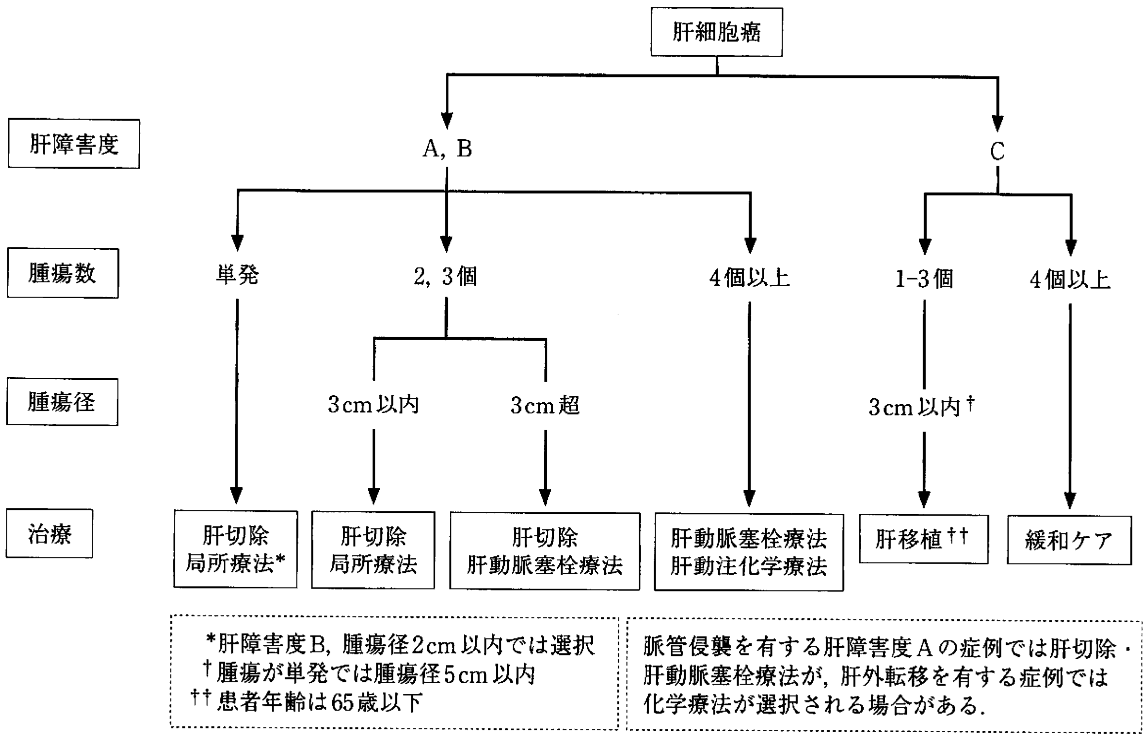

図1 肝細胞癌治療アルゴリズム(文献[1]より引用)

が2cm以内ならば経皮的局所療法も選択される), ②腫瘍数が2個または3個で腫瘍径が3cm以内ならば肝切除または経皮的局所療法が推奨される, ③同腫瘍数で腫瘍径が3cm超ならば肝切除または肝動脈塞栓療法が推奨される, ④腫瘍数が4個以上ならば肝動脈塞栓療法または肝動注化学療法が推奨される. 肝障害度Cの症例においては, ①腫瘍数が3個以下で腫瘍径が3cm以内(および腫瘍が単発で腫瘍径が5cm以内)で, 患者年齢が65歳以下ならば肝移植が推奨される, ②腫瘍数が4個以上ならば緩和ケアが推奨される. なお, 脈管侵襲を有する肝障害度Aの症例では肝切除・肝動脈塞栓療法が, 肝外転移を有する症例では化学療法が選択される場合がある.

2. 肝癌の外科治療

a. 外科治療の進歩

日本肝癌研究会による25年前の全国調査では, 原発性肝癌における肝切除症例の手術死亡率は27.5%, 5年生存率は11.8%であったが, 最近の第16回調査(2000-01年)では手術死亡率は0.9%, 5年生存率は54.6%と飛躍的に改善した. この治療成績向上は, 術前評価, 手術手技, 周術期管理などの総合的な外科診療の進歩によると考えられている.

b. 肝癌外科治療の適応決定

手術適応の決定は, 肝細胞癌の進行度評価と肝機能評価が必須である. 肝癌進行度は, 腫瘍の大きさ, 数, 血管侵襲, リンパ節転移, 遠隔転移の有無によって規定されるが, 治療法選択においては, 腫瘍の存在部位や過去の治療歴も考慮される. 肝機能からみた適応評価では, ICG 15分値(インドシアニン・グリーン15分停滞率)が単独の臨床検査値の中では優れるとされているほか, これら検査値の組み合わせによる計算式による様々な肝切除の基準が提唱され, 利用されている. 肝癌外科切除術式においては, 腫瘍根治と肝機能温存の両面から妥当と考えられる手術法が我が国から多数開発されてきた.

c. 系統的亜区域切除と部分切除

従来から議論のあった, 系統的亜区域切除の臨床的意義に関して, 同時期に2種類の見解が

示されている．一つはTanakaら[2]，Kaiboriら[3]の系統的切除の有用性を認めないとする報告であり，他は肝癌研究会集計[4]の多施設のretrospectiveな5,781例のデータである．前者のうち，Tanakaらは125例のretrospectiveなデータであり，系統的亜区域切除を行った群では再発率・生存率のいずれをとっても非解剖学的局所切除を行った群と差がないため，肝機能温存を主眼とした手術を行うことが重要としている．Jaubiriらも単一施設で247例のC型関連肝細胞癌の切除術式を検討し，解剖学的切除の有用性がなく，肝実質温存を目指す部分切除が良いとしている．一方後者の多施設データの集計では，系統的切除が行われた2-5cmの肝癌では，無再発生存率が有意に高いため，‘系統的切除の方が推奨される’と結論付けている．後者は大規模な集積比較研究であるが，相対的に再発率の低い軽度の肝障害症例に系統的切除が選ばれているという，retrospectiveな研究としてのバイアスを払拭するには至っていない．肝細胞癌治療ガイドライン[1]では，‘非肝硬変肝細胞癌に対する肝切除範囲に際しては，治癒切除が可能であれば必ずしも拡大切除は必要とせず，肝機能と腫瘍の進展に応じて部分切除でも十分’，‘肝切除において肝切離断端距離は必要最低限でよい’と記載している一方，‘予後の観点から肝切除では系統的に行うことが推奨される’としている．

3. 経皮的(穿刺)局所療法

肝癌に対する内科的な経皮的局所治療としては，ラジオ波凝固療法(RFA)，マイクロ波凝固療法(MCT)，エタノール局注療法(PEI)が保険診療で認可されているほか，最近では収束超音波治療(HIFU)，冷凍凝固治療などが応用され始めている．

a. 経皮的局所治療の適応

肝細胞癌治療ガイドライン[1]によると，穿刺局所療法の良い適応は，Child-Pugh分類AあるいはBの肝機能，腫瘍径3cm以下腫瘍数3個以下である．肝細胞癌治療における穿刺局所療法は肝切除に代わる第一選択の治療になりうるかについての結論は現在のところ出ていない．種々のデータ・報告より，経皮的局所治療の中ではRFAの成績が最も良好であるとされている[5,6]．

b. 経皮的局所治療と他治療との比較

日本肝癌研究会の解析(n=12,888)では，単発2cm未満で臨床病期(CS)Iでは肝切除の治療成績がPEIより良好(p=0.01)だが，CS II以上では肝切除とPEIで有意差はない．一方，単発で2cmより大きい症例では肝切除の治療成績が良好である．2cmより大きいCS IIにおいても肝切除の成績が良好であった[7]．国内18施設の肝癌症例(n=3,225)のretrospectiveな集計では，3cm以下3個以下の症例でCS Iでは5年生存率は肝切除とPEIで同等だがCS IIではPEIの生存率が高かった[8]．Livraghiら[9]は多施設共同研究で，2cm以下単発の218例の肝細胞癌治療について，RFAと肝切除を比較した．ここでは局所の持続制御率と治療関連合併症について検討しているが，局所の持続制御率は中央値31カ月で216例(97.2%)に得られ，治療侵襲も少ないことより，RFAが小型肝癌の基本的治療であるとしている．良質の無作為化試験で肝切除と経皮的局所治療を比較した研究は今のところ発表されていない．

Murakamiら[10]は，3cm以下3個以下もしくは5cm以下単発の肝癌に対してRFAまたは肝動脈化学塞栓療法(TACE)で治療された連続258症例について局所再発率を検討し，有意にRFAがTACEに勝っていた(p=0.013)データを示している．

c. RFAと他治療の併用，その他RFAの最近の知見

新しいタイプのRFA機器として，電極冷却型バイポーラ電極を使用した大型肝癌治療の初期成績が報告されている[11]．肝癌は5-9cmの26例で，1症例1-2回の治療が行われた．27結節中22個(81%)で完全壊死が得られ，平均14カ月の観察期間で14%の局所再発，24%の異所再発がみられたとしている．肝機能良好で脈管侵襲のない大型肝癌では，肝切除以外に治療オプションとして今後広く行われる可能性がある

と思われる.

RFA施行後の新規肝癌の増殖速度は無治療の自然経過の病変より早いことがParkら[12]により報告されている. 彼らはこのmultiphasic CTを繰り返し行って算出した増殖速度から, RFA後の画像経過観察は2.5カ月ごとに行うことが望ましいとしている.

Lencioniら[13]は, 3.3-7cm(平均5.0cm)のやや大型の肝癌に対して, ドキソルビシン溶出ビーズ(DEB: ドキソルビシン50-125mg)肝動注併用RFA治療のパイロット試験を20例に行った. RFA治療後の壊死域は48cm^{3}であったが, DEB動注後には75.5cm^{3}に増大した. 重大な合併症はなく, 肝機能の増悪もみられず, 目標病変が12例(60%)で完全壊死に陥ったとしている. Chengら[14]は, 3cmを超えるやや大型の肝癌に対して, RFA+TACE, TACE単独, RFA単独の無作為化比較試験を行った. 併用治療96例, TACE単独95例, RFA単独100例に割り付けられ, 生存率・直接治療効果が検討された. 生存期間の中央値は, それぞれ37カ月(治療4.4コース), 24カ月(3.4コース), 22カ月(3.6コース)で, 併用治療を行った群の生存率が最も良好であったとしている. Yamakadoら[15]も, RFA+TACE治療を行い, これを肝切除の成績と比較している. これは104例の早期の肝癌についてretrospectiveな検討を行ったもので, 内科的なRFA+TACE治療で無再発生存率・全体生存率ともに外科とほぼ同じ成績が得られたとしている.

Zhangら[16]は, RFAにPEI併用を行う・行わないの無作為化比較試験を行った. RFA+PEI併用群66例, RFA単独群67例での1年・2年・3年・4年・5年生存率は, 併用群で95.4%, 89.2%, 75.8%, 63.3%, 49.3%, RFA単独群で89.6%, 68.7%, 58.4%, 50.3%, 35.9%で, 併用群の生存率が有意に良好であった($p=0.04$). 全再発率に関しては, 併用群23例, 単独群33例で, 両群間に有意差はなかったが, 局所再発は, 併用群4例, 単独群14例で, RFA+PEI併用群が有意に優れていた($p=0.012$). 著者らは腫瘍径が予後に最も影響する因子であったとしており, 大型肝癌では併用療法の有用性があるとしている.

Kobayashiら[17]は, RFA時に肝動脈のバルーン閉塞を行う意義を無作為化比較試験で検討した. 動脈閉塞の意義が壊死領域を大きくすることは既に広く知られているが, 本論文では同亜区域内の再発率に影響するとする長期成績を示したところが重要である. 一方, Sudheendraら[18]は, RFA治療の際に間欠的に肝静脈をバルーン閉塞させることの意義を検討した. 中央値4.2cmの肝腫瘍8例に治療を行い, 平均径6.3cmの壊死域が作成でき, 静脈血栓などの合併症は起こらなかったとしている.

d. RFA治療の副作用・合併症

Kasugaiら[19]は, 大阪地区多施設の2,614例, 3,891回のRFA治療について副作用の頻度をまとめた. 治療症例中合併症を認めたのは207例(7.9%)であった. うち3カ月以内死亡9例(0.3%)がみられ, その死因は肝不全3例, 肝癌急速進行3例, 胆管損傷1例, 消化管出血1例, 急性心筋梗塞1例という内訳で, 治療経験数が増せばその頻度を減少させることができるとしている. Kimら[20]は, 872例1,120セッションのRFA(cool-tip電極)を行い, 20回(1.8%)の肝梗塞がみられたと報告している. このうち2例はbiloma, 2例は肝膿瘍, 1例は門脈血栓, 1例は肝葉単位の梗塞から肝不全死に至った. 多変量解析では高齢・腫瘍径が肝梗塞発症に関連し, 頻度は少ないが注意すべきであると記載している.

Kimら[21]は, 非代償期肝硬変合併肝癌19例26結節に対してcool-tip電極を用いたRFAを行った. 治療6カ月後に腫瘍が完全壊死と判定されたのは23結節(88.5%)で, 50%生存期間12カ月であった. 2例は肝不全死(1例は2カ月後, 1例は4カ月後)で, 他は6カ月以上生存した. 第1週目, 第2週目のトランスアミナーゼ・ビリルビンは有意に上昇し, 肝障害の増悪がみられることもあり, 非代償期肝硬変合併肝癌患者に対するRFAは適切な患者選択を行うべきであるとしている.

e. そのほかの肝癌局所療法

Ohmotoら[22]は，RFAとマイクロ波凝固療法(MCT)とのretrospectiveな比較で，侵襲度・局所再発の低さ・高い生存率・少ない回数で大きな壊死域など，多くの点でRFAが勝っているとした．少数例のretrospectiveな比較であり，またRFAの方が最近行われた治療という‘練習効果’の影響などがあり，長期予後までの効果は十分に示されているとはいえないが，腫瘍が大きく焼灼できるという点に加えて，RFAでは胆管障害や胸水貯留などの副作用が経皮的MCT群に比べて少なかったとしている．

経皮的治療のうち，針を穿刺しなくてもすむ無侵襲の治療として収束超音波治療(HIFU)が徐々にその臨床成績を示しつつある．Liら[23]は，これまで主として小型肝癌が対象とされてきたHIFU治療を，切除不能大型肝癌151例に対して施行し，CR 43例(28.5%)，PR 91例(60.3%)の成績を示した．これはHIFUと他の支持療法の併用での成績ではあるものの，対照群30例ではそれぞれ0%，16.7%であった結果と比較してHIFUが良好な効果を示しているとした．また，1年・2年生存率がそれぞれ50.5%・30.9%と優れていたと長期成績についても論じているが，長期成績の評価には，他の支持療法・継続的な集学的治療の効果を十分に考慮する必要があると考えられる．

Hanajiriら[24]は，治療前にmicrobubbleの投与を行うとHIFUの抗腫瘍効果を高めるとする動物実験データを示しており，将来の臨床応用への有用性を示唆した．

肝癌に対する冷凍凝固療法は1990年頃より欧米・中国などで試みられているが，機器が一般的でなく，プローブも3-10mmと太いこともあって外科的に行われるなど，我が国ではPEI・RFAなどのように広く行われていない．このようななかで，イタリアのOrlacchioら[25]は，超音波ガイド下経皮的に17ゲージの冷凍凝固プローブを複数本穿刺し，これをCTでモニターする方法を示し，経皮的な治療が可能であることを示した．

4. 肝動脈(化学)塞栓療法(TACE/TAE)

肝臓は肝硬変になっていても動脈・門脈の2血流の支配を受けているのに対し，進行した肝細胞癌は肝動脈のみの血流に依存していることを利用して，肝動脈末梢部を経カテーテル的に塞栓することで肝細胞癌のみを壊死に陥らせることを目指す治療である．TACEは肝細胞癌に特有な治療法で，原発性肝癌である胆管細胞癌や転移性肝癌での治療効果は劣る．

a. 肝動脈化学塞栓療法の有効性

我が国では1980年頃から1990年代中頃までは切除不能と判断された腫瘍濃染像を有する肝細胞癌の大多数はTACE/TAE療法が施行されていた．その後種々の経皮的凝固療法の導入により，切除不能肝細胞癌でかつ経皮的凝固療法の対象外とされている肝細胞癌がTACE/TAEの適応となってきた．1990年代のランダム化比較成績では，TACE/TAEの有用性は証明されなかったが，2000年代に入り2編の無作為化比較試験論文とメタアナリシスでTACE/TAEは切除不能肝細胞癌の予後向上に寄与することが証明された[26,27]．

b. 肝動脈化学塞栓療法の適応

肝細胞癌診療ガイドライン[1]では，腫瘍径3cm，3個以内の肝癌は肝切除や経皮的局所治療の適応，また単発では大型のものまで肝切除の適応があるので，これら‘根治的な治療’が困難な症例に対して肝動脈化学塞栓療法・肝動注化学療法が適応と考えられる．一方，門脈浸潤・閉塞が起こっている進行肝癌ではTACEにより肝不全をきたすため，禁忌である．肝機能からは，高度の腹水や肝性脳症のあるChild-Pugh分類Cの症例，ビリルビン3mg/dL以上の肝予備能不良の症例は適応外である．TACEは様々な治療法の中では，根治療法不能の‘中等度進行肝癌’の広い適応範囲をもった治療法である．

まとめて示すと，TACE/TAEはChild-Pugh分類A，Bの進行肝癌(手術不能で，かつ経皮的凝固療法の対象とならないもの)に対する治療として推奨されている．安全で有効なTACEのためには，化学塞栓される非癌部肝容積の非癌

部全肝容積に占める割合と残肝予備能を考慮したTACE/TAEが推奨される.

c. 肝動脈化学塞栓療法の具体的な方法

担癌区域(肝癌の存在する区域)・亜区域までカテーテルを挿入し,これより抗癌剤・脂溶性造影剤(リピオドール™)懸濁液を動注した後,ゼラチンスポンジ細片(ジェルパート™)により塞栓する.肝両葉に腫瘍が存在する場合には,固有肝動脈からTACEを行う場合もあるが,腫瘍の栄養血管を同定し末梢動脈までカテーテルを挿入して,抗癌剤+リピオドール懸濁液を強く注入した後に塞栓を行う(亜)区域性Lp-TACEの治療効果は高く,腫瘍の完全壊死も期待できる.

[**症例1**(通常のTACE,小型少数で限局した腫瘍)] エピルビシン40mg+リピオドール3mL,その後ジェルパートで塞栓

[**症例2**(両葉多発,進行肝障害,門脈末梢部腫瘍塞栓合併例)] シスプラチン100mg(70mL溶解液),リピオドール4mLを少量ずつ交互に肝動注

[**症例3**(両葉多発,進行肝障害,門脈末梢部腫瘍塞栓合併例)] ミリプラチン80mg+リピオドール4mL懸濁液を担癌区域動脈に動注

5. 進行肝癌に対する動注化学療法と分子標的薬

早期肝癌(3cm以下少数),中期肝癌(3cm超,3個超だが脈管浸潤なし)に対しては,肝切除・経皮的局所治療,TACEなどの有力な治療が存在するため,抗癌剤治療は主として進行肝癌に対して適応を考慮する.

肝細胞癌に対する化学療法に関する論文は,他の悪性腫瘍に対する化学療法のように多数の症例を検討したランダム化比較試験といったエビデンスレベルの高いものは少ない.進行肝癌を中心に様々な化学療法が報告されているが,対照群を伴わない症例集積研究や後ろ向き研究が多い.

a. 動注化学療法

単剤での肝動注療法による奏効率は,シスプラチンで37%,ドキソルビシンで60%というコホート研究があるが,進行肝癌に対する治療効果はこれより劣る.進行肝癌に対する良好な直接治療効果として報告されているのは,①シスプラチン肝動注とインターフェロン全身療法併用[28,29],②5-FU持続肝動注+インターフェロン全身投与併用[30,31],③5-FU持続肝動注+シスプラチン併用療法の組み合わせ[32]である.

進行肝細胞癌に対する動注化学療法は我が国を中心に盛んに行われているが,生命予後まで示した無作為化比較試験というものは報告されておらず,エビデンスとしての標準的な治療としては確立されていない.

b. 分子標的薬

2008年7月にソラフェニブ(ネクサバール™)の進行肝細胞癌への有効性が大規模二重盲検ランダム化比較試験[33]で示され,2009年5月より我が国でも進行肝癌(門脈浸潤,肝外転移のある肝機能良好例)に対する治療薬として保険診療が可能となった.しかし我が国では使用開始後短期間での肝不全・肝性脳症が多くみられる一方,2009年12月には,中期進行肝癌に対して我が国で行われた'TACE施行例に対するソラフェニブの無作為化比較試験で有効性が示されなかった'とのデータが開示された.日本人におけるソラフェニブの有効性や副作用に関してはまだ十分なデータがない状態であり,副作用に十分な注意を払いながら慎重に有用性を検討していく必要がある.

現在世界中で20種類を超す分子標的薬が肝癌に対して治験がなされている状況であり,今後の分子標的薬の位置づけは個々の薬剤で検討していく必要がある.

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総　説

肝癌の画像診断と内科的治療

池田　健次

要旨：わが国の肝癌死亡数は減少に転じたといわれているが，診療実数は増加傾向であるほか，高齢化・合併症併存など治療に影響する要因も変化しつつある．肝癌治療に際しては，生物学的悪性度の評価が欠かせず，画像診断・腫瘍マーカーの両面からの理解が必要である．経皮的ラジオ波凝固療法は集学的治療の一手段として欠かせないが，反復治療例・超音波での描出困難例が増加傾向であり，造影エコー検査・Real-time virtual sonography，炭酸ガス動注エコー検査などの補助手段の必要性が増している．

肝胆膵治研誌8（1）：5～11

Key words：肝細胞癌，ラジオ波凝固療法（RFA），生物学的悪性度，PIVKA-II

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最近の肝癌の動向

1970年代より急速な増加を示していた原発性肝癌死亡者数は2000～2005年の間に，年間死亡者数34,000人をピークにやや減少傾向に入っているといわれている．当然のことながら，新規のC型肝炎関連肝細胞癌の減少に伴う変化であるが，一方では，C型慢性肝炎から肝硬変を経ずに直接発癌してくる高齢者，非アルコール性脂肪性肝炎（NASH）からの発癌者の増加，背景に「原因」を有さない高齢肝細胞癌患者の漸増，また，糖尿病合併患者の急増など，肝細胞癌発生者の内容に若干の変化もみられる．

経年的に行われる各施設の肝切除・ラジオ波凝固療法（RFA）・肝移植症例数の推移からは，小型肝癌ではラジオ波凝固療法治療症例の漸増とともに，年間10例以上の肝移植治療を行っている外科施設の増加が目立っている．

肝癌高危険群の考え方

肝癌の背景疾患に変化がみられつつあるとはいえ，慢性肝疾患がなく肝炎ウイルス感染もない人から肝癌が発癌してくることは例外的である．わが国では，何らかの慢性肝疾患があるか，HBV・HCVのいずれかの保因者であることが第一の肝癌のハイリスク群である．C型肝硬変はその中でも発癌リスクが高く，このうち男性・高齢・低アルブミン血症・高AFP血症・低血小板血症・ICG15分値高値などの要因は，発癌率をいっそう高める．B型肝硬変はC型肝硬変の約半分の発癌率を示し，肝細胞癌発癌の明らかなハイリスク群をなす．B型慢性肝炎では，e抗原が陰性でトランスアミナーゼが安定していれば病変が進行しないことも多く，一般的には肝病変進行・肝癌発癌のリスクは少ない．しかし，発癌率は低いながらもHBs抗原が陽性であるだけの安定したキャリアから発癌することがあり，臨床的にしばしば問題となる．絶対数は多くないものの，これらHBs

Imagings and medical treatment of hepatocellular carcinoma
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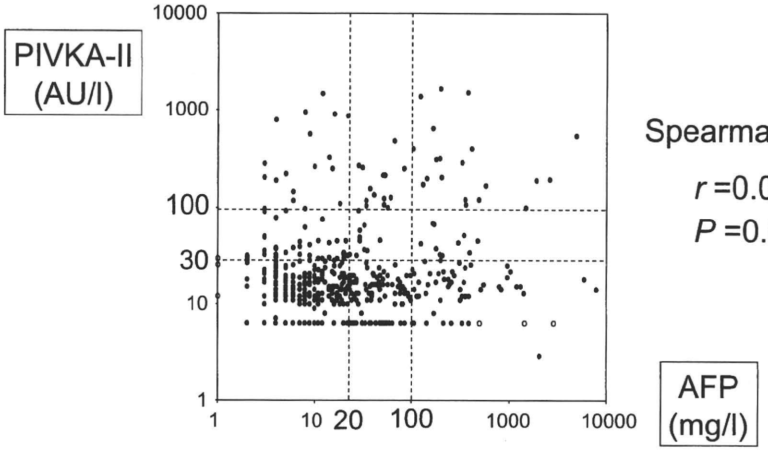

図1 小型肝癌診断時の腫瘍マーカー陽性率
根治治療(肝切除またはRFA)が行えた直径3cm以下の肝癌540例
AFPとPIVKA-IIとの間に相関はなく，両者は相補的関係にある.

抗原陽性者では，これ以上の発癌高危険群を絞り込むことがやや困難である.

以上から，肝細胞癌の危険因子としては，肝硬変，C型慢性肝炎，B型慢性肝炎，男性，高齢，アルコール摂取，アフラトキシンなどが肝発癌の危険因子として挙げられる[1].

肝癌発生監視のためのフォローアップ

2～6カ月ごとに定期的に画像スクリーニング検査(超音波検査，X線CT，MRI)を繰り返し，1～2カ月ごとに腫瘍マーカー(AFP，PIVKA-II)の測定を行うことで，単発・小結節の段階で肝癌が検出される可能性が高くなる[1]．慢性肝疾患に対する定期スクリーニングにより早期に肝癌が検出され根治的治療を受ける機会が増えるが[2,3]，このことにより予後が明らかに改善するとの根拠には比較的乏しい．Zhangら[4]は，上海都市部でB型肝炎ウイルス感染者もしくは慢性肝炎といわれたことのある18,816例を登録したprospectiveな研究を発表した．サーベイランス群(N=9,373)は6カ月ごとに超音波検査とAFP検査を施行し，対照群(N=9,443)はサーベイランスを行わない例からなり，両者の比較を行ったところ，10万人あたりの肝癌診断率はサーベイランス群で1.37倍高く，死亡率の比較ではサーベイランスを行うと死亡率が37％減少したとして，無症状期の定期的スクリーニング検査の意義を示している.

肝癌症例でのAFPとPIVKA-IIとは相関しないことが知られている．当院でも直径3 cm以下の小型肝癌のうち根治治療のできる「早期肝癌」540例について，AFPとPIVKA-IIの相関を調べた(**図1**)．AFPのカットオフは20～100 mg/L，PIVKA-IIのカットオフは30～100 AU/Lとしてみても両者には相関は全くなく，Spearman相関係数は0.005(P=0.91)であった．それゆえ，肝細胞癌の診断において，2種以上の腫瘍マーカーを測定することに意味があり，小肝癌の検出感度を向上させる．このうち，AFPとPIVKA-IIとの組み合わせの報告は多数なされており，直径2 cm，3 cmなどの小型肝癌での陽性率が1.5倍程度まで上昇するため[5]，両者を交互または同時に測定することが望ましい.

超音波検査と腫瘍マーカーの併用による肝細胞癌定期的スクリーニングを軸に，dynamic CT/dynamic MRIを併用した定期的スクリーニングを行うと，肝細胞癌が単発小結節の段階で検出される可能性が高まる[1]．これは特に，B型慢性肝疾患の経過観察や，超音波検査で死角を作りやすい肝硬変症例でのフォローアップの際に考慮すべきである．最近ではMRI検査用造影剤としてGd-EOB-DTPAが使用可能となり，動脈血流が証明されない早期の時期での肝癌検出が可能となった