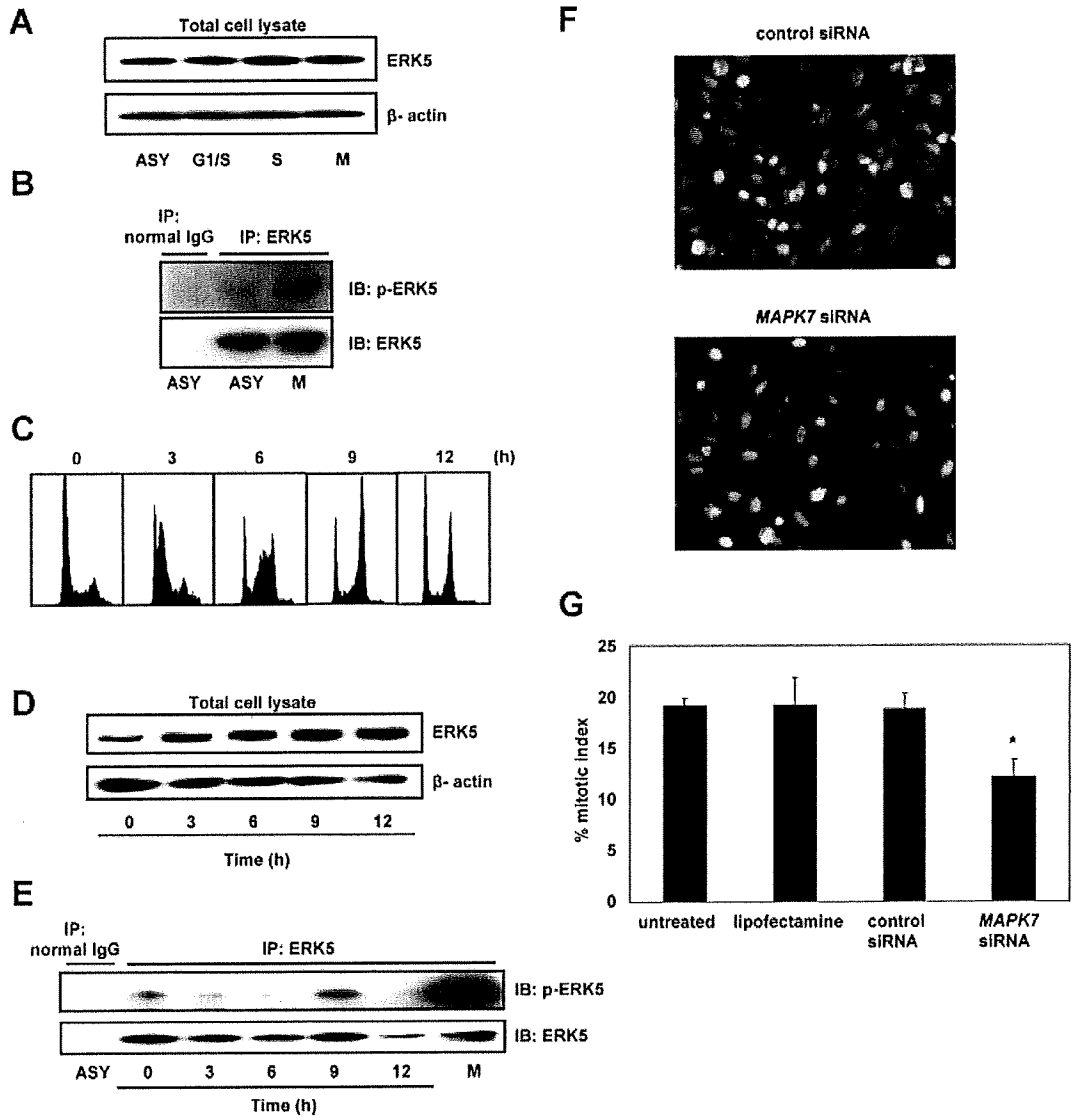


Figure 6. ERK5 is phosphorylated during the G2/M phases of the cell cycle. (A) Immunoblot analysis to detect protein levels of total ERK5 and β-actin, an internal control, in SNU449 cells that were synchronized at the G1/S, early S, or M phases using a double-thymidine, aphidicolin, or nocodazole block, respectively, or were untreated and used as an asynchronous (ASY) population. (B) Levels of phosphorylated ERK5 (p-ERK5). ERK5 was immunoprecipitated (IP) from lysates of SNU449 cells that were synchronized at the M phase (M) or from asynchronous cells (ASY). The samples were split and analyzed by immunoblotting (IB) for p-ERK5 and total ERK5. Normal rabbit immunoglobulin (normal IgG) was used as a negative control for immunoprecipitation. (C) Flow cytometric analysis. SNU449 cells were synchronized to the G1/S boundary using a double-thymidine block. Synchronized cells were released from the block and harvested at the indicated time points. The X-axis indicates DNA content and the Y-axis indicates the number of cells. (D) Time course of changes in the level of total ERK5 after release from the double-thymidine block. The level of β-actin was used as an internal control. (E) Time course of changes in the level of p-ERK5 after release from the double-thymidine block. ERK5 was immunoprecipitated from lysates of SNU449 cells harvested at the indicated times after release from the double-thymidine block. The samples were split and analyzed by immunoblotting for p-ERK5 and total ERK5. SNU449 cells, synchronized at the M phase with nocodazole, were also examined as described in (A) and (B). Normal rabbit IgG was used as a negative control for immunoprecipitation. (F) Representative images of mitotic cells in an SNU449 cell population that was transfected with *MAPK7*- or control-siRNA. SNU449 cells were treated with siRNA targeting *MAPK7*, negative control siRNA, or the transfection agent alone (Lipofectamine). Untreated cell were maintained under identical conditions. These cells were synchronized at the G1/S boundary using a double-thymidine block. The synchronized cells were released from the block and stained with anti-phospho-histone H3 9 hr after release, a time corresponding to the G2/M phase as shown in (C). Mitotic cells were identified by positive staining for phospho-histone H3 (green). Nuclear DNA was stained with propidium iodide (red). (G) The mitotic index was scored as described in Materials and Methods section. Data are presented as means ± SD (ANOVA; $*P < 0.05$).

expression using RNAi and assessed its effect on mitosis. SNU449 cells were transfected with siRNA targeting *MAPK7* and synchronized at the G1/S-phase boundary by a double-thymidine block. The synchronized cells were released from the block and harvested 9 hr after release, a time which corresponds to the G2/M phase (Fig. 6C). Finally, harvested cells were stained with anti-phospho-histone H3 antibody, which specifically detects mitotic cells (Fig. 6F). Compared with a control siRNA or transfection agent alone, transfection of *MAPK7* siRNA significantly reduced the mitotic index (Fig. 6G). These findings suggest that ERK5 regulates mitotic entry in the HCC cells.

## DISCUSSION

High-density SNP arrays are powerful tools for high-resolution analysis of DNA copy number aberrations in cancers. In the present study, using the Affymetrix GeneChip 100K and 250K SNP arrays we detected a novel amplification in HCC cells at 17p11. We were able to narrow the amplification to a 750-kb region. Notably, the amplification might have been missed using conventional analyses such as CGH. Amplification at 17p11.2-p12 has been detected in high-grade osteosarcoma using CGH (Forus et al., 1995; Tarkkanen et al., 1995). The group of van Dartel et al., (2002) established 17p11.2-p12 amplification profiles by semi-quantitative PCR using 15 microsatellite markers and seven candidate genes to assay amplification in this tumor type. They found that most of the tumors had complex amplification profiles, suggesting that multiple amplification targets, including *MAPK7*, might be present in region 17p11.2-p12. In contrast, we were able to define a smaller common region of amplification at 17p11 in two HCC cells and to determine the expression status of all genes in the amplicon. Three of the seven genes in the amplicon; *EPN2*, *EPPB9*, and *MAPK7*, were always overexpressed in cells that showed amplification in the 17p11 region. Thus, we considered these three genes as candidate targets for amplification. The function of *EPPB9* (B9 protein) is not known, and the protein encoded by *EPN2* (epsin 2) is similar to epsin 1, which plays a putative role in clathrin-mediated endocytosis (Rosenthal et al., 1999). Therefore, we focused on *MAPK7* as a target for the amplification.

Several lines of evidence implicate ERK5, which is encoded by *MAPK7*, in tumorigenesis (Wang and Tournier, 2006): (a) the ERK5 pathway is activated by Ras (English et al., 1999), ErbB (Esparis-Ogando et al., 2002; Yuste et al., 2005), Src (Sun et al., 2003), Cot (Chiariello et al., 2000), Bcr-Abl (Buschbeck et al., 2005), insulin-like growth factor-II (Linnerth et al., 2005), and interleukin-6 (Carvajal-Vergara et al., 2005); (b) ERK5 is involved in the control of breast cancer cell proliferation (Esparis-Ogando et al., 2002); (c) ERK5 mediates a survival signal that confers chemoresistance to breast cancer (Weldon et al., 2002); (d) insulin-like growth factor-II promotes cell survival via the ERK5 pathway in lung cancer cells (Linnerth et al., 2005); (e) the level of ERK5 contributes to the survival of Bcr/Abl-positive leukemic cells (Buschbeck et al., 2005); (f) ERK5 regulates cell proliferation and antiapoptotic responses in multiple myeloma (Carvajal-Vergara et al., 2005); and (g) an elevated level of MEK5, a specific activator of ERK5, is associated with metastasis and a poor prognosis in prostate cancer (Mehta et al., 2003).

The present study is the first to show the status of amplification and expression of *MAPK7* and its functional role in HCC. We found that *MAPK7* is amplified in 35 of 66 HCC tumors (53%). However, we could not determine the copy number of *MAPK7* in the nontumorous counterparts of the samples assayed because these samples were not available. Therefore, we cannot exclude the possibility that copy number polymorphism might influence the results of copy number analysis. We studied the expression of ERK5 using immunohistochemical analysis in primary HCCs and their surrounding nontumorous liver tissues. In nontumorous liver tissues, ERK5 was weakly expressed in the cytoplasm of non-neoplastic hepatocytes. Intriguingly, it was more strongly expressed in bile ducts, bile ductules, and a few small hepatocytes. In HCC tumor tissues, ERK5 was expressed in the cytoplasm of tumor cells. The level of ERK5 was elevated in 11 of 43 HCC tumors compared with their nontumorous counterparts. However, we did not observe a significant link between the level of ERK5 and any clinicopathological parameters. A recent report showed that, in prostate cancer, an increase in ERK5 cytoplasmic signals correlates with advanced disease and that strong nuclear ERK5 localization correlates with poor survival (McCracken et al., 2008).

We examined the functional roles of ERK5 in HCC cells using RNAi. Downregulation of *MAPK7* by siRNA suppressed the growth of

SNU449 cells, which had the greatest amplification and overexpression of *MAPK7* of all of the cell lines tested. These findings suggest that increased levels of ERK5 enhance the growth of HCC cells. Moreover, our results indicate that ERK5 is phosphorylated during the G2/M phases of the cell cycle and that it regulates entry into mitosis, which may explain how it promotes the growth of HCC cells.

Conflicting results have been reported by different investigators regarding the role of ERK5 in cell cycle progression. Some investigators have reported that ERK5 regulates the G1/S transition: expression of a dominant-negative form of ERK5 prevents cells from entering the S-phase of the cell cycle (Kato et al., 1998), and ERK5 can drive cyclin D1 expression (Mulloy et al., 2003). In contrast, Cude et al., (2007) and Gírio et al., (2007) recently reported that ERK5 is activated at the G2/M phases and is required for mitotic entry, findings that agree with our results.

Few molecules have been identified as direct downstream targets of ERK5. The transcriptional factors of the monocyte enhancer factor 2 family are among the best characterized substrates of ERK5. Phosphorylation of monocyte enhancer factor 2C by ERK5 enhances its transcriptional activity and subsequently leads to an increase in c-Jun gene expression (Kato et al., 1997; Wang and Tournier, 2006). A more complete identification of components downstream of ERK5 will be necessary to fully understand the role of ERK5 in carcinogenesis.

In summary, using high-density SNP arrays, we identified *MAPK7* as a probable target for the amplification events at 17p11 in HCCs. Our results suggest that the ERK5 protein product of the *MAPK7* gene plays a role in proliferation of HCC cells by regulating mitotic entry and may therefore be an optimal target for the development of novel therapies for this widespread type of cancer.

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# Expression of Aurora B and alternative variant forms in hepatocellular carcinoma and adjacent tissue

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(Received August 6, 2008/Revised November 20, 2008/Accepted November 23, 2008/Online publication January 5, 2009)

**Surgical resection is the effective treatment modality for hepatocellular carcinoma (HCC); however, rapid recurrence of the tumors are frequently observed even after apparently curative resection. The recurrence and prognostic assessment of patients with HCC after resection is an important clinical issue. We recently reported that aberrant expression of Aurora B is observed in primary HCC, and that it can be a predictive factor for HCC recurrence exceeding Milan criteria after curative hepatectomy. In this study we investigated the expression of the newly observed Aurora B splicing variant forms in HCC, and their roles in hepatocarcinogenisis. The expression of Aurora B and splicing variant forms were screened in 125 HCC patients (94 chronic hepatitis with cirrhosis background liver specimens), 18 metastatic liver cancer patients and 16 normal liver specimens by cDNA microarray, reverse transcription – polymerase chain reaction (RT-PCR) and Real time quentitative Reverse Transcription PCR (qRT-PCR). The results showed that expression of Aurora B splicing variant 2 (AURKB-Sv2) variant form was absent in normal liver and was higher in metastatic liver cancer than HCC. This aberrant expression was associated with the advanced stages of HCC ($P < 0.01$), correlated with a poor outcome ($P = 0.008$) and short disease-free period ($P = 0.018$). Furthermore, AURKB-Sv2 variant form is associated with a higher level of serum α-fetoprotein, protein induced by vitamin K absence or antagonist-II (PIVKAII), tumor capsular invasion, multiple tumor formation and at an age younger than those with other variant forms ($P < 0.05$). The results thus suggest that AURKB-Sv2 variant form is more significantly associated with the advanced stages of HCC than others and is a marker of poor prognosis. Founded in the tumor capsular invasion and multiple tumor regions, suggests that this might play a role in enhancing multiple malignant tumor formation and recurrence of HCC in hepatocarcinogenesis. This is the first study to report clinicopathological significance of aberrant expression of AURKB-Sv2 variant form in hepatocellular carcinoma. (*Cancer Sci* 2009; 100: 472–480)**

Hepatocellular carcinoma (HCC) is one of the major causes of cancer death in the world.[1,2] As a result of advances in the diagnosis and disease management of HCC, significant improvements in overall and disease-free survival rates after resection of HCC have been achieved within the past decade.[3,4] Surgical resection is the effective treatment modality for HCC; however, rapid recurrence of the tumors are observed frequently even after apparently curative resection.[5,6] Even when curative resection is performed, a considerable number of patients develop intrahepatic and/or extrahepatic recurrence postoperatively.[7–9] The recurrence and prognostic assessment of patients with HCC after resection is an important clinical issue. Hepatic recurrence has been classified as intrahepatic metastasis and multicentric recurrence and the long-term outcomes are affected mainly by metastatic recurrence.[10,11]

The development of DNA microarray technology has enabled us to analyze genome-wide profiles of gene expression specific to malignant tumors. Using this technology, we investigated the messenger RNA (mRNA) expression patterns in primary HCC, and clarified that the genes associated with cell cycle, especially Aurora kinase B (AURKB), were up-regulated significantly in hepatocellular carcinoma in relation to the invasion of the portal vein and/or hepatic vein. We recently reported that Aurora B aberrant expression in primary HCC can be the predictive factor of HCC recurrence exceeding Milan criteria after curative hepatectomy.[12]

AURKB is known as an aberrantly expressed gene in many cancer and tumor cell lines.[13–16] Aurora B exists in a complex with at least two other proteins, inner centromere protein (INCENP) and Survivin. Aurora B, INCENP, and Survivin are so-called chromosomal passenger proteins and they associate with inner centromere regions during prophase, but subsequently relocalize to the midzone of the central spindle and concentrate at the midbody.[17–20] Aurora B is localized on the controversy from prophase through the metaphase-anaphase transition. Another chromosome passenger protein is INCENP, which tightly associates with Aurora B, probably regulating its activity. It is overexpressed throughout the cell cycle in cancer cells.[21–23] Overexpression of Aurora B produces multinuclearity and induces aggressive metastasis, suggesting that overexpressed Aurora B has multiple functions in cancer development. Honda *et al.*[24] mentioned about smaller immunoreactive protein of Aurora B represents either a cleavage product or a splicing variant of Aurora B. Sistayanarain *et al.*[13] reported that Aurora B and its two variant forms are expressed in HCC (by studies with some limited samples by reverse transcription – polymerase chain reaction [RT-PCR]). However, the roles of expression of Aurora B and variant forms in HCC and other adjacent tissues are unknown. In this study we aimed to investigate the expression of the newly observed Aurora B splicing variant forms in HCC, adjacent tissue and their roles in hepatocarcinogenisis.

## Materials and Methods

**Cancer cell lines, patients and tissue samples.** The human HCC cell lines, PLC/PRF/5, SK-Hep1, HepB3, were obtained from the American Type Culture Collection (Manassas, VA, USA), JHH4, JHH5, HLE, HepG2, Huh1, Huh6, Huh7 were obtained

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Abbreviation: HCC; AURKB-WT; Aurora B kinase; Aurora B kinase splicing variants; cDNA microarray.



doi: 10.1111/j.1349-7006.2008.01068.x

Table 1. Clinicopathological data for 125 primary HCC cases

| Clinicopathologic factor | Primiry HCC $n$ = 125 |
|---|---|
| Age(y, mean ± SD) | 66.3 ± 0.9 |
| Gender (male:female) | 93:32 |
| Hepatitis virus (HBV:HCV:non-B-non-C) | 26:66:33 |
| AST (IU/L, mean ± SD) | 61.4 ± 11.1 |
| ALT (IU/L, mean ± SD) | 54.8 ± 7.4 |
| Plt ($\times 10^9$L, mean ± SD) | 14.7 ± 0.7 |
| ICG-R15(%, mean ± SD) | 19.7 ± 1.1 |
| PT% (mean ± SD) | 83.8 ± 1.1 |
| Total bilirubin (mg/dL, mean ± SD) | 0.9 ± 0.04 |
| Alb (g/dL, mean ± SD) | 3.9 ± 0.04 |
| AFP (>100 ng/mL) | 46:79 |
| AFP (>400 ng/mL) | 29:96 |
| PIVKA-II (>40mAU/mL) | 83:42 |
| PIVKA-II (>100mAU/mL) | 63:62 |
| Number of tumors (A:B:C:D) | 91:26:2:6 |
| Tumor size (A:B:C) | 14:72:39 |
| Tumor size (>5.1 cm) | 39:86 |
| Histological differentiation(well:mod:poor) | 22:53:50 |
| Histological structure(trab:pseud:comp:scir) | 95:8:21:1 |
| Growth pattern(expansive:invasive) | 108:17 |
| Capsular formation (+:) | 100:25 |
| Capsular invasion (+:) | 81:44 |
| Portal vein invasion (+:) | 56:69 |
| Hepatic vein invasion (+:) | 16:109 |
| Stages (I:II:III:IV) | 6:58:45:16 |

NOTE: AST: aspartate amino transferase, ALT: alanine aminotransferase, PLT: platelet, ICG-R15: indocyanine green retention rate at 15 min, PT%: prothrombin time, Alb: albumin, AFP: α-fetoprotein, PIVKAII, protein induced by vitamin K absence or antagonists II. Number of tumors (A:B:C:D); A = 1; B = 2; C = 3; D = 4, tumor size (A: B:C); A: = 2.0; B: = 0.2.1, = 5.0; C: = 5.1, trab: trabecular type, pseud: pseundoglandular type, comp: compact type, scir: srirrhous type, positive: +, negative: –.

from the Human Science Research Resources Bank (Osaka Japan). All cell lines were maintained in Dulbecco's minimum essential medium (DMEM: Sigma, St Louis, MO, USA) containing nonessential amino acid (Invitrogen, Carlsbad, CA, USA) and 10% heat inactivated fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA) and was grown at 37°C in 5% $CO_2$. Primary HCC and metastatic liver cancer tissues were obtained with informed consent from 125 patients and 18 patients, respectively, by surgical resection in the Department of Hepato-Biliary-Pancreatic Surgery at Tokyo Medical and Dental University Hospital between November 2005 and May 2008. This research project was approved by the local ethical committee and all samples were obtained with the patient's informed consent. A part of the resected sample was fixed in formalin and embedded in paraffin for histological diagnosis and all tissues were snap frozen in liquid nitrogen and then stored at –80°C for RNA analysis. Sections were obtained from each frozen sample before mRNA extraction. One section was stained with hematoxylin and eosin to verify the presence of viable tumor. Histological diagnosis was made when two pathologists specializing in liver disease reached the same conclusion. The patients consisted of 93 (74.4%) males and 32 (25.3%) females, 40–85 years old (mean 66.3). Other clinicopathological features are shown in Table 1.

**RNA isolation, complementary RNA (cRNA) preparation and microarray analysis.** One hundred and twenty-five primary HCC and correlated background specimens were obtained from surgically resected materials. Total RNA was extracted from tissue specimens and human HCC cell lines using RNeasy Mini kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase according to the manufacturer's instructions. Integrity of obtained RNA was assessed using Agilent Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). All samples had RNA Integrity Number (RIN) > 5.0. Using 2 μg of total tissue RNA specimens, cRNA was prepared using one-cycle target labeling and control reagents by Affymetrix, P/N 900493 (Affymetrix, Santa Clara, CA, USA). Hybridization and signal detection of HG-U133 plus 2.0 arrays (Affymetrix) were performed following the manufacturer's instruction.

**Western blot analysis.** The cells were maintained in continuous monolayer cultures at 37°C and 5% $CO_2$, expanded up to 70–80% confluence and then employed for the experiments, as described below. For Western blotting, cellular proteins were solubilized in 2× gel sample buffer, boiled for 5 min, and resolved by 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes, which were blocked in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) plus 5% non-fat dried milk and incubated with the primary antibodies antihuman Aurora B (1 : 1000; Abcam, Cambridge, UK, catalog no. ab2254) in TBST plus 1% non-fat dried milk. Secondary antibodies were examined using the ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). The expression ratio of Aurora B to the control was examined using Multi-Gage software (FUJIFILM, Tokyo, Japan).

**TaqMan® MGB probe real-time PCR.** Two micrograms of tissue RNA were reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription System (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using the TaqMan Fast Universal PCR master mix 2x (Applied Biosystems), TaqMan Gene Expression Assays AURKB (Hs00177782_m1, Context Sequence GCCGACAGACGGCTCCATCTGGCCT) for AURKB. Triplicate 20 μL RT-PCR reactions for each sample contained 10 μL of AB TaqMan Fast Universal PCR Master Mix, 1 μL of the relevant 20× assay, 1 μL of target cDNA and $dH_2O$ under following conditions: one cycle of 20 sec at 95°C, 40 cycles of 3 sec at 95°C, 30 sec at 60°C. Data was analyzed using the comparative relative quantification method and samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Quantitative real time RT-PCR.** Two micrograms of cells and tissues RNA were reverse-transcribed to cDNA with High-Capacity cDNA Reverse Transcription System (Applied Biosytems). Quantitative PCR was performed using the SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the AB 7500 Fast Real Time PCR System (Applied Biosystems) with 30-mer sense (5′-GAGAGTGCATCACACAACGAGACCTATCGC-3′) and antisense (5′-AGAAAACAGATAAGGGAACAGTTAGGGATC-3′) for AURKB-WT (Aurora B-wild type) primers, with 30-mer sense (5′-CGGCACTTCACAATTGATGACTTTGAGATT-3′) and antisense (5′-TTATCAACATCTCTGCGTCCTACAACCCTA-3′) for AURKB-Sv1 (Aurora B-splicing variant 1) primers, with 20-mer sense (5′-ATCTTAACCAGGCGGCACTT-3′) and antisense (5′-ACTCCTCCATGATTGCAGGT-3′) for AURKB-Sv2 (Aurora B-splicing variant 2) primers under the following conditions: 2 min at 50°C, 10 min at 95°C one cycle; 40 cycles of 15 s at 95°C, 60 s at 60°C. Immediately after the amplification, melt curve protocols were performed to ensure primer-dimers and other non-specific products had been minimized or eliminated. GAPDH transcript was tested as an endogenous reference to calculate the relative expression levels of target genes according to Applied Biosystems instructions. The PCR reactions were separated by gel electrophoresis and the DNA bands were visualized under ultraviolet light for photographing.

**Normalization and statistical analysis of microarray data.** Obtained 80 microarray datasets were normalized using the robust multiarray average (RMA) method (R 2.4.1 statistical software together with BioConductor package).[25] Estimated gene expression levels were $\log_2$-transformed, and 62 control probe sets were removed for further analysis. Out of 80 patients, 23

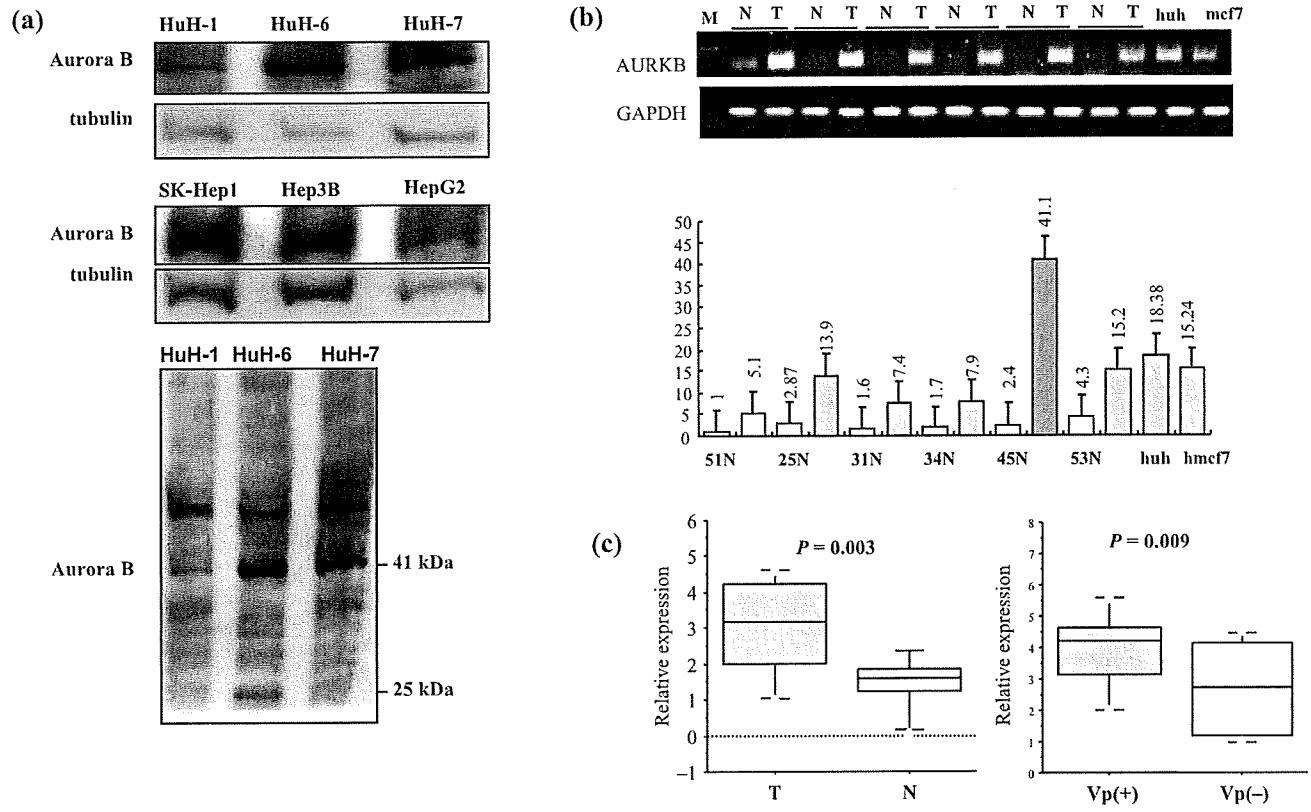


**Fig. 1.** Expression of Aurora kinase B (AURKB) in human hepatocellular carcinoma (HCC) cell lines and HCC patients. (a) Protein levels of Aurora B and alpha-tubulin (control) in HCC cell lines were examined using standard Western blot analysis on 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis. All cell lines indicated Aurora B (41 kDa). (b) TaqMan® MGB probe Gene Expression Assays for AURKB, Quantitative polymerase change reaction (PCR) was performed using the TaqMan Fast Universal PCR master mix 2× and normalized for sample variation by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (c) All changes determined by reverse transcription – PCR were statistically significant and consistent with the direction of change reported by the microarray analysis. The relative expression *P*-values were tested for significance using Wilcoxon rank sum test. (N; non-tumor, T; tumor, VP (+); portal vein positive, VP (–); portal vein negative).

showed expression of AURKB-SV2. To identify genes associated with the expression of AURKB-SV2, Wilcoxon rank-sum test was performed to estimate the significance of gene expression differences between AURKB-SV2 (+) and (–) groups for each 54 613 probe sets. Obtained *P*-values from the multiple hypothetical testing were adjusted by the false discovery rate (FDR), and probe sets with $P < 0.0005$ (FDR < 0.266) were considered for further analysis. Hierarchical clustering with the selected probe sets was performed on R software using Pearson's correlation coefficient as a similarity index and complete linkage method for agglomeration. For visualization, the expression levels were standardized by z-scores (mean = 0 and variance = 1) for each probe set.

**Statistical analysis of qRT-PCR.** A quantitative analysis of specific mRNA expression was performed by qRT-PCR using the Applied Biosystems 7500 Fast Real-Time PCR System. $C_T$ values were calculated using the 7500 SDS software. For each sample, expressions of AURKB gene and splicing variant forms were normalized with expression of control gene and fold difference between tumor and normal tissue calculated using the $??C_T$ method (Applied Biosystems User Bulletin #2, 1997).

**Statistical analysis of clinicopathogical correlation.** Student's *t*-test was used to analyze the differences in age of patients. Fisher's exact test was used to compare the categorical data between groups. Wilcoxon rank sum test was used to analyze the non-categorical data. The overall survival curve and disease-free survival rate was calculated by the Kaplan-Meier method and rates are reported with 95% confidence intervals. Differences were tested for significance using the log-rank test. The overall survival rate was measured from the date of resection until the date when the recurrence of HCC was detected or when the patient died. $P < 0.05$ was deemed to be statistically significant.

## Results

**AURKB overexpressed in tumor than adjacent-non-tumor and vascular invasion cases than-non-vascular invasion cases in hepatocellular carcinoma.** From Western blotting analysis, cultured human HCC cell lines were found to express Aurora B protein (41 kDa) Fig. 1(a). To assess the validity of our hybridization results, we examined the expressed gene using TaqMan® MGB probe Gene Expression Assays for AURKB, quantitative PCR was performed using the TaqMan Fast Universal PCR master mix 2× (Applied Biosystems). Strong correlation between PCR expression data and microarray values was found (Fig. 1b,c). All changes determined by RT-PCR were statistically significant ($P < 0.01$) and consistent with the direction of change reported by the microarray analysis.

**Detection of AURKB-WT and splicing variant forms in human HCC cell lines, HCC cases, metastatic liver cancer cases and normal liver specimens.** In order to analyze the expression of AURKB and alternative splicing variant forms in human HCC cell lines, cancer and the related adjacent tissue were screened by RT-PCR and qRT-PCR. As with eight kinds of cell lines, 125 HCC cases, of which we found 94 chronic hepatitis and cirrhosis background liver specimens, 18 metastatic liver cancer cases and 16 normal



doi: 10.1111/j.1349-7006.2008.01068.x

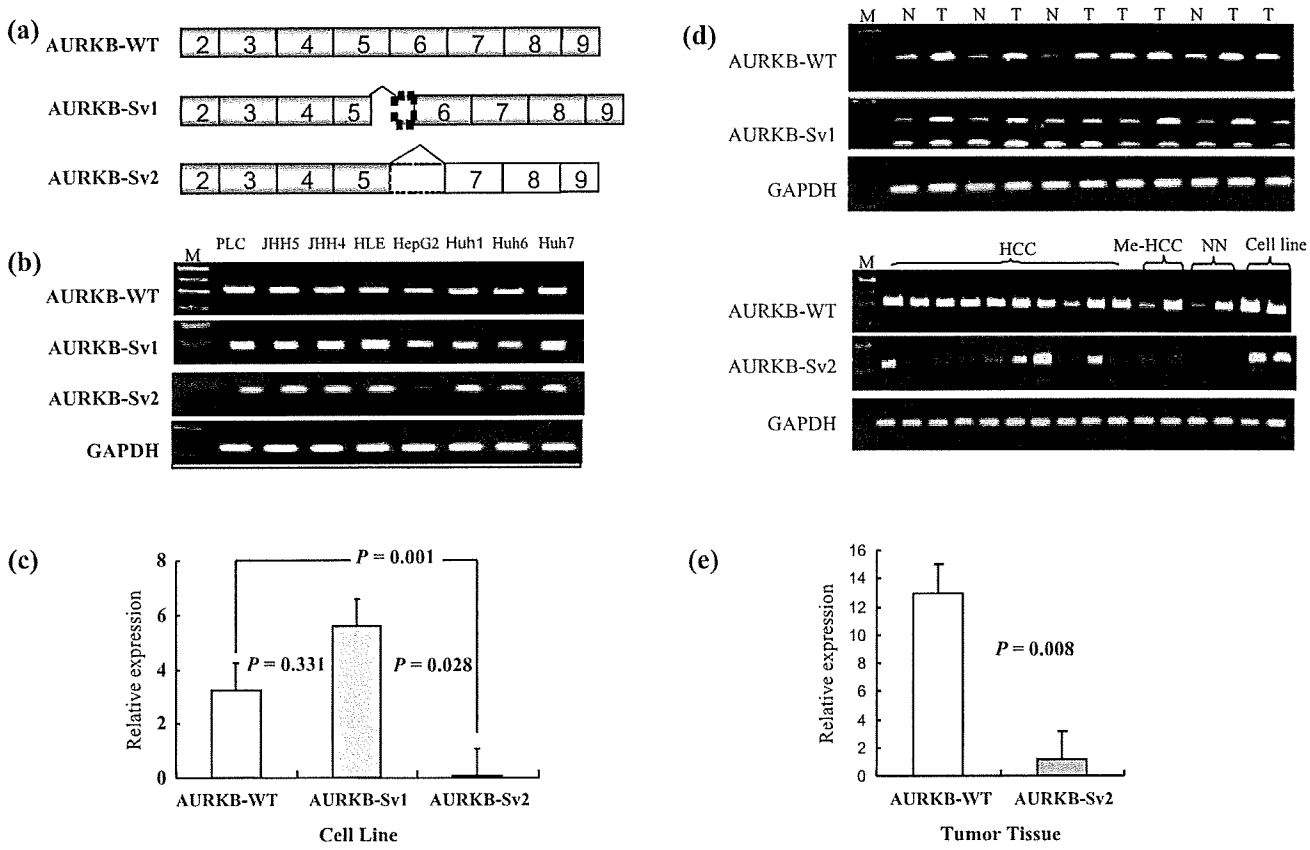


**Fig. 2.** Expression of Aurora kinase B (AURKB) and splicing variant forms in human hepatocellular carcinoma (HCC) cell lines and in human cancer tissue specimens. (a) Schematic illustration of the AURKB alternative splicing coding messenger RNA (mRNA). The boxes indicate the exons numbered on Ensemble (access ENST380101). The gray boxes are coding regions and the white boxes are not translated due to frame shift. The box with dotted line is 47 bp of intron 5–6 retained in the variant AURKB splicing variant 1 (AURKB-Sv1) and AURKB-Sv2 in which the entire sequence is missing from exon 6. (b) Expression of AURKB and alternative splicing variant forms in human HCC cell lines. Relative AURKB-WT, AURKB-Sv1, AURKB-Sv2 mRNA levels cell lines were evaluated by quantitative reverse transcription – polymerase chain reaction (qRT-PCR). (c) Relative expression ratio for all cell lines expressed AURKB and variant forms. *P*-values were evaluated by Student's *t*-test. (d) Expression of AURKB and alternative splicing variant forms in HCC, related adjacent tissue, metastasis liver cancer cases and normal liver specimens. qRT-PCR using the Applied Biosystems 7500 Fast real-time PCR System. CT values were calculated using the 7500 SDS software. The relative expression *P*-values were tested for significance using Student's *t*-test.

**Table 2. Expression of Aurora kinase B (AURKB) and variant forms in HCC, adjacent tissue, metastatic cancer and normal liver specimens**

| Specimens | AURKB-WT | | | AURKB-Sv1 | | | AURKB-Sv2 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Positive | Negative | % | Positive | Negative | % | Positive | Negative | % |
| HCC | 125 | 0 | 100% | 125 | 0 | 100% | 42 | 83 | 33.6% |
| CH + LC | 94 | 0 | 100% | 92 | 2 | 97.8% | 15 | 79 | 15.9% |
| Me-HCC | 18 | 0 | 100% | 16 | 2 | 88.9% | 11 | 7 | 61.1% |
| Normal liver | 16 | 0 | 100% | 4 | 12 | 25% | 0 | 16 | 0.0% |

NOTE: HCC: hepatocellular carcinoma, CH: chironic hepatitis, LC: liver cirrhosis; Me-HCC: metastasis liver cancer, WT: wild type; Sv1: splicing variant 1; Sv2: splicing variant 2.

liver specimens were screened by RT-PCR and qRT-PCR. Schematic illustration of the AURKB and alternative splicing variants are shown in Fig. 2(a). qRT-PCR using the Applied Biosystems 7500 Fast Real-Time PCR System is shown in Fig. 2(b,d). We found that the AURKB-WT, AURKB-Sv1 and AURKB-Sv2 forms of mRNA were expressed in all cell lines and the relative expression ratios are shown in Fig. 2(c). AURKB-WT mRNA was aberrantly expressed in all primary HCC tumor specimens (125 of 125), chronic hepatitis and cirrhosis background liver specimens (94 of 94), metastasis liver cancer cases (18 of 18) and normal liver specimens (16 of 16). AURKB-Sv1 variant form was also up-regulated at 100%, 97.8%, 88.9% and 25%, respectively. However, only 33% of primary HCC cases (42 of 125), 15% of chronic hepatitis and cirrhosis background liver specimens (15 of 94), 61% of metastasis liver cancer cases (11 of 18), 0% of normal liver specimens (0 of 16) had up-regulated AURKB-Sv2 variant form. The results are shown in Table 2 and the relative expression ratio is shown in Fig. 2(e).

**Expression of AURKB-Sv2 variant form and clinicopathologic features.** We detected AURKB-Sv2 variant form in 33% of primary hepatocellular cases (42 of 125). AURKB-WT and

Table 3. Expression of Aurora kinase B splicing variant 2 (AURKB-Sv2) variant and clinicopathological findings in hepatocellular carcinoma

| Clinicopathologic factor | AURKB (Wt/Sv2) | | |
|---|---|---|---|
| | +/+ (*n* = 42) | +/– (*n* = 83) | *P*-value |
| Age(y, mean ± SD) | 63.6 ± 1.6 | 67.7 ± 1.1 | 0.030* |
| Gender (Male:Female) | 34:8 | 59:24 | 0.282 |
| Hepatitis virus positive (HBV:HCV) | 8:24 | 18:42 | 0.808 |
| AFP(>100 ng/mL) | 23:19 | 23:60 | 0.006* |
| AFP (>400 ng/mL) | 15:27 | 14:69 | 0.025* |
| PIVKA-II(>40mAU/mL) | 31:11 | 52:31 | 0.235 |
| PIVKA-II(>100mAU/mL) | 27:15 | 36:47 | 0.037* |
| Solitary or multiple | 24:18 | 67:16 | 0.019* |
| Tumor size (>5.1 cm) | 10:32 | 29:54 | 0.227 |
| Histological differentiation(well:mod:poor) | 3:23:16 | 19:30:34 | 0.044* |
| Histological structure(trab:pseud:comp:scir) | 32:2:8:0 | 63:6:13:1 | 0.811 |
| Growth pattern(expansive:invasive) | 36:6 | 72:11 | 0.784 |
| Capsular formation (+:) | 32:10 | 68:15 | 0.453 |
| Capsular invasion (+:) | 33:9 | 48:35 | 0.029* |
| Portal vein invasion (+:) | 19:23 | 37:46 | 0.944 |
| Hepatic vein invasion (+:) | 9:33 | 7:76 | 0.050 |
| Stages (I + II:III + IV) | 14:28 | 50:33 | 0.008* |

NOTE: AFP: α-fetoprotein, PIVKAII, protein induced by vitamin K absence or antagonists II.
trab: trabecular type, pseud: pseundoglandular type, comp: compact type, scir: srirrhous type, positive: +, negative: –, *Statistically significant.

AURKB-Sv1 were also detected in all positive cases. We studied the relationship between the expression of these mRNA (regardless of their variant forms) and clinicopathologic factors of HCC. As shown in Table 3 AURKB-Sv2 was associated with the advanced stages of HCC more significantly than AURKB-WT form ($P = 0.008$). The expression of AURKB-Sv2 variant form was furthermore associated with a higher level of serum α-fetoprotein (AFP) ($P = 0.025$), protein induced by vitamin K absence or antagonists-II (PIVKAII) ($P = 0.037$), tumor capsular invasion ($P = 0.029$), histological differentiation ($P = 0.044$) and multiple tumor formation ($P = 0.017$). The AURKB-Sv2 variant patients were of an age younger than those with other variant forms ($P = 0.030$). However, the expression of AURKB-Sv2 variant did not show association with hepatitis B and hepatitis C virus infection, the histological structure patterns, tumor capsular formation, growth pattern, vascular invasion or other factors.

**Expression of AURKB-Sv2 variant form and patient survival.** We were able to follow the postoperative course of 110 patients. The follow-up period until death or the end point of this study was 4–959 days (mean 276 days, median 206 days). Eighty patients survived without recurrence of HCC within the follow-up period, and the mean survival (median, 193 days) was 276 days. Fifty-five patients lived longer than 1 year after the operation. The mean survival period was 391 days (median; 389 days). Figure 3(a) shows the overall survival rate (110 patients) and Fig. 3(b) shows disease-free survival rate (80 patients) of AURKB-Sv2 variant +/– HCC patients. There was a significant difference in the overall survival rate ($P = 0.008$) and disease-free survival rate ($P = 0.018$) between AURKB-Sv2 positive patients and negative patients.

**Gene selection and hierarchical clustering.** By evaluating gene expression changes between AURKB-SV2 (+) and (–) groups, 93 probe sets that satisfied $P < 0.0005$ (FDR < 0.266) by Wilcoxon rank sum test were identified as differently expressed genes (Supporting Information Table S1). Table 4 shows up- and down-regulated genes in 80 primary HCC with AURKB Sv2 variant (+/–) ($P < 0.0005$, FC > 1.4). Figure 3(c) shows the hierarchical clustering results using selected probe sets. All patients in the left cluster were AURKB-SV2 (–), and the majority in the right cluster was AURKB-SV2 (+).

## Discussion

We have previously identified AURKB as the only independent predictor of the aggressive recurrence of HCC.[12] AURKB is a chromosomal passenger serine/threonine protein kinase that regulates accurate chromosomal segregation, cytokinesis, protein localization to the centromere and kinetochore, correct microtubule-kinetochore attachments and regulation of the mitotic checkpoint.[26] Our previous study revealed that AURKB expression was closely associated with genetic instability of HCC tumors. More importantly, AURKB has recently received increasing attention as an eligible target of molecular cancer therapy.[27] AURKB-targeted therapy might be a promising neoadjuvant approach for the occult vascular invasion of HCC.

DNA microarray technology enabled us to analyze the genome-wide profile of gene expression specific to malignant tumor. Using this technology, we investigated the mRNA expression patterns in 80 cases of primary HCC, and clarified that the genes associated with cell cycle, especially AURKB, were significantly up-regulated in HCC in relation to the invasion of the portal vein and/or hepatic vein. From Western blotting analysis, Aurora B protein was expressed in all cultured human HCC cell lines. To assess the validity of our microarray results, we examined by the RT-PCR using TaqMan® MGB probe Gene Expression Assays for AURKB. A strong correlation between qRT-PCR expression data and microarray values was found. AURKB was overexpressed in tumor more than adjacent non-tumor specimens; overexpression was also observal in vascular invasion cases rather than non-vascular invasion cases in HCC.

To confirm the analysis of Aurora B mRNA expression pattern in human HCC and adjacent tissue, we screened eight human HCC cell lines, 125 tumor specimens, 94 chronic hepatitis and cirrhosis background liver specimens, 18 metastatic liver cancer cases and 16 normal liver specimens (based on the sequencing) for different Aurora B variants including AURKB-WT and two variant forms AURKB-Sv1, AURKB-Sv2 by RT-PCR and qRT-PCR. AURKB-WT was expressed in all HCC tumor specimens, chronic hepatitis and cirrhosis background liver specimens, metastasis liver cancer and normal liver specimens. However, the expression of AURKB-Sv1 variant form were 100%, 97.8%, 88.9% and 25%, respectively. In cases of the AURKB-Sv2 variant



doi: 10.1111/j.1349-7006.2008.01068.x

**Table 4. Up- and down-regulated genes in 80 primary hepatocellular carcinomas with Aurora kinase B splicing variant 2 (AURKB-Sv2) (+/–) ($P < 0.0005$, FC > 1.4). We used the expression data by Affymetrix HG-U133 Plus 2.0 (54 675 probe sets)**

| Gene symbol | Up-regulated gene in AURKB-Sv2(+) | | | |
|---|---|---|---|---|
| | Title | *P*-value | FC | FDR |
| 1559213_at | Homo sapiens, clone IMAGE:5394246, mRNA | 4.77E-04 | 5.34 | 2.74E-01 |
| SSX1 | synovial sarcoma, X breakpoint 1 | 3.83E-04 | 4.51 | 2.74E-01 |
| GPR88 | G protein-coupled receptor 88 | 3.51E-04 | 4.19 | 2.74E-01 |
| COCH | coagulation factor C homolog, cochlin (Limulus polyphemus) | 7.33E-05 | 3.47 | 2.16E-01 |
| COCH | coagulation factor C homolog, cochlin (Limulus polyphemus) | 2.20E-05 | 2.52 | 1.20E-01 |
| MAGEA2///2B | melanoma antigen family A, 2///melanoma antigen family A, 2B | 1.59E-04 | 2.32 | 2.35E-01 |
| DSG2 | desmoglein 2 | 2.67E-04 | 2.11 | 2.74E-01 |
| LOC133874 | hypothetical gene LOC133874 | 9.88E-05 | 1.92 | 2.16E-01 |
| EPB41L2 | erythrocyte membrane protein band 4.1-like 2 | 2.67E-04 | 1.81 | 2.74E-01 |
| TMEM48 | transmembrane protein 48 | 4.77E-04 | 1.78 | 2.74E-01 |
| CEACAM1 | carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) | 3.83E-04 | 1.61 | 2.74E-01 |
| PLXNC1 | plexin C1 | 3.67E-04 | 1.59 | 2.74E-01 |
| RAB3IP | RAB3A interacting protein (rabin3) | 2.93E-04 | 1.59 | 2.74E-01 |
| TMEM118 | transmembrane protein 118 | 1.26E-04 | 1.55 | 2.30E-01 |
| POT1 | POT1 protection of telomeres 1 homolog (S. pombe) | 3.51E-04 | 1.53 | 2.74E-01 |
| PSENEN | presenilin enhancer 2 homolog (C. elegans) | 1.58E-05 | 1.52 | 1.20E-01 |
| LOC642236 | similar to FRG1 protein (FSHD region gene 1 protein) | 5.21E-04 | 1.52 | 2.74E-01 |
| CKLF | chemokine-like factor | 2.33E-04 | 1.51 | 2.74E-01 |
| MAPRE2 | microtubule-associated protein, RP/EB family, member 2 | 2.03E-04 | 1.49 | 2.52E-01 |
| SSX3 | synovial sarcoma, X breakpoint 3 | 1.68E-04 | 1.49 | 2.35E-01 |
| CKLF | chemokine-like factor | 1.94E-04 | 1.46 | 2.46E-01 |
| UROD | uroporphyrinogen decarboxylase | 1.26E-04 | 1.44 | 2.30E-01 |
| ULK4 | unc-51-like kinase 4 (C. elegans) | 2.93E-04 | 1.42 | 2.74E-01 |
| ERGIC3 | ERGIC and golgi 3 | 5.58E-06 | 1.41 | 1.20E-01 |
| ATP6V0B | ATPase, H + transporting, lysosomal 21 kDa, V0 subunit b | 1.68E-04 | 1.41 | 2.35E-01 |
| H3F3B | H3 histone, family 3B (H3.3B) | 4.19E-04 | 1.40 | 2.74E-01 |
| MED8 | mediator of RNA polymerase II transcription, subunit 8 homolog (S. cerevisiae) | 1.46E-04 | 1.40 | 2.35E-01 |
| AIP | aryl hydrocarbon receptor interacting protein | 1.46E-04 | 1.40 | 2.35E-01 |
| Gene symbol | Down-Regulated Gene in AURKB-Sv2(+) | | | |
| | Title | *P*-value | FC | FDR |
| 1562346_at | MRNA; cDNA DKFZp313F2234 (from clone DKFZp313F2234) | 3.21E-04 | 0.89 | 2.74E-01 |
| IL1RL1 | Interleukin 1 receptor-like 1 | 4.01E-04 | 0.89 | 2.74E-01 |
| FUT6 | Fucosyltransferase 6 (alpha (1,3) fucosyltransferase) | 8.95E-05 | 0.88 | 2.16E-01 |
| IL28RA | interleukin 28 receptor, alpha (interferon, lambda receptor) | 3.06E-04 | 0.88 | 2.74E-01 |
| 1555488_at | NA | 5.21E-04 | 0.88 | 2.74E-01 |
| KCNAB3 | potassium voltage-gated channel, shaker-related subfamily, beta member 3 | 7.33E-05 | 0.88 | 2.16E-01 |
| DIXDC1 | DIX domain containing 1 | 1.41E-05 | 0.87 | 1.20E-01 |
| 1569912_at | Homo sapiens, clone IMAGE:5459012, mRNA | 1.74E-06 | 0.87 | 9.52E-02 |
| 236991_at | Transcribed locus | 1.20E-04 | 0.87 | 2.30E-01 |
| ZNF474 | zinc finger protein 474 | 9.33E-05 | 0.87 | 2.16E-01 |
| CCDC85A | Coiled-coil domain containing 85A | 4.77E-04 | 0.87 | 2.74E-01 |
| BAI2 | brain-specific angiogenesis inhibitor 2 | 2.55E-04 | 0.87 | 2.74E-01 |
| MDGA1 | MAM domain containing glycosylphosphatidylinositol anchor 1 | 1.46E-04 | 0.87 | 2.35E-01 |
| KIAA1787 | KIAA1787 protein | 5.44E-04 | 0.86 | 2.74E-01 |
| MORN1 | MORN repeat containing 1 | 4.99E-04 | 0.86 | 2.74E-01 |
| FAM41AY | family with sequence similarity 41, member A, Y-linked | 8.04E-05 | 0.86 | 2.16E-01 |
| ITGA10 | integrin, alpha 10 | 5.99E-05 | 0.85 | 2.16E-01 |
| 236541_at | Transcribed locus | 4.99E-04 | 0.84 | 2.74E-01 |
| AMACR | alpha-methylacyl-CoA racemase | 1.21E-04 | 0.84 | 2.30E-01 |
| DYNLRB2 | dynein, light chain, roadblock-type 2 | 4.57E-04 | 0.84 | 2.74E-01 |
| RBM25 | RNA binding motif protein 25 | 1.85E-04 | 0.83 | 2.46E-01 |
| 228525_at | Transcribed locus, strongly similar to XP_512572.1 similar to low density lipoprotein receptor-related protein 3 [Pan troglodytes] | 5.21E-04 | 0.81 | 2.74E-01 |
| LOC338328 | high density lipoprotein-binding protein | 3.83E-04 | 0.79 | 2.74E-01 |
| CYP46A1 | cytochrome P450, family 46, subfamily A, polypeptide 1 | 2.20E-05 | 0.77 | 1.20E-01 |
| RICS | Rho GTPase-activating protein | 3.51E-04 | 0.73 | 2.74E-01 |
| EML1 | echinoderm microtubule associated protein like 1 | 5.44E-04 | 0.73 | 2.74E-01 |
| DGCR5 | DiGeorge syndrome critical region gene 5 (non-coding) | 1.67E-04 | 0.72 | 2.35E-01 |

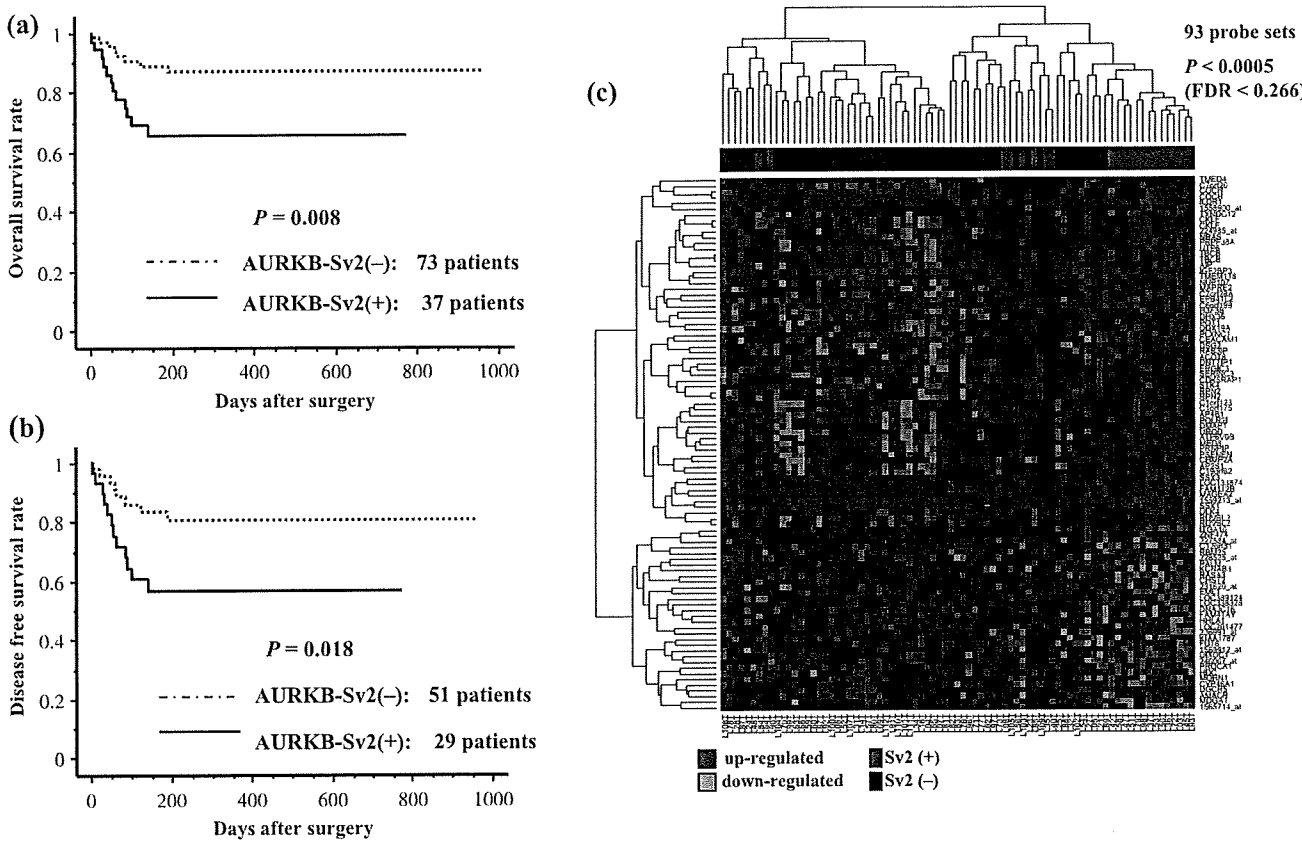


Fig. 3. Kaplan-Meier method analysis survival curves for primary hepatocellular carcinoma (HCC) with Aurora kinase B splicing variant 2 (AURKB-Sv2) (+/–) cases. Differences were tested for significance using the log-rank test. All of the microarray-examined patients with HCC classified into AURKB-Sv2 (+) group ($n$ = 37, $n$ = 29) and AURKB-Sv2 (–) group ($n$ = 73, $n$ = 51) after curative resection ($P$ = 0.008) ($P$ = 0.018). (a) Cumulative overall survival curves (110 patients). (b) Disease-free survival curves (80 patients). (c) Hierarchical clustering of gene expression profiles of selected 93 probe sets obtained from 80 HCC patients: AURKB-SV2 (+) (denoted as red in vertical side bar), and AURKB-Sv2 (–) (denoted as black). Dendrograms show the classification determined by hierarchical clustering analysis. Red and green colors indicate relative overexpression and underexpression, respectively.

form, 33.6%, 15.9%, 61.1%, 0% specimens only expressed, respectively. It would be a very interesting finding that the expression of AURKB-Sv2 was absent in normal liver and was higher in metastatic liver cancer than HCC. Sistayanarain *et al.*[13] reported that Aurora B and two variant forms were expressed in HCC, but this was studied only with some limited samples including 11 frozen tissues and six paraffin-embedded tissues by RT-PCR. In that study, they showed the two variants (B1 and B2) present in tumor specimens at 41.1%, 52.9%, respectively, but by the analysis of only 11 frozen tumor specimens showed 45% and 27.3%, respectively. In contrast, all specimens were fresh and frozen in our study. Co-expression of AURKB-WT or alternative variant AURKB-Sv1 was detected in all positive HCC cases; AURKB-Sv2 was detected in some limited cases. This result was similar to that which Sistayanarain *et al.* reported previously. Here, we also studied the significance of AURKB-Sv2 variant form from a the clinical viewpoint, for the progression of hepatocellular carcinoma, and showed that expression of AURKB-Sv2 form was associated with the advanced stage of HCC rather than others ($P < 0.01$). The AURKB-Sv2 positive cases showed a higher level of serum α-AFP and PIVKAII, poor differentiation cases, tumor capsular invasion, multiple tumor formation cases and at a younger age than with other variant forms ($P < 0.05$). Although this variant did not show the association with vascular invasion, we previously reported that the AURKB-WT was more readilyassociated with vascular invasion and recurrence cases in HCC. Then we analyzed the overall survival rate for 110 patients and disease-free survival rate for 80 AURKB-Sv2 variant positive/negative HCC patients. Statistical significance was observable for poor survivals of AURKB-Sv2 (+) cases.

We tried to detect AURKB-Sv2 protein in tissue samples, but could not detect this. As the mRNA expression level of AURKB-Sv2 was very low compared to the AURKB-WT, we assume the protein could not be detected by our antibody.

This opens a question: what are the roles of AURKB splicing variants in hepatocarcinogenesis? Recently our research group predicted the 3D-stuctures of splicing variants by their protein sequences (Kim Hyeryun, Y. Mahmut *et al.*, data not published), using computer simulation models; molecular dynamics (MD) and root means square deviation (RMSD)–time plots. AURKB-WT has α-helix domain activated by INCEP. AURKB-Sv1 lacks this domain because of its absence in some part of exon 5, but the model suggests that α-helix was newly formed by intron 5-6 and replaced the exon 5α-helix, suggesting that the conformation direction was different from AURKB-WT. This means that AURKB-Sv1 has difficulty interacting with INCENP. On the other hand, AURKB-Sv2 kept this domain, but lacked many parts of kinase domain by truncation. This suggests that if it has no kinase activity, it is possible to compete with normal AURKB in a dominant negative manner. However, we lack the functional analysis data and further experiments are necessary to elucidate this point.

Criteria of AURKB-SV2 (+)/(–) groups used for Wilcoxon rank sum test were identified as differently revealed AURKB-Sv2 associated up- and down-regulated genes (Table 4). Several genes (CEACAM1, SSX1, SSX3 and MAGEA) are up-regulated



doi: 10.1111/j.1349-7006.2008.01068.x

in AURKB-Sv2 (+) cases. Such a cell adhesion molecule CEACAM1 (Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1) gene encodes a member of the carcinoembryonic antigen (CEA) gene family, which belongs to the immunoglobulin super family. Several studies showed that multiple cellular activities have been attributed to the encoded protein, including roles in the differentiation and arrangement of tissue three-dimensional structure, angiogenesis, apoptosis, tumor suppression, metastasis, and the modulation of innate and adaptive immune responses, was down-regulated in several types of human cancers, including prostate, colorectal and breast cancers. Thies *et al.*[28] showed that expression of CEACAM1 in primary tumors in melanoma patients is associated with the subsequent development of metastatic disease. Hokari *et al.*[29] analyzed several hepatoma cell lines and found that CEACAM1 was only expressed in HepG2 cells and the cells were treated with small interfering RNA targeted against CEACAM1, the growth rate in monolayer culture was increased. In contrast, when HepG2 cells were cultured in suspension, inhibition of CEACAM1 expression significantly decreased the growth rate, suggesting a role for CEACAM1 on hepatocarcinogenesis, by showing that CEACAM1 acts as a tumor suppressor in HepG2 cells in anchorage-dependent growth conditions, while in anchorage-independent growth conditions, it augments cell proliferation by potentiating the cell-cell attachment. SSX1 and SSX3 belong to the SSX family whose transcriptional repressor and cellular immune responses in cancer patients, overexpress in synovial sarcomas and are potentially useful targets in cancer vaccine-based immunotherapy. MAGEA (melanoma-associated antigen gene) belongs to the chromosome X-clustered cancer/testis antigens that normally express in the human germ line and are overexpressed in various tumor types. Monte *et al.*[30] showed that MAGEA tumor antigen target p53 transactivation functions through histone deacetylase recruitment and confers resistance to chemotherapeutic agents. Tsai *et al.*[31] reported that differential expression profile analysis of lung cancer suggests MAGE gene is not only for immunotherapy, but also valuable markers for further diagnosis and prognosis. In constant, several genes (BAI2, CYP46A1 and DYNLRB2) are down-regulated in AURKB-Sv2 (+) cases. BAI2 (brain-specific angiogenesis inhibitor 2) a p53-target gene, encodes brain-specific angiogenesis inhibitor, a seven-span transmembrane protein, and is thought to be a member of the secretin receptor family, and plays a role in angiogenesis. BAI2 expression decreased after hypoxia and preceded the increased expression of vascular endothelial growth factor.[32] CYP46A1 (cytochrome P450, family 46, subfamily A, polypeptide 1) gene encodes a member of the cytochrome P450 super family of enzymes. The cytochrome P450 proteins are mono-oxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. DYNLRB2 (dynein, light chain, roadblock-type 2) is member of an ancient dynein light chain protein family, conserved in nematode, fruit fly, mouse and rat. The expression of DNLC2B was generally high compared with that of DNLC2A except in the liver. Jiang *et al.*[33] analyzed 68 hepatocellular carcinoma tissue samples, suggesting that down-regulation of DNLC2B and up-regulation of DNLC2A genes might be involved in tumor progression.

In conclusion, an abnormal expression of Aurora B and alterative splicing variants was found to be involved in hepatocarcinogenesis. The AURKB-Sv2 variant form is more significantly associated with advanced stages of HCC than others. It is suggested to be a marker of poor prognosis. Founded in the tumor capsular invasion and multiple tumor regions, suggests that this might play a role in enhancing multiple malignant tumor formation and recurrence of HCC in hepatocarcinogenesis. Associated up- and down-regulated genes were identified by comparison of cases where AURKB-Sv2 differentially expressed or not. Further experiments are necessary to elucidate the functional properties of this variant in HCC.

## Acknowledgments

This work was supported by Special Coordination Funds for Promoting Science and Technology (Japan Science & Technology Agency), and a Grant-in-Aid from Ministry of Education, Culture, Sports, Science & Technology of Japan.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** 93 probe sets identified as differently expressed gene that satisfied false discovery rate (FDR) < 5% ($P < 0.0005$) with Wilcoxon rank sum test between AURKB-Sv2(+) and AURKB-Sv(–) group.

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doi: 10.1111/j.1349-7006.2008.01068.x

# Surgical Contribution to Recurrence-Free Survival in Patients with Macrovascular–Invasion–Negative Hepatocellular Carcinoma

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**BACKGROUND:** Macroscopic vascular invasion (MVI) is a well-known indicator of recurrence of hepatocellular carcinoma (HCC) even after curative hepatectomy, but the clinicopathologic and molecular features of the recurrence remain unclear in MVI-negative HCC.

**STUDY DESIGN:** Two hundred seven consecutive patients with confirmed primary MVI-negative HCC were retrospectively assessed after curative resection, with special emphasis on the importance of anatomically systematized hepatectomy. HCC tissues were also analyzed for genome-wide gene expression profile of each tumor using a microarray technique.

**RESULTS:** Univariant analysis of HCC recurrence revealed multiple tumors ($p < 0.001$), moderate to poor differentiation ($p = 0.044$), Child-Pugh B/C ($p = 0.047$), $\alpha$-fetoprotein elevation ($p = 0.007$), and nonanatomic hepatectomy ($p = 0.010$) as risk factors. According to Cox hazard multivariant analysis, multiple tumors ($p = 0.002$), $\alpha$-fetoprotein elevation ($p < 0.001$), and nonanatomic hepatectomy ($p = 0.002$) were identified as independent factors of the recurrence. In the recurrent cases after anatomic hepatectomy for HCC, local recurrence was significantly infrequent compared with those after nonanatomic hepatectomy ($p < 0.001$). Network expression analysis using cDNA microarray revealed distinct signaling pathways of epithelial-mesenchymal transitions are associated with recurrence after anatomically systematized hepatectomy.

**CONCLUSIONS:** Anatomically systematized hepatectomy might contribute to recurrence-free survival of HCC patients of HCC without MVI. Local recurrence could be mostly averted by anatomic hepatectomy, although specific epithelial-mesenchymal transitions signaling might regulate the biologic aggressiveness of HCC. (J Am Coll Surg 2009;208:368–374. © 2009 by the American College of Surgeons)

Hepatocellular carcinoma (HCC) is one of the most common malignancies, accounting for nearly 1 million deaths per year.[1] The incidence is still increasing worldwide, even in the US, as a result of the high prevalence of hepatitis virus infection. Although surgical resection is the effective treatment modality for HCC, rapid recurrence of the tumors remains frequent even after apparently curative resection.[2,3] Hepatic recurrence has been classified as intrahepatic metastasis and multicentric recurrence, but longterm outcomes are affected mainly by metastatic recurrence.[4,5] Because tumor vascular invasion is regarded as a direct cause of metastatic recurrence, evidence of tumor invasion in major portal and hepatic veins is a known determinant of poor outcomes after resection for HCC.[6-8] In accordance with the architecture of the portal and hepatic veins, anatomic segmentectomy or lobectomy has been developed as one of the feasible methods of surgical treatment.[9,10] In cases of HCC without apparent macrovascular invasion (MVI), nonanatomically partial hepatectomy is often selected as the alternative procedure for surgical treatment to avoid risk of postoperative hepatic failure.[11,12] To determine

Disclosure Information: Nothing to disclose.

This work was supported by Special Coordination Funds for Promoting Science and Technology (Japan Science and Technology Agency), and a Grant-in-Aid from Ministry of Education, Culture, Sports, Science and, Technology of Japan. Dr Tanaka is a recipient of the Japan Cancer Society Incitement Award and Japan Society for the Promotion of Science Prize.

Received September 18, 2008; Revised October 9, 2008; Accepted October 29, 2008.

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ISSN 1072-7515/09/$36.00
doi:10.1016/j.jamcollsurg.2008.10.031

**Abbreviations and Acronyms**
BIND = Biomolecular Interaction Network Database
EGFR = epidermal growth factor receptor
EMT = epithelial-mesenchymal transitions
HCC = hepatocellular carcinoma
MVI = macrovascular invasion
TGF-$\beta$ = transforming growth factor-$\beta$

the importance of systematized hepatectomy in recurrence of MVI-negative HCC, the tumor characteristics should be analyzed by aspects of the clinicopathologic factors.

Genome-wide gene expression analysis by microarray offers a systematic approach to unfold comprehensive information about the transcription profiles.[13] In addition, such studies should potentially lead to development of novel molecular-targeting therapy of HCC.[14] To identify the most prominent signaling pathways, we examined the network analysis of differentially expressed genes, using Biomolecular Interaction Network Database (BIND) and Pajeck Program for Large Network Analysis.[15,16] In this study, the clinicopathologic and molecular features were evaluated with special emphasis on the importance of anatomically systematized hepatectomy for such HCC.

## METHODS

### Patients and tissue samples

Two hundred sixty consecutive patients underwent initial and curative hepatectomy for HCC from 2000 to 2006 at Tokyo Medical and Dental University Hospital. Written informed consent from these patients and institutional review board approval were obtained. Preoperative evaluation including liver function and operative procedures have been described elsewhere.[2,3-8] Indications for hepatic resection and operative procedures were determined based on Makuuchi's criteria.[17] Preoperative imaging for tumor staging included abdominal ultrasonography, CT, hepatic arteriography, indirect arterial portography, CT arteriography, CT arterial portography, and MRI. MVI indicating the presence of tumor thrombus in the major branches of the portal or hepatic vein were comprehensively evaluated. Resected tissue was fixed in 10% formaldehyde solution and embedded in paraffin for histopathologic analysis by pathologists. Evidence of tumor invasion into the portal/hepatic veins was evaluated both macroscopically and microscopically. MVI on gross examination of the resected specimen confirmed the preoperative diagnosis in all of the patients examined. On histopathologic examination of the resected specimen, microscopic invasion indicated the presence of clusters of cancer cells floating in the vascular space line by endothelial cells. Patients were followed up with assays of serum level of $\alpha$-fetoprotein and protein induced by vitamin K absence or antagonists II every month, and with ultrasonography, CT, and MRI every 3 months. When tumor recurrence was suspected, precise diagnostic imaging was performed using CT angiography. Finally, the exact diagnosis of recurrence was made with the imaging. The recurrent tumor arising in the same segment as the initial tumor, or within 2 cm from the surgical stump, was regarded as a "local" recurrence, as described by Takayama and colleagues.[18] The other intrahepatic recurrence was considered a "distant" one. Mean observation time was 3.8 years.

### RNA isolation, cRNA preparation, and microarray hybridization

Primary HCC specimens were obtained from surgically resected materials. Total RNA was extracted from tissue specimens using RNeasy kit (Qiagen). Integrity of obtained RNA was assessed using Agilent 2100 BioAnalyzer (Agilent Technologies). All samples had RNA Integrity Number >5.0. Contaminant DNA was removed by digestion with RNase-free DNase (Qiagen). Using 2 $\mu$g total RNA, cRNA was prepared using one-cycle target labeling and control reagents kit (Affymetrix). Hybridization and signal detection of HG-U133 Plus 2.0 arrays (Affymetrix) was performed following manufacturer's instruction.

### Normalization and statistical analysis of microarray data

Total microarray datasets were normalized using robust multiarray average method under R 2.4.1 statistical software together with BioConductor package, as described previously.[14] Estimated gene expression levels were $\log_2$-transformed, and 62 control probe sets were removed for additional analysis. For each 54,613 probes on HG-U133 Plus 2.0 arrays, fold-change values were calculated using ratio of geometric means of gene expression levels between the two groups. Wilcoxon rank sum test was also performed to estimate the significance levels of gene expression differences between them. Then we selected genes meeting criteria for both $\log_2$(fold-change) values and p values of Wilcoxon rank sum test, as described previously.[14] Hierarchical clustering with the selected genes was performed on R software using Euclidean distance and complete linkage method. For clustering, expression data were standardized as z scores (mean 0 and variance 1) for each probe. Differentially expressed probe sets were overlaid on a cellular pathway map using the BIND database (http://bond.unleashedinformatics.

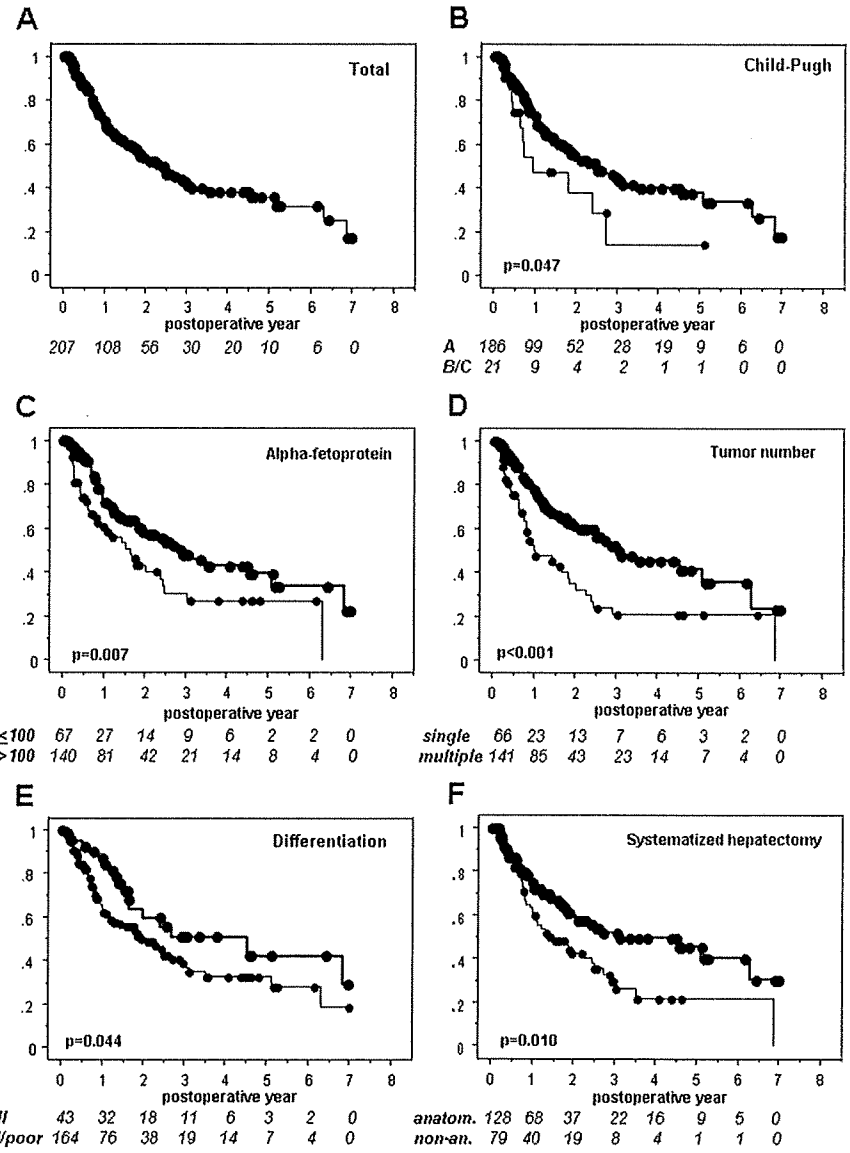


**Figure 1.** Overall recurrence-free survival curves of 207 patients with primary macroscopic vascular invasion–negative hepatocellular carcinoma after curative hepatectomy (A). A table of the actual numbers of patients at risk (in italic) shown below each survival curve. The significant differences of the recurrence-free survivals were detected in Child-Pugh classification (B) (bold = Child A, thin = Child B/C; p = 0.047), α-fetoprotein (C) (bold ≤ 100 mAU/mL, thin > 100 mAU/mL; p = 0.007), tumor number (D) (bold = single, thin = multiple; p < 0.001), histologic differentiation (E) (bold = well, thin = mod/poor; p = 0.044), and systematization of hepatectomy (F) (bold = anatomic, thin = nonanatomic; p = 0.010).

com/index.jsp).[15] The resulting networks were represented in graphic format using the Pajek software (http://vlado.fmf.uni-lj.si/pub/networks/pajek/).[16]

### Statistical analysis

Statistical comparisons of clinicopathologic characteristics for significance were made using chi-square test or Fisher's exact test with a single degree of freedom, and Student's *t*-test was used to analyze differences between continuous values. Cumulative patient survival rates were determined using the Kaplan-Meier method, and for comparisons we used the log rank test. A p value $<0.05$ was considered to have statistical significance. To investigate factors that contributed to prediction ability of the aggressive recurrence, we performed multivariant analysis by logistic regression model.

## RESULTS

### Risk factors of recurrence in HCC without vascular invasion

Among the consecutive 260 patients who underwent initial and curative hepatectomy for HCC from 2000 to 2006 at our hospital, the 207 patients with MVI-negative HCC were analyzed in this study; 28 patients at TNM stage 1, 88 at stage II, 69 at stage III, and 22 at stage IV.[19] Cumulative recurrence-free survivals of these 207 patients were 57.8% (3-year) and 35.3% (5-year), as shown in Figure 1A. Univariant analysis of HCC recurrence was shown in Table 1. Risk factors of recurrence were revealed as multiple tumors ($p < 0.001$), moderate/poor differentiation ($p = 0.044$), Child-Pugh B/C ($p = 0.047$), $\alpha$-fetoprotein elevation ($p = 0.007$), and nonanatomic hepatectomy ($p = 0.010$) (Fig. 1B–F). According to Cox hazard multivariant analysis, multiple tumors ($p < 0.001$), $\alpha$-fetoprotein elevation ($p = 0.002$), and nonanatomic hepatectomy ($p = 0.002$) were identified as independent factors for recurrence (Table 2).

### Recurrence of HCC after anatomically systematized hepatectomy

To analyze the differences in recurrence patterns, localization of the recurrent tumors was compared between cases after anatomic hepatectomy and those after nonanatomic hepatectomy. Local recurrence was detected in 27 of 45 recurrent cases after the nonanatomic hepatectomy, but in only 7 of 47 recurrent cases after anatomic hepatectomy (Table 3). There was significant infrequence of local recurrence after anatomically systematized hepatectomy ($p < 0.001$).

Recurrence after anatomic hepatectomy was evaluated by network analysis of the genome-wide gene expression of the primary HCC tissues (Suppl. Table 1 and Table 2, online only). According to network expression analysis on comparison between the recurrent and nonrecurrent cases within 2 postoperative years (Fig. 2A), distinct signals, including activating transcription factor 2 downstream of transforming growth factor-$\beta$ (TGF-$\beta$)–SMAD pathways, were activated in cases of recurrence. In addition, the differences between local and distant recurrence were assessed by network expression analysis using the BIND database[15] and Pajeck Program,[16] as described elsewhere.[20,21] Figure 2B demonstrated the E2F1 pathways downstream of epidermal growth factor receptor (EGFR)-Src signaling might regulate the biologic aggressiveness of HCC,[22] potentially leading to local recurrence after anatomically systematized hepatectomy. Because both of the pathways have recently been revealed as critical factors of epithelial-mesenchymal transitions (EMT) for cancer metastasis,[23-25] the specific EMT signaling might regulate the biologic aggressiveness of HCC.[26]

**Table 1.** Univariate Analysis on Risk Factors of Recurrence after Resection for Macroscopic Vascular Invasion–Negative Hepatocellular Carcinoma

| Variables of primary HCC | n | Recurrence probability (3-year) | p Value* |
|---|---|---|---|
| Age (y) | | | 0.704 |
| 65 or younger | 78 | 0.609 | |
| Older than 65 | 129 | 0.573 | |
| Gender | | | 0.695 |
| Male | 150 | 0.586 | |
| Female | 57 | 0.667 | |
| Hepatitis virus | | | 0.508 |
| HBV | 33 | 0.529 | |
| HCV | 107 | 0.633 | |
| NBNC | 67 | 0.558 | |
| Child-Pugh | | | 0.047 |
| A | 186 | 0.539 | |
| B/C | 21 | 0.716 | |
| AFP (mAU/mL) | | | 0.007 |
| ≤100 | 67 | 0.522 | |
| >100 | 140 | 0.732 | |
| PIVKA-II (mAU/mL) | | | 0.146 |
| ≤100 | 109 | 0.494 | |
| >100 | 98 | 0.578 | |
| Tumor number | | | <0.001 |
| Single | 66 | 0.497 | |
| Multiple | 141 | 0.789 | |
| Tumor size (cm) | | | 0.714 |
| <3 | 65 | 0.555 | |
| ≥3 | 142 | 0.609 | |
| Pathologic vascular invasion | | | 0.281 |
| Negative | 144 | 0.556 | |
| Positive | 63 | 0.634 | |
| Histologic differentiation | | | 0.044 |
| Well | 43 | 0.483 | |
| Moderate/poor | 164 | 0.621 | |
| Systematization of hepatectomy | | | 0.010 |
| Anatomic | 128 | 0.484 | |
| Nonanatomic | 79 | 0.741 | |

*Log rank test.
AFP, $\alpha$-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NBNC, non-B non-C; PIVKA-II, protein induced by vitamin K absence or antagonists-II.

## DISCUSSION

The concept of anatomically systematized hepatectomy has been proposed on the basis of hepatic sectors along Glisson's pedicles.[9,10] The systematized hepatectomy has been reported to substantially improve longterm outcomes,[17,27-29] but several reports found that major hepatectomy does not affect long-term outcomes.[30,31] Because patient survival is affected mainly by metastatic recurrence through tumor vascular invasion,[4]

**Table 2.** Cox Multivariable Analysis on Risk Factors of Recurrence after Curative Resection for Macroscopic Vascular Invasion–Negative Hepatocellular Carcinoma

| Variables of primary HCC | Coefficient | Odds ratio | 95% Confidence Interval | p Value |
|---|---|---|---|---|
| Child-Pugh B, C | 0.622 | 1.863 | 0.954–3.640 | 0.069 |
| AFP >100 mAU/mL | 0.723 | 2.299 | 1.449–3.648 | 0.002 |
| Multiple tumors | 0.833 | 2.061 | 1.300–3.266 | <0.001 |
| Moderete or poor differentiation | 0.657 | 1.928 | 0.688–5.404 | 0.212 |
| Nonanatomic | 0.686 | 1.986 | 1.275–3.095 | 0.002 |

AFP, α-fetoprotein; HCC, hepatocellular carcinoma.

**Table 3.** Recurrence Patterns of Hepatocellular Carcinoma after Anatomic or Nonanatomic Hepatectomy for Macroscopic Vascular Invasion–Negative Hepatocellular Carcinoma

| Systematization of hepatectomy | Local recurrence | Distant recurrence | Total |
|---|---|---|---|
| Anatomic | 7* | 40 | 47 |
| Nonanatomic | 27* | 18 | 45 |
| Total | 34 | 58 | 92 |

The recurrent tumor arising in the same segment as the initial tumor, or within 2 cm from the surgical stump was regarded a "local" recurrence, while the other intrahepatic recurrence was named a "distant" one, as described by Takayama and colleagues.[18]
*Chi-square test; $p < 0.001$.

surgical procedures and outcomes should be analyzed dependent on the status of the vascular invasion. Because the existence of apparent MVI obviously requires anatomic hepatectomy for HCC, systematization of resection should be argued in the patients without MVI. We focused on the cases of MVI-negative HCC to determine the effects of hepatectomy. In our study, the anatomic hepatectomy contributed substantially to recurrence-free survival of patients with MVI-negative HCC (Fig. 1F), not only by univariate analysis (Table 1), but also by multivariate analysis (Table 2).

Next, the recurrence patterns were assessed after anatomic or nonanatomic hepatectomy. Local recurrence, as shown in Table 3, was significantly suppressed by anatomic hepatectomy, compared with nonanatomic cases ($p < 0.001$). Nakashima and colleagues[32] reported the histopathologically detailed analysis of 219 small HCCs without MVI, and found pathologic invasion in the portal veins in >25% and intrahepatic micrometastasis in 10% within the sector.[32] These findings suggested that metastatic foci within the sector cannot be removed with nonanatomically partial hepatic resection. The systematized anatomic hepatectomy might contribute to recurrence-free survivals, possibly as a result of the inhibition of local recurrence potentials of HCC.

It is open to debate whether the biologic ability of the recurrence is identified in the primary MVI-negative HCC even after anatomic hepatectomy. In this study, we assessed the network expression analysis using cDNA microarray data using the BIND database[15] and Pajeck Program.[16] Signal transduction pathways play a key role in the regulation of key cellular processes of cancer, including invasion and metastasis. As demonstrated for recurrent cases within 2 postoperative years (Fig. 2A), TGF-β-SMADs–activating transcription factor 2 signaling pathways were upregulated in the primary HCC tumors. TGF-β is a polypeptides with dual tumor-suppressive and oncogenic effects, signaling through serine/threonine kinase receptor complexes, which phosphorylate cytoplasmic mediators, the SMADs.[33] On phosphorylation, SMADs translocate to the nucleus and regulate the transcriptional factors, including activating transcription factor 2, which mediates both transcription and DNA damage control in the malignant tumor development.[34] During initial tumorigenesis, malignantly transformed cells often lose the response to tumor-suppressive effects of TGF-β, which, in turn, starts to act as an autocrine tumor-promoting factor by enhancing cancer invasion and metastasis.[33,35] More important, TGF-β has been noted as one of the main inducers of EMT,[23] a process to convert epithelial cells into mesenchymal cells and control cell adhesion, motility invasion, survival, and differentiation.[26] Growing evidence points to changes in TGF-β signaling pathway that occur during HCC progression at the late stage.[36]

Hepatic recurrence of HCC is classified as intrahepatic metastasis and multicentric recurrence.[4,5] To distinguish the recurrence patterns after anatomic hepatectomy,[6,7] differences between local and distant intrahepatic recurrence were evaluated by network expression analysis (Fig. 2B). Accordingly, the EGFR-Src-E2F1 signaling pathways were upregulated in a cluster associated with the locally metastatic recurrence after anatomically systematized hepatectomy. It is of interest that EGFR-Src signaling is also known as a positive regulator of EMT.[26] Additional evidences indicated that E2F1 transcription factor, the major target of tumor suppressor Rb, might stimulate the EMT through Zeb1/ZFHX1A,[24,25] Snail,[24] or Slug repressors,[25] reported to advance the metastatic potential of HCC in our previous studies.[37] On the basis of our investigations, new therapeutic strategies targeting the EMT signals might be proposed for inhibiting HCC recurrence.

In conclusion, the surgical contribution to MVI-negative HCC might be emphasized because anatomically systematized hepatectomy could suppress the local recurrence. Network gene expression analysis revealed the distinct signaling pathways of EMT are associated with recurrence after anatomically systematized hepatectomy. Limitations of our studies are mainly related to the retrospective analysis and short observation time. Randomized trials and much longer observation time are required for proof of the critical impact of oncological profiling. Additional attention should be paid to

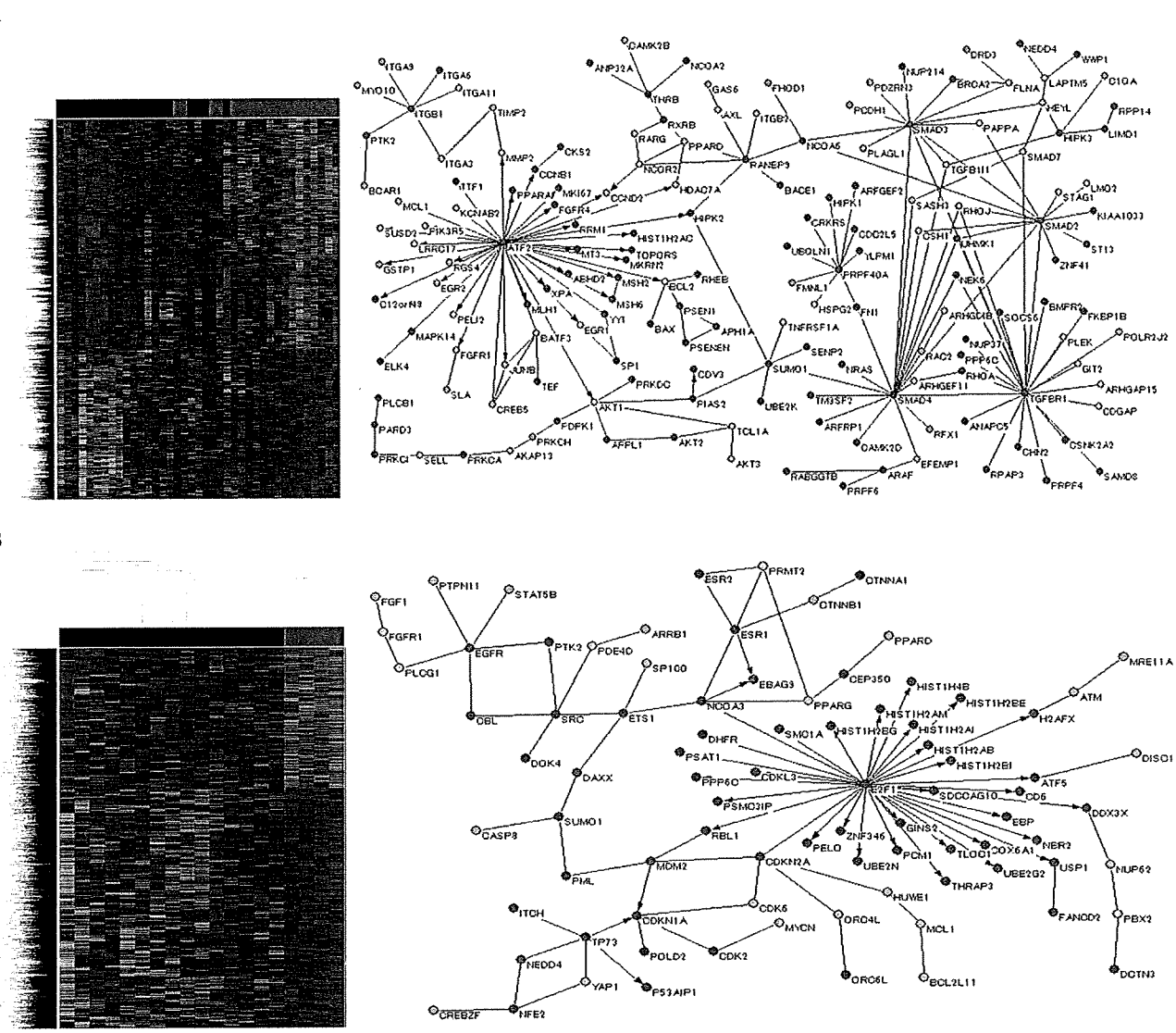


**Figure 2.** Hierarchical clustering (left) and biomolecular interaction networks (right) on the basis of cDNA microarray data of primary hepatocellular carcinoma tumors. (A) The recurrent cases (red bar) and the nonrecurrent cases (black bar) within 2 years after anatomically systematized hepatectomy. Red and green nodes represent upregulated and downregulated expression in the recurrent ones, respectively. In the network panel using Pajek software,[16] blue lines indicate the protein–protein interactions, and arrows indicate the protein–DNA interactions using Biomolecular Interaction Network Database.[15] Transforming growth factor-β–SMADs–activating transcription factor 2 signaling pathways are upregulated in a cluster associated with early recurrence after anatomically systematized hepatectomy. (B) Local recurrence cases (red bar) and distant recurrence cases (black bar) after anatomically systematized hepatectomy. Red and green nodes represent upregulated and downregulated expression in the local recurrence ones, respectively. Epithelial growth factor receptor-Src-E2F1 signaling pathways are upregulated in a cluster associated with the locally metastatic recurrence after anatomically systematized hepatectomy. Note that both of the pathways are closely related to epithelial-mesenchymal transitions.

the novel therapeutic strategies targeting the signal network for adjuvant therapy of HCC.[38,39]

## Author Contributions

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