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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 ($p = 0.047$), α-fetoprotein elevation ($p = 0.007$), and nonanatomic hepatectomy ($p = 0.010$) as risk factors. According to Cox hazard multivariant analysis, multiple tumors ($p = 0.002$), α-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

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Abbreviations and Acronyms

BIND = Biomolecular Interaction Network Database
EGFR = epidermal growth factor receptor
EMT = epithelial-mesenchymal transitions
HCC = hepatocellular carcinoma
MVI = macrovascular invasion
TGF-β = transforming growth factor-β

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 α-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 μg 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.

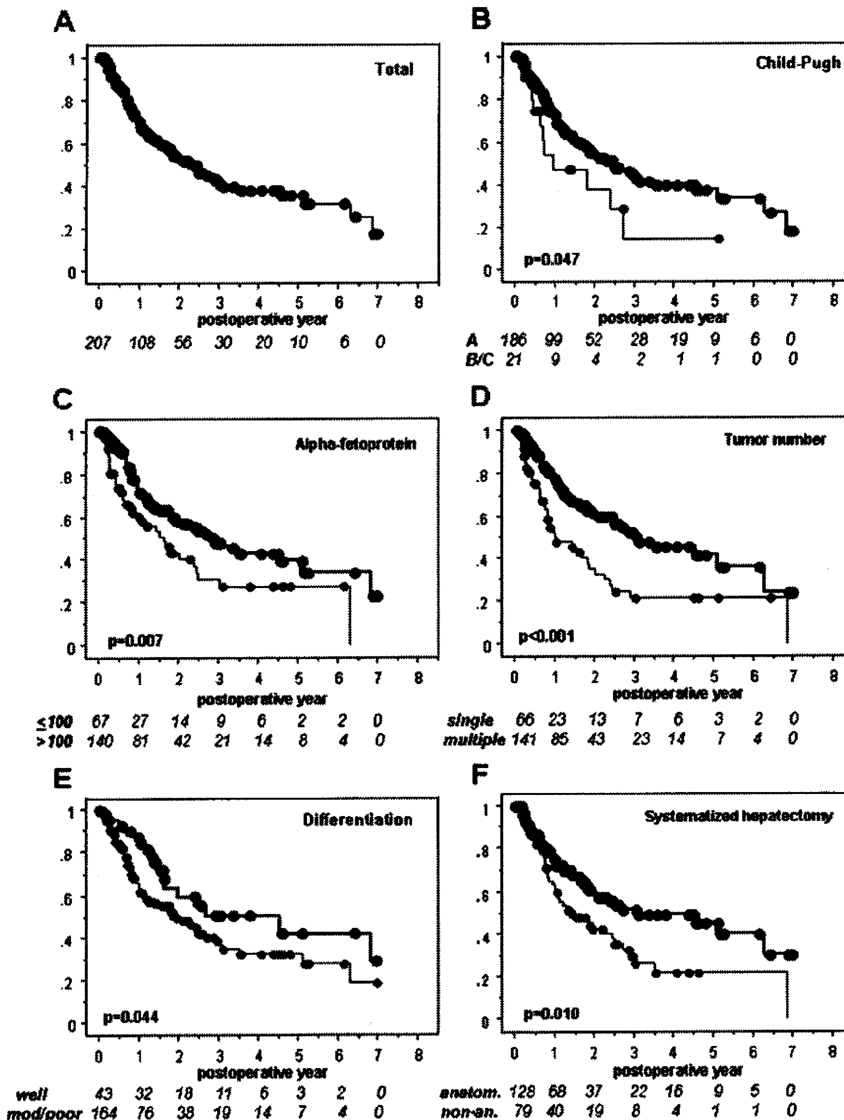


Figure 1. Overall recurrence-free survival curves of 207 patients with primary macroscopic vascular invasion–negative hepatocellular carcinoma after curative hepatectomy (A). A table of the actual numbers of patients at risk (in italic) shown below each survival curve. The significant differences of 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 ($p = 0.047$), α-fetoprotein elevation ($p = 0.007$), and nonanatomic hepatectomy ($p = 0.010$) (Fig. 1B–F). According to Cox hazard multivariant analysis, multiple tumors ($p < 0.001$), α-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-β (TGF-β)–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, α-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 longterm 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; $p < 0.001$.

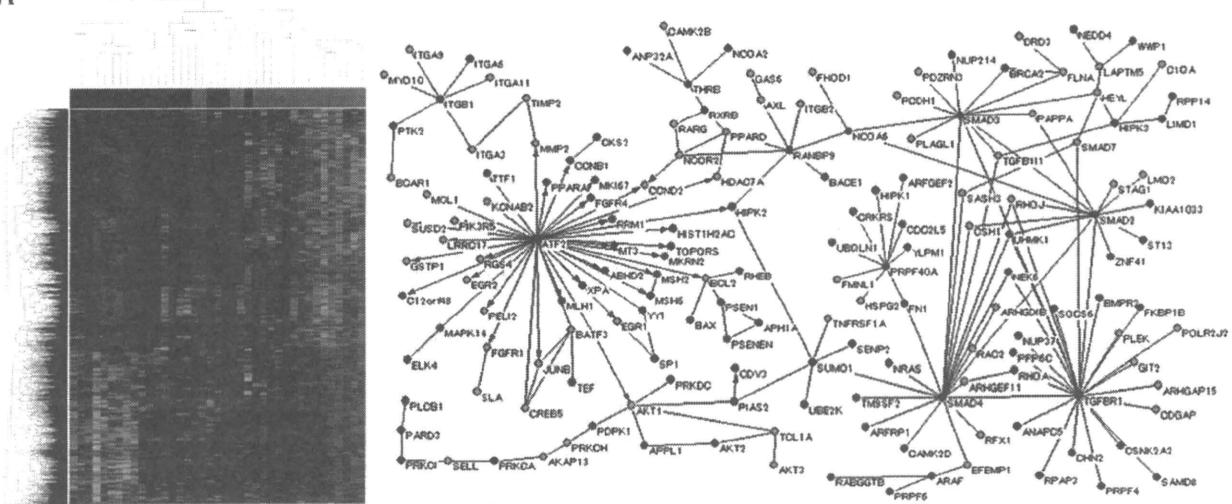
surgical procedures and outcomes should be analyzed dependent on the status of the vascular invasion. Because the existence of apparent MVI obviously requires anatomic hepatectomy for HCC, systematization of resection should be argued in the patients without MVI. We focused on the cases of MVI-negative 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



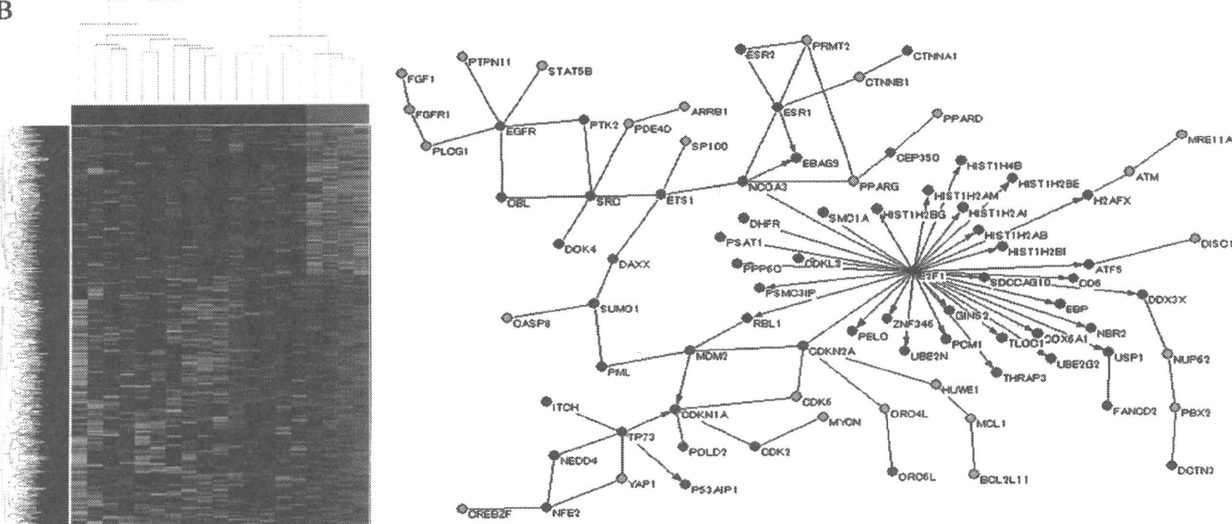


Figure 2. Hierarchical clustering (left) and biomolecular interaction networks (right) on the basis of cDNA microarray data of primary hepatocellular carcinoma tumors. (A) The recurrent cases (red bar) and the nonrecurrent cases (black bar) within 2 years after anatomically systematized hepatectomy. 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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Original article

Aurora kinase B is a predictive factor for the aggressive recurrence of hepatocellular carcinoma after curative hepatectomy

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Background: Patterns of cancer recurrence hold the key to prognosis after curative resection. This retrospective study aimed to identify a predictor and therapeutic candidate for aggressive recurrence of hepatocellular carcinoma (HCC).

Methods: Primary HCC tissues from 107 patients who had curative resection were analysed. Genome-wide gene expression profiles were investigated using a microarray technique, and clustering analysis was carried out based on the first diagnosis of recurrence according to the Milan criteria. Immunohistochemical expression and array-based comparative genomic hybridization (array-CGH) were also assessed.

Results: Microarray analysis revealed overexpression of Aurora kinase B, a chromosome passenger protein kinase, as the most significant predictor of the aggressive recurrence of HCC. Aurora kinase B protein expression was significantly associated with aggressive recurrence ($P < 0.001$) and prognosis ($P < 0.001$). Multivariable analysis identified Aurora kinase B as the only independent predictor of aggressive recurrence of HCC ($P = 0.031$). Array-CGH analysis showed that genomic instability was closely related to Aurora kinase B expression ($P = 0.011$).

Conclusion: Aurora kinase B is an effective predictor of aggressive HCC recurrence, in relation to the genomic instability. It might be worth considering as a molecular target for the adjuvant therapy of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a common fatal malignancy worldwide[1]. A major obstacle in its treatment is the high frequency of tumour recurrence even after curative resection. Indeed, the pattern of recurrence, rather than the recurrence itself, has critical effects on prognosis[2]. Prognosis has been shown to be closely associated with the pattern of recurrence of HCC defined according to the Milan criteria (solitary lesion of maximum diameter 5 cm, or up to three nodules of maximum diameter 3 cm, without major vascular invasion or distant metastasis)[3], which have been proposed for selection of patients with HCC for liver transplantation[4]. Even in the event of recurrence after curative resection, patients who meet these criteria have a good prognosis[3]. In contrast, when the recurrence exceeds the Milan criteria, treatment is limited and the prognosis poor[5].

In recent years, various molecules have been proposed as predictive markers for recurrence of HCC, but none has proved useful clinically because different patterns of recurrence were not considered[6,7]. The identification of novel biomarkers is required for the accurate prediction

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of aggressive recurrence at the first diagnosis of recurrent HCC[3].

Genome-wide gene expression analysis by microarray offers a systematic approach to gaining comprehensive information regarding transcription profiles[8]. Such studies have the potential to lead to the development of a novel molecular-targeted therapy for HCC[9]. The authors have previously analysed gene expression in HCC[10,11], and have identified novel molecules that might represent therapeutic targets[10,12]. The aim of the present study was to identify a marker associated with aggressive recurrence of HCC within 2 years of curative resection, by examination of the genome-wide expression profile of HCC generated by microarray study. The genes identified by such a comprehensive genetic analysis may shed light on the biological tumour characteristics that lead to recurrence, which might enable the development of a specific adjuvant therapy for HCC.

Methods

One hundred and seven patients underwent curative hepatectomy for HCC between 2000 and 2004 at Tokyo Medical and Dental University Hospital. Written informed consent was obtained from these patients, and the institutional review board approved the study. Forty patients were randomly assigned to a microarray training set, and the remaining 67 to a validation set. Resected tissues containing no necrosis were divided into two specimens immediately after surgery; one was snap-frozen in liquid nitrogen and stored at −80°C for microarray analysis, and the other was fixed in 10 per cent formaldehyde solution and embedded in paraffin for histopathological analysis.

Patients were followed up with assays of serum level of α-fetoprotein and protein induced by vitamin K absence or antagonists II every month, and by ultrasonography, computed tomography (CT) and magnetic resonance imaging every 3 months. The mean observation time was 4·1 years. When tumour recurrence was suspected, precise diagnostic imaging was performed by means of CT angiography. Finally, the exact diagnosis of recurrence was made by imaging, and the number and size of the tumour(s) determined. At the first diagnosis of a recurrence within 2 years after the initial operation, the pattern was defined in accordance with the Milan criteria. A comparison of the characteristics of patients in the microarray and validation groups showed no statistically significant differences in clinicopathological features. No recurrence, recurrence meeting the Milan criteria and recurrence exceeding the criteria were noted in 18, nine and 13 patients respectively in the microarray group, compared with 32, 13 and 22 patients in the validation group ($P = 0{\cdot}924$).

RNA preparation and microarray hybridization

Forty primary HCC specimens were obtained from the surgically resected materials. Total RNA was extracted from the sliced tissue specimens using an RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA obtained was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, California, USA). For the gene expression analysis, 29 samples with an RNA integrity number greater than 5·0 were further analysed. There were no statistically significant differences in the clinicopathological features between the 29 samples analysed and the 11 discarded; no recurrence, recurrence meeting the criteria and recurrence exceeding the criteria were observed in 13, six and ten samples analysed respectively, compared with five, three and three discarded ($P = 0{\cdot}869$). The rate of cancer cells was above 40 per cent (mean 81·0 per cent) in all 29 samples. Contaminating DNA was removed by digestion with RNase-free DNase (Qiagen). Complementary RNA was prepared from 2 μg total RNA, using one-cycle target labelling and a control reagents kit (Affymetrix, Santa Clara, California, USA). Hybridization and signal detection of the Human Genome U133 Plus 2.0 arrays (Affymetrix) were performed in accordance with the manufacturer's instructions.

Analysis of gene expression data

A total of 29 microarray data sets was normalized using the robust multiarray average method[13] with R 2.3.1 statistical software together with a BioConductor package[14]. The estimated gene expression levels were $\log_2$-transformed, and 62 control probe sets were removed for further analysis. For each 54 613 probes on the Human Genome U133 Plus 2.0 arrays, the fold change (FC) values were calculated using the ratio of the geometric means of the gene expression levels between the two groups. A Wilcoxon rank sum test was performed to estimate the significance levels of the differences in gene expression between them. Genes were selected for which both the $\log_2$(FC) values were greater than 1 (equivalent to FC more than 2·0 or FC less than 0·5; denoted FC $> 2{\cdot}0$ for simplicity) and $P < 0{\cdot}0002$ in the Wilcoxon rank sum test. A hierarchical clustering with the selected genes was performed with R software using the Euclidean distance and complete linkage method. For clustering, the expression data were standardized as z-scores (mean = 0 and variance = 1) for each probe.

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Immunohistochemical analysis

To validate further the expression patterns detected by the microarray, immunohistochemical studies were performed on 22 HCC samples in the microarray group and an additional 67 HCC samples for validation. The primary antibody (Novus Biologicals, Littleton, Colorado, USA) was used at 1 : 50 dilution with phosphate-buffered saline containing 1 per cent bovine serum albumin. The tissue sections were stained with an automated immunostainer (BenchMark® XT; Ventana Medical Systems, Tucson, Arizona, USA) using heat-induced epitope retrieval and a standard diaminobenzidine detection kit (Ventana). Strong, diffuse, nuclear staining in more than 10 per cent of the cells was considered a positive result. Immunohistochemical staining was evaluated under a light microscope by two independent investigators.

Analysis of array-based comparative genomic hybridization and altered fraction of genome

The in-house array MCG Cancer Array-800, which contains 800 bacterial artificial chromosome/P1-derived artificial chromosome clones carrying genes or sequence-tagged site markers of potential importance in cancer genesis or progression, was used for array-based comparative genomic hybridization (array-CGH) analysis[15]. The hybridizations were carried out as described elsewhere[16] with minor modifications, using a HYBRIMASTER® HS-300eTAC hybridization machine (Aloka, Tokyo, Japan). The hybridized slides were scanned with GenePix 4000B (Axon Instruments, Foster City, California, USA) and the acquired images were analysed with GenePix Pro 6.0 imaging software (Axon Instruments). The fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratios across the array was zero. Based on a previous control study[15], the thresholds for copy-number gain and loss were set at $\log_2$ ratios of 0·4 and −0·4 respectively; $\log_2$ ratios greater than 2·0 were defined as high-level amplifications and $\log_2$ ratios below −2·0 as homozygous deletions. To evaluate the genomic instability of each of the HCC tumours, the fraction of genome altered (FGA) was calculated based on the array-CGH data as described by Veltman and colleagues[17]. The FGA for each tumour was quantitated by assigning for each clone a distance equal to the sum of one half of the distance between its own centre and that of its neighbouring clones.

Statistical analysis

Statistical comparisons of clinicopathological characteristics were made using the χ^2 test or Fisher's exact test with a single degree of freedom, and Student's t test was used to analyse differences between continuous values. Cumulative patient survival rates were determined using the Kaplan–Meier method, and compared by means of the log rank test. $P < 0.050$ was considered statistically significant.

To investigate the factors that predicted aggressive recurrence, multivariable analysis was performed using a logistic regression model. The following six factors were considered: expression of Aurora kinase B protein (no *versus* yes), the number of tumours (one or two *versus* three or more), tumour size (at least 5 cm *versus* less than 5 cm), portal vein invasion (no *versus* yes), hepatic vein invasion (no *versus* yes) and tumour node metastasis (TNM) stage (I/II/III *versus* IV). To select a set of factors that were strongly associated with aggressive recurrence, a stepwise variable selection was performed using the six factors. The initial model contained all six factors, and at each step a variable that minimized Akaike's information criterion was added or removed until convergence was achieved.

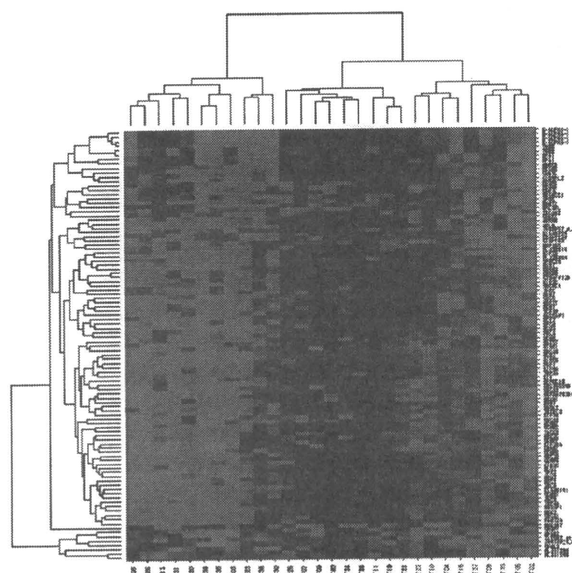

Fig. 1 Hierarchical clustering of primary hepatocellular carcinoma with recurrence exceeding the Milan criteria using probes satisfying both fold change (FC) > 2 and $P < 0.0002$. T02, T04, T05, T08, T10, T11, T15, T27, T33 and T37 are the patients with aggressive recurrence (shown in red text). Red represents upregulation in the group with recurrence, and green downregulation in the others

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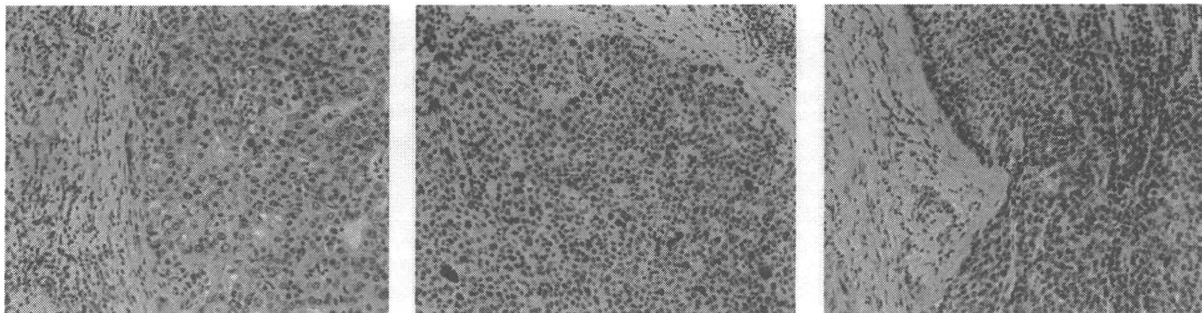
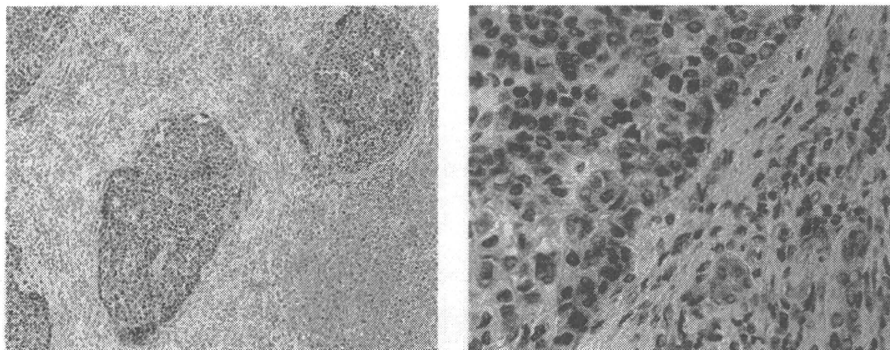


a Levels of expression of Aurora kinase B protein



b Transition zone capsule

Fig. 2 Immunohistochemical analysis of Aurora kinase B in hepatocellular carcinoma (HCC) tissues. **a** HCC tissues showing negative (less than 10 per cent; left panel), moderate (10–20 per cent; middle panel) and strong (more than 20 per cent; right panel) staining intensities of Aurora kinase B protein in the nucleus of cancer cells, but not in the adjacent non-cancerous cells (original magnification ×100). **b** The transition zone capsule invading HCC cancer cells with Aurora kinase B expression at low magnification (×20; left panel) and high magnification (×400; right panel)

Results

Aurora kinase B as a predictor of aggressive recurrence of hepatocellular carcinoma

A gene expression analysis was performed for the 29 samples in the microarray test group in relation to aggressive recurrence. Among 54 613 probes, 101 that satisfied FC > 2·0 and Wilcoxon $P < 0.0002$ were identified as differently expressed genes. *Fig. 1* shows the hierarchical clustering of the 101 probes. The patient numbers in red on the right denote patients with aggressive recurrence, indicating that the genes were divided into two clusters. As shown in *Table 1* and *Appendix 1* (available as supplementary material online; http://www.bjs.co.uk), it is of interest that the genes associated with cytokinesis (Aurora kinase B; switching/sucrose non-fermenting-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 1; minichromosome maintenance-deficient 2, mitotin (*Saccharomyces cerevisiae*); protein regulator of cytokinesis 1; microtubule-associated, homologue (*Xenopus laevis*); discs, large homologue 7 (*Drosophila*); citron (rho-interacting, serine/threonine kinase 21); and chromosome segregation 1-like) were highly upregulated in the primary HCC of patients who had aggressive recurrence exceeding the Milan criteria. Aurora kinase B, a chromosome passenger protein kinase, was potentially the strongest predictor of aggressive recurrence.

Aurora kinase B protein is overexpressed in HCC tissues in association with aggressive recurrence

To validate further the expression of Aurora kinase B as detected by microarray, immunohistochemical studies were performed on HCC tissues using Aurora kinase B antibody. Strong, diffuse, nuclear staining in more than 10 per cent of the cells, considered a positive result, was observed in tumour tissues, but not in adjacent normal liver tissue (*Fig. 2*). Aurora kinase B protein was detected in nine of 22 samples in the microarray test group. The microarray intensity of the Aurora kinase B gene was

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Table 1 List of genes differently expressed in cases of recurrence exceeding the Milan criteria ($P < 0.00003$)

No.	Probe ID	Gene symbol	Title	P	Fold change
1	209464_at	AURKB	Aurora kinase B	0·0000002	3·59
2	202503_s_at	KIAA0101	KIAA0101	0·0000007	5·24
3	201075_s_at	SMARCC1	Switching/sucrose non-fermenting (SWI/SNF)-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 1	0·0000012	2·46
4	202107_s_at	MCM2	MCM2, minichromosome maintenance-deficient 2, mitotin (*Saccharomyces cerevisiae*)	0·0000012	4·33
5	218009_s_at	PRC1	Protein regulator of cytokinesis 1	0·0000012	4·83
6	210052_s_at	TPX2	TPX2, microtubule-associated, homologue (*Xenopus laevis*)	0·0000019	3·92
7	203764_at	DLG7	Discs, large homologue 7 (*Drosophila*)	0·0000030	4·04
8	212801_at	CIT	Citron (rho-interacting, serine/threonine kinase 21)	0·0000030	2·56
9	218355_at	KIF4A	Kinesin family member 4A	0·0000030	4·11
10	201112_s_at	CSE1L	CSE1, chromosome segregation 1-like (yeast)	0·0000045	2·13
11	218755_at	KIF20A	Kinesin family member 20A	0·0000045	4·52
12	202705_at	CCNB2	Cyclin B2	0·0000097	3·22
13	204709_s_at	KIF23	Kinesin family member 23	0·0000097	3·07
14	206102_at	PSF1	DNA replication complex GINS protein PSF1	0·0000097	4·57
15	209642_at	BUB1	BUB1, budding uninhibited by benzimidazoles 1 homologue (yeast)	0·0000097	3·87
16	225687_at	C20orf129	Chromosome 20 open reading frame 129	0·0000097	4·52
17	1552619_a_at	ANLN	Anillin, actin-binding protein (scraps homologue, *Drosophila*)	0·0000097	3·38
18	202326_at	EHMT2	Euchromatic histone-lysine *N*-methyltransferase 2	0·0000139	2·14
19	206550_s_at	NUP155	Nucleoporin 155 kDa	0·0000139	2·49
20	210115_at	RPL39L	Ribosomal protein L39-like	0·0000139	2·55
21	226980_at	DEPDC1B	DEP domain-containing 1B	0·0000139	3·53
22	201111_at	CSE1L	CSE1 chromosome segregation 1-like (yeast)	0·0000194	2·26
23	201292_at	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	0·0000194	5·22
24	204603_at	EX01	Exonuclease 1	0·0000194	2·21
25	223271_s_at	CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like 2	0·0000194	2·07
26	201663_s_at	SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	0·0000269	2·89
27	207828_s_at	CENPF	Centromere protein F, 350/400 kDa (mitosin)	0·0000269	3·75
28	209714_s_at	CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)	0·0000269	4·55
29	212639_x_at	K-ALPHA-1	Tubulin, alpha, ubiquitous	0·0000269	2·14
30	214710_s_at	CCNB1	Cyclin B1	0·0000269	4·21
31	223699_at	CNDP1	Carnosine dipeptidase 1 (metallopeptidase M20 family)	0·0000269	0·21
32	224753_at	CDCA5	Cell division cycle-associated 5	0·0000269	2·74
33	1555278_a_at	CKAP5	Cytoskeleton-associated protein 5	0·0000269	2·03

significantly higher in these nine samples than in the remaining 13 (FC = 2·3; $P = 0.004$). The results of the immunohistochemical analysis confirmed the microarray findings at the protein expression level.

An additional 67 samples in the validation set were analysed. Again, nuclear staining in more than 10 per cent of the cells was noted in tumour tissue, but not in the adjacent normal liver. Immunohistochemical expression of Aurora kinase B was detected in 17 of 22 cases with aggressive recurrence, but in only eight of 45 without such recurrence ($P < 0.001$). A close relationship between Aurora kinase B expression in the primary HCC and aggressive recurrence was confirmed.

The association between Aurora kinase B protein expression and other clinicopathological variables was investigated in the 67 patients (*Table 2*). There was a statistically significant relationship between Aurora kinase B protein expression and primary tumour number, tumour size, portal vein invasion, hepatic vein invasion and TNM stage.

Fig. 3 shows the survival rates after curative surgery of the 25 patients who were positive and the 42 who were negative for Aurora kinase B expression. The 5-year survival rate of the latter group was significantly higher (79 *versus* 24 per cent; $P < 0.001$).

Multivariable analysis of aggressive recurrence and the significance of Aurora kinase B expression

Multivariable logistic regression of aggressive recurrence was performed using five clinicopathological factors and the immunohistological data (*Table 3*). The stepwise variable selection led to a model with three factors: tumour size, portal vein invasion and Aurora kinase B expression. The expression of Aurora kinase B was the only significant

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Table 2 Clinicopathological variables in hepatocellular carcinoma in relation to Aurora kinase B expression

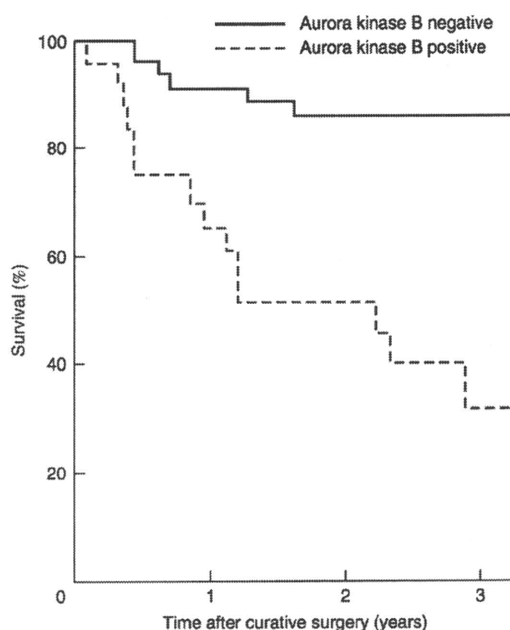
	Negative ($n = 42$)	Positive ($n = 25$)	*P*
Recurrence			< 0·001†
No	27	5	
Yes, meeting criteria	10	3	
Yes, exceeding criteria	5	17	
Tumour number (primary)			0·029†
1–2	37	16	
≥ 3	5	9	
Tumour size (primary) (cm)*	4·3(0·5)	7·1(1·1)	0·009‡
Histological differentiation (primary)			0·084†
Good	12	2	
Moderate	17	10	
Poor	13	13	
Portal vein invasion (primary)			< 0·001†
No	28	5	
Yes	14	20	
Hepatic vein invasion (primary)			0·045§
No	40	19	
Yes	2	6	
Growth (primary)			0·649†
Expansive	28	18	
Invasive	14	7	
Arterial invasion (primary)			0·373§
No	42	24	
Yes	0	1	
Capsule formation (primary)			0·427†
No	16	12	
Yes	26	13	
Capsule formation with cancer cell infiltration			0·774†
No	22	14	
Yes	20	11	
TNM stage (primary)			0·003†
II	5	0	
II	17	2	
III	13	10	
IVA	6	10	
IVB	1	3	
α-Fetoprotein (×10⁴ ng/ml)*	1·9(1·8)	2·6(1·2)	0·816‡
α-Fetoprotein lectin 3 (%)			
≤ 10	14	6	0·419†
> 10	28	19	
PIVKA-II (×10⁴ mAU/ml)*	2·2(1·8)	6·0(4·8)	0·393‡

*Values are mean(s.e.m.). TNM, tumour node metastasis; PIVKA, protein induced by vitamin K absence or antagonists; AU, arbitrary units. †χ^2 test; ‡Student's *t* test; §Fisher's exact test.

factor in both the six-factor model and the reduced three-factor model.

Higher genomic instability in Aurora kinase B-positive cases

The extent of the genome affected was defined as the FGA, as represented by the clones on the array for the



No. at risk				
Negative	42	36	27	17
Positive	25	16	8	4

Fig. 3 Cumulative survival curves of patients with primary hepatocellular carcinoma negative or positive for Aurora kinase B protein expression. $P < 0.001$ (log rank test)

Table 3 Logistic regression models for prediction of recurrence exceeding the Milan criteria

Features of primary HCC	Coefficient	Odds ratio	*P*
Six-factor model			
Expression of Aurora kinase B (no *versus* yes)	2·18	8·84 (1·21, 64·56)	0·031
No. of tumours (1–2 *versus* ≥ 3)	1·61	4·98 (0·35, 70·19)	0·234
Tumour size (≤ 5 *versus* > 5 cm)	0·67	1·95 (0·19, 19·9)	0·571
Portal vein invasion (no *versus* yes)	1·78	5·95 (0·36, 97·85)	0·212
Hepatic vein invasion (no *versus* yes)	− 0·41	0·66 (0·03, 15·13)	0·797
TNM stage (I/II/III *versus* IV)	1·19	3·29 (0·26, 41·12)	0·356
Three-factor model*			
Expression of Aurora kinase B (no *versus* yes)	2·17	8·77 (1·57, 49·78)	0·009
Tumour size (≤ 5 *versus* > 5 cm)	0·81	2·25 (0·29, 20·29)	0·471
Portal vein invasion (no *versus* yes)	0·95	2·58 (0·35, 18·88)	0·349

Values in parentheses are 95 per cent confidence intervals. *Three factors selected by stepwise variable selection. HCC, hepatocellular carcinoma; TNM, tumour node metastasis.

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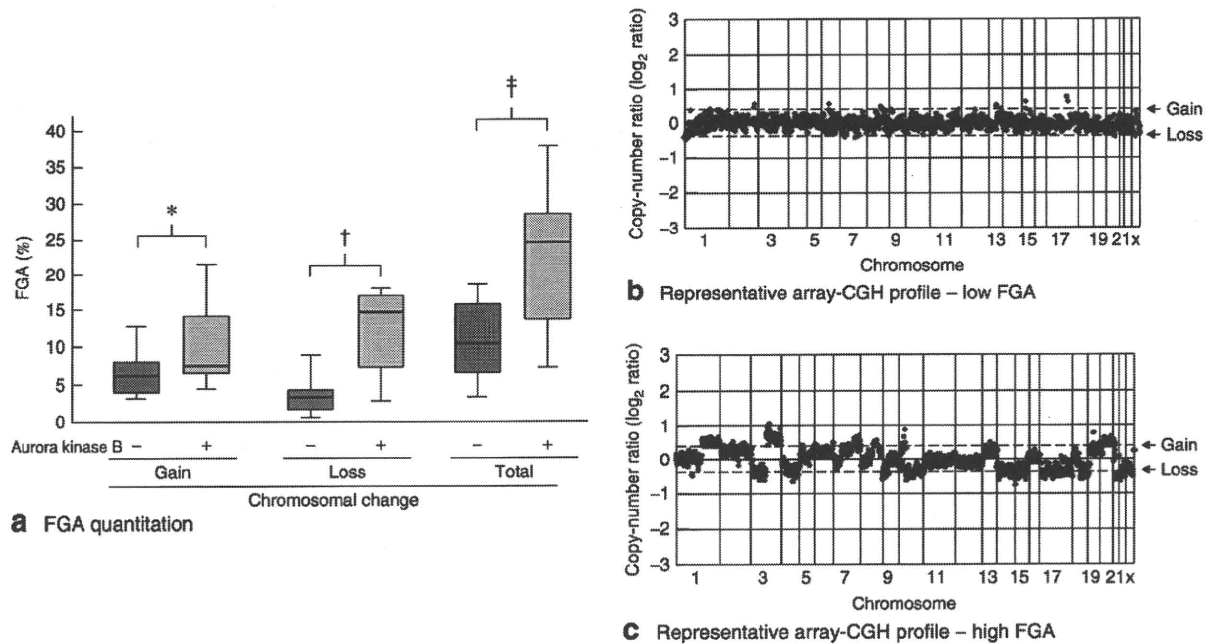

Fig. 4 a Fraction of genome altered (FGA) determined by array-based comparative genomic hybridization (array-CGH) in ten primary hepatocellular carcinomas (HCCs) without and nine with Aurora kinase B immunoreactivity. Horizontal bars within boxes, boxes and error bars represent median, interquartile range and range respectively. $^{*}P = 0{\cdot}221$, $^{\dagger}P = 0{\cdot}009$, $^{\ddagger}P = 0{\cdot}011$ (Mann–Whitney *U* test). **b,c** Representative array-CGH profiles from samples demonstrating **b** low FGA (3·4 per cent) or **c** high FGA (30·5 per cent) which showed negative and positive Aurora kinase B immunoreactivity respectively. Profiles are copy-number alterations relative to normal genomic DNA ordered by chromosome, with each spot representing one of the duplicated clones. The thresholds for determining chromosomal gain and loss were defined as $\log_2$ ratio more than +0·4 and less than −0·4 respectively[15]

19 primary HCCs in which high-quality DNA, suitable for an array-CGH analysis, were available. Nine tumours with Aurora kinase B immunoreactivity had a median FGA of 7·3, 14·4 and 24·5 per cent in gained, lost and total alterations respectively, whereas the ten without Aurora kinase B immunoreactivity had a median FGA of 5·8, 3·2 and 10·1 per cent respectively (*Fig. 4*). The FGA values for lost and total alterations in the Aurora kinase B-positive cases were significantly higher than those in the negative cases, suggesting that greater Aurora kinase B expression may contribute to the genomic instability in HCC.

Discussion

Although surgical procedures and perioperative care for patients with HCC have improved markedly, tumour recurrence remains a major obstacle to better survival. Repeat hepatectomy and salvage transplantation have both been proposed as treatment options for recurrent tumours meeting the Milan criteria[18,19]. Indeed, hepatic resection is performed as an initial treatment, followed by salvage transplantation when recurrence develops and/or liver function has deteriorated. Correct prediction of the type of recurrence at the initial operation would be useful in the selection of optimal adjuvant therapy[3,9].

In the present study a microarray technique was used to determine whether prediction of aggressive recurrence is possible, and several cytokinesis-associated genes were found to be highly upregulated in the primary HCC, resulting in recurrence exceeding the criteria. Of these, Aurora kinase B was identified as the most significant predictor of such recurrence. Univariable analysis revealed that Aurora kinase B protein expression was significantly related to aggressive recurrence and to prognosis, and additional multivariable analyses confirmed that Aurora kinase B protein was the only statistically significant predictor of such recurrence.

Aurora kinase B 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

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of the mitotic checkpoint[20]. It localizes first to the chromosomes during the prophase and then to the inner centromere region between the sister chromatids during the prometaphase and metaphase. During the prometaphase, Aurora kinase B is responsible for the correct localization and stabilization of the centromeric proteins, including Borealin, Bub1, the inner centromeric protein and survivin/baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5)[21]. Interestingly, the present microarray analysis revealed that other chromosome passenger proteins, including Bub1 and survivin/BIRC5, were also significantly overexpressed in primary HCCs with an aggressive pattern of recurrence (*Appendix*, available as supplementary material online at http://www.bjs.co.uk). One of the essential functions of Aurora kinase B is in mitotic spindle attachment. Because of its vital role in correcting chromosome–spindle attachment, deregulated expression of Aurora kinase B can be expected to impair chromosome segregation and mitotic progression, leading to aneuploidy.

Overexpression of Aurora kinase B has been reported in a variety of malignant cancers[22,23]. Several studies have used xenograft models of localized tumour formation in mice injected with cells that overexpress Aurora kinase B to demonstrate that such overexpression induces oncogenic transformation[23,24]. In these studies, oncogenic transformation appeared to be mediated by aneuploidy, and to be a consequence of Aurora kinase B overexpression in relation to genomic instability. Similarly, in the present study genomic instability was closely associated with Aurora kinase B overexpression in HCC. Future investigations should focus on the direct ability of genomic instability to progress malignant phenotypes of cancer cells, as well as on the identification of 'stem cell-ness' in the genetics[8,25].

Aurora serine/threonine kinases have recently received increasing attention as eligible targets for molecular cancer therapy[26]. Aurora kinase B inhibition results in abnormal kinetochore–microtubule attachments, failure to achieve chromosomal biorientation and failure of cytokinesis. These *in vitro* effects were greater in transformed cells than in either non-transformed or non-dividing cells[27]. Thus, targeting the Aurora kinases may achieve *in vivo* selectivity for cancer. Given their preclinical antitumour activity and potential for tumour selectivity, small-molecule pan inhibitors and even selective inhibitors of Aurora kinase B have been developed, such as MK0457/VX-680[28] and AZD1152[29]. Clinical trials of these and other Aurora kinase inhibitors are ongoing. The recent finding of an indispensable requirement for Aurora kinase B in polyploid cells[30] indicates that ploidy-specific lethality might represent the basis for a strategy to identify new cancer therapies.

This small study has identified Aurora kinase B as a predictor of the aggressive recurrence of HCC based on a genome-wide microarray study. Patients identified as being at high risk of aggressive recurrence should be followed up closely. This approach to the stratification of patients with HCC after surgical resection might provide an avenue to more rational clinical trials of postoperative adjuvant therapy using Aurora kinase inhibitors[31].

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INVITED FEATURE SECTION

Hepatocelluar nodules in liver cirrhosis: hemodynamic evaluation (angiography-assisted CT) with special reference to multi-step hepatocarcinogenesis

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Abstract

To understand the hemodynamics of hepatocellular carcinoma (HCC) is important for the precise imaging diagnosis and treatment, because there is an intense correlation between their hemodynamics and pathophysiology. Angiogenesis such as sinusoidal capillarization and unpaired arteries shows gradual increase during multi-step hepatocarcinogenesis from high-grade dysplastic nodule to classic hypervascular HCC. In accordance with this angiogenesis, the intranodular portal supply is decreased, whereas the intranodular arterial supply is first decreased during the early stage of hepatocarcinogenesis and then increased in parallel with increasing grade of malignancy of the nodules. On the other hand, the main drainage vessels of hepatocellular nodules change from hepatic veins to hepatic sinusoids and then to portal veins during multi-step hepatocarcinogenesis, mainly due to disappearance of the hepatic veins from the nodules. Therefore, in early HCC, no perinodular corona enhancement is seen on portal to equilibrium phase CT, but it is definite in hypervascular classical HCC. Corona enhancement is thicker in encapsulated HCC and thin in HCC without pseudocapsule. To understand these hemodynamic changes during multi-step hepatocarcinogenesis is important, especially for early diagnosis and treatment of HCCs.

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Key words: Hepatocellular carcinoma—Blood supply—Multi-step hepatocarcinogenesis—Early HCC—Dysplastic nodule—Liver

Hepatocellular carcinoma (HCC) is the most common primary liver cancer worldwide. Approximately 80% of Japanese HCC cases are derived from HCV-associated liver cirrhosis and chronic hepatitis, and the remaining less than 20% of the patients are HBV positive. The patients with hepatitis B or C cirrhosis are especially classified as a very high-risk group. Ultrasonography is performed every 3–4 months for the very high-risk group. Because of the introduction of this surveillance system, the size of HCCs firstly detected during 2002–2003 ($n = 33731$) was less than 2 cm in 32.5% of all cases, 2.1–5.0 cm 47.0%, respectively [1]. However, various types of hepatocellular nodules such as dysplastic nodule (DN) are also detected during screening procedures. Ultrasound and CT features of DNs and early HCCs are similar, and a precise differential diagnosis is impossible. Pathologically, human HCC develops in a multistep fashion from DN to classic hypervascular HCC. Therefore, for the early diagnosis of HCC, understanding of the concept of multi-step hepatocarcinogenesis and the sequential changes of imaging findings in accordance with multi-step hepatocarcinogenesis is important.

To understand the hemodynamics of HCC is important for the precise imaging diagnosis and treatment, because there is an intense correlation between its hemodynamic and pathophysiology. For this purpose, dynamic MDCT is most valuable because of its high

spatial and contrast resolution. However, because of the dual blood supply of the liver and intravenous injection of the contrast medium, the precise analysis of hemodynamics by conventional MDCT is often difficult. By the introduction of dynamic CT during selective arteriography, including CT during arterial portography (CTAP) [2, 3] and CT during hepatic arteriography (CTHA) [4], it has become possible to visualize the distribution of the intra-hepatic portal and arterial blood flow separately with extremely high contrast resolution, and as a result, to analyze precisely the correlation between blood supply and pathophysiology. In this article, blood flow imaging features of HCC will be discussed based on the CTAP and CTHA imaging and pathophysiologic correlations with special reference to multi-step hepatocarcinogenesis.

Classification of hepatocellular nodules and multi-step hepatocarcinogenesis

The concept of multi-step hepatocarcinogenesis and related small hepatocellular nodules in the patients with chronic liver diseases, particularly those with cirrhosis or chronic hepatitis caused by hepatitis B or C viruses, was developed mainly in Japan. However, it had not been widely accepted throughout the world and the diagnostic criteria of these nodules different even among the world specialists. However, in 2009, the International Consensus Group for Hepatocellular Neoplasia organized by the world's leading liver pathologists finally reached agreement [5].

According to this report, these nodules are divided into large regenerative nodule, low grade DN (L-DN), high-grade DN (H-DN), and HCC. In addition, small HCC (less than 2 cm) is divided into early HCC and progressed HCC. Early HCC has a vaguely nodular appearance and is well differentiated. Progressed HCC has a distinctly nodular pattern and is mostly moderately differentiated, often with evidence of microvascular invasion. L-DNs are vaguely or distinct nodular with mild increase in cell density and no cytologic atypia. H-DNs are more likely to show a vaguely nodular pattern with architectural and/or cytologic atypia, but the atypia is insufficient for a diagnosis of HCC. They show increased cell density, sometimes more than two times higher than the surrounding nontumoral liver, often with an irregular trabecular pattern. Unpaired arteries are found in most lesions, but usually not in great numbers. A nodule with largely H-DN features containing a subnodule of well-differentiated HCC can be seen. Early HCCs are vaguely nodular and are characterized by various combinations of the following major histologic features; (1) increased cell density more than two times that of the surrounding tissue, with an increased nuclear/cytoplasm ratio and irregular thin-trabecular pattern; (2) varying numbers of portal tracts within the nodule (intratumoral portal tracts); (3) pseudoglandular pattern; (4) diffuse fatty change; and (5) varying numbers of unpaired arteries. Any of the features listed above may be diffused throughout the lesion or may be restricted to an expansile subnodule (nodule-in-nodule). Most importantly, because all of these features may also be found in H-DNs, it is important to note that stromal invasion remains most helpful in differentiating early HCC from H-DNs. However, the application of these criteria is challenging because most histologic criteria are arrayed on a gradual spectrum and cannot be easily summarized as present or absent.

Because of these reasons as described above, it should be realized that there must be various degree of overlaps among imaging features of these nodules and they may show gradual changes during multi-step hepatocarcinogenesis.

Angiogenesis during multi-step hepatocarcinogenesis

Vascular endothelial growth factor (VEGF) is known to play a critical role in the neovascularization in the development and progression of malignant neoplasms [6, 7]. VEGF is produced by tumor cells, and its binding with VEGF receptors such as Flt-1 and Flk-1, which are expressed on vascular endothelial cells, leads to the proliferation and migration of endothelial cells. In addition, VEGF receptors expressed on tumor cells are involved in tumor proliferation in an autocrine loop via interaction with VEGF produced by the tumor cells themselves [7]. Park et al. [8] reported that the expression of VEGF was correlated with angiogenesis and cell proliferation in hepatocarcinogenesis. On the other hand, tumors often encounter hypoxic conditions during their growth. Under such conditions, hypoxia inducible factor-1α (HIF-1α) promotes the transcriptional activity of angiogenesis-related molecules such as VEGF and erythropoietin by affecting the hypoxia response element and HIF-1α located in nuclei [7].

We analyzed these changes of the angiogenesis during multi-step hepatocarcinogenesis by immunohistochemical and molecular studies [7]. According to our analysis, it was found that hepatocellular areas around the portal tracts in DNs, including those with sinusoidal capillarization and unpaired arteries, were strongly positive for HIF-1α, whereas this molecule was faintly expressed in the surrounding livers. Cytoplasmic overexpression and intranuclear expression of HIF-1α, a more increased expression pattern, were also observed in HCC, suggesting that cytoplasmic HIF-1α might have been moved into the nuclei in activated HCC cells. HIF-1α is involved in the upregulation of genes harboring the hypoxia response element such as VEGF, suggesting that increased expression of HIF-1α in the areas around the portal tracts of DNs may be responsible for increased expression of