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IV. 研究成果の刊行物・別刷

Gene-expression phenotypes for vascular invasiveness of hepatocellular carcinomas

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Background. Gross vascular invasion is a well-established prognostic indicator in hepatocellular carcinoma (HCC), but the biological significance of microscopic invasion remains unclear.
Methods. Curatively resected primary HCCs were classified retrospectively into 3 groups: HCCs without vascular invasion (V0), HCCs with microvascular invasion (V1), and HCCs with macrovascular invasion (V2). Microarray profiling of patients with V0, V1, and V2 using Jonckheere-Terpstra (JT) tests and Wilcoxon rank sum tests was performed.
Results. Distinct patterns of gene expression were demonstrated between V0 and V2 groups; less (L) and highly (H) invasive phenotypes, respectively. It is noteworthy that 2 dendrograms by the hierarchical clustering provided exactly the same assignment results for V1 cases that were thus separated into L and H gene-expression phenotypes. Marked differences were found in overall (P < .001) and tumor-free survival (P < .001) between L and H gene-expression phenotypes. Multivariate analyses indicated that the phenotypes of the patterns of gene expression, rather than the clinicopathologic markers of vascular invasion, were independent predictors of tumor recurrence (P = .031). Using the gene-expression patterns identified by both JT and Wilcoxon rank sum test analyses, other V1 cases validated these differences in tumor-free survivals between gene-expression phenotypes within the group (P = .039).
Conclusion. Gene profiling suggested that microvascular invasiveness consisted of a classable mixture of 2 distinct phenotypes. Thus, gene-array analyses may have clinical benefit, because they may in fact be more predictive than other clinical factors. (Surgery 2010;147:405-14.)

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HEPATOCELLULAR CARCINOMAS (HCCs) are common malignancies, and their prevalence is increasing on a worldwide basis.[1,2] Although operative resection is an effective treatment modality for HCC, the recurrence of these aggressive neoplasms is frequent even after an apparent curative resection.[2,3] The long-term outcome is affected mainly by metastatic recurrences.[4] Because malignant vascular invasion is regarded as the direct etiology of metastatic recurrences, gross and radiologic evidence suggests that tumor invasion in major veins is a known determinant of poor outcomes after resection of HCC.[5,6] In contrast to visually evident macrovascular invasion in major portal or hepatic veins, microvascular invasion, which is defined as microscopic tumor invasion into smaller intrahepatic vessels as identified on histopathologic analysis, is difficult to determine in preoperative assessments.[7,8] Although macroscopic vascular invasion is an established prognostic indicator and represents a variable in the pathologic staging of HCCs,[8] the biologic significance of microscopic vascular invasion in subjects with resectable HCCs remains obscure.

Genome-wide gene-expression analyses by microarrays offer a potentially important, systematic approach to unfold comprehensive information regarding (gene) transcription profiles.[9]

Furthermore, such studies may lead potentially to the development of novel, molecular-targeting therapies in HCCs.[10] We analyzed previously HCC gene-expression profiles[11] and identified a novel molecule as a therapeutic target. Our current study seeks to identify signatures associated with vascular invasion in HCC from genome-wide expression profiles of HCC as generated from microarray studies. The clinicopathologic features and candidate molecular pathogeneses were evaluated in subjects with HCC with a special emphasis on the significance of tumor vascular invasion.

SUBJECTS AND METHODS

Subjects and tissue samples. All 188 subjects had curative hepatectomies to treat HCC from 2001 to 2006 at the Tokyo Medical and Dental University Hospital. Written informed consent was obtained from each subject, and study procedures were approved by the Institutional Review Board. Pre-operative evaluations, including liver function tests and operative procedures, are described elsewhere.[2,3,11] Pre-operative imaging for tumor staging included abdominal ultrasonography, computed tomography (CT), hepatic arteriography, indirect arterial portography, CT-arteriography, CT-arterial portography, and magnetic resonance imaging (MRI). Radiologic vascular invasion indicated by the presence of tumor thrombi in major branches of portal or hepatic veins was evaluated comprehensively. Intra-operative ultrasonography was performed on all subjects. Resected tissues lacking necrosis were divided into 2 specimens immediately after operation; 1 specimen was snap frozen in liquid nitrogen and stored at –80°C for microarray analyses, and the other was fixed in 10% formaldehyde solution and embedded in paraffin for histopathologic analysis by a pathologist.

The evidence of portal/hepatic vein tumor invasion was evaluated both macroscopically and microscopically. Gross examinations of resected specimens confirmed the preoperative diagnosis of macroscopic invasion in all examined cases. On histopathologic examinations of resected specimens, microscopic invasion indicated the presence of clusters of cancer cells floating in the vascular space. Radiologically, macroscopic invasion was detected in 45 cases (V2); 47 cases were diagnosed as microscopic vascular invasion on histopathologic analyses (V1), and the remaining 96 cases had no vascular invasion by either macroscopic or microscopic examinations (V0). Subjects were assayed for serum levels of alpha-fetoprotein

(AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II) every month and with ultrasonography, CT, and MRI every 3 months. When tumor recurrence was suspected, diagnostic imaging was performed using a CT-angiography. Finally, the diagnosis of a recurrence was made via imaging. The mean observation time was 3.8 years.

RNA isolation, complementary RNA (cRNA) preparation, and microarray hybridization. Total RNA was extracted from HCC specimens using an RNeasy kit (Qiagen, Hilden, Germany). The integrity of the obtained RNA was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). All samples had an RNA Integrity Number greater than 5.0. Contaminant DNA was removed by digestion with RNase-free DNase (Qiagen). Using 2 μg total RNA, cRNA was prepared using 1-cycle target labeling and control reagents kit (Affymetrix, Santa Clara, CA). Hybridization and signal detection of HG-U133 Plus 2.0 arrays (Affymetrix) were performed as per the manufacturer's instructions.

Normalization and statistical analyses of microarray data. In the 188 cases, 59 were selected randomly for test analyses (V0 = 29 cases, V1 = 14 cases, and V2 = 16 cases), and another 129 cases were evaluated for validation analyses (V0 = 67 cases, V1 = 33 cases, and V2 = 29 cases). Statistical differences were not identified when clinicopathologic factors were analyzed between test and validation cases. Microarray data sets were normalized using a robust multiarray average method under R 2.4.1 statistical software together with a Bioconductor package, as previously described.[11] Estimated gene-expression levels were $\log_2$-transformed, and 62 control probe sets were removed for subsequent analyses. For each of the 54,613 probes on the HG-U133 Plus 2.0 array, fold-change (FC) values were calculated using ratios of geometric means of gene-expression levels between subtypes, and genes with an FC difference greater than 1.2-fold were selected for evaluation, according to a previous report.[12] To identify the genes associated with the development of vascular invasion in V0, V1, and V2 cases, we applied Jonckheere-Terpstra (JT) tests, which are nonparametric tests for monotone trends in terms of ordinary classes.[12] In addition, Wilcoxon rank sum tests were performed to estimate the significance levels of gene-expression differences between V0 and V2 groups for each of the 54,613 probe sets. For each statistical test, obtained P values from multiple hypothetical tests were adjusted by a false discovery rate (FDR), and probe sets with a FDR <5% were considered for subsequent analysis.
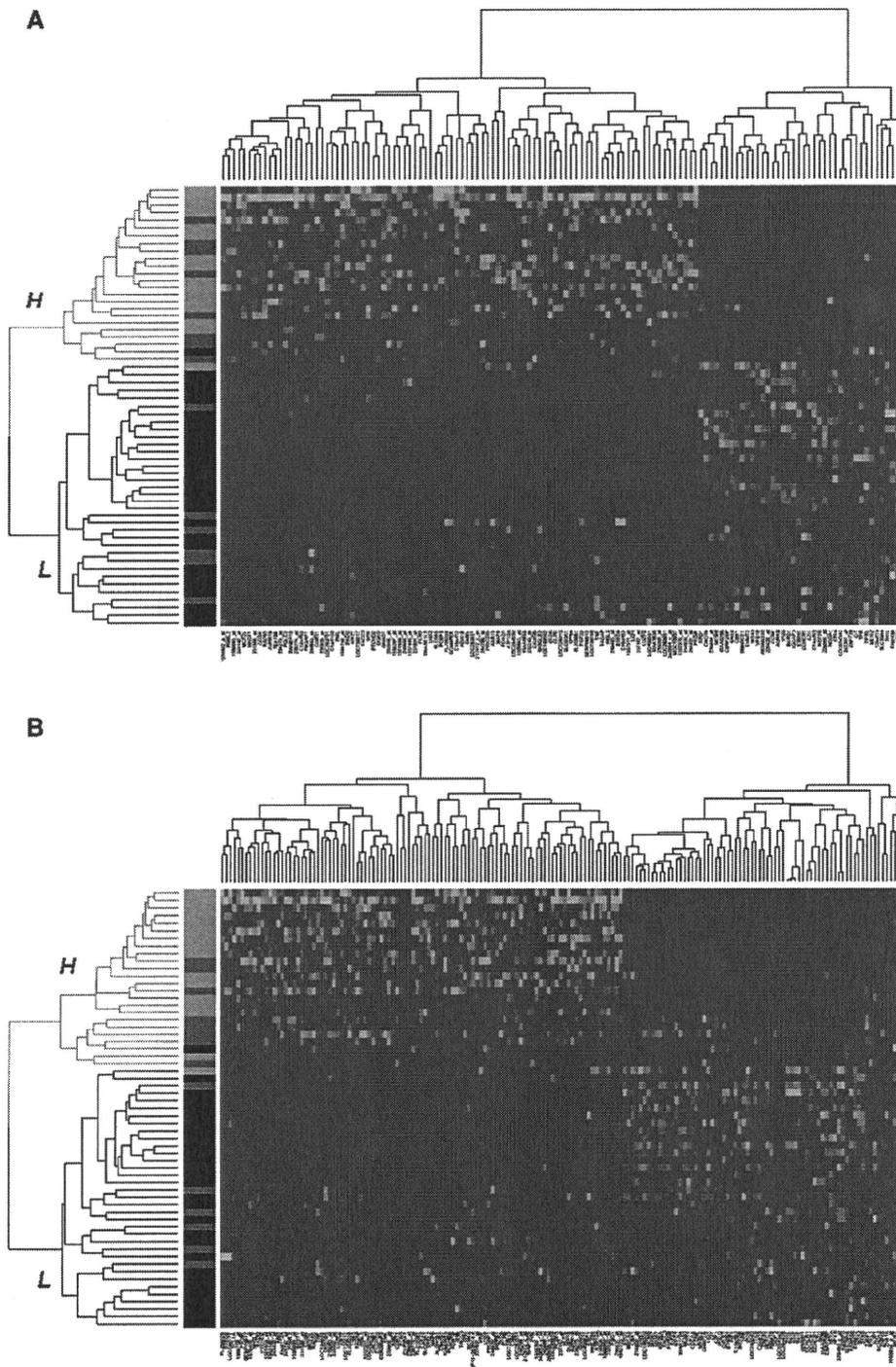
Fig 1. Hierarchical clustering of gene-expression profiles on 59 HCC subjects; 29 with no vascular invasion (V0: *black bar*), 14 with microvascular invasion (V1: *blue bar*), and 16 with macrovascular invasion (V2: *orange bar*). Dendrograms shows the classification determined by hierarchical clustering analyses; less (L) and highly (H) invasive phenotypes. Red and green colors indicate relative overexpression and underexpression, respectively. (*A*) Hierarchical clustering with 132 probe sets, which showed monotone trends with the development of vascular invasion in V0, V1, and V2 cases (JT tests; Supplementary Table I). (*B*) Hierarchical clustering using 172 probe sets, which were differentially expressed in V0 and V2 cases (Wilcoxon rank sum test; Supplementary Table II).

Table I. Comparison of the clinicopathologic factors between gene-expression phenotypes

Variables	L phenotype (n = 37)	H phenotype (n = 22)	P value
Age (>65 years)	22:15	12:10	.712
Sex (male:female)	27:10	17:5	.714
Hepatitis virus (HBV:HCV:None)	9:15:13	7:11:4	.379
Child-Pugh (A:B:C)	35:2:0	21:1:0	.999
ICG-R15	18.3 ± 2.3	19.2 ± 2.6	.804
Number of neoplasms	1.3 ± 0.1	1.6 ± 0.2	.099
Size of neoplasm	3.6 ± 0.4	6.2 ± 1.2	.022
Histologic differentiation (well:mod/poor)	12:25	0:22	.002
Growth pattern (expansive:invasive)	36:1	20:2	.549
Capsular formation (+:−)	27:10	17:5	.714
Capsular invasion (+:−)	19:18	16:6	.106
Biliary invasion (+:−)	1:36	2:20	.549
Systematized hepatectomy (+:−)	29:8	20:2	.295
AFP (>100 mAU/mL)	10:27	15:7	.002
PIVKA-II (>300 mAU/mL)	12:25	13:9	.045

Hierarchical clustering with selected genes was performed on R software using the Pearson correlation coefficient as a similarity index and a complete linkage method for agglomeration. For visualization, expression intensities were standardized by z scores (mean = 0 and variance = 1) for each probe set. Biomolecular network interaction analyses were performed using databases such as BIND and KEGG, as described previously.[12]

Immunohistochemical analyses. To validate expression patterns detected by microarrays, immunohistochemical studies were performed on 89 HCC samples. Tissue sections were stained using anti-AURKB antibodies (ab2254; Novus Biologicals Inc., Littleton, CO) at 1:50 dilutions with phosphate buffered solution (PBS) containing 1% bovine serum albumin (Sigma, St. Louis, MO),[11] followed by reactions in an automated immunostainer (Ventana XT System; Ventana, Tucson, AZ) using heat-induced epitope retrieval and a standard DAB detection kit (Ventana). Strong, diffuse, nuclear staining in >10% of cells was considered a positive result. The immunohistochemical staining was evaluated under a light microscope by 2 independent investigators.

Statistical analyses. Statistical comparisons of clinicopathologic characteristics for significance were made using Chi-square tests or the Fisher exact tests with a single degree of freedom, and Student t tests were used to analyze differences between continuous values. Cumulative survival and recurrence rates were determined using the Kaplan-Meier method, and for comparisons, we used log-rank tests. P values of <.05 were considered statistically significant.

RESULTS

Gene-expression analyses of vascular invasiveness. The molecular properties of cases with vascular invasion were analyzed in 59 test cases composed of V0 (n = 29), V1 (n = 14), and V2 (n = 16). Microarray analyses of changes in gene expression among V1, V1, and V2 groups revealed that 132 probe sets showed strong monotone trends with the development of vascular invasion at FDR <5% (P < .0001) by JT tests (see Supplementary Table I in the online version at doi: 10.1016/j.surg.2007.09.037, published on the electric version only). Similarly, by evaluating changes in gene expression between V0 and V2 groups, 172 probe sets that satisfied FDR <5% (P < .0001) with Wilcoxon rank sum tests were identified as differently expressed genes (see Supplementary Table II in the online version at doi: 10.1016/j.surg.2007.09.037).

Hierarchical clustering using selected probe sets are shown in Fig 1, A and (JT tests) B (Wilcoxon rank sum tests). Both analyses revealed that all but 1 of the V0 and V2 cases were divided clearly into 2 major clusters: A less (L) invasive phenotype (L) and a highly (H) invasive phenotype of gene expression. No cluster was evident within the V1 cases analyzed. It is noteworthy that the 2 dendrograms identified by the hierarchical clustering provided exactly the same assignment results for 14 V1 cases that were thus classified into the following 2 subtypes (Fig 1, A and B): 6 V1-cases located in the middle of a V0 cluster were denoted as the L phenotype, and the remaining 8 V1-cases located in the V2 cluster were denoted as the H phenotype.
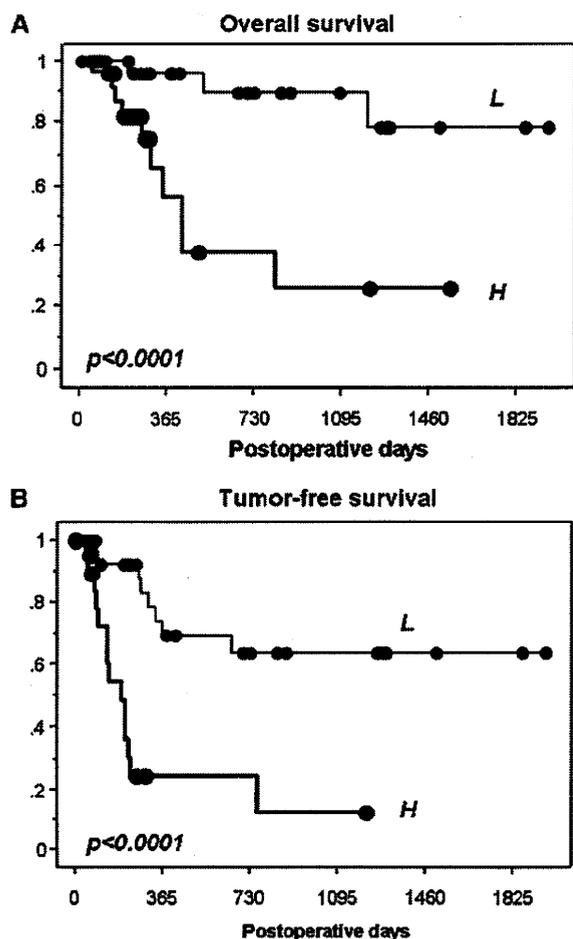
A

B

Fig 2. Cumulative overall survival curves (A) and tumor-free survival curves (B) of 59 subjects with HCC classified into the L phenotype group (thin; n = 37) and the H phenotype group (thick; n = 22) after curative resection (P < .001).

Table II. Univariate risk factors of HCC recurrence

Variables of primary HCC	Number of cases	Recurrence rate/year*	P value*
Gene-expression phenotype			<.001
L phenotype	37	0.259	
H phenotype	22	0.762	
Vascular invasion			<.001
V0 (none)	30	0.407	
V1 (microvascular invasion)	14	0.425	
V2 (macrovascular invasion)	15	0.885	
Number of neoplasms			.036
<3	50	0.414	
>3	9	0.8	
Size of neoplasm			.038
<4 cm	31	0.293	
>4 cm	28	0.74	
Histologic differentiation			.027
Well	13	0	
Moderate/poor	46	0.598	
PIVKA-II			.030
<300 mAU/mL	33	0.272	
>300 mAU/mL	26	0.721	

*Recurrence rates and P values were determined using the Kaplan-Meier method and the log-rank test.

Clinicopathologic analyses of gene-expression phenotypes. To determine differences between L and H gene-expression phenotypes, clinicopathologic analyses were performed on 59 test cases (Table I). Statistical differences were found in tumor size (P = .023), histologic differentiation (P = .002), and pre-operative tumor makers, including either AFP (P = .002) or PIVKA-II (P = .045). In total, there were differences of overall survival (P < .001) and tumor-free survival (P < .001) between L phenotypes (37 cases) and H phenotypes (22 cases; Fig 2). Statistical risk factors for HCC recurrence were determined by univariate analyses; the gene-expression phenotype (P < .001) as well as the clinicopathologic vascular invasion (P < .001), number of neoplasms (P = .036), size of neoplasm (P = .038), histologic differentiation (P =

.027), and level of PIVKA-II (P = .030) (Table II). Multivariate analysis for recurrence indicated L-H gene-expression phenotypes (P = .031) and level of PIVKA-II (P = .048) as independent (risk) factors but not macrovascular invasion (P = .124) or microvascular invasion per se (P = .274; Table III).

Validation analyses of gene-expression phenotypes. The phenotypes of vascular invasion of patients with HCC according to the pattern of gene expression were identified with 132 and 172 probe sets using JT tests (Fig 1, A, Supplementary Table I) and Wilcoxon rank sum tests (Fig 1, B, Supplementary Table II). Among these probe sets, 93 genes were identified commonly by both JT and Wilcoxon tests (see Supplementary Table III in the online version at doi: 10.1016/j.surg.2007.09.037). To validate the phenotypes of gene expression, an additional 129 HCC cases composed of V0 (n = 67), V1 (n = 33), and V2 (n = 29) were analyzed for hierarchical clustering using the 93 genes identified by 2 test analyses. As shown in Fig 3, A, no cluster was evident when V1 cases were analyzed. Two dendrograms by the hierarchical clustering provided similar assignment results for 33 V1 cases that were separated into L phenotypes (18 cases) and H

Table III. Results of Cox multivariate analysis on risk factors of HCC recurrence

Variables of primary HCC	Coefficient	Odds ratio	95% confidence interval	P value
Gene-expression phenotype	1.685	5.390	1.167–24.905	.031
Macrovascular invasion	1.033	2.809	0.755–10.455	.124
Microvascular invasion	0.822	2.274	0.521–9.919	.274
Number of neoplasms	0.436	1.546	0.436–5.483	.500
Size of neoplasm	0.390	1.477	0.519–4.201	.465
Histologic differentiation	1.238	3.450	0.400–29.777	.260
Serum level of PIVKA-II	1.009	2.744	1.007–7.476	.048

phenotypes (15 cases). Indeed, tumor recurrence in V1 subjects demonstrated significant differences between 2 gene-expression phenotypes. As shown in Fig 3, *B*, tumor-free survivals of L phenotypes were better compared with those of H phenotypes within the V1 cases (P = .039). Gene profiling revealed the microvascular invasiveness consisted of a classable mixture of L and H phenotypes, substantially affecting prognoses.

Among the 93 commonly identified genes, 28 genes were upregulated in the H phenotype (Table IV). Several cytokinesis-related genes were found, including aurora kinase B (AURKB), translocated promoter region, centromere protein I (CENPI), chromobox homolog 1 (CBX1), stathmin 1/oncoprotein 18 (STMN1), nuclear transcription factor Y alpha (NFYA), citron/rho-interacting, serine/threonine kinase 21 (CIT), cyclin F (CCNF), importin 9 (IPO9), metastasis associated 1 family member 3 (MTA3), exportin 1 (XPO1), and minichromosome maintenance 8 (MCM8). Interestingly, STMN1 is a direct substrate of AURKB,[13] and other molecules such as CENPI, CBX1, and CIT are regulated indirectly by AURKB.[14-16] According to biomolecular interaction network analyses using these cytokinesis-related genes, the AURKB pathway seems to be activated in H phenotypes with HCC vascular invasion (Fig 4, *A*).

AURKB protein overexpressed in HCC tissues in association with gene-expression phenotypes. To validate the expression of AURKB detected by microarrays, immunohistochemical studies were performed on 89 HCC cases composed of 58 L-phenotypes and 31 H-phenotypes using anti-AURKB antibodies (ab2254; Novus Biologicals Inc.) at 1:50 dilutions with PBS containing 1% bovine serum albumin (Sigma), followed by reactions in an automated immunostainer Ventana XT System. Strong, diffuse nuclear staining in AURKB was observed only in HCC tumor tissues but not in the adjacent normal tissues of the liver (Fig 4, *B*). AURKB protein was detected in 32 of 89 cases

(36%). The microarray intensity of the AURKB gene was greater in the 32 cases compared with the remaining 57 cases (FC = 2.4; P = .001). The results of immunohistochemical analyses confirmed microarray findings at the level of protein expression. The overexpression of AURKB protein was recognized in 24 of 31 H-phenotypes but only in 8 of 58 L-phenotypes in total (P < .001). Within 22 V1 cases, 8 of 12 H-phenotypes but only 1 of 10 L-phenotypes were positive for AURKB protein (P = .002).

DISCUSSION

Vascular invasiveness is a major factor that reflects the biologically aggressive phenotype in HCC.[4-8] Because various molecules seems to regulate the macrovascular invasiveness in HCC,[17-21] it may be possible to determinate biologic significances of microvascular invasion (V1) and to distinguish microvascular from macrovascular invasion (V2). In this study, we analyzed genome-wide gene expression using microarray profiling (Fig 1). As shown by hierarchical clustering, V0 and V2 cases were clearly divided into 2 major clusters (L and H) based on JT or Wilcoxon analyses; however, the V1 cases with microvascular invasion did not form a cluster (Fig 1, *A* and *B*). It is noteworthy that both hierarchical clustering analyses provided exactly the same assignment results for V1 subjects. In total, V0, V1, and V2 cases were classified into 2 dendrograms, L (37 cases) and H (22 cases) phenotypes. The prognosis was different between L and H phenotypes in overall survival (P < .001) and tumor-free survival (P < .001; Fig 2). Furthermore, a multivariate analysis for the tumor recurrence indicated that the L-H gene phenotypes of gene expression (P = .031) were an independent (risk) factor (Table III) but not the V0-V1-V2 clinicopathologic vascular invasion per se, which was identified only by univariate analyses (Table II).

Histologic V1 cases may consist of 2 components, namely, L and H phenotypes. Using gene-

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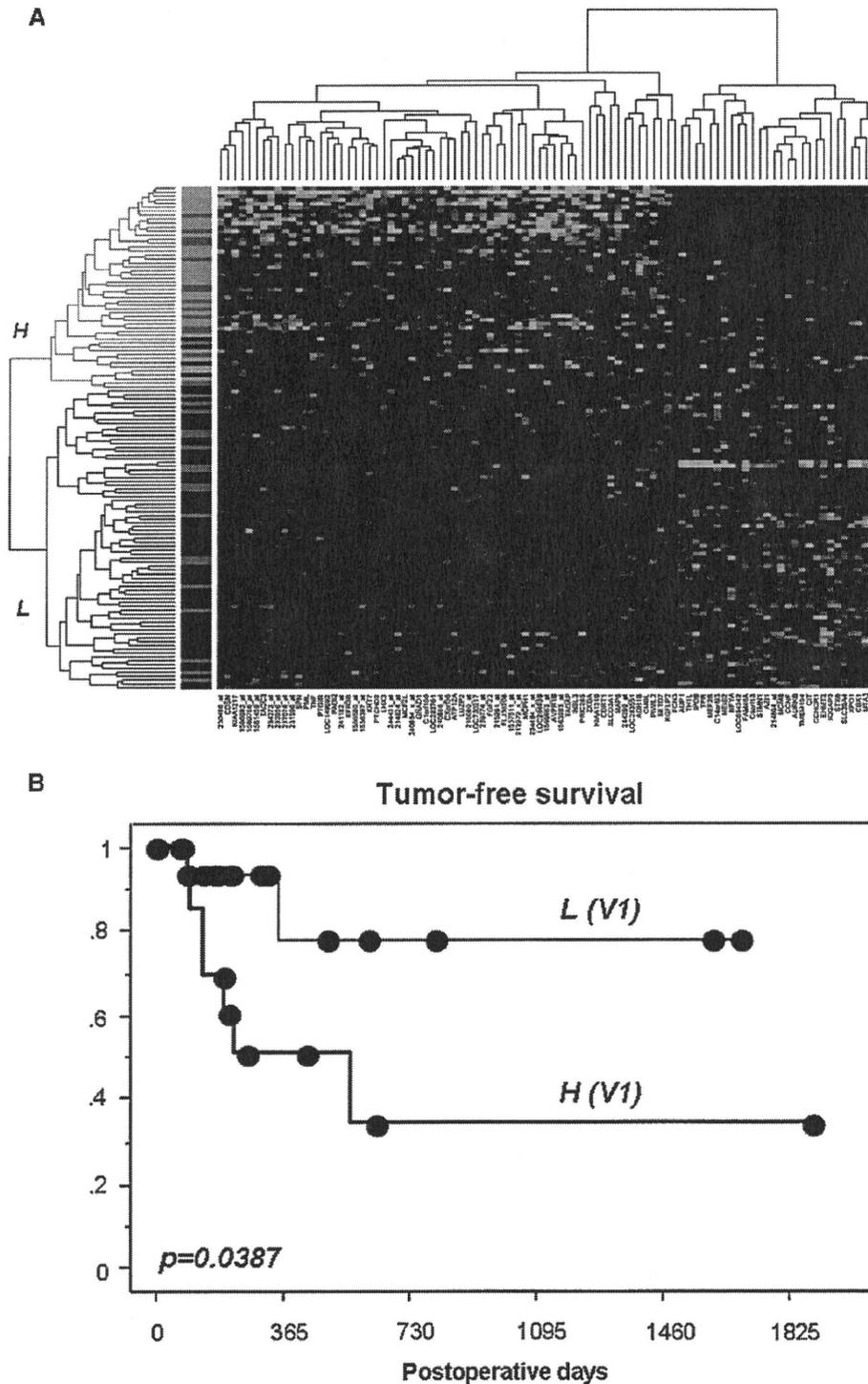
Fig 3. Validation analyses of gene-expression phenotypes using the 93 probe sets in additional 129 cases with HCC composed of 67 V0 cases (*black bar*), 33 V1 cases (*blue bar*), and 29 V2 cases (*orange bar*). (*A*) Hierarchical clustering of gene-expression profiles. Dendrograms show the classification determined by hierarchical clustering analyses: less (L) and highly (H) invasive phenotypes. Red and green colors indicate relative overexpression and underexpression, respectively. Two dendrograms by the hierarchical clustering provided similar assignment results for the 33 V1 cases that separated into the L phenotype (18 cases) and H phenotype (15 cases). (*B*) Tumor-free survival curves of 33 V1 cases with HCC classified into the L phenotype group (*thin*) and the H phenotype group (*thick*) after curative resection (*P* = .039).

Table IV. Gene lists commonly upregulated in the H phenotype by both JT and Wilcoxon rank sum tests

Probe set ID	Gene symbol	Gene title	JT P value	JT FDR	Wilcoxon P value	Wilcoxon FDR	Fold change
209464_at	AURKB	Aurora kinase B	1.07E-04	0.050	3.55E-05	0.035	2.36
214742_at	AZI1	5-azacytidine induced 1	1.20E-04	0.050	7.52E-05	0.043	1.46
242133_s_at	LOC654342	Similar to lymphocyte-specific protein 1	7.96E-05	0.049	1.21E-04	0.048	2.58
201730_s_at	TPR	Translocated promoter region (to activated MET oncogene)	7.50E-05	0.049	5.88E-05	0.038	1.86
214804_at	CENPI	Centromere protein I	8.96E-05	0.049	9.56E-05	0.045	1.69
201518_at	CBX1	Chromobox homolog 1 (HP1 beta homolog Drosophila)	9.51E-05	0.049	1.36E-04	0.049	1.68
42361_g_at	CCHCR1	Coiled-coil alpha-helical rod protein 1	8.45E-05	0.049	1.40E-06	0.010	1.57
217714_x_at	STMN1	Stathmin 1/oncoprotein 18	7.73E-05	0.049	6.65E-05	0.040	1.52
220525_s_at	AUP1	Ancient ubiquitous protein 1	8.45E-05	0.049	1.36E-04	0.049	1.33
229538_s_at	IQGAP3	IQ motif containing GTPase activating protein 3	6.27E-05	0.048	1.52E-04	0.049	2.24
215720_s_at	NFYA	Nuclear transcription factor Y, alpha	5.39E-05	0.046	1.83E-05	0.029	1.41
225865_x_at	TH1L	TH1-like (Drosophila)	5.23E-05	0.045	1.04E-05	0.020	1.54
219215_s_at	SLC39A4	Solute carrier family 39 (zinc transporter), member 4	4.09E-05	0.042	3.55E-05	0.035	4.42
207480_s_at	MEIS2	Meis homeobox 2	4.09E-05	0.042	3.11E-05	0.035	2.28
212801_at	CIT	Citron (rho-interacting, serine/threonine kinase 21)	2.19E-05	0.042	1.95E-06	0.010	2.26
212621_at	KIAA0286	KIAA0286 protein	4.09E-05	0.042	5.19E-05	0.038	1.99
226330_s_at	FAM48A	Family with sequence similarity 48, member A	2.82E-05	0.042	2.39E-05	0.034	1.83
204826_at	CCNF	Cyclin F	3.40E-05	0.042	2.32E-07	0.010	1.72
217885_at	IPO9	Importin 9	3.40E-05	0.042	5.88E-05	0.038	1.64
223311_s_at	MTA3	Metastasis associated 1 family, member 3	2.65E-05	0.042	9.56E-05	0.045	1.61
228544_s_at	CSRP2BP	CSRP2 binding protein	3.40E-05	0.042	1.49E-04	0.049	1.57
208775_at	XPO1	Exportin 1 (CRM1 homolog, yeast)	3.73E-05	0.042	1.52E-04	0.049	1.44
219009_at	C14orf93	Chromosome 14 open reading frame 93	3.73E-05	0.042	8.05E-06	0.018	1.43
230424_at	C5orf13	Chromosome 5 open reading frame 13	1.31E-05	0.034	5.82E-06	0.016	2.56
202326_at	EHMT2	Euchromatic histone-lysine N-methyltransferase 2	7.73E-06	0.034	5.88E-05	0.038	1.80
214441_at	STX6	Syntaxin 6	8.82E-06	0.034	2.73E-05	0.034	1.24
224320_s_at	MCM8	Minichromosome maintenance complex component 8	2.26E-06	0.034	4.30E-06	0.016	2.34
1553978_at	MEF2B	Myocyte enhancer factor 2B	6.32E-06	0.034	1.65E-06	0.010	1.49

expression patterns identified by both JT and Wilcoxon rank sum tests, additional analyses of hierarchical clustering on different cases validated that the other V1 subjects separated into 2 expression phenotypes as expected (Fig 3, *A*). Although 5 V0 cases were located in the H area, the number was too small to show a statistical difference in survival compared with the other V0 cases. It is noteworthy that there were differences in tumor recurrence in V1 subjects between the 2 gene-expression phenotypes of L and H (Fig 3, *B*). These novel findings may question whether operative resection or liver transplantation is the best therapeutic option for some of these HCCs.[22] Gross macrovascular invasion is a well-known independent predictor of tumor

recurrence after liver transplantations[23,24]; however, the importance of microvascular invasion remains controversial. Several recent reports suggested that microvascular invasion did not seem to be an independent factor for survival.[25,26] Furthermore, the Barcelona Group proposed salvage transplantation even in the absence of proven residual disease in subjects after hepatic resection when pathologic microvascular invasion was noted.[27] According to their results for 5 transplanted cases, 1 subject presented extrahepatic dissemination early after the transplantation and died 4 months later. Together with our microarray profiling, the H gene phenotype in a patient with microvascular invasion could not be overcome by liver transplantation.

A

B
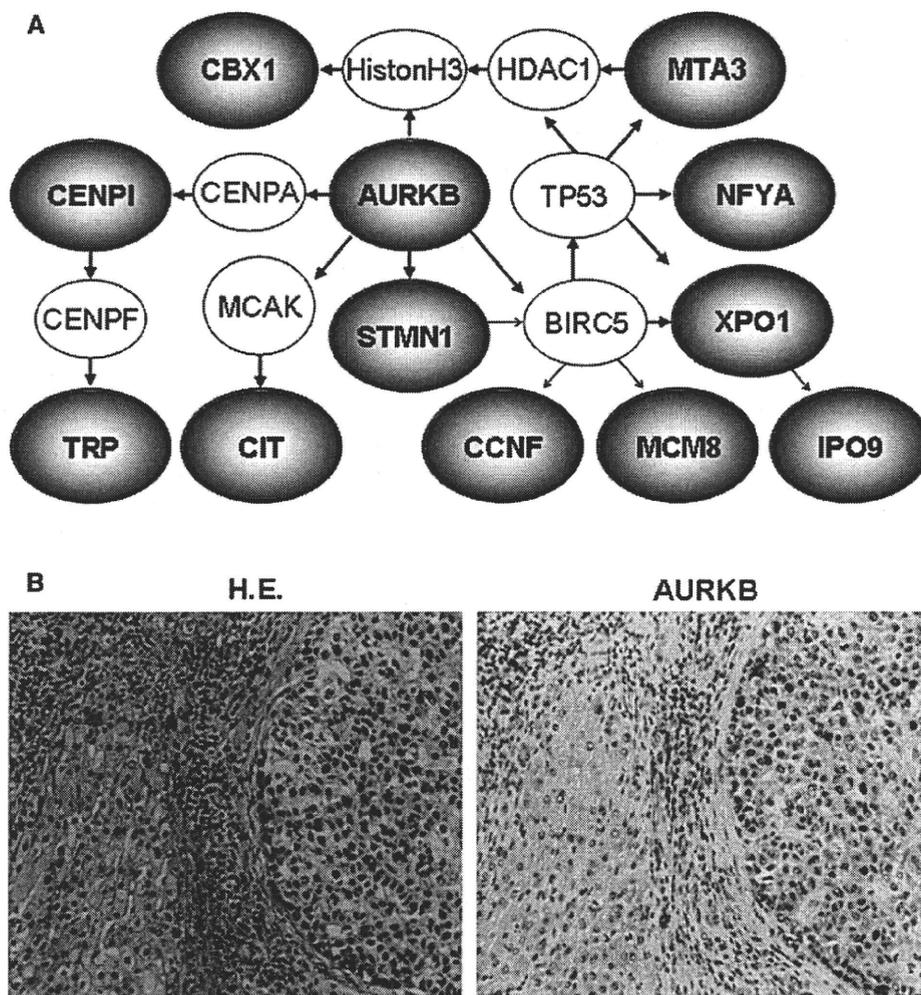
Fig 4. Network pathway and immnodetection in HCC. (*A*) Biomolecular interaction network analyses of commonly up-regulated genes by both JT and Wilcoxon rank sum tests of complementary DNA (cDNA) microarray data. The AURKB related pathway is composed of 12 upregulated genes (*red circles*). *Bold arrows* indicate direct protein–protein interactions, and *thin arrows* indicate indirect interactions. (*B*) Immunohistochemical analyses of AURKB in HCC tissues (magnification; × 100). AURKB protein in cancer cell nuclei, but not in adjacent noncancerous hepatocytes (left panel). Hematoxylin-eosin staining (H.E.; right panel).

Biomolecular network interaction analyses on criteria of H phenotypes revealed that vascular invasion may be associated with the upregulation of the AURKB-related pathway (Fig 4, *A*) and protein overexpression of AURKB was confirmed by immunohistochemistry (Fig 4, *B*). AURKB is a chromosomal passenger serine/threonine protein kinase that regulates accurate chromosomal segregation, cytokinesis, and protein localization to the centromere and kinetochore, corrects microtubule-kinetochore attachments, and regulates the mitotic checkpoint.[28] Our previous studies on HCC recurrences revealed that AURKB expression is associated closely with genetic instability in HCC.[11] Pathway genes such as CCNF and

MCM8 also relate to genetic instability.[29,30] More importantly, AURKBs have received recently increasing attention as eligible targets in molecular cancer therapies.[31,32] An AURKB-targeted therapy might be a promising, neoadjuvant approach for occult vascular invasion in HCC.

In conclusion, gene-expression profiling revealed that neoplasms containing microvascular invasion are composed of classable mixtures of less invasive and highly invasive phenotypes. Randomized trials as well as longer observation times are required to prove critical impacts of oncologic profiling. Our findings suggest that attention should be directed at the heterogeneity of HCC with microvascular invasion when considering

hepatic resection and liver transplantation. There should be additional analyses on specific gene-expression profiles that associate with an increased risk of recurrence and poor survival.

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Current status of molecularly targeted therapy for hepatocellular carcinoma: basic science

Shinji Tanaka · Shigeki Arii

Abstract Conventional systemic chemotherapy has been developed with so-called anti-cancer agents, essentially screened for cytotoxicity to cultured cancer cells. However, in patients with hepatocellular carcinoma (HCC), the role of chemotherapy is quite limited because most anti-cancer agents are ineffective and relatively toxic to HCC patients with chronic liver diseases. On the other hand, accumulated understanding of the molecular mechanisms regulating cancer progression has led to novel development of molecularly targeted therapies with cytostatic agents. Recently, a phase III clinical trial revealed a multi-kinase inhibitor, Sorafenib, as the first agent leading to improved overall survival of patients with advanced HCC. A new era of HCC treatment has arrived, based on identification of deranged signaling pathways of cancer cells or their microenvironment. This review summarizes the molecular hallmarks of HCC with a focus on angiogenesis, growth signals, and mitotic stress, and a novel concept "synthetic lethality" for the targeted therapy strategy.

Keywords Angiogenesis · Growth signals · Mitotic stress · Aurora · Synthetic lethality

Introduction

A decade ago, Hanahan and Weinberg [1] proposed six hallmarks of cancer that collectively promote survival and proliferation in foreign environments (Fig. 1). The hallmarks consist of self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, tissue invasion and metastasis, and sustained angiogenesis. Subsequently, additional hallmarks, "evading immune surveillance" and five "stress phenotypes", were expanded by Kroemer et al. [2] and Elledge et al. [3], respectively. A total of twelve hallmarks have been suggested as oncogene and non-oncogene addictions of cancer cells [3, 4] (Fig. 1). More important, these addictive hallmarks might be inextricably linked to molecular targets for cancer treatment, as the Achilles "heals" of cancer cells [5]. Numerous studies on molecular abnormalities in hepatocellular carcinoma (HCC) progression have revealed the crucial roles of such hallmarks in the pathogenesis [6]. Among these, this review focuses on three hallmarks of HCC; "sustained angiogenesis", "self-sufficiency in growth signals", and "mitotic stress", in respect of substantially and/or potentially targeted therapy for HCC [7, 8].

Sustained angiogenesis

In-vivo tumor progression requires the supply of oxygen and nutrition by the vasculature [9]. HCC is a typical angiogenic tumor, because dramatic alteration in the arterial vascularity is usually recognized in moderately-to-poorly differentiated types of HCC, leading to acquisition of the potential for vascular invasiveness and metastasis [10]. Blood vessels consist of endothelial cells and the externally shielding pericytes. Vascular endothelial growth factor (VEGF) is one of the potent factors stimulating the growth and migration of the endothelial cells [11]. We first found a close relationship between VEGF expression and

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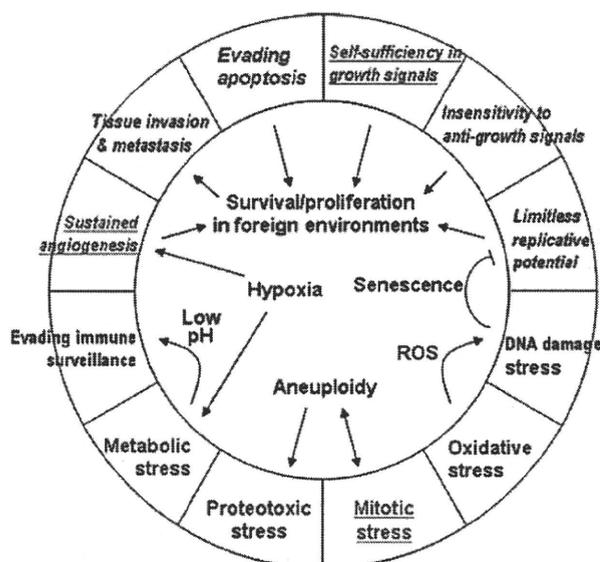
Fig. 1 Twelve hallmarks of cancer [3]. The top six hallmarks (*italics*) were originally proposed by Hanahan and Weinberg in 2000 [1]. The additional hallmark "evading immune surveillance" (*left*) and five "stress phenotypes" (*bottom*), were expanded by Kroemer et al. [2] and Elledge et al. [3], respectively. Three hallmarks "sustained angiogenesis", "self-sufficiency in growth signals", and "mitotic stress" are the focus of this review (*underlined*)

HCC progression in a clinical specimen [12]. It is of interest that the receptors VEGFR1/Flt-1 and VEGFR2/KDR/Flk-1 were recognized not only on vascular endothelial cells but also on human HCC cells [13]. According to basic studies on murine tumor models, VEGFR1-expressing hematopoietic cells might play critical roles in formation of the premetastatic niche [14, 15]. Thus, the VEGF signaling might function not only as angiogenesis but also as cancer invasion and metastasis of HCC [16].

VEGF belongs to a family of structurally and functionally related growth factors including the platelet-derived growth factors (PDGFs) that broadly stimulate various phenotypes of cells including vascular pericytes, the other components of the vasculature [17]. In drosophila, PEGF/VEGF-like factors share a single receptor [17]. Additionally, dual inhibition of VEGF and PDGF signals was revealed to have more effective anti-angiogenic effects in in-vivo tumor models [18]. Another potent stimulator of the HCC angiogenesis is fibroblast growth factor (FGF) [12]. FGF is frequently associated with the inflammatory process, and overexpression has also been observed in chronic hepatitis and cirrhotic liver tissues [19]. Serous basic FGF levels were reported to correlate with clinico-pathological progression of HCC [20], and synergetic effects of FGF and VEGF might involve HCC angiogenesis [12].

Angiogenesis is orchestrated hierarchically by numerous factors acting on these cells and progenitor cells from the tissue and/or bone marrow. Together with the VEGF/VEGFR system, a variety of angiogenic factors and their receptors have been nominated as therapeutic targets for HCC. Most of molecularly targeted agents can be broadly classified into two main categories: small-molecule kinase inhibitors and monoclonal antibodies [6]. Table 1 summarizes the main agents and the targeted molecules studied by clinical trials on the HCC patients. *Sorafenib* (Nexavar, BAY43-9006) is a unique multi-targeting small-molecule that inhibits the receptor tyrosine kinases (RTKs) of VEGFR2, VEGFR3, PDGFR-beta, c-kit, and Flt-3, and the Raf serine-threonine kinase in intracellular growth signal transduction [21] (Fig. 2). It is worthy of note that the recent phase III trial, Sorafenib HCC assessment randomized protocol (SHARP), revealed Sorafenib to be the first agent that improved overall survival of patients with advanced HCC [21]. Other multiple kinase inhibitors of VEGFRs and PDGFRs, *Sunitinib* (Sutent, SU11248), Vatalanib (PTK787/ZK222584), *Cediranib* (AZD2171), and *SU6668* (TSU-68), are now under active investigation in clinical trials. *Brivanib* (BMS-540215), another type of inhibitor of VEGFRs and FGFRs has been reported to be effective against Sorafenib-resistant HCC in the early phase of clinical trials. The other category of targeted agents includes monoclonal antibodies against VEGF-A and VEGFR-2, *Bevacizumab* (Avastin) and *Ramucirumab* (IMC-1121B), respectively. In addition to the single-agent trials, combination trials with cytotoxic drugs have also been conducted.

Furthermore, we previously isolated Angiopoietin-2 as another angiogenic pathway essential for HCC progression [22, 23]. The angiopoietin family contains specific ligands for Tie2 RTK on vascular endothelial cells [24]. Although Angiopoietin-1 makes the vasculature stable, Angiopoietin-2 destabilizes vessel formation. According to recent reports, Angiopoietin-1 stimulates only the homodimer of Tie2 tyrosine kinase whereas Angiopoietin-2 also induces hetero-dimerization of Tie2 with Tie1, leading to vascular instability [25]. Angiopoietin-2 might function as an initiator of angiogenic switch, promoting susceptibility to VEGF stimulation in the angiogenic progression [26]. It should be noted that a promising result of clinical trials on patients with renal cell carcinoma was recently reported in which an angiopoietin inhibitor AMG386 (2 × Con4[C]), a synthetic peptide with high affinity for Angiopoietin-2, fused to the constant region of human immunoglobulin G1 [27, 28]. AMG386 is being studied in combination with Sorafenib, and with cytotoxic chemotherapy, for a variety of other angiogenic tumors.

## Self-sufficiency in growth signals

In general, deregulation of RTKs occurs in HCC cells, and epidermal growth factor receptor (EGFR) and insulin-like

Table 1 Molecularly targeted agents for hepatocellular carcinoma in clinical trials

Agent	Classification	Function
Sorafenib (Nexabar, BAY43-9006)	Small molecule compound	VEGFR2, VEGFR3, PDGFR-β, Flt-3, c-KIT tyrosine kinase, Raf serine/threonine kinase inhibitor
Sunitinib (Sutent, SU11248)	Small molecule compound	VEGFR1, VEGFR2, PDGFRs, Flt-3, c-KIT tyrosine kinase inhibitor
Vatalanib (PTK787/ZK222584)	Small molecule compound	VEGFR1, VEGFR3, VEGFR3, PDGFR-β, c-KIT tyrosine kinase inhibitor
Cediranib (AZD2171)	Small molecule compound	VEGFR1, VEGFR2, VEGFR3, PDGFRs, c-KIT tyrosine kinase inhibitor
SU6688 (TSU-68)	Small molecule compound	VEGFR2, PDGF R-β, FGFRs tyrosine kinase inhibitor
Brivanib (BMS-540215)	Small molecule compound	VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3 tyrosine kinase inhibitor
Bevacizumab (Avastin)	Monoclonal antibody	VEGF-A-neutralization
Ramucirumab (IMC-1121B)	Monoclonal antibody	VEGFR2-neutralization
Gefitinib (Iressa, ZD1839)	Small molecule compound	EGFR/ErbB1/Her1 tyrosine kinase inhibitor
Erlotinib (Tarceva, OSI774)	Small molecule compound	EGFR/ErbB1/Her1 tyrosine kinase inhibitor
Lapatinib (Tykerb, GW572016)	Small molecule compound	EGFR/ErbB1/Her1 and ErbB2/Her2/Neu tyrosine kinase inhibitor
BMS-599626	Small molecule compound	EGFR/ErbB1/Her1 and ErbB2/Her2/Neu tyrosine kinase inhibitor
Cetuximab (Erbitux)	Monoclonal antibody	EGFR/ErbB1/Her1-neutralization
IMC-A12	Monoclonal antibody	IGF-IR-neutralization
AZD6244 (ARRY-142886)	Small molecule compound	MEK serine/threonine tyrosine kinase inhibitor
Everolimus (RAD001)	Small molecule compound	mTOR serine/threonine kinase inhibitor
Sirolimus (Rapamune)	Small molecule compound	mTOR serine/threonine kinase inhibitor
Bortezomib (Velcade)	Small molecule compound	Proteasome inhibitor
PXD101 (Belinostat)	Small molecule compound	HDAC inhibitor
PI-88	Small molecule compound	Heparanase inhibitor
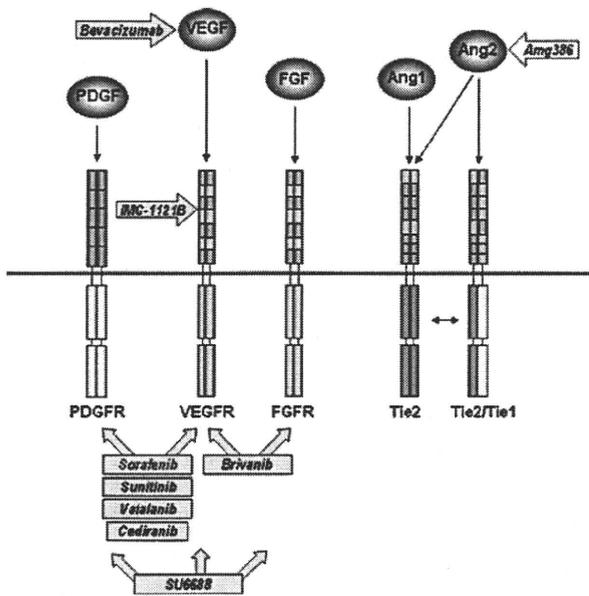
Fig. 2 Molecular targets in angiogenesis with the growth factors and the receptors. Targeted agents are indicated by *arrows*
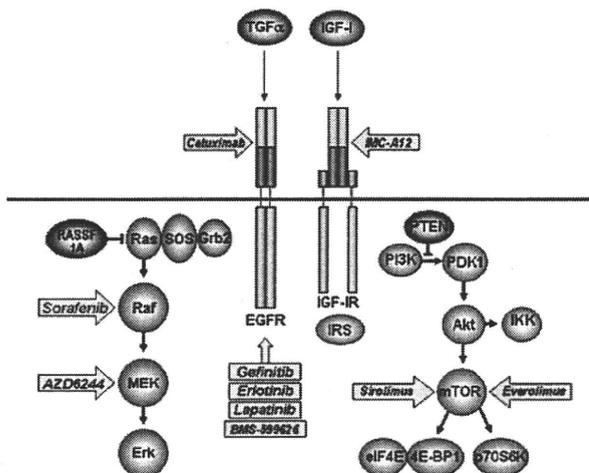
Fig. 3 Molecular targets in HCC cells with the growth factors, receptors, and intracellular signaling. Targeted agents are indicated by *arrows*

growth factor-1 receptor (IGF-1R) are regarded as molecular targets for HCC treatment [6, 7] (Fig. 3). Clinical trials on HCC patients are ongoing with several tyrosine kinase inhibitors of EGFR—*Gefintinib* (Iressa, ZD1839), *Erlotinib* (Tarceva, OSI774), Lapatinib (Tykerb, GW572016), and BMS-599626. Monoclonal antibodies against EGFR (Cetuximab [Erbitux]) and IGF-1R (IMC-A12) are also being evaluated for HCC in clinical trials.

The activated RTKs then stimulate several intracellular signal-transduction pathways including Ras/Raf/MEK/MAPK and PI3K/Akt/mTOR [29, 30] (Fig. 3). In a series

of specific phosphorylation events, an adaptor protein Grb2 stimulates SOS, leading to activation of oncogenic Ras, which is usually mutated in various malignancies. Despite the low incidence of Ras mutations in HCC, silencing with DNA methylation of the RASSF1A gene, a member of the RAS inhibitor family, was detected in human HCC [31]. The silencing of the RAS inhibitor might result in persistent activation of the downstream pathway during hepatocarcinogenesis. The activated form of Ras then stimulates Raf serine–threonine kinase that phosphorylates MEK kinases, and finally it activate the extracellular regulated kinase Erk of the MAPK family. The activated, Erk translocates to the nucleus where it acts as a regulator of gene expression of various proteins, including those of cell cycle progression, apoptosis resistance, and cellular motility [32]. As mentioned above, Raf kinase is one of the targets of the multi-kinase inhibitor *Sorafenib* [21]. Additionally, an MEK kinase inhibitor, *AZD6244* (ARRY-142886), has been evaluated for HCC in clinical trials.

The PI3K/Akt/mTOR pathway has emerged as a contributor to hepatocarcinogenesis [33]. After association of PI3K with the intracellular domain of several RTKs or specific substrates such as IRS, PI3K phosphorylates PIP2 to generate PIP3 which transduces PDK, which activates a serine/threonine kinase Akt [32]. PIP3 is dephosphorylated by PTEN, a tumor suppressor which reverses this pathway. The activated Akt regulates multiple cellular target proteins such as the mammalian target of rapamycin (mTOR) [34]. The mTOR protein regulates the phosphorylation of p70 S6 serine–threonine kinase and the translational repressor protein 4E-BP1 [35]. As rapamycin-derivates, mTOR inhibitors temsirolimus (CCI-779) and *everolimus* (RAD001) are now under active investigation in clinical trials for patients with HCC.

Mitotic stress

Aneuploidy was noticed as one of the essential properties of cancer cells in the late nineteenth century. Recent studies clarified the increased rates of chromosome missegregation in mitosis, referred to as the chromosome instability (CIN) phenotype [36]. Cancer cells bearing mitotic abnormalities such as aneuploidy are likely to be more dependent on stress-support pathways for specific segregation of the chromosomes. In addition, the CIN phenotype could result from stresses placed on the mitotic apparatus because of the need to segregate abnormal chromosomes. Furthermore, mitotic stress might arise indirectly as a result of double-strand breaks and genomic instability after oncogene activation [3].

We have identified Aurora kinase B, a mitotic checkpoint kinase, as a unique molecule for predicting the lethal