event in the early stage of hepatocarcinogenesis.[19,20] Moreover, some studies show that a *p16* deficiency does not fully restore the self-renewal capability of *Bmi-1*. In addition, reduced stem cell frequency occurs in *Bmi-1*-deficient neural stem cells, even when p16Ink4a and p19Arf are not expressed.[4,29,30] These studies indicate that there are additional downstream pathways that might mediate the effect of *Bmi-1* on self-renewal and cell proliferation.

From our gene expression analysis, we found that induction of *Bmi-1* in bone marrow cells resulted in an upregulation of *ABCB1*.[6] *ABCB1* appears to be a consistent feature of mammalian cells displaying resistance to multiple anticancer drugs, and has been postulated to mediate drug resistance.[31,32] Interestingly, recent findings also show expression of *ABCB1* in various stem cells,[33–35] which might make them less sensitive to cancer treatment. Increased expression of *ABCB1* was observed in HCC, particularly in early and well differentiated HCC, compared with the surrounding noncancerous region. *ABCB1* expression decreases with the progression of HCC, suggesting a reflection of tumor dedifferentiation.[36] We showed here that *ABCB1* expression was clearly altered in parallel with *Bmi-1* expression. High expression of both *Bmi-1* and *ABCB1* was observed in the early stage of hepatocarcinogenesis, which suggests their collaboration in maintaining the cell's ability for self-renewal, proliferation, and increased resistance from apoptosis. Although it is possible that *ABCB1* represents a novel downstream target for *Bmi-1*, further analysis is necessary to clarify the mechanism underlying the link between *Bmi-1* and *ABCB1* expression.

In summary, we evaluated the expression and involvement of the "stemness" gene, *Bmi-1*, in HCC, particularly in early stage hepatocarcinogenesis. The strong correlation observed between *Bmi-1* and *ABCB1* expression in HCC indicates a new regulatory pathway for *Bmi-1*, and reveals a potential novel target for enhancing future HCC treatment strategies.

Acknowledgments

This work was supported in part by a Keio University Grant-in-Aid for Encouragement of Young Medical Scientists. Grant-in-aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to T.M.; Grants for the Health Labour Sciences Research and the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan; Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to M.S. Our sincere thanks to H. Suzuki, H. Abe, S. Kusakari, N Hashimoto, M. Konno, and T. Nagai for support throughout the work, M. Fujiwara and M. Iwata, for providing technical assistance, and also Dr. Toru Kiyono (Virology Division, National Cancer Center Research Institute of Japan).

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Sakamoto M, Hirohashi S, Shimosato Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. *Hum Pathol* 1991; **22**: 172–8.
3 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105–11.
4 Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* 2003; **3**: 895–902.
5 van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell* 1991; **65**: 737–52.
6 Mori T, Kiyono T, Imabayashi H *et al.* Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. *Mol Cell Biol* 2005; **25**: 5183–95.
7 Haga K, Ohno S, Yugawa T *et al.* Efficient immortalization of primary human cells by p16INK4a-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT. *Cancer Sci* 2007; **98**: 147–54.
8 Dimri GP, Martinez JL, Jacobs JJ *et al.* The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. *Cancer Res* 2002; **62**: 4736–45.
9 Song LB, Zeng MS, Liao WT *et al.* Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. *Cancer Res* 2006; **66**: 6225–32.
10 Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 1999; **397**: 164–8.
11 Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev* 1999; **13**: 2678–90.
12 van Kemenade FJ, Raaphorst FM, Blokzijl T *et al.* Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. *Blood* 2001; **97**: 3896–3901.
13 Bea S, Tort F, Pinyol M *et al.* BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. *Cancer Res* 2001; **61**: 2409–12.
14 Vonlanthen S, Heighway J, Altermatt HJ *et al.* The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 2001; **84**: 1372–6.
15 Kim JH, Yoon SY, Kim CN *et al.* The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. *Cancer Lett* 2004; **203**: 217–24.
16 Qin ZK, Yang JA, Ye YL *et al.* Expression of Bmi-1 is a prognostic marker in bladder cancer. *BMC Cancer* 2009; **9**: 61.
17 Wang H, Pan K, Zhang HK *et al.* Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2008; **134**: 535–41.
18 Sasaki M, Ikeda H, Itatsu K *et al.* The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. *Lab Invest* 2008; **88**: 873–82.
19 Hui AM, Sakamoto M, Kanai Y *et al.* Inactivation of p16INK4 in hepatocellular carcinoma. *Hepatology* 1996; **24**: 575–9.
20 Kaneto H, Sasaki S, Yamamoto H *et al.* Detection of hypermethylation of the p16(INK4A) gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. *Gut* 2001; **48**: 372–7.
21 Genda T, Sakamoto M, Ichida T *et al.* Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. *Hepatology* 1999; **30**: 1027–36.
22 Hirohashi S, Ishak K, Kojiro M *et al.* Tumours of the liver and intrahepatic bile ducts. In: Hamilton SR, Aaltonen LA, eds. *Pathology and Genetics of Tumours of the Digestive System.* Lyon: IARC Press, 2000; 157–202.
23 Neo SY, Leow CK, Vega VB *et al.* Identification of discriminators of hepatoma by gene expression profiling using a minimal dataset approach. *Hepatology* 2004; **39**: 944–53.
24 Francis NJ, Kingston RE. Mechanisms of transcriptional memory. *Nat Rev Mol Cell Biol* 2001; **2**: 409–21.
25 Buchenau P, Hodgson J, Strutt H, Arndt-Jovin DJ. The distribution of polycomb-group proteins during cell division and development in Drosophila embryos: impact on models for silencing. *J Cell Biol* 1998; **141**: 469–81.
26 Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. Stem cells and cancer: the polycomb connection. *Cell* 2004; **118**: 409–18.
27 Nagao K, Tomimatsu M, Endo H, Hisatomi H, Hikiji K. Telomerase reverse transcriptase mRNA expression and telomerase activity in hepatocellular carcinoma. *J Gastroenterol* 1999; **34**: 83–7.
28 Oh BK, Jo Chae K, Park C *et al.* Telomere shortening and telomerase reactivation in dysplastic nodules of human hepatocarcinogenesis. *J Hepatol* 2003; **39**: 786–92.
29 Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev* 2005; **19**: 1432–7.
30 Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 2003; **425**: 962–7.
31 Kartner N, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* 1985; **316**: 820–3.

doi: 10.1111/j.1349-7006.2009.01431.x

32 Riordan JR, Deuchars K, Kartner N, Alon N, Trent J, Ling V. Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* 1985; **316**: 817–9.
33 Bunting KD, Galipeau J, Topham D, Benaim E, Sorrentino BP. Effects of retroviral-mediated MDR1 expression on hematopoietic stem cell self-renewal and differentiation in culture. *Ann NY Acad Sci* 1999; **872**: 125–40. discussion 40–1.
34 Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002; **20**: 11–20.
35 Keshet GI, Goldstein I, Itzhaki O *et al.* MDR1 expression identifies human melanoma stem cells. *Biochem Biophys Res Commun* 2008; **368**: 930–6.
36 Vander Borght S, Komuta M, Libbrecht L *et al.* Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin. *Liver Int* 2008; **28**: 1370–80.

Supporting Information

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

Fig. S1. ATP-binding cassette transporter B1 (*ABCB1*) expression in hepatocellular carcinoma (HCC) cell lines and clinical samples. (a) Quantitative real-time PCR and Western blot of *ABCB1* in HCC cell lines. Expression of *ABCB1* was significantly higher in KIM-1 cells compared with the other cell lines. (b) *ABCB1* mRNA expression levels in HCC clinical cases. The relative mRNA expression levels in tumor tissues (black column, T) and corresponding non-cancerous, background liver tissues (gray column, N) (left panel). High levels of *ABCB1* expression were observed in well differentiated HCC. The average expression levels of *ABCB1* were higher in tumor tissues than in the non-cancerous background liver tissues (2.30 *vs* 1.23, $P = 0.21$) (right panel). (c) Immunostaining of ABCB1 in well differentiated HCC. An irregular and thicker form of canalicular pattern with cytoplasmic staining was observed in the tumor region compared with the non-cancerous background region (magnification, ×100). Black arrows outline the border between the non-cancerous background region (N) and the tumor region (T). (d) ABCB1 expression in moderately differentiated HCC (magnification, ×200). Only an irregular canalicular pattern was observed (a, H&E stain; b, corresponding ABCB1 staining).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

J Gastroenterol 2009; 44[Suppl XIX]:108–111
DOI 10.1007/s00535-008-2245-y

Journal of Gastroenterology

Early HCC: diagnosis and molecular markers

MICHIIE SAKAMOTO

Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. HCC occurs mainly in patients with chronic liver disease such as in hepatitis B and C infection. These high-risk patients are closely followed up, and increasing numbers of small equivocal lesions are detected by imaging diagnosis. They are now widely recognized as a precursor or early stage of HCC and are classified as dysplastic nodules or early HCC. It is considered that early HCC is a key step in the process of HCC development and progression. However, the molecular mechanisms of early hepatocarcinogenesis are far from clear. Specific mutations of classical oncogenes or tumor suppressor genes have not been identified in early HCC so far. Recent progress in comprehensive analysis of gene expression is shedding some light on this issue. It has been reported that HSP70, CAP2, glypican 3, and glutamine synthetase could serve as molecular markers for early HCC. Further analysis is expected to evaluate their usefulness in routine pathological diagnosis including biopsy diagnosis and also as serum markers for early detection of HCC.

Key words: hepatocellular carcinoma, HSP70, CAP2, GPC3

Introduction

Hepatocellular carcinoma (HCC) occurs mainly in livers that are chronically diseased as a result of hepatitis B or C virus infection. As is known for other cancers, HCC is also characterized by an obvious multistage process of tumor progression. Histopathological and molecular biological studies have revealed the multistep development of human HCCs.[1–6] Precancerous lesions known as adenomatous hyperplasia (AH, also called dysplastic nodule) or atypical adenomatous hyperplasia (AAH, AH with focally increased atypia but too indefinite for a diagnosis of HCC, also called high-grade dysplastic nodule) appear in the chronically diseased liver. These lesions develop into early HCC, which corresponds to in situ or microinvasive carcinoma, then develop into progressive HCC through the stage of "nodule-in-nodule"-type HCC (progressed HCC within early HCC), which indicates a transition from early to progressed HCC. Clinical findings, including radiologic findings, also support these pathological findings.[7–10] However, most early HCCs are asymptomatic clinically, negative for serum markers (AFP and PIVKA-II), lack typical radiologic findings, and show minimal histological atypia. Lack of typical features of ordinary HCC causes difficulty for early detection of small early-stage HCC. In this review, I describe histological features of early HCC and focus on present candidate molecular markers applicable for its histological diagnosis.

Received: June 10, 2008 / Accepted: July 2, 2008
Reprint requests to: M. Sakamoto

Histological features of early HCC

Early HCC is defined as vaguely nodular well-differentiated HCC.[6] It shows preservation of the preexisting liver structure within the nodule and is vaguely nodular with macroscopically indistinct margins. It is a hypercellular nodular lesion showing structural atypia such as the formation of acini, thin trabeculae, and remodeling of the cord structure. Diffuse fatty change of tumor cells is frequently observed. Corresponding to macroscopic features, many portal tracts are present within the tumor nodule, and tumor cell invasion into some portal tracts can be seen. At the tumor–nontumor boundary, tumor cells show replacing growth, and there is no capsule formation. Based on these features, early HCC is considered to correspond to "carcinoma in situ" or "micro-

invasive carcinoma" of the liver. In parallel to these histological features, early HCC receives a portal blood supply and does not show tumor blushing in angiographic examination. In contrast, classical HCCs, even if small and well differentiated, show tumor blushing without portal flow. Intrahepatic extension including vascular invasion and intrahepatic metastases is exceptional.[5] Moreover, these HCCs are locally curable, have a favorable long-term outcome, and can be defined clinically as "early HCC."[9,10]

Molecular markers

HSP70

Expression profiles among seven early components and seven progressed components of "nodule-in-nodule"-type HCCs and their corresponding noncancerous live tissues were compared using an oligonucleotide array.[11] Of the approximately 12600 genes that were analyzed, a set of 95 genes provided a molecular signature which distinguished between early HCC components and their noncancerous liver tissues, and a set of 92 genes distinguished between progressed and early HCC components. Of these genes, the most abundantly upregulated gene in early HCC components was heat-shock protein 70 (HSP70), which was confirmed by real-time quantitative reverse transcription-polymerase chain reaction. Further immunohistochemical examination of HSP70 revealed its significant overexpression in early HCC compared with precancerous lesions and in progressed HCC compared with early HCC. Hepatocytes in noncancerous liver tissue showed no immunostaining or only focal and faint staining in the nucleus, whereas we detected immunoreactivity ranging up to 80% in most cases of early HCC (Fig. 1A). The percentage of positive cells was significantly different between the precancerous lesions AH + AAH (3.49 ± 10.9) and early HCC (32.9 ± 24.3) ($P < 0.001$).

We are now widely applying this HSP70 staining for suspicious cases and consultation cases. Although we have not systemically evaluated this staining for biopsy specimens, it seems that it is helpful to support diagnosis of cancer. It is also noted that HSP70 are negative in other benign nodular lesions, hepatocellular adenoma, and focal nodular hyperplasia (unpublished observations). Thus, HSP70 might be useful as a histological molecular marker for the differentiation of benign and malignant in liver nodules.

CAP2

Using the same DNA array database mentioned above, we also found that cyclase-associated protein 2 (CAP2) expression is upregulated in a stepwise manner in multistage hepatocarcinogenesis.[12] CAP was originally identified from budding yeast as a downstream of the *ras* and as a factor associated with adenylyl cyclase.[13] CAP also binds monomeric actin and, therefore, also possesses a cytoskeletal function.[14] However, only a few studies on CAP have been performed in mammalian

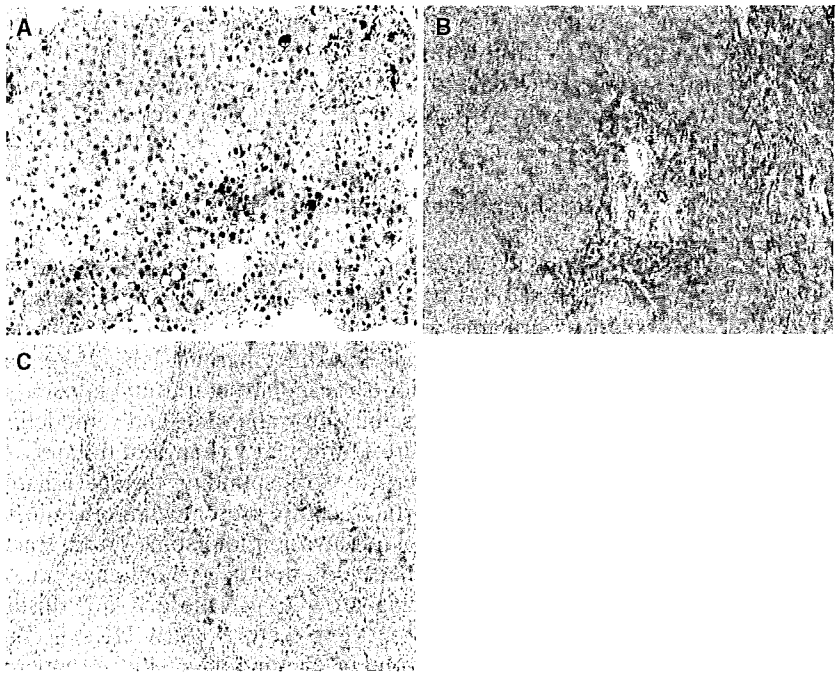

Fig. 1. Applications of molecular markers for diagnosis of early hepatocellular carcinoma (HCC). Representative figures of (**A**) heat-shock protein (HSP)70, (**B**) cyclase-associated protein (CAP)2, and (**C**) glypican-3 (GPC3) immunostaining. *Bars*, 100 μm

cells, and its overexpression has not been previously reported in human cancer. We originally raised a polyclonal antibody specific for CAP2 and applied it for immunohistochemical examination. CAP2 was strongly expressed in smooth muscle cells but was negative in normal hepatocytes. However, parts of the damaged liver samples, especially the periphery of regenerative nodules of liver cirrhosis, were focally and weakly positive for CAP2. All cases of precancerous lesions were negative or focally positive (~5%–10%); in contrast, most cases of early HCC were positive for CAP2 to some extent, and 12 of 29 cases showed 70% to 100% positivity. Frequently, tumor cells invading the stroma are positively stained and clearly highlighted in early HCC, which further supports the diagnosis of cancer in early lesions (Fig. 1B).

GPC3 and GS

Glypican-3 (GPC3) has been reported as a novel serum and histochemical marker for HCC by several groups.[15–17] It is an oncofetal protein and is expressed abundantly in the fetal liver, is inactive in the normal adult liver, and is frequently reactivated in HCC. Immunohistochemical expression of GPC3 was much higher in small HCCs than in cirrhosis and dysplastic nodules (Fig. 1C). Another group reported that positive staining was observed in 48% of high-grade dysplastic nodules or early HCCs and in 3% of benign or low-grade dysplastic nodules. It was also demonstrated that a soluble NH_2-terminal fragment of GPC3 is cleaved and can be detected in the sera of patients with HCC. They suggested GPC3 as a serological marker for early-stage HCC.

Glutamine synthetase (GS) catalyzes the synthesis of glutamine, which is the major energy source of tumor cells. Accumulation of GS was first found through analyzing increased ubiquitinated protein in HCC, and its stepwise increase in expression from precancerous lesions to early to advanced HCC was shown by immunohistochemistry.[18] GS is expressed in hepatocytes surrounding the terminal hepatic venules. Positive areas of GS were less than 10% in cirrhotic livers, so staining in 10% or more was considered as positive expression. It was positive in 1 of 23 AHs, 4 of 31 early HCCs, and 19 of 49 advanced HCCs. It is noteworthy that GS has been reported as a novel target of beta-catenin signaling, which is implicated in development of HCC.[19]

Discussion

Here, the present status of histological and molecular diagnosis of early HCC has been briefly overviewed. However, we have to recognize that inconsistency of terminology and definition of early-stage HCC and precancerous lesions still exists and causes difficulty for simple comparison and interpretation of published works reporting sensitivity and specificity of molecular markers. Still, it is obvious that immunohistochemical applications of the aforementioned molecules could be sensitive markers for the differential diagnosis of early HCC from precancerous lesion or noncancerous liver, which is difficult even for hepatopathologists because of the very well differentiated histology with little atypia in early HCC. Usefulness of combination of these molecules was also reported, leading to further increase of sensitivity and specificity for detection of early HCC.[20]

In conclusion, several candidate molecular markers have been identified as useful for histological diagnosis of early HCC. It is necessary to evaluate their usefulness in routine pathological diagnosis, including biopsy diagnosis. Moreover, further evaluation of these markers and discovery of other molecular markers are expected to establish serum markers useful for early detection of HCC.

Acknowledgments. The work discussed in this review was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan.

References

1. Arakawa M, Kage M, Sugihara S, Nakashima T, Suenaga M, Okuda K. Emergence of malignant lesions within an adenomatous hyperplastic nodule in a cirrhotic liver. Observations in five cases. Gastroenterology 1986;91:198–208.
2. Tsuda H, Hirohashi S, Shimamoto Y, Terada M, Hasegawa H. Clonal origin of atypical adenomatous hyperplasia of the liver and clonal identity with hepatocellular carcinoma. Gastroenterology 1988;95:1664–6.
3. Takayama T, Makuuchi M, Hirohashi S, Sakamoto M, Okazaki N, Takayasu K, et al. Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. Lancet 1990;336:1150–3.
4. Sakamoto M, Hirohashi S, Shimamoto Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. Hum Pathol 1991;22:172–8.
5. Hirohashi S, Ishak KG, Kojiro M, Puig PL, Wanless IR, Fischer HP, et al. Hepatocellular carcinoma. In: Hamilton SR, Aaltonen LA, editors. Pathology and genetics of tumors of the digestive system. Lyon: IARC Press; 2000. p. 159–72.
6. Kojiro M. Pathology of hepatocellular carcinoma. Oxford: Blackwell; 2006.
7. Matsui O, Kadoya M, Kameyama T, Yoshikawa J, Takashima T, Nakanuma Y, Unoura M, et al. Benign and malignant nodules in cirrhotic livers: distinction based on blood supply. Radiology 1991;178:493–7.
8. Kudo M. Morphological diagnosis of hepatocellular carcinoma: special emphasis on intranodular hemodynamic imaging. Hepatogastroenterology 1998;45:1226–31.
9. Takayama T, Makuuchi M, Hirohashi S, Sakamoto M, Yamamoto J, Shimada K, et al. Early hepatocellular carcinoma as an

entity with a high rate of surgical cure. Hepatology 1998;28: 1241–6.
10. Sakamoto M, Hirohashi S. Natural history and prognosis of adenomatous hyperplasia and early hepatocellular carcinoma: multiinstitutional analysis of 53 nodules followed up for more than 6 months and 141 patients with single early hepatocellular carcinoma treated by surgical resection or percutaneous ethanol injection. Jpn J Clin Oncol 1998;28:604–8.
11. Chuma M, Sakamoto M, Yamazaki K, Ohta T, Ohki M, Asaka M, Hirohashi S. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. Hepatology 2003;37:198–207.
12. Shibata R, Mori T, Du W, Chuma M, Gotoh M, Shimazu M, et al. Overexpression of cyclase-associated protein 2 in multistage hepatocarcinogenesis. Clin Cancer Res 2006;12(18):5363–8.
13. Fedor-Chaiken M, Deschenes RJ, Broach JR. SRV2, a gene required for RAS activation of adenylate cyclase in yeast. Cell 1990;61:329–40.
14. Baum B, Li W, Perrimon N. A cyclase-associated protein regulates actin and cell polarity during *Drosophila* oogenesis and in yeast. Curr Biol 2000;10:964–973.
15. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, Filmus J. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125(1): 89–97.
16. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306(1):16–25.
17. Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, et al. Identification of soluble NH_2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64(7):2418–23.
18. Osada T, Sakamoto M, Nagawa H, Yamamoto J, Matsuno Y, Iwamatsu A, et al. Acquisition of glutamine synthetase expression in human hepatocarcinogenesis: relation to disease recurrence and possible regulation by ubiquitin-dependent proteolysis. Cancer (Phila) 1999;85(4):819–31.
19. Cadoret A, Ovejero C, Terris B, Souil E, Lévy L, Lamers WH, Kitajewski J, et al. New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. Oncogene 2002; 21(54):8293–301.
20. Di Tommaso L, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, et al. Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. Hepatology 2007;45(3):725–34.

SPECIAL ARTICLE

Pathologic Diagnosis of Early Hepatocellular Carcinoma: A Report of the International Consensus Group for Hepatocellular Neoplasia

International Consensus Group for Hepatocellular Neoplasia

See Editorial on Page 355

Advances in imaging techniques and establishment of surveillance protocols for high-risk populations have led to the detection of small hepatic nodules in patients with chronic liver diseases, particularly those with cirrhosis or chronic hepatitis caused by hepatitis B or C viruses. These nodules, comprising a broad range of diagnostic entities—some benign and some with malignant potential—are currently defined histologically, and their clinical management often depends on the ability to make a reliable histologic diagnosis.

Evidence accumulated in the last two decades strongly favors the existence of a sequence of events in hepatic nodules that precedes the emergence of hepatocellular carcinoma (HCC),[1-10] and these lesions are recognized as precursors of HCC. However, from the beginning of their recognition, there has been considerable confusion concerning nomenclature and diagnostic approaches to these hepatic nodules. To clarify these issues, an International Working Party (IWP) of the World Congresses of Gastroenterology proposed a consensus nomenclature and diagnostic criteria for hepatocellular nodular lesions in 1995.[11] The IWP classified nodular lesions found in chronic liver disease into large regenerative nodule, low-grade dysplastic nodule (L-DN), high-grade dysplastic nodule (H-DN), and HCC; this nomenclature has been widely adopted. In addition, the IWP introduced the concept of dysplastic focus as a cluster of hepatocytes with features of early neoplasia (in particular small cell change or iron-free foci in a siderotic background) measuring less than 0.1 cm, and defined small HCC as a tumor measuring less than 2 cm.

More recent studies support the division of small HCC into two clinico-pathological groups that have been termed 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.[12] Early HCC has a longer time to recurrence and a higher 5-year survival rate compared with progressed HCC.[13]

Small lesions with malignant potential have only subtle differences from the surrounding parenchyma, making them difficult to assess reproducibly. Differences in the application of diagnostic criteria between Western and Eastern pathologists has been a persistent difficulty in research and clinical management of these lesions.[14] In order to obtain a refined and up-to-date international consensus on the histopathologic diagnosis of nodular lesions, such as dysplastic nodules and early HCC, the International Consensus Group for Hepatocellular Neoplasia (ICGHN) was convened in April 2002 in Kurume, Japan. The group has met several times up to July 2007 under the auspices of the Laennec Liver Pathology Society. The ICGHN is currently comprised of 34 pathologists and two clinicians from 13 countries. It includes most members of the original IWP who are still active and all the participants from the first ICGHN meeting. This consensus document summarizes the results of our meetings.

Materials and Methods

Twenty-six resected cases of nodules from 23 patients with chronic hepatitis or cirrhosis caused by hepatitis B or

Abbreviations: GPC3, glypican-3; GS, Glutamine synthetase; HCC, hepatocellular carcinoma; H-DN, high-grade dysplastic nodule; HSP70, heat shock protein 70; ICGHN, The International Consensus Group for Hepatocellular Neoplasia; IWP, International Working Party; L-DN, low-grade dysplastic nodule.

From the Department of Pathology, Kurume University, Kurume, Japan.

Received June 11, 2008; accepted October 16, 2008.

The International Consensus Group for Hepatocellular Neoplasia consists of: Masamichi Kojiro, Ian R. Wanless, Venancio Alves, Sunil Badve, Charles Balabaud, Pierre Bedosa, Prithi Bhathal, Paulette Bioulac-Sage, Elizabeth M. Brunt, Alastair D. Burt, John R. Craig, Amar Dhillon, Linda Ferrell, Stephen A. Geller, Zackary D. Goodman, Annette S. H. Gouw, Maria Guido, Maha Guindi, Prodromos Hytiroglou, Masayoshi Kage, Fukuo Kondo, Masutoshi Kudo, Gregory Y. Lauwers, Masayuki Nakano, Valerie Paradis, Young-Nyun Park, Alberto Quaglia, Massimo Roncalli, Tania Roskams, Boris Ruebner, Michiie Sakamoto, Romil Saxena, Neil D. Theise, Swan Thung, and Dina Taniakos.

Address reprint requests to: Masamichi Kojiro, M.D., Department of Pathology, Kurume University, 67 Asahi-machi, Kurume-shi 830-0011, Japan. E-mail: kojiro@med.kurume-u.ac.jp; fax: (81)-942-31-7726.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22709

Potential conflict of interest: Nothing to report.

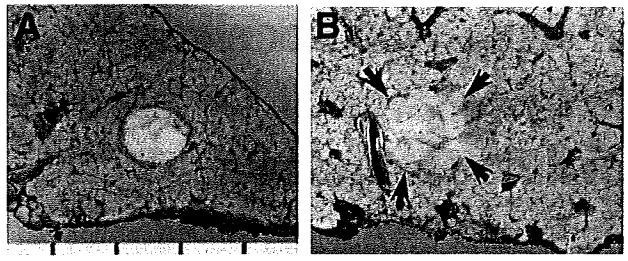

Fig. 1. (A) HCC of distinctly nodular type (progressed HCC), 12 mm in diameter. There was no discrepancy in the diagnosis of HCC with this growth pattern despite the small tumor size. (B) Small HCC of vaguely nodular type (early HCC) (arrows). These lesions were often a diagnostic problem, solved in part by recognition of the histologic features of stromal invasion.

C virus were selected from one Korean and two Japanese medical centers. All the lesions measured less than 2 cm in diameter. One hematoxylin and eosin–stained slide comprising the entire width of each lesion, a gross picture, and brief clinical data were reviewed by each pathologist individually, and the lesions were classified according to the IWP criteria. The group met at Kurume University Medical School, Kurume, Japan, in April 2002 to review all the lesions with photographs and by group review of relevant slides with a projecting microscope. The histologic diagnostic criteria were discussed, focusing on cases with marked discrepancies in initial, premeeting diagnosis. The second meeting was held at the University of Leuven, Belgium, in May 2004. The members discussed the diagnosis of an additional set of 22 resected small nodules. The third meeting was held at the Aristotle University of Thessaloniki, Greece in May 2006, and histopathologic consensus on both dysplastic nodules and early HCC was obtained. Kappa statistics were obtained from the comparative diagnostic panels of the first two of these meetings using SAS software version 8.2 (SAS Institute Inc., Cary, NC).

Summary of Comparative Diagnosis Data from Two Rounds of Slide Circulation

There was little difficulty in agreeing on the diagnosis of well-differentiated, small HCC of the distinctly nodular type or when the tumor was moderately differentiated HCC (Fig. 1A). The overwhelming diagnostic challenge was the differentiation of H-DNs from well-differentiated, small HCC of the vaguely nodular type (early HCC) (Fig. 1B). These lesions showed the lowest kappa value at the first conference with wide interobserver variation on initial review; the variation was diminished, but not totally resolved after the first conference. Initially, Asia-trained pathologists generally diagnosed HCC more frequently than Western pathologists. After the first conference, this discrepancy decreased, and kappa values for HCC rose from 0.30 to 0.49 (though with different slide sets), with most Western pathologists ultimately agreeing with the diagnosis of HCC. The improvement of diagnostic agreement after the initial conference was due to the recognition of stromal invasion as a criterion for diagnosis of well-differentiated HCC. Stromal invasion is defined as tumor cell invasion into the portal tracts or fibrous septa within vaguely nodular lesions[15,16] (Fig. 2).

Current Suggestions for Diagnostic Criteria

Gross and Radiographic Features

It is often possible to make a presumptive diagnosis of HCC when a small lesion is distinctly nodular and is hypervascular on contrast-enhanced imaging in the setting of cirrhosis.[17,18] However, errors will occur occasionally with this approach. It has been reported that a small but significant proportion of explant livers was misdiagnosed as HCC.[19] Any focal lesion containing a large arterio-venous shunt may be hypervascular (for example, focal nodular hyperplasia[20] or similar lesions[21]). A hypovascular lesion less than 2 cm having a vaguely nodular appearance cannot be accurately diagnosed by gross examination or imaging. Such lesions should undergo guided needle core biopsy.

Some small nodules have a "nodule-in-nodule" appearance either radiologically or on gross examination.[22] In this situation, the subnodule usually represents dedifferentiation of the "parent" nodule. The parent nodule may be a dysplastic nodule or well-differentiated HCC, and the subnodule is invariably a less-differentiated lesion. In these situations, the entire nodule is classified by the worst component. Typically, the less-differentiated component is more vascular than the parent component.[23-27] However, if the parent nodule is a dysplastic nodule and the subnodule is well-differentiated HCC, the subnodule may not be hypervascular, because unpaired arteries have not yet developed. Such unpaired arteries are small arteries (unaccompanied by bile duct) occurring

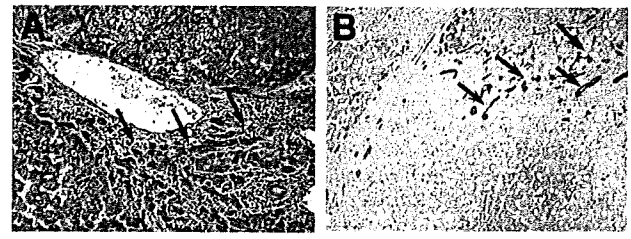

Fig. 2. (A) Stromal invasion in early HCC. The tumor cells (arrows) are invading an intratumoral portal tract. (B) CK19 immunostaining of another lesion. The ductular reaction (arrows) is mimicking stromal invasion and is prominent at the stroma-parenchymal interface. Well-differentiated HCC with fatty change is located at the bottom half of the image.

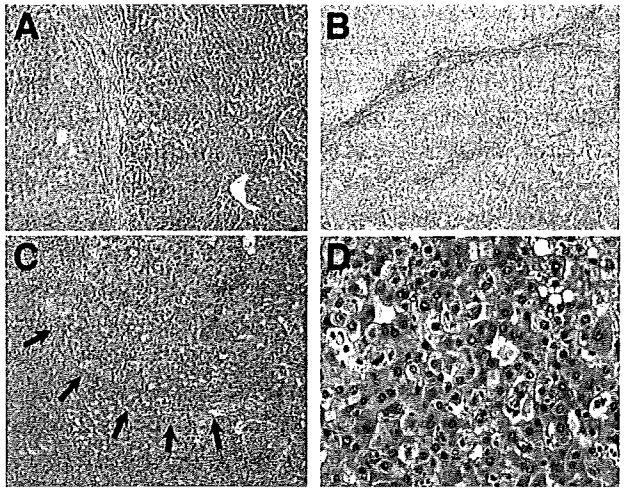

Fig. 3. (A) Low-grade dysplastic nodule (right two-thirds) shows mild increase in cell density with a clearer trabecular arrangement than the adjacent parenchyma. (B) High-grade dysplastic nodule. The cell density in this example is more than 1.5 times higher than that in the surrounding tissue (upper left). Irregularity of the trabecular pattern is remarkable, but there is no obvious infiltrative growth. (C, D) Small, well-differentiated HCC of vaguely nodular type (early HCC). The tumor shows replacing growth at the boundary (arrows), and the cell density is more than 2 times higher than that in the surrounding tissue. The tumor cells show an irregular thin-trabecular pattern with occasional pseudoglands. Stromal invasion was present elsewhere in the tumor.

outside the original portal tracts, and are indicative of neovascularization. They have a thin muscular wall that can be recognized in more detail via immunostaining for α-smooth muscle actin. Regardless of vascularity, a nodule-in-nodule appearance suggests the presence of HCC.

Pathologic Features

Low-Grade Dysplastic Nodules. L-DNs are sometimes vaguely nodular but are often distinct from the surrounding cirrhotic liver because of the presence of peripheral fibrous scar. This is not a true capsule, but rather condensation of scarring as is seen around all cirrhotic nodules. L-DNs show mild increase in cell density with a monotonous pattern, and they have no cytologic atypia, though they may have large cell change (formerly referred to as large cell dysplasia[28]). Architectural changes beyond clearly regenerative features are not present; these lesions do not contain pseudoglands or markedly thickened trabeculae (Fig. 3). Unpaired arteries are sometimes present in small numbers.[29] Nodule-in-nodule lesions are not present in L-DNs. L-DNs may have diffuse siderosis or diffusely increased copper retention.

Among members of the consensus panels, there was no serious difficulty in differentiating L-DNs from early HCC. At the opposite end of the spectrum, distinction between L-DNs and large regenerative nodules was often found to be difficult or impossible. Therefore, there is currently consensus that distinction between these two diagnostic categories cannot be made confidently by morphology alone and remains a task for the future. Fortunately, this distinction does not appear to have significant practical consequences at present.

High-Grade Dysplastic Nodules. H-DNs may be distinctly or vaguely nodular in the background of cirrhosis, although they also lack a true capsule, similar to L-DNs; however, they are more likely to show a vaguely nodular pattern than L-DNs. An H-DN is defined as having architectural and/or cytologic atypia, but the atypia is insufficient for a diagnosis of HCC. These lesions most often show increased cell density, sometimes more than 2 times higher than the surrounding nontumoral liver, often with an irregular trabecular pattern (Fig. 3). Small cell change (also known as small cell dysplasia[30]) is the most frequently seen form of cytologic atypia in H-DNs. This form of atypia may also occur in small hepatocellular foci outside of H-DNs; the term dysplastic focus[11] may be appropriately used for such lesions. Large cell change may or may not be present in H-DNs. Unpaired arteries are found in most lesions, but usually not in great numbers. A nodule-in-nodule appearance is occasionally found in H-DNs, and subnodules often have a higher labeling index of Ki-67 or proliferating cell nuclear antigen than that of H-DN parenchyma. When a nodule with largely H-DN features contains a subnodule of HCC, the subnodule of HCC is usually well-differentiated with a well-defined margin.

The diagnostic discrepancy between H-DN and early HCC was frequent at the first consensus meeting, but was remarkably improved at the second meeting due to the recognition of stromal invasion as a diagnostic criterion for the differentiation of H-DN from early HCC. If areas of questionable invasion are present, immunostaining for keratins 7 or 19 may be useful; if such staining demonstrates a ductular reaction, the focus is considered a pseudoinvasion and does not warrant a diagnosis of HCC[31] (Fig. 2B).

Early HCC (Small Well-Differentiated HCC of Vaguely Nodular Type)

Early HCC tumors are vaguely nodular and are characterized by various combinations of the following major histologic features[6,22,32] (Fig. 3):

(1) increased cell density more than 2 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;

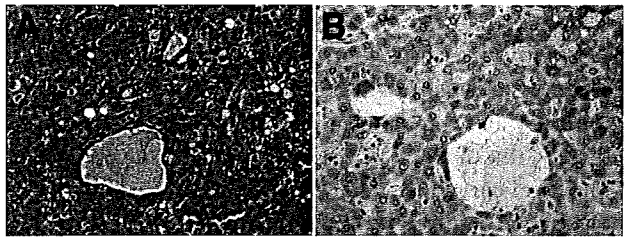

Fig. 4. GPC3 expression in small, well-differentiated HCC of vaguely nodular type (early HCC). (A) Hematoxylin-eosin stain. (B) Immunostaining for GPC3 shows expression in the cytoplasm of tumor cells.

(4) diffuse fatty change; and
(5) varying numbers of unpaired arteries.

Among these features, diffuse fatty change is observed in approximately 40% of cases.[33] The characteristic features of early HCC are sometimes seen in larger tumors as well—that is, well-differentiated tumors that measure over 2 cm and thus do not qualify for the designation of small HCC set forth by the IWP. The prevalence of fatty change decreases along with increasing tumor size; therefore, fatty change is uncommon in tumors larger than 3 cm. Fatty change is also uncommon in moderately differentiated HCCs. Any of the features listed above may be diffuse 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.

Emerging Tumor Markers

Alpha-fetoprotein is a well-established serum marker for HCC. However, elevated levels are rarely found in early HCCs. Alpha-fetoprotein is not useful as a tissue marker because of low sensitivity (25% to 30%), even with moderately differentiated HCC.

Glypican-3 (GPC3), a cell-surface heparan sulfate proteoglycans that is secreted into the plasma, has recently become established as a serum and tissue marker for HCC.[34-39] GPC3 immunoreactivity has a reported sensitivity of 77% and specificity of 96% in the diagnosis of small HCC; therefore, GPC3 positivity is a strong argument for malignancy.[40,41] The staining pattern is usually cytoplasmic but may be membranous or canalicular (Fig. 4). The monoclonal antibody from Biomosaics (IG12 clone) at a dilution of 1/50 to 1/100 as amplified with the new short polymer systems (Advance [Dako], Novolink [Novocastra], and Super-picture + [Zymed]) yields reliable results. Because GPC3 staining may be only focal, additional markers or a panel of markers may be necessary. GPC3 staining must be interpreted in context, because it may also be seen in regenerating hepatocytes in a setting of hepatitis[42] and in melanocytic lesions.[43]

Heat shock protein 70 (HSP70) belongs to a class of genes (heat shock proteins) implicated in the regulation of cell cycle progression, in apoptosis, and in tumorigenesis.[44-46] Most HCCs are associated with chronic inflammation and fibrosis acting as stressful conditions that lead to heat shock protein synthesis. HSP70 is, in particular, a potent antiapoptotic survival factor. Chuma et al.[47] reported HSP70 as the most abundantly up-regulated gene among a set of 12,600 genes in early HCC. Furthermore, it was significantly overexpressed in progressed HCC as compared with early HCC, and in the latter as compared with precancerous lesions. HSP70 immunoreactivity was recently reported in the majority of HCCs, including early and well-differentiated forms, but not in nonmalignant nodules,[48] thus suggesting its use as a marker of malignancy. HSP70 immunoreactivity (SC-24, dilution 1:250 to 1:500 amplified with short polymer systems; Santa Cruz Biotechnology, Santa Cruz, CA) is nucleocytoplasmic and mostly focal with 70% sensitivity for HCC detection in surgically resected specimens.[49]

Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonia in the mammalian liver[49] where it has been shown to be restricted to hepatocytes surrounding the terminal hepatic venules.[50] It is known that glutamine, the end product of GS activity, is the major energy source of tumor cells.[51] Most importantly, GS is a target gene of β-catenin so that its overexpression is associated with mutations of β-catenin or with activation of this pathway.[52-54] Up-regulation of GS messenger RNA, protein, and activity were shown by Christa et al.[52] in human HCC, while Osada et al.[55] reported the stepwise increase in GS immunoreactivity from precancerous lesions to early HCC to progressed HCC. The monoclonal antibody from Chemicon International (clone MB302) at a dilution of 1/500 to 1/1000 and amplified with a new short polymer system yields reliable results. In order to increase its specificity as a marker of malignancy, GS immunostaining should be diffuse and of strong intensity, a pattern that can be seen in 50% of HCCs, including early forms.[49]

The combination of more than one putative marker of malignancy raises the overall accuracy. When applying a panel of these three markers (GPC3, HSP70, and GS) to resected small lesions, the finding of any two positive markers had a sensitivity of 72% and a specificity of 100% to detect malignancy.[48] The diagnostic accuracy of this panel of markers in liver biopsies of hepatocellular nodules has not been yet tested.

Comment

The IWP criteria of 1995 have led to remarkable progress in global standardization of nomenclature of liver nodules.[11] However, although these criteria have been widely adopted, their application is challenging in equivocal lesions. Perhaps the most significant problem is that most histologic criteria are arrayed on a gradual spectrum and cannot be easily summarized as present or absent. Additionally, the number of criteria suggested in the literature are too numerous to achieve interobserver consensus, and the diagnostic weight carried by each of these criteria is uncertain. Frequently used criteria for malignancy in other tissues, such as mitotic activity and cellular atypia, are not represented to a significant degree in well-differentiated HCC. In addition, because the liver lacks a layered structure as seen in the gastrointestinal tract, it is difficult to determine the presence of destructive growth in early HCCs.

Despite these difficulties, current histologic criteria for these nodules clearly yield reliable diagnoses at both ends of the spectrum; most pathologists will correctly identify nodules up to L-DN as benign, whereas even small well-differentiated nodules with distinct nodular pattern or small moderately differentiated HCCs will be correctly identified as malignant. The remaining gray zone includes H-DN and early HCC. In evaluation of these lesions, the presence of stromal invasion is a useful criterion of malignancy.[15,16] Accordingly, pathologists can decide whether the equivocal tumor is HCC or H-DN by recognizing the presence or absence of tumor cell invasion into the intratumoral portal tracts. When obvious stromal invasion is not found in an equivocal tumor, the lesion may be diagnosed as either H-DN or early HCC without detectable invasion. The diagnosis of stromal invasion is subjective and may require the assistance of histochemical (Victoria Blue or reticulin stains[16]) and immunohistochemical stains (keratin 7 or 19) for differentiation from pseudoinvasion.[29] New immunohistochemical and molecular markers are still under investigation and are likely to prove useful.[46,54,56]

Role of Liver Biopsy. Regarding the application of biopsy for small nodules, the American Association for the Study of Liver Diseases recommends that biopsy should be performed for nodules less than 2 cm if their radiologic findings are not characteristic of HCC, whereas biopsy is not needed for lesions showing characteristic radiologic findings.[17] This recommendation has been supported by prospective validation.[18] Biopsy diagnosis of equivocal nodules remains a challenge, because minute biopsy specimens may not contain intratumoral portal tracts, thus precluding the detection of stromal invasion.

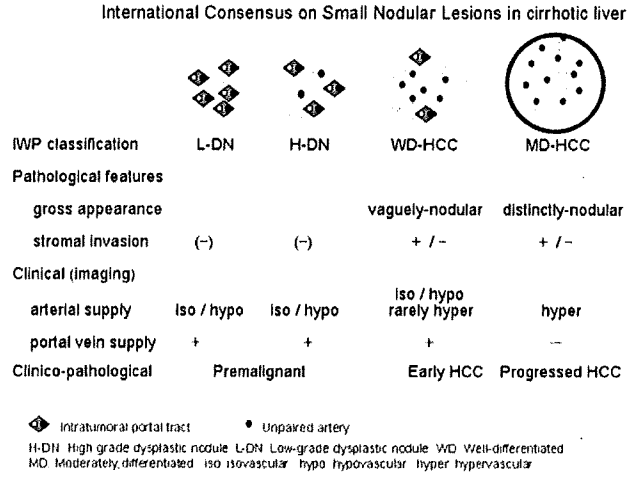

Fig. 5. Diagram summarizing clinical and pathological correlations. The cartoons in the top row show the anatomic changes that are found with the evolution of fully malignant HCC. Because early HCCs grow in a replacing pattern at the boundary, with tumor cells replacing the surrounding liver cell cords, they show a vaguely nodular appearance. When the tumors reach 1.5 to 2 cm in diameter, they tend to de-differentiate, becoming moderately differentiated and growing in an expansile fashion with formation of a fibrous capsule. Hypovascularity, hypervascularity, and isovascularity are understood to mean the signal intensity in the arterial phase of contrast-enhanced imaging relative to the nontumorous liver. Hypervascularity is related to the development of unpaired arteries, the absence of portal vein supply, and the distinctly nodular growth. The diagnosis must consider the context of the lesion, especially the presence of cirrhosis, the imaging findings, and the growth rate. In the appropriate context, a lesion with decreased portal vein supply without hypervascularity is suggestive of early HCC.

Similarly, the detection of unpaired arteries, mitoses, and various immunohistochemical markers are prone to sampling error. Core liver biopsy is definitely superior to fine needle aspiration, because the specimen obtained is suitable for the assessment of both architectural and cytologic features. Furthermore, the tissue block obtained provides materials for marker studies. Fine needle aspiration is usually adequate for the evaluation of large lesions that are likely to be moderately to poorly differentiated, where diagnostic criteria are easier to evaluate.

Clinico-pathological Correlation. Clinical and pathological features of early hepatocellular neoplasia are summarized in Fig. 5. The characteristic imaging appearance of HCC is a hypervascular lesion that shows washout in the portal venous phase. This appearance is also typical in small HCC of the distinctly nodular type and most moderately differentiated small HCCs. Dysplastic nodules and most early HCCs are hypovascular lesions. These classic images are explained by the anatomic features of the lesions. Taken together, the pathologic and imaging features define three phases in the evolution of neoplasia in cirrhotic liver, where dysplastic nodules represent the premalignant phase, well-differentiated HCC of the

vaguely nodular type represents early carcinoma, and small HCCs of the distinctly nodular type and moderately differentiated HCCs represent progressed carcinoma. In the noncirrhotic liver, however, the developmental process of HCC in humans has not been clarified.

Acknowledgment: The authors thank Mr. Kazuhiro Abe for kappa statistics.

References

1. Arakawa M, Kage M, Sugihara S, Nakashima T, Suenaga M, Okuda K. Emergence of malignant lesions within an adenomatous hyperplastic nodules in a cirrhotic liver. Observation in five cases. Gastroenterology 1986; 91:198-208.
2. Sakamoto M, Hirohashi S, Shimamoto Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. Hum Pathol 1991;22:172-178.
3. Borzio M, Bruno S, Roncalli M, Mels GC, Ramella G, Borzio F, et al. Liver cell dysplasia is a major risk factor for hepatocellular carcinoma in cirrhosis: a prospective study. Gastroenterology 1995;108:812-817.
4. Le Bail B, Bernard PH, Balabaud C, Bioulac-Sage P. Prevalence of liver cell dysplasia and association with HCC in a series of 100 cirrhotic liver explants. J Hepatol 1997;27:835-842.
5. Libbrecht L, Craninx M, Nevens F, Desmet V, Roskams T. Predictive value of liver cell dysplasia for development "of hepatocellular carcinoma in patients with non-cirrhotic and cirrhotic chronic viral hepatitis. Histopathology 2001;39:66-73.
6. Hytiroglou P. Morphological changes of early human hepatocarcinogenesis. Semin Liver Dis 2004;24:65-75.
7. Libbrecht L, Desmet V, Roskams T. Preneoplastic lesions in human hepatocarcinogenesis. Liver Int 2005;25:16-27.
8. Theise ND, Park YN, Kojiro M. Dysplastic nodules and hepatocarcinogenesis. Clin Liver Dis 2002;6:497-512.
9. Paradis V, Laurendeau I, Vidaud M, Bedossa P. Clonal analysis of macronodules in cirrhosis. HEPATOLOGY 1998;28:953-958.
10. Plentz RR, Park YN, Lechel A, Kim H, Nellessen F, Langkopf BH, et al. Telomere shortening and p21-checkpoint inactivation characterize multistep hepatocarcinogenesis in humans. HEPATOLOGY 2007;45:968-976.
11. International Working Party. Terminology of nodular hepatocellular lesions. HEPATOLOGY 1995;22:983-993.
12. Kojiro M, Nakashima O. Histopathologic evaluation of hepatocellular carcinoma with a special reference to small early stage tumor. Semin Liver Dis 1999;19:287-296.
13. Takayama T, Makuuchi M, Hirohashi S, Sakamoto M. Early hepatocellular carcinoma as an entity with high rate of surgical cure. HEPATOLOGY 1998;28:1241-1246.
14. Kojiro M. Diagnostic discrepancy of early hepatocellular carcinoma between Japan and West. Hepatology Res 2007;37:S249-S252.
15. Kondo F, Kondo Y. Nagato Y, Tomizawa M, Wada K. Interstitial tumor cell invasion in small hepatocellular carcinoma. Evaluation in microscopic and low magnification view. J Gastroenterol Hepatol 1994;9:604-612.
16. Nakano M, Saito A, Yamamoto M, Doi M, Takasaki K. Stromal invasion and blood vessel wall invasion in well differentiated hepatocellular carcinoma. Liver 1997;17:41-46.
17. Bruix J, Sherman M. Management of hepatocellular carcinoma. HEPATOLOGY 2005;42:1208-1236.
18. Forner A, Vilana R, Ayuso C, Bianchi L, Sole M, Ramon J, et al. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. HEPATOLOGY 2008;47:97-104.
19. Hayashi PH, Trotter J, Forman L, Kugelmas M, Strinberg T, Russ P, et al. Impact of pretransplant diagnosis of hepatocellular carcinoma on cadveric liver allocation in the era of MELD. Liver Transpl 2004;10:42-48.
20. Wanless IR, Sapp H, Guindi M, Olshansky D, Takayama A. The pathogenesis of focal nodular hyperplasia: an hypothesis based on histologic review of 20 lesions including 3 occurring in early biliary cirrhosis. HEPATOLOGY 2006;44:491A.
21. Libbrecht L, Cassiman D, Verslype C, Maleux G, Van Hees D, Porenne J, et al. Clinicopathological features of focal nodular hyperplasia-like nodules. Am J Gastroenterol 2006;101:2341-2346.
22. Kojiro M. Pathology of Hepatocellular Carcinoma. Oxford: Blackwell Publishing, 2006.
23. Matsui O, Takashima T, Kadoya M, Ida M, Suzuki M, Kitagawa K, et al. Dynamic computed tomography during arterial portography: the most sensitive examination for small hepatocellular carcinoma. J Comput Assist Tomogr 1985;9:19-24.
24. Kudo M, Tomita S, Tochio H, Mimura J, Okabe Y, Kashida H, et al. Small hepatocellular carcinoma: diagnosis with US angiography with intra-arterial CO2 gas microbubles. Radiology 1992;182:155-160.
25. Bennett GL, Krinsky GA, Abitbol RJ, Kim SY, Theise ND, Teperman LW, et al. Ultrasound detection of hepatocellular carcinoma and dysplastic nodules in patients with cirrhosis: correlation of pretransplant ultrasound findingss and liver explant pathology in 200 patients. AJR Am J Roentgenol 2002;179:75-80.
26. Krinsky GA, Lee VS, Theise ND, Weinreb J, Morgan GR, Diflo T, et al. Hepatocellular carcinoma and dysplastic nodules in patients with cirrhosis: prospective diagnosis with MR imaging and explant correlation. Radiology 2001;219:445-454.
27. Van den Bos IC, Hussain SM, Terkivatan T, Zondervan PE, de Man RA. Stepwise carcinogenesis of hepatocellular carcinoma in the cirrhotic liver: demonstration on serial MR imaging. J Magn Reson Imaging 2006;24: 1071-1080.
28. Anthony PP, Vogel CL, Barker LE. Liver cell dysplasia: a premalignant condition. J Clin Pathol 1973;26:217-223.
29. Park YN, Yang C-P, Fernandez GJ, Cubukcu O, Thung SN, Theise ND. Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver. Am J Surg Path 1998;22:656-662.
30. Watanabe S, Watanabe S, Okita K, Harada T, Kodama T, Numa Y, et al. Morphologic studies of the liver cell dysplasia. Cancer 1983;51:2197-2205.
31. Park YN, Kojiro M, Di Tommaso L, Dhillon AP, Kondo F, Nakano M, et al. Ductular reaction is helpful in defining early stromal invasion, small hepatocellular carcinomas, and dysplastic nodules. Cancer 2007;109:915-923.
32. Hytiroglou P, Park YN, Krinsky G, Theise ND. Hepatic precancerous lesions and small hepatocellular carcinoma. Gasteroenterol Clin N Am 2007;36:867-887.
33. Kutami R, Nakashima Y, Nakashima O, Shiota K, Kojiro M. Pathomorphologic study on the mechanism of fatty change in small hepatocellular carcinoma of humans. J Hepatol 2000;33:282-289.
34. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89-97.
35. Midorikawa Y, Ishikawa S, Iwanari H, Imamura T, Sakamoto H, Miyazono K, et al. Glypican-3, overexpressed in hepatocellular carcinoma, modualates FGF2 and BMP-7 signaling. Int J Cancer 2003;103:455-465.
36. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003; 306:16-25.
37. Sung YK, Hwang SY, Park YN, Farooq M, Han IS, Bae HI, et al. Glypican-3 is overexpressed in human hepatocellular carcinoma. Cancer Sci 2003;94:259-262.
38. Moriguch H, Sato C. The values and limitations of glypican-3 as a novel tumor marker for hepatocellular carcinoma from clinical and economic viewpoints. Gastroenterology 2004;127:679-680.
39. Kandil D, Leiman G, Allegretta M, Trotman W, Pantanowitz L, Goulart R, et al. Glypican-3 immunohistochemistry in liver fine-needle aspirates; a novel stain to assist in the differentiation of benign and malignant liver lesions. Cancer 2007;111:316-322.
40. Libbrecht L, Severi T, Cassiman D, Vander Borght S, Pienne J, Nevens F, et al. Glypican-3 expression distinguishes small hepatocellular carcinomas

from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules. Am J Surg Pathol 2006;30:1405-1411.
41. Wang XY, Degos F, Dubois S, Tessiore S, Allegretta M, Guttmann RD, et al. Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. Hum Pathol 2006; 37:1435-1441.
42. Abdul-Al HM, Makhlou HR, Wang G, Goodman ZD. Glypican-3 expression in benign liver tissue with active hepatitis C: implications for the diagnosis of hepatocellular carcinoma. Hum Pathol 2008;39:209-212.
43. Nakatsuka T, Kageshita T, Ito S, Wakamatsu K, Monji M, Ikuta Y, et al. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004;10:6612-6621.
44. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 2001;286:433-442.
45. Helmbrecht K, Zeise E, Rensing L. Chaperones in cell cycle regulation and mitogenic signal transduction: a review. Cell Prolif 2000;33:341-365.
46. Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 2000;92: 1564-1572.
47. Chuma M, Sakamoto M, Yamazaki K, Ohta T, Ohiki M, Asaka M, et al. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. HEPATOLOGY 2003;37:198-207.
48. Di Tommaso L, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, et al. Diagnostic value of HSP70, glypican 3 and glutamine synthetase in hepatocellular nodules in cirrhosis. HEPATOLOGY 2007;45:725-734.
49. Haussinger D, Sies H, Gerok W. Functional hepatocyte heterogeneity in ammonia metabolism. The intercellular glutamine cycle. J Hepatol 1985; 15:3-14.
50. Moorman AF, de Boer PA, Geerts WJ, van de Zande L, Lamers WH, Charles R. Complementary distribution of carbamoylphosphate synthetase (ammonia) and glutamine synthetase in rat liver acinus is regulated at a pretranslational level. J Histochem Cytochem 1988;36:751-755.
51. Moorman AF, Vermeulen JL, Charles R, Lamers WH. Localization of ammonia-metabolizing enzymes in human liver: ontogenesis of heterogeneity. HEPATOLOGY 1989;9:367-372.
52. Christa L, Simon MT, Flinois JP, Gebhardt R , Brechot C, Lasserre C. Overexpression of glutamine synthetase in human primary liver cancer. Gastroenterology 1994;106:1312-1320.
53. Zucman-Rossi J, Benhamouche S, Godard C, Boyault S, Grimber G, Balabaud C, et al. Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. Oncogene 2007;26:774-780.
54. Audard V, Grimber G, Elie C, Radenen B, Audebourg A, Letourneur F, et al. Cholestasis is a marker for hepatocellular carcinomas displaying beta-catenin mutations. J Pathol 2007;212:345-352.
55. Osada T, Sakamoto M, Nagawa H, Yamamoto J, Matsuno Y, Iwamatsu A, et al. Acquisition of glutamine synthetase expression in human hepatocarcinogenesis. Relation to disease recurrence and possible regulation by ubiquitin-dependent proteolysis. Cancer 1999;85:819-831.
56. Llovet JM, Chen Y, Wurmbach E, Roayaie S, Fiel MI, Schwarts M, et al. A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis. Gastroenterology 2006;131:1758-1767.

Intraductal carcinoma component as a favorable prognostic factor in biliary tract carcinoma

Hidenori Ojima,[1] Yae Kanai,[1,5] Motoki Iwasaki,[2] Nobuyoshi Hiraoka,[1] Kazuaki Shimada,[3] Tsuyoshi Sano,[3] Yoshihiro Sakamoto,[3] Minoru Esaki,[3] Tomoo Kosuge,[3] Michiie Sakamoto[4] and Setsuo Hirohashi[1]

[1]Pathology Division, National Cancer Center Research Institute, [2]Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, [3]Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [4]Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-0016, Japan

(Received July 5, 2008/Revised September 9, 2008/Accepted September 16, 2008/Online publication November 25, 2008)

The aim of this study is to evaluate the prognostic impact of an intraductal carcinoma component and bile duct resection margin status in patients with biliary tract carcinoma. An intraductal carcinoma component was defined as carcinoma within the bile duct outside the main tumor nodule consisting of a subepithelial invasive component. Surgically resected materials from 214 patients were evaluated by histological observations. Seventy-nine patients (36.9%) with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion and venous, lymphatic and perineural involvement in the main tumor nodule. An intraductal carcinoma component was inversely correlated with advanced clinical stage, and was shown to be a significantly favorable prognostic factor by both univariate and multivariate analyses. Proximal (hepatic) side bile duct resection margin status was categorized into negative for tumor cells, positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). Forty-five patients (21.0%) with an R1 resection margin had a poorer prognosis than 148 patients (69.2%) with a negative resection margin, whereas 21 patients (9.8%) with an R1 (is) resection margin did not. In patients with an R1 resection margin, the risk of anastomotic recurrence was higher, and the period until anastomotic recurrence was shorter, than in patients with an R1 (is) resection margin. Surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications. (*Cancer Sci* 2009; 100: 62–70)

Biliary tract carcinoma still has a poor prognosis, and most cases are at an advanced stage when patients present with symptoms. Previous studies of extrahepatic bile duct carcinoma and hilar cholangiocarcinoma have indicated that surgical resection is the only curative treatment for affected patients.[1–10] Biliary tract carcinoma is remarkable because of its tendency for superficial extension by wide intraductal carcinoma.[11–14] However, it is difficult to accurately estimate the extent of the intraductal carcinoma component in the biliary tract on the basis of preoperative imaging studies.[13,15–18] It is feasible that intraoperative histological diagnosis using frozen sections may detect tumor involvement at the bile duct resection margin. Surgeons are required to make an immediate decision about the resection area based on intraoperative frozen section diagnosis. However, to our knowledge, no previous study has examined the clinicopathological significance and prognostic impact of an intraductal carcinoma component with reference to bile duct resection margin status in patients with biliary tract carcinoma.

In this retrospective study, the presence or absence of an intraductal carcinoma component and bile duct resection margin status were evaluated by histological observations of all surgically resected materials from 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent. In order to provide a yardstick for surgeons who depend on the results of frozen section diagnosis during surgery, we examined the correlation between an intraductal carcinoma component and bile duct resection margin status on the one hand, and clinicopathological parameters on the other, and also the prognostic impact of an intraductal carcinoma component and bile duct resection margin status.

Materials and Methods

Patients and specimens. The study included 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent at the National Cancer Center Hospital, Tokyo, Japan, between May 1965 and December 2003. Patients who died in hospital or within 100 days after surgery, and patients who underwent biopsy or palliative surgery, were not included. The included patients comprised 150 men and 64 women, ranging in age from 33 to 83 (mean 63.4) years.

The main tumor nodule was located in the lower, middle and upper thirds of the extrahepatic bile duct, the entire extrahepatic bile duct, the hilar bile duct, and intrahepatic bile duct adjacent to the hilar area in 27, 38, 14, 5, 77, and 53 patients, respectively. Patients with carcinoma of the peripheral intrahepatic bile duct were excluded. Pancreatoduodectomy (PD), extrahepatic bile duct resection (EHBD), hepatic resection with extrahepatic bile duct resection (HR+EHBD), hepatic resection (HR) and combined hepatectomy and pancreatoduodectomy (HPD) were performed in 47, 19, 124, 16 and 8 patients, respectively. The formalin-fixed surgically resected specimens were cut into slices at intervals of 0.5–0.7 cm, and all the sections were embedded in paraffin and routinely processed for microscopical examination. All tumors were classified according to the pathological tumor-node-metastasis (TNM) classification.[19] Intrahepatic bile duct carcinomas adjacent to the hilar area, for which TNM criteria have never been established, were classified according to the TNM classification for extrahepatic bile duct carcinoma. This study was approved by the Ethical Committee of the National Cancer Center, Tokyo.

Evaluation of an intraductal carcinoma component and bile duct resection margin status. The intraductal carcinoma component was defined as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component (Fig. 1). For cases in which intraoperative frozen section diagnosis of the ductal stump had been performed, the proximal (hepatic) side bile duct resection margin status was histologically assessed by review of the frozen section and its re-fixed permanent section with reference

[5]To whom correspondence should be addressed. E-mail: ykanai@ncc.go.jp

doi: 10.1111/j.1349-7006.2008.01009.x

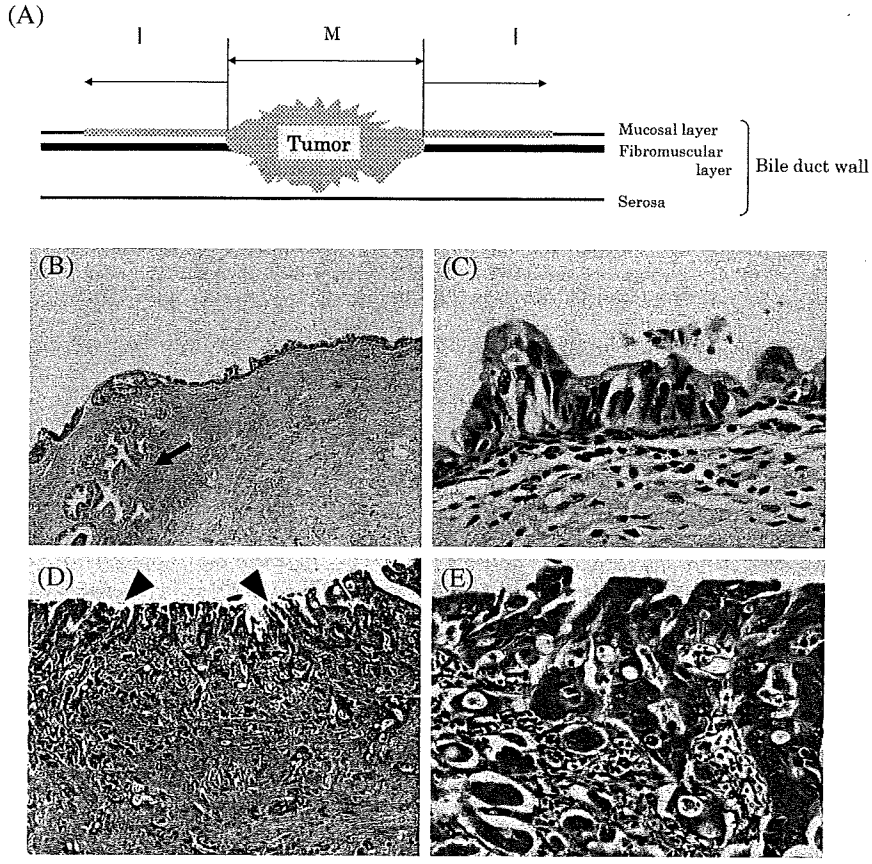

Fig. 1. Definition of an intraductal carcinoma component (I). (A) I is defined as intraductal carcinoma in the bile duct and its small branch outside the main tumor nodule (M) consisting of a submucosal invasive component. (B and C) Microscopic view of an example of I in the bile duct and its small branch (arrow). Hematoxylin and eosin (H&E) stain, original magnification ×40 (B) and ×400 (C). (D and E) Microscopic view of an example of M. Carcinoma *in situ* inside M (arrow heads) is not considered as I in this study. H&E stain, original magnification ×40 (D) and ×200 (E).

to the extent of the tumor in formalin-fixed surgically resected specimens. For cases in which intraoperative frozen section diagnosis of the ductal stump had not been performed, proximal side bile ductal resection margin status was histologically assessed by review of the formalin-fixed surgically resected specimens.

Follow-up and assessment of anastomotic recurrence at the bile duct resection margin. All 214 patients were followed for more than 100 days, and the mean duration of follow-up was 1215 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels every 3–6 months by surgeons. Anastomotic recurrence at the proximal side of the bile duct resection margin was diagnosed only in patients with a positive resection margin. In such patients, when a mass lesion was detected after dilatation of the bile duct in the residual liver because of obstruction of the anastomosis site, using radiological evaluation including computed tomography and ultrasonography, surgeons considered that the patient had anastomotic recurrence (not local recurrence in which perineural invasion around the hepatic artery and/or involved regional nodes first formed a mass lesion). Causes of death were determined from the medical records.

Statistical analyses. Correlations between presence or absence of an intraductal carcinoma component and bile duct resection margin status on the one hand and clinicopathological parameters on the other were analyzed using chi-squared test.

Person-days of follow-up were calculated from the date of surgical resection until date of death or end of the study period (March 8, 2005), whichever occurred first. The crude rate of all-cause deaths was calculated by dividing the number of deaths by the number of person-days. Similarly, person-days of follow-up were calculated from the date of surgical resection until date of death, date of diagnosis of anastomotic recurrence, or end of the study period (March 8, 2005), whichever occurred first. The crude rate of recurrence at the proximal side bile duct resection margin was calculated by dividing the number of cases with recurrence by the number of person-days. Survival curves were constructed using the Kaplan–Meier method, and differences in survival were evaluated using the log-rank test. The Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence interval (CI) of death or anastomotic recurrence by clinicopathologic factors using the SAS program (PROC PHREG) (SAS Institute Inc., Cary NC, US). All tests were two-sided and differences at $P < 0.05$ were considered statistically significant.

Results

Univariate analysis of correlation between an intraductal carcinoma component and clinicopathological parameters. An intraductal carcinoma component was positive in 79 (36.9%) of the 214 examined patients. Correlations between an intraductal carcinoma component and clinicopathological parameters were examined using univariate analysis (Table 1). Location of the main tumor nodule ($P = 0.007$), and histologic type ($P < 0.0001$) were significantly correlated with an intraductal carcinoma component (Table 1). Tumor size ($P = 0.01$), depth of invasion ($P < 0.0001$), venous involvement ($P < 0.0001$), lymphatic involvement ($P = 0.0006$), perineural involvement ($P < 0.0001$), the pathological assessment of the primary tumor (pT) ($P < 0.0001$), and pathological TNM stage ($P < 0.0001$) were each inversely correlated with presence of an intraductal carcinoma component: patients with an intraductal carcinoma component infrequently developed large tumors, infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule, and were infrequently at an advanced stage when diagnosed (Table 1).

Table 1. Correlation between an intraductal carcinoma component and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases		*P* for difference*
	Intraductal carcinoma component		
	Negative ($n = 135$)	Positive ($n = 79$)	
Age (years)			0.01
<65	73	29	
≥65	62	50	
Sex			0.85
Male	94	56	
Female	41	23	
Location of the main tumor nodule			0.007
Lower third of extrahepatic bile duct	15	12	
Middle third of extrahepatic bile duct	17	21	
Upper third of extrahepatic bile duct	6	8	
Entire extrahepatic bile duct	2	3	
Hilar bile duct	54	23	
Intrahepatic bile duct	41	12	
Histologic type			<0.0001
Adenocarcinoma	129	55	
Papillary adenocarcinoma	1	21	
Others	5	3	
Tumor size (cm)			0.13
<3	54	40	
≥3	81	39	
Differentiation of adenocarcinoma			0.50
Well	34	18	
Moderate	80	29	
Poor	15	8	
Depth of invasion			<0.0001
Carcinoma *in situ* or invasion to fibromuscular layer	1	16	
Invasion into subserosa or beyond bile duct wall	134	63	
Venous involvement			<0.0001
Absent	6	19	
Present	129	60	
Lymphatic involvement			0.0006
Absent	9	18	
Present	126	61	
Perineural involvement			<0.0001
Absent	10	24	
Present	125	55	
pT classification			<0.0001
pT1-2	11	40	
pT3-4	124	39	
pN classification			0.06
pN0	64	48	
pN1	71	31	
TNM stage			<0.0001
0, IA, IB	8	32	
IIA	53	14	
IIB	62	28	
III	12	5	

*Chi-squared test.

Univariate analysis of correlation between an intraductal carcinoma component or clinicopathological parameters on the one hand and prognosis of patients on the other. Overall survival rates after resection were 33.2% at 5 years and 22.9% at 10 years. Hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and other clinicopathological parameters were examined using univariate analysis (Table 2). Patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 2).

Multivariate analysis of prognostic impact of an intraductal carcinoma component. When all 214 patients were examined by multivariate analysis adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement and TNM stage, patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 3). When only the 117 patients who underwent complete resection (proximal side bile duct resection margin for all

doi: 10.1111/j.1349-7006.2008.01009.x

Table 2. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate†	Crude HR	95% CI	*P* for trend
Intraductal carcinoma component						
Negative	96	136 804	70.2	1.00		
Positive	35	123 209	28.4	0.39	0.27, 0.58	
Age (years)						
<65	58	137 562	42.2	1.00		
≥65	73	122 451	59.6	1.33	0.94, 1.87	
Sex						
Male	94	179745	52.3	1.00		
Female	37	80 268	46.1	0.84	0.57, 1.23	
Location of the main tumor nodule						
Lower third of extrahepatic bile duct	17	43 899	38.7	1.00		
Middle third of extrahepatic bile duct	23	43 696	52.6	1.04	0.56, 1.96	
Upper third of extrahepatic bile duct	10	18 228	54.9	1.17	0.54, 2.56	
Entire of extrahepatic bile duct	3	4837	62.0	1.07	0.31, 3.67	
Hilar bile duct	46	87 502	52.6	1.09	0.63, 1.91	
Intrahepatic bile duct	32	61 851	51.7	1.14	0.63, 2.06	
Histologic type						
Adenocarcinoma	123	211 330	58.2	1.00		
Papillary adenocarcinoma	5	37 000	13.5	0.25	0.10, 0.62	
Others	3	11 683	25.7	0.51	0.16, 1.61	
Tumor size (cm)						
<3	46	134 392	34.2	1.00		
≥3	85	125 621	67.7	1.82	1.27, 2.61	
Differentiation of adenocarcinoma						
Well	36	70 278	51.2	1.00		
Moderately	67	126 210	53.1	1.13	0.75, 1.69	
Poorly	20	14 842	134.8	2.56	1.47, 4.44	
Depth of invasion						
Carcinoma *in situ* or invasion to fibromuscular layer	3	37 435	8.0	1.00		
Invasion into subserosa or beyond bile duct wall	128	222 578	57.5	6.44	2.04, 20.3	
Venous involvement						
Absent	6	63 305	9.5	1.00		
Present	125	196 708	63.5	5.80	2.54, 13.3	
Lymphatic involvement						
Absent	9	76 294	11.8	1.00		
Present	122	183 719	66.4	4.67	2.25, 9.67	
Perineural involvement						
Absent	11	72 565	15.2	1.00		
Present	120	187 448	64.0	3.67	1.95, 6.89	
pT classification						
pT1-2	23	89 367	25.7	1.00		
pT3-4	108	170 646	63.3	2.32	1.47, 3.66	
pN classification						
pN0	57	176 738	32.3	1.00		
pN1	74	83 275	88.9	2.56	1.80, 3.65	
TNM stage						
0,IA,IB	15	77 359	19.4	1.00		<0.01
IIA	39	91 428	42.7	2.26	1.24, 4.12	
IIB	65	75 858	85.7	4.21	2.37, 7.46	
III	12	15 368	78.1	3.80	1.77, 8.15	

†per 100 000 person-days.

patients, distal [duodenal] side bile duct resection margin for patients who underwent HR + EHBR, resected margin of the pancreas for patients who underwent PD were all negative) were examined in order to eliminate the effect of surgical curability, an intraductal carcinoma component was still a favorable prognostic factor (Table 3).

Correlation between an intraductal carcinoma component and bile duct resection margin status. Although an intraductal carcinoma component has been proven to be a favorable prognostic factor, it is feasible that patients with such components frequently have tumor involvement at the bile duct resection margin. Therefore, the correlation between an intraductal carcinoma component and proximal side bile duct resection margin status (negative or positive) was examined statistically (Table 4). An intraductal carcinoma component was found to be correlated with bile duct resection margin status: patients with an intraductal carcinoma component more frequently had a positive resection margin than patients without such a component ($P = 0.0192$, Table 4). In

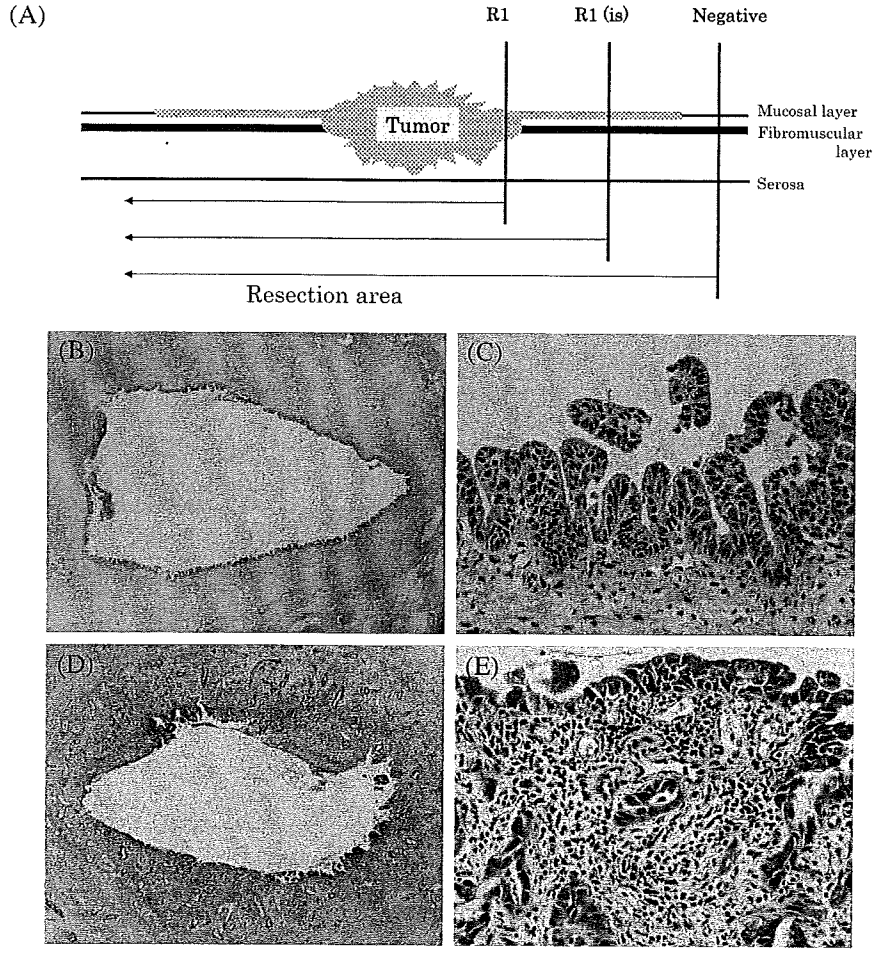

Fig. 2. Definition of bile duct resection margin status. (A) Bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). (B and C) Microscopic view of an example of an R1 (is) bile duct resection margin. Hematoxylin and eosin (H&E) stain, original magnification ×12.5 (B) and ×200 (C). (D and E) Microscopic view of an example of an R1 bile duct resection margin. H&E stain, original magnification ×12.5 (D) and × 200 (E).

Table 3. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by an intraductal carcinoma component

	Adjusted HR	95% CI
Total (214 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.50	0.31, 0.79
Complete resection cases (117 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.31	0.13, 0.73

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement, and TNM stage.

Table 4. Correlation between an intraductal carcinoma component and proximal side bile duct resection margin status

	No. of cases		*P* for difference*
	Bile duct resection margin status		
	Negative (*n* = 148)	Positive (*n* = 66)	
Intraductal carcinoma component			0.0192
Negative	101	34	
Positive	47	32	

*Chi-squared test.

order to further examine the clinicopathological significance and prognostic impact of bile duct resection margin status with reference to an intraductal carcinoma component, proximal side bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], or positive with a subepithelial invasive component (R1) (Fig. 2). According to the International Union Against Cancer, when invasive carcinoma is completely resected but histology shows an *in situ* component at the resection margin, the residual tumor is defined as R1 (is).[20] When the surgeon considers that resection has been complete but histology shows invasive carcinoma at the resection margin, the residual tumor is defined as R1.[20]

Univariate analysis of correlations between bile duct resection margin status and clinicopathological parameters. Bile duct resection margin status was negative, R1 (is) and R1 in 148 (69.2%), 21 (9.8%) and 45 (21.0%) of the 214 examined patients, respectively. Correlations between bile duct resection margin status and clinicopathological parameters were examined by univariate analysis (Table 5). Location of the main tumor nodule ($P = 0.0004$), histological type ($P = 0.008$) and venous involvement ($P = 0.009$) were each significantly correlated with bile duct resection margin status (Table 5).

doi: 10.1111/j.1349-7006.2008.01009.x

Table 5. Correlation between bile duct resection margin status and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases			*P* for difference*
	Proximal side ductal resection margin			
	Negative ($n = 148$)	R1 (is) ($n = 21$)	R1 ($n = 45$)	
Age (years)				0.09
< 65	77	6	19	
≥ 65	71	15	26	
Sex				0.81
Male	103	16	31	
Female	45	5	14	
Location of the main tumor nodule				0.004
Lower third of extrahepatic bile duct	23	2	2	
Middle third of extrahepatic bile duct	25	8	5	
Upper third of extrahepatic bile duct	8	1	5	
Entire of extrahepatic bile duct	0	1	4	
Hilar bile duct	53	7	17	
Intrahepatic bile duct	39	2	12	
Histologic type				0.008
Adenocarcinoma	129	13	42	
Papillary adenocarcinoma	15	6	1	
Others	4	2	2	
Tumor size (cm)				0.34
< 3	65	12	17	
≥ 3	83	9	28	
Differentiation of adenocarcinoma				0.16
Well	33	7	12	
Moderately	78	4	27	
Poorly	18	2	3	
Depth of invasion				0.06
Carcinoma *in situ* or invasion to fibromuscular layer	14	3	0	
Invasion into subserosa or beyond bile duct wall	134	18	45	
Venous involvement				0.009
Absent	20	5	0	
Present	128	16	45	
Lymphatic involvement				0.18
Absent	22	3	2	
Present	126	18	43	
Perineural involvement				0.57
Absent	26	3	5	
Present	122	18	40	
pT classification				0.08
pT1-2	37	8	6	
pT3-4	111	13	39	
pN classification				0.48
pN0	80	12	20	
pN1	68	9	25	
TNM stage				0.35
0, IA, IB	28	7	5	
IIA	48	5	14	
IIB	59	9	22	
III	13	0	4	

*Chi-squared test.

Univariate and multivariate analysis of prognostic impact of bile duct resection margin status. Univariate analysis revealed that although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 6). Surgical resection procedure, which was not examined in Table 3, is addressed in Table 6. None of the 66 patients with a positive resection margin [both R1 (is) and R1] had received any adjuvant therapy until recurrence was diagnosed.

When adjusted for age, operation day, surgical resection procedure, tumor size, histologic type, tumor differentiation, depth of invasion and venous involvement, although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 7).

Table 6. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by bile duct resection margin status and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate*	Crude HR	95% CI	*P* for trend*
Bile duct resection margin status						
Negative	82	209 492	39.1	1.00		<0.01
R1 (is)	11	22 476	48.9	1.00	0.53, 1.88	
R1	38	28 045	135.5	2.80	1.88, 4.18	
Surgical resection procedure						
PD	29	62 430	46.5	1.00		
EHBR	14	26 842	52.2	1.03	0.54, 1.94	
HR+EHBR	75	129 281	58.0	1.08	0.70, 1.67	
HR	11	32 569	33.8	0.86	0.43, 1.73	
HPD	2	8891	22.5	0.45	0.11, 1.87	

*per 100 000 person-days.
PD: pancreatoduodectomy, EHBD: extrahepatic bile duct resection, HR + EHBD: hepatic resection with extrahepatic bile duct resection, HR: hepatic resection, HPD: combined hepatectomy and pancreatoduodectomy. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

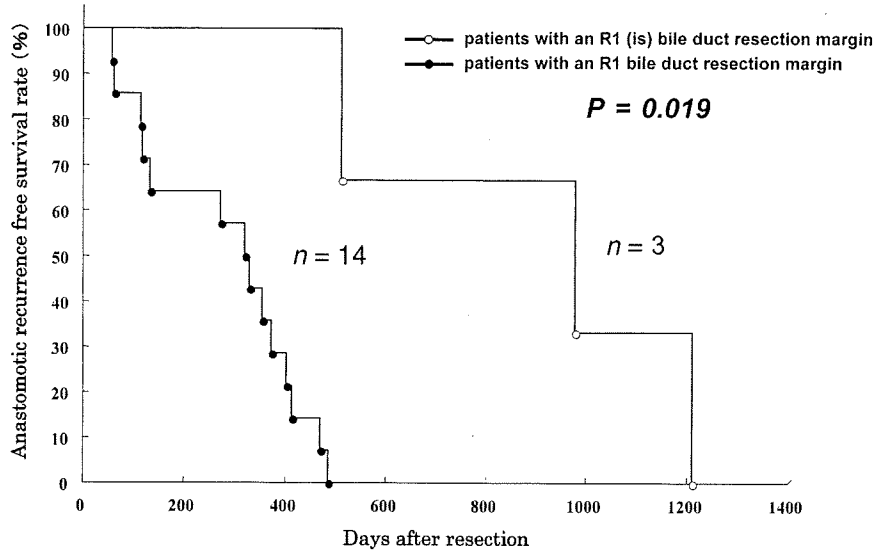

Fig. 3. Anastomotic recurrence-free survival rate of patients with a positive bile duct resection margin. Kaplan-Meier analysis revealed that the period until anastomotic recurrence at the bile duct resection margin after surgical resection in patients with a subepithelial invasive component bile duct resection margin (solid circles) was significantly shorter than that in patients with an intraductal carcinoma component bile duct resection margin (clear circles) ($P = 0.019$).

Table 7. Adujusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by bile duct resection margin status

	Adjusted HR†	95% CI
Total (214 cases)		
Bile duct resection margin status		
Negative	1.00	
R1 (is)	1.06	0.53, 2.10
R1	1.95	1.27, 3.00

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, and venous involvement. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

Correlation between bile duct resection margin status and anastomotic recurrence at the bile duct resection margin. In order to understand the background factors responsible for the difference in prognostic impact between R1 (is) and R1 bile duct resection margins, the correlation between bile duct resection margin status [R1 (is) *vs* R1] and anastomotic recurrence at the bile duct resection margin was examined by multivariate analysis. The risk of anastomotic recurrence in patients with an R1 bile duct resection margin was 4.5 times higher than that in patients with an R1 (is) bile duct resection margin (Table 8). In addition, the Kaplan-Meier method revealed that the period until anastomotic recurrence after surgical resection in patients with an R1 bile duct resection margin was significantly shorter than that in patients with an R1 (is) bile duct resection margin (Fig. 3, $P = 0.019$).

Discussion

Unlike several previously published studies analyzing the prognostic parameters in small series of patients with biliary tract carcinoma,[21–28] the present study examined in detail the clinicopathological parameters of 214 patients who underwent radical surgery with curative intent and had been strictly followed up at a single institution.

It has recently been reported that biliary tract carcinoma has a marked tendency for superficial extension by wide intraductal carcinoma[11–14] In this study, we defined the intraductal carcinoma component as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component. Surprisingly, an intraductal carcinoma component outside the main tumor nodule was significantly correlated with lower aggressiveness in the main tumor nodule:

doi: 10.1111/j.1349-7006.2008.01009.x