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Original Paper

Dicer is required for proper liver zonation

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Abstract

A number of genes and their protein products are expressed within the liver lobules in a region-specific manner and confer heterogeneous metabolic properties to hepatocytes; this phenomenon is known as 'metabolic zonation'. To elucidate the roles of Dicer, an endoribonuclease III type enzyme required for microRNA biogenesis, in the establishment of liver zonation, we examined the distribution of proteins exhibiting pericentral or periportal localization in hepatocyte-specific *Dicer1* knockout mouse livers. Immunohistochemistry showed that the localization of pericentral proteins was mostly preserved in *Dicer1*-deficient livers. However, glutamine synthetase, whose expression is normally confined to a few layers of hepatocytes surrounding the central veins, was expressed in broader pericentral areas. Even more striking was the observation that all the periportal proteins that were examined, including phosphoenolpyruvate carboxykinase, E-cadherin, arginase 1, and carbamoyl phosphate synthetase-I, lost their localized expression patterns and were diffusely expressed throughout the entire lobule. Thus, with regard to periportal protein expression, the consequences of Dicer loss were similar to those caused by the disruption of β-catenin. An analysis of livers deficient in β-catenin did not identify the down-regulation of *Dicer1* or any microRNAs, indicating that they are not directly activated by β-catenin. Thus, the present study illustrates that Dicer plays a pivotal role in the establishment of liver zonation. Dicer is essential for the suppression of periportal proteins by Wnt/β-catenin/TCF signalling, albeit it likely acts in an indirect manner.

Keywords: Dicer; β-catenin; liver; zonation

Introduction

Although hepatocytes are uniform at a histological level, they differ in a number of metabolic functions [1,2]. For example, pericentral hepatocytes are active in glutamine and bile acid synthesis and the metabolism of xenobiotics, whereas periportal hepatocytes are more active in cholesterol, urea, and glucose synthesis [1,2]. The metabolic heterogeneity of hepatocytes enables multiple and occasionally antagonistic metabolic functions to be performed efficiently in the liver. A number of genes and their protein products involved in these metabolic processes are expressed in a region-specific manner along the porto-central axis within the liver lobule and their respective functions confer the heterogeneous metabolic properties of hepatocytes [1–3].

Recent studies have revealed that the Wnt signalling pathway plays a key role in the establishment of liver zonation [4,5]. The Wnt signalling pathway is activated by the binding of secreted Wnt ligands to Frz and Lrp receptors on cell membranes. This leads to the stabilization of β-catenin through the inhibition of proteosomal degradation, and the translocation of the protein to the nucleus, where it activates TCF-dependent transcription [6]. β-Catenin/TCF-dependent transcription is normally active in the pericentral hepatocytes, where it induces pericentral gene expression while suppressing periportal gene expression [4,7]. The hepatocyte-specific ablation of Apc, leading to the constitutive activation of β-catenin/TCF-dependent transcription, resulted in the diffuse expression of pericentral genes and the down-regulation of periportal genes throughout the entire liver lobule [4,8]. Conversely, the suppression of Wnt signalling by the overexpression of Dkk1 or the conditional ablation of β-catenin caused a loss of pericentral gene expression and the diffuse expression of periportal genes [4,9]. Braeuning *et al* further showed that activation of the Ras/MAPK pathway by the oncogenic form of H-ras or serum components suppressed pericentral genes and induced periportal genes through the inhibition of β-catenin/TCF-dependent transcription [10,11]. Thus, the expression of pericentral and periportal genes is coordinately and inversely regulated by Wnt/β-catenin/TCF signalling.

Dicer is an essential component of microRNA biogenesis that cleaves pre-microRNAs into mature microRNAs. Since Dicer is encoded by a single locus in the mouse genome, the disruption of the single *Dicer1* gene results in the loss of virtually all microRNAs [12–14]. Here, we demonstrate that Dicer plays an essential role in the establishment of proper liver zonation. Remarkably, the loss of Dicer impairs the localization of periportal proteins, leaving the expression of pericentral proteins mostly intact. Thus, our results reveal the novel finding that microRNAs appear to be specifically required for the suppression of periportal protein expression.

Materials and methods

Mice

Alb-Cre [15,16], *Dicer1*$^{flox/flox}$ [12], *Ctnnb1*$^{flox/flox}$ [17], *Alb-Cre;Ctnnb1*$^{flox/flox}$ [7,9], and *Alb-Cre;Dicer*$^{flox/flox}$ [18] mice have been previously described. The mice used in the present study were maintained in barrier facilities and all studies were conducted in compliance with the University of California IACUC (Institutional Animal Care and Use Committee) guidelines and according to protocols approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center, Japan.

Immunohistochemistry

Liver tissue samples were fixed with 10% buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections. Immunohistochemistry was performed by an indirect immunoperoxidase method using peroxidise-labelled anti-mouse, -rabbit or -goat polymers (Histofine Simple Stain, Nichirei, Tokyo, Japan). 3,3′-Diaminobenzidine tetrahydrochloride was used as a chromogen. The primary antibodies that were used are listed in Table 1. For double immunofluorescence staining, anti-mouse IgG antibody conjugated with Alexa Fluor 488 and anti-rabbit IgG antibody conjugated with Alexa Fluor 594 were used as secondary antibodies. The sections were analysed using a confocal microscope (LSM5 Pascal; Carl Zeiss Jena GmbH, Jena, Germany) equipped with a 15 mW Kr/Ar laser.

Quantitative PCR

RNA extraction and the reverse-transcription reaction were performed using standard protocols. Quantitative PCR reactions were performed using SYBR Green PCR master mix (Applied Biosystems, CA, USA). The expression of *Dicer1* was compared with the expression level of *Gusb*, as previously described [9]. The primer sequences were as follows: *Dicer1*: GAACGAAATGCAAGGAATGGA and GGGACTTCGATATCCTCTTCTTTCTC; *Gusb*: ACGGGATTGTGGTCATCGA and TCGTTGCCAAAACTCTGAGGTA.

Microarray analysis

RNA samples were prepared from liver tissues of 6-week-old female *Alb-Cre;Ctnnb1*$^{flox/flox}$ and *Ctnnb1*$^{flox/flox}$ mice. The samples were labelled with a miRNA Labeling Reagent & Hybridization Kit (Agilent Technologies, CA, USA) based on the manufacturer's instructions. The labelled RNA samples were hybridized with a mouse miRNA microarray (Agilent Technologies) containing 566 mouse miRNA probes based on Sanger miRBase v10.0. MicroRNAs that showed more than two-fold changes with $p < 0.05$ (Welch t-test) were considered significant.

Results

Localization of pericentral proteins is only marginally affected in Dicer mutant mice

To elucidate the roles of Dicer in liver zonation, liver samples from *Alb-Cre;Dicer*$^{flox/flox}$ mice and their control littermates (*Dicer*$^{flox/flox}$) were immunohistochemically examined. As previously reported, the efficient deletion of *Dicer1* in hepatocytes was achieved in 3-week-old *Alb-Cre;Dicer*$^{flox/flox}$ mice; however, *Dicer1*-deficient hepatocytes were prone to apoptosis and the complete disruption of *Dicer1* was followed by repopulation with *Dicer1*-expressing hepatocytes that had escaped Cre-mediated recombination [18]. We therefore examined the livers from 3-week-old *Alb-Cre;Dicer*$^{flox/flox}$ and *Dicer*$^{flox/flox}$ mice (hereafter referred to as Dicer-deficient and control livers).

Immunohistochemistry showed that the localization of pericentral proteins was mostly maintained

Table 1. Antibodies used for immunohistochemistry

Antigen	Clone	Dilution	Source
Glutamine synthetase	6	1:1000	Becton Dickinson, Franklin Lakes, USA
GLT-1	Polyclonal	1:500	Dr Masahiko Watanabe [24]
OAT	Polyclonal	1:500	Santa Cruz Biotechnologies, Santa Cruz, USA
CYP2E1	Polyclonal	1:500	Dr Magnus Ingelman-Sundberg [25]
E-cadherin	36	1:250	Becton Dickinson, Franklin Lakes, USA
PEPCK	Polyclonal	1:200	Santa Cruz Biotechnologies, Santa Cruz, USA
CPS1	Polyclonal	1:500	Santa Cruz Biotechnologies, Santa Cruz, USA
Arginase I	19	1:2500	Becton Dickinson, Franklin Lakes, USA

OAT = ornithine aminotransferase; PEPCK = phosphoenolpyruvate carboxykinase; CPS1 = carbamoyl phosphate synthetase-I.

in the absence of Dicer. The distributions of GLT-1, ornithine aminotransferase (OAT), and CYP2E1 were unaltered in Dicer-deficient livers. GLT-1 and OAT were expressed in a few layers of hepatocytes surrounding the central veins (Figures 1A, 1B, 1D, and 1E). CYP2E1 was expressed in broader pericentral areas (Figures 1J and 1K). Expression of glutamine synthetase (GS) was observed in the pericentral areas of both mice; however, the GS-positive areas were significantly broader in the Dicer-deficient livers (Figures 1G and 1H).

The altered localization of GS was confirmed by double immunofluorescence staining for GS and CYP2E1. In control mouse livers, distinct distributions of these proteins were evident: GS expression was restricted to a few layers of hepatocytes surrounding the central veins, whereas CYP2E1 expression was extended to the distal pericentral

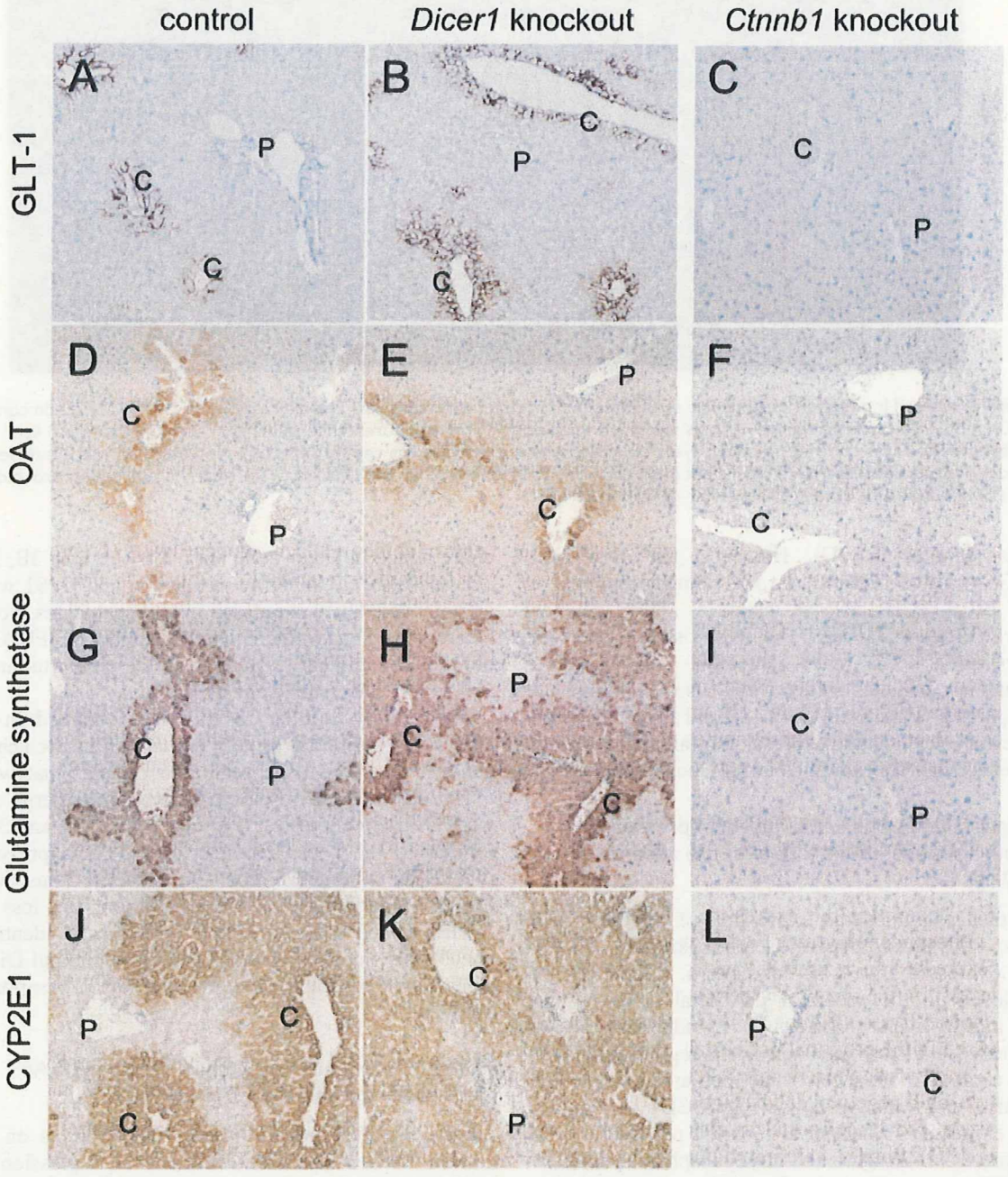

Figure 1. Expression of pericentral proteins in Dicer-deficient liver. Pericentral protein expression was examined using immunohistochemistry. The distributions of GLT-1, OAT, and CYP2E1 were unaltered in Dicer-deficient liver (B, E, K) compared with those in control mouse liver (*Dicer*$^{flox/flox}$) (A, D, J). Glutamine synthetase maintained its pericentral localization in Dicer-deficient liver (H), but its expression extended beyond its normal boundary and encompassed a broader area than that observed in control mouse liver (G). Pericentral protein expression was completely lost in β-catenin-deficient livers (C, F, I, L). C = pericentral vein; P = portal tract

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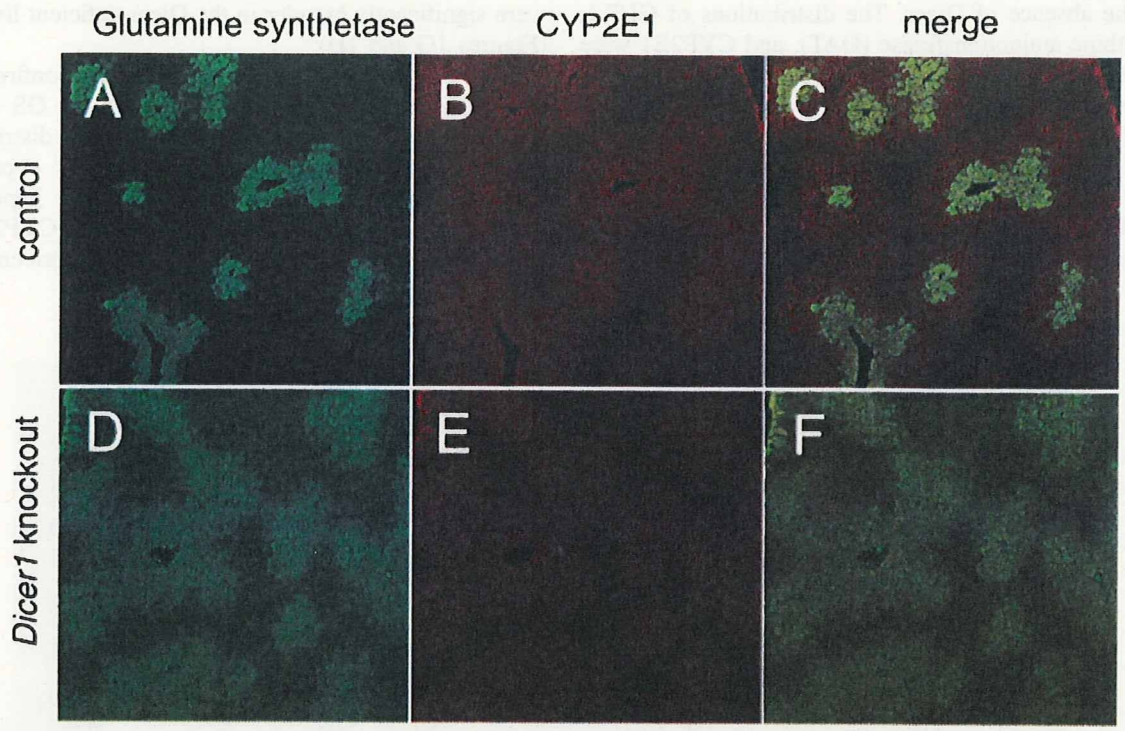

Figure 2. Altered localization of glutamine synthetase (GS) in Dicer-deficient liver. The expression of GS and CYP2E1 in control (A–C) and Dicer-deficient liver (D–F) was examined using double immunofluorescence staining. In the control mouse liver, the expression of GS was confined to a few layers of hepatocytes surrounding the central veins (A). However, GS was expressed in broader pericentral areas in Dicer-deficient livers (D). The distributions of GS and CYP2E1 were clearly distinct in control mouse liver (C) but were almost identical in Dicer-deficient liver (F)

areas (Figures 2A–2C). However, the distribution of GS-positive hepatocytes was almost identical to that of CYP2E1-positive cells in *Dicer1*-deficient livers (Figures 2D–2F). The liver tissues from *Alb-Cre;Ctnnb1*$^{flox/flox}$ mice (hereafter referred to as β-catenin-deficient livers) were also examined for comparison (Figures 1C, 1F, 1H, and 1K). β-Catenin-deficient livers lost the expression of all the pericentral proteins that were examined as previously reported [9].

Periportal proteins are diffusely expressed throughout the entire lobule in the absence of Dicer

We then examined the expression of periportal proteins. Phosphoenolpyruvate carboxykinase (PEPCK) was expressed in a gradient pattern, with the highest levels in the proximal periportal areas in control mouse livers (Figure 3A). E-cadherin was also expressed in the proximal periportal regions but exhibited a more pronounced sharp demarcation between positive and negative cells (Figure 3D). Arginase 1 was expressed in periportal to distal pericentral areas (Figure 3G). Finally, carbamoyl phosphate synthetase-I (CPS1) expression was found throughout the liver lobules, with the exception of a few layers of perivenous hepatocytes; this distribution was complementary to that of GS (Figure 3J). Remarkably, all of these periportal proteins lost their localized expression patterns and appeared in a diffuse pattern throughout the entire lobule in Dicer-deficient livers (Figures 3B, 3E, 3H, and 3K). E-cadherin, arginase 1, and CPS1 were all homogeneously expressed in all hepatocytes. Some heterogeneity was noted in the staining for PEPCK, but the predominant expression in the periportal areas was lost in Dicer-deficient livers.

Interestingly, similar results were obtained in an analysis of periportal protein expression in β-catenin-deficient livers. All the periportal proteins that were examined lost their restricted expression patterns and were diffusely expressed (Figures 3C, 3F, 3I, and 3L). Similar to the Dicer-deficient livers, the expression of PEPCK exhibited minor heterogeneity. Thus, with regard to periportal protein expression, the loss of β-catenin and Dicer resulted in virtually identical phenotypes, suggesting that both β-catenin and Dicer are required for the localized expression of periportal proteins.

Neither *Dicer1* nor any individual microRNAs are directly activated by β-catenin/TCF

Previous studies and the present observations on β-catenin-deficient livers showed that the expression of pericentral proteins is dependent on active Wnt/β-catenin signalling [4,9]. The conserved pericentral protein expression therefore indicates that Wnt signalling is still active in the pericentral hepatocytes of Dicer-deficient livers. On the other hand, periportal proteins are diffusely expressed throughout the liver lobules in

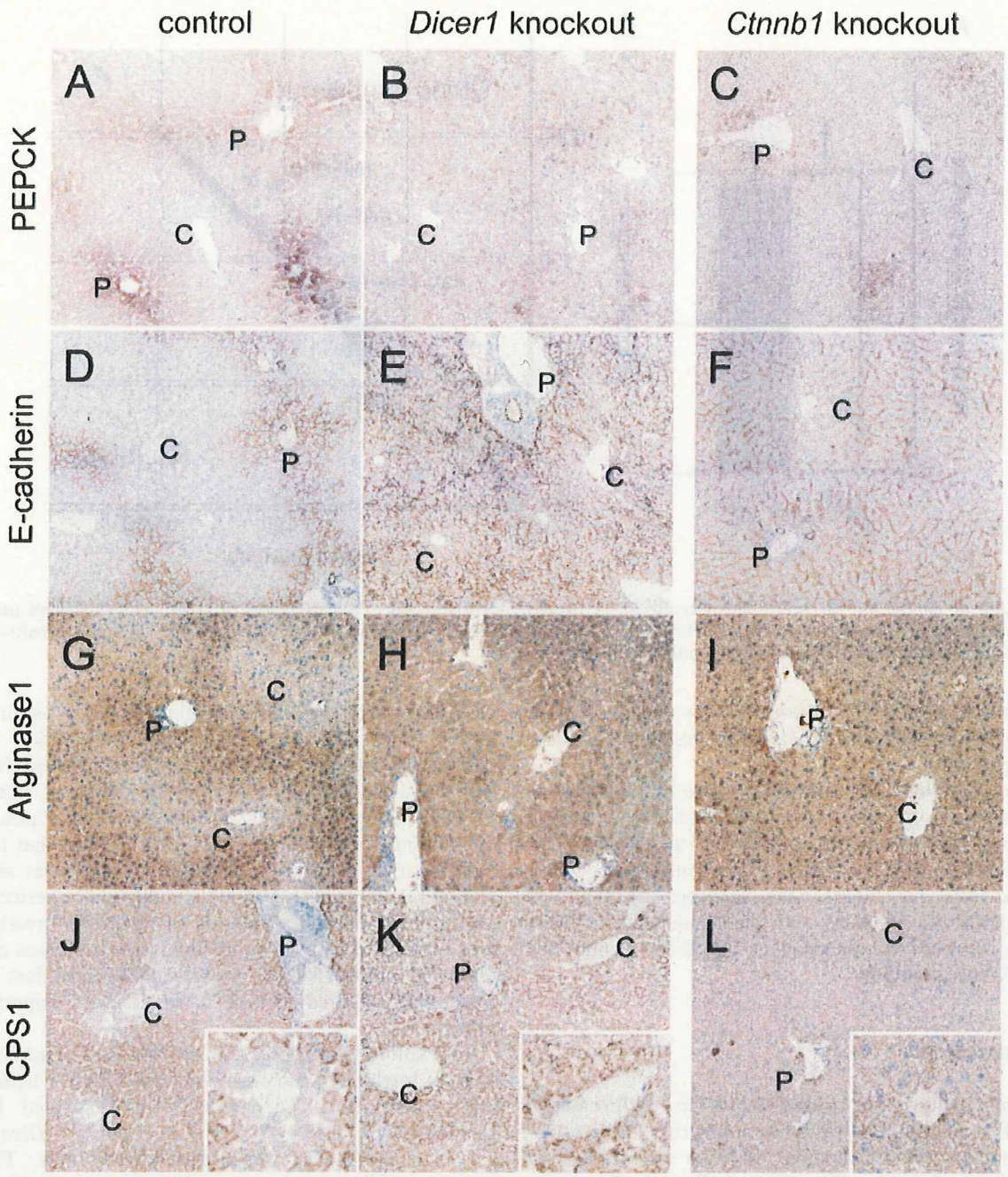

Figure 3. Expression of periportal proteins in Dicer-deficient liver. Periportal protein expression was examined using immunohistochemistry. The characteristic distributions of the periportal proteins in the control mouse liver (A, D, G, J) were completely lost in the Dicer-deficient (B, E, H, K) and β-catenin-deficient livers (C, F, I, L). High-magnification views of the pericentral areas are presented as insets for CPS1 (J–L). C = pericentral vein; P = portal tract

Dicer-deficient livers. This finding suggests that Dicer is essential for the repression of periportal proteins achieved by active Wnt signalling and that Dicer may act downstream of β-catenin/TCF. However, *Dicer1* expression was not affected in β-catenin-deficient livers, indicating that *Dicer1* itself is not involved immediately downstream of Wnt signalling (Figure 4A).

Furthermore, we performed a microarray analysis to test whether individual microRNAs are regulated by β-catenin. Similarly, a comparison of β-catenin-deficient and control livers identified no microRNAs whose expression levels were down-regulated in β-catenin-deficient livers. Thus, we did not find any microRNAs that were directly activated by β-catenin/TCF signalling (Figure 4B and Supporting information, Supplementary Table 1). On the other hand, the analysis identified four microRNAs that were up-regulated in β-catenin-deficient livers: miR-31 (2.84-fold), miR-34a (2.77-fold), miR-31* (2.91-fold), and miR-193b (2.21-fold). However, considering the modest increase

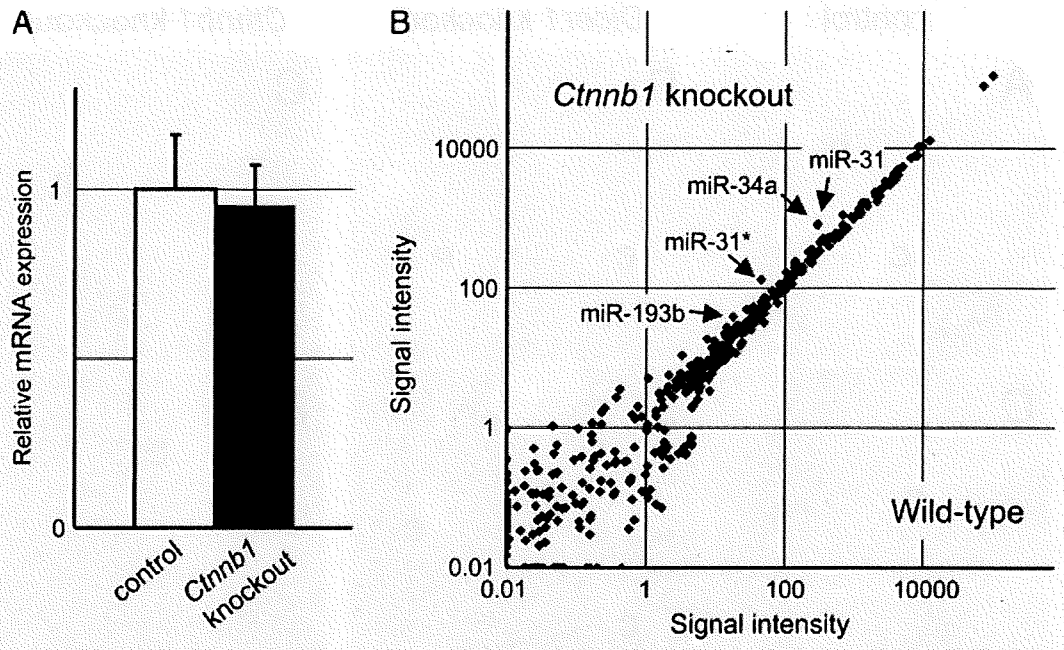

Figure 4. Expression of *Dicer1* and microRNAs in β-catenin-deficient liver. (A) Expression of *Dicer1* as determined using quantitative PCR ($n = 6$ per group). (B) Microarray analysis of microRNA expression ($n = 3$ per group). The four microRNAs with significantly altered expressions are indicated by the arrows

of these microRNAs, these changes are unlikely to explain the dramatically altered expression of periportal proteins.

In summary, our data demonstrate that neither the expression of *Dicer1* nor that of individual microRNAs is dependent on β-catenin/TCF signalling. Thus, while Dicer and β-catenin elimination results in similar defects with regard to the inappropriate expression of periportal proteins, our results indicate that Dicer and microRNA expression is not directly controlled by Wnt signalling.

Discussion

Recent studies have suggested that the Wnt/β-catenin/TCF signalling pathway plays a key role in the establishment of liver zonation [4,5]. As observations of β-catenin-deficient liver have indicated, β-catenin-mediated signalling is essential for both the expression of pericentral proteins and the repression of periportal proteins in pericentral hepatocytes. Even though MAPK signalling has been reported to affect zonation through the modulation of β-catenin/TCF-dependent transcription [11], the mechanisms underlying the establishment of zonation remain largely undefined. The present study identified Dicer as a novel and essential component in the establishment of one aspect of liver zonation, the repression of periportal proteins in pericentral areas.

The hepatocyte-specific loss of Dicer resulted in the diffuse expression of proteins that are normally localized to the periportal areas. On the other hand, the localization of pericentral proteins was left mostly unaltered. Since pericentral protein expression requires active Wnt signalling [4,8,9], the conservation of pericentral protein expression in Dicer-deficient livers indicates that the loss of Dicer does not affect Wnt activity in pericentral hepatocytes. In contrast, our findings in Dicer-deficient livers suggest that the repression of periportal proteins by active Wnt signalling requires Dicer. While the induction of pericentral proteins and the repression of periportal proteins are coordinated by Wnt signalling, these processes are regulated independently of each other, and Dicer is selectively required for the repression of periportal proteins.

To explore how Dicer is involved in the repression of periportal proteins, we first tested whether the expression of Dicer itself was regulated by β-catenin/TCF; however, the expression of *Dicer1* was not altered in β-catenin-deficient livers. The primary physiological role of Dicer is microRNA processing [12,19]. While Dicer has microRNA-independent functions, such as endogenous siRNA processing in at least some organs [20,21], Dicer's functions are generally thought to be largely mediated by microRNAs. Since microRNA precursors are mostly transcribed by RNA polymerase II [22], some microRNAs might be transactivated by β-catenin/TCF, resulting in suppression of periportal genes. Nevertheless, we could not identify any microRNAs that were down-regulated in β-catenin-deficient livers. Collectively, these observations imply that Dicer plays an essential role in the repression of periportal proteins at some point downstream of β-catenin/TCF signalling, albeit this effect likely occurs through an indirect mechanism.

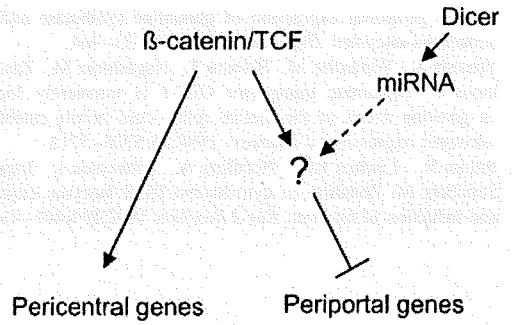

Figure 5. Model of the regulation of zonal gene expression. β-Catenin/TCF transactivates pericentral genes as well as represses periportal genes. Dicer and microRNAs are essential for the repression of periportal genes, but are not directly regulated by β-catenin

The disruption of Dicer did not have a major effect on the localization of pericentral proteins, but it did result in the expression of GS in a broader area. This finding indicates that a suppressive signal mediated by Dicer is required for the repression of GS in distal pericentral areas. A previous study reported that loss of Hnf4a also caused aberrant GS expression [23]. However, the loss of Hnf4a resulted in weak expression of GS in the entire lobule, unlike in Dicer-deficient livers, and the expression of Hnf4a was not altered in Dicer-deficient livers [18]. While the loss of Dicer and Hnf4a both affected the localization of GS, these two molecules seem to regulate GS expression through independent mechanisms.

In summary, the present study shows that Dicer is required for the establishment of proper liver zonation. Dicer is essential for the suppression of periportal proteins by Wnt/β-catenin/TCF signalling, albeit neither Dicer itself nor any individual microRNAs are directly activated by β-catenin/TCF. Our results suggest that Dicer regulates factor(s) that suppress periportal genes at some point downstream of β-catenin (Figure 5). However, the individual microRNAs responsible for the repression of periportal proteins remain elusive at present. Further studies of individual microRNAs should help to elucidate the precise mechanisms by which these factors regulate zonal gene expression in the liver.

Acknowledgements

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Table S1. MicroRNA expression in β-catenin-deficient liver

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

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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

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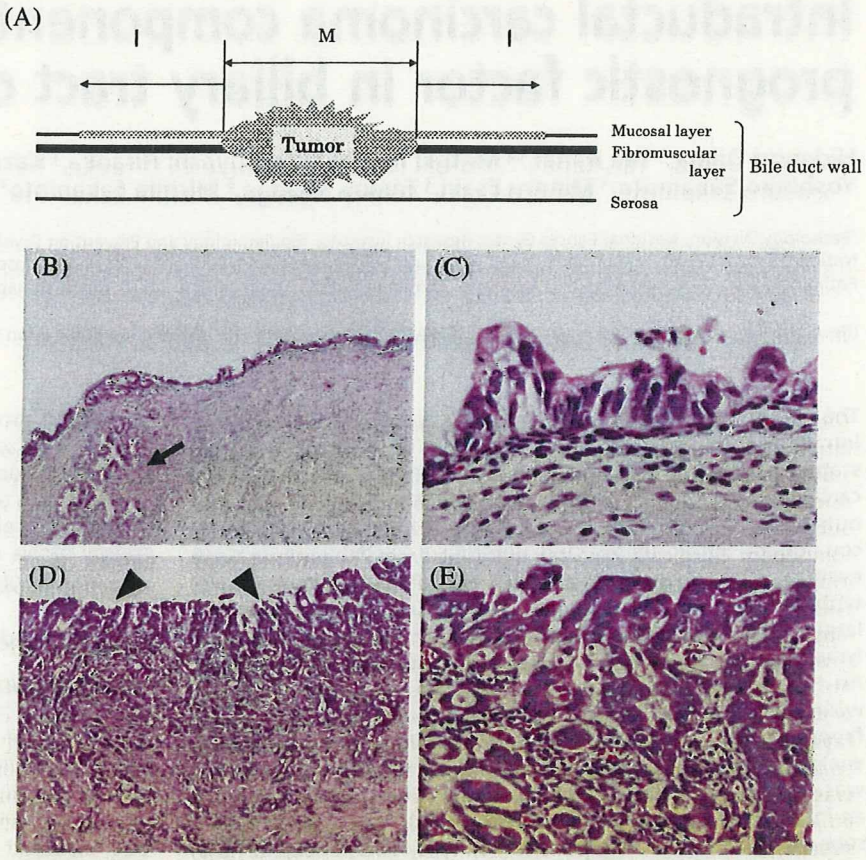

Fig. 1. Definition of an intraductal carcinoma component (I). (A) I is defined as intraductal carcinoma in the bile duct and its small branch outside the main tumor nodule (M) consisting of a submucosal invasive component. (B and C) Microscopic view of an example of I in the bile duct and its small branch (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

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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

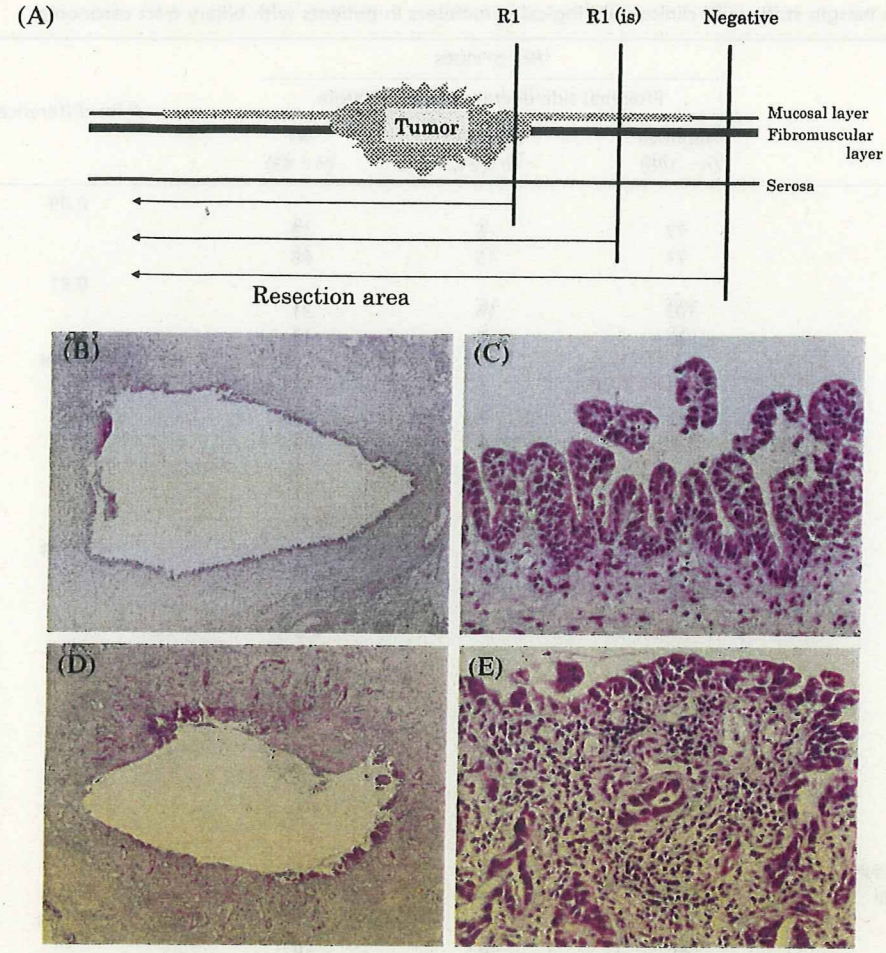

Fig. 2. Definition of bile duct resection margin status. (A) Bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). (B and C) Microscopic view 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).

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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.

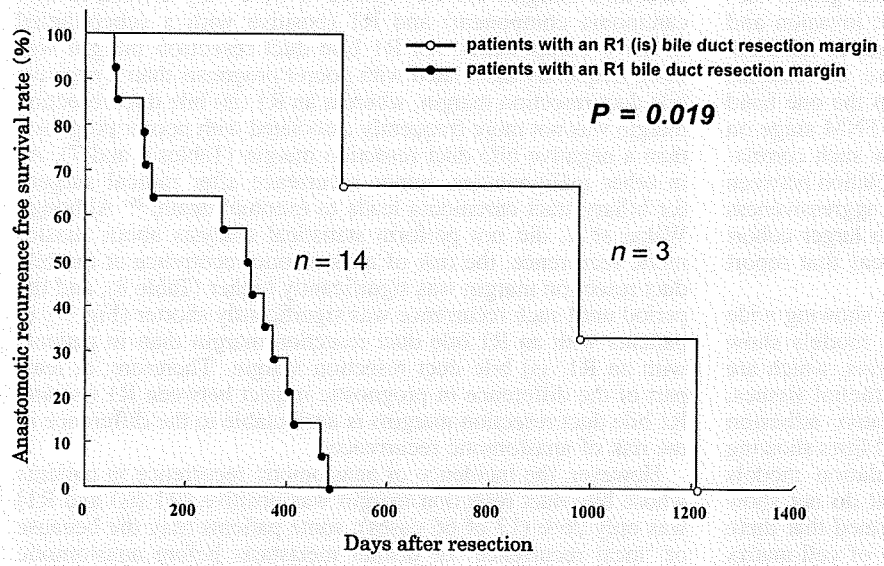

Fig. 3. Anastomotic recurrence-free survival rate of patients with a positive bile duct resection margin. Kaplan-Meier analysis revealed that the period until anastomotic recurrence at the bile duct resection margin after surgical resection in patients with a subepithelial invasive component bile duct 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. Adjusted 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:

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Table 8. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of anastomotic recurrence by bile duct resection margin status in patients with a positive bile duct resection margin

	No. of recurrent cases	Person-days	Adjusted HR†	95% CI
Bile duct resection margin status				
R1 (is)	3	22 029	1.00	
R1	14	25 237	4.48	1.09, 18.5

†Adjusted for operation day and type of surgical resection. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

patients with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule (Table 1). Furthermore, patients with an intraductal carcinoma component were infrequently at the advanced stage when diagnosed (Table 1). During the preparation of this paper, Nakanishi *et al.*[29] reported that cases of extrahepatic bile duct carcinoma with intraepithelial spread showed a more differentiated histological grade, less deep invasion, infrequent portal vein or hepatic invasion and a better prognosis than cases without such spread. In addition, we demonstrated statistically significant inverse correlations between the intraductal carcinoma component on the one hand and lymphatic and perineural involvement and TNM stage on the other, whereas Nakanishi *et al.* failed to show such correlations. Moreover, we demonstrated an inverse correlation between the intraductal carcinoma component and tumor aggressiveness by both univariate and multivariate analyses in a larger cohort than that reported by Nakanishi *et al.*[29], whereas that report performed only univariate analysis.

We have revealed that human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models show strong adhesiveness to extracellular matrix proteins, which are components of the basement membrane of epithelial tissues, *in vitro*.[30] The expression patterns of cell-matrix adhesion molecules, such as integrins, in human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models differ from those in human cancer cell lines that do not show such spreading.[30] In addition, it has been confirmed that there is a similar difference in the expression pattern of cell-matrix adhesion molecules between cancer cells showing, and not showing, such spreading in surgically resected clinical samples.[30] Cell-matrix adhesion molecules generally participate in cancer-stromal interactions and determine the invasiveness of human cancers.[31] Therefore, it is feasible that cancer cells showing wide intraepithelial spreading also show strong adhesiveness to the basement membrane of cancer nests and a less invasive tendency. This may be the reason why an intraductal carcinoma component was inversely correlated with aggressiveness in the main tumor nodule in this study. Although the molecular mechanism responsible for such an inverse correlation in biliary tract carcinoma needs to be further clarified, the presence of an intraductal carcinoma component may become an indicator of lower tumor aggressiveness. In fact, patients with an intraductal carcinoma component showed a significantly better prognosis than patients without such a component, irrespective of whether all patients from the present cohort or only patients who underwent complete resection were examined. The correlation between an intraductal carcinoma component and a favorable prognosis is consistent with the similar correlation observed in ductal carcinoma of the pancreas: the presence of an intraductal carcinoma component is reportedly a significantly good prognostic parameter for patients with invasive ductal carcinoma of the pancreas after surgical resection.[32–34]

On the other hand, the presence of an intraductal carcinoma component was significantly correlated with a positive bile duct resection margin (Table 4). Recently, Wakai *et al.* have reported that invasive carcinoma at the ductal resection margin appears to have a strong impact on patient survival, whereas residual carcinoma *in situ* does not, after surgical resection for extrahepatic bile duct carcinoma,[14] although they did not mention any background factors for the difference in prognostic impact between invasive carcinoma and carcinoma *in situ* at the ductal resection margin. We also defined two types of positive bile duct resection margin: R1 (is) (positive with only an intraductal carcinoma component) and R1 (positive with a subepithelial invasive component). An R1 bile duct resection margin was more frequently associated with poorer prognosis than a negative bile duct resection margin, whereas an R1 (is) bile duct resection margin was not more frequently associated with poorer prognosis than a negative bile duct resection margin (Tables 6 and 7). As in other malignancies, tumor recurrence after radical surgery for biliary tract carcinoma leads to eventual death.[26] Although Wakai *et al.* did not perform statistical analysis about anastomotic recurrence, the risk of anastomotic recurrence at the bile duct resection margin was significantly higher (Table 8), and the period until such recurrence was significantly shorter (Fig. 3), in patients with an R1 bile duct resection margin than in patients with an R1 (is) bile duct resection margin. Therefore, at least part of the difference in prognostic impact between R1 (is) and R1 bile duct resection margins is attributable to the difference in the risk of anastomotic recurrence.

However, the incidence of anastomotic recurrence in patients whose bile duct resection margin was positive [R1 (is) and R1] was only 26% (17 of 66 cases); some patients may die because of 'local recurrence' or distant metastasis before anastomotic recurrence becomes clinically obvious. Sakamoto *et al.* have proposed that anastomotic recurrence should be distinguished from 'local recurrence' derived from perineural invasion around the hepatic artery and/or involved regional nodes.[35] It is self-evident that all patients with an R1 (is) bile duct resection margin possess an intraductal carcinoma component that is inversely correlated with tumor aggressiveness, including perineural and lymphatic involvement and clinical stage. The intraductal carcinoma component may be partly responsible for the difference in prognostic impact between R1 (is) and R1 with reference not only to 'anastomotic recurrence' but also to 'local recurrence' derived from perineural invasion and/or involved regional nodes.

We analyzed both the intraductal carcinoma component and resection margin status in the same cohort and examined in detail the inconsistency of less tumor aggressiveness and a positive surgical margin in patients with intraductal carcinoma components. Surgeons are frequently required to decide the resection area based on the results of intraoperative histological diagnosis of bile duct resection margin status using frozen sections, and generally intend to achieve a negative bile duct resection margin. If the frozen section diagnosis is R1, the risk of death will be actually reduced if surgeons make efforts to achieve a negative bile duct resection margin. However, if the frozen section diagnosis is R1 (is), an intraductal carcinoma component is present and the prognosis for such patients will be

favorable. Moreover, if surgeons perform additional resection to achieve a negative bile duct resection margin, then the prognosis for patients whose initial bile duct resection margin is R1 (is) may not be improved significantly. Therefore, 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.

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CME/SAM

Prognostic Significance of CXCL12 Expression in Patients With Colorectal Carcinoma

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Key Words: CXCL12; Invasive front; Immunohistochemistry; Colorectal carcinoma; Prognosis

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Upon completion of this activity you will be able to:
- define the World Health Organization criteria for the histologic grading of the differentiation of colorectal carcinoma.
- discuss the role of examination of the invasive front of colorectal carcinoma for tumor budding and how this may pertain to prognosis.
- discuss the potential application of immunohistochemical staining for CXCL12 to highlight tumor budding in colorectal carcinoma.

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Questions appear on p 307. Exam is located at www.ascp.org/ajcpcme.

Abstract

The present study investigated the protein expression level of CXCL12 in colorectal cancer and aimed to elucidate its association with prognosis. CXCL12 positivity in 50% or more of tumor cells was defined as high expression and that in less than 50% of the tumor cells as low expression. CXCL12+ tumor budding at the invasive front was divided into 2 grades: high with 10 or more budding foci per ×200 field of view and low grade with fewer than 10 budding foci. Patients with high expression (72.7%) and high grade CXCL12+ tumor budding (43.0%) had significantly shorter survival than patients with low expression (P = .014) and low grade (P = .003), respectively. Patients with a combination of high expression and high grade had the worst outcome (P < .001). Our study demonstrated that CXCL12 expression in colorectal cancer cells and at sites of budding were significant prognostic factors. Furthermore, together with lymph node metastasis, a combination of both expression patterns was a more powerful independent prognostic factor.

Several studies have revealed that the establishment of metastasis is the final outcome of a series of phenomena including tumor cell deposition in distant organs, clonogenic growth, and angiogenesis. These processes are fundamental for tumor cell survival and tumor metastasis and are regulated by interactions of cancer cells with the host microenvironment.[1-3] The CXC chemokine ligand-12 (CXCL12), stromal cell–derived factor-1, is a member of the CXC chemokine family, which has been initially cloned from murine bone marrow and characterized as a pre–B-cell growth-stimulating factor.[4-6] CXCL12 exerts effects through its physiologic cognate receptor, CXC chemokine receptor 4 (CXCR4), and is known to have roles in chemotaxis,[7] hematopoiesis,[8] and angiogenesis.[9,10] In addition, CXCR4 is involved in tumor cell homing to specific organs and in tumor progression.[11-13] CXCL12/CXCR4 also has a critical role in determining the metastatic destination of breast cancer cells.[12] It is also evident that some CXCR4+ tumors, including colorectal cancer,[1,13,14] exhibit marked malignant behavior.[12,15,16] So far, few studies have focused on chemokine expression in cancer cells,[17-21] and little is known about the clinicopathologic significance of CXCL12 expression in patients with colorectal cancer.

In this study, we attempted to evaluate the clinicopathologic significance of CXCL12 expression in patients with colorectal cancer by using immunohistochemical analysis together with examination of conventional histopathologic features.

Materials and Methods

Between 1996 and 1997 at the National Cancer Center Hospital, Tokyo, Japan, 165 patients underwent surgery for primary colorectal carcinoma, including 100 colon (60.6%)

and 65 rectal (39.4%) cancers. Sample selection was restricted to consecutive cases diagnosed as stages II and III according to the International Union Against Cancer–TNM classification.[22] Of the 165 cases, 72 (43.6%) were classified as stage II and 93 (56.4%) as stage III; 116 cases (70.3%) were T2-3, and 49 were T4 (29.7%).

All patients underwent curative resection, defined as the removal of gross cancer and the demonstration of tumor-negative surgical margins by histopathologic examination of the total circumference. No patients received preoperative chemotherapy, and all patients were free of distant visceral metastases. The patients comprised 101 men and 64 women, ranging in age from 32 to 93 years (mean ± SD, 61.8 ± 11.2 years). Postsurgical follow-up studies were completed for all patients, with follow-up periods ranging from 3 to 2,544 days (median, 1,844 days). Postsurgical recurrence was diagnosed by ultrasonography and computed tomography. This study was approved by the National Cancer Center Ethics Committee, Tokyo, Japan.

All available routinely processed, formalin-fixed, and paraffin-embedded blocks of colorectal carcinoma were obtained. Sections containing the maximum diameter of the tumor were used in the present study. Age, sex, tumor location, tumor size, macroscopic type, depth of tumor invasion, tumor differentiation, tumor budding grade by H&E staining, lymphatic vessel invasion, blood vessel invasion, lymph node metastasis, liver metastasis, and lung metastasis were subjected to statistical analyses ▮Table 1▮.

The grade of tumor differentiation was decided on the basis of the predominant component in the tumor according to the World Health Organization classification: the percentage of the tumor showing formation of gland-like structures can be used to define the grade; well differentiated (grade 1) lesions exhibit glandular structures in more than 95% of the tumor; moderately differentiated (grade 2) adenocarcinoma has 50% to 90% glands; poorly differentiated (grade 3) adenocarcinoma has 5% to 50%; and undifferentiated (grade 4) carcinoma has less than 5%. Mucinous adenocarcinoma and signet-ring cell carcinoma, by convention, are considered poorly differentiated (grade 3).[23]

The existence of tumor budding at the invasive front was also evaluated. The invasive front in this study was defined as all regions of the border area between the primary tumor and interstitium in the submucosa, muscularis propria, subserosa, or nonperitonealized pericolonic/perirectal tissues. In accordance with previous studies, an isolated single cancer cell or a cluster composed of fewer than 5 cancer cells observed in the stroma of the actively invasive region was defined as a budding focus.[24-26] After reviewing the H&E-stained slides from each case, a field where the budding foci were most intense was selected. The number of budding foci was counted using a 20× microscope objective, giving a final magnification of ×200. A count ranging from 0 to 9 budding foci per field was designated as low grade and a count of 10 or more as high grade, in accordance with previous studies.[25,26]

Immunohistochemical Studies

After deparaffinization, all sections were pretreated in citrate buffer (10 mmol/L, pH 6.0) at 121°C for 10 minutes for antigen retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes. The sections were then incubated with anti-CXCL12/SDF-1 monoclonal antibody (0.5 ng/mL; R&D Systems, Minneapolis, MN) at 4°C overnight. The immunostained sections were washed with phosphate-buffered saline and processed with an EnVision+ kit (DakoCytomation, Carpinteria, CA) in accordance with the manufacturer's instructions. Immunoproducts were visualized by using diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. As an internal positive control for CXCL12 staining, the immunopositivity of normal colorectal epithelia adjacent to the tumors and endothelial cells of blood vessels was used. Intensity of positive staining in tumor cells that was the same as or more than that in normal colorectal epithelia was considered positive. Sections from each paraffin block were used as negative control samples by replacing the primary antibody with normal mouse immunoglobulin. After immunohistochemical analysis, the tumors were categorized according to the ratio and localization of immunopositive tumor cells in the sections containing the maximum tumor diameter.

We examined the relationship between the CXCL12 positivity rate in tumor cells and various clinicopathologic factors and found that a cutoff value of 50% was the most powerful discriminatory factor. Accordingly, a cutoff index of 50% was selected for our study. When the proportion of tumor cells positive for CXCL12 was 50% or more, the tumor was defined as showing high CXCL12 expression, whereas tumors in which fewer than 50% of the cells were positive were defined as showing low CXCL12 expression.

In addition, CXCL12 expression in foci of tumor budding at the invasive front was quantified and scored by the same method as for evaluation of tumor budding grade using H&E-stained sections. After scanning each CXCL12-immunostained slide, a field where immunopositivity in the budding foci was the most intense was selected, and the number of CXCL12+ budding foci was counted at ×200 magnification. For all histopathologic variables, each macroscopic record and microscopic slide was analyzed by 2 experienced pathologists (Y.A.-F. and Y.N.) to reach a consensus.

Statistical Analysis

The relationships between clinicopathologic characteristics and the number of immunopositive tumor cells were analyzed by variance tests when appropriate. The χ^2 test was

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used to analyze variables such as sex, tumor location, depth of tumor invasion, tumor differentiation, tumor budding grade assessed by H&E staining, lymphatic vessel invasion, blood vessel invasion, lymph node metastasis, liver metastasis, and lung metastasis. The Student *t* test was used for statistical comparisons of variables such as age and tumor size, and the Mann-Whitney *U* test was applied to compare the variables of macroscopic type. Survival was measured from the date of surgery to the end of the follow-up period or death. Overall survival curves were determined by using the Kaplan-Meier method and were analyzed by using the log-rank test. Univariate and multivariate survival analysis was performed by using the Cox proportional hazards regression model with the StatView, version 5.0 software package (SAS Institute, Cary, NC), in a stepwise manner.

Results

Expression of CXCL12 in Colorectal Cancer

Immunoreactivity of CXCL12 was observed in the cell membrane and/or cytoplasm of tumor cells (Image 1A). The endothelial cells of blood vessels and normal intestinal epithelia, especially in the middle to upper third portion of the crypt,

Table 1
Correlation of CXCL12 Expression With Clinicopathologic Features*

Variable	CXCL12 Expression			CXCL12+ Tumor Budding Grade			CXCL12 Expression and Tumor Budding Grade		
	Low (n = 45)	High (n = 120)	*P*	Low (n = 94)	High (n = 71)	*P*	Others (n = 99)	High Expression With High Grade (n = 66)	*P*
Age (y)	62.9 ± 12.7	61.4 ± 10.7	.319	61.9 ± 11.5	61.6 ± 11.0	.778	62.4 ± 11.5	60.9 ± 10.8	.346
Sex			.986			.192			.096
Male	27	74		53	48		55	46	
Female	18	46		41	23		44	20	
Tumor location			.525			.622			.625
Colon	25	75		59	41		62	38	
Rectum	20	45		35	30		37	28	
Maximum tumor diameter (cm)	4.7 ± 2.1	5.1 ± 1.9	.155	5.1 ± 2.1	4.7 ± 1.6	.232	5.0 ± 2.1	4.8 ± 1.6	.589
Macroscopic type†			.943			.256			.287
1	3	9		10	2		10	2	
2	42	109		84	67		89	62	
3	0	2		0	2		0	2	
4	0	0		0	0		0	0	
Tumor depth‡			.001			<.001			<.001
T2, T3	40	76		78	38		81	35	
T4	5	44		16	33		18	31	
Tumor differentiation§			>.999			>.999			>.999
Grade 1/2	44	116		91	69		96	64	
Grade 3/4	1	4		3	2		3	2	
Tumor budding grade (H&E staining)			.013			<.001			<.001
Low	36	69		78	27		80	25	
High	9	51		16	44		19	41	
Lymphatic vessel invasion			.984			.324			.559
Present	36	94		71	59		76	54	
Absent	9	26		23	12		23	12	
Blood vessel invasion			>.999			.096			.150
Present	29	79		56	52		60	48	
Absent	16	41		38	19		39	18	
Lymph node metastasis			.411			.003			.022
Present	29	67		45	51		50	46	
Absent	16	53		49	20		49	20	
Liver metastasis			.044			.101			.052
Present	1	18		7	12		7	12	
Absent	44	102		87	59		92	54	
Lung metastasis			.071			.030			.014
Present	1	16		5	12		5	12	
Absent	44	104		89	59		94	54	

* Data are given as number of cases or mean ± SD. *P* values were calculated by using the Mann-Whitney *U* test for age and maximum tumor diameter, the Student *t* test for macroscopic type, and the χ^2 test for all others.
† According to the World Health Organization classification: 1, polypoid; 2, ulcerating circumscribed; 3, ulcerating infiltrative; 4, diffusely infiltrative.
‡ According to the International Union Against Cancer TNM classification: T2, tumor invades the muscularis propria; T3, tumor invades through the muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforates the visceral peritoneum.
§ According to the World Health Organization classification: grade 1, well-differentiated adenocarcinoma; grade 2, moderately differentiated adenocarcinoma; grade 3, poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet-ring cell carcinoma; grade 4, undifferentiated carcinoma.

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