A

Lane No.	1	2	3
NPC1L1-HA	-	-	+
NPC2-Myc-His	-	+	+

NPC1L1

NPC2 — HMW / LMW

NPC1

Cathepsin D

α-tubulin

B

NS

Relative mRNA level of NPC2 (% of Control)

120
100
80
60
40
20
0

Control NPC1L1

C

Ad-NPC1L1	-	-	5 MOI	10 MOI	20 MOI
Ad-NPC2-Myc-His	-	5 MOI			

NPC1L1

Cellular NPC2 — HMW / LMW

Secreted NPC2 — HMW / LMW

α-tubulin

D

Endo H	-	+					-	+				
Ad-NPC1L1	-	-	5 MOI	10 MOI	20 MOI	-	-	-	5 MOI	10 MOI	20 MOI	-
Ad-NPC2-Myc-His	5 MOI					-	5 MOI					-

HMW →
LMW →
Nonglycosylated →

— 26
— 23
— 19

Cellular NPC2   Secreted NPC2

PNGase F	-	+					-	+				
Ad-NPC1L1	-	-	5 MOI	10 MOI	20 MOI	-	-	-	5 MOI	10 MOI	20 MOI	-
Ad-NPC2-Myc-His	5 MOI					-	5 MOI					-

HMW →
LMW →
Nonglycosylated →

— 26
— 23
— 19

Cellular NPC2   Secreted NPC2

E

NPC1L1	-					+				
Pulse time (minutes)	0	5	10	30	60	0	5	10	30	60

Cellular NPC2 — HMW / LMW

Secreted NPC2 — HMW

Control cells

Cellular ratio of HMW NPC2 (% of HMW + LMW NPC2)

100
80
60
40
20
0

5 10 30 60
Pulse time (minutes)

NPC1L1 cells

Cellular ratio of HMW NPC2 (% of HMW + LMW NPC2)

100
80
60
40
20
0

5 10 30 60
Pulse time (minutes)

■: HMW NPC2
□: LMW NPC2

Fig. 2. Effect of NPC1L1 on the expression of NPC2. (A) CHO-K1 cells were transfected with the indicated vectors or an empty control vector. Twenty-four hours after transfection, the cells were harvested and subjected to an immunoblot analysis. (B) The mRNA levels of NPC2 in CHO-K1 cells that were cultured for 24 hours after transient transfection with the NPC2-Myc-His vector and either the NPC1L1-HA vector or an empty control vector were determined with quantitative real-time PCR. The relative mRNA level of NPC2 in each cell was normalized to the level of β-actin mRNA. The columns and vertical bars represent the means and standard deviations of three determinations. (C) CHO-K1 cells were infected with Ad-NPC1L1 at the indicated MOI together with Ad-NPC2-Myc-His at 5 MOI. Twenty-four hours after infection, the total cell lysate and the concentrated media were subjected to an immunoblot analysis. Ad-GFP was used to equalize the total amount of the infected adenovirus. (D) The aforementioned total cell lysate and concentrated media were deglycosylated with either Endo H or PNGase F (see the supporting information). The deglycosylated and undigested proteins were subjected to immunoblot analyses. (E) CHO-K1 cells were infected with Ad-NPC1L1 or Ad-GFP (the control) at 10 MOI together with Ad-NPC2-Myc-His at 5 MOI. Twenty-four hours after infection, ^{35}S-labeling was performed for the indicated times. The ^{35}S-labeled NPC2 protein was detected with a phosphor imager. The lower panels show the ratio of the ^{35}S-labeled HMW NPC2 radioactivity to the total ^{35}S-labeled NPC2 radioactivity. The data points and bars represent the means and standard deviations of three specimens on different images. Abbreviation: NS, not significant.

overexpression, and this was consistent with the decrease in the intracellular NPC2 protein level.

A glycosidase digestion assay was performed next. After digestion with endoglycosidase H (Endo H; upper panels, Fig. 2D) or peptide *N*-glycosidase F (PNGase F; lower panels, Fig. 2D), intracellular NPC2 was detected at 18 kDa, which corresponded to the nonglycosylated form of the protein. This result suggests that HMW NPC2 and LMW NPC2 are glycosylation variants. In addition, the secretion of maturely glycosylated HMW NPC2, which corresponded to a 23-kDa band after digestion with Endo H, was concomitantly reduced with the increase in NPC1L1 expression (upper panels, Fig. 2D). These results raise the possibility that NPC1L1 inhibits the maturation of NPC2.

To test this hypothesis, we characterized NPC2 maturation over time with a [^{35}S]methionine/cysteine pulse-chase experiment. In control cells, the amounts of both cellular and secreted ^{35}S-labeled HMW NPC2 increased in a time-dependent manner (upper panels, Fig. 2E). However, in NPC1L1-overexpressing cells, very little cellular or secreted ^{35}S-labeled HMW NPC2 was detected, whereas the amount of ^{35}S-labeled LMW NPC2 increased in a time-dependent manner (upper panels, Fig. 2E). Furthermore, the ratio of the cellular amount of ^{35}S-labeled HMW NPC2 to the amount of total ^{35}S-labeled NPC2 (HMW + LMW) increased time-dependently in control cells, whereas this ratio did not change in NPC1L1-overexpressing cells (lower panels, Fig. 2E). These results support the hypothesis that NPC1L1 inhibits the maturation of NPC2 from LMW forms to HMW forms.

NPC1L1 Interacts With NPC2 in Prelysosomal Compartments. To clarify the cellular compartment in which the interaction between NPC1L1 and NPC2 occurs, we performed immunohistochemical staining for markers of various organelles. As shown in Fig. 3, NPC2 was localized in vesicle-like structures that were partially costained for the lysosomal marker cathepsin D (Fig. 3A) but not for the endoplasmic reticulum (ER) marker calnexin (Fig. 3B). However, in agreement with the results of the immunoblot analyses (Figs. 1 and 2A), when NPC1L1 was overexpressed, the expression of NPC2 protein was reduced, and minimal staining of NPC2 was observed (Fig. 3C).

Because treatment with *N*-(benzyloxycarbonyl)leucinylleucinylleucinal (MG132), a proteasome inhibitor, could inhibit the degradation of NPC2 and increase LMW NPC2 even when NPC1L1 was overexpressed (Supporting Fig. 2), immunohistochemical staining was performed in the presence of MG132. Figure 3D

shows that MG132-sensitive NPC2 was colocalized with NPC1L1 in intracellular compartments that were partially costained for calnexin (Fig. 3E) but not cathepsin D (Fig. 3F). The observation that NPC1L1 could be coimmunoprecipitated with LMW NPC2 (Fig. 1) and the observation that most of the MG132-sensitive NPC2 was LMW NPC2 (Supporting Fig. 2) suggest that NPC1L1 interacts with LMW NPC2 in prelysosomal compartments, including the ER.

Degradation of NPC2 Is Accelerated in the Presence of NPC1L1. Because NPC1L1 reduces the expression of NPC2 protein, it was hypothesized that the degradation of NPC2 protein is accelerated by NPC1L1. To test this hypothesis, we analyzed the degradation rate of NPC2 protein. As shown in Fig. 4, the degradation of LMW NPC2 was more rapid in NPC1L1-overexpressing cells (half-life = 2.1 ± 0.1 hours) versus control cells (half-life = 7.1 ± 2.5 hours). In contrast, the half-life of HMW NPC2 was hardly affected by the overexpression of NPC1L1 (3.2 ± 0.8 hours in control cells and 2.3 ± 0.1 hours in NPC1L1-overexpressing cells). These results suggest that the ability of NPC1L1 to accelerate the degradation of LMW NPC2 may contribute to the lower expression of NPC2 protein in NPC1L1-overexpressing cells and that the reduced expression of HMW NPC2 in NPC1L1-overexpressing cells (Figs. 1 and 2A,C) could be explained by the inhibition of NPC2 maturation rather than the difference in the degradation speed of HMW NPC2.

Endogenous NPC1L1 Regulates the Protein Expression and Secretion of NPC2. Although the overexpression of NPC1L1 caused a reduction in the protein expression and secretion of NPC2, an overexpression model is fraught with potential artifacts. To eliminate this possibility, we investigated the ability of endogenous NPC1L1 to regulate the expression and secretion of endogenous NPC2. For this purpose, HepG2 cells in which both NPC1L1 and NPC2 were expressed endogenously were transfected with a small interfering RNA targeted against Niemann-Pick C1-like 1 (siNPC1L1), and changes in NPC2 expression and secretion were analyzed. As shown in Fig. 5, after the transfection of siNPC1L1, the mRNA levels of endogenous NPC1L1 (Fig. 5A) and its protein levels (Fig. 5B) were reduced to approximately 40% and 60%, respectively, of the levels in cells transfected with the control small interfering RNA (siControl). Under these conditions, although the expression of NPC2 mRNA was unaltered (Fig. 5A), the level of NPC2 protein increased more than 1.9-fold (cellular NPC2, Fig. 5B). In addition, the amount of NPC2 secreted
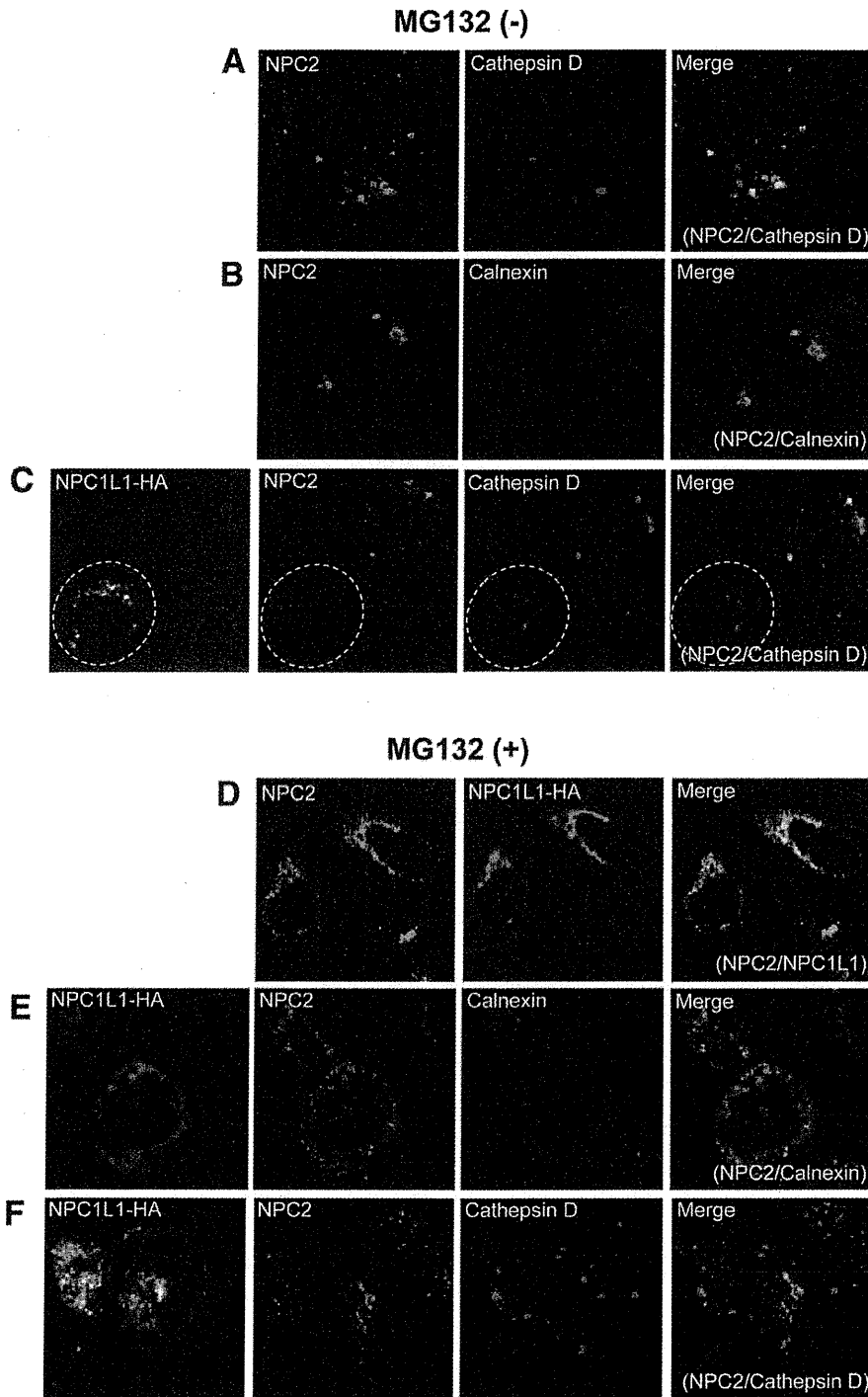
MG132 (-)

MG132 (+)

Fig. 3. Intracellular colocalization of NPC1L1 with NPC2 in HepG2 cells. The cellular localization of NPC1L1-HA and NPC2 was examined with immunohistochemical staining. HepG2 cells were transfected with (A,B) the control vector or (C) the NPC1L1-HA vector. Twenty-four hours after transfection, the cells were stained for an immunohistochemical analysis. NPC1L1-expressing cells are indicated by circles. (D-F) HepG2 cells were transfected with the NPC1L1-HA vector. Twenty-four hours after transfection, the cells were treated with MG132 (10 μM) for 6 hours, and they were then subjected to immunohistochemical staining. Representative images are shown. In each panel, colocalization appears yellow in the merged image.

into the media also increased more than 2.5-fold after the suppression of NPC1L1 expression (secreted NPC2, Fig. 5B). However, transfection with siNPC1L1 had no effect on NPC1 protein expression (Fig. 5B). These results suggest that the expression and secretion of endogenous NPC2 are negatively regulated by endogenous NPC1L1.

Suppression of Intracellular Cholesterol Trafficking by NPC1L1 Overexpression Can Be Rescued by the Addition of Purified NPC2 Protein. Because the loss of intracellular NPC2 suppresses intracellular cholesterol trafficking and causes cholesterol accumulation within lysosomes,[10] the cholesterol distribution was analyzed in NPC1L1-overexpressing cells. In agreement
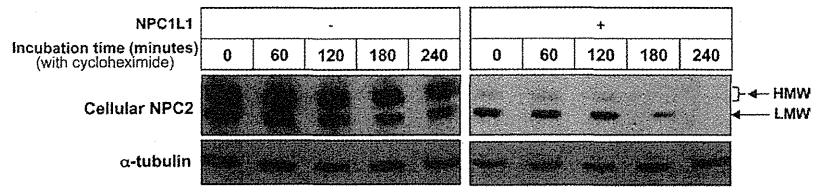
Fig. 4. Effect of NPC1L1 on the degradation rate of NPC2 protein. CHO-K1 cells were infected with Ad-NPC1L1 or Ad-GFP (the control) at 10 MOI together with Ad-NPC2-Myc-His at 5 MOI. Twenty-four hours after infection, the cells were incubated with cycloheximide (100 μM). The total cell lysate, which was prepared at the indicated time points, was examined with an immunoblot analysis. The lower panels show quantitative comparisons of the levels of NPC2 protein normalized to the level of α-tubulin. The data points and bars represent the means and standard deviations of three specimens on different immunoblots. *Significantly different from control cells according to the Student t test ($P < 0.05$). **Significantly different from control cells according to the Student t test ($P < 0.01$).
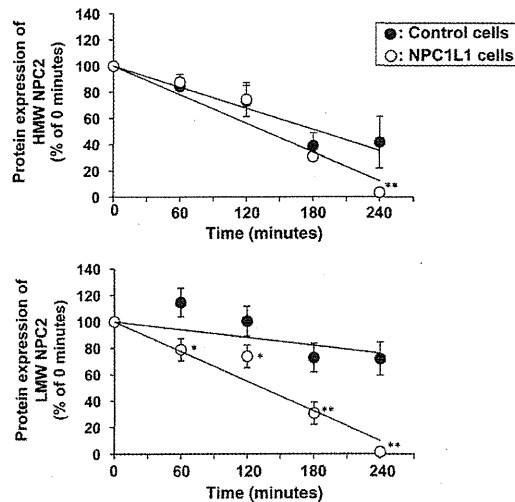
with the previous results, the amount of endogenous NPC2 protein in HepG2 cells was reduced along with the increase in NPC1L1 expression, whereas the level of endogenous NPC1 protein was hardly affected (Fig. 6A). Because extracellular (secreted) NPC2 can be taken up into lysosomes via receptor-mediated endocytosis,[26] NPC1L1-overexpressing cells were cultured in media containing exogenous NPC2 protein, and the amount of cellular NPC2 was examined. Exogenous NPC2 protein was purified from cell culture media containing secreted HMW NPC2. Figure 6B shows that the reduction of NPC2 in NPC1L1-overexpressing cells could be recovered by culturing with purified NPC2 protein, and this suggests that NPC1L1 may only minimally affect the protein stability of endocytosed NPC2. The endocytosed NPC2 protein in NPC1L1-overexpressing cells was colocalized with the lysosomal marker cathepsin D by immunohistochemistry (data not shown). These results are consistent with the observation that NPC1L1 interacts with NPC2 in prelysosomal compartments (Fig. 3E).

Filipin staining was next performed in order to analyze the cellular distribution of cholesterol. As shown in Fig. 6C, in comparison with control cells, NPC1L1-overexpressing cells exhibited increased staining for intracellular cholesterol, whereas staining for cholesterol in the plasma membrane was reduced. A similar pattern was observed in cells treated

with 3-beta-(2-diethylaminoethoxy)androst-5-en-17-one hydrochloride (U18666A), an inhibitor of intracellular cholesterol trafficking. When NPC1L1-overexpressing cells were incubated with purified wild-type (WT) NPC2, intracellular NPC2 levels were restored to the same levels found in control cells (Fig. 6B); intracellular cholesterol accumulation was significantly decreased, and in turn, the distribution of cholesterol in the plasma membrane was restored (Fig. 6C). This rescue effect was not observed when cells were cultured with NPC2 D72A, a loss-of-function mutant.[27] These results suggest that the altered distribution of cholesterol in NPC1L1-overexpressing cells is mostly caused by the reduced expression of lysosomal NPC2 and not by NPC1L1 itself. In addition, NPC1L1 only slightly affects the function of lysosomal NPC2.

NPC2 Expression Is Negatively Correlated With NPC1L1 in Human Liver Specimens. Finally, the correlation between the levels of NPC2 and NPC1L1 in human liver specimens was determined with immunoblot analyses and quantitative real-time PCR. In agreement with the *in vitro* results, the protein levels of NPC2 were negatively correlated with NPC1L1 levels in human liver specimens (Fig. 7A), although there was no significant correlation between the mRNA levels (Fig. 7B). Furthermore, the translational efficiency of NPC2, which is expressed as the ratio of the NPC2 protein level to the NPC2 mRNA level, was also
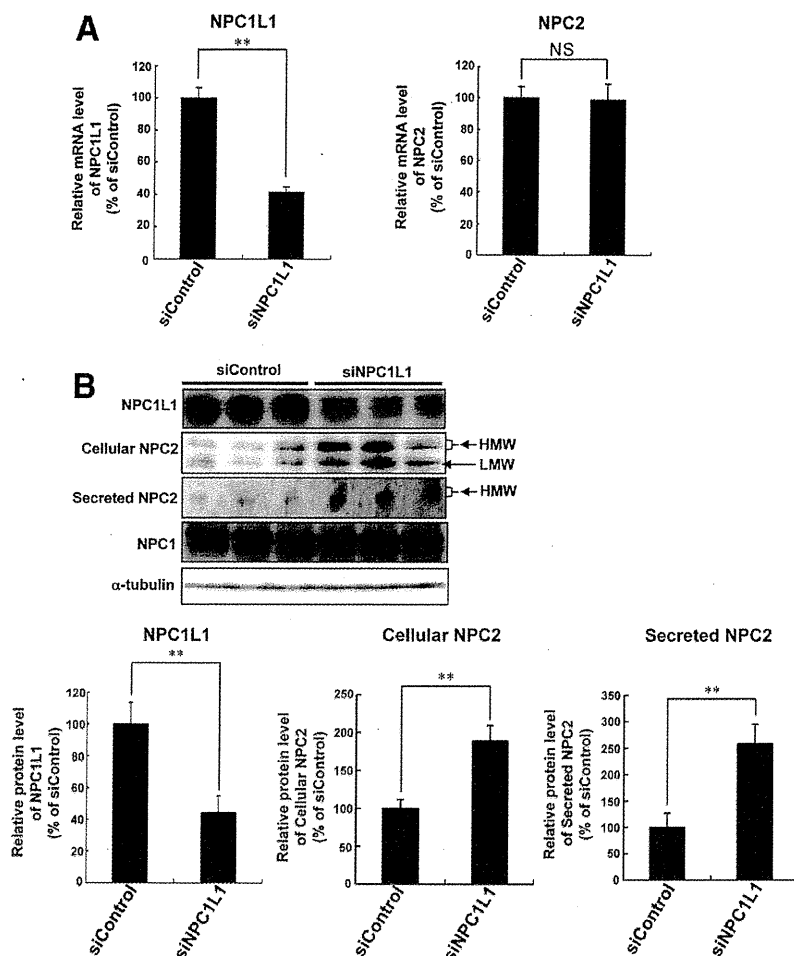
Fig. 5. Effect of siNPC1L1 on the endogenous expression of NPC2. HepG2 cells were transfected with siNPC1L1 or siControl (see the supporting information) and cultured for 2 days. The extracted RNA and the total cell lysate were then analyzed with (A) quantitative real-time PCR and (B) an immunoblot analysis, respectively. (A) The relative mRNA levels of NPC1L1 and NPC2 were normalized to the β-actin mRNA levels. (B) The relative levels of the NPC1L1 and NPC2 proteins were calculated as the band densities of the proteins and were normalized to the α-tubulin protein level in each specimen. The columns and vertical bars represent the means and standard deviations of the three specimens in the immunoblot. **Significantly different according to the Student t test ($P < 0.01$). Abbreviation: NS, not significant.

negatively correlated with the protein level of NPC1L1 (Fig. 7C). These results support the hypothesis that the protein expression of NPC2 is posttranscriptionally regulated by NPC1L1 in the human liver.

Discussion

According to this study, in addition to its well-known role as a cholesterol importer, NPC1L1 has a novel function as a negative regulator of the expression and secretion of NPC2, which is based on the ability of NPC1L1 to inhibit the maturation of NPC2 protein and stimulate the degradation of LMW NPC2 protein. Because treatment with ezetimibe, an inhibitor of NPC1L1-mediated cholesterol import, does not affect the binding of NPC1L1 and NPC2 and cannot reverse the reduction in the NPC2 protein level in NPC1L1-coexpressing cells (Supporting Fig. 3), this novel function of NPC1L1 as a negative regulator of NPC2 protein is independent of its well-known role as a cholesterol importer.

Previously, it has been reported that NPC2 is degraded by the proteasome system.[22] Therefore, we investigated whether treatment with MG132, a proteasome inhibitor, could inhibit the NPC1L1-mediated down-regulation of NPC2. In agreement with the previous report,[22] in the absence of NPC1L1, the levels of both HMW NPC2 and LMW NPC2 were increased by MG132 treatment (Supporting Fig. 2). However, when NPC1L1 was coexpressed, MG132 treatment hardly increased the expression of HMW NPC2, although the expression of LMW NPC2 clearly increased (Supporting Fig. 2). This observation is in agreement with the finding that NPC1L1 inhibits the maturation of NPC2 from LMW forms to HMW forms (Fig. 2C,E). Furthermore, the stability of LMW NPC2 was altered by NPC1L1 coexpression (Fig. 4), and LMW NPC2 coimmunoprecipitated with NPC1L1 (Fig. 1). These data imply that NPC1L1 interacts with LMW NPC2, inhibits its maturation, and simultaneously promotes its degradation. As a
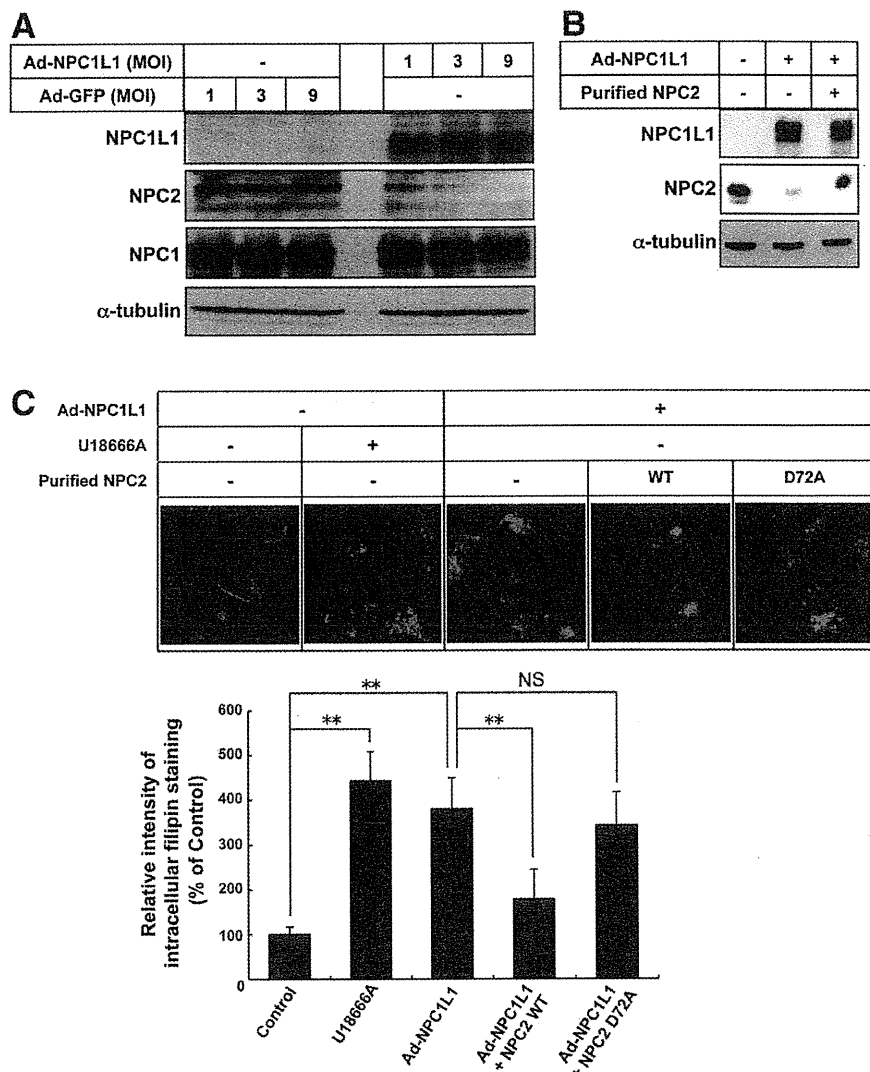
Fig. 6. Effect of NPC1L1 on intracellular cholesterol trafficking. (A) HepG2 cells were infected with Ad-NPC1L1 or Ad-GFP at the indicated MOI. Twenty-four hours after infection, the cells were collected and subjected to an immunoblot analysis. (B) HepG2 cells were infected with Ad-NPC1L1 or Ad-GFP (the control) at 3 MOI and were cultured together with purified NPC2 protein (10 nM). Twenty-four hours after infection, the cells were collected and subjected to an immunoblot analysis. (C) HepG2 cells were infected with Ad-NPC1L1 or Ad-GFP (the control) at 3 MOI and were cultured together with the indicated type of purified NPC2 protein (10 nM) or with U18666A (1.25 μM) for 8 hours. Twenty-four hours after infection, the cells were stained with filipin. The upper panels show the results of filipin staining. Representative images are shown. The lower panel shows a quantitative comparison of filipin staining in intracellular compartments. The columns and vertical bars represent means and standard deviations of three different images. **Significantly different according to an analysis of variance followed by Dunnett's test ($P < 0.01$). Abbreviation: NS, not significant.

result, intracellular NPC2 is reduced, and this in turn leads to a decrease in NPC2 secretion (Fig. 8).

In contrast to LMW NPC2, a molecular association between HMW NPC2 and NPC1L1 was not detected by coimmunoprecipitation (Fig. 1). Because HMW NPC2 and LMW NPC2 are glycosylation variants (Fig. 2D), it is possible that the complex glycosylation of HMW NPC2 may inhibit its binding to NPC1L1, whereas core-glycosylated NPC2 can interact with NPC1L1. This idea is consistent with the observation that in addition to the expression of WT NPC2, the expression of NPC2 mutants (in which one or both of the glycosylation sites of NPC2 are mutated[24]) was reduced by the coexpression of NPC1L1 (Supporting Fig. 4). Because NPC1L1 colocalizes with NPC2 in prelysosomal compartments (Fig. 3) and the reduced expression of NPC2 can be rescued by exogenous

secreted NPC2 (Fig. 6B), it seems that NPC1L1 interacts with NPC2 during the process of complex glycosylation.

The RNA interference studies using HepG2 cells (Fig. 5) and the correlation analysis of the expression levels of NPC1L1 and NPC2 in human liver specimens (Fig. 7) have revealed that endogenous NPC1L1 negatively regulates the expression of NPC2 posttranscriptionally. It has recently been reported that in addition to the NPC1L1-mediated regulation of NPC2, Nogo-B receptor (NgBR) interacts with NPC2 at the ER and enhances NPC2 protein stability by inhibiting its proteasomal degradation.[22] Because NgBR is known to be expressed in the liver,[28] NgBR may function as a positive regulator of NPC2 protein expression, whereas NPC1L1 acts as a negative regulator. The balance of the expression levels of NPC1L1 and

A

$R_s = -0.75$
$P = 0.020$

Relative NPC1L1 protein level
Relative NPC2 protein level

B

$R_s = -0.40$
$P = 0.286$

Relative NPC1L1 mRNA level
Relative NPC2 mRNA level

C

$R_s = -0.73$
$P = 0.024$

Relative NPC1L1 protein level
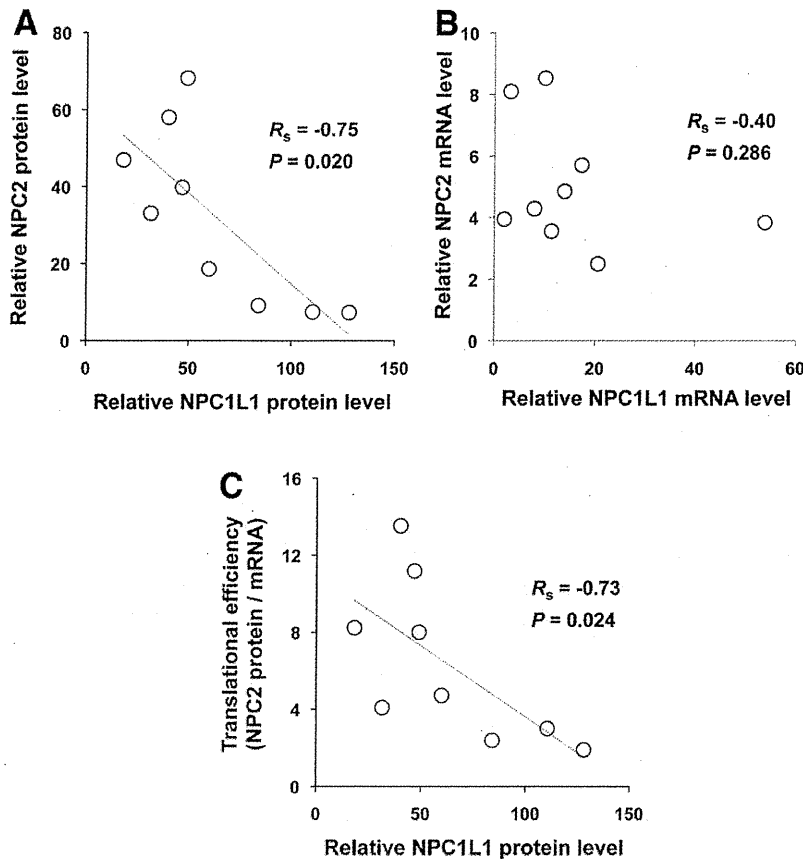Translational efficiency (NPC2 protein / mRNA)

Fig. 7. Correlation between the levels of NPC1L1 and NPC2 in human liver specimens. Homogenates and extracted RNA from human liver specimens were subjected to (A) an immunoblot analysis and (B) quantitative real-time PCR, respectively. (A) The relative protein levels of NPC1L1 and NPC2 were calculated as the band densities of the proteins and were normalized to the α-tubulin protein level in each specimen. (B) The relative mRNA levels of NPC1L1 and NPC2 were normalized to the β-actin mRNA level in each specimen. (C) The translational efficiency of NPC2 was calculated as the ratio of the NPC2 protein level to the NPC2 mRNA level. A correlation analysis was performed with Spearman's rank method. Abbreviation: R_s, Spearman's rank correlation coefficient.

NgBR may, therefore, determine the hepatic expression of NPC2 protein.

In addition to posttranscriptional modification, several groups have studied the transcriptional regulation of the NPC2 gene. For instance, Rigamonti et al.[29] reported that in human macrophages, NPC2 mRNA is induced by activators of liver X receptor (LXR). Because LXR is activated by cellular cholesterol-related compounds, the expression of NPC2 mRNA may be positively regulated by cellular cholesterol levels. On the other hand, cholesterol has been shown to down-regulate the expression of NPC1L1 by transcriptional regulation via sterol regulatory element binding protein 2 and hepatocyte nuclear factor 4α.[30,31] Taken together, these data suggest that when the cellular cholesterol level increases, the expression of NPC2 protein is effectively elevated by a combination of positive transcriptional regulation via the LXR pathway and reduced posttranscriptional regulation via the interaction with NPC1L1. Because NPC2 is a crucial protein for intracellular cholesterol trafficking, which affects the regulation of cholesterol synthesis and uptake by delivering cholesterol to the sterol-sensing machinery in the ER,[32] the expression level of NPC2 protein must be tightly regulated by various steps.

(A) NPC1L1 inhibits maturation and secretion of NPC2
(B) NPC1L1 accelerates degradation of NPC2
(C) Cholesterol efflux is reduced by the decrease in biliary NPC2
(D) NPC1L1 imports cholesterol from bile to hepatocytes

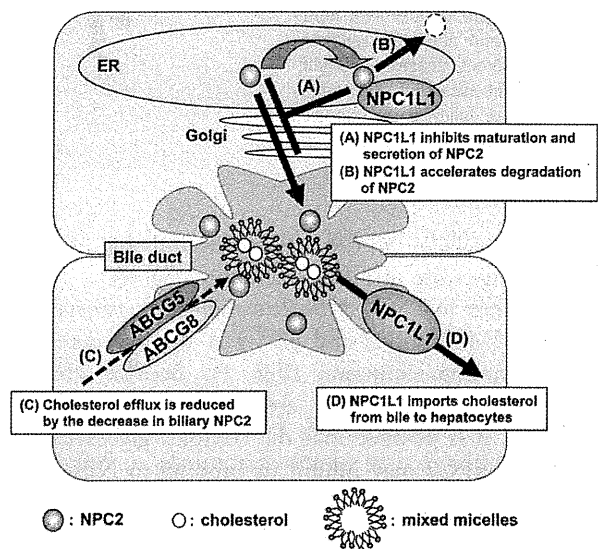⊘ : NPC2   ○: cholesterol   ❋ : mixed micelles

Fig. 8. Suggested function of hepatic NPC1L1 in the regulation of biliary cholesterol secretion. (A) NPC1L1 inhibits the maturation and secretion of NPC2. (B) NPC1L1 accelerates the degradation of NPC2. (C) By reducing NPC2 secretion, NPC1L1 indirectly suppresses ABCG5/ABCG8-mediated cholesterol efflux. (D) In addition to its well-known function as a cholesterol importer, NPC1L1 contributes to the regulation of biliary cholesterol secretion by negatively regulating NPC2 secretion.

NPC1L1 affects the secretion of NPC2 protein by inhibiting the maturation and expression of intracellular NPC2 (Figs. 2C and 5). This regulatory mechanism of NPC2 secretion would be relevant in specific tissues such as the liver and intestine because NPC1L1 is predominantly expressed in these tissues in humans. In fact, because of the negative correlation between the protein levels of NPC1L1 and NPC2 in human liver specimens (Fig. 7), it is possible that hepatic NPC1L1 negatively regulates the biliary secretion of NPC2. Our recent study revealed the physiological function of biliary NPC2 as a positive regulator of biliary cholesterol secretion mediated by ABCG5/ABCG8 on the bile canalicular membrane of hepatocytes.[15] Together with the results from this study, the data suggest that hepatic NPC1L1 may also indirectly affect ABCG5/ABCG8-mediated transport by decreasing biliary NPC2. In addition to its direct role in cholesterol re-uptake from the bile by hepatocytes,[6] NPC1L1 may down-regulate the biliary secretion of NPC2 and, consequently, reduce NPC2-mediated cholesterol efflux by ABCG5/ABCG8 from hepatocytes into the bile[15] (Fig. 8). When we consider that ABCG5 and ABCG8 are predominantly expressed in the liver and intestine and that ABCG5/ABCG8-mediated cholesterol excretion is an important process in cholesterol homeostasis, it makes sense that the regulatory mechanism for the secretion of NPC2 is working in these tissues to maintain adequate cholesterol levels in response to the dynamic cholesterol fluctuations in the body.

Collectively, the results of the present study suggest that NPC1L1 down-regulates the expression and secretion of NPC2 by interacting with NPC2 during its maturation process. Through this regulatory function, hepatic NPC1L1 is suggested to suppress the hepatic expression and biliary secretion of NPC2. In addition to its direct role in cholesterol re-uptake, hepatic NPC1L1 may effectively control biliary cholesterol secretion by negatively regulating NPC2 secretion because biliary NPC2 stimulates ABCG5/ABCG8-mediated cholesterol efflux.[15] This is the first report suggesting a function for NPC1L1 besides its activity as a (re-)uptake transporter.

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Original

Expression of *N*-acetylglucosaminyltransferase V in Intrahepatic Cholangiocarcinoma and Its Association with Clinicopathological Findings

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Purpose: *N*-acetylglucosaminyltransferase V (GnT-V), an enzyme that catalyzes the β1-6 branching of *N*-acetylglucosamine on asparagine-linked oligosaccharides of cellular proteins, enhances the malignant behaviors of carcinoma cells in experimental models. We previously reported that GnT-V expression in pathological tumor stage II gallbladder carcinoma (GBC) correlates with postsurgical survival and postsurgical recurrence of distant organ metastasis. This study aimed to examine GnT-V expression in intrahepatic cholangiocarcinoma (ICC) and determine its association with clinicopathological findings. **Method:** GnT-V expression was evaluated by immuno-histochemistry in each curatively resected ICC specimen from 72 patients (72 cases). Thereafter, the association of GnT-V expression with clinicopathological findings was examined. **Results:** Of the 72 ICC cases, 42 showed positive staining for GnT-V and the remaining 30 demonstrated negative staining. Patients with ICCs having low GnT-V expression had shorter survival time than patients with ICCs having high GnT-V expression, although the difference was not statistically significant (p = 0.31). In all of the 72 cases, the frequency of postsurgical recurrence was not significantly different in terms of GnT-V expression. **Conclusion:** Although GnT-V was found to be expressed in more than half of the ICC cases in this study, it may not be a useful marker for scaling the aggressiveness of ICC because the mechanisms underlying ICC metastasis possibly involve different molecular pathways that are not affected by GnT-V expression.

Key Words: intrahepatic cholangiocarcinoma, *N*-acetylglucosaminyltransferase V, malignant behavior

Introduction

Intrahepatic cholangiocarcinoma (ICC) is a fatal cancer, and the only curative treatment for patients with ICC is surgery[1]. However, the recurrence rate is high even after curative resection. According to the American Joint Committee on Cancer (AJCC) 7th Edition of TNM Staging, the 5-year survival rate of stage I to IV was 62%, 27%, 14%, and 0%, respectively[2]. Previous studies have shown that the most common site of recurrence is the liver[3]~[5]. Thus, if postoperative recurrence of ICC can be prevented, patient survival could be greatly improved.

Among several kinds of glycosyltransferases, *N*-acetylglucosaminyltransferase V (GnT-V) is one of the most important enzymes which are associated with carcinogenesis and tumor aggressiveness (i.e., invasion and metastasis)[6]~[8]. GnT-V is involved in the synthesis of β1-6 GlcNAc branching formation on *N*-glycans. GnT-V is also essential for tumor growth and metastasis, as previously shown in GnT-V-deficient mice[9]. The mechanisms underlying the modulation of tumor metastasis by GnT-V may involve the upregulation of signaling of many growth factor receptors on the cell surface by the suppression of receptor endocytosis[10], the enhancement of certain kinds of protease activity[11], and the

Table 1 Patient description and number of cases for each intrahepatic cholangiocarcinoma (ICC) stage (2000-2008)

Patient description n or mean ± SD	M/F	Age	Surgical procedures		
			Hep[a]	Hep with EBR[b]	Hep with PV, IVC[c]
Intrahepaticcholangiocarcinoma (n = 72)	52/20	64.8 ± 11.1	41	20	11
ICC stage*					
Stage I (n = 20)	13/7	64.7 ± 12	13	7	0
Stage II (n = 17)	11/6	65.7 ± 12.5	14	1	2
Stage III (n = 32)	26/6	64.8 ± 10.5	14	10	8
Stage IV (n = 3)	2/1	62 ± 4.3	0	2	1

*Based on the American Joint Committee on Cancer classification.
[a]: hepatectomy alone.
[b]: hepatectomy with extrahepatic bile duct resection.
[c]: hepatectomy with partial resection of the portal vein or inferior vena cava.

stimulation of angiogenesis as a cofactor[12].

Immunohistochemical studies of GnT-V have shown that GnT-V expression is positively correlated with the poor prognosis of certain kinds of cancer[13)14)]. GnT-V is one of the Golgi enzymes that modulate branching of oligosaccharides in cells. In fact, an immunofluorescent study of GnT-V in B16 mouse melanoma cells showed its localization in Golgi apparatus[13]. Our study also showed Golgi localization of GnT-V in cancer tissues.

Furthermore, we previously reported the strong positive correlations of GnT-V expression with postsurgical survival and the recurrence of distant organ metastasis in 90 cases of pathological tumor stage II (pT2) gallbladder carcinoma (GBC)[15]. However, GnT-V expression has not yet been investigated in human cholangiocarcinoma.

In this retrospective analysis, we investigated the immunohistochemical expression of GnT-V in each ICC specimen resected from 72 patients (72 cases) and determined the association of GnT-V expression with clinicopathological findings.

This study aimed to evaluate whether GnT-V is an important prognostic factor and a useful marker for scaling the aggressiveness of ICC.

Materials and Methods
Patients

Patient description and number of cases for each ICC stage are shown in Table 1. ICC specimens from 72 patients (52 men and 20 women) were analyzed. The study was approved by the Research and Ethics Committee of Tokyo Women's Medical University. They were selected consecutively by reviewing the pathologic findings, and patients who had died from other diseases were excluded. The ICC specimens of all the patients were curatively resected with a free surgical margin. The mean age of the patients was 64.8 ± 11.1 years (range, 26-83 years). These patients underwent surgery for ICC between 2000 and 2008 at the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University Hospital. ICC was definitively diagnosed on the basis of histological findings and classified according to the tumor-node-metastasis classification of the AJCC 6th Edition[16]. Of the 72 cases, 20 were in stage I, 17 stage II, 32 stage III, and 3 stage IV. The surgical procedures were as follows: hepatectomy alone in 41 patients, hepatectomy with extrahepatic bile duct resection in 20 patients, and hepatectomy with partial resection of the portal vein or inferior vena cava in 11 patients (Table 1). Fourteen patients had hepatitis C virus, 3 patients had hepatitis B virus, and 1 had both B and C virus. Eleven patients had liver cirrhosis, and 5 patients had chronic hepatitis. No patients had certain other diseases such as hepatolithiasis or primary sclerosing cholangitis. Before surgery, no patient had undergone chemotherapy or radiotherapy.

The follow-up periods until April 2008 ranged from 1 to 79 months (mean, 14 months). Of the 72 patients who had undergone curative resection with a free surgical margin, 42 were alive as of April 2008 and 30 died from intrahepatic metastasis,
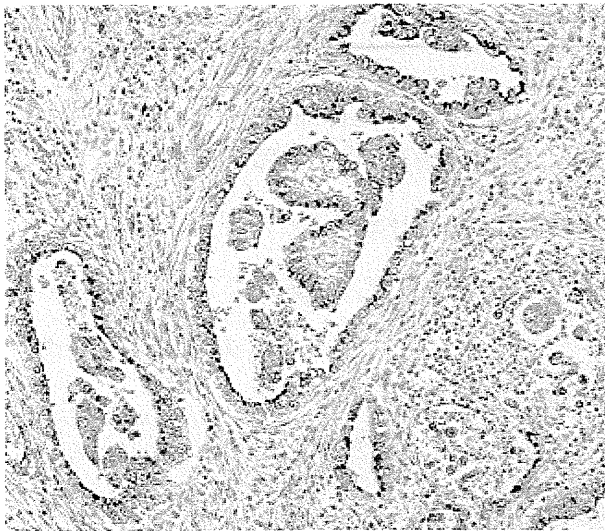
Fig. 1 Immunostaining of GnT-V
Typical positive case which shows Golgi localization of GnT-V in cancer tissues.

Table 2 GnT-V expression rates in the negative and positive staining cases according to tumor stage

	Negative	Positive	p*
ICC (n = 72)	30	42	0.861
Stage I (n = 20)	8	12	
Stage II (n = 17)	6	11	
Stage III (n = 32)	15	17	
Stage IV (n = 3)	1	2	

The GnT-V expression rates between the negative and positive staining cases were not significantly different for all tumor stages.
*X^2 for independence test.

Table 3 Association between histopathological findings and immunohistochemical localization of GnT-V

GnT-V	Negative	Positive	p*
Number	30 (100%)	42 (100%)	
Histological grade			
G1	1 (3)	3 (7)	0.862
G2-4	29 (97)	39 (93)	
Intrahepatic metastasis			
Negative	25 (83)	32 (76)	0.462
Positive	5 (17)	10 (24)	
Vascular invasion			
Negative	10 (33)	13 (31)	0.831
Positive	20 (67)	29 (69)	
Lymph node metastasis			
Negative	19 (63)	25 (60)	0.744
Positive	11 (37)	17 (40)	

There were no significant differences in the parameters of pathological malignancies between the 2 staining groups.
*X^2 for independence test.

distant organ metastasis, lymph node metastasis, or peritoneal dissemination. No patient had died from other disease or had been lost to follow-up.

GnT-V immunostaining

ICC tissues that had been preserved in 10% formalin and then embedded in paraffin were serially sectioned at 2 μm thickness, mounted on silane-coated slides, and deparaffinized. The slides were immersed in 0.3% hydrogen peroxide in methanol for 20 min to deplete endogenous peroxidase. After washing with phosphate buffered saline (PBS), the slides were incubated with a protein blocking agent for 5 min at room temperature in a humidity chamber. The slides were then stained by the indirect immunoperoxidase method using an anti-GnT-V antibody, 22G12 (Fuji-revio, Tokyo, Japan) at a 1 : 3,000 dilution rate. A negative control was prepared using 2% bovine serum albumin (BSA) instead of the mAb. Details of the procedure were described previously[13].

Evaluation of the tissue sections was performed by a single pathologist who was blinded to the clinical characteristics and histopathological findings. Immunohistochemical analysis of the total number of cancerous epithelia in each section of surgical specimens was evaluated. The immunohistochemical expression of GnT-V was classified into positive or negative (Fig. 1).

Statistical analysis

Statistical evaluations of data were analyzed using the X^2 for independence test. Survival curves of the postsurgical outcome of the 72 patients who underwent curative resection of ICC were analyzed using the Kaplan-Meier method. Differences in the survival of the 72 patients were analyzed by the log-rank test. Several clinicopathological factors were subjected to multivariate regression analysis using the Cox proportional hazards regression model. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Immunohistochemical staining and GnT-V expression in ICC

Of the 72 ICC cases, there were 42 cases (58%) in

Table 4 Postsurgical recurrence patterns of ICC

GnT-V	Negative	Positive	p*
Number	30 (100%)	42 (100%)	
Total number of recurrence	21 (70)	20 (48)	0.06
Liver metastasis	10 (33)	8 (19)	0.168
Distant organ metastasis	4 (13)	9 (21)	0.379
Lymph node metastasis	7 (23)	3 (7)	0.06
Peritoneal dissemination	3 (10)	1 (2)	0.164

There were no significant differences in the frequencies and patterns of recurrence between the 2 staining groups.
*X^2 for independence test.
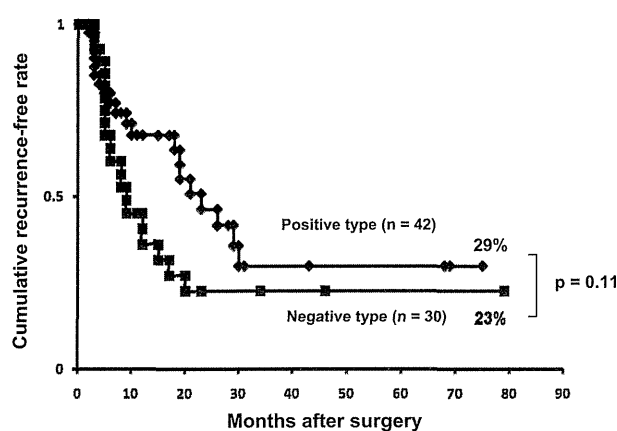
Fig. 2 Association between postsurgical recurrence-free rate and GnT-V expression
There was no significant difference in the cumulative recurrence-free rates between the positive type and the negative type.

Fig. 3 Association between postsurgical survival rate and GnT-V expression
There was no significant difference in the cumulative survival rates between the positive type and the negative type.

which GnT-V was expressed in cancer tissues, and they were defined as positive. The remaining 30 cases (42%) that did not express GnT-V in cancer tissues were defined as negative.

The GnT-V expression rates between the negative and positive staining cases were not significantly different for all tumor stages (Table 2).

Association between parameters of pathological malignancies and GnT-V expression in patients with ICC

A comparison of the positive staining and negative staining of GnT-V was made with particular reference to the parameters of pathological malignancies, namely, histological grade, intrahepatic metastasis, vascular invasion, and lymph node metastasis (Table 3). There were no significant differences in the parameters of pathological malignancies between GnT-V expression types.
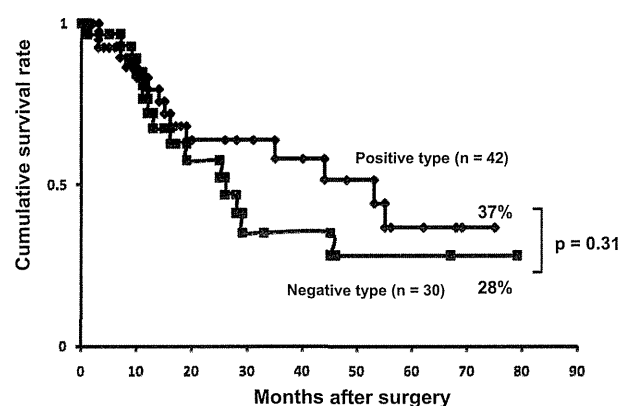
Association between pattern of recurrence in patients with ICC and GnT-V expression in the specimens

The postsurgical pattern of recurrence of the 72 ICC cases was compared in terms of GnT-V expression (Table 4). Of the 42 cases showing positive staining, 8 had liver metastasis, 9 had distant organ metastasis, 3 had lymph node metastasis, and 1 had peritoneal dissemination. Of the 30 cases showing negative staining, 10 had liver metastasis, 4 had distant organ metastasis, 7 had lymph node metastasis, and 3 had peritoneal dissemination. There were no significant differences in the frequencies and patterns of recurrence between the 2 staining groups.

Association between postsurgical recurrence-free rate of patients with ICC and GnT-V expression in the specimens

The overall postsurgical recurrence-free rate of

Table 5 Multivariate regression analysis of prognostic factors

Variable	Hazard ratio	C.L.	p
Histology*	-	-	0.983
Intrahepatic metastasis	3.670	1.644-8.195	0.002
Vascular invasion	2.471	0.691-8.834	0.164
Lymph node metastasis	2.851	1.237-6.573	0.014
GnT-V expression	0.458	0.206-1.017	0.055

*Histology: poorly differentiated adenocarcinoma or others.

Table 6 Stepwise multivariate regression analysis of prognostic factors

Variable	Hazard ratio	C.L.	p
Intrahepatic metastasis	4.932	1.241-31.975	0.026
Vascular invasion	3.355	0.898-23.613	0.077
Lymph node metastasis	11.241	2.532-34.578	0.001
GnT-V expression	4.165	0.061-0.945	0.041

Variable	Hazard ratio	C.L.	p
Intrahepatic metastasis	6.236	1.561-40.299	0.013
Lymph node metastasis	2.851	3.282-41.966	0.001
GnT-V expression	0.458	0.077-1.000	0.060

the 72 patients with ICC was compared in terms of GnT-V expression (Fig. 2). The cumulative recurrence-free rate of patients in the negative staining group tended to be lower than that of patients in the positive staining group. However, the difference was not statistically significant (23% vs 29%; p = 0.11).

Association between postsurgical survival rate of patients with ICC and GnT-V expression in the specimens

The overall postsurgical survival rate of the 72 patients with ICC was compared in terms of GnT-V expression (Fig. 3). The cumulative survival rate of patients in the negative staining group tended to be lower than that of patients in the positive staining group respectively. However, the difference was not statistically significant (28% vs 37%; p = 0.31).

A summary of the results of the multivariate regression analysis of prognostic factors for ICC is shown in Table 5. In the analysis, intrahepatic metastasis (p = 0.002) and lymph node metastasis (p = 0.014) were found to be statistically significant independent risk factors. GnT-V was not a statistically significant independent risk factor (Table 5). Furthermore, intrahepatic metastasis (p = 0.013) and

lymph node metastasis (p = 0.001) were found to be important risk factors by stepwise multivariate regression analysis (Table 6).

Discussion

GnT-V expression is closely associated with the survival outcomes of patients with several kinds of cancers[13)14)]. We have recently reported that in pT_2 GBC, the survival rate of patients in the positive staining group was significantly lower than that of patients in the negative staining group[15)]. In this study, we investigated relations between clinicopathological features, surgical outcomes, and the expression of GnT-V in ICC. The results of the present study showed a tendency for GnT-V expression to inversely correlate with postsurgical recurrence and survival outcomes of patients with ICC (Tables 4, 5). In particular, the results of multivariate regression analysis showed a higher correlation of GnT-V expression with post surgical prognosis than with histology or vascular invasion (Table 5).

Malignant transformation of glandular epithelia is accompanied by alterations in the biochemical and biological characteristics of glycoproteins. Elevated levels of β1-6 branching of *N*-acetylglucosamine, transferred by GnT-V, are shown to be positively

correlated with metastatic potential and tumor invasiveness in several reports. Moreover, GnT-V functions as an angiogenesis inducer that has a completely different function from the original function of glycosyltransferase[12]. A secreted type of GnT-V protein has specifically been shown to promote angiogenesis *in vitro* and *in vivo*[17][18]. In agreement with the above-mentioned biological roles of GnT-V, our recent *in vitro* and *in vivo* experiments using GBC cells have also shown that the cellular expression levels of GnT-V are positively correlated with malignant behaviors, such as rapid cell growth, potent angiogenic capability, and high metastatic potential (unpublished results).

The reasons why GnT-V expression showed a tendency to inversely correlate with ICC aggressiveness in this study remain unknown. Several reports showed that GnT-V expression was inversely correlated with poor prognosis of certain kinds of cancer[19][20]. This discrepancy might be dependent on whether or not cancer cells have target proteins of GnT-V or a protease involved in GnT-V cleavage.

It was reported that the expression and immunohistochemical localization of a cancer-associated glycoprotein, mucin core polypeptide 1 (MUC1), correlated with the aggressiveness of ICC[21] and with that of GBC[22]. Carcinoma cells expressing MUC1 are less sensitive to cytolysis by human lymphokine-activated killer lymphocytes[23]~[26] and MUC1 overexpression inhibits integrin-mediated extracellular matrix interaction[27]. Moreover, MUC1 on the cell membrane destabilizes cell-to-cell adhesion and allows carcinoma cells to migrate and metastasize[28]. These reports may therefore be helpful to speculate that the mechanisms underlying ICC metastasis possibly involve molecular pathways that are not affected by GnT-V expression.

On the other hand, more advanced cases were included in this retrospective analysis than in other studies[13][15] so it may be difficult to evaluate the biological role of GnT-V in ICC correctly.

In conclusion, GnT-V was found to be expressed in more than half of the ICC cases and GnT-V expression showed a tendency to inversely correlate with ICC aggressiveness, so it may be a useful marker for scaling the low malignant potential of ICC. However, because of the large number of advanced cases in this study, caution is needed to draw any conclusion regarding the correlation between GnT-V expression and ICC biological malignancy. Thus far, lymph node metastasis is considered to be the most important prognostic factor for ICC[29]~[32]. In this study, intrahepatic metastasis and lymph node metastasis were evaluated as important prognostic factors. However, as the accurate assessment of ICC intrahepatic metastasis and lymph node metastasis is very difficult in daily clinical practice, there is a crucial need to accumulate more cases to further investigate the biological markers that correlate with ICC lymph node or distant organ metastasis.

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肝内胆管癌における糖転移酵素 N–アセチルグルコサミン転移酵素 V（GnT-V）発現とその臨床病理学的意義

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〔目的〕癌細胞表面の糖鎖構造はその悪性挙動に深く関与する．糖転移酵素 GnT-V は，ノックアウトマウスの解析結果より，癌の増殖・転移に必須の分子であることが証明されており，様々な癌種において生物学的悪性度との関連性が報告されている．以前我々は，pT2 胆嚢癌（GBC）の GnT-V 陽性例において，術後遠隔臓器転移再発が多く，陰性例と比較し有意に予後不良であることを報告した．しかし，他の胆道癌に関しての GnT-V 発現と臨床病理学的意義に関する報告は過去にない．そこで，肝内胆管癌（ICC）における GnT-V 発現と予後および術後再発との関連性について検討を行った．〔方法〕ICC 治癒切除 72 例を対象とし，GnT-V 発現を免疫組織学的にて解析し，その結果を臨床病理学的因子および術後予後と比較検討した．〔結果〕GnT-V の免疫組織学的発現は陽性 42 例，陰性 30 例であった．GnT-V 発現の有無と病理組織学的因子との間に有意な相関関係は認められなかった．GnT-V 陽性例は陰性例と比較し術後 5 年生存率は高い傾向にあったが有意差を認めなかった．（p＝0.31）また，GnT-V 発現の有無と術後再発率，再発様式との間に有意差は認めなかった．〔結語〕ICC においては，GnT-V と関連のない機序の癌増殖・転移経路の存在が示唆された．

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95：749

<症例報告>

総胆管結石内視鏡的治療1年後に発見された上部胆管早期癌の1例

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要旨：症例は70歳代，男性．主訴は右上腹部痛．腹痛精査のための腹部CTで総胆管結石を疑われたため当科を紹介された．第1回入院時腹部CT，MRCP，ERCPで上部胆管に不整形の総胆管結石を認め，十二指腸乳頭切開術後総胆管結石切石を行った．胆嚢結石は認めなかった．退院1年後に右上腹部痛再燃し当科入院．腹部CTで胆管内腔にはわずかな造影効果を伴う腫瘍を認めた．ERCPでは上部胆管に乳頭状腫瘍を認め胆管生検で高分化腺癌を得た．肝外胆管切除術を施行し病理組織診断ではfm, fStage IAであった．胆管癌に胆管結石を合併することがあり，不整形の胆管結石の内視鏡治療時には特に注意して詳細な観察を行う必要があると考えられた．

索引用語：　ERCP　　胆管癌　　胆管結石症

はじめに

　胆管結石に胆管癌を合併することは多いとはいえずその頻度は不明である[1,2]．一方，胆管癌に胆管結石を合併することも報告されているがその頻度も不明である[3]〜[15]．今回，我々は総胆管結石に対する内視鏡治療1年後に上部胆管癌を発見された1例を経験したため報告する．

症　　例

症　例：70歳代，男性．

主　訴：右上腹部痛

既往歴：慢性閉塞性肺疾患，狭心症，前立腺癌，慢性B型肝炎

家族歴：特記すべきことなし

嗜好歴：飲酒歴：機会飲酒，喫煙歴：なし．

　第1回入院時現症：血圧116/72mmHg，脈拍49回/分・整，体温34.8℃．右上腹部に軽度の自発痛・圧痛を

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認めるが，腹部は平坦・軟で筋性防御は認めない．眼瞼結膜に貧血無く，眼球結膜に黄疸を認めない．

　現病歴：2006年7月通院していた近医で軽度右上腹部痛のため腹部CT検査施行．CT検査で総胆管結石を認めたため当科第1回紹介入院となった．

　第1回入院時検査所見：γGTPの軽度上昇を認めたが，黄疸・炎症所見は認めなかった（表1）．

　腹部CT検査：胆管上・中部に不整形の結石を認めた．腹部CT，腹部超音波検査で胆嚢結石を認めなかった．肝内胆管の軽度拡張を認めたが（図1a，矢頭），胆管壁および胆管内に造影効果を示す病変はみられなかった（図1a，b，c，矢印）．

　ERCP検査：ERCP施行時に観察された十二指腸乳頭形態に異常は認めなかった（図1d）．胆管造影検査ではCT・MRCPと同様に上・中部胆管に可動性を有する不整形の陰影欠損（図1d，矢印）を認めた．胆嚢管は造影された（図1d矢頭）．また，胆管造影で膵・胆管合流異常は認めなかった（図1e）．

　第1回入院後経過：内視鏡的乳頭切開術（EST）・内視鏡的総胆管結石切石術（EML）後の胆管造影では上部胆管のごくわずかな狭窄（図1f，矢印）が認められたが，表面は平滑で胆管壁不整を認めず胆管炎による変化であると判断された（図1f）．胆管炎は改善し退院した．

　2007年9月腹痛・食欲不振のため当科第2回紹介入院となった．

　第2回入院時現症：血圧112/70mmHg，脈拍88回/

表1　第1回入院時検査所見

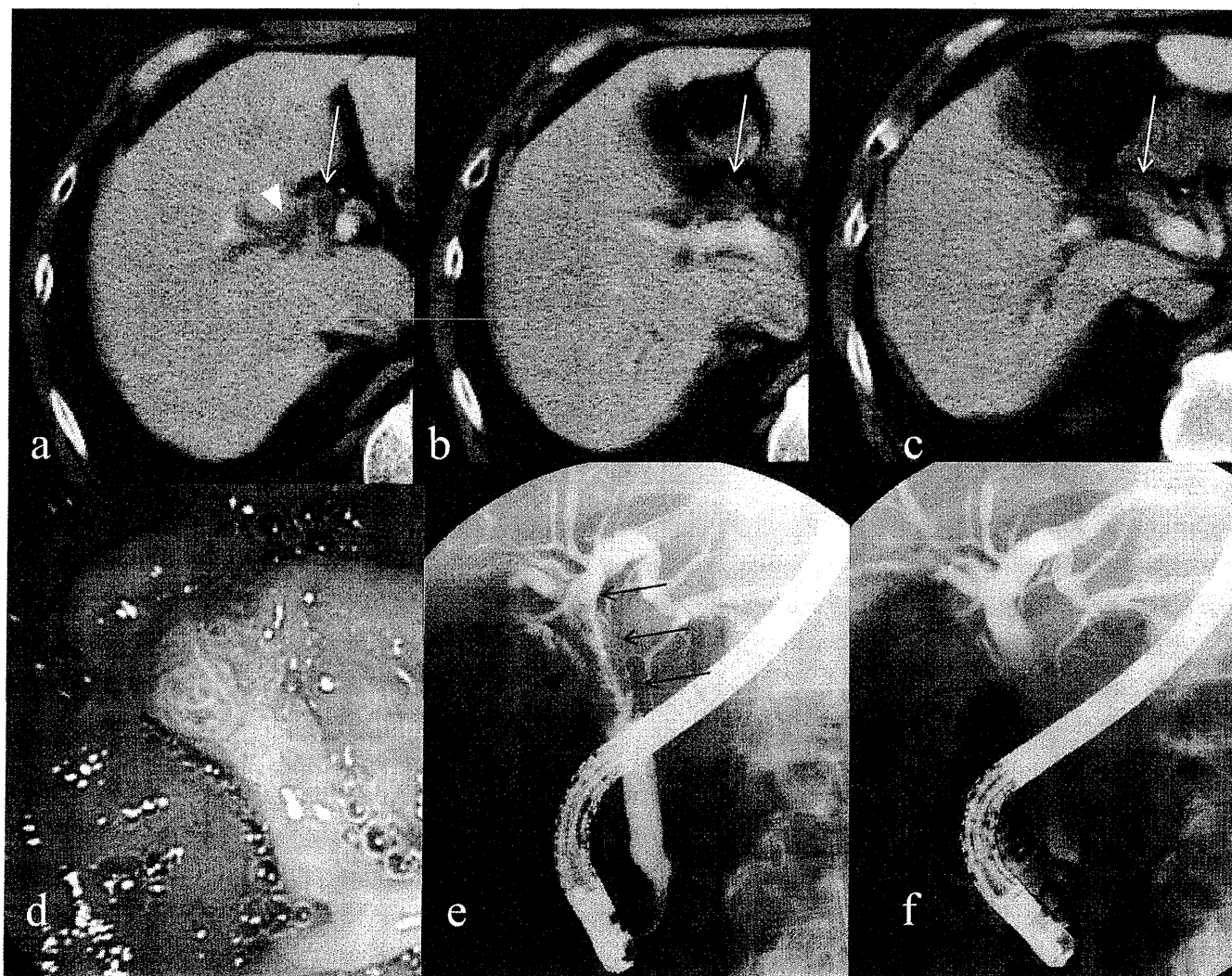| WBC	3400 /μl	T.Bil	0.6 mg/dl	APTT	30.0 sec
RBC	401×10⁴ /μl	D.Bil	0.3 mg/dl	PT	11.7 sec
Hb	13.4 g/dl	ALP	98 IU/l	PT-INR	1.15
Ht	39.5 %	γGTP	102 IU/l		
Plt	13.0×104 /μl	AST	22 IU/l	CEA	1.3 mg/ml
		ALT	24 IU/l	CA19-9	5.2 mg/ml
Na	142 mEq/l	CK	107 IU/l		
K	4.2 mEq/l	LDH	164 IU/l		
Cl	105 mEq/l	TP	6.8 g/dl		
Ca	9.4 mg/dl	ALB	4.2 g/dl		
UN	15.7 mg/dl	AMY	83 IU/l		
Cr	0.94 mg/dl	CRP	0.08 mg/dl		

図1　総胆管結石治療時画像所見
a　第1回入院時腹部CT．肝内胆管の軽度拡張を認めた（矢頭）．
a, b, c　胆管壁ならびに胆管内腔に造影効果を示す病変はみられなかった（矢印）．
d　第1回入院時ERCP．十二指腸乳頭形態は正常．
e　第1回入院時ERCP．上中部胆管に不整形の陰影欠損（矢印）を認めた．胆嚢管は描出された（矢頭）．
f　第1回入院時総胆管結石切石術後ERCP．上部胆管は軽度狭窄のみで胆管壁不整は認められない（矢印）．

表2　第2回入院時検査所見

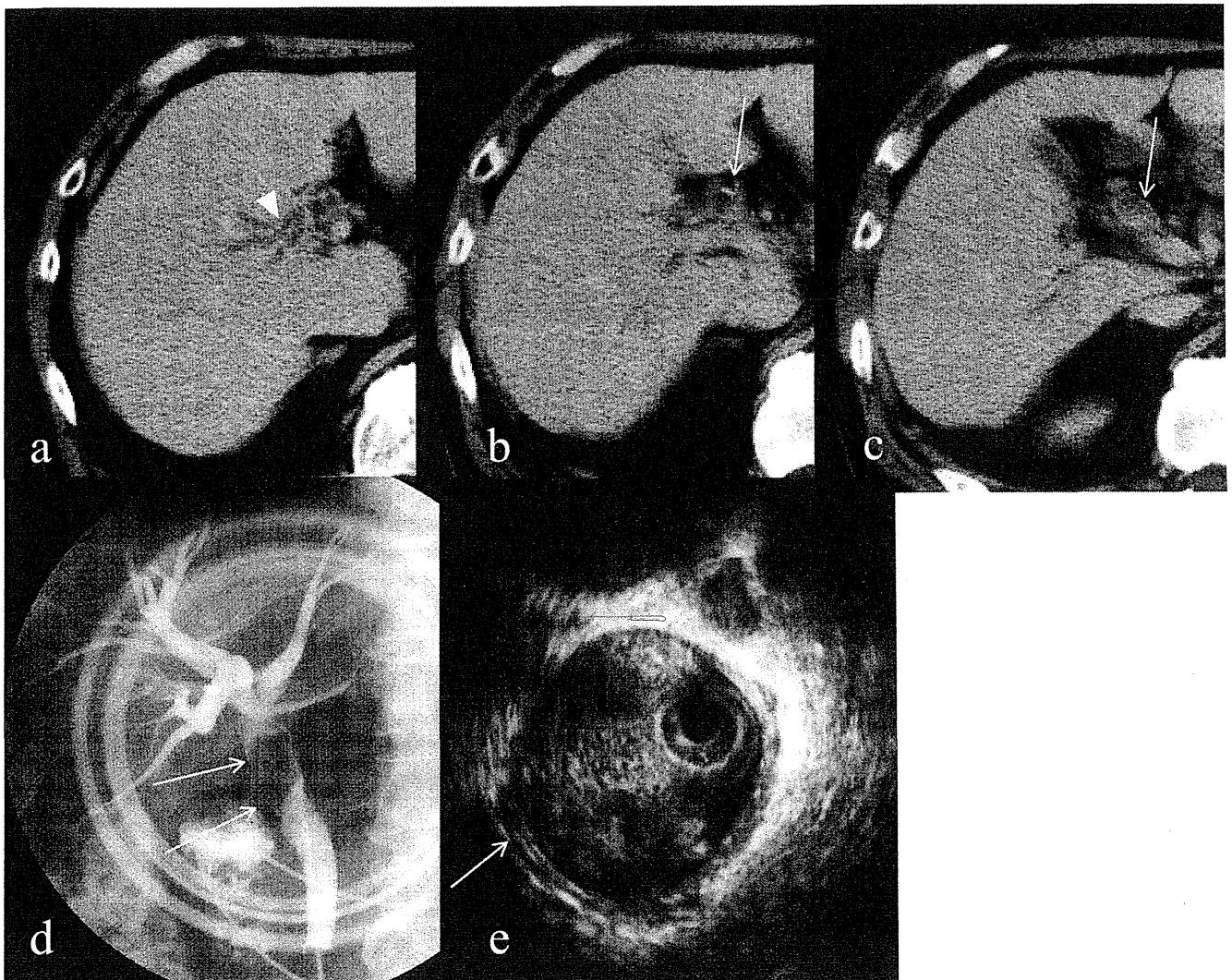|					
WBC	5320 /μl	T.Bil	2.0 mg/dl	APTT	28.8 sec
RBC	395×10⁴ /μl	D.Bil	1.2 mg/dl	PT	11.7 sec
Hb	12.9 g/dl	ALP	685 IU/l	PT-INR	1.15
Ht	39.1 %	γGTP	1204 IU/l		
Plt	14.8×104 /μl	AST	149 IU/l	CEA	1.9 mg/ml
		ALT	150 IU/l	CA19-9	14.4 mg/ml
Na	141 mEq/l	CK	59 IU/l		
K	4.1 mEq/l	LDH	183 IU/l		
Cl	103 mEq/l	TP	7.4 g/dl		
Ca	9.0 mg/dl	ALB	4 g/dl		
UN	14.9 mg/dl	AMY	62 IU/l		
Cr	0.88 mg/dl	CRP	0.92 mg/dl		

図2　胆管癌発見時画像所見
　a　第2回入院時腹部CT．肝内胆管の軽度拡張を認めた（矢頭）．
　b　第2回入院時腹部CT．胆管壁左側にわずかな造影効果を認めた（矢印）．
　c　第2回入院時腹部CT．軽度の造影効果を示す胆管腫瘍を認めた（矢印）．
　d　第2回入院時ENBD造影で上部胆管に乳頭状隆起性病変を認めた（矢印）．
　e　第2回入院時IDUSで胆管壁外側高エコー層は保たれていた（矢印）．