Identification of antibody against wingless-type MMTV integration site family member 7B as a biliary cancer tumor marker

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Abstract. Biliary cancer has a poor prognosis due to a lack of specific biomarkers and difficulty in diagnosis. The present study aimed to identify serum tumor markers for the diagnosis of biliary cancer via serological identification of antigens by recombinant cDNA expression cloning. Wingless-type MMTV integration site family, member 7 (WNT7B) was identified as a target antigen, suggesting the presence of serum antibodies against this antigen. Deletion mutants were then prepared to evaluate the response to serum antibodies. When serum antibody levels against WNT7B deletion mutants ($WNT7B_{-92\text{-}2}$, $_{-92\text{-}260}$, $_{2\text{-}260}$ and $_{184\text{-}260}$) were examined using amplified luminescence proximity homogeneous assay-linked immunosorbent assay, the levels of the antibody against WNT7B with amino acids 184-260 were higher in patients with biliary cancer than in healthy donors. Therefore, the region covering residues 184-260 of WNT7B was decomposed to generate seven peptides, and the levels of antibodies against these peptides were measured. Among them, the levels of antibodies against $WNT7B_{234\text{-}253}$ and $WNT7B_{244\text{-}260}$ were higher in patients with biliary cancers than in healthy donors ($WNT7B_{234\text{-}253}$, P=0.0009; $WNT7B_{244\text{-}260}$, P=0.0005). The levels of the antibody against the former were specifically high in patients with biliary cancer but not in those with esophageal, gastric, colorectal, pancreatic, or breast cancer. Furthermore, analysis by the cutoff value of $WNT7B_{234\text{-}253}$ defined by ROC showed a high sensitivity of 70% in patients with biliary cancer. Therefore, the serum levels of the antibody against $WNT7B_{234\text{-}253}$ may be useful as a marker for biliary cancer diagnosis.

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Abbreviations: AlphaLISA, amplified luminescence proximity homogeneous assay-linked immunosorbent assay; CA19-9, carbohydrate antigen 19-9; GST, glutathione S-transferase; SEREX, serological identification of antigens by recombinant cDNA expression cloning; *E. coli*, *Escherichia coli*; WNT7B, wingless-type MMTV integration site family, member 7; BLAST, Basic Local Alignment Search Tool

Key words: AlphaLISA, biliary cancer, SEREX, tumor marker, WNT7B

Introduction

Biliary cancer has a poor prognosis. The 5-year survival rate ranges from 24.2 to 61.3% (39.8% for gallbladder cancer, 24.2% for perihilar bile duct cancer, 39.1% for distal bile duct cancer, and 61.3% for ampullary region cancer) (1). Biliary cancer is difficult to detect and is often already advanced at the time of diagnosis. Therefore, it is necessary to develop simple diagnostic tools for detection. One such method is tumor marker measurement, which is possible with a minimally invasive blood test. However, tumor markers are primarily used for identifying advanced cancers, specifically for monitoring

therapeutic effects and possible recurrence. Yamada *et al* (2) emphasized the significance of serum carbohydrate antigen 19-9 (CA19-9) as a tumor marker with high diagnostic accuracy for para-aortic lymph node and regional lymph node metastases in intrahepatic cholangiocarcinoma. However, CA19-9 levels may also be abnormally high in response to inflammation (3). Thus, identifying new tumor markers is important because CA19-9 alone has a limited diagnostic ability for biliary cancer. Serum p53 antibodies are highly expressed in esophageal, colorectal and uterine cancers (4). Zhang *et al* (5) also reported that serum p53 antibodies may be a tumor marker for various cancers, including breast, colon, lung, and ovarian cancers. Previously, these antibodies have been recognized as markers for extrahepatic cholangiocarcinoma (6). Therefore, it was sought to identify tumor markers for biliary cancer.

Serological identification of antigens by recombinant cDNA expression cloning (SEREX), a screening method that utilizes the antigen-antibody response, employs cDNA libraries from tumor specimens expressed in *Escherichia coli* (*E. coli*) to screen for clones reactive to patient serum IgG. This platform has enabled the large-scale screening of tumor antigens and has identified numerous novel antigens (7). SEREX is an effective screening method to isolate tumor markers that elicit high-titer IgG response, which can be measured in patient serum. SEREX has been used for various malignancies, such as esophageal cancer (8,9), gastric cancer (10,11), colon cancer (12,13), pancreatic cancer (14,15) and hepatocellular carcinoma (16,17). Thus far, SEREX has not yet been employed to identify markers for biliary cancer.

In the present study, SEREX was performed to search for novel tumor markers of biliary cancer. The levels of the antibody against the tumor antigen obtained by SEREX were measured in the serum using amplified luminescence proximity homogeneous assay-linked immunosorbent assay (AlphaLISA).

Materials and methods

Human samples. Sera were obtained from 56 patients with biliary cancer who underwent surgical intervention at the Department of Gastroenterological Surgery II, Faculty of Medicine, Hokkaido University between 2014 and 2016; biliary cancer was pathologically diagnosed in these patients using their surgical specimens or biopsy tissue. The inclusion criteria were histologically diagnosed intrahepatic biliary cancer, extrahepatic biliary cancer, gallbladder cancer, cystic ductal cancer, or cancer of the ampulla of Vater. Patients with other synchronous cancers or autoimmune diseases were excluded. The patients in the present study were classified according to the tumor-node-metastasis classification of the Union for International Cancer Control, 7th edition as follows: stages 0 (n=3), I (n=2), II (n=22), III (n=18), IV (n=9), and X (stage unknown; n=2). These patients underwent surgical methods such as radical operation, palliative operation, and exploratory laparotomy. Blood was collected in tubes without ethylenediaminetetraacetic acid from the 56 patients who provided written informed consent upon admission. The blood samples were then centrifuged at 3,000 x g for 10 min at room temperature, and the supernatants (sera) were stored at -80˚C. Sera from 30 healthy donors were obtained from Tokyo Future Style (Tsukuba, Japan). Serum samples were also collected from 14 patients with biliary cancer, 87 with esophageal cancer, 92 with gastric cancer, 93 with colorectal cancer, 39 with pancreatic cancer, and 91 with breast cancer and from 114 healthy donors from the Department of Frontier Surgery, Graduate School of Medicine, Chiba University. The study protocol was approved by the Ethics Committee of Hokkaido University Hospital (approval no. 015-0098; Sapporo, Japan) and Chiba University Graduate School of Medicine (approval no. 973; Chiba, Japan).

Screening of cDNA libraries by SEREX. The cDNA libraries were screened for clones as previously reported (7-9,18,19) using a commercially available λ ZAP II phage-based human testis cDNA library (Stratagene; Agilent). XL1-Blue MRF' (Stratagene; Agilent) *E. coli* cells infected with the λ ZAP II phage were cultured at 37˚C on NZY agar plates for 4-5 h until plaques appeared. NitroBind nitrocellulose transfer membrane (cat. no. 1215471; GVS Japan; gvsjapan.co.jp) pretreated with isopropyl ß-D-1-thiogalactopyranoside for 30 min was placed on the NZY agar plates and incubated for 2 h to transfer expressed proteins from the agar plates. The membranes were washed thrice with TBS-T (0.05% Tween 20; 150 mM NaCl; 20 mM Tris-HCl, pH 7.5) and incubated for 1 h at room temperature with biliary cancer patient sera (1:2,000 dilution), which served as the source of the antibodies. After three washes with TBS-T, the membranes were incubated with alkaline phosphatase-conjugated AffiniPure goat anti-human IgG (1:5,000 dilution; RRID: AB_2337577; Jackson ImmunoResearch Laboratories, Inc.). Positive reactions were visualized using a color development solution (0.3 mg/ml nitroblue tetrazolium chloride; 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate; 100 mM NaCl; 5 mM $MgCl_2$; 100 mM Tris-HCl, pH 9.5). The positive plaques were further screened twice, after which the clones were monoclonalized.

Identification of antigen-encoding genes by sequence analysis. Screened monoclonal phage cDNA clones were converted to pBluescript phagemid constructs via *in vivo* excision using the ExAssist Helper Phage (Stratagene; Agilent). Plasmid DNA obtained from the *E. coli* SOLR (Stratagene; Agilent) strain was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI PRISM 3130-Avant Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). A search using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the resulting sequences was performed to identify the genes encoded by candidate antigens.

Expression and purification of glutathione S-transferase (GST)-fusion antigenic proteins. Recombinant GST-tagged proteins were constructed by recombining the insertion sequence of pBluescript with that of the pGEX-4T-1 vector plasmid (Cytiva). The pGEX-4T-1 vector was digested using EcoRI and XhoI (Nippon Gene Co., Ltd.). Inserted cDNAs in the pBluescript plasmids were amplified via polymerase chain reaction using PrimeSTAR HS (premix; Takara Bio,

Inc.). The DNA fragments of the insert cDNAs and the vector pGEX-4T-1 were isolated using QIAquick Gel Extraction kit (Qiagen GmbH) and ligated using In-Fusion HD Cloning Kit (Takara Bio, Inc.) according to the manufacturer's protocol. ECOS competent *E. coli* JM109 cells (Nippon Gene Co., Ltd.) were transformed with the ligation mixtures and selected on Luria-Bertani agar plates containing 50 μg/ml ampicillin and incubated overnight at 37˚C. Plasmid DNA was purified, and successful recombinants were confirmed via DNA sequencing. The recombinant plasmids were introduced into ECOS competent *E. coli* BL21 (DE3; Nippon Gene Co., Ltd.) cells for protein expression. The expression of GST-fusion proteins was induced by incubating the cells with 0.5 mM isopropyl ß-D-1-thiogalactopyranoside for 4 h at 25˚C. GST-fusion proteins were purified using the GSTrap FF column (Cytiva), and the buffer was exchanged with phosphate-buffered saline using Amicon Ultra-15 Centrifugal Filters (MilliporeSigma).

Solubilization of insoluble proteins. Insoluble GST-fusion proteins were dissolved using 8 M urea in TED buffer (50 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid; and 1 mM dithiothreitol), and the samples were dialyzed stepwise against 4 M urea in TED buffer, 2 M urea in TED buffer, and 50 mM NaCl in TED buffer.

Preparation of antigenic proteins and peptides. Wingless-type MMTV integration site family, member 7 (WNT7B) was selected as the target antigen. One GST-tagged recombinant protein ($WNT7B_{245-353}$; GST + WNT7B residues 245-353) was purchased from Abnova (cat. no. NP_478679), and four deletion mutants ($WNT7B_{-92-2}$, $_{-92-260}$, $_{2-260}$ and $_{184-260}$) were constructed using the KOD-Plus-Mutagenesis kit (Toyobo Life Science) according to the manufacturer's protocol. The other four mutants, apart from $WNT7B_{245-353,}$ were used to transform ECOS competent *E. coli* BL21 (DE3) cells to express the cDNA products. N-terminal biotinylated peptides were purchased from Peptide 2.0, Inc. The names and residue numbers of the GST-tagged recombinant proteins and N-terminal biotinylated peptides are shown in Table SI.

Analysis of serum antibody levels by AlphaLISA. The serum levels of antibodies against candidate antigens in the patient and healthy donor groups were compared using AlphaLISA immunoassay. Serum samples (2.5 μl, 1:100 dilution) with AlphaLISA ImmunoAssay Buffer (PerkinElmer, Inc.) and 2.5 μl of GST, GST-fusion protein (10 μg/ml), biotin, or N-terminal biotinylated peptides (400 ng/ml) were placed in 384-well microtiter plates (white opaque OptiPlate). The mixture was incubated for at least 3 h at room temperature in the dark. AlphaLISA anti-human IgG Acceptor Beads (2.5 μl of 40 μg/ml) and Glutathione-Donor Beads (2.5 μl of 40 μg/ml) or Streptavidin-Donor Beads were added, followed by 1 to 14 days of incubation. The Alpha photon counts representing the antigen-antibody reaction were measured using an EnSpire Alpha microplate reader (PerkinElmer, Inc.). The serum levels of antibodies against the GST-tagged proteins were determined by subtracting the Alpha photon counts for GST from those for the GST-tagged proteins. The serum levels of antibodies against the N-terminal biotinylated peptides were determined by subtracting the Alpha counts for biotin from those for N-terminal biotinylated peptides (20). Each measurement was performed in triplicate.

Analysis of the tertiary structure of WNT7B. The tertiary structures of $WNT7B_{234-253}$ and $WNT7B_{244-260}$ were predicted using computational modeling (I-TADSSER: Iterative Threading ASSEmbly Refinement, https://zhanggroup.org/I-TASSER/) (21). A peptide consisting of 20 amino acid residues was applied to the prediction. Hence, residues 234-253 were assigned to the model for the $WNT7B_{234-253}$ domain and residues 244-263 for $WNT7B_{244-260}$.

Cell lines. Human biliary cancer cell lines, G-415, HuCCT1, TFK-1, and YSCCC, were purchased from RIKEN. A normal human dermal fibroblast (NHDF) cell line was purchased from Takara Bio, Inc. Biliary cancer cell lines were cultured at 37˚C in RPMI-1640 (Nacalai Tesque, Inc.), while NHDF was cultured in DMEM (Nacalai Tesque, Inc.). All media were supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol and used for cDNA synthesis (PrimeScript RT Master Mix; Takara Bio, Inc.). cDNA was then used to amplify target genes using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) and gene-specific primers. PCR reactions and data analysis were performed via the StepOne Real-Time PCR System (Thermo Fisher Scientific, Inc.), using the comparative $2^{-\Delta\Delta Cq}$ method, normalized against the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta Cq}$ method was based on a previous study (22). The thermocycling conditions of qPCR were as follows: 95˚C for 20 sec, followed by 40 cycles of denaturation, 3 sec each at 95˚C, and annealing/extension at 60˚C for 30 sec. Primer specificity was confirmed by peaks in the melting curve. All experiments were performed in triplicate for each sample. The primers used in the present study were as follows: GAPDH forward, 5'-GAA GGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGT GATGGGATTTC-3'; WNT7B forward, 5'-TGGCGTCCT GTACGTGAA-3' and reverse, 5'-TCTTGTTGCAGATGA TGTTGG-3'.

Western blotting of whole cell lysates. Total cell lysates were prepared using radioimmunoprecipitation buffer with phenylmethylsulfonyl fluoride. Protein samples (10 μg/sample) were resolved using Mini-PROTEAN TGX Precast Gels 4-20% (Bio-Rad Laboratories, Inc.) and transferred to a polyvinylidene difluoride membrane (Merck Millipore). Membranes were probed at 25˚C with target-specific primary antibodies [WNT7B (1:5,000; cat. no. ab155313; Abcam) for 1 h and β-actin (1:5,000; cat. no. MAB1501; Merck Millipore) for 16 h] followed by incubation at 25˚C for 1 h with secondary antibodies [horseradish peroxidase (HRP)-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (1:30,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.) against the β-actin and HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (1:30,000; cat. no. 111-035-003; Jackson

Table I. Genes identified by SEREX screening of sera of patients with biliary cancer.

Clone name	Gene identify	NCBI Accession no.
BC1B1A2-3	Zinc finger and SCAN domain-containing 18	XM_017027170.2
BC2H1C2-3	Wingless-type MMTV integration site family, member 7B	XM_011530366.1
BC3B1-3	Cilia- and flagella-associated protein 53	NM_145020.4
BC6D1-3	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.6
BC8A1-3	Nascent polypeptide-associated complex subunit alpha	NM_001320193.1
BC9C1, BC9E1	BRCA1-associated protein	NM_006768.4

ImmunoResearch Laboratories, Inc.) against the WNT7B antibody]. Immunoreactivity was detected with an Enhanced Chemiluminescence Detection System (GE Healthcare Life Sciences).

Statistical analyses. All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc.). Significant differences in serum antibody levels between healthy donors and patients with cancer were analyzed using Wilcoxon rank-sum test, which was also used to analyze the correlation among sex, age, and serum antibody levels. The serum antibody level cutoff value was fixed based on the receiver operating characteristic curve analysis. The correlation between the serum level of CA19-9 and that of the antibody against $WNT7B_{234-253}$ was evaluated using Spearman's rank correlation coefficient. Overall survival was analyzed via the Kaplan-Meier method and compared using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of biliary cancer-associated antigens by SEREX. A total of $1x10^6$ clones in the testis cDNA library were screened using sera from 10 biliary cancer patients, and seven immunoreactive clones were isolated. DNA sequence analysis and BLAST search identified six distinct antigens: zinc finger and SCAN domain-containing 18 (ZSCAN18), WNT7B, cilia- and flagella-associated protein 53 (CFAP53), GAPDH, nascent polypeptide-associated complex alpha subunit (NACA), and BRCA1-associated protein (BRAP) (Table I). The following nucleotide regions were isolated from each gene: 82-2596 in *ZSCAN18*, 164-3683 in *WNT7B*, 47-1828 in *CFAP53*, 77-1084 in *GAPDH*, 311-1038 in *NACA*, and 799-2035 and 286-2100 in *BRAP*.

mRNA and protein expression of WNT7B in human cell lines. *WNT7B* mRNA was not detected in the non-biliary cancer cell line (NHDF) but was observed in all biliary cancer cell lines (G-415, HuCCT1, TFK-1 and YSCCC) (Fig. S1A). WNT7B protein expression was not observed in the non-biliary cancer cell line but was detected in all biliary cancer cell lines (Fig. S1B).

Quantification of antibodies against WNT7B antigens using AlphaLISA. Serum levels of antibodies against WNT7B of the following three cohorts were analyzed depending on the step (Table II): Cohort 1 consisted of sera from 44 biliary cancer patients and 30 healthy donors, cohort 2 consisted of sera from 44 biliary cancer patients and 44 healthy donors, and cohort 3 consisted of sera from 70 biliary cancer patients, 114 healthy donors, and 402 patients with other kinds of cancer. GST-fused full-length WNT7B protein was expressed in *E. coli* and purified by affinity chromatography. AlphaLISA immunoassay revealed that the serum levels of the antibody against full-length WNT7B were not significantly different between the healthy donors and patients with biliary cancer (P=0.9649; Fig. 1A). However, the identification of WNT7B by SEREX screening indicated the presence of anti-WNT7B antibodies in the sera of patients with biliary cancer. A total of 3 deletion mutants were then prepared ($WNT7B_{-92-2}$, $WNT7B_{-92-260}$ and $WNT7B_{245-353}$; Fig. 2) to evaluate the response to serum antibodies. There was no significant difference in the serum levels of antibodies against $WNT7B_{-92-2}$ (P=0.5704, Fig. 1B) and $WNT7B_{245-353}$ (P=0.5089, Fig. 1C) between healthy donors and patients with biliary cancer, whereas a significant difference was observed in the serum levels of anti-$WNT7B_{-92-260}$ antibodies (P=0.0369, Fig. 1D). To further narrow down the epitope site, two deletion mutants, $WNT7B_{2-260}$ and $WNT7B_{184-260}$, were prepared, and the serum levels of the antibodies against these proteins were measured (Fig. 2). The serum levels of the antibody against $WNT7B_{2-260}$ (P=0.6949, Fig. 1E) did not differ significantly between healthy donors and patients with biliary cancer, whereas the serum levels of the antibodies against $WNT7B_{184-260}$ did (P=0.0056, Fig. 1F). This suggested that the serum antibodies recognize the epitope comprising the amino acid residues 184-260 of WNT7B. The cDNA clone isolated by SEREX contained the pre-splicing sequence of WNT7B X1 mRNA at the 5'-end of WNT7B X1 cDNA (Fig. 2). However, the serum levels of the antibodies against $WNT7B_{-92-2}$, which contained only this pre-splicing sequence, did not differ significantly between healthy donors and patients with biliary cancer (P=0.5704; Fig. 1B). Therefore, this pre-splicing sequence site did not affect the serum levels of antibodies against WNT7B.

To further narrow down the epitope site, the region was decomposed into seven peptides, and the serum levels of antibodies against these were measured. The serum levels of antibodies against $WNT7B_{194-213}$, $WNT7B_{214-233}$, $WNT7B_{234-253}$ and $WNT7B_{244-260}$ were significantly higher in patients with biliary cancer than in healthy donors ($WNT7B_{194-213}$, P=0.0310; $WNT7B_{214-233}$, P<0.0001; $WNT7B_{234-253}$, P=0.0009; $WNT7B_{244-260}$, P=0.0005) (Fig. 3). By contrast, no significant difference was observed in the levels of antibodies against other peptides ($WNT7B_{184-203}$, P=0.0595; $WNT7B_{204-223}$,

Table II. The background of healthy donors and patients with cancer using AlphaLISA analysis.

	Backgroud of HD and patients used AlphaLISA analysis										
	Cohort 1 (n=74)		Cohort 2 (n=88)		Cohort 3 (n=586)						
	HD (30)	BC (44)	HD (44)	BC (44)	HD (114)	BC (70)	EC (87)	GC (92)	CC (93)	PC (39)	BrC (91)
Sex											
Male	20 (66.7)	29 (65.9)	25 (56.8)	29 (65.9)	64 (56.1)	47 (67.1)	76 (87.4)	62 (67.4)	55 (59.0)	23 (59.0)	0 (0)
Female	10 (33.3)	15 (34.1)	19 (43.2)	15 (34.1)	50 (43.9)	23 (32.9)	11 (12.6)	30 (32.6)	38 (40.9)	16 (41.0)	91 (100)
Age											
<65	7 (23.3)	11 (25.0)	22 (50.0)	11 (25.0)	95 (83.3)	20 (28.6)	25 (28.7)	24 (26.1)	24 (25.8)	10 (25.6)	62 (68.1)
≥65	23 (76.7)	33 (75.0)	22 (50.0)	33 (75.0)	19 (16.7)	50 (71.4)	62 (71.3)	68 (73.9)	69 (74.2)	29 (74.4)	29 (31.9)
Stage		3 (6.8)		3 (6.8)		3 (4.3)	4 (4.6)		3 (3.2)		23 (25.3)
I		1 (2.3)		1 (2.3)		3 (4.3)	6 (6.9)	47 (51.0)	19 (20.4)	2 (5.1)	28 (30.8)
II		19 (43.2)		19 (43.2)		22 (31.4)	12 (13.8)	15 (16.3)	28 (30.1)	0 (0)	22 (24.2)
III		15 (34.1)		15 (34.1)		20 (28.6)	25 (28.7)	5 (5.4)	25 (26.9)	4 (10.3)	2 (2.2)
IV		5 (11.4)		5 (11.4)		20 (28.6)	21 (24.1)	17 (18.5)	15 (16.1)	28 (71.8)	2 (2.2)
X		1 (2.3)		1 (2.3)		2 (2.9)	19 (21.8)	8 (8.7)	3 (3.2)	5 (12.8)	14 (5.4)
Location											
G		8 (18.2)		8 (18.2)		9 (12.9)					
C		0 (0)		0 (0)		1 (1.4)					
Bh		1 (2.3)		1 (2.3)		7 (10.0)					
Bp		24 (54.4)		24 (54.4)		36 (51.4)					
Bd		10 (22.7)		10 (22.7)		15 (21.4)					
A		1 (2.3)		1 (2.3)		2 (2.9)					

HD, healthy donor; BC, biliary cancer; EC, esophageal cancer; GC, gastric cancer; CC, colorectal cancer; PC, pancreatic cancer; BrC, breast cancer; G, gall bladder; C, cystic duct; Bh, intrahepatic bile duct; Bp, perihilar bile duct; Bd, distal bile duct; A, ampulla of Vater.

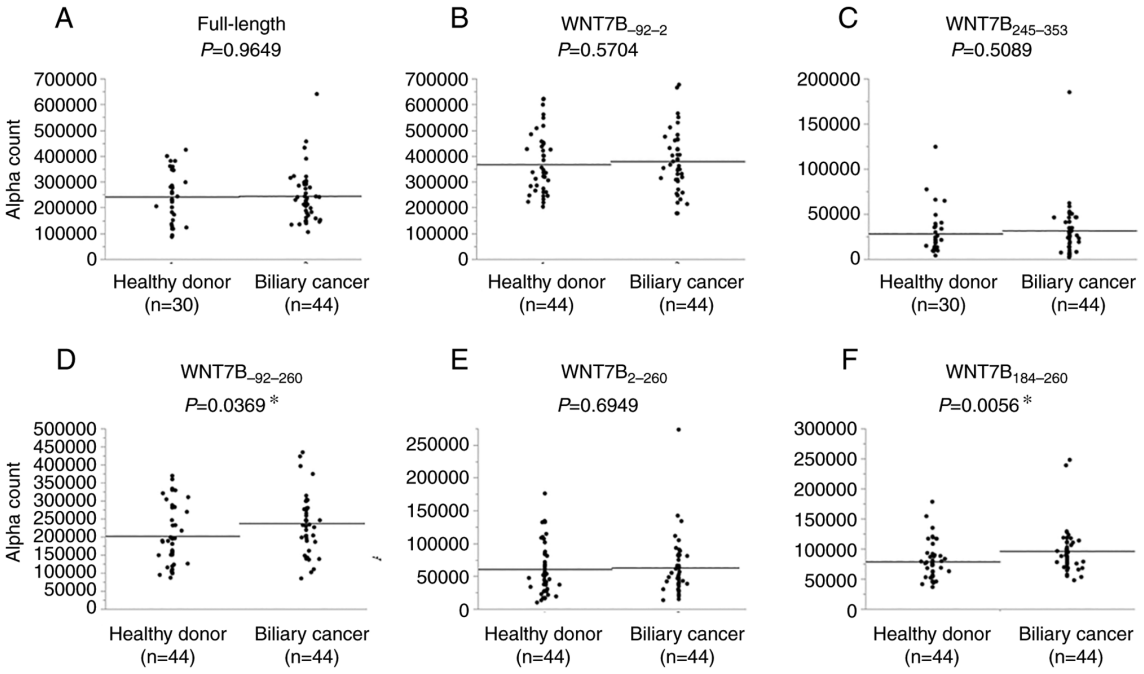

Figure 1. Comparison of serum levels of antibodies against WNT7B and WNT7B mutants between healthy donors and patients with biliary cancer. (A-F) Serum levels of antibodies against the full-length WNT7B protein and each deletion mutant were compared based on Alpha count using amplified luminescence proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) and shown in scatter dot plots. Dots represent the antibody levels, and the average value of each group is shown by a horizontal bar. P-values examined with Wilcoxon rank-sum test are shown. Full-length WNT7B and $WNT7B_{245-353}$ were analyzed in cohort 1, and other WNT7B mutants were analyzed in cohort 2. *P<0.05 vs. healthy donors. WNT7B, wingless-type MMTV integration site family, member 7.

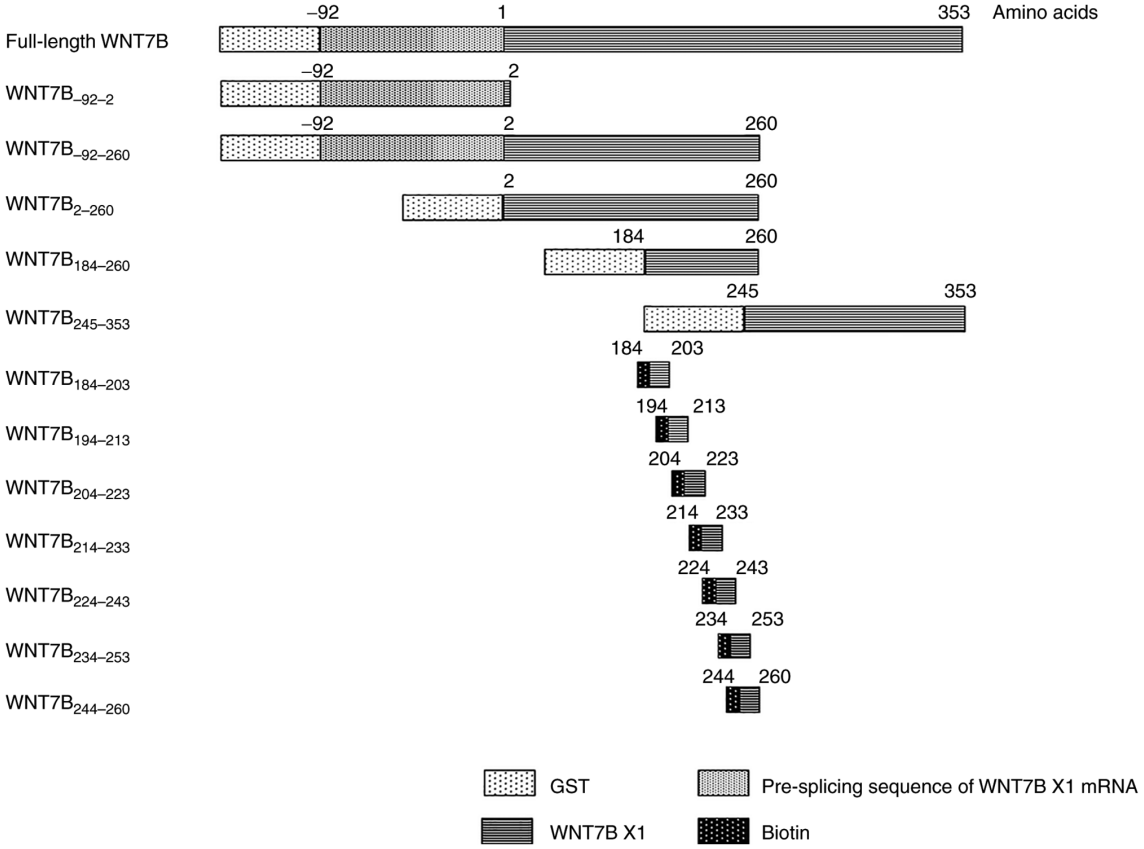

Figure 2. Schemas of deletion mutants and peptides. To investigate the antibody response to WNT7B, full-length WNT7B and WNT7B deletion mutants covering all coding sequences ($WNT7B_{92-2}$, $_{92-260}$, $_{2-260}$, $_{184-260}$ and $_{245-353}$) were prepared. The epitope site was inferred from the antibody reaction and divided into peptides ($WNT7B_{184-203}$, $_{194-213}$, $_{204-223}$, $_{214-233}$, $_{224-243}$, $_{234-253}$ and $_{244-260}$). GST-tag was used for proteins and biotin tag was used for peptides. The proteins identified in the present study also include the pre-splicing sequences of *Homo sapiens* WNT7B X1, which are indicated by '- (minus)'. WNT7B, wingless-type MMTV integration site family, member 7; GST, glutathione S-transferase.

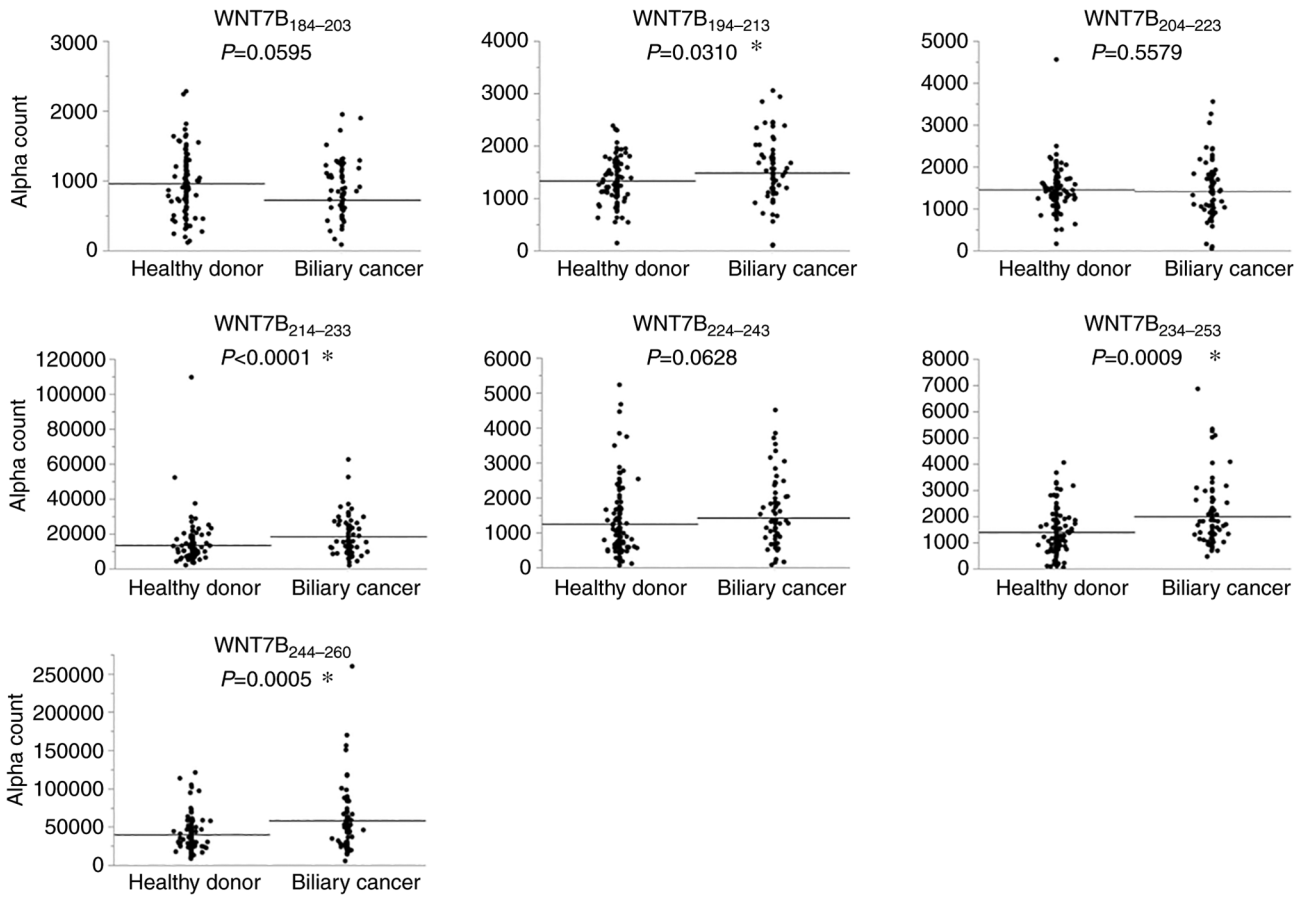

Figure 3. Comparison of serum levels of antibodies against biotinylated WNT7B peptides between healthy donors and patients with biliary cancer. Epitope sites were searched by dividing $WNT7B_{184-260}$ into 17-20 aminoacid-long peptides and comparing serum antibody levels between healthy donors and patients with biliary cancer using the sera of cohort 3. P-values determined with Wilcoxon rank-sum test are shown. *P<0.05 vs. healthy donors. WNT7B, wingless-type MMTV integration site family, member 7.

P=0.5579; $WNT7B_{224-243}$, P=0.0628) between healthy donors and patients with biliary cancer. The serum levels of anti-$WNT7B_{234-253}$ antibodies ($WNT7B_{234-253}$-Abs) and anti-$WNT7B_{244-260}$ antibodies ($WNT7B_{244-260}$-Abs) were then examined in patients with other types of cancer, including esophageal, gastric, colorectal, pancreatic and breast cancer. $WNT7B_{234-253}$-Ab levels were significantly increased only in patients with biliary cancer compared with those in healthy donors (Fig. 4A). Furthermore, $WNT7B_{244-260}$-Ab levels were significantly higher in patients with esophageal, gastric, and colorectal cancer than in healthy donors but not in patients with pancreatic or breast cancer (Fig. 4B).

Correlation between $WNT7B_{234-253}$-Ab levels and characteristics of patients with biliary cancer. The correlation between sex and age and $WNT7B_{234-253}$-Ab levels was analyzed in 70 patients with biliary cancer (Fig. S2). There was no significant difference in $WNT7B_{234-253}$-Ab levels between the sexes (P=0.0549). In addition, there was no significant difference in $WNT7B_{234-253}$-Ab levels between the two age groups: ≥65 and <65 years (P=0.8585). The correlation between $WNT7B_{234-253}$-Ab and CA19-9 levels was analyzed in 55 patients with biliary cancer whose CA19-9 data could be confirmed, and no correlation was found between $WNT7B_{234-253}$-Ab and CA19-9 levels (ρ=-0.0029; P=0.9831).

The cutoff value for $WNT7B_{234-253}$-Ab levels. The cutoff value for $WNT7B_{234-253}$-Ab levels fixed based on receiver operating characteristic curve analysis was 1271 Alpha count (area under the curve=0.646; Fig. 5A). Based on this cutoff value, $WNT7B_{234-253}$-Ab levels ≥1271 Alpha count were defined as positive, and those <1271 Alpha count were defined as negative. Thus, the positivity rate of $WNT7B_{234-253}$-Abs was 66.7, 33.3, 81.8, 70 and 70% for Stages 0, I, II, III, and IV of biliary cancer, respectively.

Diagnostic accuracy of CA19-9 and $WNT7B_{234-253}$-Ab levels in detecting biliary cancer. The accuracy of CA19-9 and serum $WNT7B_{234-253}$-Ab levels to serve as a tumor marker for biliary cancer was examined. Preoperative CA19-9 measurements were evaluated based on a cutoff value of 37 U/ml, which is our facility criteria. The sensitivity of $WNT7B_{234-253}$-Abs alone was analyzed in 70 patients with biliary cancer. The sensitivity of CA19-9 alone and that of $WNT7B_{234-253}$-Ab- and/or CA19-9-positivity were analyzed in 55 patients with biliary cancer for whom CA19-9 data was available. The specificity of $WNT7B_{234-253}$-Abs was analyzed using the sera of 144 healthy donors. The sensitivity of CA19-9 alone in diagnosing biliary cancer was 40% (22 of 55 cases), and that of $WNT7B_{234-253}$-Abs alone was 70% (49 of 70 cases). Conversely, the sensitivity of CA19-9- and/or $WNT7B_{234-253}$-Ab-positivity in detecting

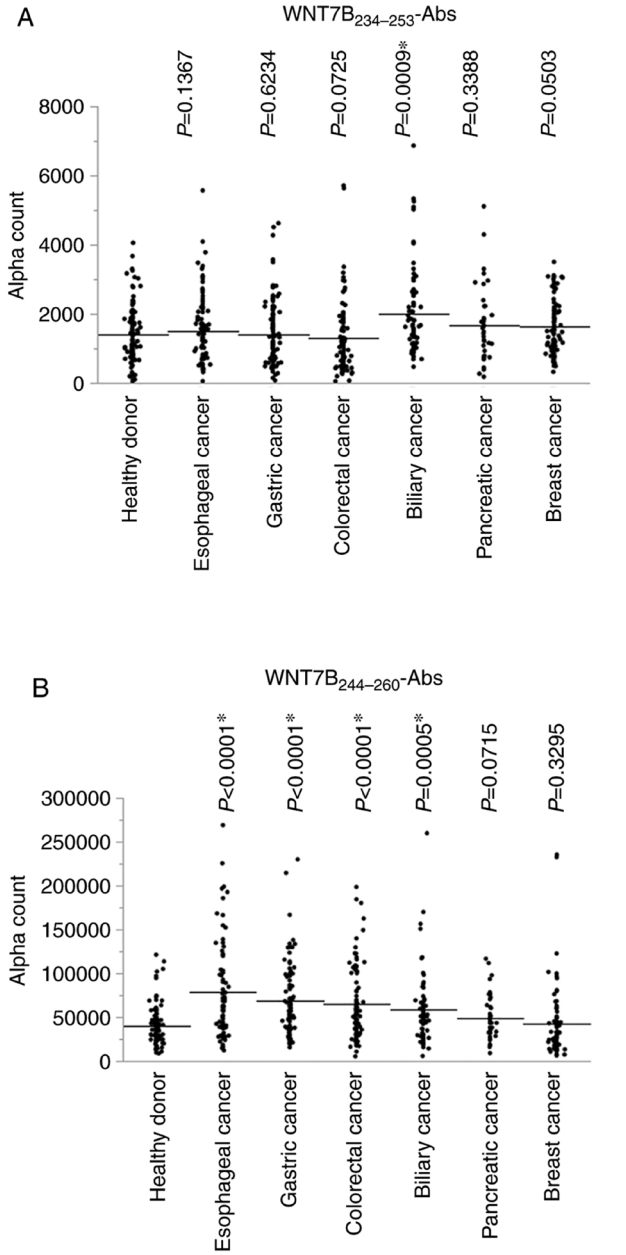

Figure 4. Comparison of serum levels of anti-WNT7B$_{234-253}$ (WNT7B$_{234-253}$-Abs) and anti-WNT7B$_{244-260}$ antibodies (WNT7B$_{244-260}$-Abs) between sera from healthy donors and patients with various solid cancers. (A) To verify the disease specificity of WNT7B$_{234-253}$-Abs, antibody serum levels of patients with esophageal (n=87), gastric (n=92), colorectal (n=93), pancreatic (n=39) and breast (n=91) cancer were compared with those of healthy donors using AlphaLISA. P-values determined with Wilcoxon rank-sum test are shown. *P<0.05 vs. healthy donors. (B) Verification of WNT7B$_{244-260}$-Abs for disease specificity. WNT7B, wingless-type MMTV integration site family, member 7.

biliary cancer was 83.6% (46 of 55 cases) (Fig. 5B). In addition, the sensitivity of WNT7B$_{234-253}$-Abs in diagnosing relatively early-stage biliary cancer (Stage 0-II) was 75% (21 of 28 cases). The specificity of WNT7B$_{234-253}$-Abs was 51.8% (59 of 114 cases).

Relationship between WNT7B$_{234-253}$-Ab levels and biliary cancer prognosis. The association between WNT7B$_{234-253}$-Ab levels and the prognosis of biliary cancer was analyzed in 52 patients with a known prognosis. A total of 36 patients with biliary cancer were WNT7B$_{234-253}$-Ab-positive and 16 were WNT7B$_{234-253}$-Ab-negative. Overall survival analysis demonstrated a lower survival rate in the WNT7B$_{234-253}$-Ab-positive group than in the WNT7B$_{234-253}$-Ab-negative group, but there was no significant difference (P=0.2555, Fig. 6).

Discussion

The purpose of the present study was to discover novel serum markers for biliary cancer by measuring serum antibody levels. SEREX analyses of serum from patients with gastrointestinal cancers have previously been used to identify antigens against which the antibodies produced can serve as useful tumor markers (9,18,23). Hence, in the present study, target antigen proteins were identified via SEREX using sera from patients with biliary cancer. Among these candidate antigens, WNT7B were targeted in the present study because RT-qPCR showed high mRNA levels of *WNT7B* in biliary cancer cell lines, which was further verified via western blotting (Fig. S1). It was revealed that the serum levels of antibodies against WNT7B$_{-92-260}$ and WNT7B$_{184-260}$ proteins were significantly increased in patients with biliary cancer compared with those in healthy donors. Further analysis using peptide antigens showed that the serum levels of WNT7B$_{234-253}$-Abs were exclusively higher in biliary cancer patients than in healthy donors. Therefore, WNT7B$_{234-253}$-Abs may have the potential to be a specific biliary cancer marker.

The serum levels of antibodies (Alpha counts) against WNT7B$_{244-260}$ were higher than those against WNT7B$_{234-253}$. Such a difference in specificity and reactivity between the two peptide antigens may be attributable to the tertiary structure of peptides and may cause a difference in disease specificity. The tertiary structure of WNT7B$_{234-253}$ and WNT7B$_{244-260}$ was then analyzed using a computational model. Although both have a helical structure, WNT7B$_{244-263}$ but not WNT7B$_{234-253}$ has a bend at the amino acid sequence portion 'RQP' (Fig. S3). BLAST search found five proteins that had the same sequence as the bend. Among them, protein kinase C is carcinogenic and widely studied (24,25). According to the Expression Atlas (https://www.ebi.ac.uk/gxa/home), a high expression of protein kinase C epsilon-type was confirmed in numerous gastrointestinal cancers. Antibodies developed against this sequence of protein kinase C in numerous gastrointestinal cancers may cross-react with WNT7B$_{244-260}$.

Notably, the present study found that the levels of the antibody against WNT7B in the sera of patients with biliary cancer were high and that the specific epitope was present among the amino acids 234-253. It was demonstrated that WNT7B$_{234-253}$-Abs are more sensitive than CA19-9 in detecting biliary cancer, and the combination of WNT7B$_{234-253}$-Abs and CA19-9 yielded a high sensitivity of 83.6%, rendering it useful as a novel tumor marker. In addition, relatively early-stage biliary cancer can also be identified with high sensitivity (75%), and improved prognosis can be expected through early diagnosis and treatment. Nevertheless, the low specificity of WNT7B$_{234-253}$-Abs makes it difficult to use them as tumor markers in clinical practice. If the number of cases analyzed could be increased to set a more appropriate cutoff value, WNT7B$_{234-253}$-Abs could be expected to play a major role in diagnosing biliary cancer in clinical practice.

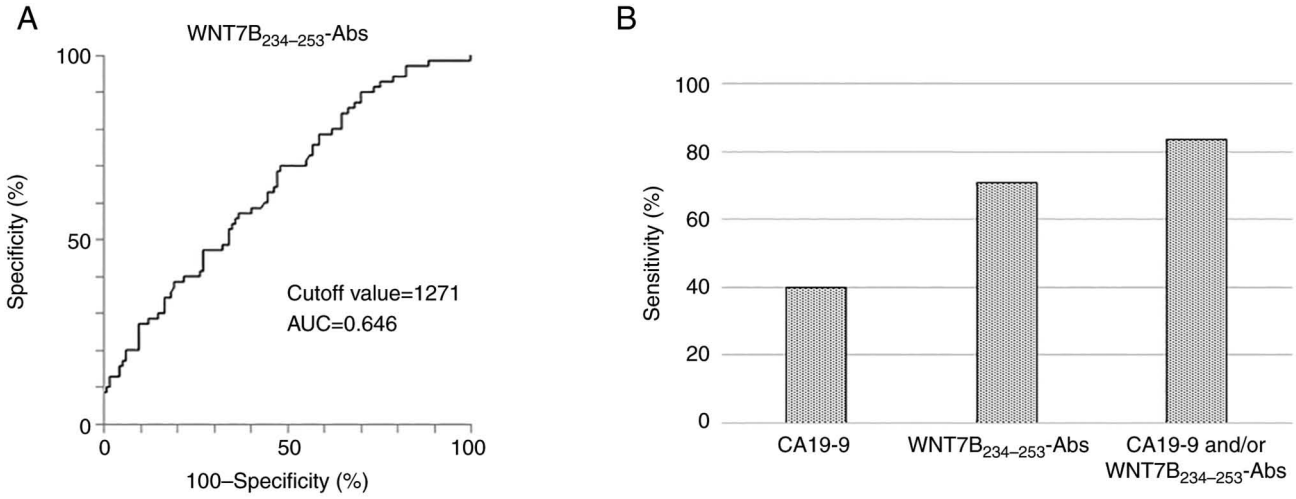

Figure 5. Diagnostic accuracy of $WNT7B_{234-253}$-Abs in biliary cancer. (A) The cutoff level of $WNT7B_{234-253}$-Abs in diagnosing biliary cancer was set based on receiver operating characteristic curve analysis. (B) The sensitivity of CA19-9 alone, $WNT_{234-253}$-Abs alone, and CA19-9 and/or $WNT_{234-253}$-Ab-positivity in detecting biliary cancer was analyzed. 'CA19-9 and/or WNT7B234-253-Abs' describes positive results for either or both CA19-9 and WNT7B234-253-Abs. WNT7B, wingless-type MMTV integration site family, member 7, CA19-9, carbohydrate antigen 19-9.

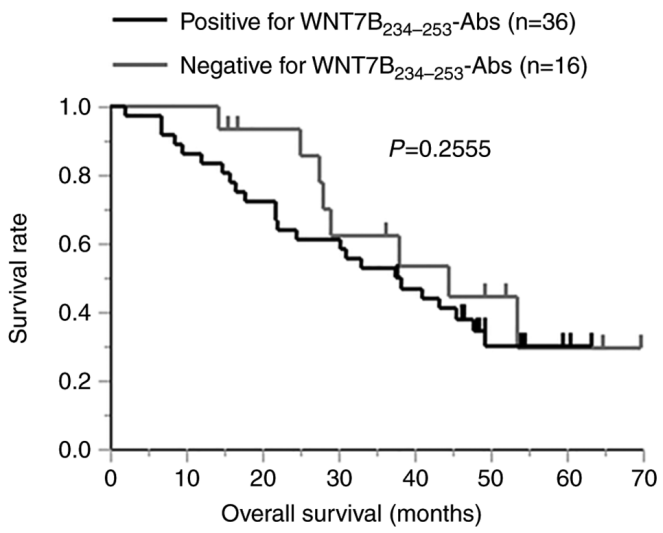

Figure 6. Relationship between $WNT7B_{234-253}$-Abs and overall survival. $WNT7B_{234-253}$-Abs were classified as either positive or negative according to the cutoff value (1271 Alpha count), and overall survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. WNT7B, wingless-type MMTV integration site family, member 7.

Since the first discovery of WNT1 (initially named *int-1*) in 1982 (26), 19 human WNT members have been identified, including *WNT7B*. When Wnt binds to its receptors, the β-catenin, planar cell polarity, and Ca^{2+} pathways become activated (27). The β-catenin pathway, which controls cell proliferation and differentiation, has been linked to carcinogenesis. In fact, mutations and abnormal accumulation of β-catenin have been reported in various types of cancer, including colorectal, liver, gastric, ovarian, prostate and pancreatic cancer (28). Mila *et al* (29) reported that the nuclear expression of β-catenin in gallbladder cancer correlates with tumor grade and depth of invasion, thus suggesting the role of this gene in tumor progression.

The anti-WNT7B antibody may also offer additional benefits in terms of applicability aside from serving as a tumor marker. In the β-catenin pathway, one of the intracellular signal transduction pathways of WNT7B, stabilized β-catenin enters the nucleus and stimulates the transcription of target genes, such as the oncogene cyclin D1 and c-myc, and exerts an important function in carcinogenesis (30,31). In biliary cancer, suppression of β-catenin by siRNA has been shown to inhibit the expression of cyclin D1, leading to decreased viability of biliary cancer cell lines. Therefore, the WNT/β-catenin pathway is considered to contribute to cell proliferation in biliary cancer (32). In the present study, significantly elevated levels of anti-WNT7B antibodies were observed in the sera of patients with biliary cancer compared with those in healthy donors and patients diagnosed with other types of cancer.

A major limitation to the present study was that most of the cases analyzed were stage II or higher, primarily because few cases of biliary cancer are detected at an early stage. Hence, in the future, further examination of $WNT7B_{234-253}$-Abs in patients at an early stage of biliary cancer is warranted for a more precise and comprehensive analysis. Another drawback is the low specificity of the $WNT7B_{234-253}$-Abs for clinical applications. Further investigation of optimal cutoff values by increasing the number of cases may increase their clinical significance.

In conclusion, it was found that $WNT7B_{234-253}$-Ab is a possible new tumor marker for biliary cancer and the appropriate cutoff value for serum anti-$WNT7B_{234-253}$ levels to diagnose biliary cancer was determined.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MT made substantial contributions to the conceptualization, design, acquisition, analysis and interpretation of data for the work. TT, TNa, KM, HM, MO, HS, KT, YN, TA, TNo, KO, TS and SH made substantial contributions to the conceptualization and design of the work. THi, KH, YH and THo made substantial contributions to the design, acquisition, analysis, and interpretation of data for the work. TK, KI and HT made substantial contributions to acquisition and analysis. All authors read and approved the final version of the manuscript. MT and TT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Hokkaido University Hospital (approval no. 015-0098; Sapporo, Japan) and Chiba University Graduate School of Medicine (approval no. 973; Chiba, Japan). Informed consent was obtained from all participants of the present study. Written consent or opt-out consent was provided by the 56 patients with biliary cancer in our institute. For specimens from collaborating institutions, consent was obtained in accordance with the standards of the collaborating institutions. It was determined that there are no ethical issues with the 30 healthy donor sera purchased from Tokyo Future Style.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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