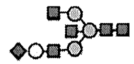
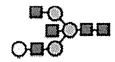
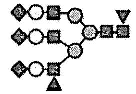
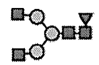
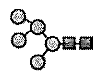
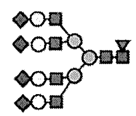
G2114	2114.778		88.46	75.88	2.208	0.839
G1809	1809.666		84.62	72.9	0.679	0.838
G3341	3341.221		84.62	69.92	0.086	0.821
G1590	1590.592		80.77	69.92	10.696	0.817
G1362	1362.481		65.38	87.26	1.381	0.813
G3865	3865.407		92.31	56.37	0.121	0.812

Table 1

List of the 14 serum N-glycans which were evaluated to be specific for hepatocellular carcinoma compared with normal controls by receiver operating characteristic (ROC) analysis. The area-under-the-curve (AUC) values of these 14 serum N-glycan were greater than 0.80.

These glycan structures are represented with the symbol nomenclature explained in http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml.

		(n)	PS Hazard Ratio	PS p-value	DFS Hazard Ratio	DFS p-value
G2032	Low	206	1	0.9362	1	0.1054
	High	163	1.017		1.243	
G2890	Low	152	1	<0.0001	1	0.0001
	High	217	3.044		1.705	
G1793	Low	112	1	0.6829	1	0.2897
	High	257	1.095		1.168	
G1708	Low	145	1	0.0016	1	0.0043
	High	224	2.017		1.485	
G1870	Low	151	1	0.5552	1	0.4008
	High	218	1.132		1.122	
G1955	Low	113	1	0.4213	1	0.795
	High	256	1.2		1.038	
G3195	Low	206	1	<0.0001	1	0.0001
	High	163	3.238		1.662	
G3560	Low	246	1	<0.0001	1	<0.0001
	High	123	4.209		1.74	
G2114	Low	275	1	0.0056	1	0.1627
	High	94	1.776		1.232	
G1809	Low	238	1	0.0027	1	0.055
	High	131	1.824		1.306	
G3341	Low	188	1	<0.0001	1	0.0005
	High	181	3.185		1.592	
G1590	Low	167	1	0.0956	1	0.9102
	High	202	1.413		0.985	
G1362	Low	261	1	0.0399	1	0.0004
	High	108	1.526		1.634	
G3865	Low	192	1	<0.0001	1	0.0014

	High	177	3.145	1.532

Table2

Univariate analysis of predictive values (the selected 14 *N*-glycans) of patient survival (PS) and disease free survival (DFS).

		(n)	PS Hazard Ratio	PS p-value	DFS Hazard Ratio	DFS p-value
sex	Male	301	1	0.7486	1	0.6535
	Female	68	0.913		0.943	
age(years)	<=62	160	1	0.3272	1	0.6320
	62<	209	1.211		1.106	
HBV	positive	176	1.259	0.1911	1.007	0.8093
	negative	192	1		1	
HCV	positive	119	1.291	0.2433	1.008	0.8183
	negative	250	1		1	
Albumin(mg/dl)	<=4.05	147	2.128	<0.0001	1.626	0.0001
	4.05<	222	1		1	
Total bilirubin(mg/dl)	<=0.82	235	1	0.5831	1	0.5241
	0.82<	134	1.122		1.128	
ICGR15(%)	<=16.7	223	1	0.1223	1	0.0106
	16.7<	146	1.349		1.375	
Child-Pugh	A	358	1	<0.0001	1	0.0374
	B	11	4.292		2.169	
Anatomical resection	Anatomical	282	1	0.8569	1	0.1435
	Non anatomical	87	0.949		1.225	
AFP(ng/ml)	<=20	183	1	<0.0001	1	0.0008
	20<<=1000	115	2.395		1.449	
	1000<	71	4.433		1.870	
AFP-L3(%)	<=15	255	1	<0.0001	1	0.0567
	15<	113	2.366		1.285	
PIVKA-II(mAU/ml)	<=40	109	1	<0.0001	1	0.0095
	40<<=1000	133	1.593		1.240	
	1000<	123	3.784		1.635	
Number	Single	235	1	<0.0001	1	<0.0001
	2,3	89	3.731		2.252	

	4<=	45	7.299		3.788	
Size(cm)	<=3	116	1	<0.0001	1	0.0086
	3<<=5	96	2.688		1.260	
	5<	157	4.049		1.570	
differebntiation	well	17	1	0.0003	1	0.0002
	moderetely	190	2.568		2.990	
	poorly	159	5.358		4.361	
vp	positive	94	4.630	<0.0001	2.156	<0.0001
	negative	275	1		1	
vv	positive	35	5	<0.0001	1.969	0.0004
	negative	334	1		1	
Macroscopic vascular invasion	positive	48	6.135	<0.0001	1.961	<0.0001
	negative	321	1		1	
Stage	1	26	1	<0.0001	1	<0.0001
	2	172	2.844		1.206	
	3	111	9.901		2.404	
	4A	60	15.625		3.106	
Non cancerous liver	Chirosis	120	1.199	0.3105	1.293	0.0398
	Non chirosis	249	1		1	

Table 3

Univariate analysis of predictive values (clinical and tumor associated factors) for patient survival (PS) and disease free survival (DFS). AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

		P value	Hazard ratio	95%Confidence Interval	
ICGR15(%)	16.7<	0.000209	2.435	1.5213	3.898
Child-Pugh	B	0.011136	3.007	1.2852	7.037
AFP(ng/ml)	20<<=1000	0.0003	2.558	1.5372	4.256
	1000<	0.000217	2.782	1.6177	4.786
Tumor number	2,3	0.011844	1.937	1.1575	3.241
	4<=	<0.0001	2.989	1.7693	5.049
Size(cm)	3<<=5	0.278625	1.483	0.7269	3.026
	5<	0.016071	2.237	1.1613	4.307
vp	positive	<0.0001	2.982	1.8446	4.822
C3560	>0.158	<0.0001	2.52	1.6191	3.923

Table 4

Multivariate analysis of values that is predictive for overall HCC patient survival. ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

		P value	Hazard ratio	95%Confidence Interval	
ICGR15(%)	16.7<	0.00334	1.519	1.149	2.008
AFP(ng/ml)	20<<=1000	0.04904	1.366	1.001	1.864
	1000<	0.01851	1.591	1.081	2.342
Tumor number	2,3	0.0072	1.551	1.126	2.135
	4<=	<0.0001	2.649	1.704	4.118
Differenciation	moderately	0.01495	2.838	1.225	6.577
	poor	0.00501	3.398	1.446	7.984
vp	positive	0.01023	1.544	1.108	2.152
C2890	>1.12	0.01125	1.443	1.087	1.915

Table 5

Multivariate analysis of values that are predictive of disease free survival in HCC patients. ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

		G2890			G3560		
		High(n=217)	Low(n=152)	p	High(n=123)	Low(n=246)	p
Sex	Male	184	117	0.0767	105	196	0.2286
	Female	33	35		18	50	
Age	≦62	90	70	0.4433	49	111	0.393
	>62	127	82		74	135	
HBV	positive	107	69	0.5254	59	117	0.9706
	negative	110	83		64	129	
HCV	positive	63	56	0.1425	32	87	0.0904
	negative	154	96		91	159	
Albumin(mg/dl)	≦4.05	109	38	<0.0001	73	74	<0.0001
	>4.05	108	114		50	172	
Total bilirubin(mg/dl)	≦0.82	136	99	0.7088	82	153	0.4671
	>0.82	81	53		41	93	
ICGR15(%)	≦16.7	125	98	0.2224	77	146	0.6246
	>16.7	92	54		46	100	
Child-Pugh	A	206	152	0.0034	115	243	0.008
	B	11	0		8	3	
Anatomical resection	Anatomical	172	110	0.1583	106	176	0.0028
	Non anatomical	45	42		17	70	
AFP(ng/ml)	≦20	102	81		52	131	
	20< & ≦1000	64	51	0.0461	30	85	<0.0001
	>1000	51	20		41	30	
AFP-L3(%)	≦15	143	112	0.1147	68	187	<0.0001
	>15	74	40		55	59	
PIVKA II(mAU/ml)	≦40	52	58		22	88	
	40< & ≦1000	74	60	0.0001	33	101	<0.0001
	>1000	91	34		68	57	

Number	Single	122	113		68	167	
	2, 3	60	29	0.0009	27	62	<0.0001
	≧4	35	10		28	17	
Size(cm)	≦3	48	68		15	101	
	3< & ≦5	60	36	<0.0001	21	75	<0.0001
	>5	109	48		87	70	
Differentiation	well	12	8		6	14	
	moderately	102	88	0.0981	46	144	0.0003
	poorly	103	56		71	88	
vp	positive	67	27	0.0065	49	45	<0.0001
	negative	150	125		74	201	
vv	positive	29	6	0.0043	24	11	<0.0001
	negative	188	146		99	235	
Macroscopic vascular invasion	positive	43	5	<0.0001	32	16	<0.0001
	negative	174	147		91	230	
Stage	1	7	19	<0.0001	3	23	<0.0001
	2	88	84		45	127	
	3	71	40		35	76	
	4A	51	9		40	20	
Non cancerous liver	Cirrhosis	71	49	0.9876	35	85	0.2888
	Non cirrhosis	146	103		88	161	

Table 6

Correlation between the G2890 and G3560 *N*-glycans and clinical and tumor associated factors in HCC cases.

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

Fig1

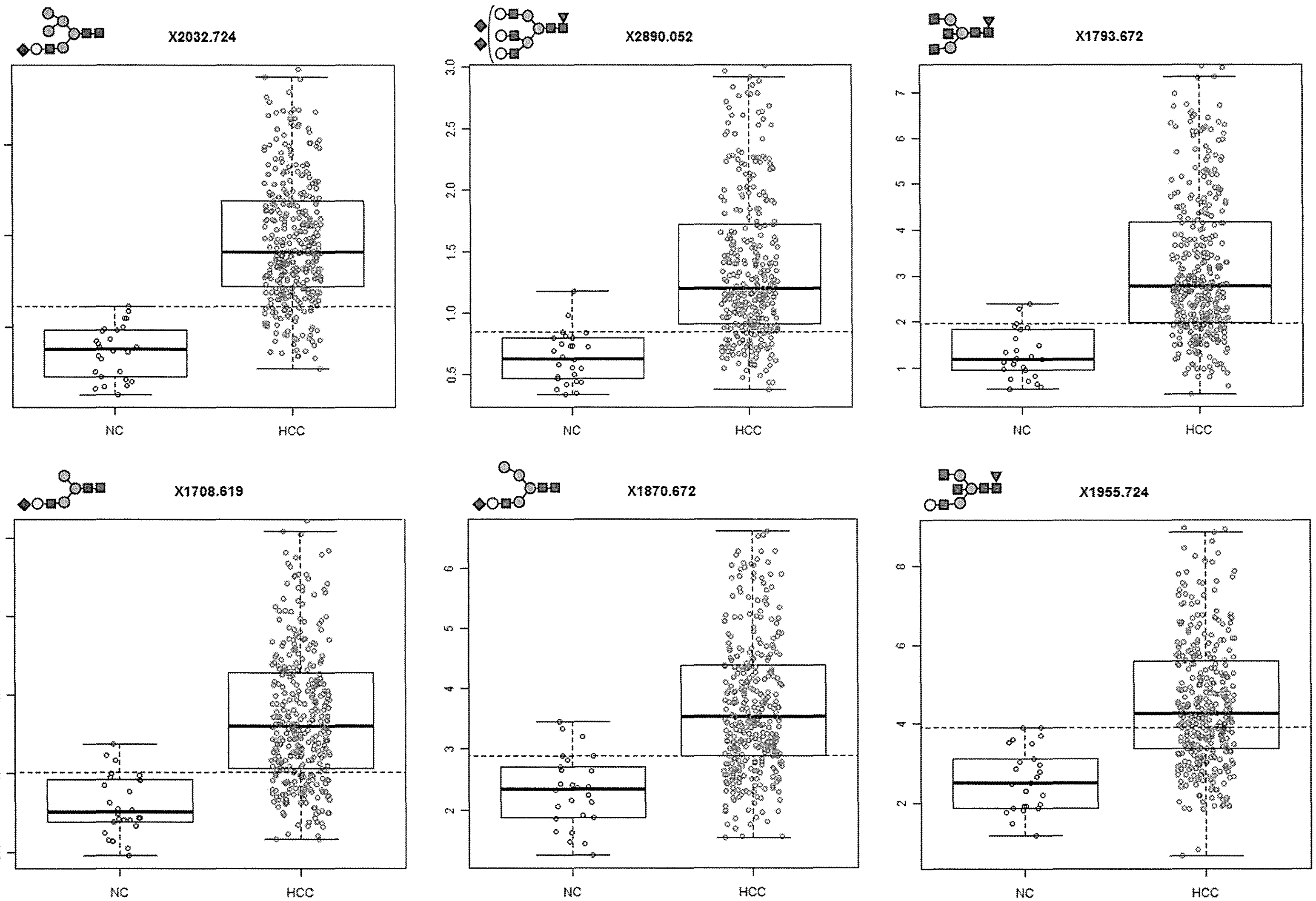

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Fig1

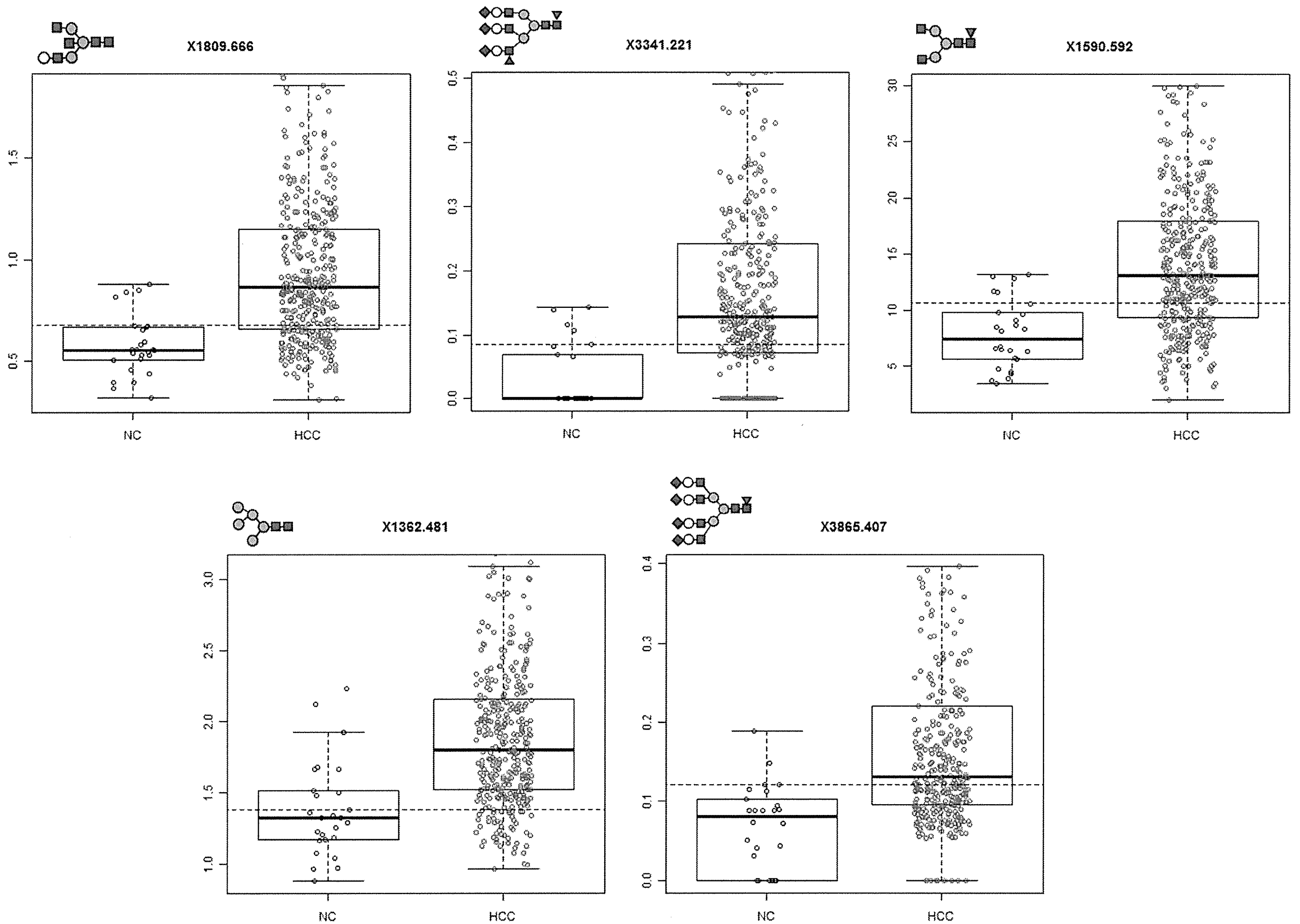
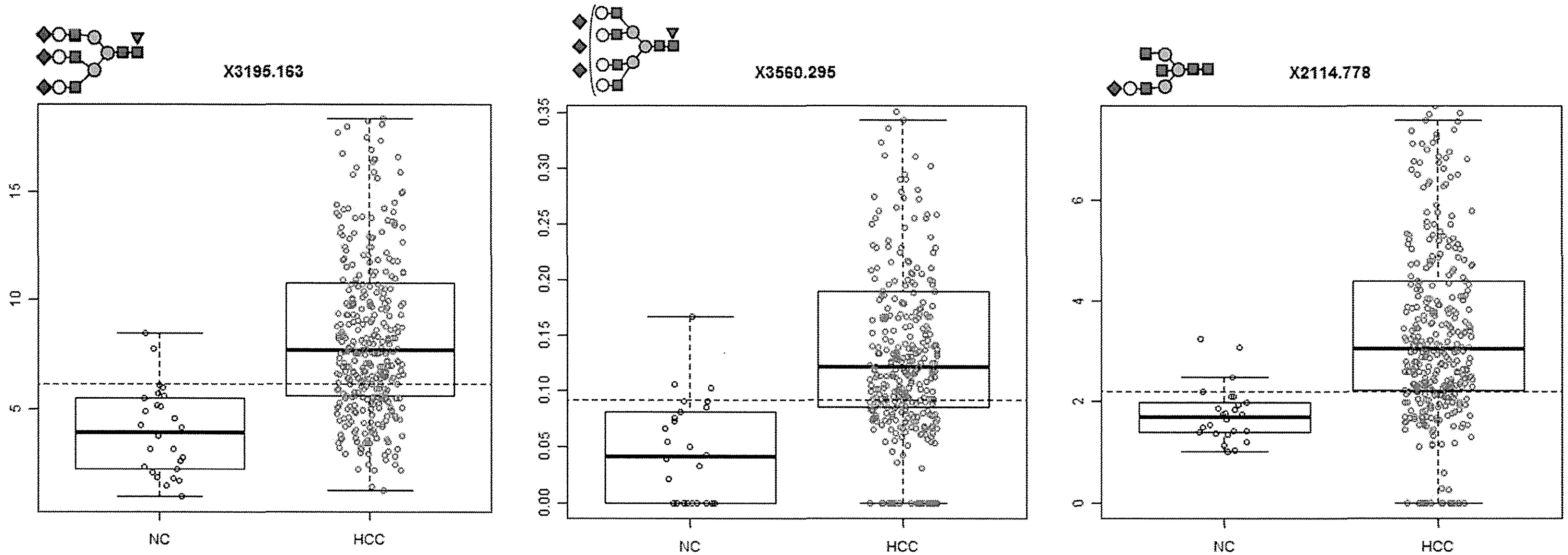
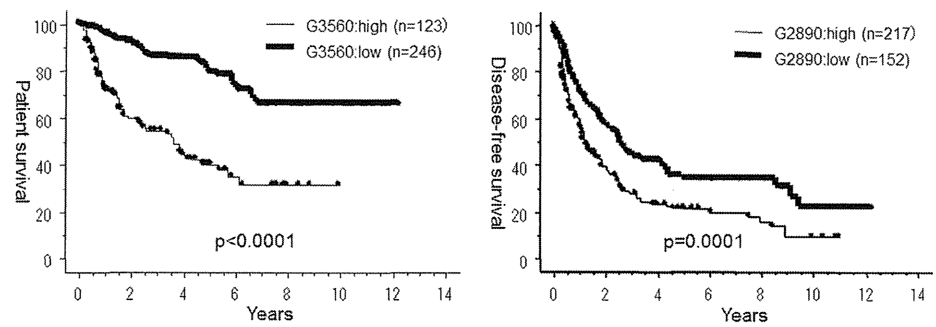


Fig1





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Intracellular localization of mesothelin predicts patient prognosis of extrahepatic bile duct cancer

FUTOSHI KAWAMATA[1], HIROFUMI KAMACHI[1], TAKAHIRO EINAMA[1], SHIGENORI HOMMA[1], MUNENORI TAHARA[1], MASAYA MIYAZAKI[3], SHINYA TANAKA[3], TOSHIYA KAMIYAMA[1], HIROSHI NISHIHARA[2], AKINOBU TAKETOMI[1] and SATORU TODO[1]

Departments of [1]General Surgery, [2]Translational Pathology and [3]Cancer Pathology, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

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Abstract. Mesothelin is expressed in various types of malignant tumors, and we recently reported that the expression of mesothelin was related to unfavorable patient outcome in pancreatic ductal adenocarcinoma and gastric adenocarcinoma. In this study, we examined the clinicopathological significance of mesothelin expression in extrahepatic bile duct cancer (EHBDCA), especially in terms of its association with the staining pattern. Tissue samples from 61 EHBDCA (16 hilar cholangiocarcinoma, 17 upper bile duct adenocarcinoma, 20 middle bile duct adenocarcinoma and 8 distal bile duct adenocarcinoma) were immunohistochemically examined. The expression levels of mesothelin in tumor cells was classified into the localization of mesothelin in luminal membrane and/or cytoplasm, in addition to high and low according to the staining intensity and proportion as a conventional analysis. 'High-level expression' of mesothelin (47.5%) was statistically correlated with liver metastasis (P=0.013) and poorer patient outcome (P=0.022), while 'luminal membrane positive' of mesothelin (52.5%) was more significantly correlated with liver metastasis (P=0.006), peritoneal metastasis (P=0.024) and unfavorable patient outcome (P=0.017). Moreover, we found that 'cytoplasmic expression' isolated from 'luminal membrane negative' of mesothelin represented the best patient prognosis throughout this study. We describe the expression pattern level of mesothelin, i.e., in luminal membrane or cytoplasm both high and low level, evidently indicate the patient prognosis of EHBDCA, suggesting the pivotal role of mesothelin in cancer promotion depending on its intracellular localization.

Correspondence to: Dr Hiroshi Nishihara, Department of Translational Pathology, Hokkaido University School of Medicine, Kita-Ku, Kita 14, Nishi 7, Sapporo 060-8638, Japan
E-mail: hnishihara@s5.dion.ne.jp

Key words: mesothelin, intracellular localization, luminal membrane expression

Introduction

Extrahepatic bile duct cancer (EHBDCA), consisting of hilar cholangiocarcinoma and distal bile duct adenocarcinoma (excluding gallbladder cancer), is a rare disease in the United States with an incidence of 1-2/100,000/year (1). It occurs with great frequency in Asian countries, and is one of the common causes of cancer death in Japan, with near to 17,000 deaths annually (2). The 5-year survival rate of EHBDCA, even after the surgical resection is poor, ranging from 20 to 45% (3-5). The incidence of EHBDCA is increasing throughout the world with a high fatality rate; therefore, new prognostic markers and treatment for EHBDCA patients are urgently needed.

Mesothelin is expressed on normal mesothelial cells lining the pleura, pericardium and peritoneum (6,7). In addition, the overexpression of mesothelin has been found in several cancer types, including malignant mesothelioma, ovarian cancer and pancreatic cancer (8-11,12). The full length of human *mesothelin* gene codes the primary product, which is a 71-kDa precursor protein. This protein can be physiologically cleaved by certain furin-like proteases into a 40-kDa *C*-terminal fragment that remains membrane-bound and a 31-kDa *N*-terminal fragment, which is secreted into the blood (6). The *C*-terminal 40-kDa fragment is named mesothelin and is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor (13). The biological functions of mesothelin are not clearly understood, although recent studies have suggested that enforced expression of mesothelin increases cell proliferation and migration (14). In ovarian cancers, higher mesothelin expression was found to be associated with chemoresistance and shorter patient survival (15). In pancreatic cancer, mesothelin expression was immunohistochemically observed in all cases, while its absence was noted in non-cancerous pancreatic ductal epithelium, with or without pancreatitis (8,12,16,17). We recently found that the expression of mesothelin was related to an unfavorable patient outcome in pancreatic ductal adenocarcinoma (12), while the opposite result was reported in gastric cancer, in which the mesothelin expression was correlated with prolonged patients' survival (18). However, our consecutive investigation for mesothelin expression patterns in gastric cancer recently discovered that luminal membrane expression, not cytoplasmic expression of mesothelin is a prominent negative prognostic factor for gastric cancer (19), suggesting the significance of expression pattern of mesothelin in clinicopathological analysis of cancer. In EHBDCA, Zhao *et al*, who first studied mesothelin expression in dysplasia and carcinoma of external bile duct, reported that mesothelin was expressed in 5 of 10 adenocarcinomas (50%) in cell membranes and cytoplasm (20); however, the detailed clinicopathological analysis of mesothelin expression in EHBDCA, especially with large number of the cases, has not yet been performed.

In this study, we investigated the mesothelin expression in 61 EHBDCA cases by immunohistochemistry, and its clinicopathological significance associated with patients' outcome was analyzed. Moreover, we focused on the intracellular localization of mesothelin, i.e., in luminal membrane and/or cytoplasm, and its clinicopathological significance associated with the patients' outcome.

Materials and methods

Patients' demography and tumor specimens. This study was performed with the approval of the Internal Review Board on Ethical Issues of Hokkaido University Hospital, Sapporo, Japan. The samples and the patient information were obtained under a blanket written informed consent. The subjects of this study were 61 patients who underwent radical surgery for bile duct adenocarcinoma between the years 2000 and 2008 at Hokkaido University Hospital by the Department of General Surgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan. The clinicopathological characteristics of these cases are summarized in Table I.

Mean age of patients was 67.5 years [±9.0 standard deviation (SD)]; 47 patients (77.0%) were male and 14 patients (23.0%) were female. The predominant sites of the cancer were the hilar bile duct in 16 cases (26.2%), upper bile duct in 17 cases (27.9%), middle bile duct in 20 cases (32.8%) and distal bile duct in 8 cases (13.1%). The surgical procedures consisted of the standard pancreatoduodenectomy in 21 (34.4%) cases, the pylorus-preserving pancreatoduodenecomy in 5 cases (8.2%), the extended right or left hemihepatectomy with extrahepatic bile duct resection in 28 cases (45.9%), and the extrahepatic bile duct resection in 7 cases (11.5%). Intraoperative diagnosis of the ductal resection margins was performed using frozen sections. When a positive margin was found, additional resection of marginal bile duct was performed to the maximum extent possible. R0 curative resection was achieved in 39 cases (63.9%), and R1 resection was achieved in 22 cases (36.1%). T-factor, N-factor, M-factor and clinical stage were assigned according to the TNM classification of the Union Internationale Contre le Cancer (UICC) (21). The median survival time of patients was 29.8 months (±3.5 SD).

Formalin-fixed paraffin-embedded tissue blocks were prepared from surgical specimens and sections were sliced and stained with hematoxylin and eosin (H&E) for routine histopathological examination. All specimens were diagnosed as EHBDCA.

Immunohistochemical evaluation. Immunohistochemical staining against mesothelin was performed as described previously (12). In brief, the tissue sections were incubated with a mouse monoclonal antibody against mesothelin (clone 5B2 diluted 1:50; Novocastra, Newcastle Upon Tyne, UK) at a 1:50 dilution, and reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako). All assessments were made

Table I. Clinicopathological characteristics of 61 patients with EHBDCA in this study.

Parameter	No. of cases
Age (years)	
<60	11
≥60	50
Mean ± SD	67.5±9.0
Gender	
Male	47
Female	14
Location	
Hilar	16
Upper	17
Middle	20
Distal	8
Surgical procedure	
Pancreatoduodenectomy	21
Pylorus-preserving pancreatoduodenectomy	5
Extended right or left hemihepatectomy with bile duct resection	28
Extrahepatic bile duct resection	7
Resection status	
R0	39
R1	22
T-factor	
T1	5
T2	27
T3	19
T4	10
N-factor	
N0	25
N1	36
M-factor	
M0	58
M1	3
Stage	
IA	4
IB	14
IIA	4
IIB	28
III	8
IV	3
Median survival (months)	29.8±3.5

SD, standard deviation.

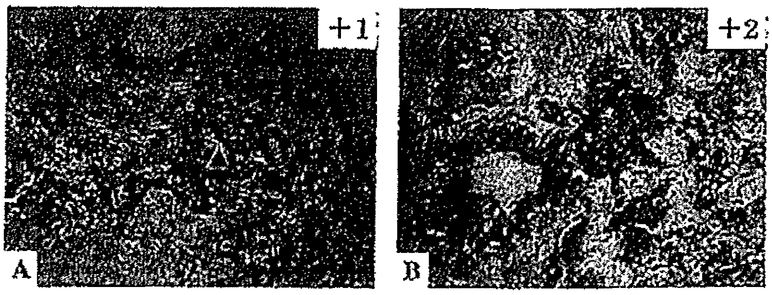

Figure 1. Representative cases of 'low-level expression' (A) and 'high-level expression' (B) of mesothelin in EHBDCA specimens by immunoistochemistly. (A) Partial luminal membrane staining (arrowhead; intensity, +1) and the weak cytoplasmic staining were observed in <50% area (proportion, +2). (B) Entire circumference of the luminal membrane was strongly positive in >50% tumor cells (intensity, +2; proportion, +3). (Magnification, x200).

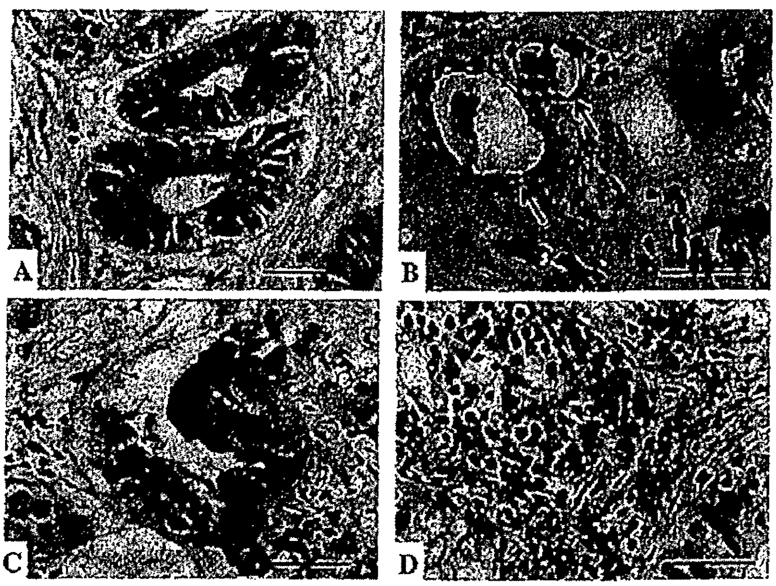

Figure 2. Representative cases of 'luminal membrane positive' (A, B) and 'luminal membrane negative' (C, D) of mesothelin in EHBDCA specimens by immunohistochemistry. (A) Granular cytoplasmic staining was observed (arrowheads; intensity, +2) and luminal membrane was also stained partially (arrows). (B) Entire circumference of the luminal membrane was explicitly stained (arrows). (C) Granular cytoplasmic, but no membranous staining in cancer cells was observed. (D) No expression of mesothelin was found in tumor cells, also designated 'mesothelin negative'. (Magnification, x400; scale bars, 50 μm).

in the tumor region of the specimen (x400). Each slide was evaluated independently by three pathologists (F. Kawamata, M. Miyazaki and H. Nishihara) who did not know the clinical outcomes. Immunostaining for mesothelin was evaluated for both the proportion and staining intensity of tumor cells in each case. The proportion of mesothelin expression was assessed according to the percentage of mesothelin-positive cells as follows: 0, 0%; +1, 1<10%; +2, 10-50%; and +3, >50%. The staining intensity of mesothelin was evaluated as weak (+1) and moderate to strong (+2) (Table II). The final evaluation of mesothelin expression was assessed using the following scoring system: 'high-level expression' of mesothelin was defined as ≥+3 of the proportion score and/or +2 of the intensity score, while a 'low-level expression' of mesothelin was given when the total score was ≤+3 except in cases when the proportion score was +1 and the intensity score was +2 (Fig. 1).

Furthermore, among the 61 cases of EHBDCA, the staining localization of mesothelin was evaluated in luminal membrane or cytoplasm. Cases in which the luminal membrane was stained even partially or faintly (Fig. 2A), or the entire circumference of the luminal membrane was explicitly stained

Table II. Immunohistochemical findings of mesothelin expression.

	No. of cases (%)			
	Percentage of mesothelin-positive cells			
Staining intensity on tumor cells	0	1-10%	10-50%	>50%
Score 0	17 (27.9)	0 (0.0)	0 (0.0)	0 (0.0)
Score 1	0 (0.0)	13 (21.3)	2 (3.3)	1 (1.6)
Score 2	0 (0.0)	6 (9.8)	12 (19.7)	10 (16.4)

Table III. Correlation between mesothelin expression levels and clinicopathological features.

		Mesothelin			Luminal membrane expression		
Parameter	Total	High-level (n=29)	Low-level (n=32)	P-value	Positive (n=32)	Negative (n=29)	P-value
Histopahological grade							
1 or 2	54	26	28	1.000	28	26	1.000
3	7	3	4		4	3	
pT-factor							
pT1-2	32	13	19	0.310	19	13	0.301
pT3-4	29	16	13		13	16	
pN-factor							
Negative	25	11	14	0.795	16	9	0.198
Positive	36	18	18		16	20	
pStage							
I-IIB	50	24	26	1.000	26	24	1.000
III-IV	11	5	6		6	5	
Lymphatic permeation							
Negative	23	10	13	0.792	12	11	1.000
Positive	38	19	19		20	18	
Blood vessel permeation							
Negative	26	11	15	0.606	11	15	0.200
Positive	35	18	17		21	14	
Perineural invasion							
Negative	9	3	6	0.478	3	6	0.287
Positive	52	26	26		29	23	
Resection margin							
pR0	39	20	19	0.594	24	15	0.069
pR1	22	9	13		8	14	
Recurrence							
No	18	6	12	0.172	6	12	0.090
Yes	43	23	20		26	17	
Liver metastasis							
No	47	18	29	0.013	20	27	0.006
Yes	14	11	3		12	2	
Local recurrence							
No	46	22	24	1.000	25	21	0.761
Yes	15	7	8		7	8	
Peritoneal metastasis							
No	49	20	29	0.052	22	27	0.028
Yes	12	9	3		10	2	

(Fig. 2B) were judged as 'luminal membrane positive'. In cases with no membrane staining (Fig. 2D) and those in which only cytoplasmic staining (Fig. 2C) was observed in any intensity level, the term 'luminal membrane negative' was given.

Statistical analysis. We used the χ^2 test or Fisher's exact test to determine the correlation between mesothelin and clinicopathologic data. Survival curves for patients were drawn by the Kaplan-Meier method. Differences in survival curves were analyzed by the log-rank test. Prognostic implications of mesothelin expression and clinicopathologic parameters were analyzed by Cox univariate and multivariate proportional hazards models. All differences were considered significant at a P-value of <0.05. All statistical analyses were performed using the Ekuseru-Toukei 2010 software for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

High-level expression of mesothelin was correlated with liver metastasis and poor patient outcome. The overexpression of mesothelin has been found in several cancer types, including

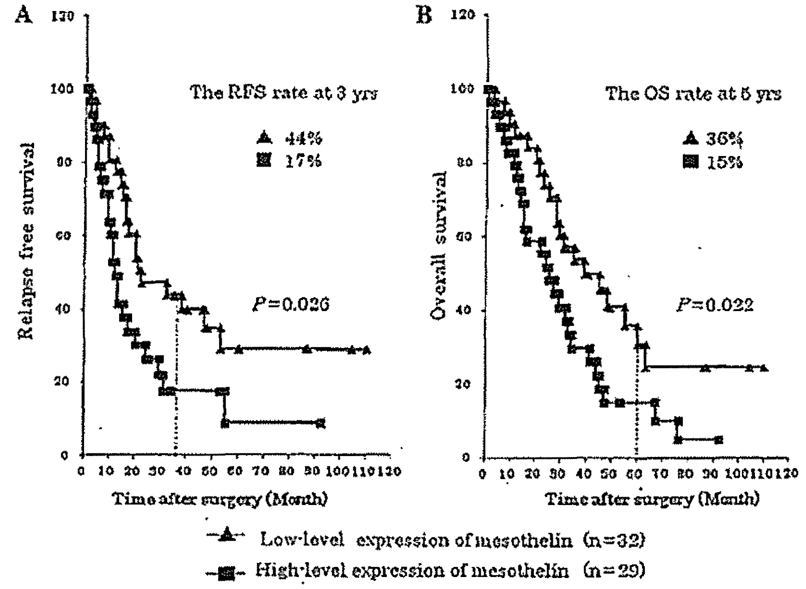

Figure 3. Relapse-free survival (RFS) and overall survival (OS) curves of EHBDCA patients according to the expression levels of mesothelin. The group of 'high-level expression' of mesothelin represented a statistically significantly unfavorable outcome compared to the group of 'low-level expression' (P=0.026 and 0.022, respectively).

malignant mesothelioma, ovarian cancer, and pancreatic cancer (11,12); thus, we first evaluated the comprehensive expression of mesothelin in EHBDCA. As described in Materials and Methods, 'high-level expression' and 'low-level expression' of mesothelin was attributed to all 61 cases of EHBDCA (Fig. 1). As summarized in Table II, 'high-level expression' was detected in 29 cases (47.5%), whereas 'low-level expression' was detected in 32 cases (52.5%). The statistical analysis for the clinicopathological parameters such as histological grade, T-factor and metastasis revealed that 'high-level expression' of mesothelin was significantly correlated with liver metastasis (P=0.013, Table III). Furthermore, recent studies reported that higher mesothelin expression was found to be associated with shorter patient survival; therefore, we examined the correlation of mesothelin overexpression with relapse-free survival (RFS) and overall survival (OS) in the EHBDCA patients. The group of 'high-level expression' of mesothelin had a significantly poorer RFS than the group of 'low-level expression' of mesothelin (P=0.026). In addition, the group of 'high-level expression' of mesothelin had a significantly poorer OS than the group of 'low-level expression' of mesothelin (P=0.022) (Fig. 3).

Luminal membrane expression of mesothelin is a prominent negative prognostic factor for the patients with EHBDCA. During our previous studies on pancreatic adenocarcinoma and gastric adenocarcinoma, we already noted that expression of mesothelin was found in the luminal membrane as well as the cytoplasm (19). Mesothelin was reported to attach to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor after being physiologically cleaved by some furin-like proteases (22), which are involved in the translocation of mesothelin, although the biological functions of mesothelin associated with its intracellular localization are not fully understood. Thus, we analyzed the intracellular localization of mesothelin by immunostaining to explore the clinicopathological significance of its translocation.

As shown in Table III, the group 'luminal membrane positive', which consisted of the cases with luminal membrane staining even partially, was 32 (52.5%) cases, while the group 'luminal membrane negative', which contained 17 cases which were completely mesothelin negative was comprised of 29 (47.5%) cases. The statistical analysis revealed that the incidence of luminal membrane positivity was significantly correlated with peritoneal metastasis (P=0.024) in addition to liver metastasis (P=0.006) (Table III). The analysis of the patients' overall survival showed that 'luminal membrane positive' of mesothelin indicated a significantly unfavorable RFS (P=0.012) and OS (P=0.017) compared to 'luminal membrane negative' of mesothelin (Fig. 4).

To clarify the mesothelin expression as an independent prognostic factor, we performed a univariate analysis of the 61 EHBDCA using the Cox proportional hazards model, the result indicated that resection margin, 'high-level expression' and 'luminal membrane positive' of mesothelin were significantly correlated with risks of cancer mortality. Multivariate analysis also confirmed that resection margin (RR 3.361, 95% CI, 1.670-6.763, P=0.0007) and 'luminal membrane positive' of mesothelin (RR 2.964, 95% CI, 1.401-6.296, P=0.0045) were independent predictors of the overall patient survival (Table IV).

Isolation of 'cytoplasmic expression' of mesothelin potentiates more exquisite prediction of prognosis in EHBDCA. To explore the clinicopathological value of the cytoplasmic expression of mesothelin, we performed a sub-analysis in 'luminal membrane negative', dividing the group into 17 cases of 'mesothelin negative' and 12 cases of 'cytoplasmic expression'. The P-value (OS, P=0.0085) between 'luminal membrane positive' and 'cytoplasmic expression' was minimum in these

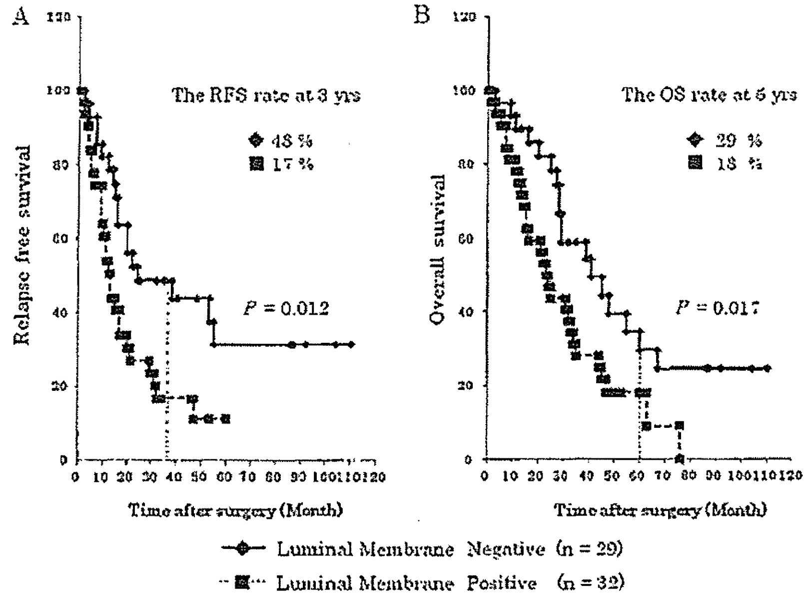

Figure 4. Relapse-free survival (RFS) and overall survival (OS) curves of EHBDCA patients according to the expression pattern of mesothelin. The grou 'luminal membrane positive' represented a statistically significantly unfavorable outcome compared to the group of 'luminal membrane negative' (P=(and 0.017, respectively).

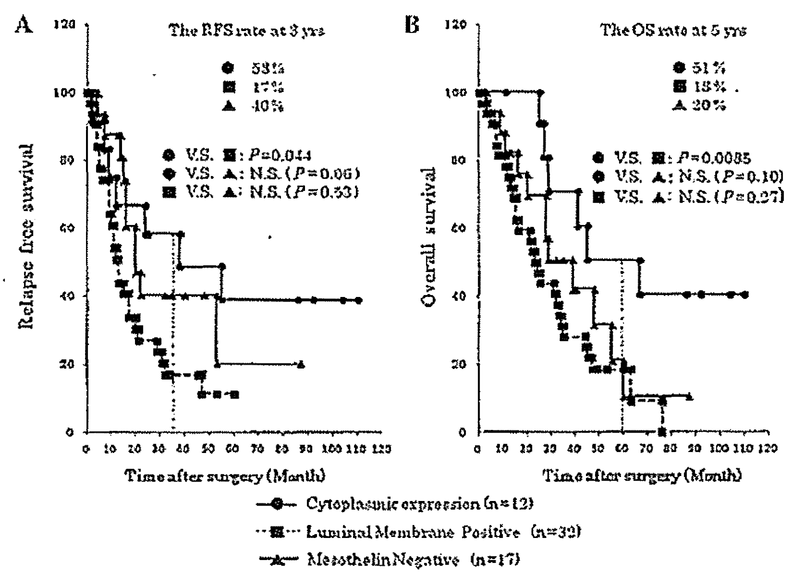

Figure 5. Relapse-free survival (RFS) and overall survival (OS) curves of EHBDCA patients among three groups of detailed expression patterns of mesotl 'Cytoplasmic expression' of mesothelin represented the best prognosis among the 3 groups.

survival analyses, suggesting the clinical benefit of isolation of 'cytoplasmic expression' of mesothelin (Fig. 5). Interestingly, 'cytoplasmic expression' of mesothelin represented relatively favorable patients' prognosis compared to 'mesothelin negative', although it was statistically not significant (RFS, P=0.06; OS, P=0.10).

Discussion

In this study, we confirmed that mesothelin expression is a prominent prognostic factor for EHBDCA patients as well as for other tumors such as pancreatic cancer and ova carcinoma described previously (12,15,23). Furtherm we revealed that the expression pattern of mesothelir luminal membrane or cytoplasm, could be a more evi prediction factor for these patients. These results evide support our recent report of mesothelin expression patt in gastric cancer in which luminal membrane expression cytoplasmic expression of mesothelin is a prominent neg prognostic factor for gastric cancer (19).

The mechanism for the membranous localization of m thelin should be explained as follows: the full length o

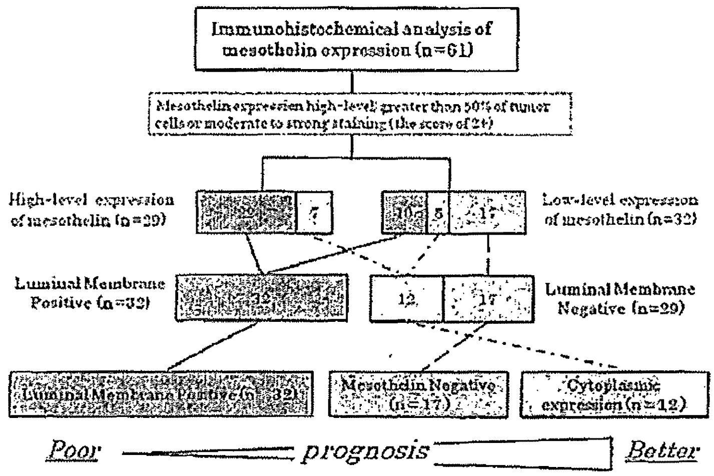

Figure 6. Flow chart of immunohistochemical evaluation of mesothelin expression and the prognostic aspect. The P-value (OS, P=0.0085) between 'luminal membrane positive' and 'cytoplasmic expression' was minimum in our survival analyses, suggesting the clinical benefit of isolation of 'cytoplasmic expression' of mesothelin.

Table IV. Univariate and multivariate analysis of patients' survival in EHBDCA.

		Univariate analysis		Multivariate analysis		
Factor	n=61	P-value	RR (95% CI)	RR (95% CI)	Hazard ratio	P-value
Histopahological grade						
1 or 2	54	0.3931	1		NC	
3	7		1.508 (0.588-3.871)			
pT-factor						
pT1-2	32	0.4264	1		NC	
pT3-4	29		1.266 (0.708-2.262)			
pN-factor						
Negative	25	0.3639	1		NC	
Positive	36		1.314 (0.729-2.368)			
pStage						
I-IIB	50	0.2026	1		NC	
III-IV	11		1.608 (0.774-3.339)			
Lymphatic permeation						
Negative	23	0.1908	1		NC	
Positive	38		1.537 (0.807-2.924)			
Blood vessel permeation						
Negative	26	0.2999	1		NC	
Positive	35		1.370 (0.756-2.482)			
Perineural invasion						
Negative	9	0.4733	1		NC	
Positive	52		0.728 (0.306-1.732)			
Resection margin						
pR0	39	**0.0398**	1	1.670-6.763	1	**0.0007**
pR1	22		1.859 (1.029-3.356)		3.361	
Mesothelin expression						
Low-level	32	**0.0236**	1	0.864-3.067	1	0.1317
High-level	29		1.968 (1.095-3.538)		1.621	
Luminal membrane expression of mesothelin						
Negative	29	**0.0175**	1	1.401-6.296	1	**0.0045**
Positive	32		2.078 (1.137-3.798)		2.964	

RR indicates relative risk/hazard ratio; CI, confidence interval. NC, not calculable.