Nuclear Expression of Thioredoxin-1 in the Invasion Front is Associated with Outcome in Patients with Gallbladder Carcinoma

Short title: Thioredoxin-1 expression in gallbladder cancer

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Original article

Abstract

Background: Multifunctional redox protein human thioredoxin (TRX-1) is reduced by thioredoxin reductase (TRX-R). This study examined distribution of TRX-1 and TRX-R expressions in gallbladder carcinoma (GBC) to clarify their usefulness as prognostic factors after surgical resection.

Methods: Immunohistochemical staining for TRX-1 and TRX-R was performed in GBC tissue from 38 patients who underwent surgical resection, and TRX-1/TRX-R localization in relation to outcome was examined.

Results: TRX-1 protein levels were significantly higher in GBC samples than in cholecystolithiasis samples (P=0.0174). TRX-1 expression was observed in 100% (38/38) of tumor samples and in the nucleus in 76% (29/38), with nuclear expression in the invasion front observed in 45% (13/29). TRX-R expression was only detected in cytoplasm of cancer cells and in the invasion front in 28 samples. In all samples, the depth of tumor invasion, lymph node metastasis, surgical margin, curability, and nuclear expression of TRX-1 in the invasion front were significant prognostic factors by univariate analysis. In selected 27 patients who underwent curative resection, both TRX-1 nuclear expression and TRX-R cytoplasmic expression in the invasion front was significantly prognostic factor.

Conclusion: TRX-1 nuclear expression in the GBC invasion front is a significant prognostic marker. Patients with both TRX-1 nuclear expression and TRX-R cytoplasmic expression in the tumor invasion front should be observed carefully even if after curative resection.

Keywords Thioredoxin, Thioredoxin reductase, Gallbladder carcinoma

Introduction

Gallbladder carcinoma (GBC) is the most frequently occurring of the biliary cancers [1, 2]. Resection in numerous cases is impossible because there are few symptoms, and early diagnosis remains difficult despite recent progress in several diagnostic modalities [2, 3]. It is generally accepted that the outcome of surgery for GBC is strongly determined by the depth of tumor invasion (T), lymph node metastasis (N), and stage [3-7]. In addition to these factors, it is important to clarify the independent molecular biological markers influencing the prognosis of GBC invading the subserosal layer or deeper because prognosis of early GBC restricted to the mucosa or proper muscle layer is comparatively good.

The cellular redox state is a important mediator of various metabolic, signaling, and transcriptional processes in cells, and a fine balance between reducing and oxidizing conditions is essential for the normal function and survival of cells [8, 9]. Accumulating evidence indicates that cellular redox status is involved substantially in growth promotion and drug resistance of cancer cells [10, 11]. Moreover, redox mechanisms play a key role in regulating the resistance of cancer cells to apoptosis and angiogenesis [12-15]. Thioredoxin (TRX) is a multifunctional redox protein found in both prokaryotic and eukaryotic cells. Human thioredoxin (TRX-1) is a low molecular weight (12 kDa) protein with 27% amino acid identity to *Escherichia coli* TRX. TRX

was originally studied for its ability to act as a reducing co-factor for ribonucleotide reductase, the first unique step in DNA synthesis in *E. coli* [16]. The oxidized TRX is reduced by an NADPH-dependent thioredoxin reductase (TRX-R), and the reduced TRX is a very effective protein disulphide reductase. TRX-Rs are the only enzymes known that can reduce the active site of TRX. TRX-1 was subsequently shown to exert redox control over a number of transcription factors including NF-kB, AP-1, p53, and indirectly through the nuclear redox protein Redox factor-1 [17]. TRX modulates the binding of these transcription factors to DNA and thus regulates gene transcription.

TRX expression is induced by various kinds of oxidative stresses including viral infection, mitogens, X-ray and UV irradiation, hydrogen peroxide, and post-ischemic reperfusion [8]. Regulation of the intracellular redox environment is critical for activation and proliferation of tumor cells [18]. Both the overexpression of TRX-1 in various human malignant tumors and the association of TRX-1 with growth stimulation, anti-apoptosis, and angiogenesis have been reported [19, 20]. Retrospective analyses in colorectal carcinoma and non-small cell lung carcinoma have shown that TRX-1 overexpression may be an independent prognostic factor of poor survival [21-23].

In the present study, to clarify the role of TRX-1 expression in GBC, we examined both the relation between TRX-1 and TRX-R expression by immunohistochemical analysis and the prognosis of patients with GBC.

Patients and Methods

Patients

Thirty-eight patients with GBC except for pT1 cancer restricted to the mucosa or muscle layer who had undergone surgical resection from 1990 to 2006 at Miyazaki University Hospital were enrolled in this study. The patients included 17 men and 21 women with a median age of 68.5 years (range 40 to 89 years) (Table1). The end point was the evaluation of disease specific survival after date of surgery. Median follow up time was 34.6 months (range, 3.9-109 months). Pathological findings of T, N, M, stage and final curability were classified based on the Japanese Society of Biliary Surgery classification system [24] (Table 2).

The depth of primary tumor invasion (pT) was classified into the following four groups as pT1: tumors restricted to the mucosa or muscle layer; pT2: tumors invading the perimuscular connective tissue; pT3: tumors perforating the serosa and/or slightly invading the liver and the hepatoduodenal ligament; and pT4: tumors extending more than 5 mm into the liver parenchyma and/or invading the left margin of the hepatoduodenal ligament, and/or invading the portal veins or hepatic arteries. Lymph node metastasis (pN) was classified as pN0: no regional lymph node metastasis; pN1: metastasis in the cystic duct and/or pericholedochal node; pN2: metastasis in the

hepatoduodenal ligament except pN1, posterosuperior pancreas head, along the common hepatic artery; and pN3: metastasis in the peripancreatic, celiac, superior mesenteric, paraaortic lymph nodes. Final curability (fCur) were classified according to the final histopathological diagnosis. A curative resection was defined as a complete removal of the cancer cells with negative histological margins without the presence of any residual tumor.

All tissue samples were fixed with 10% formalin for immunohistochemical investigation and Western blot analysis. Fifteen samples from patients with cholecystolithiasis who had undergone cholecystectomy were used as normal controls.

This study was conducted according to the ethical principles stated in the latest version of the Helsinki Declaration and the applicable guidelines for good clinical practice. The experimental design in this study was approved by the ethics committee of Miyazaki University Hospital, article No. 763.

Western blot analysis of TRX-1

Western blot analysis was performed as previously described [25]. Tissues taken from GBC and cholecystolithiasis specimens were homogenized in protein lysis buffer. After centrifugation of the crude homogenate, protein concentration was measured. Samples containing 10 μg protein were applied/lane to gel, and the gel was electrophoresed.

Proteins were transferred electrophoretically onto membranes. The blotted membranes were incubated with monoclonal antibodies against human TRX-1 (Redox Bio Science, Kyoto, Japan) (1:2500) for 16 hours at 4°C. The membranes were incubated with antimouse secondary antibody conjugated to mouse peroxidase for 1 hour at room temperature. ECL Plus was used to detect the proteins, and the luminol excitation was imaged. β-actin expression was detected with the same membranes after stripping them of bound antibodies. Detection and imaging were performed as described for TRX-1 [26, 27].

Immunohistochemical analysis for TRX-1 and TRX-R

Formalin-fixed paraffin-embedded tumor sections were mounted on glass slides, dewaxed with xylene, and then transferred to alcohol. To enhance immunoreactivity of TRX-1, we retrieved antigens by autoclaving at 121°C for 12 minutes in citrate buffer (pH=6.0). The primary antibodies used were TRX-1 monoclonal antibody (Redox Bio Science, Kyoto, Japan, dilution 1:500) [21, 22]. The appearance of TRX-1 was confirmed with the dyed specimen, and the expression pattern was determined. TRX-R expression was analyzed similarly with TRX-R 2 polyclonal antibody [26].

Statistical analysis

The difference in clinicopathological factors between patients with and without TRX-1 nuclear expression was examined by the Fisher's exact text. Survival rates were calculated by the Kaplan-Meier method, and statistical differences were examined by the log-rank test. Probability values of <0.05 were considered statistically significant. Analyses were performed with JMP for Macintosh (SAS Institute, Cary, NC, USA).

Results

Western blot analysis for TRX-1

The specificity of TRX-1 antibody in GBC was determined by Western blot analysis. A clear single TRX-1 protein band was shown at the molecular weight of approximately 12 kDa, and TRX-1 protein levels of GBC samples were significantly higher than those of samples of cholecystolithiasis (P=0.0174) (Fig. 1).

Immunohistochemical analysis for TRX-1

TRX-1 was detected in 8 of 15 samples of cholecystolithiasis (53% positivity rate), with staining located mainly in the cytoplasm of mucosal epithelial cells (Fig. 2A). In contrast, TRX-1 expression was confirmed in all samples of GBC (100% positivity rate). TRX-1 expression in the cytoplasm of the cancer cells was observed in all samples (Fig.

2B). TRX-1 nuclear expression was confirmed in 29 of the 38 samples of GBC (76%), not in the entire tumor but in a part of the tumor. The invasion front was defined as the deepest cancerous lesion infiltrated. TRX-1 nuclear expression only in the invasion front of the tumor was confirmed in 4 samples (Fig. 2C), in both the invasion front and the tumor center in 9 samples, and only in the tumor center in 16 samples (Fig. 2D). Thus, TRX-1 nuclear expression in the invasion front was observed in 13 of the 29 samples.

Relation between the localization of TRX-1nuclear expression and outcome

There were no statistically significant differences in clinicopathological characteristics between the patients with TRX-1 nuclear expression only in the invasion front (n=4) and those with TRX-1 nuclear expression in both the invasion front and tumor center (n=9). Prognosis of these 13 patients with TRX-1 nuclear expression in the invasion front was poor, and none of these patients survived for more than 5 years (Fig. 3). Patient outcome was significantly poorer (P=0.0387) in patients with TRX-1 nuclear expression observed in the invasion front (n=13) than in patients with TRX-1 nuclear expression observed only in the tumor center (n=16) (Fig. 4). Differences in clinicopathological factors and curability (presence or absence of the residual tumor) are shown in Table 3. There were no statistically significant differences between the two

groups.

TRX-R expression

TRX-R expression in GBC was confirmed in 36 of the 38 samples. TRX-R was expressed in the cytoplasm of the cancer cells, whereas its nuclear expression was not observed. TRX-R cytoplasmic expression was confirmed only in the invasion front in 4 samples, in both the invasion front and the tumor center in 24 samples, and only in the tumor center in 8 samples. Thus, TRX-R cytoplasmic expression in the invasion front was confirmed in 28 of the 38 samples. No significant difference in postoperative survival rate was observed between patients with and without TRX-R expression in the invasion front (P=0.986) (Fig. 5).

Prognostic factors of GBC

Clinicopathological characteristics of GBC that can predict poor prognosis were identified (Table 4). Univariate analysis showed that depth of tumor invasion, lymph node metastasis, surgical margin, and curability were all significant prognostic factors. Similarly, the presence of TRX-1 nuclear expression in the invasion front was also a significant prognostic factor by univariate analysis.

Since curability as an operative factor was a significant prognostic factor,

prognostic factor in selected 27 patients who underwent curative resection (fCurA, B) were examined. Postoperative survival was significantly worse in the patients with the presence of both TRX-1 nuclear expression and TRX-R cytoplasmic expression in the invasion front than in those without this expression ($p<0.012$, Table 5). There were no statistically significant differences in several clinicopathological factors including the depth of tumor, lymph node metastasis and stage between these two groups (data not shown). The presence of TRX-1 nuclear expression in the invasion front significantly worsened the outcome (Table 5).

Discussion

Recently, several reports have been published concerning factors predictive of poor prognosis in GBC, such as p53 and COX-2 [28, 29]. It is important to predict postoperative prognosis of GBC so that the appropriate patients can be recommended for combined adjuvant therapy. The results of the present study suggest that nuclear expression of TRX-1 in the tumor invasion front may be a significant prognostic marker of survival in patients with GBC.

In our study, we first analyzed the mode and localization of TRX-1 expression in GBC. Previous studies have reported the overexpression of TRX-1 in various malignant

tumors such as malignant melanoma and lung and breast carcinoma [26, 27, 30-33]. Yoon et al also reported TRX overexpression in cholangiocarcinoma by Western blot analysis [34]. The present study indicated that TRX-1 was overexpressed in all of the cases of GBC.

TRX-1 expression was detected in the cytoplasm in all GBC samples, whereas nuclear expression was confirmed in 76% of samples. The extent of TRX-1 nuclear expression differed depending on its location in the tumor, whether in the invasion front of the tumor, in the tumor tissues, or in both. In previous studies, it was reported that TRX-1 mainly appears in the cytoplasm, and its expression was also seen in the nuclei of colorectal and lung carcinomas [21-23]. In breast cancer, cytoplasmic staining for TRX-1 has been reported to vary between 48-67%, and nuclear staining varies between 59-63% [33, 35].

In the present study, TRX-1 nuclear expression in the invasion front was a significant prognostic factor in GBC. In the cytoplasm, TRX-1 works as an antioxidant and a reducing cofactor, whereas in the nucleus, it regulates transcription factors, and this is probably the most important role of TRX-1. TRX activity has also been detected in the extracellular space, and it stimulates cell growth by sensitizing the cell itself [18]. TRX-1 has also been shown to translocate into the nuclei of normal endothelial and tumor cells, and treatments with H_2O_2, hypoxia, nitric oxide, ionizing radiation, and anticancer drugs such as cisplatin, for example, further increase this translocation [36-40].

It has been suggested that the translocation of TRX-1 into the nucleus strongly correlates with p53 expression and poor prognosis in breast cancer [33]. Moreover, it was reported that TRX-1 expression relates to poor prognosis of lung cancer and liver metastasis from colorectal cancer [22, 23].

We showed that the prognosis of the patients with the presence of TRX-1 nuclear expression in the invasion front was significantly worse compared with its absence. Jung et al reported that the oncogene ß-catenin is found in the nuclear compartment of tumor cells in the invasion front of well-differentiated colorectal adenocarcinomas [41]. Under these conditions, ß-catenin can function as a transcription factor and thus activate target genes. One of these target genes, cyclin D_1, is known to reduce tumor cell proliferation. It is suggested that translocation of TRX-1 in the invasion front into the nucleus invests high infiltration and/or metastatic capability to the cancer cell. However, the details of the mechanisms of TRX-1 nuclear expression associated with poor prognosis require further study.

Prognosis of GBC remains poor even in patients after curative surgical resection. In the present study, presence of both TRX-1 nuclear expression and TRX-R cytoplasmic expression in the invasion front was a prognostic factor for survival after curative resection. TRX-1 nuclear expression in the invasion front may be a useful prognostic marker of GBC. In addition, we propose that patients with both TRX-1

nuclear expression and TRX-R cytoplasmic expression in the invasion front of the tumor should be treated with adjuvant therapy even if after curative resection. Recently, it was reported that cisplatin plus gemcitabine is an appropriate option for the treatment of patients with advanced biliary cancer [42].

More extensive studies will be required to determine whether TRX-1 nuclear expression can be used reliably as a prognostic marker for GBC. TRX-1 inhibitors are being developed as anticancer agents to stimulate spontaneous and drug-induced apoptosis and to inhibit tumor growth [43-46]. Therefore, in patients with GBC in which TRX-1 is overexpressed, TRX-1 inhibitors may be a promising treatment for GBC.

Conclusions

TRX-1 nuclear expression in the tumor invasion front of patients with GBC may be a useful prognostic marker for survival. We propose that patients with both TRX-1 nuclear expression and TRX-R cytoplasmic expression in the invasion front of the tumor should be observed carefully even if after curative surgical resection. Additional studies including a larger number of patients need to be done to confirm the clinical significance of thioredoxin.

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Figure legends

Figure 1 Western blot analysis for TRX-1 protein in tissue samples of advanced gallbladder carcinoma (GBC) and cholecystolithiasis. Note the clear bands made by the same antibody used for immunohistochemical analysis. Quantitative analysis indicated that TRX-1 protein level increased significantly by about 2.1-fold in the tissue of GBC compared with that of cholecystolithiasis ($P<0.05$). *means ± standard deviation of the mean.

Figure 2 Immunohistochemical staining for TRX-1 in advanced gallbladder carcinoma (GBC) and cholecystolithiasis. A: TRX-1 expression is revealed by immunohistochemical staining in the cytoplasm of mucosal epithelial cells of cholecystolithiasis. B: All samples of advanced GBC showed cytoplasmic expression of TRX-1 in the tumor center. C: TRX-1 nuclear expression is confirmed in the invasion front of advanced GBC. D: TRX-1 nuclear expression is confirmed in the tumor center of advanced GBC.

Figure 3 Survival curves of the 4 groups of patients with advanced gallbladder carcinoma after surgical resection according to the presence or absence of TRX-1 nuclear expression and location are shown. There is a significant difference in survival between the patients with TRX-1 nuclear expression only in the invasion front and the patients with TRX-1 nuclear expression only in tumor center ($P<0.05$).

Figure 4 Survival curves of the patients with advanced gallbladder carcinoma (GBC) after surgical resection according to the location of TRX-1 nuclear expression of GBC are shown. The postoperative survival rate of the patients with TRX-1 nuclear expression in the invasion front of GBC (n=13) was significantly worse than that of the patients with TRX-1 nuclear expression only in the tumor center (n=16) (P=0.0387).

Figure 5 Survival curves of the patients with advanced gallbladder carcinoma (GBC) after surgical resection according to the presence or absence of TRX-R expression in the invasion front of GBC are shown. There was no significant difference in postoperative survival rates between the patients with and without the presence of TRX-R expression.

Figure1

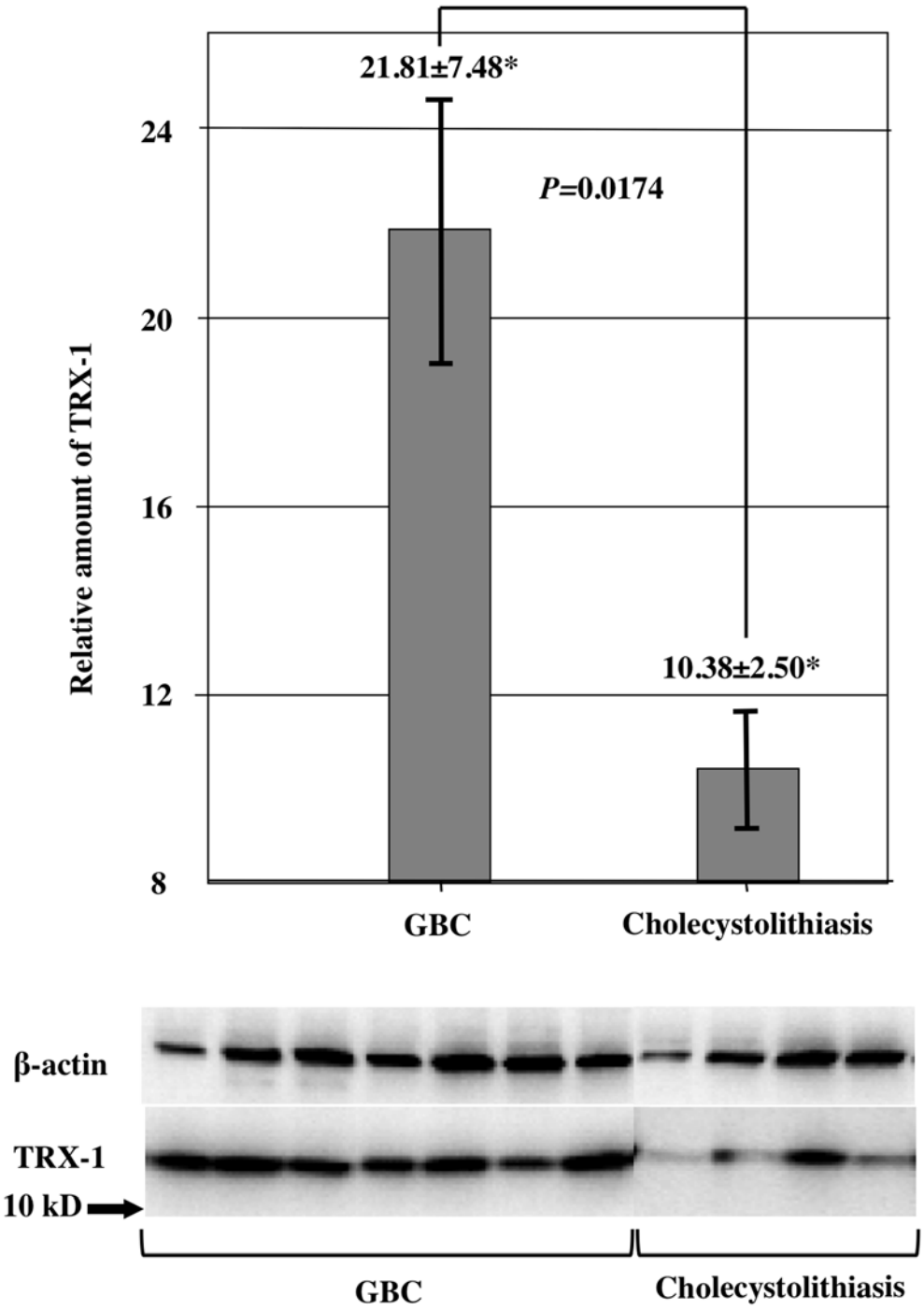

21.81±7.48*
P=0.0174
10.38±2.50*
Relative amount of TRX-1
24
20
16
12
8
GBC
Cholecystolithiasis
β-actin
TRX-1
10 kD
GBC
Cholecystolithiasis

Figure2

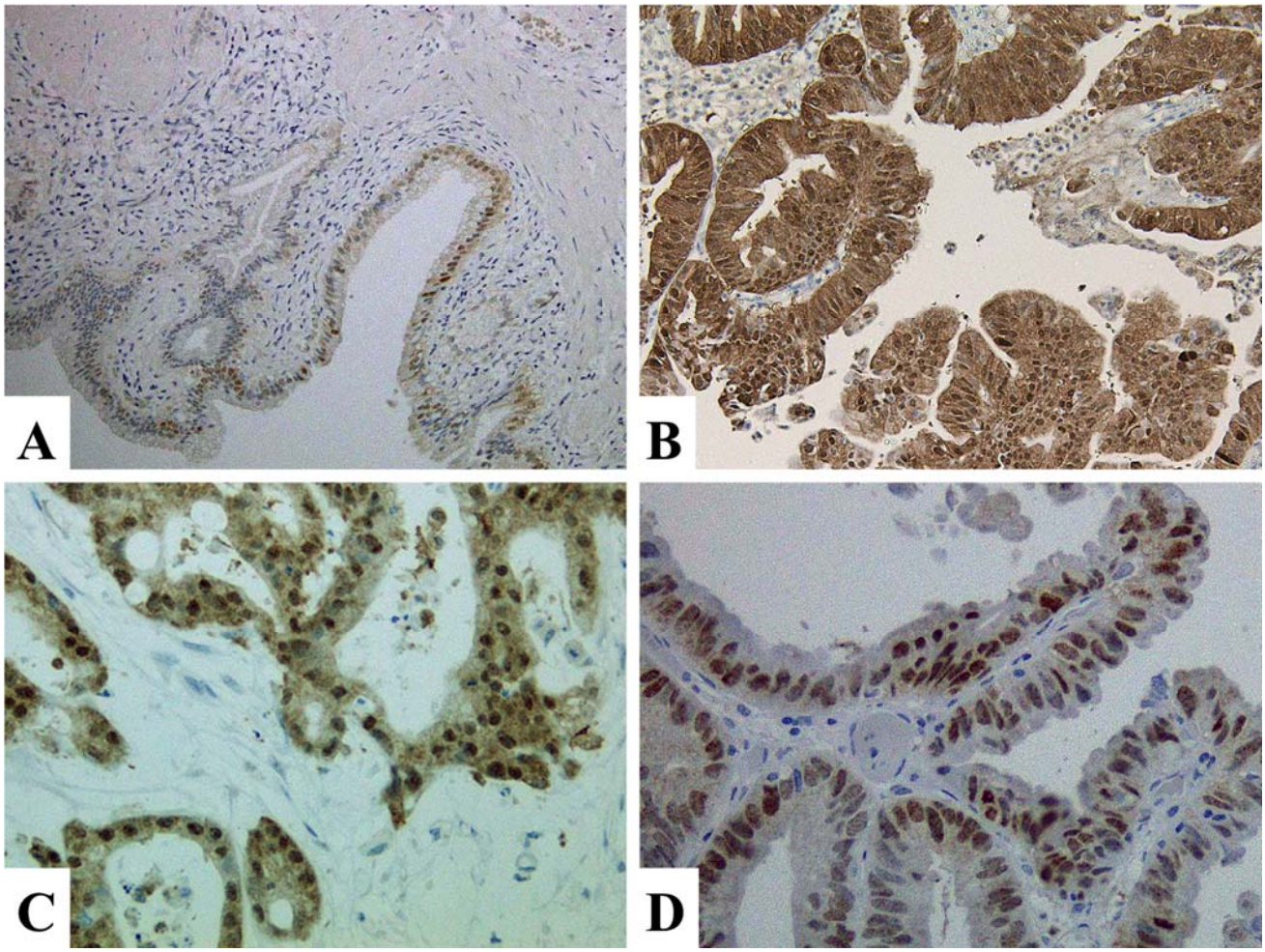
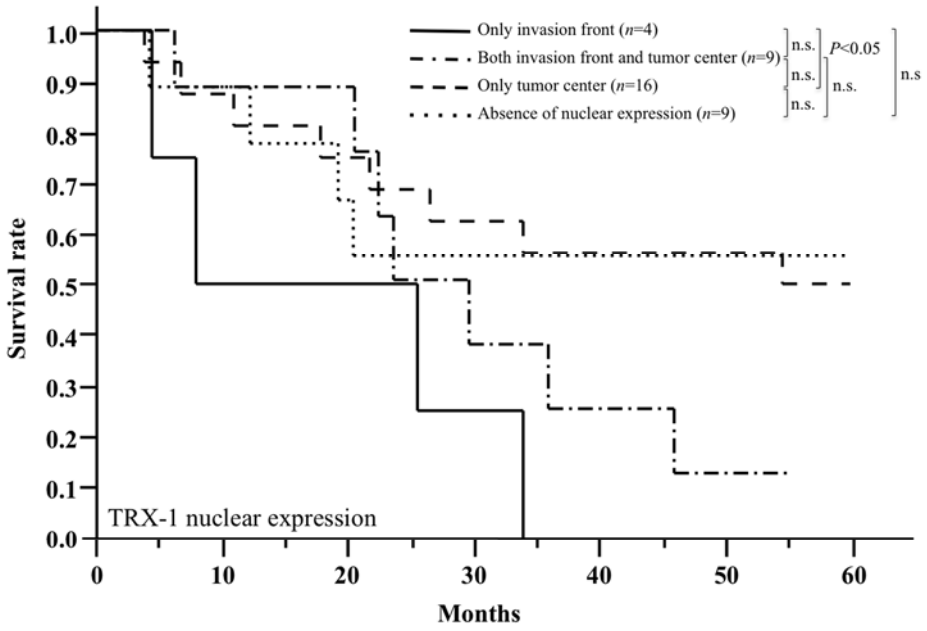


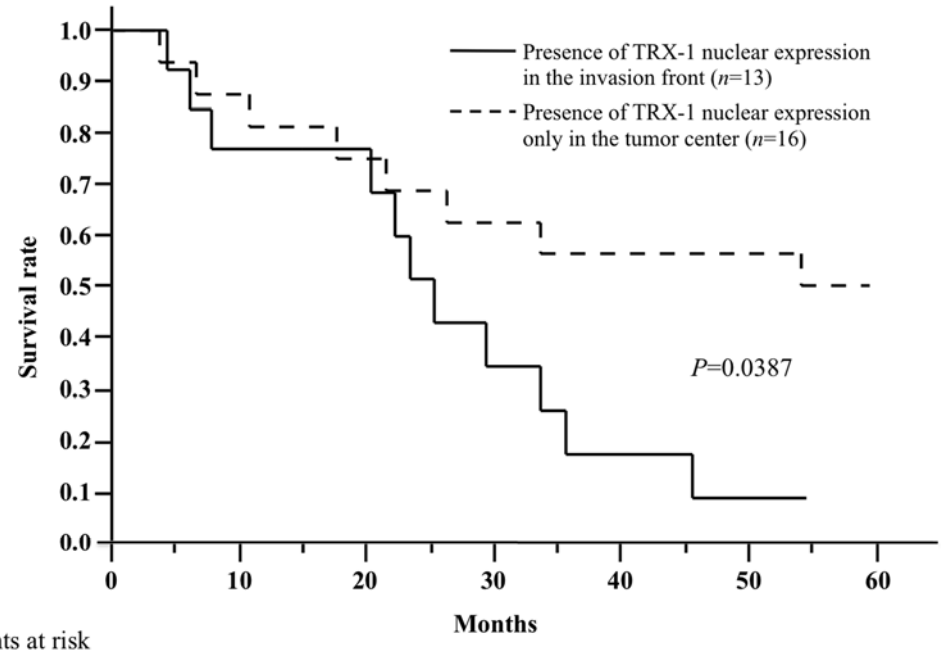
Figure3



No. of patients at risk

Only invasion front	4	2	2	0		
Both invasion front and tumor center	9	8	4	3	1	0
Only tumor center	16	13	11	9	9	8
Absence of nuclear expression	9	8	5	4	4	4

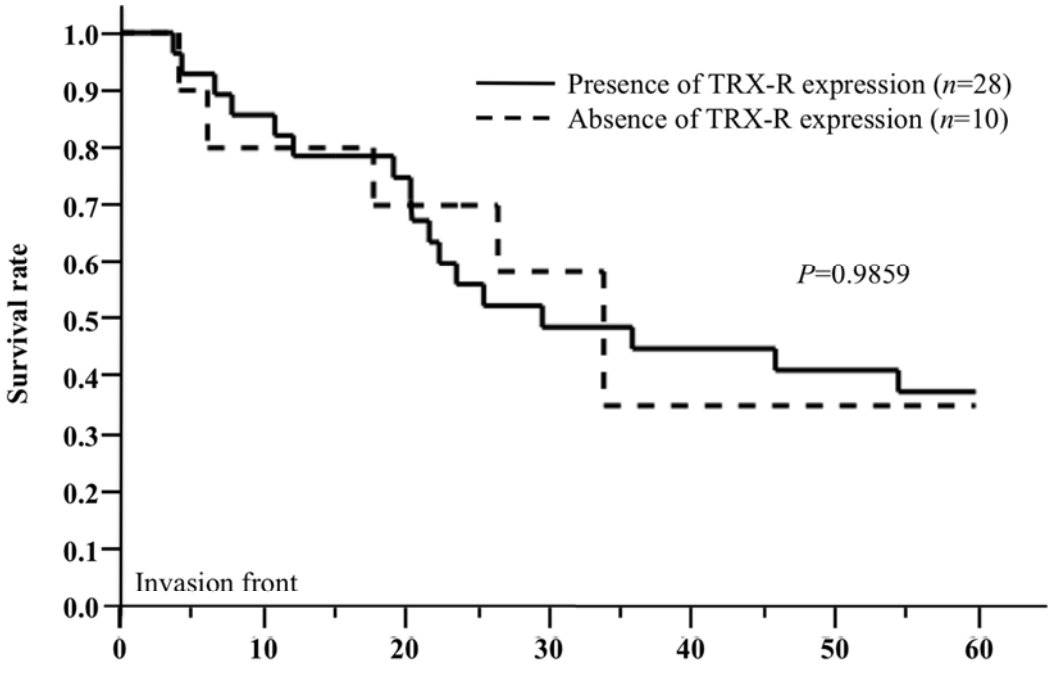
Figure4



No. of patients at risk

TRX-1 nuclear expression in the invasion front	13	10	6	3	1	0
TRX-1 nuclear expression only in the tumor center	16	14	12	10	10	8

Figure5



No. of patients at risk

Presence	28	23	15	13	11	10
Absence	10	8	7	3	3	2

Table 1 Clinicopathological characteristics in Patients with GBC

Variable	
Patient background	
Sex M : F	17 :21
Median Age (range)	68.5 (40-89)
Tumor factors	
Histological type	
Pap / tub1 / tub2 / tub3/ other	9 / 5 / 12 / 9 / 3
Tumor invasion	
pT2 / pT3 / pT4	17 / 3 / 18
Lymph node metastasis	
Negative / Positive	17 / 21
Stage	
II / III / IVa / IVb	11 / 7 / 8 / 12
Operative factor	
Surgical margin	
Negative / Positive	31 / 7
Final curability	
fCurA / B / C	14 /13/ 11

GBC gallbladder cancer

Table 2 Classification Systems for Staging, Curability by the JSBS

Final stage					
	H(-), P(-), M(-)				H(+), P(+), M(+)
	pN_0	pN_1	pN_2	pN_3	
pT_1	I	II	III	IVa	IVb
pT_2	II	III	III	IVa	
pT_3	III	III	IVa	IVb	
pT_4	IVa	IVa	IVb	IVb	

Final curability			
	pN-D		surgical margin
Final curability A, B	pN≦D	and	negative
Final curability C	pN>D	and/or	positive

JSBS Japanese Society of Biliary Surgery, H liver metastasis, P metastasis to the peritoneum, M distant metastasis other than peritoneal and/or liver metastases, pN histological lymph node metastasis, D lymph node dissection,

Surgical margin: microscopic surgical margin

Table 3 Clinicopathological Factors of Patients with and without TRX-1 Nuclear Expression in the Invasion Front

Variable	No. of patients	TRX-1 nuclear expression in the invasion front		*P* value
		Absence	Presence	
Sex				0.897
M	13	7	6	
F	16	9	7	
Age (y)				0.5335
<65	13	8	5	
≥65	16	8	8	
Histological type (pap+tub1 vs tub2,3)				0.3754
pap	8	5	3	
tub1	4	3	1	
tub2	10	4	6	
tub3	6	4	2	
Tumor invasion (pT2 vs pT3, 4)				0.5335
pT2	13	8	5	
pT3	3	1	2	
pT4	13	7	6	
Lymph node metastasis				0.6381
Negative	12	6	6	
Positive	17	10	7	
Stage (Stage II vs III, IV)				0.2373
II	8	3	5	
III	6	5	1	
IVa	5	5	0	
IVb	10	3	7	
Final curability				0.4364
fCurA, B	20	12	8	
fCurC	9	4	5	

TRX thioredoxin

Table 4 Clinicopathological Factors Influencing Postoperative Survival in Patients with GBC

Variable	No. of patients	3-year survival rate (%)	5-year survival rate (%)	*P* value
Patient background				
Sex				
M	17	35	29	0.4888
F	21	48	43	
Age (y)				
<65	14	29	29	0.5182
≥65	24	53	43	
Tumor factors				
Histological type				
pap, tub1	14	55	47	0.0787
tub2, tub3	21	31	26	
Tumor invasion				
pT2	17	69	63	0.0050
pT3, pT4	21	26	16	
Lymph node metastasis				
Negative	17	63	56	0.0248
Positive	21	26	21	
Stage (Stage II vs III-IV)				
II	11	70	60	0.0616
III	7	57	57	
IVa	8	45	45	
IVb	12	8	0	
TRX-1 nuclear expression in the invasion front				
Absence	25	55	51	0.0391
Presence	13	17	0	
TRX-R cytoplasmic expression in the invasion front				
Negative	10	35	35	0.9859
Positive	28	45	37	
TRX-1 nuclear and TRX-R cytoplasmic expression in the invasion front				
Absence	28	53	49	0.0257
Presence	10	11	0	
Operative factor				
Surgical margin				
Negative	31	49	46	0.0362
Positive	7	14	0	
Final curability				
fCurA, B	27	57	53	0.0013
fCurC	11	9	0	

GBC gallbladder cancer, *TRX* thioredoxin, *TRX-R* thioredoxin reductase, Surgical margin: microscopic surgical margin

Table 5 Clinicopathological Factors Influencing Postoperative Survival in Patients with GBC After Curative Resection

Variable	No. of patients	3-year survival rate (%)	5-year survival rate (%)	*P* value
Patient background				
Sex				
M	9	61	61	0.5827
F	18	55	50	
Age (y)				
<65	10	40	40	0.3546
≥65	17	70	63	
Tumor factors				
Histological type				
pap, tub1	10	80	69	0.1322
tub2, tub3	15	36	36	
Tumor invasion				
pT2	17	69	63	0.1650
pT3, pT4	10	36	36	
Lymph node metastasis				
Negative	15	72	65	0.1432
Positive	12	39	39	
Stage (Stage II vs III-IV)				
II	11	70	60	0.3974
III	7	57	57	
IVa	6	62	62	
IVb	3	0	0	
TRX-1 nuclear expression in the invasion front				
Absence	19	68	68	0.0465
Presence	8	29	0	
TRX-R cytoplasmic expression in the invasion front				
Negative	7	51	51	0.7938
Positive	20	58	53	
TRX-1 nuclear and TRX-R cytoplasmic expression in the invasion front				
Absence	21	66	66	0.0117
Presence	6	21	0	

GBC gallbladder cancer, *TRX* thioredoxin, *TRX-R* thioredoxin reductase