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RT-PCR of peritoneal washings predicts peritoneal pancreatic cancer recurrence

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ABSTRACT

Background: Peritoneal recurrence of pancreatic cancer is a frequent and lethal outcome after R0 resection. A method to predict peritoneal recurrence could be helpful in its prevention.

Materials and methods: Peritoneal washings were prospectively obtained from 29 patients in whom R0 resection was performed. Cytological examination (CY) and real-time reverse transcription polymerase chain reaction (RT-PCR) of the peritoneal washing for the detection of cancer-related genes, CEACAM5, KRT7, KRAS, and MUC1, were performed. Clinicopathological characteristics and real-time RT-PCR results of the peritoneal washing were compared between patients whose pancreatic cancer recurred peritoneally ($n = 7$) and those patients who it did not recur ($n = 22$).

Results: Only one CY-positive (CY^+) case was detected, and that patient recurred. MUC1 mRNA expression was significantly higher in the recurrence group ($P = 0.015$). Cumulative incidence-function analysis demonstrated that peritoneal recurrence rate was significantly higher in MUC1-positive ($MUC1^+$) patients ($P = 0.044$). $MUC1^+$ patients had significantly decreased disease-free survival ($P = 0.009$) and disease-specific survival ($P = 0.031$). MUC1 protein was detected in the primary tumor in 18 of 29 patients. However, no significant difference was observed in the expression of MUC1 protein in peritoneal washings from the primary tumor ($P = 0.579$).

Conclusions: High expression of MUC1 mRNA in peritoneal washings is a significant risk factor for peritoneal recurrence of pancreatic cancer after R0 resection along with poor disease-specific survival. RT-PCR of MUC1 mRNA in peritoneal washing may be useful for individualization of adjuvant chemotherapy.

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Introduction

Pancreatic cancer is recalcitrant with the lowest 5-year survival rate of the major types of cancer.[1] Pancreatic cancer has low response rates to chemotherapy and radiotherapy.[2] Long-term survival is therefore contingent on R0 resection performed at an early stage of the disease. Despite advances in preoperative treatment and adjuvant chemotherapy, results in an 80% recurrence rate within 2 years,[3] including distant recurrence in approximately 75% of cases. The liver and the peritoneal cavity are the most common sites of recurrence. Local recurrence only occurs in nearly one-third of all cases.[4]

Prediction of postoperative peritoneal recurrence is very important in pancreatic cancer. In some cancers, such as gastric cancer, which has a high rate of postoperative peritoneal recurrence, cytological examination (CY) is often used to predict the risk of this outcome. Positive CY (CY^+) is an independent risk factor for disease recurrence and poor overall survival.[5] CY^+ status is categorized as M1 disease in the TNM Classification of Malignant Tumors (7th edition). However, the sensitivity of CY is controversial because peritoneal recurrence has been observed with CY use.[6] For patients with CY^+ pancreatic cancer, who otherwise qualify as curable based on the absence of other associated risk factors, there is no consensus on their suitability for radical resection.[7] The development of a precise method to predict the risk of postoperative peritoneal recurrence could enable more effective treatment strategies for pancreatic cancer.

Dalal *et al.*[8] used quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) of peritoneal washings in patients with pancreatic cancer, who are undergoing staging laparoscopy to detect tumor markers, including CEA, as an indicator of the presence of peritoneal micrometastasis. This method was comparable to previous cytology results. However, this study included patients with peritoneal dissemination, which therefore precluded the study from being predictive for this outcome. Therefore, we restricted our study to patients with pancreatic cancer who had undergone R0 resection to investigate the ability of real-time RT-PCR of cancer-marker genes in floating cancer cells in peritoneal washings to predict peritoneal recurrence.

Materials and methods

From September 2011 to February 2013, peritoneal washings were obtained prospectively from 36 patients with pancreatic cancer who underwent R0 resection at the Yokohama City University Hospital. Of these, R0 resection was not performed in five patients due to the presence of distant metastatic lesions discovered at surgery including four patients with liver metastasis and one with peritoneal dissemination. In addition, two patients died of other causes in the early postoperative period at our hospital. One patient died of sepsis from a liver abscess, while the other died of pneumonia. The analysis was performed on the remaining 29 patients who underwent R0 resection, which was defined as resection with a tumor-free margin of 1 mm or more.

The right upper abdomen, left upper abdomen, and pelvis were washed with 600 mL saline during laparotomy. Peritoneal lavage fluid (300 mL) was collected from each site. Half of this fluid was used for routine cytology, and the other half was used for real-time RT-PCR. Peritoneal washing was performed at the beginning of surgery to avoid the contamination of the lavage fluid with blood cells.

Positive cytology (CY^+) of intraoperative peritoneal washings was detected with the Papanicolaou stain. The results of RT-PCR were not used for therapeutic decision-making for any patient. Follow-up data were obtained from the patients' medical records. To assess recurrence, physical examination and laboratory tests, including tumor markers, were performed every month. Computed tomography (CT) was performed every 3 months. If CT was inconclusive and an increase in tumor markers occurred, positron emission tomography-CT (PET-CT) was performed.

The definition of peritoneal recurrence included ascites and peritoneal nodules detected by CT or PET-CT.

The 29 patients were divided into two groups, based on the development of peritoneal recurrence during the 2-year follow-up period. Of the 29 patients, peritoneal recurrence occurred in seven patients and was not observed in the remaining 22 patients. We compared gene expression in peritoneal washings from the two groups. In addition, we explored the association between MUC1 expression in peritoneal washings from the primary lesion tumor and subsequent peritoneal recurrence.

The study protocol was approved by the Institutional Ethical Committee at Yokohama City University (B111110029) and written informed consent was obtained from all patients before their enrollment in the study.

Real-time reverse transcription polymerase chain reaction

The genes investigated by Dalal *et al.*,[8] *CEACAM5, CK7, KRAS,* and *MUC1,* were also detected in our study using RT-PCR of peritoneal washings. Since KRAS expression was not significantly different between pancreatic cancer patients with and without peritoneal recurrence, KRAS mutational status was not analyzed in this study.

Peritoneal washings to be used for real-time RT-PCR were centrifuged at 2000 rpm for 10 min, and the supernatant was removed. After the addition of 1 mL phosphate-buffered saline, the specimens were centrifuged again at 10,000 rpm for 5 min at 4°C. Total RNA was extracted from the remaining pellet after homogenization with QIAzol (QIAGEN, Valencia, CA), followed by on-column clean-up with the miRNeasy Mini Kit (QIAGEN). Total RNA (2 μg) was reverse transcribed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) for complementary DNA synthesis. Complementary DNA (2 μL) was amplified in a final volume of 20 μL, with the following TaqMan Gene Expression Assays (Applied Biosystems): *MUC1* (Hs00159357_m1), *CEACAM5* (Hs00944025_m1), *KRT7* (Hs00559840_m1), *KRAS* (Hs00364284_g1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control (Hs99999905_m1). All

Table 1 – The comparison of the patient clinicopathological charecteristics.

Variables	Recurrence group ($n = 7$)	Nonrecurrence group ($n = 22$)	P value
Age (y) [median (range)]	71 (53-79)	65.5 (37-81)	0.940
Sex			0.758
Male	4	14	
Female	3	8	
Tumor marker [median (IQR)]			
CEA (ng/mL)	3.8 (1.7-5.8)	2.7 (2.3-6.2)	0.823
CA19-9 (U/mL)	25.0 (9.0-129.0)	22.5 (10.0-76.0)	0.901
SPan-1 (U/mL)	26.0 (3.4-43.0)	23.5 (7.2-35.0)	0.823
DUPAN-2 (U/mL)	25.0 (25.0-120.0)	114.5 (29.5-412.5)	0.110
Tumor location			0.087
Head	7	15	
Body/tail	0	7	
Tumor size (cm) [median (IQR)]	3.0 (2.1-3.3)	2.7 (2.0-3.5)	0.627
Neoadjuvant chemoradiotherapy			0.484
No	1	6	
Yes	6	16	
Adjuvant chemotherapy			0.086
No	4	5	
Yes	3	17	
Operative procedures			0.230
Pancreaticoduodenectomy	7	15	
Distal pancreatectomy	0	4	
Total pancreatectomy	0	3	
UICC T-stage			0.853
T1	0	1	
T2	1	2	
T3	6	18	
T4	0	1	
Lymph node metastasis			0.331
Negative	3	14	
Positive	4	8	
Pathological type			0.824
Well	1	5	
Moderate	6	14	
Poor	0	1	
Anaplastic	0	1	
Adenosquamous	0	1	
Neural invasion			0.080
No	2	15	
Yes	5	7	
Vascular invasion			0.223
No	4	7	
Yes	3	15	
UICC stage			0.766
I A	0	1	
I B	0	2	
II A	3	10	
II B	4	8	
III	0	1	

(continued)

Table 1 – (*continued*)

Variables	Recurrence group ($n = 7$)	Nonrecurrence group ($n = 22$)	P value
CY			0.071
Negative	6	22	
Positive	1	0	

UICC = Union for International Cancer Control; IQR = interquartile range.
There was no significant difference between two groups.

reactions were performed in triplicate using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems), and the mean values were analyzed to prevent dispersion of results.

Immunohistochemical methods

Tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. The sections were subsequently washed with ultrapure water and unmasked in citrate antigen unmasking solution (Mitsubishi Kagaku Iatron, Tokyo, Japan) in an autoclave for 10 min at 121°C. The sections were washed with ultrapure water and phosphate-buffered saline and then treated for 30 min with 0.03% hydrogen peroxide to block endogenous peroxidase activity. The sections were incubated with anti-MUC1 (1:100; Fitzgerald) for 60 min at 37°C. The bound primary antibody was detected by incubating an anti-mouse secondary antibody and avidin/biotin/horseradish peroxidase complex (Dako Cytomation, Kyoto, Japan) with the sections for 10 min at room temperature. The labeled antigens were visualized by staining with the DAB kit (Dako Cytomation). Finally, the sections were counterstained with hematoxylin and examined under a microscope.

Analysis of immunohistochemically-stained slides was performed as previously described.[9] Cytoplasmic or membranous reactivity was classified as follows: 0 (no reactivity), 1 (reactivity in <10% of cancer cells), 2 (reactivity in >10% but <40% of cancer cells), or 3 (reactivity in >40% of cancer cells). For statistical analysis, classes 0 and 1 were defined as negative, and classes 2 and 3 were defined as positive.

Statistical analysis

Data were analyzed using SPSS, version 21, for Windows (SPSS, Chicago, IL). For univariate analysis, binomial variables were compared using the chi-squared test. Continuous variables were compared using the Mann–Whitney U test. We did not perform multivariate analysis due to the small sample

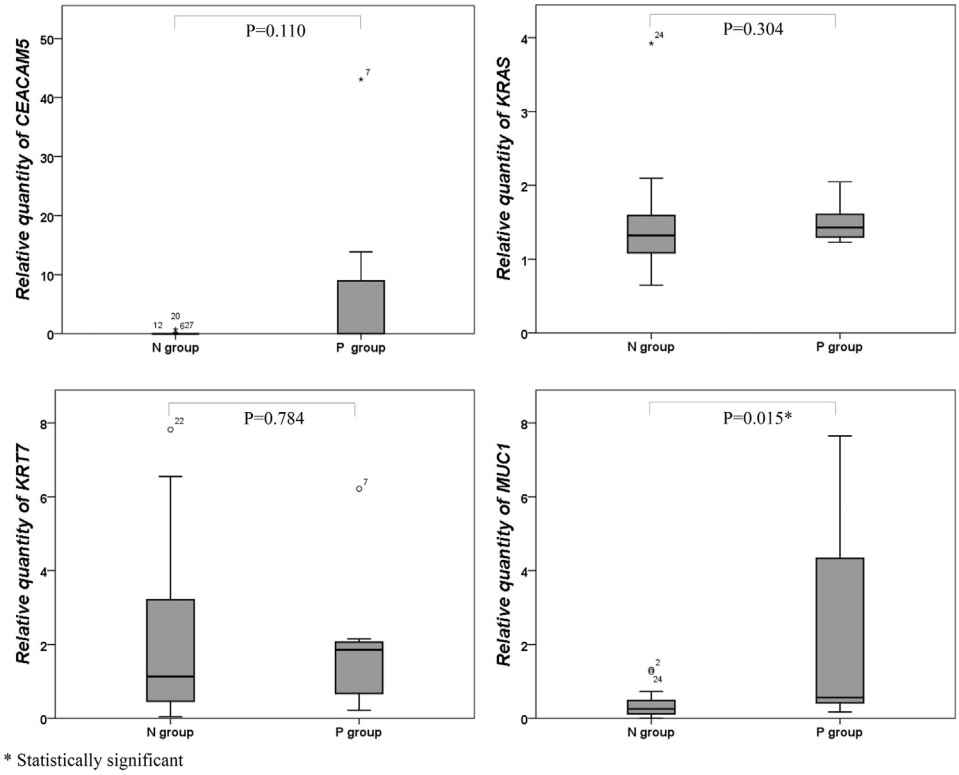

Fig. 1 – Gene expression analysis. The expression of MUC1 was higher in the recurrence group than in the nonrecurrence group. However, there was no significant difference in the expression of the other three genes tested. *Statistically significant.

Table 2 – The comparison of the patient clinicopathological characteristics between MUC1$^+$ group and MUC1$^-$ group.

Variables	MUC1$^+$ ($n = 14$)	MUC1$^-$ ($n = 15$)	P value
Age (y) [median (range)]	61 (53-75)	70 (64-79)	0.123
Sex			0.268
Male	10	8	
Female	4	7	
Tumor marker [median (IQR)]			
CEA (ng/mL)	3.3 (2.0-7.5)	2.7 (2.4-5.8)	0.683
CA19-9 (U/mL)	11.5 (1.8-59.0)	56.0 (14.0-129.0)	0.093
SPan-1 (U/mL)	16.0 (2.9-32.3)	30.0 (11.0-45.0)	0.270
DUPAN-2 (U/mL)	34.0 (25.0-315.0)	79.0 (31.0-410.0)	0.134
Tumor location			0.166
Head	9	13	
Body/tail	5	2	
Tumor size (cm) [median (IQR)]	2.9 (5.3-7.5)	3.0 (2.2-3.5)	0.949
Neoadjuvant chemoradiotherapy			0.224
No	2	5	
Yes	12	10	
Adjuvant chemotherapy			0.599
No	5	4	
Yes	9	11	
Operative procedures			0.363
Pancreaticoduodenectomy	9	13	
Distal pancreatectomy	3	1	
Total pancreatectomy	2	1	
UICC T-stage			0.481
T1	1	0	
T2	1	2	
T3	11	13	
T4	1	0	
Lymph node metastasis			0.176
Negative	10	7	
Positive	4	8	
Pathological type			0.530
Well	3	3	
Moderate	9	11	
Poor	0	1	
Anaplastic	1	0	
Adenosquamous	1	0	
Neural invasion			0.587
No	8	9	
Yes	6	6	
Vascular invasion			0.442
No	6	5	
Yes	8	10	
UICC stage			0.199
I A	1	0	
I B	0	2	
II A	8	5	
II B	4	8	
III	1	0	

(continued)

Table 2 – (continued)

Variables	$MUC1^+$ ($n = 14$)	$MUC1^-$ ($n = 15$)	P value
CY			0.292
Negative	13	15	
Positive	1	0	

UICC = Union for International Cancer Control; IQR = interquartile range.
There was no significant difference in the clinicopathological factors between the two groups.

size. The selected continuous variables used for univariate analysis were converted to dichotomous variables using receiver-operating-characteristic curve analysis. The cumulative incidence rate for the recurrence of peritoneal dissemination was determined (instead of Kaplan–Meier analysis) due to recurrence at other sites than the peritoneum. Gray's test[10] was used to determine between-group differences. Survival curves were constructed using the Kaplan–Meier method and compared using the log-rank test. P values < 0.05 were considered significant.

Results

Patient outcome

The mean follow-up duration was 26.6 mo (range, 3.9–38.9 mo). Of the 29 patients, peritoneal recurrence was observed in seven patients within 2 y of resection (recurrence group). One patient in the recurrence group was diagnosed due to the presence of ascites. The remaining patients in the recurrence group were diagnosed based on CT or PET. These patients died within 6 mo of detection of recurrence. Peritoneal recurrence was not observed in the remaining 22 patients (nonrecurrence group). There were no significant baseline differences between the two groups (Table 1).

Cytological examination of peritoneal washings

Only one CY^+ patient (in the recurrence group) was found among the entire cohort, thereby precluding CY status from being predictive in the present study.

Expression of cancer-related genes

The expression of CEACAM5, KRAS, KRT7, and MUC1 in peritoneal washings was compared between the recurrence and nonrecurrence groups. No significant difference was observed between the two groups for the expression of CEACAM5 ($P = 0.110$), KRAS ($P = 0.304$), and KRT7 ($P = 0.784$). However, the expression of MUC1 in the recurrence group was significantly higher than in the nonrecurrence group ($P = 0.015$) (Fig. 1).

Cutoff value for the selected continuous variable for univariate analysis

The cutoff value for the selected continuous variables for predicting peritoneal recurrence on univariate analysis was estimated using receiver-operating-characteristic curves. The best cutoff value for MUC1/GAPDH was 3.45×10^{-2}. Using this cutoff value, we converted the quantity of MUC1/GAPDH to a binary variable (+ or –). Of the 29 patients, 14 were $MUC1^+$ and 15 were $MUC1^-$. There was no significant difference between the $MUC1^+$ and $MUC1^-$ groups, with respect to clinicopathological characteristics (Table 2).

Predictive value of MUC1 expression for peritoneal recurrence

Among the 22 patients in the nonrecurrence group, six patients had recurrence at distant sites: four patients had liver metastasis, one had lung metastasis, and one had brain metastasis. Sixteen patients did not have recurrence within the follow-up period. However, three of these 16 patients died of other causes. The mean follow-up period for the remaining 13 patients was 24.5 mo (range, 12–38.9 mo). The cumulative incidence rate for peritoneal recurrence in the $MUC1^+$ group was significantly higher than for the $MUC1^-$ group ($P = 0.044$) (Fig. 2). However, the cumulative incidence rate for recurrence at other sites was not significantly different between the two groups ($P = 0.315$). The predictive values of $MUC1^+$ for peritoneal recurrence were as follows: sensitivity of 85.7%, specificity of 63.6%, and accuracy of 68.9%.

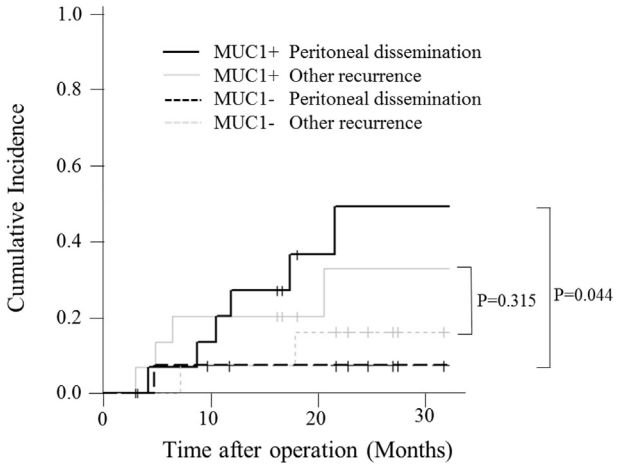

Fig. 2 – Cumulative incidence function for peritoneal recurrence. The peritoneal recurrence rate was significantly higher in the $MUC1^+$ group than in the $MUC1^-$ group ($P = 0.044$). However, there was no significant difference with respect to recurrence at sites other than the peritonium ($P = 0.315$).

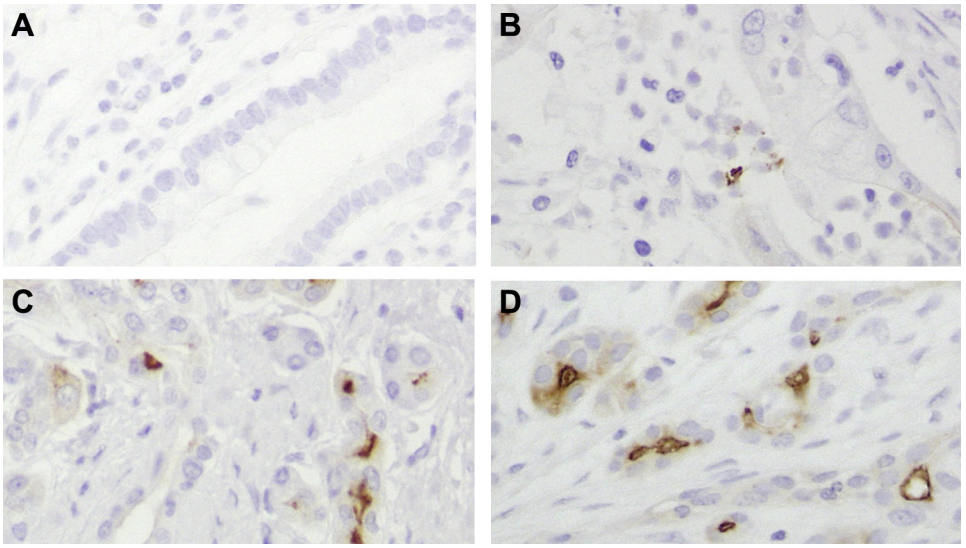

Fig. 3 – Representative images of pancreatic cancer tissues immunostained for MUC1. (A) No reactivity; (B) reactivity in < 10% of tumor cells; (C) reactivity in > 10% but < 40% of tumor cells; (D) reactivity in > 40% of tumor cells. (Color version of figure is available online.)

Expression of MUC1 protein in the primary tumor

Expression of the MUC1 protein in the primary tumor was observed in 18 (62%) of the 29 patients. Figure 3 shows representative images of positive and negative MUC1 protein expression. The occurrence of MUC1 mRNA expression in peritoneal washings and MUC1 protein expression in the primary tumor were not significantly different ($P = 0.597$).

Patient survival

The median overall survival ($n = 29$) was 24.8 mo. We compared disease-free survival and disease-specific survival (DSS). Disease-free survival ($P = 0.009$) and DSS ($P = 0.031$) of $MUC1^+$ patients were significantly shorter than those of $MUC1^-$ patients (Fig. 4).

Discussion

Overexpression of MUC1 in pancreatic cancer was previously shown to be an independent indicator of poor prognosis. MUC1 overexpression was associated with cancer-cell invasion and metastasis.[11,12] In the present study, high expression of MUC1 mRNA in peritoneal washings was found to be a predictive factor for peritoneal recurrence, and for the overall prognosis of pancreatic cancer patients undergoing R0 resection. However, it was not possible to clarify the relationship between MUC1 expressions in peritoneal washings and in the primary tumor. Neoadjuvant chemoradiotherapy, which was administered to most patients in the present study, may have affected MUC1 expression in the primary tumor.

Risk prediction for peritoneal recurrence by RT-PCR–based analysis has been reported in gastrointestinal carcinomas.[13-18] However, there are few such reports in pancreatic cancer.[19-22] Dalal *et al.*[8] reported that RT-PCR of a panel of tumor markers, including CEA, could be a sensitive method for the detection of subclinical peritoneal-tumor dissemination in pancreatic cancer. Kelly *et al.*[21] also reported that RT-PCR for CEA is a sensitive and specific method for the detection of clinically significant, peritoneal micrometastases of pancreatic cancer. In the present study, it is unclear why MUC1, rather than CEA, was useful for the prediction of peritoneal recurrence. However, our results are consistent with previous results demonstrating that the overexpression of MUC1 in cancer cells was associated with increased invasiveness and metastatic properties.[11,12]

CY^+ has been previously useful for the prediction of peritoneal recurrence in gastrointestinal cancer. However, the usefulness of CY^+ for pancreatic cancer is yet to be established. Clark *et al.*[7] reported that CY^+ in locally-advanced pancreatic cancer is a common finding and is associated with shortened survival. However, Yoshioka *et al.*[23] stated that CY^+, in the absence of distant metastasis, should not necessarily preclude resection in patients with pancreatic cancer. In the present study, it was not possible to draw a conclusion regarding the usefulness of CY^+ because only one patient was CY^+.

Individualized treatment based on the expression of biomarkers is being increasingly used. For example, Motoi *et al.* reported that sustained elevation of serum tumor markers after resection is an important prognostic factor for pancreatic cancer.[24] The present study indicates that $MUC1^+$ expression in peritoneal washings is predictive for peritoneal recurrence of pancreas cancer after R0 resection. In the present study, several patients in the $MUC1^+$ group received gemcitabine (GEM) + tegafur/gimeracil/oteracil as neoadjuvant chemotherapy. Our findings suggest that neoadjuvant GEM + tegafur/gimeracil/oteracil chemotherapy in $MUC1^+$ patients is not effective in preventing recurrence.

The following treatment strategy is therefore proposed: diagnostic laparoscopy should be performed in patients with potentially resectable pancreatic cancer, and patients at a high risk of recurrence should be identified based on MUC1 mRNA expression in peritoneal washings. Subsequently,

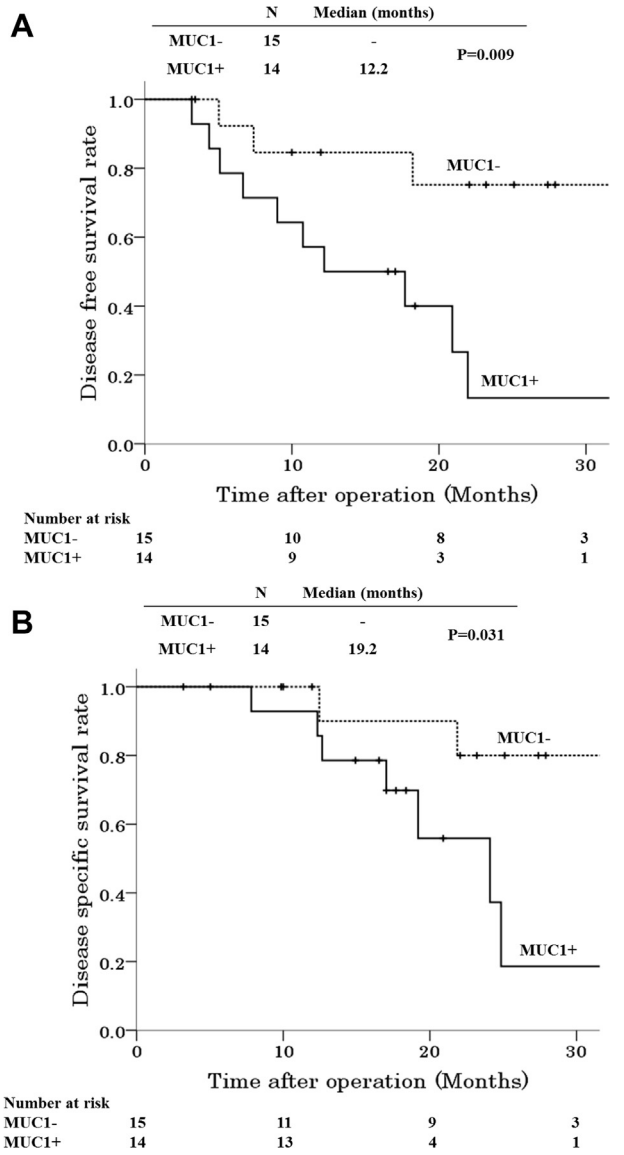

Fig. 4 – Correlation between MUC1 status and prognosis. (A) Disease-free survival and (B) DSS of MUC1$^+$ patients were significantly shorter than those of MUC1$^-$ patients.

neoadjuvant chemotherapy with newer regimens such as GEM + nanoparticle albumin-bound paclitaxel or FOLFILINOX should be used for MUC1$^+$ patients to improve outcomes.

In this study, all patients with peritoneal recurrence had pancreatic head cancer. However, there were five patients in the MUC1$^+$ group with pancreatic body and tail cancer. If the follow-up period was longer, it is possible that these five patients would have developed recurrence with peritoneal dissemination. Even in patients with pancreatic body and tail cancer that is resectable, if the patient is MUC1$^+$, it may be necessary to devise a treatment strategy that includes improved neoadjuvant therapy.

Some limitations of our study need to be taken into account while interpreting our findings. These include the small sample size and a relatively short follow-up period. Further studies on larger number of patients are warranted with longer follow-up periods.

In conclusion, high expression of MUC1 mRNA in peritoneal washings was associated with a higher risk of peritoneal recurrence of pancreatic cancer after R0 resection along with poor DSS. Individualized preoperative and postoperative chemotherapy protocols may be useful to prevent recurrence in these patients.

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Conflict of interest: The authors have no conflicts of interest.

Authors' contributions: Conception and design of the study was performed by R.Mo., Y.H., and I.E. Analysis and interpretation of data were carried out by K.S. and M.S.O. Collection and assembly of data were carried out by K.S., R.Mo., and R.Ma. Drafting of the article was performed by K.S. Critical revision of the article for important intellectual content was given by K.S., R.M.H., and M.B. The final approval of the article was done by I.E.

Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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