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Significance of Intraperitoneal-free *KRT20* and *CEACAM6* mRNA Expression for Peritoneal Recurrence of Gastric Cancer

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Abstract. *Background/Aim: Peritoneal lavage cytology is widely used to predict peritoneal recurrence after surgery, but cases of peritoneal recurrence are often recognized in patients with peritoneal lavage cytology negativity (CY0) who underwent no residual tumour (R0) surgery. We used peritoneal lavage fluid before and after gastric cancer surgery to detect cytokeratin 20 (KRT20) and carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) mRNA by RT-PCR. Materials and Methods: We collected peritoneal lavage fluid before and after surgery from 58 patients who underwent gastrectomy. RNA was extracted from these samples and RT-PCR was performed. RNA expression was defined as positive and negative in cases with values higher or lower than the median value. We investigated the relationship between mRNA expression and clinicopathological and surgical factors and prognosis. Results: Tumour invasion to the sub-serosa (T3) or penetration of the serosa (T4a), lymph node metastasis, and more than 150 ml intraoperative bleeding were significantly correlated with KRT20 mRNA expression. Multivariate analysis of its relationship with peritoneal recurrence showed that the odds ratio of CEACAM6 mRNA for recurrence was high (odds ratio=24.753; 95%CI=0.883-694.06; p=0.0592). All cases with peritoneal recurrence were CEACAM6-positive at pre- or post-surgery. The prognosis of peritoneal recurrence for both KRT20- and CEACAM6-positive cases was significantly poorer than that of other cases. The recurrence-free survival of the CEACAM6-positive group was significantly poorer than that of the CEACAM6-negative group. Conclusion: Measurement of CEACAM6 mRNA in peritoneal lavage fluid at pre- and post-surgery may be useful as a predictor of peritoneal recurrence.*

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Key Words: Gastric cancer, peritoneal recurrence, *KRT20*, *CEACAM6*, peritoneal lavage fluid.

Peritoneal recurrence is the most common type of gastric cancer recurrence, but the prognosis is very poor because early detection is difficult and systemic chemotherapy is ineffective (1-3). The mechanism is thought to involve the release of cancer cells into the abdominal cavity directly from the gastric serosal surface, metastatic lymph nodes, and omentum, or through the subperitoneal lymph vessels, their attachment to peritoneal mesothelial cells, retraction of the mesothelial cells, and exposure of the basement membrane, attachment to the basement membrane, degradation in the extracellular matrix, and proliferation (4). In Japan, intraperitoneal lavage cytology (CY) is widely performed to predict minute peritoneal dissemination, and CY positivity is classified as pathological distant metastasis (pM1) according to the 13th edition of the Japanese Classification of Gastric Carcinoma and is classified as Stage IV disease (5). Because intraperitoneal lavage cytology is diagnosed by microscopy, smaller lesions are difficult to detect, and patients often die of cancer due to peritoneal recurrence despite curative resection and peritoneal cytology negative for carcinoma cells (CY0). As a more sensitive diagnostic method, some reports used cytokeratin 20 (*KRT20*) mRNA, which is an epithelial cell marker in intraperitoneal lavage fluid, and carcinoembryonic antigen related cell adhesion molecule 6 (*CEACAM6*) mRNA, which is a cancer cell marker, but they were measured preoperatively (6, 7), and few reports, in which *KRT20* and *CEACAM6* mRNA expression was measured in intraperitoneal lavage fluid after surgery as well as before surgery are available. In this study, we investigated whether *KRT20* and *CEACAM6* mRNA expression in intraperitoneal lavage fluid collected before and after surgery could predict peritoneal dissemination recurrence.

Materials and Methods

Patients. This study has been approved by the institutional review board of Kurume University School of Medicine in accordance with the ethics guidelines for clinical research by the Ministry of Health, Labour, and Welfare (approval number: 18108). Patients with gastric cancer who underwent gastrectomy at Kurume University Hospital gave informed consent in writing for the use of their sample data for research purposes. Of the patients who underwent no residual tumour

(R0) or microscopic residual tumour (R1) surgery at our hospital from April 2018 to March 2021, the intraperitoneal lavage fluid of 58 patients could be collected before and after surgery (Table I).

Sample collection. Immediately after the start of surgery, 100 ml saline was injected into the Douglas Fossa and stirred slowly, and 50 ml injected saline was collected as a sample. At the time of wound closure, the abdominal cavity was washed with 2,000 ml saline, and then 100 ml saline was injected into the Douglas Fossa after washing and 50 ml of injected saline was collected as a sample.

RT-PCR. We centrifuged the collected samples at 280 × *g* for 5 min, removed the supernatant, added 350 μl buffer solution (10 μl β-mercaptoethanol per 1 ml Buffer RLT; Qiagen, Hilden, Germany) into the precipitate, and used 350 μl with the All Prep DNA/RNA Mini Kit, which allowed the simultaneous purification of genomic DNA and total RNA from the same biological sample (Qiagen). Superscript Vilo (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate the cDNA. Primers were as follows: Beta-actin (*ACTB*) (Hs01060665_g1; Thermo Fisher Scientific), *KRT20* (Hs00300643_m1; Thermo Fisher Scientific), and *CEACAM6* (Hs03645554_m1; Thermo Fisher Scientific); Quantitative PCR was performed using the Step One Real Time PCR system (Thermo Fisher Scientific) to detect RNA. The PCR temperature cycling conditions were as follows: initial denaturation at 95˚C for 1 min followed by 35 cycles at 95˚C for 30 s, 55˚C for 1 min, and 72˚C for 2 min, and a final extension step at 72˚C for 10 min.

The expression level of each gene was measured, and the median value was used as the cutoff value; patients with values higher than the median value were classified as the positive group and those with values below the median value were classified as the negative group.

Clinicopathological and surgical factors and recurrence-free survival. We investigated the association between clinicopathological factors, surgical factors, recurrence prognosis, and gene expression. The clinicopathological terms are based on the 15th edition of the Japanese Classification of Gastric Carcinoma (8). The following items were examined as clinicopathological factors and surgical factors: gross type (localized *vs*. diffuse), histological type (differentiated *vs*. undifferentiated), tumour size (less than 60 mm *vs*. 60 mm or more), depth of tumour invasion [pathologically diagnosed mucosal or submucosal tumour (pT1) or tumour invading the muscularis propria (pT2) *vs*. tumour invading the subserosa or more (≥pT3)], pathological lymph node metastasis [lymph node metastasis-negative (pN0) *vs*. lymph node metastasis-positive (≥pN1)], lymphatic invasion (no lymphatic invasion (Ly0) *vs*. lymphatic invasion-positive (Ly1)), venous invasion [no venous invasion (V0) *vs*. venous invasion-positive (V1)], infiltrative growth pattern (INFa or INFb *vs*. INFc), surgical procedure (total gastrectomy vs. distal gastrectomy or proximal gastrectomy), and intraoperative bleeding (less than 150 ml *vs*. more than 150 ml). The relationship between the above factors and the expression of *KRT20* and *CEACAM6* mRNA was investigated. Furthermore, the recurrence-free survival (RFS) of the *KRT20*- and *CEACAM6*-positive and -negative groups was compared.

Statistical analyses. The association of *KRT20* and *CEACAM6* mRNA expression with clinicopathological and surgical factors was investigated by bivariate analysis using the χ^2 test. Firth's logistics analysis detected factors involved in recurrence. First, in the bivariate analysis, factors significantly involved in recurrence were detected,

Table I. *Patient characteristics*.

	n	%
Sex		
Male	42	72.4
Female	16	27.6
Macroscopic type		
Localized	34	58.6
Diffuse	24	41.4
Histology		
Differentiated	29	50
Undifferentiated	29	50
T		
1a	4	6.9
1b	9	15.5
2	9	15.5
3	13	22.4
4a	21	36.2
4b	2	3.4
N		
0	17	29.3
1	13	22.4
2	14	24.1
3a	7	12.1
3b	7	12.1
Stage		
1a	9	15.5
1b	6	10.3
2a	4	6.9
2b	12	20.7
3a	13	22.4
3b	6	10.3
3c	4	6.9
4	4	6.9
Ly		
Ly0	23	39.7
Ly1	35	60.3
V		
V0	19	32.8
V1	39	67.2
INF		
a, b	47	81
c	11	19
Type of gastrectomy		
DG	5	8.6
TG	8	13.8
LDG	31	53.4
LTG	7	12.1
LPG	7	12.1
Lymph node dissection		
D1	4	6.9
D1+	11	19
D2	43	74.1
Volume of bleeding (ml)	61 (2-1492)	
Recurrence*		
+	9	15.5
–	49	84.5

*All recurrences were peritoneal. T: Depth of tumor invasion; N: lymph node metastasis; Ly: lymphatic invasion; V: venous invasion; INF: tumor infiltrative pattern; DG: distal gastrectomy; TG: total gastrectomy; LDG: laparoscopic distal gastrectomy; LTG: laparoscopic total gastrectomy; LPG: laparoscopic proximal gastrectomy.

Table II. *Clinicopathological and surgical factors of the KRT20-positive and KRT20-negative groups.*

	KRT20-positive (n=33)	*KRT20*-negative (n=25)	*p*-Value
Macroscopic type			
Localized	18	16	0.4680
Diffuse	15	9	
Histology			
Differentiated	14	15	0.1837
Undifferentiated	19	10	
Tumor size			
<60 mm	18	19	0.0883
≥60 mm	15	6	
T			
<T3	9	13	0.0496
≥T3	24	12	
N			
N0	5	12	0.0062
≥N1	28	13	
Ly			
Ly0	13	10	0.9627
Ly1	20	15	
V			
V0	12	7	0.4998
V1	21	18	
INF			
a, b	24	23	0.0534
c	9	2	
Type of gastrectomy			
TG	21	3	0.0302
DG or PG	12	22	
Intraoperative bleeding			
<150 ml	19	21	0.0272
≥150 ml	14	4	

CK: Cytokeratin; T: depth of tumor invasion; <T3: tumor invaded less than subserosa; ≥T3: tumor invaded into subserosa or more; N: lymph node metastasis; Ly: lymphatic invasion; V: venous invasion; INF: tumor infiltrative pattern; TG: total gastrectomy; DG: distal gastrectomy; PG: proximal gastrectomy.

Table III. *Clinicopathological and surgical factors of the CEACAM6-positive and CEACAM6-negative groups.*

	CEACAM6-positive (n=39)	*CEACAM6*-negative (n=19)	*p*-Value
Macroscopic type			
Localized	26	8	0.0755
Diffuse	13	11	
Histology			
Differentiated	18	11	0.4006
Undifferentiated	21	8	
Tumor size			
<60 mm	25	12	0.9440
≥60 mm	14	7	
T			
<T3	17	5	0.1964
≥T3	22	14	
N			
N0	11	6	0.7918
≥N1	28	13	
Ly			
Ly0	18	5	0.1408
Ly1	21	14	
V			
V0	16	3	0.9895
V1	23	16	
INF			
a, b	31	16	0.3619
c	8	3	
Type of gastrectomy			
TG	8	7	0.1898
others	31	12	
Intraoperative bleeding			
<150 ml	26	14	0.5846
≥150 ml	13	5	

T: Depth of tumor invasion; <T3: tumor invaded less than subserosa; ≥T3: tumor invaded into subserosa or more; N: lymph node metastasis; Ly: lymphatic invasion; V: venous invasion; INF: tumor infiltrative pattern; TG: total gastrectomy; DG: distal gastrectomy; PG: proximal gastrectomy.

and multivariate analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Using JMP software, version 16 (SAS Institute), a survival curve was created by the Kaplan–Meier method, a log-rank test was performed, and RFS was compared. *p*-Values less than 0.05 were considered statistically significant.

Results

Clinicopathological and surgical factors. There were four cases of Stage IV disease, and in each case, only CY1 was the Stage IV factor. There were nine recurrences, all of which were peritoneal recurrences (Table I). The relationship between *KRT20* mRNA expression and clinicopathological and surgical factors was investigated. There were 33 (56.9%) *KRT20*-positive cases, and there were significantly more cases of ≥pT3, ≥pN1, total gastrectomy, and intraoperative bleeding of 150 ml or more in the *KRT20*-positive group than in the KRT20-negative group (p=0.0496, p=0.0062, p=0.00302, and p=0.0272, respectively) (Table II). We also investigated the relationship between *CEACAM6* expression and clinicopathological and surgical factors. There were 39 (67.2%) patients with *CEACAM6* positivity, and no association between *CEACAM6* mRNA expression and clinicopathological or surgical factors was recognized (Table III).

Peritoneal recurrence. The relationship between peritoneal recurrence and clinicopathological factors, surgical factors, and gene expression was investigated (Table IV). In univariate analysis, INFc and intraoperative bleeding of 150 ml or more were significantly correlated with peritoneal recurrence (p=0.0472 and p=0.0225 respectively). A tumour diameter of

Table IV. *Clinicopathological factors and gene expression in the recurrence and non-recurrence groups.*

	Recurrence (n=9)	Non-recurrence (n=49)	Univariate *p*-Value	Odds ratio (95%CI)	Multivariate *p*-Value
Tumor size					
<60 mm	3	34	0.0561	1.024 (0.173-6.059)	0.9792
≥60 mm	6	15			
T					
<T3	0	22	0.0694	16.475 (0.61-445.26)	0.0958
≥T3	9	27			
N					
N0	0	17	0.1266		
≥N1	9	32			
Ly					
Ly0	1	22	0.106		
Ly1	8	27			
V					
V0	3	16	0.9682		
V1	6	33			
INF					
a, b	5	42	0.0472	1.624 (0.272-9.69)	0.5949
c	4	7			
Intraoperative bleeding					
<150 ml	3	37	0.0225	2.919 (0.556-15.318)	0.2054
≥150 ml	6	12			
KRT20					
Negative	2	23	0.1554		
Positive	7	26			
CEACAM5					
Negative	0	19	0.0997	24.753 (0.883-694.062)	0.0592
Positive	9	30			
KRT20^{+}/*CEACAM6*$^{+}$	7	19	0.0529	0.535 (0.062-4.64)	0.5704
KRT20^{+}/*CEACAM6*$^{-}$, *KRT20*$^{-}$/*CEACAM6*$^{+}$, *KRT20*$^{-}$/*CEACAM6*$^{-}$	2	30			

T: Depth of tumor invasion; <T3: tumor invaded less than subserosa; ≥T3: tumor invaded into subserosa or more; N: lymph node metastasis; Ly: lymphatic invasion; V: venous invasion; INF: tumor infiltrative pattern; *KRT20*$^{+}$/*CEACAM6*$^{+}$ both *KRT20*- and *CEACAM6*-positive (n=26); *KRT20*$^{+}$/*CEACAM6*$^{-}$: *KRT20*-positive and *CEACAM6*-negative (n=7); *KRT20*$^{-}$/*CEACAM6*$^{+}$: *KRT20*-negative and *CEACAM6*-positive (n=13); *KRT20*$^{-}$/*CEACAM6*$^{-}$: both *KRT20*- and *CEACAM6*-negative (n=12).

60 mm or more, ≥pT3, *CEACAM6* positivity, and both *KRT20* and *CEACAM6* positivity tended to correlate with peritoneal recurrence (p=0.0561, p=0.0694, p=0.0997, and p=0.0529, respectively). In multivariate analysis, the odds ratio (OR) of *CEACAM6* positivity was the highest and was considered to be a factor involved in recurrence [OR=25.743 (95%CI=0.883-694.062), p=0.0592] (Table IV).

Prognosis of peritoneal recurrence. No significant difference in the recurrence prognosis was found between the *KRT20*-positive and -negative groups (Figure 1A). The *CEACAM6*-positive group had a significantly worse prognosis for recurrence than the *CEACAM6*-negative group (p=0.0482) (Figure 1B). Twenty-six patients (44.8%) were positive for both *CEACAM*6 and *KRT20*, and their prognosis for recurrence was significantly worse than that of other patients (p=0.0458) (Figure 1C).

Table V shows nine cases of peritoneal recurrence. All cases were *CEACAM6*-positive, with four cases being positive before and after surgery, four cases being preoperatively negative and postoperatively positive, and only one case being preoperatively positive and postoperatively negative. Seven of the nine cases (77.8%) were *KRT20*-positive, three were positive before and after surgery, four were negative before and positive after surgery, and two were negative before and after surgery. There were three cases of CY1, all of which were *CEACAM6*-positive before and after surgery (Table V).

Patients who were *KRT20*-negative both preoperatively and postoperatively (25 cases, 43.1%) had a significantly better peritoneal recurrence prognosis than patients who were *KRT20*-positive both preoperatively and postoperatively (nine cases, 15.5%) (p=0.0312) (Figure 2A). Preoperative and postoperative *CEACAM6*-positive patients (12 patients, 20.7%) had a significantly poorer prognosis for recurrence than *CEACAM6*-

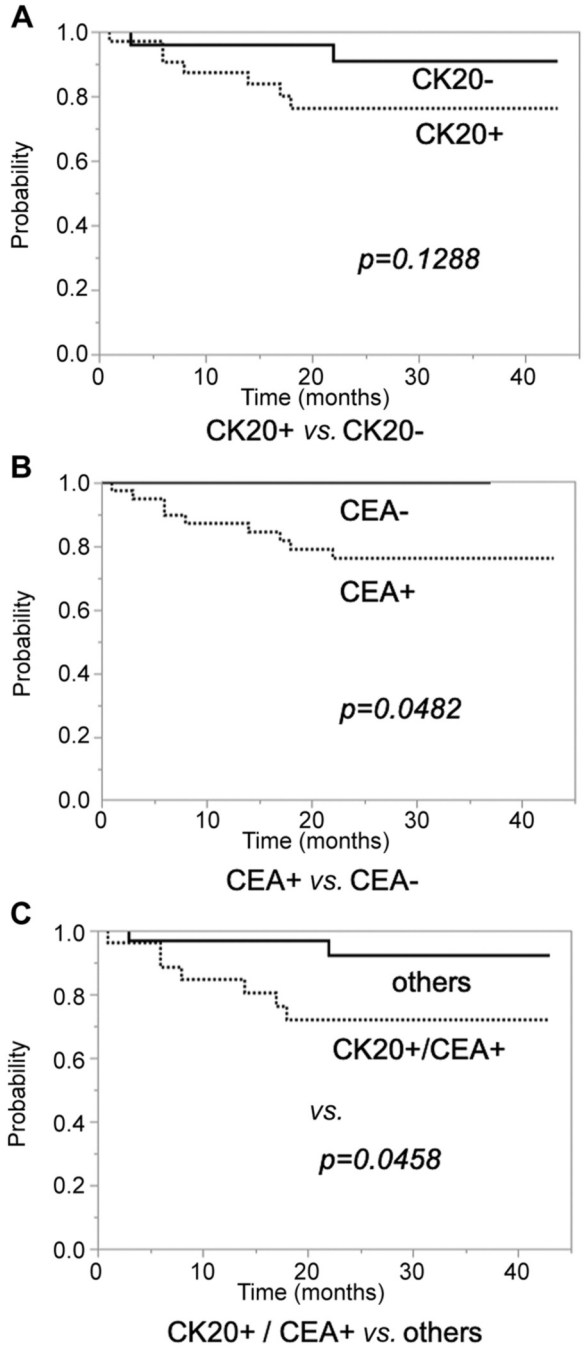

Figure 1. *Relationship of RFS with KRT20 and CEACAM6 mRNA expression. A) KRT20(−) (n=25) and KRT20(+) (n=33). There was no significant difference in the survival curves between KRT20(−) and KRT20(+). Three-year RFS rate: KRT20(−), 90.3%; KRT20(+), 75.3%. B) CEACAM6(−) (n=19) and CEACAM6(+) (n=39). Survival curves of CEACAM6(+) were significantly poorer than those of CEACAM6(−) (p=0.0482). Three-year RFS rate: CEACAM6(−), 100%; CEACAM6(+), 75.3%. c. KRT20(+)/CEA(+) (n=26), CK20(+)/CEA(−) (n=7), CK20(−)/CEA(+) (n=13), and CK20(−)/CEA(−) (n=12). Survival curves of the KRT20- and CEACAM6-positive group were significantly poorer than those of other groups (p=0.0458). Three-year RFS rate: CK20(+)/CEA(+), 71.5%; others, 91.5%. RFS: Recurrence-free survival.*

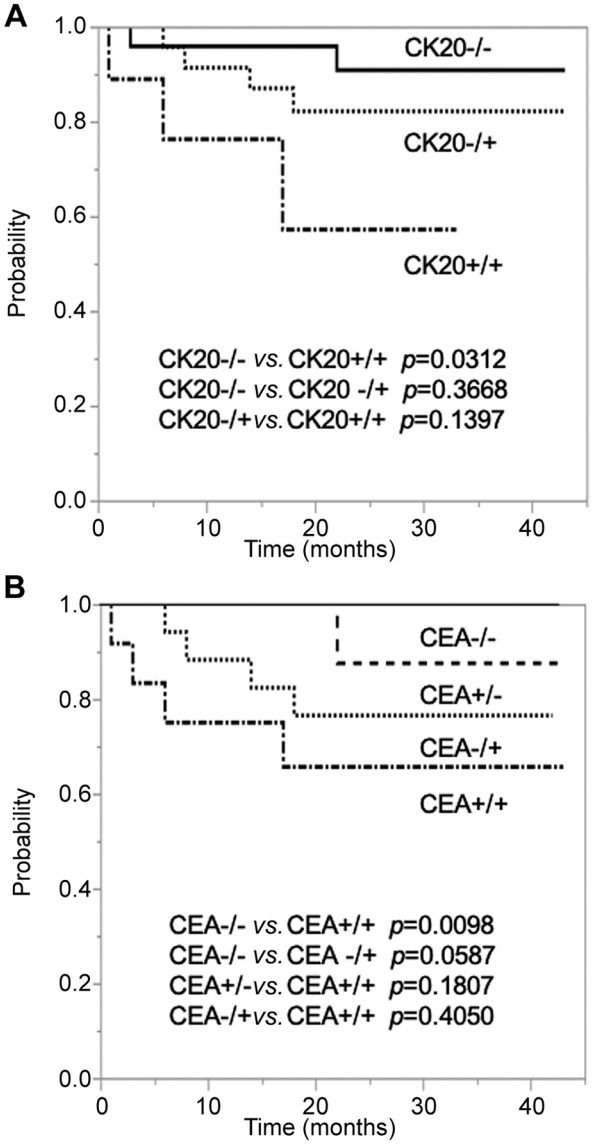

Figure 2. *Relationship of RFS with KRT20 and CEACAM6 mRNA expression pre- and post-surgery. A) KRT20+/+ (n=9), KRT20+/− (n=0), KRT20−/+ (n=24), and KRT20−/−(n=25). Survival curves of KRT20+/+ were significantly poorer than those of KRT20−/− (p=0.0312). Three-year RFS rate: KRT20−/−, 90.8%; KRT20−/+, 82.1%; KRT20+/+, 57.1%. B) CEACAM6+/+ (n=12), CEACAM6+/− (n=10), CEACAM6−/+ (n=17), and CEACAM6−/− (n=19). Survival curves of CEACAM6+/+ were significantly poorer than those of CEACAM6−/− (p=0.0098). Survival curves of CEACAM6-/+ tended to be poorer than those of CEA−/− (p=0.0587). Three-year RFS rate: CEACAM6−/−, 100%; CEACAM6+/−, 86.5%; CEACAM6−/+, 76.0%; CEACAM6+/+, 64.8%. RFS: Recurrence-free survival.*

negative patients (19 patients, 32.7%) both preoperatively and postoperatively (p=0.0098) (Figure 2B). In addition, patients who were negative for preoperative *CEACAM6* mRNA and positive for postoperative *CEACAM6* mRNA tended to have a

Table V. *Gene expression in patients with peritoneal recurrence (n=9).*

Age	Sex	Macro	Histology	Size	T	N	CY	Stage	Type	D	*KRT20* pre+	*KRT20* post+	*CEACAM6* pre+	*CEACAM6* post−
28	F	2	por2	50	4a	3a	1	4	TG	2	+	+	+	+
68	M	2	tub1	45	4a	1	0	3a	LDG	2	−	+	−	+
63	M	2	muc	120	4a	3b	0	3c	LPG	2	−	−	+	+
82	M	2	tub2	50	4a	3a	0	3b	LDG	2	−	+	−	+
60	F	4	por2	100	4a	3b	1	4	TG	2	+	+	+	+
59	M	4	por2	80	4a	3b	1	4	TG	2	+	+	+	+
85	F	2	por2	90	4a	3b	0	3c	LDG	2	−	−	+	−
80	M	3	tub2	70	3	3b	0	3c	LDG	2	−	+	−	+
88	F	3	por2	60	4a	3b	0	3c	DG	2	−	+	−	+

M: Male; F: female; por2: poorly differentiated adenocarcinoma (non-solid type); tub1: tubular adenocarcinoma (well differentiated type); muc: mucinous adenocarcinoma; tub2: tubular adenocarcinoma (moderately differentiated type); T: depth of tumor invasion; N: lymph node metastasis; CY: peritoneal lavage cytology; TG: total gastrectomy; LDG: laparoscopic distal gastrectomy; LPG: laparoscopic proximal gastrectomy; DG: distal gastrectomy; D: extent of lymph node dissection.

poorer prognosis for recurrence than those who were negative both before and after surgery (p=0.0587) (Figure 2B).

Discussion

Peritoneal recurrence is the most common form of postoperative gastric cancer recurrence, and ascites lavage cytopathology is widely used to detect it (1, 2). In recent years, advances in chemotherapy have led to the practice of conversion surgery for stage IV gastric cancer. Suzuki *et al*. reported that conversion surgery improved the prognosis of patients with stage IV gastric cancer in whom chemotherapy can achieve pathological disappearance of metastatic lesions including peritoneal metastases (9). However, we often experience cases of peritoneal recurrence after curative resection with CY0. Some reports have investigated the relationship between the expression of *KRT20* and *CEACAM6* mRNA and peritoneal recurrence as a more sensitive index (6, 7), but most of them are only studies of preoperative intraperitoneal lavage fluid. We also used postoperative intraperitoneal lavage fluid, taking tissue damage resulting from surgery into account, and the possibility that cancer cell dissemination due to intraoperative manipulation may be involved in peritoneal recurrence. CK20 is present in the gastrointestinal epithelium and Merkel cells of skin (10) and is not originally present in the abdominal cavity, and our study suggested a relationship between the expression of *KRT20* mRNA and the progression of gastric cancer and the amount of intraoperative bleeding. In addition, all cases that were *KRT20*-positive before surgery were also *KRT20*-positive after surgery, and the prognosis for recurrence was significantly worse than in cases that were *KRT20*-negative both before and after surgery. These results suggest that intraoperative dissemination from blood or tissue in advanced gastric cancer led to a poor prognosis for recurrence of peritoneal dissemination. Intraoperative bleeding is an independent risk factor for peritoneal dissemination recurrence and prognosis (11), and more advanced tumours have been reported to be associated with more bleeding (12). However, studies have reported that CK20 positivity in ascites can be a predictor of micro lymph node metastasis and recurrence of peritoneal metastasis (13, 14). Another study has also reported that it can be a prognostic factor for recurrence when combined with CEA (15). In our study, the *KRT20*-positive group had many patients with deeper T3, positive lymph node metastasis, intraoperative bleeding of 150 ml or more, and total gastrectomy, and careful surgical skills are required in highly advanced cases.

No association with pathological and surgical factors was recognized for *CEACAM6*; however, all nine patients with postoperative peritoneal recurrence were positive, suggesting that it may be an independent factor involved in recurrence. Oue *et al*. also demonstrated overexpression of *CEACAM6* in tumour cells in gastric cancer but also found no association between the expression levels of *CEACAM6* and clinicopathological features (16). In addition, the prognosis of peritoneal dissemination recurrence tended to be worse in all cases of *CEACAM6*-positive conversion after surgery than in negative patients. It was considered that the cause was tissue damage during surgery and dissemination of tumour cells due to lumen release. However, not all disseminated cells led to recurrence because besides viable cancer cells, dead cells, epithelial cells, and mesothelial cells may have been detected. Takebayashi *et al*. cultured intra-abdominal lavage fluid and reported that 24 (68.6%) of 35 cases that were negative before surgery and turned positive after surgery had the ability to proliferate in the cell medium (17). The involvement of CEA in the infiltration and metastasis of gastric cancer may also need to be considered. *CEACAM6* is a member of the CEA family, and one study has reported that it is expressed in cell

membranes, overexpressed in the tumour tissues of gastric cancer, and closely associated with angiogenesis and metastasis (10). Over-expression of *CEACAM6* has been reported to positively correlate with epithelial–mesenchymal transition markers such as N-cadherin and vimentin, and negatively correlate with E-cadherin (18). It was suggested that high *CEACAM6* levels in intraperitoneal lavage fluid may lead to peritoneal recurrence. Previous reports have reported that CEA positivity in preoperative ascites is an independent factor of peritoneal recurrence (6, 7), and CEA measurement in postoperative ascites may be an important predictor of peritoneal dissemination in the future. In conclusion, measuring *KRT20* and *CEACAM6* mRNA in intraperitoneal lavage fluid before and after surgery is considered to be useful as a method for predicting peritoneal recurrence.

This study has several limitations. First, it was a single-centre study, and the number of samples was small. Second, the follow up period was short, with an average follow-up period of 26 months (range=4-43 months), and because only approximately half of the recommended postoperative surveillance for gastric cancer was followed, recurrence in late-stage disease was not investigated. In addition, RT-PCR takes a long time to perform, and thus it is not suitable for rapid intraoperative diagnosis, plus it is difficult to widely perform in any hospital from the viewpoint of equipment.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

The conception or design of the work, or acquisition, analysis or interpretation of data: Hideaki Kaku, Keishiro Aoyagi, Tomoya Sudo, Yuya Tanaka, Taizan Minami, Taro Isobe, Junya Kizaki, Yuki Umetani. Drafting the work or revising it critically for important intellectual content: Hideaki Kaku, Keishiro Aoyagi. Final approval of the version to be published: Keishiro Aoyagi, Naotaka Murakami, Fumihiko Fujita, Yoshito Akagi. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work: Yoshito Akagi.

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