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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Viable Cancer Cells in the Remnant Stomach are a Potential Source of Peritoneal Metastasis after Curative Distal Gastrectomy for Gastric Cancer

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ABSTRACT

Background. The mechanisms underlying peritoneal metastasis (PM) after curative gastrectomy for gastric cancer (GC) are not well elucidated. This study assessed whether viable cancer cells, including cancer stemlike cells (CSCs), were present in the remnant stomach immediately before gastrointestinal (GI) tract reconstruction because these could be a source of PM after gastrectomy.

Methods. Saline fluid used for remnant stomach lumen irrigation before GI reconstruction was prospectively collected from 142 consecutive patients undergoing distal gastrectomy for GC and cytologically examined. Proliferative activity (Ki67 staining) and stemness (expression of the CSC surface markers CD44s or CD44v6) were evaluated in detected cancer cells.

Results. Viable cancer cells were detected in 33 (23.2 %) of the 142 remnant stomachs. These cells formed clusters and stained positively for Ki67, indicating proliferation. Cancer cells in remnant stomachs and surface cancer cells in primary GCs from 10 (30.3 %) of these 33 cases also stained positively for CD44s or CD44v6. In a multiple logistic regression analysis, advanced cancer (odds ratio [OR], 4.65; 95 % confidence interval [CI], 1.32–16.4; $P = 0.017$), tumor size of 40 mm or larger (OR, 3.78; 95 % CI, 1.12–12.8; $P = 0.033$), and histologic differentiation (OR, 3.10; 95 % CI, 1.30–7.40; $P = 0.011$) were associated independently with the presence of cancer cells in the remnant stomach.

Conclusion. Viable, proliferative, and clustered cancer cells, including CSCs, were found in remnant gastric lumens immediately before GI reconstruction, indicating a possible cellular source of PM after curative gastrectomy for GC. Dissemination of gastric contents into the peritoneal cavity should be avoided during GI reconstruction.

Curative gastrectomy with D2 lymph node dissection (curative D2 gastrectomy) and subsequent adjuvant chemotherapy have been shown to prolong survival of gastric cancer (GC) patients.[1,2] However, among GC patients who undergo potentially curative gastric surgery, relapse most frequently occurs in the form of peritoneal metastasis (PM),[3] with reported median survival durations of 3–10 months.[4–6] Approximately 50 % of patients undergoing potentially curative surgery for serosa-invasion-positive GCs without visible evidence of metastases at the time of diagnosis will experience the development PMs, and most of these patients with PM will die within 2 years.[7,8]

Peritoneal recurrence is thought to occur after the invasion of exfoliated cancer cells from the primary tumor into the serosal layer of the stomach or from extranodal extensions of lymphatic metastases. However, peritoneal recurrences after curative surgery may develop as non-serosal invasive or nonlymphatic metastatic cancers.

Recently, we showed that cancer cells that had disseminated into the peritoneal cavity during curative D2

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gastrectomy for GC were viable, proliferative, and tumorigenic and could give rise to PMs.[9] These findings indicate that surgery for GC can induce peritoneal recurrence. In most cases, cancer cell dissemination during surgery could be explained by serosal invasion or lymphatic involvement. However, 1 (4.16 %) of the 24 patients whose perioperative peritoneal washing samples contained viable cancer cells exhibited nonserosal invasive disease [pT1(SM)] with no lymph node involvement (pN0) and no vascular invasion (ly0, v0), and thus lacked potential channels for cancer cell dissemination. In addition, peritoneal recurrences, although rare, have even been reported in pT1 and pN0 cases[10–12] and could not be explained by cancer infiltration through the gastric wall or lymphatic involvement. Accordingly, other causes of peritoneal recurrence after potentially curative surgery should be investigated.

Dissemination of free cancer cells from the gastric lumen into the peritoneal cavity during gastrointestinal (GI) tract reconstruction represents a potential cellular source of PM and is thus of interest. A previous report described free cancer cells in the gastric lumen of GC patients that were detected cytologically in gastric fluid aspirated through a nasogastric tube before gastric resection.[13] Although the authors of that report suggested the possibility of cancer cell dissemination, they demonstrated the presence of both the primary cancer and free cancer cells in the stomach before resection. Therefore, it remains unclear whether free cancer cells reside in the remnant stomach after resection but immediately before GI reconstruction. In addition, more detailed analysis of the free cancer cells in the gastric lumen is needed to establish whether they give rise to PM.

In this study, we examined the presence of viable cancer cells or cancer stem-like cells (CSCs) in the remnant stomach lumen immediately before GI reconstruction. Our findings suggest that cancer cells may disseminate into the peritoneal cavity when the remnant stomach wall is opened and could be a source of peritoneal recurrence after potentially curative gastrectomy for GC.

METHODS

Patients

Patients with GC histologically confirmed via endoscopic biopsy who underwent distal gastrectomy at Shiga University of Medical Science Hospital between May 2010 and December 2014 were enrolled in this study. Informed consent was obtained before surgery. Tumor stage and pathologic classification were described according to the Japanese Classification of Gastric Carcinoma.[14] The primary tumor size was defined as the maximum diameter. If more than two tumors were found, then tumor size was calculated as the sum of the maximum diameters of each tumor. Continuous variables (age and tumor size) were divided into two subgroups based on the median values of all the patients.

Surgical Procedure

Radical distal gastrectomy was performed for cancers located in the lower third and distal part of the middle third of the stomach. Gastrectomy and lymph node dissection were performed according to the recommendations in the Japanese Gastric Cancer Treatment Guidelines 2010 (version 3).[15] Patients with clinical T1 (M or SM) N0M0 disease underwent laparoscopically assisted distal gastrectomy (LADG). Other patients underwent open distal gastrectomy (ODG). In both procedures, GI reconstruction was performed extracorporeally through the medial abdominal wall incision using the Billroth I reconstruction technique. Roux-en-Y reconstruction was selected for cases involving a small remnant stomach, short duodenal margin, reflux esophagitis, or hiatal hernia. Per our standard practice when opening a GI lumen, we placed a thick layer of gauze under the opened GI tract to prevent the dissemination of GI contents, including bacteria, into the abdominal cavity.

Sampling Methods

After stomach resection and immediately before GI tract anastomosis, the remnant stomach wall was cut to allow anastomosis stapler insertion, and then 50 mL of saline was introduced into the remnant gastric lumen through a soft catheter placed into the incision. The maximum possible amount of this fluid was aspirated after repeated irrigation and then cytologically analyzed.

Pathologic Examination

The saline fluid used for remnant stomach irrigation was collected and examined cytologically using Papanicolaou staining. Detected cancer cells were subjected to Ki67 staining to determine proliferative activity, and their stemness was evaluated via staining for the CD44 standard variant (CD44s) and CD44 variant exon 6 (CD44v6) proteins, which are known GC stem cell surface markers.[16–18] Due to the limited number of samples with positive cancer cells, we could conduct staining for only one of the CD44 variant surface markers for each patient. Thus, we evaluated CD44s expression in the first five samples with positive cancer cells and CD44v6 expression in subsequent cases. Immunohistochemical and immunocytochemical staining were performed using an autostainer (Benchmark

XT System; Ventana Medical System, Tucson, AZ, USA) with the following reagents: primary antibodies against Ki67 (dilution 1:50; Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), CD44s (H-CAM, 1:200 dilution; Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), and CD44v6 (VFF-18, 1:500 dilution; Abcam, Cambridge, UK).

Cell Culture

We evaluated the proliferative activity of cancer cells obtained from cell culture. Cells obtained from the saline fluid used for remnant stomach irrigation were maintained in RPMI-1640 medium (Nihon Seiyaku, Tokyo, Japan) supplemented with 15 % heat-inactivated fetal bovine serum (Gibco, Uxbridge, UK), 2 mmol of glutamine, and penicillin (50 IU/mL) and streptomycin (50 μg/mL) in a CO_2 incubator at 37 °C. If cell samples of individual cases were contaminated or if the cells did not proliferate within 4 weeks, the evaluation of the proliferative activity was discontinued.

Statistical Analysis

Uni- and multivariate logistic regression models were used to evaluate the associations of clinicopathologic variables with viable cancer cell detection in the remnant stomach and to determine odds ratios (ORs) and 95 % confidence intervals (CIs). Independent effects of significant or almost significant ($P < 0.1$) variables in the univariate analysis were subsequently assessed using multiple logistic regression analysis. A P value lower than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS, version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 149 liquid samples of gastric contents were prospectively collected from the remnant stomachs of consecutive patients undergoing distal gastrectomy for GC. Of these samples, 142 were analyzed. The median age of the patients was 68 years (range, 37–93 years), and the median primary GC tumor size was 40 mm (range, 5–130 mm) (Table 1).

For a negative control, samples were obtained after endoscopic submucosal dissection from seven patients who had undergone pathologically complete removal of the primary tumor but were found to have lymphatic or vessel invasion and who subsequently underwent gastrectomy and lymph node dissection for the treatment of tumor vascular invasion.

TABLE 1 Clinicopathologic characteristics of patients undergoing distal gastrectomy

Variables	Cancer cells in the remnant stomach ($n = 142$) n (%)	
	Negative, $n = 109$ (76.8 %)	Positive, $n = 33$ (23.2 %)
Gender		
Male	72 (66.1 %)	24 (72.7 %)
Female	37 (33.9 %)	9 (27.3 %)
Age (years)		
Mean (range)	66.2 ± 12.4 (37–93)	69.1 ± 9.63 (48–85)
<68	57 (52.3 %)	13 (39.4 %)
≥68	52 (47.7 %)	20 (60.6 %)
Location[a]		
Middle	54 (49.5 %)	11 (33.3 %)
Lower	55 (50.5 %)	22 (66.7 %)
Depth of tumor invasion[b]		
Early GC		
pT1(M)	36 (33.0 %)	1 (3.00 %)
pT1(SM)	25 (22.9 %)	6 (18.2 %)
Advanced GC		
pT2(MP)	13 (11.9 %)	5 (15.2 %)
pT2(SS)	14 (12.8 %)	11 (33.3 %)
pT3(SE)	20 (18.3 %)	10 (30.3 %)
pT4(SI)	1 (0.90 %)	0 (0.00 %)
Tumor size (mm)		
Mean (range)	42.8 ± 23.1 (5–130)	55.9 ± 20.7 (25–110)
<40	55 (50.5 %)	6 (18.2 %)
≥40	54 (49.5 %)	27 (81.8 %)
Histologic type		
Undifferentiated	67 (61.5 %)	12 (36.4 %)
Differentiated	42 (38.5 %)	21 (63.6 %)
Distal gastrectomy		
ODG	71 (65.1 %)	27 (81.8 %)
LADG	38 (34.9 %)	6 (18.2 %)
GI reconstruction		
Billroth-I	78 (71.6 %)	22 (66.7 %)
Roux-en-Y	31 (28.4 %)	11 (33.3 %)

GC gastric cancer, *ODG* open distal gastrectomy, *LADG* laparoscopically assisted distal gastrectomy

[a] The terms middle or lower indicate cancers located in the middle or lower third of the stomach, respectively

[b] Pathologic tumor stages: *pT1(M)* tumor invades the mucosa, *pT1(SM)* tumor invades the submucosa, *pT2(MP)* tumor invades the muscularis propria, *pT2(SS)* tumor invades the subserosa, *pT3(SE)* tumor invasion is contiguous with or extends beyond the serosa, *T4(SI)* tumor invades adjacent structures

Viable cancer cells were detected in 33 (23.2 %) of the 142 samples, many of which had formed clusters (Fig. 1a, b). Of these 33 samples, 28 (84.8 %) contained cells that

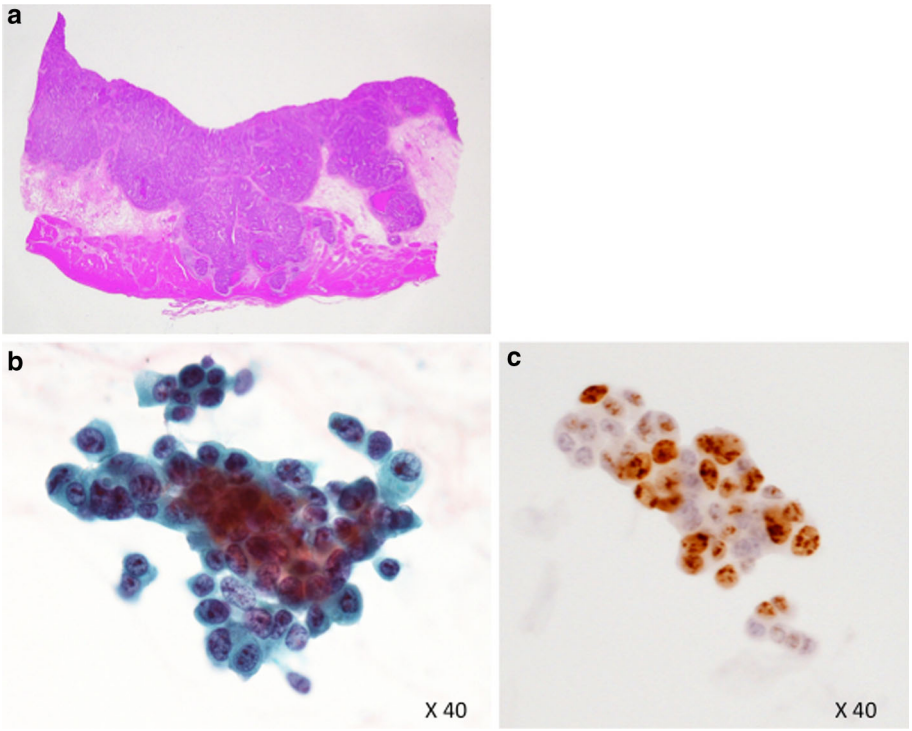

FIG. 1 **a** Moderately differentiated tubular adenocarcinoma (tub2) invasion of the proper muscle layer: pT2(MP). **b** Clustered cancer cells with cellular atypia found in the remnant stomach after distal gastrectomy for the case shown in (**a**) (Papanicolaou stain). **c** The cancer cells shown in (**b**) stained positively for Ki67, indicating proliferative ability

stained positively for Ki67, indicating proliferative activity (Fig. 1c). In addition, when the cancer cells of these 33 samples were cultured in vitro, 15 (45.4 %) were contaminated, but 9 (50 %) of 18 samples that did not show the signs of contamination demonstrated adhesion and spread of cancer cells.

Free cancer cells detected in remnant stomach samples were subjected to staining for the GC stem cell markers CD44s or CD44v6 to determine the presence of CSCs. Of 33 cases with free cancer cells, 10 (30.3 %) exhibited CD44s (2 [40 %] of 5 cases) or CD44v6 positivity (8 [28.6 %] of 28 cases) (Fig. 2b, d). Surface cells from the primary GC tumors in these 10 cases also exhibited positive CD44s or CD44v6 staining (Fig. 2a, c).

A univariate analysis of clinicopathologic variables showed that advanced cancer [≥pT2(MP); $P = 0.001$], tumor size of 40 mm or larger($P = 0.002$), and differentiated histologic type ($P = 0.013$) were significantly associated with the presence of free cancer cells in the remnant stomach lumen (Table 2). A multiple logistic regression analysis also identified advanced cancer (OR, 4.65; 95 % CI, 1.32–16.4; $P = 0.017$), tumor size of 40 mm or larger (OR, 3.78; 95 % CI, 1.12–12.8; $P = 0.033$), and differentiated histologic type (OR, 3.10; 95 % CI, 1.30–7.40; $P = 0.017$) as independent risk factors for this occurrence.

Free cancer cells were detected even in the lumen of the remnant stomachs of 6 (19.4 %) of 31 patients with early GC [pT1(SM)] (Table 1). Among these patients, 5 (38.5 %) of 14 patients who underwent LADG and 1 (5.9 %) of 17 patients who underwent ODG had free cancer cells in the remnant stomach lumen at the time of GI reconstruction. Relative to ODG, LADG was significantly associated with the presence of cancer cells in the remnant gastric lumen of patients with pT1(SM) early GC (OR, 10.6; 95 % CI, 1.06–106; $P = 0.045$). Additionally, 1 (2.7 %) of 37 patients with pT1(M) GC, who underwent LADG, was found to harbor free cancer cells in the remnant stomach (Table 1).

DISCUSSION

This study demonstrated the presence of clustered, proliferating cancer cells, including CSCs, in the lumen of the remnant stomach after distal stomach resection for GC. Accordingly, we suggest caution regarding the possible dissemination of cancer cells from the remnant stomach into the peritoneal cavity during this procedure because they could seed PM.

Approximately half of patients with serosa-invasive GC but no detected cancer cells in the peritoneal cavity (CY0) experience peritoneal recurrences after curative gastric surgery, suggesting that unidentified cancer cells remained elsewhere in the peritoneal cavity. However, patients with non-serosa-invasive cancers also experience peritoneal recurrences after curative surgery. Furthermore, patients with early GC also may experience peritoneal recurrences, although this is rare. According to previous reports, 0.4 %

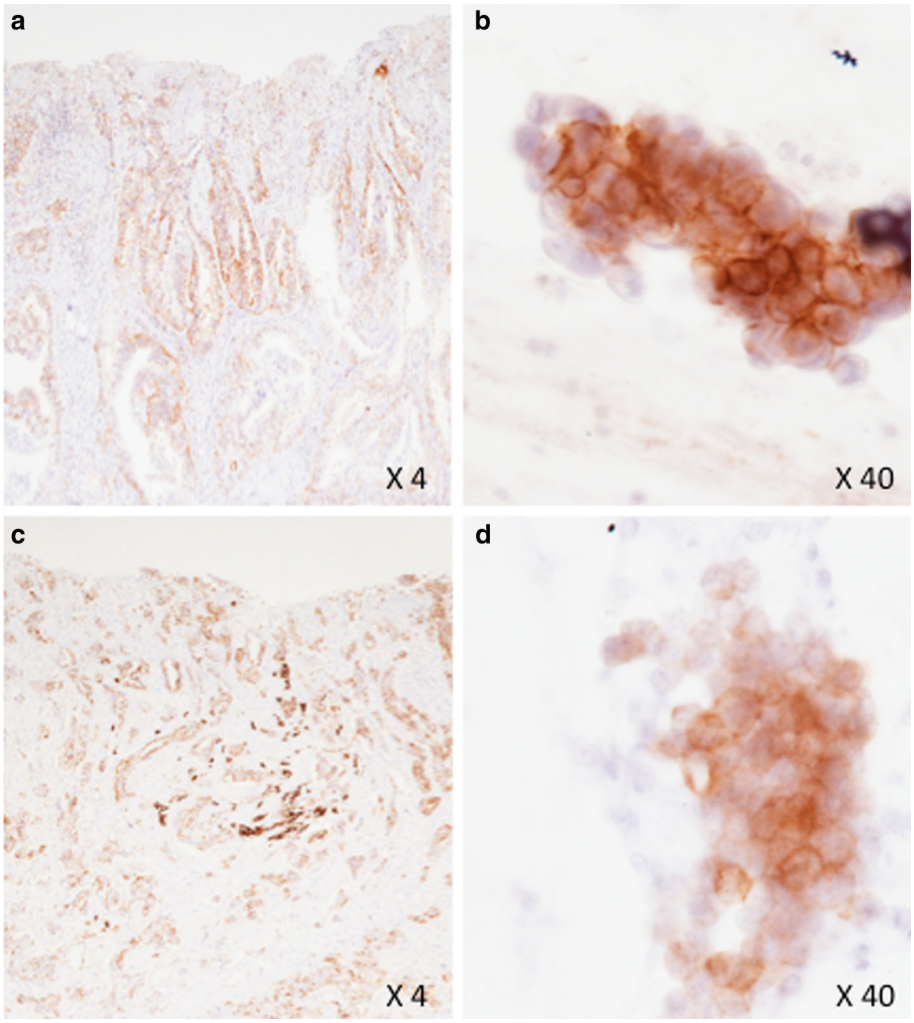

FIG. 2 Cancer cells from the surface of a primary gastric cancer lesion stained positively for CD44 standard variant (CD44s) (**a**) or CD44 variant exon 6 (CD44v6) (**c**), which are surface markers for gastric cancer stem cells. Cancer cells found in the remnant stomach after primary lesion resection **a** or **c** also stained positively for CD44s (**b**) or CD44v6 (**d**), respectively

to 2.3 % of cases involving pathologically submucosal invasive tumors [pT1(SM)] and 4.4 % to 9.3 % of cases involving proper muscular invasive tumors [pT2(MP)] had peritoneal recurrences after curative gastrectomy for GC.[3,7,10–12,19, 20] Moreover, 0.28 % to 0.31 % of cases involving pT1(SM) tumors without lymph node metastases (pN0) had peritoneal recurrences.[10–12] Marutsuka et al.[19] further reported that 0.51 % of GC patients with no pathologic signs of lymph node metastasis or lymphatic invasion, despite the presence of carcinoembryonic antigen and keratin 20 mRNA in lavage fluid collected immediately after lymph node dissection, experienced PMs after potentially curative surgery. These findings suggest the introduction of an unknown source of PM during surgery for GC.

We then focused on the potential flow of gastric juices from the remnant stomach into the peritoneal cavity when the gastric wall was opened during GI reconstruction. Among the examined cases, the gastric juices collected from the remnant stomachs of 23.2 % of the patients contained viable cancer cells. Interestingly, 19.4 % of the patients with submucosa-invasive early GC harbored viable cancer cells in the remnant stomach.

The cancer cells detected in the remnant stomachs of the patients with GC were found to cluster, a cytologic feature of peritoneal washing fluid that has been associated with poorer survival and a high incidence of PM after gastrectomy for GC.[21] Clustered cancer cells can adhere to the peritoneum and thus proliferate more easily than scattered cells. In addition, surgical trauma and the concomitant wound-healing process impair tissue integrity and promote the production of inflammatory mediators and angiogenic factors, leading to tumor cell adhesion, immune suppression, and tumor growth.[22] These postsurgical changes in the tumor microenvironment can increase the risk that disseminated tumor cells will develop into PMs. Moreover, 30.3 % of cases in which free cancer cells were detected in the remnant gastric contents also harbored CSCs with surface expression of CD44s or CD44v6, and these cells may play significant roles in tumorigenesis, metastasis, and recurrence.[23,24] Reports have shown CD44 to be a CSC surface marker in several tumor types, including

TABLE 2 Association between clinicopathologic factors and cancer cells in the remnant stomach after distal gastrectomy

Variables	Univariate			Multivariate		
	OR	95 % CI	*P* value	OR	95 % CI	*P* value
Gender						
Male (referent)	1.00					
Female	0.73	0.31–1.73	0.474			
Age (years)						
<68 (referent)	1.00					
≥68	1.69	0.76–3.73	0.196			
Location[a]						
Middle (referent)	1.00					
Lower	1.96	0.87–4.44	0.105			
Early or advanced GC						
Early GC (referent)	1.00			1.00		
Advanced GC	4.72	1.89–11.8	0.001[b]	4.65	1.32–16.4	0.017[b]
Tumor size (mm)						
<40 (referent)	1.00			1.00		
≥40	4.58	1.75–12.0	0.002[b]	3.78	1.12–12.8	0.033[b]
Histologic type						
Undifferentiated (referent)	1.00			1.00		
Differentiated	2.79	1.25–6.26	0.013[b]	3.10	1.30–7.40	0.011[b]
Distal gastrectomy						
ODG (referent)	1.00			1.00		
LADG	0.42	0.16–1.09	0.075	2.85	0.64–12.7	0.170
Reconstruction						
Billroth-I (referent)	1.00					
Roux-en-Y	1.26	0.55–2.90	0.590			

OR odds ratio, *CI* confidence interval, *GC* gastric cancer, *ODG* open distal gastrectomy, *LADG* laparoscopy-assisted distal gastrectomy

[a] The terms middle or lower indicate cancers located in the middle or lower third of the stomach, respectively

[b] Statistically significant

GC,[18,25–27] and CD44 variant isoforms (CD44v) have recently been reported to affect GC tumor initiation and cancer cell maintenance[28] and may play a role in the protection of CSCs from high levels of reactive oxygen species in the tumor microenvironment.[16] Therefore, the leakage of gastric juices containing CSCs within cancer cell clusters could potentially induce tumors such as PMs in an inflammatory tumor microenvironment after GC surgery.

Suture materials used for anastomosis, especially staples, can allow the engraftment of exfoliated malignant cells[29,30] and hence local recurrences along the suture line. An intraoperative rectal washout with cytocidal agents has been recommended before anastomosis during rectal cancer surgery as a preventive measure[31,32] because implantation of these cells is recognized as a possible mechanism of local recurrence at the site of colorectal anastomosis. However, unlike rectal cancer surgery, anastomotic recurrence rarely occurs after colonic cancer surgery and GC surgery. In rectal cancer, the stapler introduced transanally through a narrow rectal lumen collects exfoliated cancer cells at the anastomosis site,[31] which are more frequently found close to the primary tumor.[32] Many collected cancer cells could thus be implanted into the anastomotic wall during stapling, leading to local recurrence at the site of colorectal anastomosis.[31] Bacteria within the rectum and chronic inflammation might promote cancer cell growth.

In contrast, for GC surgery, a stapler is introduced through a wide gastric lumen, resulting in fewer cancer cells at the anastomosis site than in rectal surgery. Furthermore, bacteria in the gastric lumen and exposure to digestive juice might inhibit cancer cell growth, and thus local recurrence at the GI anastomosis site may be rare. However, spillage of exfoliated cancer cells into the abdominal cavity and a damaged peritoneum during surgery involving inflamed tissue may enhance their engraftment in the peritoneum and hence promote tumor growth.

In this study, advanced GC, a large tumor, and a histologically differentiated tumor type were independent risk factors for the dissemination of free cancer cells in the remnant stomach. Tumors with these factors might be subject to intraoperative breakage as a result of external surgical forces acting on the stomach wall because differentiated tumors are more fragile, containing relatively more tumor cells and less fibrous tissue than undifferentiated tumors. Consequently, the surface of advanced tumors with necrotic or ulcerative tissues is easily broken, and large tumors are more susceptible to external forces.

A subclass analysis of cases involving submucosal invasive tumors [pT1(SM)] showed a significant association of LADG (relative to ODG) with the presence of free cancer cells in the remnant stomach after gastrectomy. In addition, among cases involving mucosal invasive tumors [pT1(M)], 1 (3.7 %) of 27 cases treated with LADG harbored free cancer cells in the remnant stomach, whereas no similar cases treated with ODG harbored free cancer cells. This difference between surgical procedures with respect to the presence of free cancer cells suggests that surgery can release cancer cells from the primary tumor and facilitate their spread throughout the gastric lumen. A laparoscopic technique for distal gastrectomy may increase mechanical contact by raising, holding, or tugging the stomach to obtain an effective visual field and to secure a working space in a restricted surgical site, which could detach more cancer cells and thus allow them to enter the gastric lumen. Further investigation is needed to clarify the influence of laparoscopic procedures on cancer cell spillage in the remnant stomach, particularly among cases of advanced, invasive GC.

During extracorporeal GI reconstruction, contamination from GI bacteria has conventionally been prevented by placement of thick gauze or a similar material under the reconstructed organ, a process that might have unintentionally prevented cancer cells in the gastric lumen from spilling into the abdominal cavity. The findings of the current study demonstrate that surgeons should carefully manage gastric contents not only to preclude bacterial contamination, but also to avoid spillage of free cancer cells. It should be noted that intracorporeal anastomosis via total laparoscopic surgery for GC is a potentially defenseless procedure with regard to the spillage of gastric contents from the remnant gastric lumen.

This study had a number of limitations. First, the tumorigenic potential and in vivo PM-forming capacities of viable and proliferating cancer cells in the remnant stomach were not confirmed. Cancer cells from the remnant stomach were found to be contaminated in 45 % of cases by gastric contents during culture to expand cancer cells, and these cancer cells cannot be easily administered intraperitoneally to immunodeficient animals, such as Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse, which are unable to tolerate infection.

Second, the clinical relevance of the cancer cells in this study has not been proved in terms of patient outcomes. All the patients in this study underwent careful extracorporeal GI anastomosis with sufficient suction, and a thick gauze was placed under the opened GI tract to avoid gastric content dissemination into the peritoneal cavity. The association between cancer cells in the remnant stomach and patient outcomes could not be clarified in this study.

In conclusion, a population of viable cancer cells that includes CSCs, a potential source of PM, is present in the gastric contents of the remnant stomach during distal gastrectomy for GC. Surgeons should carefully control gastric fluids to avoid spillage into the peritoneal cavity during GI reconstruction, especially in cases involving advanced, large, or histologically differentiated GC, and during intracorporeal anastomosis.

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