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# Surgery-Induced Peritoneal Cancer Cells in Patients Who Have Undergone Curative Gastrectomy for Gastric Cancer

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## ABSTRACT

**Background.** Some patients who undergo curative gastrectomy with lymph node dissection (LND) for gastric cancer (GC) show subsequent peritoneal metastasis. The source of these metastatic cells remains unclear.

**Methods.** Curative gastrectomy with LND was performed in 102 patients with GC. Peritoneal washing was collected before and after gastrectomy. Cytology, reverse transcription-polymerase chain reaction, and cell culture were used to determine the presence of cancer cells. The proliferative potential of tumor cells was evaluated using Ki-67 staining. Tumorigenic capacity was assessed by cell injection into the peritoneal cavity of NOD/ShiJic-scid mice. Peritoneal recurrence-free survival (RFS) and peritoneal recurrence rate (RR) were examined to determine the clinical relevance of detected cancer cells.

**Results.** Of 102 peritoneal washing samples obtained before gastrectomy, 57 showed no CEA or CK20 mRNA amplification. After gastrectomy, CEA or CK20 mRNA was detected in 35 of these 57 samples, and viable cancer cells were identified in 24. The viable cancer cells in all 24 cases showed Ki-67 positivity, indicating proliferative activity. Cultured viable cancer cells generated peritoneal nodules after spilling over the peritoneal cavity in NOD/ShiJic-scid mice in 4 cases. The peritoneal RFS of patients with CEA or CK20 mRNA amplification after gastrectomy was significantly poorer than that of patients with negative amplification ($p < .05$). The 24 patients with viable cancer cells in the peritoneal cavity after gastrectomy showed higher peritoneal RR than those without them ($p = .033$).

**Conclusions.** Viable tumorigenic cancer cells spilled into the peritoneal cavity during surgery, indicating that surgery induces peritoneal metastasis.

In gastric cancer (GC) patients, curative gastrectomy with lymph node dissection (LND) prolongs survival, and adjuvant chemotherapy after curative surgery improves outcomes.[1,2] Remarkably, recurrence in GC patients occurs most frequently in the form of peritoneal metastasis, despite treatment with curative gastric surgery.[3,4] The median survival time of such patients is 3–6 months.[5] Moreover, ~50 % of patients with serosal invasion-positive GC have been found to develop peritoneal recurrence and die during the first 2 years despite treatment with curative surgery.[6–8] The mechanism underlying peritoneal recurrence following curative surgery remains unclear. Peritoneal metastasis is considered to be caused by free cancer cells exfoliated from tumors invading the serosal layer. However, patients without serosal invasion have also been reported to have died of peritoneal recurrence.[9] Additionally, some patients with early GC developed peritoneal recurrence after curative surgery, even though free cancer cells were not detected in the cytological diagnoses of these cases.[9]

Reverse transcription polymerase chain reaction (RT-PCR) is used for screening the presence of small numbers of tumor cells cytologically undetectable in circulating blood and peritoneal washings (PW).[6,8,10–14] Patients with positive PCR results and negative cytology findings at laparotomy have a greater tendency to develop peritoneal metastasis than those with negative PCR and cytology results at laparotomy, suggesting a small number of free



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cancer cells undetectable by cytology are present in the peritoneal cavity.[6,9,11] In GC patients, the negative results of PCR analysis of peritoneal lavage fluid at laparotomy have been reported to become positive after gastrectomy, suggesting the spilling over of a small amount of free cancer cells during surgery.[11,15] However, it remains unknown whether these spilled free cancer cells are viable.

Here, we investigated the mechanism of surgery-induced peritoneal metastasis through examining the viability, proliferative activity, and in vivo tumorigenicity of free cancer cells spilled during surgery.

## MATERIALS AND METHODS

### *Patients and PW*

We investigated the PW from 102 GC patients who underwent curative surgery with LND between November 2009 and September 2012 at the Department of Gastrointestinal Surgery, Shiga University of Medical Science Hospital, Japan, but who did not show the presence of cancer cells by cytological examination (CY0) at laparotomy. Ultrasonically activated shears were used for LND and vessel sealing. Informed consent was obtained from all patients.

The PW of 102 GC patients (59 men, 43 women; mean age, 67.9 years; range, 36–88 years) were obtained before (PW-before) and after gastrectomy (PW-after). In brief, 100-mL aliquots of saline were introduced into the peritoneal cavity both at the beginning of the operation and immediately after it and were collected soon after gentle stirring. Afterward, 50 mL of the PW was analyzed cytologically, while another 50 mL was used for cell culture and cDNA extraction. The pellet obtained after centrifugation (at 3,500 rpm at 10 min) was used for further analysis.

The present study conformed to the ethical standards of the World Medical Association Declaration of Helsinki. Tumor stage and pathological classification were described according to the *Japanese Classification of Gastric Carcinoma*.[16]

After surgery, we performed peritoneal lavage 5 times using 1 L of physiological saline at each time.

### *Quantitative Real-Time RT-PCR*

Total RNA was extracted from PW samples, and cDNA was produced using the Superscript III Cell Direct cDNA synthesis kit (Invitrogen, Carlsbad, CA). One-step real-time quantitative RT-PCR of carcinoembryonic antigen (CEA), cytokeratin 20 (CK20), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was performed using LightCycler (Roche Diagnostics, Mannheim, Germany), as described previously.[11,17,18] GC cell lines (MKN7, MKN45, and MKN74) were used as positive controls for the detection of CEA and CK20, and cases of partial gastrectomy for gastrointestinal stromal tumor or sleeve gastrectomy for morbid obesity were used as negative controls.

### *Viability of the Cell Culture*

We evaluated the viability of PW-before and PW-after free cancer cells obtained from the cell culture. Cells obtained from PW were maintained in RPMI-1640 medium (Nihon Seiyaku, Tokyo, Japan) supplemented with 15 % heat-inactivated fetal bovine serum (Gibco, Uxbridge, UK), 2 mM glutamine, and penicillin-streptomycin (50 IU/mL and 50 μg/mL, respectively) at 37.0 °C in an atmosphere of humidified air with 5 % $CO_2$. If cell samples of individual cases did not proliferate within 4 weeks, the evaluation was discontinued.

### *Proliferative Potential of Free Cancer Cells*

We evaluated the proliferative activity of free cancer cells through the staining of Ki-67 with a primary antibody of Ki-67 (dilution 1:50; Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), an avidin-biotin immunoperoxidase staining kit (Dako Japan Co. Ltd., Kyoto, Japan), and 3,3′-diaminobenzidine tetra-hydrochloride for visualization. Positive controls for immunohistochemical staining were lymph nodes with active germinal centers. All pathological specimens were reviewed independently by 2 pathologists.

### *Tumorigenic Capacity of the Spilled Cancer Cells In Vivo*

For the study, 24 athymic female NOD/ShiJic-scid mice (8 weeks old) were obtained from CLEA (Tokyo, Japan). The mice were housed in cages under specific pathogen-free conditions and provided with sterilized food and water ad libitum at the Shiga University of Medical Science (Shiga, Japan). All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Shiga University of Medical Science.

For assessing the tumorigenic capacity of cancer cells in PW-after samples harvested from subconfluent cultures, mice were injected intraperitoneally with $1 \times 10^7$ cells per 100 μL, maintained on experimental diets for an additional 6 weeks, and sacrificed using sodium pentobarbital. After laparotomy, the excised peritoneal nodules were used for histopathological examinations.

**TABLE 1** Characteristics of patients with gastric cancer ($n = 102$) who underwent gastrectomy and lymph node dissection

| Patient with gastric cancer | ($n = 102$) |
|---|---|
| Age (median, range) (years) | 67.9 (36–88) |
| Sex | |
| Male | 59 (57.8 %) |
| Female | 43 (42.2 %) |
| Tumor invasion | |
| m | 16 (15.7 %) |
| sm | 17 (16.7 %) |
| mp | 17 (16.7 %) |
| ss | 25 (24.5 %) |
| se | 27 (26.4 %) |
| Lymph node metastasis | |
| 0 | 52 (51 %) |
| 1 | 17 (16.7 %) |
| 2 | 19 (18.6 %) |
| 3 | 14 (13.7 %) |
| Lymphatic invasion | |
| 1y0 | 25 (24.5 %) |
| 1y1 | 50 (49 %) |
| 1y2 | 17 (16.7 %) |
| 1y3 | 10 (9.8 %) |
| Vessel invasion | |
| v0 | 44 (43.2 %) |
| v1 | 40 (39.2 %) |
| v2 | 14 (13.7 %) |
| v3 | 4 (3.9 %) |
| Cytology | |
| Negative | 102 (100 %) |
| Positive | 0 (0 %) |
| Surgery | |
| Distal gastrectomy | 84 (82.4 %) |
| Total gastrectomy | 18 (17.6 %) |
| Histological type | |
| Differentiated | 56 (55 %) |
| Undifferentiated | 46 (45 %) |

*m* mucosa, *sm* submucosa, *mp* muscularis propria, *ss* subserosa, *se* serosa-exposed

### *Postoperative Clinical Outcome*

The clinical significance of CEA or CK20 mRNA amplification by RT-PCR and the presence of viable cancer cells in the PW samples were determined on the basis of peritoneal recurrence-free survival (RFS) and the peritoneal recurrence rate (RR), respectively. The 102 patients were separated into a PCR −/− group (PCR results were negative before and after surgery) a PCR −/+ group (PCR results changed from negative to positive after surgery), and a PCR +/+ group (PCR results were positive before and after surgery). Peritoneal RFS was estimated using the Kaplan–Meier method.

**TABLE 2** Characteristics of cancer cells spilled during surgery

| | CEA or CK20 mRNA[a] (PW-after samples, $n = 57$) | | $p$ value |
|---|---|---|---|
| | Positive ($n = 35$) | Negative ($n = 22$) | |
| Tumor invasion | | | |
| m | 1 (9.1 %) | 11 (90.9 %) | |
| sm | 7 (53.8 %) | 6 (46.2 %) | |
| mp | 6 (75 %) | 2 (25 %) | |
| ss | 13 (91.3 %) | 3 (18.7 %) | |
| se | 8 (100 %) | 0 (0 %) | |
| Lymph node metastasis | | | <.001* |
| Negative | 14 (40 %) | 21 (60 %) | |
| Positive | 21 (95.5 %) | 1 (4.5 %) | |
| Lymphatic invasion | | | <.001* |
| Negative | 2 (11.3 %) | 15 (88.7 %) | |
| Positive | 33 (82.5 %) | 7 (17.5 %) | |
| Vessel invasion | | | <.001* |
| Negative | 12 (41.4 %) | 17 (58.6 %) | |
| Positive | 23 (82.1 %) | 5 (17.9 %) | |
| Surgery | | | .54 |
| Distal gastrectomy | 32 (61. %) | 19 (38.5 %) | |
| Total gastrectomy | 3 (50 %) | 3 (50 %) | |
| Histological type | | | .91 |
| Differentiated | 18 (62.1 %) | 11 (37.9 %) | |
| Undifferentiated | 17 (61.0 %) | 11 (39 %) | |

*m* mucosa, *sm* submucosa, *mp* muscularis propria, *ss* subserosa, *se* serosa-exposed, *PW-before* peritoneal washings extracted before gastrectomy, *PW-after* peritoneal washings extracted after gastrectomy

[a] Scored as positive for cancer cells if 1 or 2 marker transcripts were deleted

* Statistically significant

### *Statistical Analyses*

We conducted the following analyses using Excel (Microsoft, Redmond, WA) and Statcel2 (OMS Publisher, Saitama, Japan). The $\chi^2$ test was used to compare data obtained by evaluation of the PW and RR. Differences in the peritoneal RFS were analyzed using the log-rank test. A $p$ value of <.05 was considered statistically significant.

## RESULTS

### *Evaluation of Cancer Cells Spilled During Surgery*

The clinical characteristics of the 102 GC patients are shown in Table 1. We examined whether viable cancer

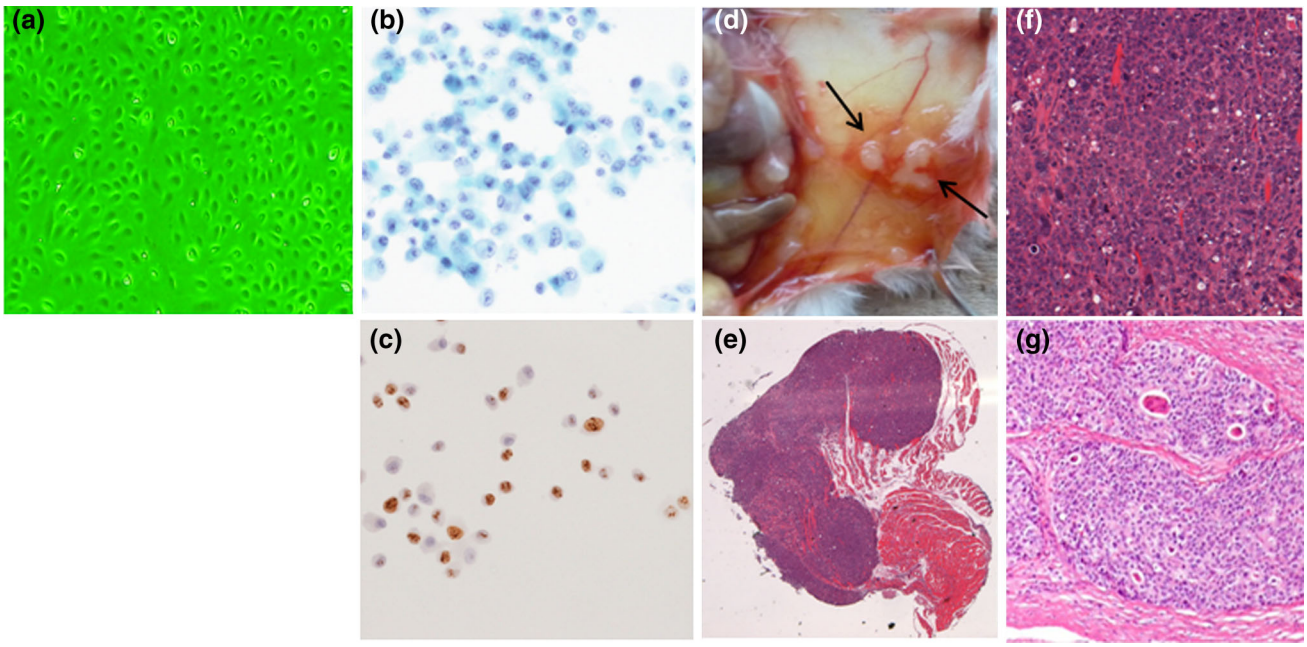


**FIG. 1** Viable and proliferative cancer cells spilled into the peritoneal cavity during surgery. Cancer cells were obtained from the peritoneal washings after surgery. **a** Proliferation of viable cells in cultured medium (×100). The cells were obtained from peritoneal washing after surgery and tested positive for Ki-67 staining, as shown by the strong nuclear staining. **b** Proliferating viable cells were defined as cancer cells by using Papanicolaou staining (×100). **c** Viable cancer cells were defined as proliferative by using Ki-67 staining (×100). Peritoneal metastasis from the surgically spilled cancer cells. Viable cancer cells obtained from peritoneal washings after gastrectomy were injected into the peritoneal cavity of NOD/Scid mice. **d** White, hard nodules were formed on the mice peritoneum that was injected with viable free cancer cells (*arrows*). **e** Whole-mount view of a surgically excised peritoneal nodule. **f** Pathological finding of the peritoneal nodule revealed a poorly differentiated adenocarcinoma [hematoxylin-eosin (HE) staining, ×100]. **g** Poorly differentiated adenocarcinoma of the stomach in a patient with primary gastric cancer (HE staining, ×100)

cells were present in the PW-after samples by cytological examination; however, all PW-after samples tested negative because the samples contained inflammatory and mesothelial cells, preventing detection of the cancer cells. Thus, we performed CEA and CK20 mRNA amplification in the PW-before and PW-after samples using RT-PCR. Of the 102 PW-before samples, 45 showed CEA or CK20 mRNA amplification. For these 45 cases, CEA or CK20 mRNA amplification was also found in the PW-after samples. Also, 57 of the 102 PW-before samples of CY0 did not show CEA or CK20 mRNA amplification. Further, 35 of these 57 (61.4 %) samples showed CEA or CK20 mRNA amplification in the PW-after samples. Detection of CEA or CK20 mRNA amplification was significantly higher in cases of advanced tumor invasion or lymph node metastasis ($p < .001$; Table 2).

### *Viability and Proliferative Activity of Free Cancer Cells Spilled During Surgery*

Because the CEA or CK20 mRNA amplification products could have been derived from dead cells, we cultivated the cancer cells extracted from the PW-after samples and tested their viability. Of the 57 cases with negative amplification of the PW-before samples, 35 showed CEA or CK20 mRNA amplification of the PW-after samples. When these 35 samples were used for cell culture, 24 (68.6 %) showed viable cancer cells in cultured medium (Fig. 1a). Meanwhile, none of the 22 samples lacking CEA and CK20 mRNA amplification showed viable cancer cells. Spilled viable cancer cells into the peritoneal cavity just after gastrectomy occurred more frequently in cases with advanced tumor invasion or lymph node metastasis than in cases with mucosal (m) or submucosal (sm) invasion of the cancer and without lymph node metastasis (Table 3).

The cultured cells from the 24 PW-after samples showing cancer cell viability tested positive for Ki-67 staining, indicating proliferative activity (Fig. 1b, c).

### *Tumorigenicity of Free Cancer Cells Spilled During Surgery*

Peritoneal nodules were observed in 4 of the 24 NOD/ShiJic-scid mice injected with cultured cancer cells obtained from the 24 PW-after samples (Fig. 1d). Histologically, the excised peritoneal nodules showed poorly differentiated adenocarcinoma (Fig. 1e, f), compatible with the histology of primary GC (Fig. 1g).

**TABLE 3** Characteristics of the viable cancer cells spilled during surgery

| | Viable cancer cells | | *p* value |
|---|---|---|---|
| | Positive ($n = 24$) | Negative ($n = 33$) | |
| Tumor invasion | | | |
| m | 1 (8.3 %) | 11 (91.7 %) | |
| sm | 3 (23.1 %) | 10 (76.9 %) | |
| mp | 4 (50 %) | 4 (50 %) | |
| ss | 12 (75 %) | 4 (25 %) | |
| se | 4 (50 %) | 4 (50 %) | |
| Lymph node metastasis | | | .001* |
| Negative | 9 (25 %) | 27 (75 %) | |
| Positive | 15 (68.2 %) | 7 (31.8 %) | |
| Lymphatic invasion | | | .0028* |
| Negative | 1 (5.9 %) | 16 (94.1 %) | |
| Positive | 23 (57.5 %) | 17 (42.5 %) | |
| Vessel invasion | | | <.001* |
| Negative | 1 (3.4 %) | 28 (96.6 %) | |
| Positive | 23 (82.1 %) | 5 (17.9 %) | |
| Surgery | | | .18 |
| Distal gastrectomy | 23 (45.1 %) | 28 (54.9 %) | |
| Total gastrectomy | 1 (16.7 %) | 5 (83.3 %) | |
| Histological type | | | .063 |
| Differentiated | 14 (48.3 %) | 15 (51.7 %) | |
| Undifferentiated | 10 (35.7 %) | 18 (64.3 %) | |
| Ki-67 stain | | | |
| Negative | 24 | ND | |
| Positive | 0 | ND | |

*m* mucosa, *sm* submucosa, *mp* muscularis propria, *ss* subserosa, *se* serosa-exposed

* Statistically significant

### *Patient Outcome*

The peritoneal RFS was worse in patients positive for CEA or CK20 mRNA amplification in the PW-before samples than in those with negative PCR results ($p < .05$). Further, the peritoneal RFS of PCR −/+ and PCR +/+ patients was poorer than that of PCR −/− patients ($p < .01$). Additionally, the peritoneal RFS of PCR −/+ patients was nearly equivalent to that of PCR +/+ patients (Fig. 2). In contrast, other RFS excluding peritoneal metastasis of PCR −/− patients was equivalent to that of PCR −/+ ($p = .45$) and PCR +/+ patients ($p = .12$).

Of the 24 patients for which viable cancer cells were detected after surgery, 11 (45.8 %) developed peritoneal recurrence. Meanwhile, only 1 patient (9.0 %) showed peritoneal recurrence among the 11 patients for which viable cancer cells were not detected after surgery. Peritoneal RR was significantly higher in patients with viable cancer cells after surgery than in those without viable cancer cells ($p = .033$). Of 4 cases that generated tumor formation on mice, 3 were associated with peritoneal recurrence in the patients.

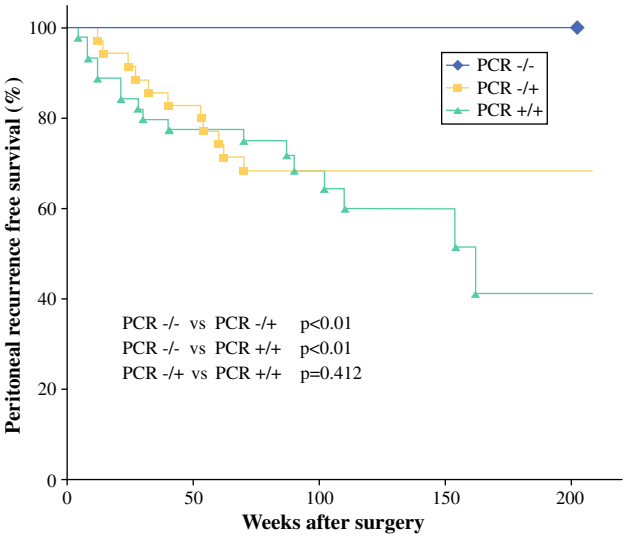


**FIG. 2** Curves for survival after curative surgery for gastric cancer are plotted using the Kaplan–Meier method and analyzed using the log-rank test. Survival curves according to the PCR results are presented. PCR −/−, PCR results were negative before and after resection; PCR −/+, PCR results changed from negative to positive after resection; PCR +/+, PCR results were positive before and after resection

## DISCUSSION

Here, we found that cancer cells spilled into the peritoneal cavity during curative gastrectomy with LND for GC were viable, proliferative, and tumorigenic and showed the clinical potential to develop into peritoneal metastasis. These findings indicate surgery for GC is a cause of peritoneal recurrence.

Some studies have shown that RT-PCR for CEA or CK20 mRNA is useful for the molecular detection of a small number of cancer cells in the PW. Patients for which the PW tested positive for CEA and CK20 mRNA at laparotomy have been reported to show significantly poorer RFS and overall survival rates than those for which the PW tested negative.[17–19] Consistent with these reports, we showed that patients with CY0 but positive for CEA or CK20 mRNA in the PW-before samples had significantly poorer peritoneal RFS than those with negative amplification. Moreover, we found that some patients were PCR −/+ and that these patients had significantly poorer peritoneal RFS than PCR −/− patients. Together, these results suggest that the detection of CEA or CK20 mRNA in PW samples has clinical relevance in predicting peritoneal recurrence.

In addition to the RT-PCR method, we cultivated cells with positive results for CEA or CK20 mRNA amplification in PW-after. Of the 35 cases with positive CEA or CK20 amplification in PW-after, 11 were negative for viable cancer cells in our study. Although RT-PCR has a high sensitivity, false positive results may be obtained as a result of DNA contamination, dead tumor cells, or epithelial cells from the gastrointestinal tract, indicating a potential limitation of the PCR method. However, the cancer cells spilled into the peritoneal cavity were viable and proliferative in 24 of the PCR −/+ cases. These patients had a significantly increased incidence of peritoneal recurrence when compared with those without viable cancer cells. Collectively, these results suggest that spilled cancer cells during surgery have the ability to develop peritoneal metastasis not only in immunodeficient mice but also in patients with curative gastrectomy for GC.

An important concern is whether the free cancer cells spilled during surgery possess tumorigenic potential. Generally, a single free cancer cell easily undergoes apoptosis in vivo. Tumor cells are exposed to both mechanical forces and immunological defenses, which may eliminate most of the disseminated tumor cells. Under these severe circumstances, it is difficult for free cancer cells to initiate metastasis. However, an alternative route of metastasis initiated after surgery has recently been proposed.[20,21] Surgical trauma impairs tissue integrity and induces inflammatory mediators and angiogenic factors, leading to immune suppression, enhanced tumor cell adhesion, and augmented tumor growth. Thus, surgery induces both local and systemic changes; hence, although surgery greatly reduces tumor mass and is potentially curative, it also paradoxically facilitates metastasis. These postsurgical changes help surgery-induced peritoneal free cancer cells establish peritoneal metastasis. As an alternative explanation for peritoneal metastasis, cancer stem cells (CSCs), capable of continuous proliferation and self-renewal, are proposed to play significant roles in tumorigenesis, metastasis, and recurrence.[22,23] CSCs can generate tumors via the stem cell processes of self-renewal and differentiation into multiple cell types. Cell reprogramming, which generates undifferentiated cancer-stemlike cells from differentiated cancer cells, has been reported to be associated with transforming growth factor beta (TGF-β)-induced epithelial-mesenchymal transition (EMT).[24,25] Recently, EMT has become recognized as an important step in cancer invasion and metastasis.[24,25] Surgery induces the production of adhesion molecules, cytokines, vascular endothelial growth factors, immunosuppressive agents, and TGF-β. Therefore, the relationships among surgery-induced free cancer cells, CSCs, and peritoneal metastasis should be investigated.

Many of the study patients who underwent standard curative gastrectomy with LND for GC possessed surgery-induced free cancer cells. The evidence reported here indicates the need for establishing new strategies that include a potentially curative resection for advanced GCs. One important strategy is to prevent the dissemination of cancer cells from the lymphatic and blood vessels by vessel-sealing devices. Various energy-based devices have been used for vessel sealing and tissue dissection. In comparison with electrocautery, vessel-sealing devices have been reported to reduce the rate of lymphorrhea.[26] The use of ultrasonically activated shears for LND in GC patients has been shown to reduce operative blood loss and postoperative lymphorrhea.[27] However, in this study, the free cancer cells frequently spread into the peritoneal cavity during surgery even when ultrasonically activated shears were used, indicating the existing energy-based devices may be insufficient for vessel sealing. New vessel-sealing devices should be developed from the viewpoint of preventing surgery-induced peritoneal metastasis. Another strategy is to treat the cancer cells spilled during surgery. Frequent washing of the peritoneal cavity, intraoperative chemotherapy, and hyperthermic intraperitoneal chemotherapy (HIPEC) are some of the prophylactic methods.[28–30] Kuramoto et al. reported the efficacy of extensive intraoperative peritoneal lavage followed by intraperitoneal chemotherapy (EIPL-IPC) and reported EIPL-IPC improved the 5-year survival rate of GC patients positive for CY and negative for peritoneal dissemination.[28] Previously, we showed that HIPEC was useful for the prevention of peritoneal metastasis in patients who underwent curative gastrectomy for advanced GC.[30] More advanced treatments should be developed on the concept that the cancer cells disseminated during surgery should be eradicated during surgery. In our study, viable cancer cells spilled during surgery occurred more frequently in cases with advanced tumor invasion, lymph node metastasis, lymphatic invasion, or vessel invasion, indicating free cancer cells spilled from the exposed primary cancer involved lymphatic tissues, fat tissues, or involved venous vessels. However, no direct evidence of the origin of the peritoneal free cancer cells was found. Moreover, 1 of the 35 samples with viable cancer cells during surgery showed early GC without any lymph node metastasis or vessel invasion, suggesting the presence of other channels for the spilling of cancer cells.

One limitation of our study is that the correlation between the surgery-induced local and systemic changes and the generation of spilled cancer cells-induced peritoneal metastasis remains unclear. Moreover, the NOD/scid mouse used in this study is not suitable for examining several surgery-induced factors, including inflammatory mediators or angiogenic factors, because of its severe combined immunodeficiency. In the future, we hope to elucidate how spilled free cancer cells initiate peritoneal metastasis during surgery.

In conclusion, the free cancer cells spilled during curative surgery were found to be viable, proliferative, tumorigenic, and a source of peritoneal metastasis. This evidence of surgery-induced peritoneal metastasis may give rise to a paradigm shift in the surgical procedures for cancer therapy. Researchers and physicians must aim to develop advanced devices that prevent spilling over of cancer cells from the cancerous tissues and establish an intraoperative intraperitoneal therapy that can eradicate surgery-induced peritoneal cancer cells.

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**DISCLOSURE** Katsushi Takebayashi and the other co-authors have no commercial conflicts of interest in the subject of study.

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