Mechanisms of Peritoneal Metastasis after Operation for Non-Serosa-invasive Gastric Carcinoma: An Ultrarapid Detection System for Intraperitoneal Free Cancer Cells and a Prophylactic Strategy for Peritoneal Metastasis

Takashi Marutsuka, Shinya Shimada, Kenji Shiomori, Naoko Hayashi, Yasushi Yagi, Takaaki Yamane, and Michio Ogawa[1]

Department of Surgery II, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan [T. M., S. S., K. S., N. H., M. O.]; Department of Surgery, Yatsushiro Health Insurance General Hospital, Kumamoto 866-8660, Japan [S. S.]; Department of Surgery, Kumamoto Regional Hospital, Kumamoto 860-0811, Japan [Y. Y.]; and Department of Surgery, Kumamoto Red Cross Hospital, Kumamoto 862-0939, Japan [T. Y.]

ABSTRACT

Purpose: **This aims of this study are to establish an ultra-rapid quantitative reverse transcription-PCR (RT-PCR) protocol that enables the diagnosis of i.p. cancer spread during operation, to reveal the mechanisms of peritoneal metastasis from non-serosa-invasive gastric carcinoma, and to evaluate the effect of the extensive intraoperative peritoneal lavage (EIPL) using the ultra-rapid quantitative RT-PCR as a prophylactic strategy for peritoneal metastasis.**

Experimental Design: **Peritoneal lavage samples from 63 patients with non-serosa-invasive gastric carcinoma were obtained at laparotomy and immediately after lymph node dissection. To identify the free cancer cells in the samples, carcinoembryonic antigen- and cytokeratin 20-specific RT-PCRs were performed using the LightCycler method in combination with an automated mRNA extractor. In addition, EIPL was performed in five cases with serosa-invasive gastric carcinoma, and its efficacy was evaluated by the ultra-rapid quantitative RT-PCR protocol.**

Results: **The method enabled us to complete the detection of cancer cells within approximately 70 min. Both the carcinoembryonic antigen and cytokeratin 20 mRNA in i.p. lavages after lymph node dissection were identified in three (14.3%), four (26.7%), and six (46.2%) patients with submucosal, muscularis propria, and subserosal tumors, respectively. Lymph node metastasis was the independent predictor of the existence of i.p. free cancer cells. The ultrarapid quantitative RT-PCR demonstrated that EIPL reduced free cancer cells from $3.8 \times 10^5 \pm 1.4 \times 10^5$ cells to 2.8 ± 1.5 cells/100 ml lavage after six to eight washes, and they disappeared after seventh to ninth wash.**

Conclusions: **The present study proved that lymph node dissection opened lymphatic channels and spread viable cancer cells into the peritoneal cavity. It is suggested that the combination of the novel detection system with the intraoperative therapy of EIPL can be a useful prophylactic strategy for peritoneal metastasis from gastric carcinoma.**

INTRODUCTION

Peritoneal metastasis is the most frequent pattern of recurrence in patients with gastric carcinoma (1, 2). About half of patients with serosa-invasive gastric carcinoma develop peritoneal recurrence and die of this disease during the first 2 years, even if curative resection is performed (3, 4). The 5-year survival rate of patients with positive peritoneal lavage cytology is only 2% (5). Peritoneal metastasis is considered to be caused by free cancer cells exfoliated from serosa-invasive tumors. Nevertheless, some cases are found even in those without serosal invasion. It has been reported that approximately 0.5% of patients with early gastric carcinoma and 5% of patients with MP[2]-invasive gastric carcinoma developed peritoneal recurrence after a curative operation (6–8). None of these cases had free cancer cells in the peritoneal cavity by cytological diagnosis, and they had no macroscopic dissemination at laparotomy. Although the reasons for peritoneal dissemination in non-serosal-invasive carcinoma have been postulated as lymph node dissection opening lymphatic channels and spreading viable cancer cells (9) and metastatic lymph nodes shedding cancer cells (10), a clear and definite reason has not been fully established thus far.

Recently, a RT-PCR has been developed for screening a small amount of tumor cells in circulating blood (11–15), bone marrow (16), lymph nodes (17, 18), and peritoneal lavage fluid (19–21). Its sensitivity is higher than that of conventional and immunohistochemical cytological examinations (20). Therefore, the RT-PCR technique is suitable for detecting minute quantities of i.p. free cancer cells. However, RT-PCR has its own drawbacks, such as the following: (*a*) results of RT-PCR are not

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[1] To whom requests for reprints should be addressed, at Department of Surgery II, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto 860-0811, Japan. Phone: 81-96-373-5213; Fax: 81-96-371-4378; E-mail: tmaru10@hotmail.com.

[2] The abbreviations used are: MP, muscularis propria; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CEA, carcinoembryonic antigen; CK, cytokeratin; M, mucosal; SM, submucosal; SS, subserosal; EIPL, extensive intraoperative peritoneal lavage.

Table 1 Clinicopathological background

	Depth of tumor invasion				
	M	SM	MP	SS	SE[a]
No. of patients	14	21	15	13	5
Age in yrs (mean ± SD)	62.0 ± 12.9	67.0 ± 12.4	70.1 ± 6.9	66.0 ± 7.2	60.0 ± 10.3
Sex (male/female)	9/5	13/8	10/5	9/4	4/1
Tumor size, mm (mean ± SD)	24.4 ± 12.9	26.3 ± 11.5	46.0 ± 23.2	58.3 ± 33.5	75.2 ± 26.5
Histological type (differentiated/undifferentiated)	8/6	16/5	8/7	8/5	3/2
Extent of lymph node metastasis (N0/N1/N2)	14/0/0	16/4/1	8/5/2	6/5/2	0/2/3
Lymphatic invasion (ly0/ly1/ly2/ly3)	14/0/0/0	14/6/1/0	7/7/1/0	3/9/0/1	0/0/1/4
Vessel invasion (v0/v1/v2/v3)	14/0/0/0	16/4/0/1	5/8/2/0	2/7/4/0	0/1/2/2
Peritoneal metastasis (P0/P1)	14/0	21/0	15/0	13/0	5/0
Liver metastasis (H0/H1)	14/0	21/0	15/0	12/1	5/0

[a] SE, serosa.

available during surgery because of the time-consuming gene amplification and subsequent data analysis; and (*b*) results of RT-PCR involve, even in low frequency, false-positive results exist by DNA contamination or pseudogenes (22). In the present study, we have established a combined system of an ultra-rapid RT-PCR using a fully automated mRNA extractor and a real-time one-step RT-PCR system with hybridization probe format for detection of peritoneal free cancer cells. This new method enabled us to obtain the results of RT-PCR with approximately 70 min after sampling. Furthermore, we carried out multiple marker RT-PCR assay for a combination of CEA and CK20 to eliminate false-positive results and to improve specificity.

The reported results of randomized trials of adjuvant perioperative i.p. chemotherapy have shown no significant improvement in survival compared with surgery alone (23–25), except in a few studies (26, 27). Because those studies suggest the lack of any fully effective therapy for peritoneal recurrence, it is important to prevent peritoneal metastasis before implantation of the i.p. free cancer cells. We have reported previously that EIPL and chemotherapy are useful to prevent peritoneal metastasis with a statistically improved survival rate (28).

In the present study, (*a*) an ultra-rapid quantitative RT-PCR system for intraoperative detection of i.p. free cancer cells was established, (*b*) we elucidated the cause of peritoneal recurrence after curative operation for patients with non-serosa-invasive gastric cancer, and (*c*) EIPL therapy is suggested to be useful for prophylaxis of peritoneal recurrence from the results of evaluation using the ultra-rapid quantitative RT-PCR system.

PATIENTS AND METHODS

Patients. Between January 2000 and January 2002, we observed 63 patients with gastric carcinoma. All had histologically proven gastric carcinoma and underwent a potentially curative operation with extended lymph node dissection (D2) at the Department of Surgery II, Kumamoto University Medical School, Yatsushiro Health Insurance General Hospital, Kumamoto Regional Hospital, and Kumamoto Red Cross Hospital. No patients had received preoperative radiation therapy or chemotherapy. No patients had an intestinal perforation. D2 lymphadenectomy, a standard procedure in Japan, denotes extended systemic lymphadenectomy with resection of perigastric lymph nodes and the nodes at the base of the left gastric artery and along the common hepatic and splenic arteries. The resected lymph nodes were cut into two equal parts under sterile conditions to prevent RNA contamination between specimens. One-half of the lymph node was fixed in 10% buffered formalin for routine histological examination, and the other half was frozen in liquid nitrogen for the RT-PCR assay. The surgically resected stomach was fixed in 10% buffered formalin in preparation for histological diagnosis with standard H&E staining. Pathological diagnosis and classifications were made based on the Japanese Classification of Gastric Carcinoma by the Japanese Research Society for Gastric Cancer (29). The patients included 14, 21, 15, and 13 patients with M, SM, MP, and SS tumors, respectively. Clinicopathological features are shown in Table 1. In addition, five patients had serosa-invasive tumors; they received EIPL and intraoperative i.p. chemotherapy. Informed consent was obtained from each patient before his/her participation in the study, and the study protocol was in accordance with the Ethics Committee guidelines for Kumamoto University School of Medicine.

Peritoneal Lavage. Peritoneal lavage was performed twice during the operation (at the time of laparotomy and immediately after lymph node dissection). At the beginning of the operation, before the manipulation of the tumor, 100 ml of physiological saline was introduced into the upper abdominal cavity and recovered after being gently stirred. Furthermore, immediately after lymph node dissection, the lymph node-dissected area around the base of the left gastric artery and the common hepatic artery was washed with 100 ml of physiological saline. To prevent contamination of gastric mucosa, the residual gastrointestinal edges were covered by thick gauze during the wash. The lavage fluids were collected in tubes with EDTA disodium salt and centrifuged at 3000 rpm for 3 min. After removing RBCs by Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany), they were stored at −80°C until use.

Ultra-rapid RT-PCR. mRNA was extracted using the MagNA Pure LC system (Roche) according to the manufacturer's instructions. In brief, the isolation procedure is based on magnetic bead technology. The mRNA is released from the cells by the lysis step, and biotin-labeled oligo(dT) is hybridized to the poly(A) residue of mRNA, and this complex is immobilized

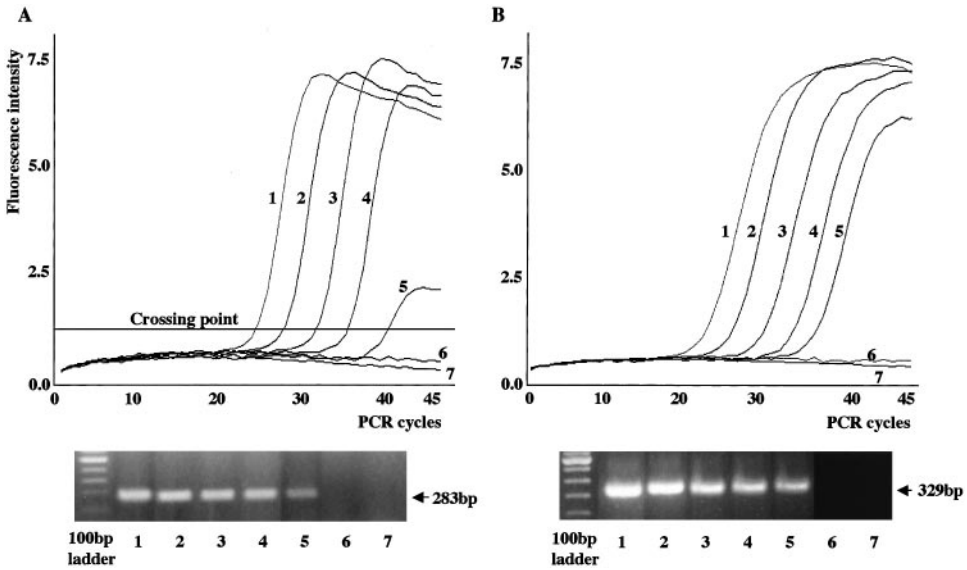

Fig. 1 Sensitivity of ultra-rapid quantitative RT-PCR assay by LightCycler using CEA (*A*) and CK20 (*B*) mRNA markers. *Curves* and *Lanes 1–5* were serially diluted 10^5 cells to 10 cells of WiDr colon carcinoma cells in 10^7 leukocytes from healthy volunteer, respectively; *curve* and *Lane 6*, 10^7 leukocytes from healthy volunteer; *curve* and *Lane 7*, no template. This assay system could detect at least 10 WiDr colon carcinoma cells in 10^7 leukocytes. The PCR products were analyzed by 2% agarose gel electrophoresis, and they were matched to the expected sizes of CEA and CK20. Crossing points were used to establish an external standard curve for quantification. Forty-five rounds of amplification were completed within 30 min.

onto the surface of streptavidin-coated magnetic beads. Contaminating components are washed away by repeated steps of separation and resuspension in wash buffer. Finally, the purified mRNA is eluted from the particles. The MagNA Pure LC system automatically performed all steps of the procedure in 40 min.

Real-time, one-step, no-nested RT-PCR for CEA mRNA and CK20 mRNA was realized on the LightCycler instrument (Roche) with the LightCycler RNA Amplification Kit for Hybridization Probes (Roche Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. In this system, the PCR is monitored using two hybridization probes labeled with fluorescein (donor dye) or LC Red 640 (acceptor dye), allowing a fluorescence resonance energy transfer after hybridization to the target sequence in a head-to-tail arrangement on the same strand of amplified DNA fragment. The intensity of the light emitted by LC Red 640 is proportional to DNA formation and measured at 640 nm (30). In this analysis, the background fluorescence was removed by setting a noise band. We classified a sample as positive if the intensity of fluorescence exceeded the noise band.

The primer sequences used for CEA amplification were 5′-GACGCAAGAGCCTATGTATG and 5′-GGCATAGGTCCCGTTATTA. The probe sequences used for CEA identification were 5′-CCCAGACTCGTCTTACCTTTCGG-FL and 5′-LC-AGCGAACCTCAACCTCTCCTGC-P (in sequences, FL means fluorescein, LC means LC Red 640 labeling, and P means phosphate group to block extension). The primer sequences used for CK20 amplification were 5′-GAAGTCGATGGCCTACACAA and 5′-AACGGGCCTTGGTCTCCTCTA. The probe sequences used for CK20 identification were 5′-CTTTGGCCTCTTGAAGGTTCTTCTG-FL and 5′-LC-GCCATGACTTCATACTTCTGCCTCA-P. All primers and probes were synthesized and purified by reverse-phase high-performance liquid chromatography by Nihon Gene Research Laboratories (Sendai, Japan). After reverse transcription for 10 min at 50°C, the following temperature profile was used for amplification: denaturation for 1 cycle at 95°C for 30 s and 45 cycles at 95°C for 1 s, 55°C for 10 s, and 72°C for 10 s. Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification. To prove the integrity of isolated RNA, a PCR assay with primers and probes specific for the gene GAPDH mRNA was carried out in each case under the same conditions as described above. The primer sequences used for GAPDH amplification were 5′-TGAACGGGAAGCTCACTGG and 5′-TCCACCACCCTGTTGCTGTA. The probe sequences used for GAPDH identification were 5′-TCAACAGCGACACCCACTCCT-FL and 5′-LC-CACCTTTGACGCTGGGGCT-P. Each series of RT-PCR reactions included RNA-negative samples as negative control, and mRNA from WiDr cells, which is well known to express high amounts of CEA and CK20, was considered a positive control.

Quantitative RT-PCR Analysis. Quantification data were analyzed using LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. To ascertain the reliability of this assay system, 10-fold serial dilutions from 10^1 to 10^5 WiDr colon cancer cells in 1×10^7 leukocytes obtained from healthy volunteers were studied. To identify the PCR products of this system, we applied them in gel electrophoresis. The obtained results were used as external controls for delineating a standard curve for quantitative analysis. Quantitation of cell number by the LightCycler was assessed by determination of the "crossing point," making the cycle when fluorescence of a given sample rose above the background level to give the maximal slope by log-linear amplification. The standard curve was made from plots of crossing points *versus* the initial number of WiDr cells. The cell number of clinical samples with unknown concentration was calculated with reference to this standard curve.

EIPL. In patients with serosa-invasive tumors and no macroscopically detected peritoneal dissemination, CEA and CK20 mRNA were detected in the lavage at laparotomy. After

Table 2 Correlation between clinicopathological factors and i.p. lavage assay by LightCycler

	CEA mRNA			CK20 mRNA			Multimarker RT-PCR[a]		
	Positive	Negative	*P*	Positive	Negative	*P*	Positive	Negative	*P*
No. of patients	20	48		19	49		18	50	
Sex									
Male	13 (28.9%)	32 (71.1%)		13 (28.9%)	32 (71.1%)		12 (26.6%)	33 (73.4%)	
Female	7 (30.4%)	16 (69.6%)	>0.99	6 (26.1%)	17 (73.9%)	>0.99	6 (26.1%)	17 (73.9%)	>0.99
Age (yrs)	65.0 ± 9.4	66.3 ± 11.3	0.637	64.7 ± 9.3	66.4 ± 11.2	0.562	69.2 ± 9.5	66.4 ± 12.6	0.475
Tumor invasion									
M	0 (0%)	14 (100%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
SM	3 (14.3%)	18 (85.7%)		3 (14.3%)	18 (85.7%)		3 (14.3%)	18 (85.7%)	
MP	5 (33.3%)	10 (66.7%)	0.235	5 (33.3%)	10 (66.7%)	0.235	4 (26.7%)	11 (73.4%)	0.418
SS	7 (53.8%)	6 (46.2%)		6 (46.2%)	7 (53.8%)		6 (46.2%)	7 (53.8%)	
SE[b]	5 (100%)	0 (0%)		5 (100%)	0 (0%)		5 (100%)	0 (0%)	
Tumor size, mm[c]	35.9 ± 10.9	30.6 ± 11.1	0.241	35.6 ± 10.8	30.6 ± 10.8	0.271	35.0 ± 11.5	31.0 ± 11.2	0.398
Lymph node metastasis[c]									
Positive	7 (58.3%)	5 (41.7%)		7 (58.3%)	5 (41.7%)		6 (50.0%)	6 (50.0%)	
Negative	1 (4.2%)	23 (95.8%)	<0.001	1 (4.2%)	23 (95.8%)	<0.001	1 (4.2%)	23 (95.8%)	0.003
Histological type[c]									
Well diff.	2 (8.3%)	22 (91.7%)		2 (8.3%)	22 (91.7%)		2 (8.3%)	22 (91.7%)	
Poorly diff.	6 (50.0%)	6 (50.0%)	0.009	6 (50.0%)	6 (50.0%)	0.009	5 (41.7%)	7 (58.3%)	0.029
Lymphatic invasion[c]									
Positive	6 (40.0%)	9 (60.0%)		6 (40.0%)	9 (60.0%)		5 (33.3%)	10 (66.7%)	
Negative	2 (9.5%)	19 (90.5%)	0.046	2 (9.5%)	19 (90.5%)	0.046	29 (9.5%)	19 (90.5%)	0.103
Venous invasion[c]									
Positive	3 (20%)	12 (80.0%)		4 (26.7%)	11 (73.3%)		4 (26.7%)	11 (73.3%)	
Negative	5 (23.8%)	16 (76.2%)	>0.99	4 (19.0%)	17 (81.0%)	0.694	3 (14.3%)	18 (85.7%)	>0.99
Interstitium[c]									
Medullary	0 (0%)	15 (100%)		1 (6.7%)	14 (93.3%)		0 (0%)	15 (100%)	
Intermediate	7 (38.9%)	11 (61.1%)		6 (33.3%)	12 (66.7%)		6 (33.3%)	12 (66.7%)	
Scirrhous	1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)	
Infiltrating pattern[c]									
Localized	2 (10.5%)	17 (89.5%)		3 (15.8%)	16 (84.2%)		2 (10.5%)	17 (89.5%)	
Diffuse	6 (35.2%)	11 (64.8%)	0.114	5 (29.4%)	12 (70.6%)	0.433	5 (29.4%)	12 (70.6%)	0.218

[a] Scored as positive for cancer cells if both of the two marker transcripts were detected.
[b] SE, serosa; diff., differentiated.
[c] Subjected to SM and MP cases only.

the potentially curative operation was performed, the peritoneal cavity was washed extensively and aspirated completely using 10 liters of physiological saline (1 liter/wash, 10 washes; Ref. 28), and 100 ml of each individual liter were analyzed by ultra-rapid quantitative RT-PCR by LightCycler.

Statistical Analysis. Statistical comparisons were made using Student's *t* test and Fisher's exact probability test. To clarify the predictor of i.p. free cancer cells, multivariate analysis was performed by logistic regression analysis. The level of significance was set at $P < 0.05$. The entire statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC).

RESULTS

Sensitivity and Time of Ultra-rapid Quantitative RT-PCR. Molecular detections of CEA mRNA, CK20 mRNA, and GAPDH mRNA were performed in 70.1 ± 2.6 min (mean ± SD) using the combination system with the fully automated RNA extractor and one-step, real-time PCR. This assay system was able to detect at least 10 cancer cells in 1×10^7 leukocytes, indicating comparable sensitivity to conventional nested RT-PCR. The PCR products were analyzed by 2% agarose gel electrophresis, and they were matched to the expected sizes (Fig. 1, *A* and *B*). The time of approximately 70 min for completing the analytical process and its sensitivity were applicable for intraoperative detection. The relationship between the crossing points of ultra-rapid quantitative RT-PCR and the initial cell concentrations was found to be linear on logarithmic scales in the range of 10 to 10^5 WiDr cells. The correlation coefficient (r) was 1.0.

Detection of CEA mRNA and CK20 mRNA Using Ultra-rapid Quantitative RT-PCR Analysis in the Lavage Samples. In the samples of lavage at laparotomy, GAPDH mRNA was detected in all of the patients we analyzed. However, CEA mRNA and CK20 mRNA were not detected in patients without serosal invasion, although they were detected in all patients with serosal invasion. As shown in Table 2, in the peritoneal lavage samples from non-serosa-invasive cases after lymph node dissection, CEA mRNA product was detected in 15 of 63 patients (23.8%; Fig. 2). It was not observed in M tumors, but it was identified in three (14.3%), five (33.3%), and seven (53.8%) patients with SM, MP, and SS tumor, respectively. With regard to CK20 mRNA, the product was identified in 14 of 63 patients (22.2%). Like CEA mRNA, CK20 mRNA was not detected in M tumors but was identified in three (14.3%), five (33.3%), and six patients (46.2%) with SM, MP, and SS tumor, respectively. Both CEA mRNA and CK20 mRNA were detected in three (14.3%), four (26.7%), and six patients (46.2%) with

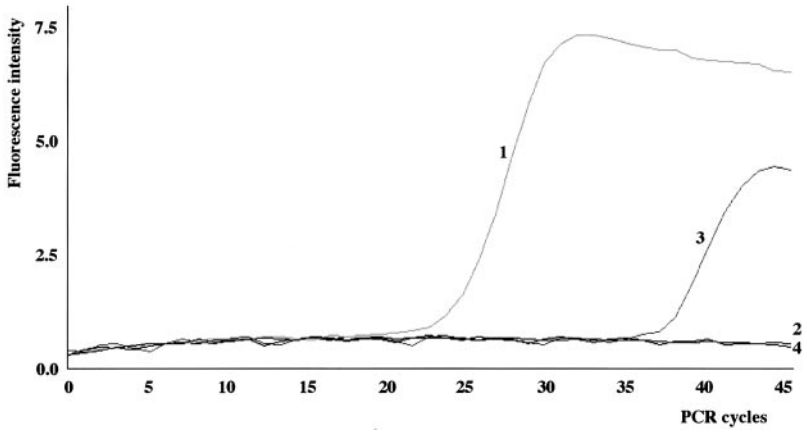

Fig. 2 Representative results of ultra-rapid RT-PCR by LightCycler in a patient with SM tumor of well-differentiated adenocarcinoma with lymph node metastasis and lymphatic invasion. *Curve 1*, WiDr colon cancer cells as a positive control; *curve 2*, i.p. lavage sample at laparotomy; *curve 3*, i.p. lavage sample immediately after lymph node dissection; *curve 4*, no template as a negative control.

Table 3 Multivariate analysis with respect to peritoneal lavage assay

	χ^2	*P*	Risk ratio	95% Confidence interval
Lymph node metastasis	4.147	0.041	12.935	1.100–152.032
Histological type	0.538	0.463	2.233	0.261–19.129

SM, MP, and SS tumor, respectively. Either CEA mRNA or CK20 mRNA was found in three (14.3%), six (40.0%), and seven patients (53.8%) with SM, MP, and SS tumor, respectively. The number of free cancer cells, calculated by the standard curve as described in "Patients and Methods," was 3.5 ± 3.7 (mean $\pm$ SD), 12.1 ± 9.6, and 124.8 ± 224.0 cells/100 ml in the lavage after lymph node dissection from SM, MP, and SS tumor, respectively. Because the number of free cancer cells was calculated based on the standard curve of WiDr colon cancer cells, the expression of mRNA may vary depending on the cells. In addition, because there is no data about conventional cytology in the present study, the expressions of CEA mRNA or CK20 mRNA in i.p. lavages at the time of operation may not actually result in peritoneal metastasis.

Relationship between Clinicopathological Factors and Detection of Tumor-related Messages. Detection of i.p. free cancer cells had significant correlation with lymph node metastasis ($P = 0.003$) and histological type of poorly differentiated adenocarcinoma ($P = 0.029$; Table 2). Multivariate analysis revealed that lymph node metastasis was the independent factor related to the existence of i.p. free cancer cells after lymph node dissection (Table 3). There were two patients in whom CEA mRNA was detected in lavage fluid, despite neither the presence of lymph node metastasis nor lymphatic invasion. Therefore, we analyzed micrometastases by RT-PCR in the lymph nodes without histopathological cancer metastasis. Consequently, we identified CEA mRNA in one patient who had micrometastasis in the sentinel lymph nodes (Fig. 3). The number of metastatic lymph nodes was 2.57 ± 1.50 and 0.34 ± 0.80 in patients with the expression of mRNA-positive and -negative lavage, respectively ($P < 0.001$). Location of the metastatic lymph nodes in the patients with positive lavage was as follows: 7 nodes were along the lesser curvature, 4 were in the root of the left gastric artery, 2 were in the suprapyrolus, one was along the right gastroepiploic vessels, and one was along the common hepatic artery.

Ultra-rapid Quantitative RT-PCR Analysis of Lavage Fluid after EIPL. In all five patients with serosal tumors without macroscopic peritoneal dissemination, CEA mRNA and CK20 mRNA were detected in the lavage fluid at laparotomy. The serial extensive lavages of EIPL were analyzed by ultra-rapid quantitative RT-PCR analysis. A representative case is shown in Fig. 4. The quantitative analysis by LightCycler demonstrated that the number of free cancer cells in lavage fluids was serially diluted $3.8 \times 10^5 \pm 1.4 \times 10^5$ to 2.8 ± 1.5 cells/100 ml by 6 to 8 liters of saline. Eventually, CEA mRNA disappeared entirely in washing fluid after seven to nine washes.

DISCUSSION

Cytological analysis has been the gold standard for detecting cancer cells in the peritoneal lavage during operation. A close association has been demonstrated between positivity for peritoneal free cancer cells and low survival rate (3, 5, 10). However, cytological examination lacks sensitivity for detecting i.p. free cancer cells (31). Over the last decade, molecular approaches using the PCR technique have been applied to various clinical fields and have become one of the most useful diagnostic tools. In particular, RT-PCR-based screening methods, which target cancer cell-related mRNA, have developed. Although RT-PCR has a high sensitivity, false-positive results ensue, caused by the DNA contamination or pseudogenes, although this is infrequent. Broll *et al.* (22) reported that RT-PCR analysis had an unusually high detection rate caused by an illegitimate transcription and expression of CEA in macrophages, leukocytes, and hematopoietic cells, and that these cells are present primarily in the abdominal cavity by contamination during laparotomy. In the present study, however, both CEA mRNA and CK20 mRNA were not detected from lavage fluid at laparotomy, nevertheless it contained numerous blood cells. In addition, our system excluded cross-contamination by automatic mRNA extraction and replicated examinations. Furthermore, false-positive results could be averted when using target sequence-specific fluorescence-labeled hybridization probes and two overlapping markers (*i.e.*, CEA mRNA and CK20

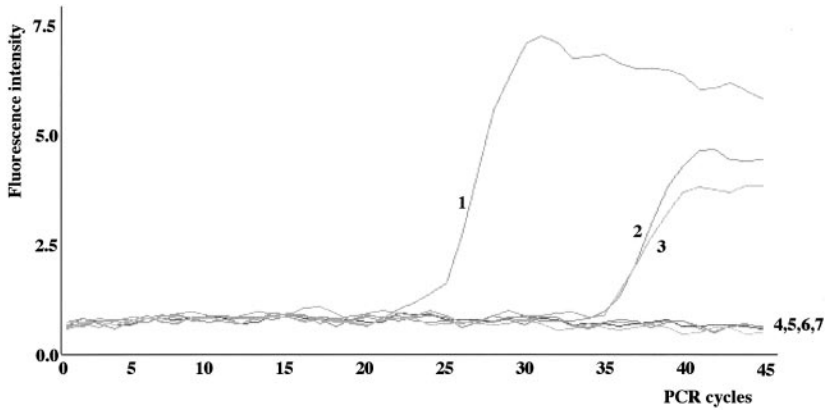

Fig. 3 Ultra-rapid RT-PCR for detection of micrometastasis in lymph nodes by LightCycler in a patient with SM tumor of poorly differentiated adenocarcinoma without pathological lymph node metastasis or lymphatic invasion. *Curve 1,* WiDr colon cancer cells; *curves 2* and *3,* sentinel lymph nodes along the lesser curvature; *curve 4,* a suprapyloric lymph node; *curves 5* and *6,* lymph nodes along the left gastric artery; *curve 7,* no template. There were two micrometastatic lymph nodes.

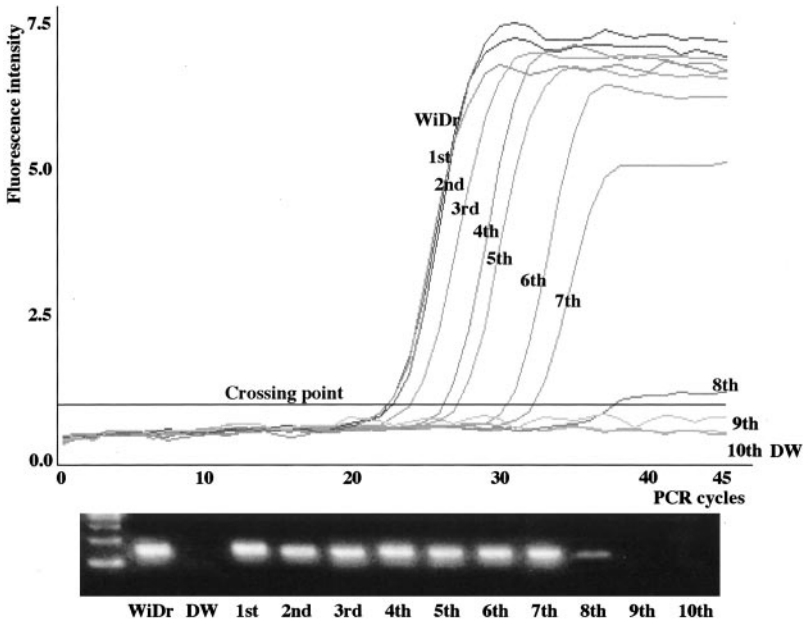

Fig. 4 Representative results of ultra-rapid quantitative RT-PCR of the serial extensive lavages by LightCycler. *Curves* and *Lanes 1st-10th* were 100-ml samples from the first to the tenth wash, each using 1 liter of saline. *WiDr* was a positive control containing 10^5 cells in 100 ml of saline, and *DW* contained no template. Crossing points were used to calculate the cell concentration of the samples with reference to the standard curve. The free cancer cells in the lavage fluids were serially diluted by 8 liters of saline and disappeared in washing fluids after the eighth wash.

mRNA) to detect cancer cells. Despite this technology, the possibility that noncancerous cells in the peritoneal cavity may express CEA mRNA or CK20 mRNA cannot be excluded. Conventional RT-PCR had another problem in that it was time-consuming and involved laborious procedures. In the present study, we succeeded in establishing an ultra-rapid detection system for i.p. free cancer cells. This new method, one-step real-time RT-PCR in combination with automated mRNA extraction, allowed rapid sample preparation, mRNA amplification, and on-line data analysis without the post-PCR steps. The measurement can be completed within approximately 70 min after sample collection. Accordingly, this new RT-PCR method can be a reliable and quick method to detect the minimal amount of i.p. free cancer cells, offering the possibility of a real-time clinical use.

Although curative surgery has been performed for patients with non-serosa-invasive gastric cancer, some patients die of peritoneal recurrence. One of the reasons postulated for peritoneal dissemination in non-serosa-invasive gastric carcinoma is that lymph node dissection might open lymphatic channels and spread viable cancer cells (9), although no convincing evidence has been presented for this supposition. The present study clearly revealed that free cancer cells were found in the lavage fluid after lymph node dissection of 14.3% and 26.7% of patients with SM and MP tumors, respectively. Statistical analysis demonstrated that lymph node metastasis was the independent predictor for the existence of i.p. free cancer cells after lymph node dissection. From our previous study for 1272 cases of gastric carcinoma between 1980 and 2000, 1 of 257 cases (0.4%) with SM tumor and 6 of 136 cases (4.4%) with MP tumor developed peritoneal metastasis after potentially curative operation (8). Among them, six patients (86%) had lymph node metastasis and/or lymphatic invasion. Nevertheless, in the present study, there were two cases who were pathologically negative for lymph node metastasis and lymphatic invasion, despite identification of CEA mRNA and CK20 mRNA in lavage fluid. One of the reasons could be that, just by happenstance, there

were no cancer cells in the sample slices for microscopic diagnosis for lymph nodes, although micrometastasis existed. However, we clearly detected the presence of cancer cells by RT-PCR in such lymph nodes. On the other hand, we could not detect CEA mRNA and CK20 mRNA in some cases with lymph node metastasis or lymphatic invasion. In these cases, it is assumed that the dissected lymphatic vessels were completely blocked up by surgical ligation or coagulation. Our results elucidated that lymph node dissection is the major factor to spread viable free cancer cells into the peritoneal cavity.

To date, there are no effective therapies for peritoneal carcinomatosis. Therefore, attention has been paid to detecting peritoneal free cancer cells in patients with advanced gastric carcinoma without overt peritoneal metastasis and to preventing peritoneal metastasis (3, 19–21). The existence of i.p. free cancer cells without macroscopic dissemination involves the condition where peritoneal implantation has not yet occurred. We have previously proposed that EIPL, *i.e.*, extensively repeated dilution and complete suction, is quite a formidable method for reducing the number of cells to potentially zero, just like the so-called "limiting dilution" (28). In the present study, we verified the effectiveness of EIPL by ultra-rapid quantitative RT-PCR. Sequential washing of i.p. free cancer cells of $3.8 \times 10^5 \pm 1.4 \times 10^5$/100 ml lavage decreased the number to 2.8 ± 1.5 cells by six to eight washes. Free cancer cells were not detected in washing fluid after that. Treatment with i.p. chemotherapy after EIPL may have the additional effect of preventing peritoneal metastasis by killing any remaining free cancer cells. We have reported that the peritoneal recurrence rate was substantially reduced by the combination of these procedures (28). This therapy can be applied to non-serosa-invasive gastric cancer with free cancer cells spilled during surgery by using the ultra-rapid detection method.

In conclusion, we clarified the development process of peritoneal recurrence after curative operation for gastric carcinoma in patients without serosal invasion and also established an ultra-rapid and quantitative diagnosis system to detect i.p. free cancer cells during operation at molecular level. Furthermore, we clearly demonstrated that the EIPL method was effective to ultimately reduce the number of i.p. free cancer cells. Combination of our rapid diagnosis system with the EIPL method and i.p. chemotherapy can play an important role for the prophylaxis of peritoneal recurrence.

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Mechanisms of Peritoneal Metastasis after Operation for Non-Serosa-invasive Gastric Carcinoma: An Ultrarapid Detection System for Intraperitoneal Free Cancer Cells and a Prophylactic Strategy for Peritoneal Metastasis

Takashi Marutsuka, Shinya Shimada, Kenji Shiomori, et al.

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