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**Hayao Nakanishi · Yasuhiro Kodera**
**Yoshitaka Yamamura · Kazuo Kuzuya**
**Tooru Nakanishi · Takayuki Ezaki**
**Masae Tatematsu**

# Molecular diagnostic detection of free cancer cells in the peritoneal cavity of patients with gastrointestinal and gynecologic malignancies

**Abstract** Free cancer cells exfoliated from cancer-invaded serosa contribute to peritoneal dissemination, the most frequent pattern of recurrence in patients with gastric and ovarian cancers. This study was designed to evaluate the prognostic significance of free cancer cells in peritoneal washes detected using the reverse transcriptase-polymerase chain reaction (RT-PCR) and cytology. RT-PCR analysis with primers specific for the carcinoembryonic antigen (CEA) gene was found to be more sensitive than cytology for detection of free tumor cells in the peritoneal washes, collected at laparotomy from 199 gastric carcinoma patients, with higher detection rates for each of the T-categories in the TNM classification. Six patients with synchronous and 5 with recurrent peritoneal dissemination were found among 25 advanced cancer patients with positive PCR and negative cytology results. Positive PCR results were significantly associated with poor survival of curatively resected advanced gastric carcinoma patients ($P < 0.001$). A rapid method for detecting CEA mRNA using the LightCycler and the dsDNA binding dye SYBR green I was also developed. The results obtained using this technique were essentially the same as those obtained using the conventional RT-PCR method. Furthermore, RT-PCR analysis with primers specific for MUC1 epithelial mucin were performed on peritoneal washes from patients with ovarian cancer. Peritoneal washes from 21 of 25 ovarian carcinoma patients, including all 17 with positive cytology results, were positive for MUC1 mRNA, again indicating a higher sensitivity using this method than conventional cytology. Highly sensitive and rapid detection of free cancer cells in peritoneal washes, most reliably by RT-PCR, is a powerful technique to predict peritoneal dissemination in patients with gastric and ovarian cancers.

**Key words** Peritoneal metastasis · Gastric cancer · Ovarian cancer · Peritoneal wash cytology · RT-PCR · LightCycler

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H. Nakanishi · M. Tatematsu
Laboratory of Pathology,
Aichi Cancer Center Research Institute,
Nagoya, Japan

Y. Kodera · Y. Yamamura
Department of Gastroenterological Surgery,
Aichi Cancer Center Hospital, Nagoya, Japan

T. Nakanishi · K. Kuzuya
Department of Gynecology,
Aichi Cancer Center Hospital, Nagoya, Japan

T. Ezaki
Department of Microbiology,
Gifu University School of Medicine, Gifu, Japan

H. Nakanishi (✉)
Laboratory of Pathology,
Aichi Cancer Center Research Institute,
1-1 Kanokoden, Chikusa-ku, Nagoya 464, Japan
e-mail: hnakanis@aichigw.aichi-cc.pref.aichi.jp
Tel.: +81-52-762-6111; Fax: +81-52-763-5233

## Introduction

Peritoneal metastasis is the type of recurrence observed most frequently after curative resection in patients with gastric and ovarian cancers in Japan and the West [2, 3]. Free cancer cells derived from serosal or capsular invasion might be an indicator of early peritoneal seeding with subsequent formation of metastatic colonies [4]. Therefore their detection is likely to be a useful tool for prediction of outcome in such cases.

Cytological examination of peritoneal washes has been the gold standard for assessment of peritoneal recurrence in gastric and ovarian cancer patients [5, 10, 22]. Nevertheless, some patients with negative cytology results die of peritoneal metastases after curative surgery [1]. Molecular approaches using the highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique have been developed; these allow

detection of carcinoma cells expressing epithelial cell-specific mRNA in the peripheral blood [6, 20], bone marrow [8], and lymph nodes [13, 17]. However, no molecular diagnostic detection methods have been reported for peritoneal washes until recently.

To increase the sensitivity of the conventional peritoneal lavage cytology, we have established an RT-PCR method using the gene encoding carcinoembryonic antigen (CEA) as a target to detect intraperitoneal tumor cells in gastric cancer patients. This method has been shown to be highly specific and sensitive [9, 15]. One problem with these molecular diagnostic techniques is that they are time-consuming and relatively laborious compared with conventional cytological methods. However, recent advances in PCR technology have allowed the time required for amplification and detection of specific mRNA to be reduced significantly [21].

In this paper, the prognostic relevance of sensitive molecular diagnostic detection of free cancer cells in the peritoneal cavity in patients with gastric and gynecologic malignancies is reported. Development of a rapid and convenient method for detecting specific mRNA in peritoneal washes using a capillary PCR technique is also reported. Finally, the clinical significance of selecting patients at high risk of peritoneal recurrence who might benefit from adjuvant therapy after surgery is discussed.

## Prognostic significance of free cancer cells in the peritoneal cavity detected by RT-PCR in gastric cancer patients

More sensitive detection of free tumor cells in the peritoneal cavity can be achieved through amplification of CEA mRNA using RT-PCR. CEA was first confirmed to be present in all gastric and colorectal cancer cell lines examined, irrespective of the degree of differentiation, and absent from blood and mesothelium, demonstrating the specificity of this approach for detection of carcinoma cells in peritoneal lavage fluids. In a sensitivity test, CEA RT-PCR proved to be capable of detecting as few as 10 carcinoma cells per sample [15]. As shown in Fig. 1, all patients with positive cytology results and some with negative cytology results were positive for CEA mRNA. In contrast, none of 5 patients with benign disease was positive.

The positivity rate with the RT-PCR assay correlated significantly with the depth of invasion in terms of the t number, ranging from 6% at the t1 stage to 93% at the t4 stage. At all stages, the positivity rate with the PCR assay was significantly higher than with conventional cytology, confirming the greater sensitivity of this method [9].

Prognostic analysis was performed with peritoneal recurrence as the endpoint. A close association between detection of free cancer cells and eventual peritoneal metastasis, with emphasis on the fate of 30 patients with negative cytology and positive PCR, is indicated by the

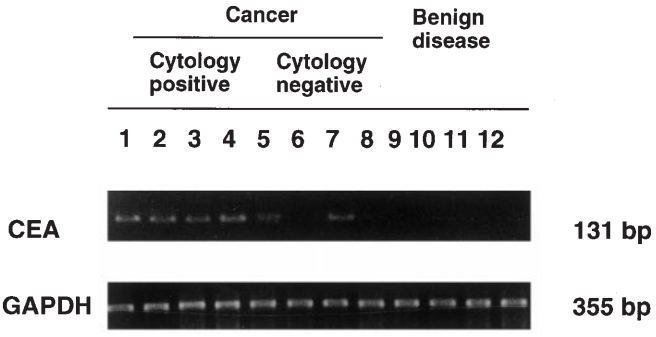


**Fig. 1** Representative RT-PCR results for peritoneal washes from patients with gastric cancers. Specific 131-bp CEA bands are evident in all washes from cytology-positive patients (*lanes 1–4*) and 2 of 4 washes from cytology-negative patients (*lanes 5–8*), but not washes from patients with benign disease (*lanes 9–12*). The integrity of extracted RNA was confirmed by RT-PCR amplification of GAPDH mRNA

Kaplan-Meier curves (Fig. 2). Six patients with synchronous and 5 patients with recurrent peritoneal metastasis were identified among 25 advanced cancer patients with negative cytology and positive PCR. Of the 139 patients with negative PCR results, 3 patients had peritoneal dissemination at the time of surgery and free cancer cells in these patients may have been overlooked even by the PCR. Intraperitoneal recurrence has so far been observed in 2 of 139 patients with negative PCR results. Both of these patients had extensive hepatic metastases at the time of surgery, and postoperative spread from the hepatic lesions to the diaphragm or peritoneum might have been the source of free cancer cells. In contrast, of the 169 patients with negative cytology results, 9 had peritoneal metastasis at the time of surgery and 8 were diagnosed with recurrences with peritoneal dissemination shortly after surgery. Thus the

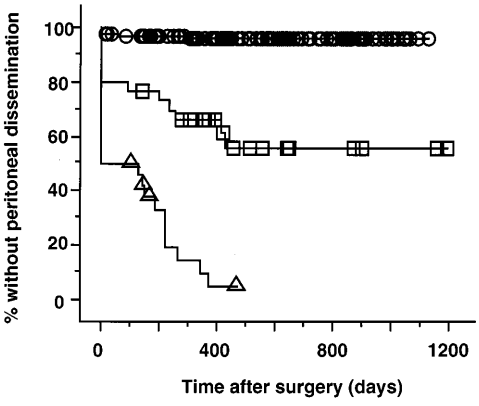


**Fig. 2** Synchronous peritoneal metastasis and peritoneal recurrence observed among patients positive and negative for RT-PCR and cytology, respectively. Curves were drawn by the Kaplan-Meier method with peritoneal dissemination as the endpoint. Death due to other types of recurrence without peritoneal dissemination were treated as censored. ○ PCR negative ($n$ = 139); □ PCR positive, cytology negative ($n$ = 30); △ PCR positive, cytology positive ($n$ = 30)

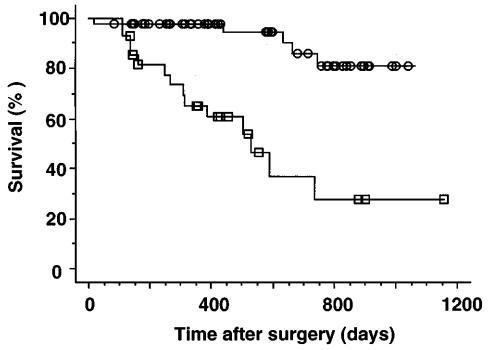


**Fig. 3** Survival of 82 curatively resected advanced gastric cancer patients positive and negative for free cancer cells by CEA RT-PCR analysis of peritoneal washes. The difference is statistically significant ($P < 0.01$). ○ PCR negative ($n = 53$); □ PCR positive ($n = 29$)

incidence of false negatives may be lower with the PCR method than with conventional cytology.

Free cancer cell detection by RT-PCR analysis was significantly associated with poor prognosis in advanced gastric cancer patients. The prognostic value of positive PCR results remained significant in the 82 patients who underwent potentially curative surgery (Fig. 3). These results indicate that there is true residual disease in this subset of patients with negative cytology and positive PCR, although the possibility cannot be excluded that positive free cancer cells reflect transient cell shedding from the primary tumor and not minimal residual disease in some cases [18]. Thus detection of free cancer cells in peritoneal washes, most reliably by RT-PCR, has promise as a predictor of poor prognosis in patients with gastric cancer. While our results revealed that conventional cytologic examination was also relatively effective, the sensitivity of peritoneal cytology reportedly varied greatly among institutions, ranging from 16% to 43% in patients with gastric carcinoma involving the serosa without peritoneal dissemination [1, 3, 22]. Therefore we recommend RT-PCR analysis of peritoneal washes as a highly sensitive and reproducible procedure.

## A rapid method for detection of CEA mRNA in peritoneal washes using a LightCycler

One difficulty with the sensitive CEA RT-PCR method is that it is relatively time-consuming and laborious. If it were possible to detect CEA mRNA in peritoneal washes during surgery, more useful information on the choice of optimal surgery would be available. To overcome this problem, we developed a rapid and convenient assay for detection of CEA mRNA using a LightCycler (Idaho Technology, Idaho Falls, ID, USA), which is a rapid thermal cycler, combined with a microvolume fluorimeter. Figure 4 illustrates real-time monitoring of a PCR reaction with the LightCycler using the SYBR green I dye, which binds preferentially to dsDNA. Fluorescent signals are proportional to the concentration of

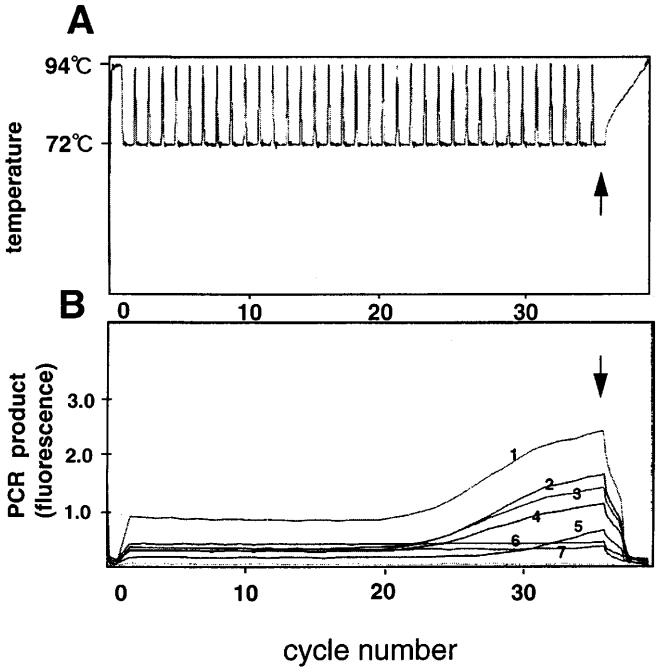


**Fig. 4A,B** Representative LightCycler results for peritoneal washes from gastric cancer patients. After CDNA synthesis from total RNA, nested primer RT-PCR was performed with the LightCycler. The second-round PCR reactions are monitored in real time with the LightCycler using SYBR green I dye. (**A**) Run profile of temperature vs PCR cycle. The reactions were cycled 35–40 times with a 94°C denaturation for 0 s and 72°C combined annealing/extension for 15 s (*arrow*), followed by cooling the sample to 72°C and then increasing the temperature to 95°C to obtain melting curves. (**B**) Run profile of fluorescence vs cycle. Amplification of CEA mRNA in peritoneal washes from RT-PCR-positive patients (*lines 2–5*) and from a RT-PCR-negative patient (*line 6*) are evident as increasing fluorescence signal with time. *Line 1* and *line 7* are positive and negative controls, respectively. At the end of amplification (*arrow*), melting curves are produced and the specificity of the product is confirmed based on the pattern of the curves or Tm of the PCR product

the product and can be measured once per cycle and immediately displayed on a computer screen, permitting real-time monitoring of the PCR reaction [21]. This technique enables 35 cycles to be performed in less than 20 min.

After amplification, the temperature is slowly elevated above the melting temperature (Tm) of the PCR product to measure the fluorescence for melting curve. This permits the identification of specific CEA transcripts because specific and nonspecific products have different Tms depending on their nucleotide composition [14]. The process can be completed in approximately 2 h without gel electrophoresis.

The LightCycler findings for peritoneal washes from 18 patients with gastric cancers are summarized in Table 1. Peritoneal washes from 6 of 13 patients with advanced gastric cancer proved positive for CEA mRNA, whereas none of 5 patients with early gastric cancer was positive. This result is essentially identical to that of conventional RT-PCR, confirming that the rapid method employing the LightCycler is reliable. In

**Table 1** LightCycler results for peritoneal washes from gastric cancer patients

| Cancer stage | LightCycler | RT-PCR | Cytology |
|---|---|---|---|
| Early | 0/5 | 0/5 | 0/5 |
| Advanced | 6/13 | 5/13 | 3/13 |

addition to being a good prognostic indicator, rapid and convenient detection of minimal residual disease in the peritoneal cavity with the LightCycler may have additional therapeutic implications.

## Detection of free cancer cells in the peritoneal cavity by RT-PCR in ovarian cancer patients

Since ovarian cancers are well known to be sensitive to several kinds of anticancer agent [11, 12], sensitive detection of micrometastasis may be of therapeutic value for patients with this disease. We compared the sensitivity and specificity of RT-PCR assays using primers for various epithelial cell-specific marker genes. Epithelial mucin, encoded by the MUC1 gene, is a large, heavily glycosylated molecule that shows a high level of expression in many types of epithelial cancer, including ovarian cancer [7]. MUC1 transcripts [16], but not CEA and cytokeratin 20 (CK20) mRNA were found to be weakly expressed by leukocytes and mesothelial cells, indicating that CEA and CK20 have higher specificity than MUC1 for RT-PCR. However, the frequency of CEA and CK20 expression in ovarian cancer tissues is 72% and 39%, respectively, and is low compared with MUC1 (89%). Moreover, we found that the MUC1 positivity rate in peritoneal washes from patients with early gastric cancers, used as negative controls, is low (8%) and similar to that with CEA RT-PCR (6%). This is probably because contamination of peritoneal washes with leukocytes is usually less extensive and suggests that the incidence of false positive results with the MUC1 RT-PCR assay for peritoneal washes obtained from ovarian cancer patients is relatively low if present. From these results, we selected MUC1 as the target gene for RT-PCR for ovarian cancers in this study.

RT-PCR analysis of 25 patients with epithelial ovarian cancers yielded 21 positives (84%) and cytology 17 (68%). This indicates the greater sensitivity of RT-PCR. As shown in Fig. 5, the positivity rate with the MUC1 RT-PCR assay tended to increase with clinical stage by the FIGO classification, ranging from 5 of 7 (71%) at stage I, to one of one (100%) at stage II, 11 of 13 (85%) at stage III, and 4 of 4 (100%) at stage IV. The detection rate with RT-PCR was the same as or slightly higher than that with cytology for each category. It has been reported that negative peritoneal cytology in patients with peritoneal dissemination is relatively common, occurring in 22–85% of patients with ovarian cancers [19]. Therefore MUC1 RT-PCR is more advantageous with respect to sensitive detection of free

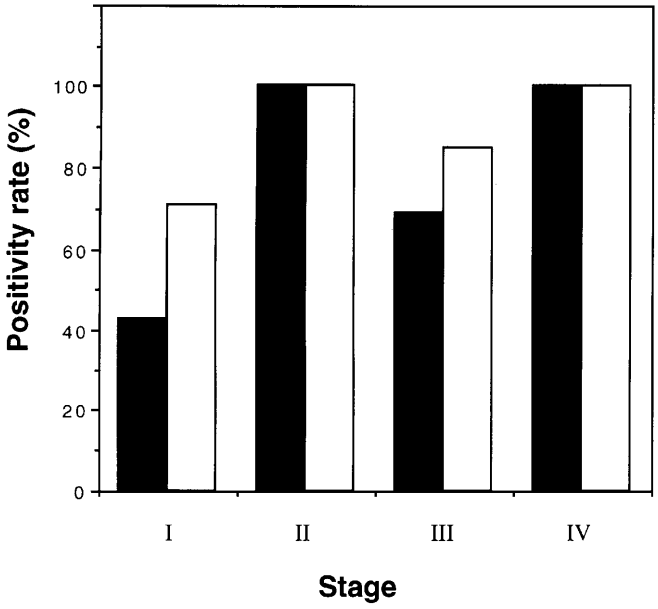


**Fig. 5** Free cancer cell detection rate by RT-PCR analysis using primers specific for MUC1 and cytology in ovarian cancer patients stratified according to clinical stage. ■ cytology; □ RT-PCR. Stage I, $n = 7$; stage II, $n = 1$; stage III, $n = 13$; and stage IV, $n = 4$

cancer cells in the peritoneal cavity of patients with advanced ovarian cancers as well as early (stage I) ovarian cancers. A clinical follow up study is now ongoing in our hospital to elucidate the prognostic significance of this method.

## Conclusions

In addition to being a good prognostic indicator, detection of minimal residual disease in the peritoneal cavity in gastric and ovarian cancer patients might have additional therapeutic implications. While attempts to improve outcome for patients with macroscopic peritoneal metastasis have failed [24], the survival of gastric cancer patients with serosal invasion, but without macroscopic evidence of peritoneal dissemination is significantly improved by intraperitoneal chemotherapy [23]. Adjuvant chemotherapy is a costly procedure with no benefit for those without residual disease and is not without toxicity and adverse side effects. Therefore highly sensitive and rapid detection of free cancer cells in the peritoneal cavity can be of great value in identifying patients who may benefit from adjuvant therapy.

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