Abstract. Background: Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for detection of carcinoembryonic antigen (CEA) mRNA in the peritoneal lavage of gastric cancer patients is now recognized as a useful method for the prediction of peritoneal recurrence after curative surgery. One problem with this method is that it is time-consuming and difficult to perform an intraoperative diagnosis, which is essential for intraperitoneal adjuvant chemotherapy. Patients and Methods: In order to overcome these problems, we introduced a transcription-reverse transcription concerted reaction (TRC), which is a non-PCR-based, isothermal mRNA amplification method, as an ultra-rapid diagnostic method, and compared its diagnostic power with qRT-PCR for peritoneal washes from 112 gastric cancer patients. Results: TRC measurement could be completed within 1.0-1.5 h and showed the same detection sensitivity ranging from 10^2 to 10^6 copies for standard CEA mRNA as qRT-PCR. The CEA mRNA copy number, as determined by TRC, was well correlated with the depth of tumor invasion (pT category), similar to the result obtained using qRT-PCR. With CEA mRNA copy numbers of 100 as a TRC cut-off value, the resultant sensitivity and specificity of TRC (85% and 100%, respectively) were higher than for cytology (62%, 100%) and comparable to qRT-PCR (92%, 100%). Conclusion: TRC has a diagnostic power almost equivalent to qRT-PCR but with the advantage of ultra-rapid detection. TRC would therefore be available for intraoperative sensitive diagnosis of occult tumor cells in the peritoneal cavity of gastric cancer patients.

The prognosis for advanced gastric cancer has not improved drastically despite recent advances in multimodal treatment strategy. Peritoneal carcinomatosis is the most frequent pattern of recurrence after curative surgery, and therefore the most important prognostic factor (1-3). We previously demonstrated from animal models of peritoneal micrometastasis that only early onset of chemotherapy targeting micrometastasis could effectively eliminate peritoneal metastasis and improve survival of mice remarkably, or effect a complete cure in some animals (4, 5). Therefore, development of a sensitive detection method for a small number of cancer free cells or micrometastases in the peritoneal cavity is essential for the prevention of peritoneal relapse and for establishing individualized therapy for high-risk populations of gastric cancer patients who may benefit from adjuvant chemotherapy after macroscopically complete surgical resection.

Cytological examination of the peritoneal washes sampled for the prediction of peritoneal recurrence is already an established prognostic factor (6, 7). However, conventional cytology lacks sensitivity and peritoneal recurrence has been predicted in only 50% of patients by this modality (8-10). This is because of the difficulty of detecting a small number of tumor cells at the micrometastasis level in the peritoneal washes. Qualitative and quantitative RT-PCR (qRT-PCR) with carcinoembryonic antigen (CEA) as a genetic marker is recognized to be the most reliable method to allow such sensitive detection (11-13). Cumulative evidence from many retrospective studies, as well as a prospective study, indicate...
that this method is more sensitive and useful for the prediction of peritoneal recurrence after curative surgery than conventional cytology in gastric cancer patients (14-17). In a limited number of facilities in Japan, it is now clinically applied as a diagnostic tool (17, 18). One problem with this method is that it is still time-consuming and complete measurement is not possible within the 1-2 h required for decision-making during surgery, e.g., the setting or not of a reservoir port for postoperative intraperitoneal chemotherapy.

Recently, ultra-rapid methods for the sensitive detection of particular microorganisms or a small number of cancer cells have been developed (19, 20). These include one-step RT-PCR (21), and non PCR-based, RNA-specific amplification methods, such as the transcription-reverse transcription concerted method (TRC) (22) and the nucleic acid sequence-based amplification method (NASBA) (23). TRC is an isothermal direct RNA amplification method which consists of a sequence of steps including cDNA synthesis with reverse-transcriptase, double-stranded DNA (dsDNA) synthesis by DNA polymerase activity of reverse-transcriptase, and subsequent transcription (mRNA amplification) of promoter-bearing dsDNA with T7 RNA polymerase. Detection of amplified CEA mRNA is achieved with a CEA-specific intercalation activating fluorescence (INAF) hybridization probe which is a DNA oligomer linked with a fluorescent dye, oxazole yellow, and allows homogeneous real-time monitoring of mRNA amplification (24). Advantages of TRC over qRT-PCR include no need for cDNA synthesis and subsequent thermal cycling for amplification thus enabling ultra-rapid detection of CEA mRNA in a single tube for approximately 60 minutes, leading to an intraoperative genetic diagnosis (20). In the present study, we introduce the TRC method for the intraoperative quantitation of CEA mRNA in the peritoneal washes from gastric cancer patients and evaluate its diagnostic power in comparison with qRT-PCR using peritoneal wash samples.

Patients and Methods

Patients. From December 2004 to December 2005, 112 gastric cancer patients who underwent laparotomy at the Aichi Cancer Center Hospital and Nagoya University Hospital with written informed consent were enrolled in this study for analyses of CEA mRNA in the peritoneal washes with both the TRC and qRT-PCR methods. Cytological examination with Papanicolaou staining was also performed. There were 74 males and 38 females, with ages ranging from 34 to 85 years. The depth of cancer invasion (pT category) was evaluated histologically according to the TNM classification (UICC classification for gastric cancer). Of the 112 patients, there were 26 patients with T1 (mucosal to submucosal invasion), 44 with T2 (muscularis propria to subserosal invasion), 35 with T3 (serosal invasion), and 7 with T4 (invasion to adjacent tissues) stage tumors. Thirteen patients with synchronous peritoneal metastasis were included in the population. No patients with synchronous liver or distant metastasis were enrolled in this group.

Peritoneal washes. At the beginning of each laparotomy, 100 ml saline was introduced into the Douglas cavity and left subphrenic space and aspirated after gentle stirring. A half of each sample was sent to the Division of Cytology at the Central Clinical Laboratory, Aichi Cancer Center Hospital for routine cytopathology with conventional Papanicolaou staining. The other half was sent to the Division of Oncological Pathology, Aichi Cancer Center Research Institute for measurement of CEA mRNA levels.

Intact cells collected from the lavages by centrifugation at 1,800 rpm for 5 min were rinsed with phosphate-buffered saline (PBS), and one half of the cell fraction was dissolved in ISOGEN-LS RNA extraction buffer (Nippon gene, Tokyo, Japan) and the remaining half in RNA extraction buffer of RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and stored at –80°C until analysis.

RNA extraction. Total RNA from samples dissolved in ISOGEN-LS was extracted using the guanidinium isothiocyanate-phenol-chloroform method as described elsewhere (13). In brief, 1.0 ml of the sample solution in ISOGEN-LS was mixed with 0.2 ml of chloroform and centrifuged at 15,000 rpm for 10 min. Subsequently 0.5 ml of supernatant was transferred to a fresh tube and mixed with an equal volume of 100% isopropyl alcohol. RNA was precipitated by centrifugation, washed with 75% ethanol and was dissolved in RNase-free water after drying with vacuum centrifugation. Since cells are usually few in wash fluids, 2 µl of glycogen solution (20 mg/ml) (Boehringer Mannheim, Germany) per tube was added as a carrier to improve RNA recovery before isopropanol precipitation. RNA was also extracted with the RNeasy Mini Kit (Qiagen) in the cases of ultra-rapid measurement according to the manufacturer’s instruction. The entire procedure takes approximately 40-60 min depending on the RNA extraction method. The quality and quantity of isolated RNA were checked using spectrophotometry. A part of this total RNA preparation was directly used for the TRC method and the remainder in the synthesis of cDNA for the real-time quantitative RT-PCR with the LightCycler as described below.

TRC reaction. The principle of direct amplification of specific mRNA by TRC is schematically represented in Figure 1. A scissor probe is used to initiate TRC reaction and the promoter and antisense primers are the pair of primers for amplification. An INAF probe is used to detect the mRNA amplicons. Sequences of primers and probes used in this study for amplification and detection of CEA mRNA are listed in Table I. The TRC reaction was carried out based on the protocol as described elsewhere (20) and was performed using TRCRtest CEA-m (Tosoh, Tokyo, Japan) with slight modification in this study.

The procedure is briefly as follows: 10 µl of substrate solution containing deoxynucleoside triphosphate, nucleoside triphosphate and inosine triphosphate and 10 µl of primer/probe sets containing scissor probe, antisense primer, promoter primer and INAF probe were mixed well and added to 5 µl of the RNA samples with unknown concentration and the standard mRNA solutions in a thin-walled PCR tube, followed by pre-warming at 43°C for 5 min. Five microliters of the enzyme mixture containing avian myeloblastosis virus (AMV) reverse transcriptase and T7 RNA polymerase were pre-warmed at 43°C for 2 min and then added to the reaction mixture for incubation at 43°C in a TRC real-time monitor instrument (TRCRapid-160, Tosoh, Tokyo, Japan) to enhance the enzyme reaction and monitor the fluorescence of the
reaction mixture simultaneously (excitation wavelength, 470 nm and emission wavelength, 520 nm).

Each run consisted of patient samples with unknown CEA mRNA concentrations (up to 12 samples), a negative control without a template and CEA mRNA standards. Standard mRNA containing the near full-length CEA mRNA was synthesized via in vitro transcription of promoter bearing double-stranded DNA as a template with SP6 RNA polymerase. Two external CEA mRNA standards (low copy standard: 1x10^2 copies and high copy standard: 1x10^6 copies) were used for making a calibration curve. Quantitation of CEA mRNA in each sample was performed automatically by reference to this standard curve constructed each time using the TRCR-160 software. With this software, the samples calculated below 10 copies are displayed <10, because the dynamic range for the detection of CEA mRNA was from 10^2 to 1x10^6 copies.

Real-time quantitative RT-PCR. cDNA was synthesized from total RNA using random hexanucleotide primers (Pharmacia, Biotech, Uppsala, Sweden) and SuperScript II RNase H-reverse transcriptase (Invitrogen, Carshad, CA, USA) according to the manufacturer’s instructions. The resultant first-strand cDNA was stored at –80°C until analysis.

Single-step real-time RT-PCR for CEA mRNA was performed using CEA-specific oligonucleotide primers and two fluorescent hybridization probes on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described elsewhere (13). To quantify and demonstrate the integrity of the isolated RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also analyzed with real-time RT-PCR using the appropriate primers and

<table>
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<th>Table I. Oligonucleotide sequence of promoter primer, antisense primer, scissor probe and INAF probe for TRC method used in this study</th>
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<tr>
<td>Promoter primer (1508-1530)(^a) 23 mer</td>
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<tr>
<td>5’-AAT TCT AAT ACG ACT CAC TAT AGG GAG ACC AAC ATC ACT GAG AAG AAC AGC-3’</td>
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<tr>
<td>Antisense primer (1668-1687) 20 mer</td>
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<tr>
<td>5’-GTT CAC AGG TGA AGG CCA CA-3’</td>
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<td>Scissor probe (1490-1513) 24 mer</td>
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<tr>
<td>5’-TGT TGG AGA TAA AGA GCT CTT GTG-3’</td>
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<tr>
<td>INAF probe (1582-1601) 20 mer</td>
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<tr>
<td>5’-ACT GTG ATT GTC TTG ACT GT-3’</td>
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\(^a\)Numbers in parentheses indicate corresponding position of the target genome sequences (GeneBank Accession No. M29540); sequence of the promoter primers in italics is the T7 RNA polymerase binding sequence.
hybridization probes. Samples with low GAPDH mRNA levels (4 of 224) were discarded from this analysis. All primers and probes were synthesized and purified using reverse-phase HPLC at Nihon Gene Research Laboratories (Sendai, Japan). Six external CEA mRNA standards were prepared by 10-fold serial dilution (1-10⁵ cells) of equivalent to 1x10⁶ COLM-2 cells (CEA high expressing colon cancer cell line) spiked into 1x10⁷ peripheral blood leucocytes. Each run consisted of six external standards, a negative control without a template and patient samples with unknown mRNA concentrations. The amount of mRNA in each sample, which is relative mRNA value, not absolute mRNA copy number because of the use of cells as CEA mRNA standards, was then automatically measured by reference to the standard curve constructed each time using the LightCycler software. The higher CEA mRNA value of two washes (Douglas cavity and subphrenic space) from each patient was selected.

A cut-off value (0.1) for CEA mRNA as measured by qRT-PCR was previously determined based on the ROC (Receiver Operating Characteristic) curve analysis performed in a retrospective study of gastric cancer patients (17). A CEA mRNA value more or less than 0.1 was judged as positive or negative for qRT-PCR, respectively. The rationale of this cut-off value of CEA mRNA to select high-risk patients for peritoneal relapse was validated by a prospective study of gastric cancer patients (17).

Statistical analysis. Statistical comparison was made using Student’s t-test and Fisher’s exact probability test. Spearman’s rank correlation was introduced to assess the significance of group associations. A p-value of <0.05 was considered significant. The statistical analysis was performed using StatView software (SAS Institute Inc, Cary, NC, USA).

Results

Amplification profile of CEA mRNA using TRC. The amplification profile of the TRC method with time measuring standard CEA mRNA ranging from 1x10 to 1x10⁶ copies is shown in Figure 2A. The negative control gave no rise from the baseline fluorescence and 1x10 copy of CEA mRNA proved to be an unreproducible result depending on the assay time. The time to reach the value of 1.2 times the starting fluorescent intensity (reaction-time) was calculated from this profile. The standard curve was then constructed by plotting the log of the CEA mRNA copy number against the respective reaction-time (Figure 2B). This calibration curve shows a log-linear relationship between the two parameters ranging from...
1x10^2 to 1x10^6 copies of CEA mRNA, indicating the proof of quantification of sample with unknown messages by this TRC method.

Comparison of detection sensitivity and time between TRC and qRT-PCR. Standard CEA mRNA (1x10^6 copies) and cDNA synthesized from this mRNA preparation with reverse transcriptase were serially diluted (1x10^-1 to 1x10^-6 copies) and then subjected to measurement with TRC and qRT-PCR, respectively. Figure 2C shows the log-linear correlation of CEA mRNA values measured by both TRC and qRT-PCR ranging from 1x10^2 to 1x10^6 copy, indicating almost the equivalent detection sensitivity and dynamic range of both methods. Total assay time for qRT-PCR, including pretreatment of peritoneal washes (10 min), RNA extraction and cDNA synthesis (90 min), along with amplification and subsequent real-time data analysis (70 min), was approximately 3 hours, whereas the entire reaction time with TRC, including pretreatment of peritoneal washes (10 min), RNA extraction (30-50 min), amplification and subsequent real-time data analysis (20 min), was only 1.0-1.5 hours, less than half the period required for qRT-PCR.

CEA mRNA level in peritoneal washes according to depth of tumor invasion (pT category). The average CEA mRNA copy numbers of the peritoneal washes as measured by TRC (T1: 7.2, T2: 1,700, T3: 105,027 and T4: 147,051) were well correlated with the depth of tumor invasion (Figure 3A), similar to the average CEA mRNA values (T1: 0.29, T2: 14.4, T3: 1,755 and T4: 1,368) as determined using qRT-PCR (Figure 3B). Median CEA mRNA copies as assessed using TRC and qRT-PCR were (T1, T2, T3: 0 and T4: 37.18), respectively. The mean relative CEA mRNA value of peritoneal washes as measured with TRC in the 9 mucosa-confined gastric cancer patients, which were considered clinically benign in terms of peritoneal metastasis and therefore as negative controls in this study, was 11.9±28.5 (SD). No CEA mRNA was detected using TRC in the peripheral blood leukocytes from 10 healthy volunteers or primary human cultured mesothelial cells, the 2 major cellular constituents in peritoneal washes. CEA mRNA values of the patients with synchronous peritoneal metastasis with TRC ranged from 10 to 1,000,000.

Cut-off value of the TRC method. In the present study, the cut-off value of CEA mRNA copies with the TRC method
(100) was calculated using the formula (mean±2xSD) of the negative control patients along with the dynamic detection range (10^2-10^6 copies). Any samples with a CEA mRNA copy number below 100 were classified as negative. With this cut-off value, the positivity rate for CEA mRNA using TRC was 0%, 6.8%, 48.6%, and 42.9% for T1, T2, T3 and T4 gastric cancer patients, respectively, whereas the positivity rate for CEA mRNA as measured using qRT-PCR was 11.5% for T1 (0% for mucosa-confined cancer), and 20.5%, 48.6% and 85.7% for T2, T3 and T4 gastric cancer patients, respectively. On the other hand, the positivity rate for cytology was 0% for T1 and T2, 25.7% for T3 and 42.9% for T4. Thus, the specificity of cytology and the TRC and qRT-PCR methods determined for negative controls (mucosa-confined cancers) was 100% for all. Furthermore, based on the results obtained from macroscopic peritoneal metastasis-positive patients, the sensitivity of conventional cytology, TRC and qRT-PCR was calculated to be 61.5%, 84.6% and 92.3%, respectively (Figure 4 and Table II).

Comparison of CEA mRNA values in peritoneal washes of gastric cancer patients between TRC and qRT-PCR methods. CEA mRNA values of all 224 peritoneal washes (Douglas cavity and left subphrenic space) from 112 patients measured by both the TRC and qRT-PCR methods are represented in Figure 5A. In the wash samples that were judged double positive for both methods, the CEA mRNA copies determined using TRC was significantly correlated with those assessed by qRT-PCR (R²=0.744, p<0.0001) (Figure 5B). Concordance of the overall results obtained from two methods was 83.9% (94/112 patients). Fifteen discordant patients who were TRC-negative and qRT-PCR-positive included 3 T1, 6 T2, 3 T3 and 3 T4 stage gastric cancer patients, in which 2 of 3 patients with
serosa positive cancer (T3) had simultaneous peritoneal metastasis. Another 3 discordant patients who were positive with TRC and negative with RT-PCR were patients with T3 stage cancer, one of whom had simultaneous peritoneal metastasis (Figure 5A and Table III).

Discussion

Sensitive detection of free cancer cells in the peritoneal washes using RT-PCR has now been recognized as a more powerful method for risk assessment of peritoneal recurrence after curative surgery than conventional cytology in gastric cancer (25). However, qRT-PCR still has the following shortcomings: i) cDNA synthesis and subsequent amplification using thermal cycling are so time-consuming that the results are not available during surgery, and ii) the instruments for qRT-PCR are relatively costly, the procedure is somewhat laborious, and therefore still limited to use at general hospitals with research institutions. TRC is one potential modality to overcome these problems. It was first introduced for ultra-rapid genetic diagnosis of occult cancer cells of gastric cancer patients by Ishii et al. (20) and offers high detection sensitivity. However, a detailed comparative analysis of TRC and qRT-PCR with a large number of peritoneal wash samples has not previously been meticulously explored. In the present study, we analyzed peritoneal washing samples collected from 112 patients with early and advanced gastric cancer with both the TRC and qRT-PCR methods. TRC proved to be much faster than qRT-PCR, close to intraoperative conventional cytology. The sensitivity and specificity of the TRC method (85% and 100%, respectively) was superior to cytology (62%, 100%) and was comparable to qRT-PCR (92%, 100%), indicating its availability as an intraoperative sensitive tool for genetic diagnostics in case of peritoneal micrometastasis. However, several points must be discussed and improved in the TRC method.

Determination of the cut-off value of CEA mRNA with TRC is one such issue. We previously determined the optimal cut-off value of CEA mRNA for qRT-PCR using ROC curve
analysis which was constructed by plotting sensitivity/1-specificity pairs based on the prognostic data (peritoneal recurrence) from the retrospective training set (17). However, prognostic data are not yet available in the present study, including that for peritoneal recurrence after surgery. Thus, we here temporarily determined the CEA mRNA cut-off value (100) based on the mean±2xSD (standard deviation) of control samples derived from patients with mucosa-confined gastric cancer patients instead of true benign counterparts (13, 15). The resultant sensitivity and specificity is almost equivalent to those determined with qRT-PCR. Though further study is needed to determine the optimal cut-off value of CEA mRNA with TRC that could more adequately evaluate the peritoneal recurrence risk based on the ROC curve analyses after several years follow-up, this preliminary cut-off value can be considered not far off the mark.

Another issue is the concordance between the results of the TRC and qRT-PCR methods for the detection of CEA mRNA. Agreement between methods such as TRC(+) qRT-PCR(+) and TRC(−)/qRT-PCR(−) were observed in 83.9% cases, and CEA mRNA values in the former cases showed a good correlation between the two methods as reported previously (20). However, there is some discrepancy between the two methods. Discordant cases including TRC(−)/qRT-PCR(+) and TRC(+) qRT-PCR(−) were observed in 13.4% (15/112) and 2.7% (3/112) among the total 112 patients, respectively. TRC(−)/qRT-PCR(+) cases, a major type of discordance, may be mainly due to the presence of possible false-positive results with qRT-PCR against 3 T1 patients and false-negative results with the TRC method against 2 peritoneal metastasis-positive patients. In one of the two latter TRC(−)/qRT-PCR(+) p(+) cases, the amount of mRNA obtained from the peritoneal wash sample was very small. Therefore, it seems possible that the false-negative results with TRC may be due to greater difficulty in amplifying mRNA by RNA polymerase from a very small copy of an initial mRNA template than qRT-PCR, which can amplify a cDNA template two-fold each cycle. However, there were no cytology and/or peritoneal metastasis-positive patients with double negative results with TRC and qRT-PCR, therefore, such false-negative results can be reduced or eliminated by the complementary use of both methods.

Intraoperative sensitive diagnosis for peritoneal metastasis is essential for the intraperitoneal adjuvant chemotherapy (26, 27), because decision making is required during the operation as to whether or not to place a reservoir for repeated intraperitoneal administration of the anticancer drug (28, 29). We previously demonstrated that micrometastasis is more sensitive to anticancer drugs such as oral S-1, a 5-FU derivative, than gross metastasis (4). We also demonstrated high efficacy of intraperitoneal paclitaxel rather than intravenous paclitaxel on the peritoneal micrometastasis using GFP-tagged human gastric cancer micrometastasis model (5), suggesting that peritoneal micrometastasis as detected by sensitive genetic diagnosis in the absence of macroscopic peritoneal deposits is a good target for anticancer treatments, especially intraperitoneal chemotherapy, besides being a prognostic factor. The efficacy of intraperitoneal cisplatin and paclitaxel was also reported in clinical ovarian cancer whose common pattern of disease failure is also peritoneal carcinomatosis (30, 31). These findings suggest the possibility that early onset of intraperitoneal chemotherapy following sensitive genetic diagnosis of micrometastasis leads to a breakthrough in the protection of gastric cancer patients from peritoneal recurrence. To examine such a possibility, a phase I clinical trial to explore a maximal tolerated dose of oral S-1 to be given in combination with intraperitoneal paclitaxel is currently ongoing in our institute.

Conclusion

We confirmed that the TRC method is sufficiently rapid and sensitive. TRC is therefore now available as an intraoperative sensitive diagnostic tool for micrometastasis in the peritoneal cavity for clinical trial of intraperitoneal chemotherapy. A multi-disciplinary approach with surgery and intraperitoneal chemotherapy may well be a potential breakthrough for the prevention and treatment of peritoneal carcinomatosis following curative resection of the primary cancer.

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