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Rapid detection of CEA mRNA in peritoneal washes using One-Step Nucleic acid Amplification (OSNA®) for gastric cancer patients

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ABSTRACT

Background: Carcinoembryonic antigen (CEA) mRNA expression in peritoneal washes from gastric cancer patients has been reported as an indicator for survival or peritoneal recurrence. The whole process of CEA mRNA detection is time- and labor-intensive. We report the potential of One-Step Nucleic acid Amplification (OSNA) as a rapid and simple system for CEA mRNA detection in peritoneal washes.
Methods: A total of 128 peritoneal washes were analyzed by cytological examination including immunocytochemistry. After the cytological examination, the CEA mRNA concentration in the residual cells was measured using the OSNA system. The CEA mRNA concentration in peritoneal washes was compared with the results of the cytological examination.
Results: CEA mRNA at concentrations from 10 to 10^7 copies/μL was detected by the OSNA system within 10 min, and an excellent correlation was observed between the logarithmic CEA mRNA concentration and the detection time ($r = 0.998$). The CEA mRNA cutoff value for distinguishing positive and negative cases through cytological examination was identified as 25 copies/μL. At this cutoff value, the concordance rate with the cytological examination was 93.8%. The overall survival in CEA mRNA-positive versus -negative cases identified using the OSNA system was statistically significant.
Conclusion: This CEA mRNA detection system shows potential for cancer cell detection and for routine use in the clinical laboratory because of its simplicity and rapidity.

1. Introduction

Peritoneal dissemination is the most frequent type of recurrence in gastric cancer patients and is considered to arise from free cancer cells in the peritoneal cavity exfoliated from the serosal surface of the stomach penetrated by the primary tumor. The presence of free cancer cells in peritoneal washes has been used to predict the risk of peritoneal recurrence and overall survival [1–3] and is usually detected by cytology. For cytology-positive cases, the patients are categorized as Stage IV in the TNM classification system [4,5].

Although positive cytological findings in peritoneal washes are a strong risk factor for peritoneal recurrence and worse survival in gastric cancer patients, peritoneal recurrence or reduced survival has been observed even with negative cytological findings [6,7]. One potential explanation is the non-detection of a trace amount of cancer cells in peritoneal washes under the technical limitations of cytology. In such cases, the sensitivity depends largely on the evaluation method or the technical skills of the cytologist. Indeed, the sensitivity of cytological examination for predicting peritoneal recurrence has been reported to be between 11.1 and 80.0% [7].

To increase the sensitivity and objectivity of cancer cell detection in peritoneal washes, the usefulness of immunocytochemical [3,8,9] or molecular biological methods [10–15] has been investigated. Some groups have reported that immunocytochemistry with various antibody panels improves the detection rate of cancer cells, increasing it by 5% to 15% compared with standard cytology [3,8,9]. Kodera et al. [16] clearly showed that the presence of carcinoembryonic antigen (CEA) mRNA in peritoneal washes correlates with survival and peritoneal recurrence. Many researchers have reported the usefulness of molecular biological techniques for the detection of CEA and/or other markers, such as cytokeratin (CK) 20 mRNA, in peritoneal washes by quantitative reverse-transcription PCR (QRT-PCR) [11,13,14,16–20]. However, these molecular biological techniques do exhibit some disadvantages

Abbreviations: CEA, carcinoembryonic antigen; OSNA, One-Step Nucleic acid Amplification; QRT-PCR, quantitative reverse-transcription PCR; RT-LAMP, reverse transcription loop-mediated amplification; Pap, Papanicolaou; ROC, receiver operating characteristic; CK, cytokeratin; TRC, transcription reverse-transcription concerted.

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for routine use in general clinical laboratories because they are time- and labor-intensive.

Previously, we and other researchers have reported a new rapid gene detection system, One-Step Nucleic acid Amplification (OSNA), for lymph node metastasis detection in breast, colorectal, gastric, and lung cancer patients [21–24]. This method consists of a simple assay sample preparation and automated rapid gene detection using a reverse transcription loop-mediated amplification (RT-LAMP) method [25]. In particular, the target mRNA detection step of this approach could contribute to a more accurate, simple, and objective detection of cancer cells in peritoneal washes from gastric cancer patients.

This method should allow the detection of trace amounts of CEA mRNA. Thus, based on this aim, we modified both the sample preparation and gene amplification steps of the OSNA system. In the sample preparation step, RNA purification was carried out to concentrate the CEA mRNA from trace amounts of cancer cells in peritoneal washes. The reaction mixture of the RT-LAMP method was optimized for CEA mRNA detection. Our results show the usefulness of the OSNA system for CEA mRNA detection even with trace amounts of cancer cells in peritoneal washes.

2. Material and methods

2.1. Peritoneal washes

The study was carried out under the approval of the IRB of Osaka Police Hospital and Sysmex Corporation. Peritoneal washes were obtained from gastric cancer patients with informed consent. The peritoneal washes used were collected during surgery or laparoscopy. Two peritoneal washes were collected from the Douglas' pouch of each patient and from the left subphrenic cavity or the right subphrenic cavity. The peritoneal washes were first subjected to cytological examination (see Section 2.2) for routine diagnostics, and the residual part was then subjected to the OSNA assay. The peritoneal washes with a small amount of cells were excluded from evaluation for CEA mRNA measurement by the OSNA system. A total of 128 peritoneal washes obtained from 75 gastric cancer patients were obtained. The number of samples in each tumor invasion category (T category) was as follows: 10 T1a, 13 T1b, 7 T2, 14 T3, 16 T4a, 3 T4b, and 12 unknown.

The abdominal cavity was washed with 100 mL of physiological saline solution, and a cell pellet from the peritoneal washes was obtained by centrifugation at 1500 ×*g*. The pellet was used for routine diagnosis through cytological examination, and the residual pellet was stored at −80 °C until use.

2.2. Cytological examination

The slide was prepared from approximately 30 μL of the cell pellet. Two pairs of slides were prepared from one patient, and each slide was then investigated through Papanicolaou (Pap) staining and immunocytochemistry using anti-CEA (Clone no. CEA010: Mochida, Takara, Shiga, Japan) antibody or anti-epithelial-related antigen (Clone no. MOC31: DAKO, Glostrup, Denmark) antibody.

2.3. RNA extraction

RNA extraction from residual cells was carried out using the RNeasy Mini Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. The purified RNA was eluted in 50 μL of RNase-free water and then stored at −80 °C until use.

2.4. Quantitative RT-PCR (QRT-PCR)

QRT-PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The RNA was subjected to QRT-PCR with the TaqMan One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems) according to the manufacturer's instructions. The primers and TaqMan probes were designed using the ABI Primer Express Version 2.0 software. The primer and probe sequences were as follows: forward CEA primer, 5′-AGACAATCACAGTCTCTGCGGA-3′; reverse CEA primer, 5′-ATCCTTGTCCTCCACGGGTT-3′; and CEA probe, 5′-FAM-GCCCAAGCCCTCCATCTCCAGCAACAACTC-TAMRA-3′. The QRT-PCR cycle parameters were as follows: 48 °C for 30 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each QRT-PCR assay was performed at least twice. The expression levels of CEA mRNA were quantified from a CEA QRT-PCR standard curve by inserting the standard CEA mRNA threshold.

2.5. CEA mRNA detection with the OSNA system

CEA mRNA detection was carried out using the OSNA system. The following oligonucleotides for RT-LAMP were used: inner primer (forward), GGCACGTATAGGATCCACTCCAGCAATCCACCCAAGA; inner primer (reverse), CAGACACTGGCCTCAATAGGAGGTTTGGGTGGCTCTGCAT; outer primer (forward), TGCACAGTACTCTTGGTTTG; outer primer (reverse), ACGGGGTTGGAGTTGTTGG; loop primer (forward), TCACAGTGATGTTGGGGAT; and loop primer (reverse), ACAGTCACGACGATCACA. Each LAMP reaction was performed in a 25-μL reaction mixture containing 1.1 μM of each inner primer, 0.1 μM of each outer primer, 0.8 μM of each loop primer, 0.8 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 50 mM Tris–HCl, 10 mM KCL, 10 mM $(NH4)_2SO_4$, 5 mM $MgSO_4$, 0.1% Triton X-100, 5.0 mM DTT, 0.2% Tergitol NP-40 (Sigma-Aldrich, St. Louis, MO, USA), 1.4 U of AMV reverse transcriptase (Promega, Madison, WI, USA), 45 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), 25 U of RNasin Plus (Promega, Madison, WI, USA), and extracted RNA. The reaction mixture was incubated at 65 °C for 16 min in the RD-100*i*. The amplification DNA derived from the target mRNA in the RT-LAMP assay was detected on RD-100*i* by monitoring the change in turbidity due to the production of the by-product, pyrophosphate magnesium [26]. The detection time of CEA mRNA was defined as the time at which the turbidity reached 0.1, and the CEA mRNA expression level was quantified from a standard curve of CEA mRNA by inserting the standard mRNA threshold. Each RT-LAMP assay was performed with standard CEA mRNA added at 10^3, 10^5, and 10^7 copies/μL. The standard CEA mRNA was synthesized using a T7 RiboMAX In Vitro Transcription System (Promega) from human CEA mRNA cloned into the pGEM-32 Vector (Promega).

2.6. Statistical analysis

The statistical analyses, including t-test, Kaplan–Meyer analysis and log-rank test, were carried out using the StatMate Ver.4.01 software (ATMS, Tokyo, Japan).

3. Results

3.1. CEA mRNA detection by the OSNA system

To investigate the assay performance in terms of CEA mRNA detection, various concentrations of CEA mRNA solutions, including 10^7, 10^5, 10^3, 10^2 and 10 copies/μL, were measured using the OSNA system. The detection time ranged from 6.7 to 10.5 min (Fig. 1a). Previously, we and other researchers reported that the detection time in the CK19 mRNA solution depended on the amount of CK19 mRNA [21]. The correlation between the detection time and the CEA mRNA concentration is shown in Fig. 1b. As expected, the detection time correlated with the logarithmic value of the CEA mRNA concentration, with a correlation efficient of 0.998. This result indicates that the CEA mRNA can be detected in a wide range from 10 to 10^7 copies/μL with the OSNA system.