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The detection of gastric cancer cells in intraoperative peritoneal lavage using the reverse transcription–loop-mediated isothermal amplification method

Akira Yoneda, MD, PhD, Ken Taniguchi, MD, PhD, Yasuhiro Torashima, MD, PhD, Seiya Susumu, MD, PhD, Kengo Kanetaka, MD, PhD, Tamotsu Kuroki, MD, PhD, and Susumu Eguchi, MD, PhD*

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

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ABSTRACT

Introduction: To detect a small number of malignant cells, we used a highly sensitive detection system that measures the expression levels of cytokeratin (CK) 19 messenger RNA by reverse transcription–loop-mediated isothermal amplification (RT–LAMP).

Materials and methods: We evaluated the clinical relevance of our novel diagnostic method with an RT–LAMP assay using CK19 as a target gene for the detection of free cancer cells in peritoneal lavage and assessed the clinical significance of the molecular diagnosis by survival analysis and frequency of recurrence, with a median follow-up period of 39 mo. We observed 52 patients with gastric cancer who underwent gastrectomy, bypass operation, and exploratory laparotomy.

Results: Those 52 patients, who were subjected to both RT–LAMP and cytologic examination, were divided into the following three groups: (1) patients positive by cytology and RT–LAMP (CY+/LAMP+) ($n = 9$), (2) patients positive by LAMP and negative by cytology (CY−/LAMP+) ($n = 12$), and (3) patients negative by both cytology and LAMP (CY−/LAMP−) ($n = 31$). All patients with simultaneous peritoneal dissemination and positive cytology were positive on RT–LAMP. The results of RT–LAMP were statistically significant for recurrence by univariate analysis ($P < 0.005$). Cytology-positive cases had a very poor prognosis, and RT–LAMP-positive cases had a worse prognosis than RT–LAMP-negative cases.

Conclusions: Our findings suggest that CK19 RT–LAMP would be useful as an intraoperative diagnostic modality to detect patients with a high risk of recurrence even after clinically curative surgery, who thus require proper adjuvant therapy.

1. Introduction

Peritoneal carcinomatosis is the most frequent pattern of recurrence in patients with gastric cancer [1,2]. The prognosis of patients with advanced gastric cancer invading the gastric serosa is very poor even after curative resection, mainly because of the high incidence of peritoneal recurrence [3]. Recurrence with this pattern is most likely caused by the presence of free cancer cells in the abdominal cavity exfoliated from the serosal surfaces of the primary gastric tumor [4]. Therefore, detection of such micrometastatic cells in the peritoneal cavity is likely to be a useful tool in the selection of

* *Corresponding author*. Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: +81 95 819 7316; fax: +81 95 819 7319.
E-mail address: dm06034e@cc.nagasaki-u.ac.jp (A. Yoneda).
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intra or postoperative chemotherapy and for predicting the outcome of such therapy in these cases [5]. In this regard, cytologic examination of lavage fluid obtained at the time of surgery is a conventional method for detecting free cancer cells in peritoneal space. However, the sensitivity of this assay has been reported to be relatively low, ranging from 19% to 30% in gastric cancer invading the serosa [6–9]. As a result, some patients with negative cytology have nevertheless developed peritoneal recurrence. Therefore, there is an urgent need for more sensitive methods to detect micrometastasis in the peritoneal cavity. To detect small numbers of malignant cells among the cytologically negative cases, we developed a highly sensitive detection system that measures the expression levels of cytokeratin (CK) 19 messenger RNA (mRNA) by reverse transcription–loop-mediated isothermal amplification (RT–LAMP). The RT–LAMP method is a new method of gene amplification, the efficacy of which has been reported [10,11]. The reaction is accelerated by the use of two additional loop primers (called loops F and B) [11]. The LAMP method can be conducted simultaneously with reverse transcription from mRNA (RT–LAMP) [10–14]. There are several practical advantages to the RT–LAMP technique: it requires only simple reaction procedures, the compact and inexpensive incubator or turbidimeter equipment costs <$5000, and <1 h is needed to obtain the final results [11–15]. Application of the LAMP technique has been reported for breast and lung cancers [16–18]. This technique might be one of the most promising candidates for analyzing the genetic features of samples obtained during surgery.

CK proteins of the intermediate filaments of epithelial cells have been used as specific markers for tumor cells of epithelial origin [19,20]. In the present study, we evaluated the clinical relevance of a new diagnostic method using an RT–LAMP assay with CK19 as the target gene for the detection of free cancer cells in the peritoneal lavage and assessed the clinical significance of the molecular diagnosis by survival analysis and frequency of recurrence.

2. Materials and methods

2.1. Cell lines

A sensitivity assay for detecting a gastric cancer cell line was performed. The human gastric cancer cell line MKN-45, obtained from the Riken Cell Bank (Institute of Physical and Chemical Research, Saitama, Japan), was incubated in RPMI-1640 medium containing 10% fetal calf serum (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2.

2.2. Patients

Between May 2007 and November 2008, we observed 52 patients (35 males and 17 females; mean age, 67.5 ± 2.8 y) with gastric cancer who underwent gastrectomy ($n = 45$), bypass operation ($n = 2$), and exploratory laparotomy ($n = 7$) for histologically proven gastric cancer at the Department of Surgery, Nagasaki University. Written informed consent for participation in this study was obtained from all the patients. All were followed up for a median of 39 mo (range, 6–51 mo) or until death. The primary tumor was resected in 45 of the 52 patients (five patients had peritoneal dissemination but underwent resection of their primary tumor because of the stenosis and bleeding caused by primary tumor as a palliative treatment) but was unresectable in seven patients because of peritoneal dissemination and positive cytology. These seven patients underwent a bypass operation or exploratory laparotomy. The resected specimens were histologically examined by hematoxylin and eosin staining according to the general rules of the Japanese Classification of Gastric Carcinoma [21]. Clinicopathologic features of the patients are shown in Table 1.

2.3. RT–LAMP reaction

LAMP primers targeting the CK19 complementary DNA were designed based on a past report [22] (Fig. 1). To quantify and prove the integrity of isolated RNA, we also performed RT–LAMP for β-actin.

The RT–LAMP method was carried out on 25 μL of the total reaction mixture with a Loopamp RNA amplification kit (Eiken Chemical Co, Tokyo, Japan) containing 40 pmol each of the forward and backward inner primers, 5 pmol each of the outer primers F3 and B3, 20 pmol each of the loop primers loops F and B, 35 pmol of dNTPs, 20 μL of Betamine, 0.5 μM Tris–HCL (pH 8.8), 0.25 μM KCL, 0.25 μM (NH4)SO4, 0.2 μM MgSO4, 0.2% Tween 20, 1.0 μL of Enzyme Mix (*Bst* DNA polymerase and

Table 1 – Clinicopathologic factors were determined according to the Japanese classification of gastric carcinoma.

	Number of patients
Total cases	52
Age (y)	67.5 ± 2.8
Sex: male/female	35/17
Depth of tumor invasion	
M	11
SM	13
MP	6
SS	9
SE and SI	13
Lymph node metastasis	
N0	22
N1	13
N2	13
N3	4
Peritoneal metastasis	
Absent	40
Present	12
Cytology	
Negative	43
Positive	9
Stages	
IA	18
IB	7
II	8
III	4
IV	15

M = mucosa; SM = submucosa; MP = muscularis propria; SS = subserosa; SE = serosa exposed; SI = serosa infiltrating.