The detection of gastric cancer cells in intraoperative peritoneal lavage using the RT-LAMP method

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Running title: The detection of gastric cancer cells using the RT-LAMP

Abstract

Introduction

   To detect a small number of malignant cells, we used a highly sensitive detection

system that measures the expression levels of cytokeratin 19-mRNA 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 cytokeratin 19 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 months. 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 cytological

examination, were divided into the following three groups: (1) patients who were

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.

Conclusion

Our findings suggest that CK-19 RT-LAMP would be useful as an intraoperative

diagnostic modality in order to detect patients with a high risk of recurrence even after

clinically curative surgery, who thus require proper adjuvant therapy.

Key words: gastric cancer, RT-LAMP, peritoneal lavage

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

intra/post-operative chemotherapy as well as 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-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

(reverse transcriptase-LAMP: 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/turbidimeter equipment costs less than $5,000, and less than

1 hr is needed to obtain the final results [11-15]. Application of the LAMP technique has

been reported for breast cancer and lung cancer [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 employing 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.

Materials and methods

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, USA) at 37°C in 5% CO_2.

Patients

Between May 2007 and November 2008, we observed 52 patients (35 males and 17 females; mean age, 67.5 ± 2.8 years) 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 patients. All were followed up for a median of 39 months (range, 6-51 months) or until death. The primary tumor was resected in 45 of the 52 patients (5 patients had peritoneal dissemination, but underwent resection of their primary tumor because of the stenosis and bleeding caused by primary tumor as a pariative treatment) but was unresectable in 7 patients due to peritoneal dissemination and positive cytology. These 7 patients underwent a bypass

operation or exploratory laparotomy. The resected specimens were histologically

examined by hematoxilin and eosin staining according to the general rules of the

Japanese Classification of Gastric Carcinoma [21]. Clinicopathological features of the

patients are shown in Table 1.

RT-LAMP reaction

LAMP primers targeting the CK19 cDNA 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 (FIP) and backward inner primers (BIP), 5 pmol each of the

outer primers F3 and B3, 20 pmol each of the loop primers loop F and loop B, 35 pmol

dNTPs, 20-μl Betamine, 0.5 μM Tris-HCL (pH 8.8), 0.25 μM KCL, 0.25 μM (NH4)SO4,

0.2μM MgSO4, 0.2% Tween20, 1.0 μl Enzyme Mix (*Bst* DNA polymerase and avian

myeloblastosis virus (AMV) reverse transcriptase) and 5 μl RNA at a constant

temperature of 63.5°C for 60 min. Temperature control for the LAMP reaction and

turbidity measurement was achieved using a turbidimeter (LA-200; Teramecs Co.,

Kyoto, Japan) especially developed for DNA analysis by LAMP reaction.

Sensitivity evaluation of RT-LAMP on CK19 mRNA detection

A fundamental experiment was performed to determine the sensitivity of the

RT-LAMP method for detecting gastric cancer cells in peripheral blood mononuclear

cells (PBMCs). Peripheral venous blood was obtained from healthy volunteers. Gastric

cancer MKN-45 cells were serially diluted from 1×10^6 cells to one cell per 1×10^7

PBMCs. The mRNA was extracted from each cell fraction, and RT-LAMP for CK19

mRNA was performed.

Preparation of peritoneal floating cells

At the beginning of the operation, before the manipulation of the tumor, 400 ml of

physiological saline was introduced into the upper abdominal cavity and recovered after

being gently stirred. Part of the peritoneal lavage fluid was subjected to conventional

cytology after standard Papanicolaou staining, and the remaining fluid was immediately

centrifuged at 2000 rpm for 10 min. The pellets of lavaged fluid were rinsed with

phosphate-buffered saline (PBS), dissolved in RNAlater^{TM} solution (Ambion, Austin,

TX) and stored at -80°C until use.

mRNA extraction

mRNA was extracted using the Dynabeads mRNA DIRECT kit (Veritas, Tokyo)

according to the manufacturer's instructions. Briefly, collected cells were lysed by the

Lysis/binding buffer, and the released mRNA,and its poly (A) residue were hybridized

with oligo (dT) conjugated with Dynabeads; then this complex was immobilized onto

the surface of magnetic beads. Contaminating components were washed away by

repeated steps of separation and resuspension in washing buffer. Finally, the purified

mRNA was eluted from the particles.

RT-LAMP on mRNA derived from peritoneal lavage

25 µl of reaction mixture was applied to each reaction. Positive control primers (β-actin)

and a negative control mixture (PBMC-derived sample) were employed for all

reactions.

Statistical analysis

All statistical analyses were performed using StatView 5.0 software (SAS Institute,

Cary, NC, USA). The chi-squared test was used to evaluate the correlation between

positive results and clinicopathological factors, and univariate analysis was used to

evaluate the results of RT-LAMP and recurrence. Survival curves were calculated using

the Kaplan-Meier method. Survival curves were compared using the log rank test.

$P<0.05$ was considered statistically significant.

Results

Evaluation of the sensitivity of RT-LAMP for identifying gastric carcinoma cell line

As few as 10^0 MKN45 cells in 10^7 normal PBMCs were detectable with the

RT-LAMP procedures targeting CK19 mRNA using extracted mRNA of cell mixtures

lysate (Fig. 2).

Detection of cancer cells in intraoperative peritoneal lavage

The mRNA of β-actin, a housekeeping gene, was detected in all of the present

samples. RT-LAMP reaction diagnosis significantly correlated with lymph node

metastasis, depth of invasion, lymphatic invasion and vessel invasion (Table 2). The

total of 52 patients who were subjected to both RT-LAMP and cytological examination

were divided into the following three groups: (1) patients who were 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). The stage of each groups were showed on Table2.

There were no patients negative by LAMP and positive by cytology; all patients with

simultaneous peritoneal dissemination and positive cytology were positive on RT-LAMP.

Simultaneous peritoneal dissemination at surgery or staging laparoscopy was detected in

12 patients (9 in the group CY+/LAMP+ and 3 in the group CY-/LAMP+). No patients

with negative RT-LAMP and negative CY status had recurrence after surgery. The

results of RT-LAMP were statistically significant for recurrence by univariate analysis

($P<0.005$). Fig. 3 shows the overall survival (Fig. 3A), recurrence-free survival (Fig.

3B) and peritoneal recurrence-free survival (Fig. 3C) curves for the three patient groups

subjected to both RT-LAMP and cytological examination. Fig. 3 shows that

cytology-positive cases had a very poor prognosis and that cases with negative cytology

but positive RT-LAMP had a worse prognosis than that of cases who were

RT-LAMP-negative.

Discussion

Although the standard surgical lymphadenectomy for gastric cancer has been

established and applied, patients with advanced stages of this cancer continue to face a

poor prognosis. A previous study suggested that the presence of peritoneal free cancer

cells was responsible for the formation of micrometastasis and subsequent extensive

dissemination [23-24]. Cytological examination aimed at the detection of these cells,

therefore, has been generally accepted as the golden criterion for the prediction of

peritoneal recurrence, and this procedure has been incorporated in the Japanese staging

system for gastric cancer [21]. However, peritoneal recurrence sometimes occurs in

patients with negative cytology, which indicates the lack of sensitivity of conventional

cytological examination for the prediction of peritoneal recurrence. We believe that it is

difficult to confirm peritoneal metastasis from cytology because of its low sensitivity.

Jung et al. reported that even in patients with clinically diagnosed carcinomatosis with

ascites, only 54% of patients were positive for cytology. [25]

Thus, a more sensitive assay for the detection of peritoneal micrometastasis is required.

New, simple and rapid molecular techniques for the detection of target genes have

been developed in recent years, and one of these is the LAMP reaction. This reaction is

a novel approach to the DNA amplification of target sequences, providing high

sensitivity, specificity and rapidity under isothermal conditions. The LAMP reaction

relies on auto-cycling strand displacement DNA synthesis that is performed with a DNA

polymerase with high strand displacement activity and four specific primers recognizing

six independent sequences. Specifically, it synthesizes a large amount of amplification

products, comprising a mixture of stem-loop DNA and cauliflower-like structures with

multiple loops (except for the loops that are hybridized by the inner primer) and prime

strand displacement DNA. When the target DNA is amplified by LAMP reaction, a

white precipitate derived from magnesium pyrophosphate (a byproduct of LAMP

reaction) is observed [10]; thus, the LAMP method does not require special reagents or

electrophoresis to detect the amplified DNA. Amplification of the targeted gene is

detectable in real-time fashion by an increase of the turbidity of the solution.

CK19 has been shown to be widely expressed by cancer cells of epithelial origin

but not by lymphoid or hematopoietic cells [26]. Among some 20 different isotypes,

CK19 and CK20 are expressed more selectively by mucosal epithelial cells than are the

others [27]. It has been reported that CK19 is superior to CK20 in detecting circulating

cancer cells using RT-PCR in peripheral blood from patients with gastric cancer [28].

Thus, this molecule may be a suitable general marker of micrometastasis in peritoneal

lavage of patients with gastric cancer. LAMP primers were generated to detect the

CK19 sequence, and the performance of the RT-LAMP reaction to detect gastric cancer

cells was tested. Only cells of epithelial origin were detected by this technique, with

sensitivity to concentrations as low as one cell per 10^7 normal PBMCs. CK expression

was observed in the RT-LAMP reaction, presumably due to its high specificity, which

requires the recognition of six independent sequences within the target molecule [22].

The development of a rapid technique for detecting cancer cells in peritoneal

lavage of patients with gastric cancer using the RT-LAMP reaction is herein described.

Among the patients with negative cytology, those with a positive RT-LAMP reaction

had a poorer prognosis than that of those with negative RT-LAMP reaction results. In

three patients, laparotomy was performed preceding the staging laparoscopy to verify

negative cytology and the lack of obvious peritoneal dissemination. However, peritoneal

dissemination was found after open exploration, and further surgery was cancelled. For

all of these patients, the results of the RT-LAMP reaction were positive. As these results

indicated, even when cancer cells are not found by cytology, the RT-LAMP method

could be used to determine the necessity of surgery. Our results demonstrated

significant correlations between the CK19 findings and the depth of cancer invasion, the

presence and extent of lymph node metastasis, and the vessel and lymphatic invasion. In

this sense, CK19 expressed by peritoneal free cancer cells could be a candidate

molecular marker indicating high invasive potential and aggressive behavior in gastric

cancers. It is remarkable that the results of the RT-LAMP method correlated not only

with the peritoneal recurrences but also with other fashions without tumor exposure to

the serosa. Minor diffusion of tumor cells might occur during the migration through the

lymphatic vessels [29-31], and further evaluation would be needed around the origin.

The benefit of adjuvant chemotherapies in solid tumors is known to be related to the

amount of remnant tumor burden. Therefore, patients with positive RT-LAMP without

macroscopic peritoneal dissemination seem good candidates for a cure through

appropriate adjuvant therapy. Kodera et al. reported that postoperative S-1 monotherapy

could make no difference in survival between patients with visible peritoneal deposits

and patients with only positive cytology [32]. To improve the prognosis, it is crucial to

identify high-risk patients at a much earlier phase of peritoneal dissemination. As

molecular approaches such as RT-LAMP and RT-PCR analysis have the potential to do

just that, adjuvant therapy could eliminate remnant cancer cells detected only by

molecular diagnosis. Limitations include a small sample size.

The RT-LAMP technique could be performed as an alternative to an intraoperative

cytologic examination. Because of its high sensitivity and rapidity, this method could

provide an opportunity to perform reliable tailor-made surgery for gastric cancers as a

common procedure in general hospitals. However, the present study has some

limitations. First, this study included a too small sample size to assess whether

RT-LAMP positivity could be independent prognostic biomarker in cytology negative

patients. Second, this molecular-based method is known to be concerned with a high

false positive rate. Kodera et al reported that when the cutoff value was set at 0.1 for

CEA mRNA, the false positive rate of CEA RT-PCR exceeded 10%. [33]   To confirm the

efficacy of the RT-LAMP method, additional efforts are necessary to assess the outcome

of the treatment of patients with RT-LAMP-positive peritoneal lavage findings in larger

sample size.

Conclusion

  Our findings suggest that CK-19 RT-LAMP would be useful as an intraoperative

diagnostic modality in order to detect potential high-risk patients who may develop

recurrence even after clinically curative surgery, and to guide choices about proper

adjuvant therapy.

Figure legends

Figure 1: Primer design for detection of CK19 mRNA by RT-LAMP.

Figure 2: Sensitivity analysis of the RT-LAMP method. As few as 10^0 MKN45 cells in

10^7 normal PBMCs were detectable with the RT-LAMP procedures targeting

CK19 mRNA using extracted mRNA of the cell mixture lysate.

Figure 3A: Overall survival curves of patients stratified according to results of cytology

and RT-LAMP. Significant differences between the CY-LAMP- and

CY-LAMP+ groups were found (P<0.05).

Figure 3B: Recurrence-free survival curves of patients stratified according to the results

of cytology and RT-LAMP. Significant differences between the CY-LAMP-

and CY-LAMP+ groups were found (P<0.05).

Figure 3C: Peritoneal recurrence-free survival curves of patients stratified according to

results of cytology and RT-LAMP. Significant differences between the

CY-LAMP- and CY-LAMP+ groups were found (P<0.05).

References

1.   Yoo CH, Noh SH, Shin DW, et al. Recurrence following curative resection for

gastric carcinoma. Br J Surg 2000;87: 236-242.

2.   Maehara Y, Hasuda S, Koga T, et al. Postoperative outcome and sites of

recurrence in patients following curative resection of gastric cancer. Br J Surg

2000;87: 353-357.

3.   Baba H, Korenaga D, Okamura T, et al. Prognostic factors in gastric cancer with

serosal invasion. Univariate and multivariate analyses. Arch Surg 1989;124:

1061-1064.

4.   Koga S, Kaibara N, Iitsuka Y, et al. Prognostic significance of intraperitoneal

free cancer cells in gastric cancer patients. J Cancer Res Clin Oncol 1984;108:

236-238.

5.   Bonenkamp JJ, Songun I, Hermans J, et al. Prognostic value of positive cytology

findings from abdominal washings in patients with gastric cancer. Br J Surg

1996;83: 672-674.

6.   Ikeguchi M, Oka A, Tsujitani S, et al. Relationship between area of serosal

invasion and intraperitoneal free cancer cells in patients with gastric cancer.

Anticancer Res 1994;14: 2131-2134.

7.   Burke EC, Karpeh MS, Jr., Conlon KC, et al. Peritoneal lavage cytology in

       gastric cancer: an independent predictor of outcome. Ann Surg Oncol 1998;5:

       411-415.

8.   Kodera Y, Yamamura Y, Shimizu Y, et al. Peritoneal washing cytology:

       prognostic value of positive findings in patients with gastric carcinoma

       undergoing a potentially curative resection. J Surg Oncol 1999;72: 60-64;

       discussion 64-65.

9.   Suzuki T, Ochiai T, Hayashi H, et al. Importance of positive peritoneal lavage

       cytology findings in the stage grouping of gastric cancer. Surg Today 1999;29:

       111-115.

10.   Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal

       amplification of DNA. Nucleic Acids Res 2000;28: E63.

11.   Nagamine K, Hase T, Notomi T Accelerated reaction by loop-mediated

       isothermal amplification using loop primers. Mol Cell Probes 2002;16: 223-229.

12.   Hirayama H, Kageyama S, Moriyasu S, et al. Rapid sexing of bovine

       preimplantation embryos using loop-mediated isothermal amplification.

       Theriogenology 2004;62: 887-896.

13.   Parida M, Posadas G, Inoue S, et al. Real-time reverse transcription

loop-mediated isothermal amplification for rapid detection of West Nile virus. J

Clin Microbiol 2004;42: 257-263.

14.   Ushio M, Yui I, Yoshida N, et al. Detection of respiratory syncytial virus genome

by subgroups-A, B specific reverse transcription loop-mediated isothermal

amplification (RT-LAMP). J Med Virol 2005;77: 121-127.

15.   Mori Y, Nagamine K, Tomita N, et al. Detection of loop-mediated isothermal

amplification reaction by turbidity derived from magnesium pyrophosphate

formation. Biochem Biophys Res Commun 2001;289: 150-154.

16.   Tsujimoto M, Nakabayashi K, Yoshidome K, et al. One-step nucleic acid

amplification for intraoperative detection of lymph node metastasis in breast

cancer patients. Clin Cancer Res 2007;13: 4807-4816.

17.   Maeda J, Inoue M, Nakabayashi K, et al. Rapid diagnosis of lymph node

metastasis in lung cancer with loop-mediated isothermal amplification assay

using carcinoembryonic antigen-mRNA. Lung Cancer 2009;65: 324-327.

18.   Tamaki Y, Akiyama F, Iwase T, et al. Molecular detection of lymph node

metastases in breast cancer patients: results of a multicenter trial using the

one-step nucleic acid amplification assay. Clin Cancer Res 2009;15: 2879-2884.

19.   Moll R, Franke WW, Schiller DL, et al. The catalog of human cytokeratins:

patterns of expression in normal epithelia, tumors and cultured cells. Cell

1982;31: 11-24.

20.   Osborn M, van Lessen G, Weber K, et al. Differential diagnosis of

gastrointestinal carcinomas by using monoclonal antibodies specific for

individual keratin polypeptides. Lab Invest 1986;55: 497-504.

21.   Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer

14: 101-112.

22.   Horibe D, Ochiai T, Shimada H, et al. Rapid detection of metastasis of gastric

cancer using reverse transcription loop-mediated isothermal amplification. Int J

Cancer 2007;120: 1063-1069.

23.   Wong SC, Yu H, So JB Detection of telomerase activity in gastric lavage fluid: a

novel method to detect gastric cancer. J Surg Res 2006;131: 252-255.

24.   Ishigami S, Sakamoto A, Uenosono Y, et al. Carcinoembryonic antigen

messenger RNA expression in blood can predict relapse in gastric cancer. J Surg

Res 2008;148: 205-209.

25.   Jung M, Jeung HC, Lee SS, et al. The clinical significance of ascitic fluid CEA

in advanced gastric cancer with ascites. J Cancer Res Clin Oncol 136: 517-526.

26.   Ruud P, Fodstad O, Hovig E Identification of a novel cytokeratin 19 pseudogene

that may interfere with reverse transcriptase-polymerase chain reaction assays used to detect micrometastatic tumor cells. Int J Cancer 1999;80: 119-125.

27.   Moll R, Lowe A, Laufer J, et al. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol 1992;140: 427-447.

28.   Majima T, Ichikura T, Takayama E, et al. Detecting circulating cancer cells using reverse transcriptase-polymerase chain reaction for cytokeratin mRNA in peripheral blood from patients with gastric cancer. Jpn J Clin Oncol 2000;30: 499-503.

29.   Yamagata K, Kumagai K Experimental study of lymphogenous peritoneal cancer dissemination: migration of fluorescent-labelled tumor cells in a rat model of mesenteric lymph vessel obstruction. J Exp Clin Cancer Res 2000;19: 211-217.

30.   Sleeman JP, Thiele W Tumor metastasis and the lymphatic vasculature. Int J Cancer 2009;125: 2747-2756.

31.   Hirakawa S From tumor lymphangiogenesis to lymphvascular niche. Cancer Sci 2009;100: 983-989.

32.   Kodera Y, Ito S, Mochizuki Y, et al. A phase II study of radical surgery followed

by postoperative chemotherapy with S-1 for gastric carcinoma with free cancer cells in the peritoneal cavity (CCOG0301 study). Eur J Surg Oncol 2009;35: 1158-1163.

33.   Kodera Y, Nakanishi H, Ito S, Mochizuki Y, Ohashi N, Yamamura Y, et al. Prognostic significance of intraperitoneal cancer cells in gastric carcinoma: analysis of real time reverse transcriptase-polymerase chain reaction after 5 years of followup. J Am Coll Surg 2006;202:231-6.

Figure 1

F3：CCTCCTACCTGGCAAGGT

B3：ATGCGCAGAGCCTGTC

FIP（F1c+F2):TAGTGGCTGTAGTCGCGG

GAAACGGCGAGCTAGAGGAGAA

BIP（B1c+B2):CGGGACAAGATTCTTGGTGCCAAAGT

CATCTGCAGCCAGACG

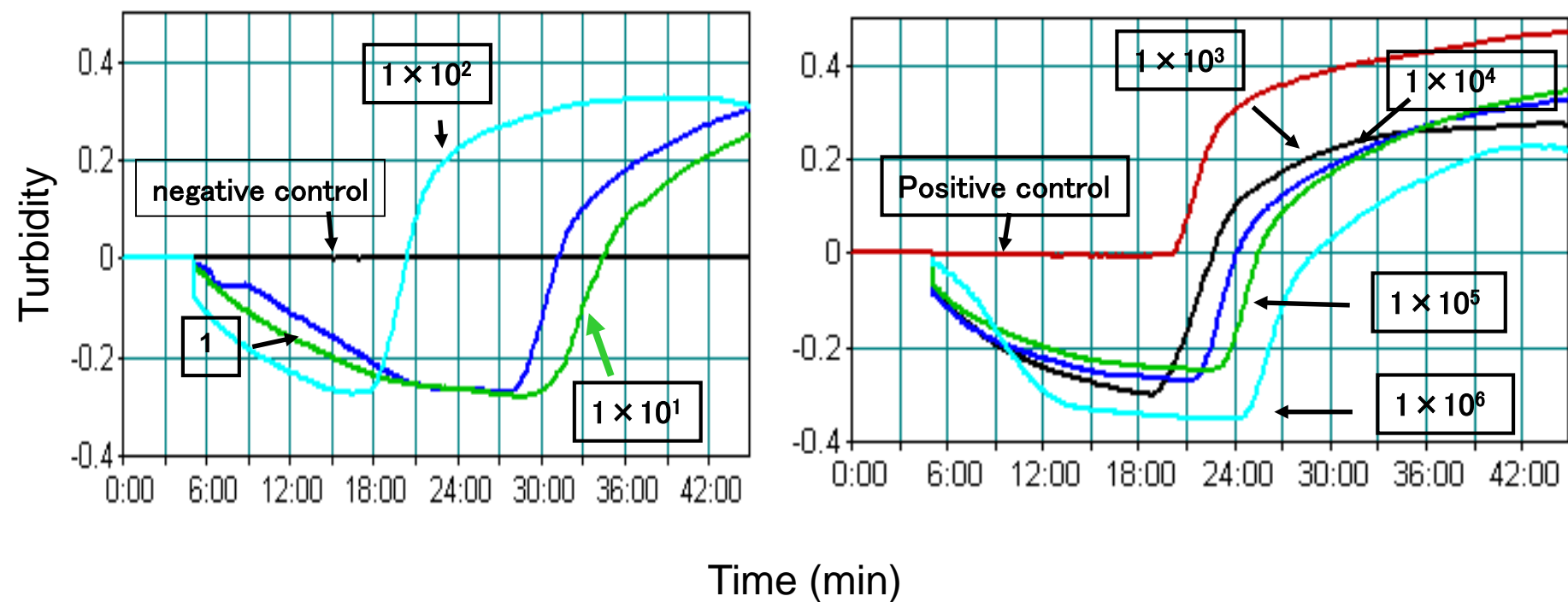loopF：AGGCCCCTGCTTCTGGTAC

loopB：CCTGCAGATCGACAATGCC
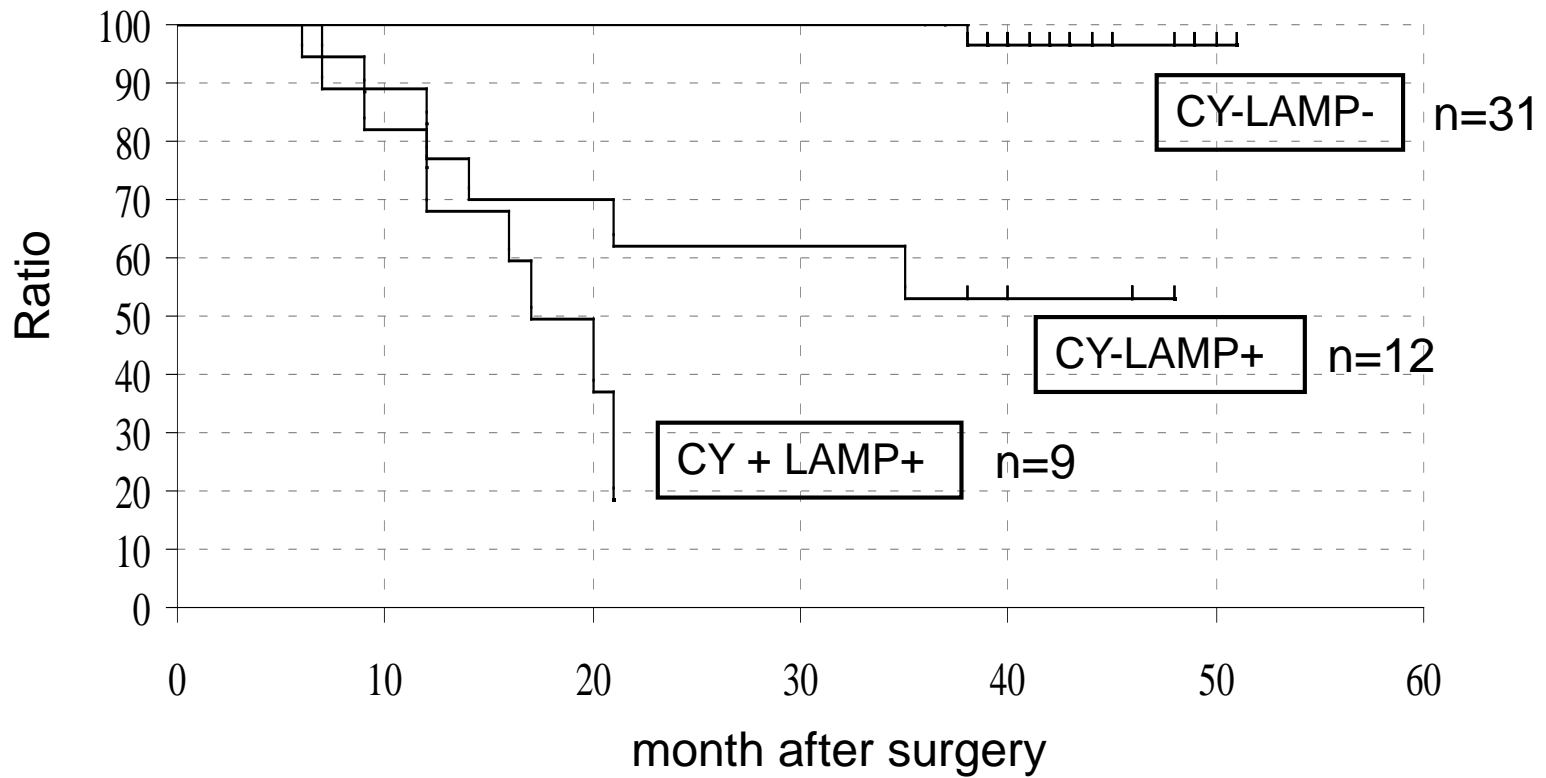
Figure 2

Figure 3A

Overall survival of patients

CY-LAMP-   n=31

CY-LAMP+   n=12

CY + LAMP+   n=9

month after surgery

Figure 3B

Recurrence-free survival of patients

CY-LAMP-   n=31

CY-LAMP+   n=12

CY + LAMP+   n=9

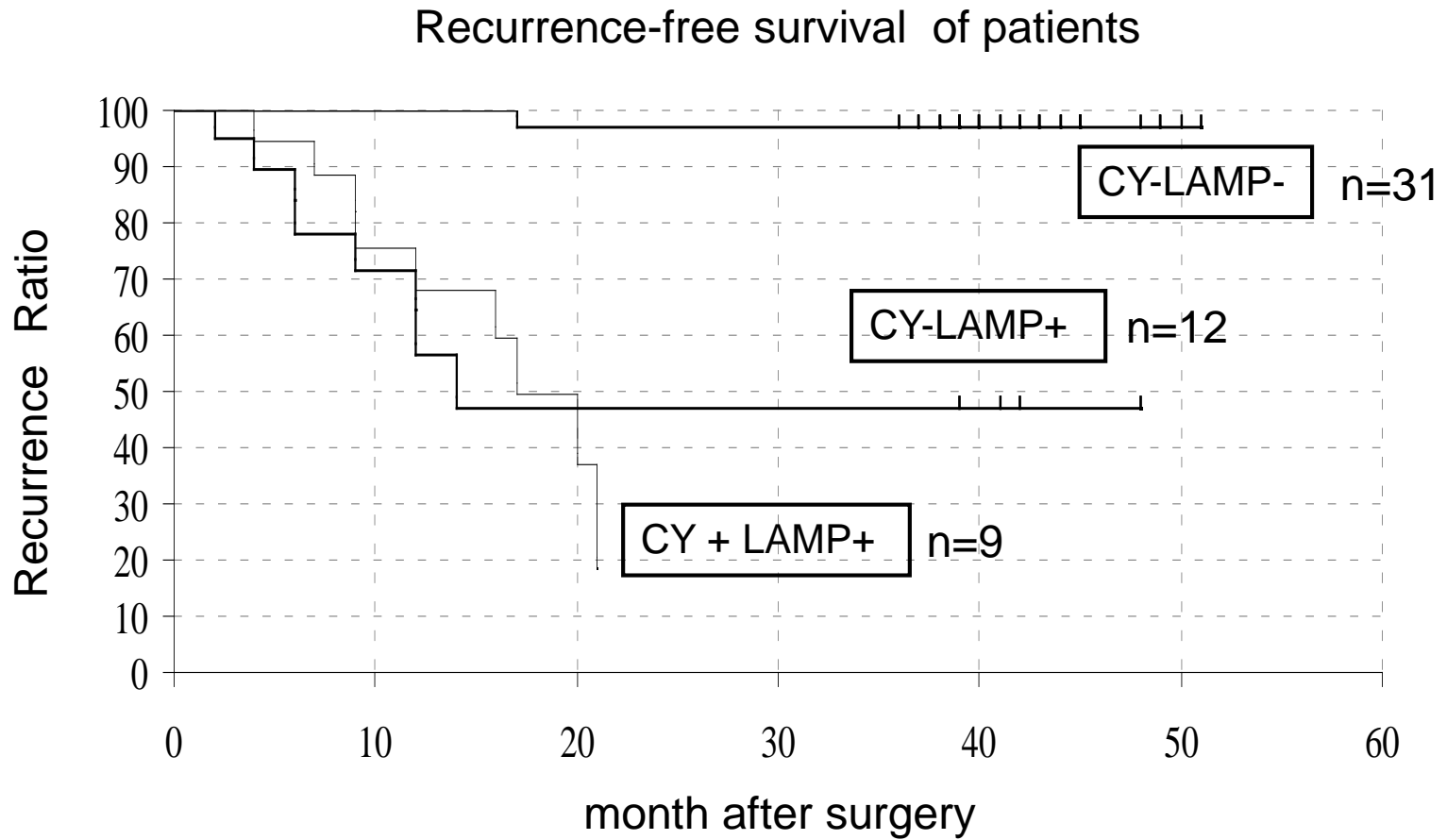Recurrence Ratio

month after surgery
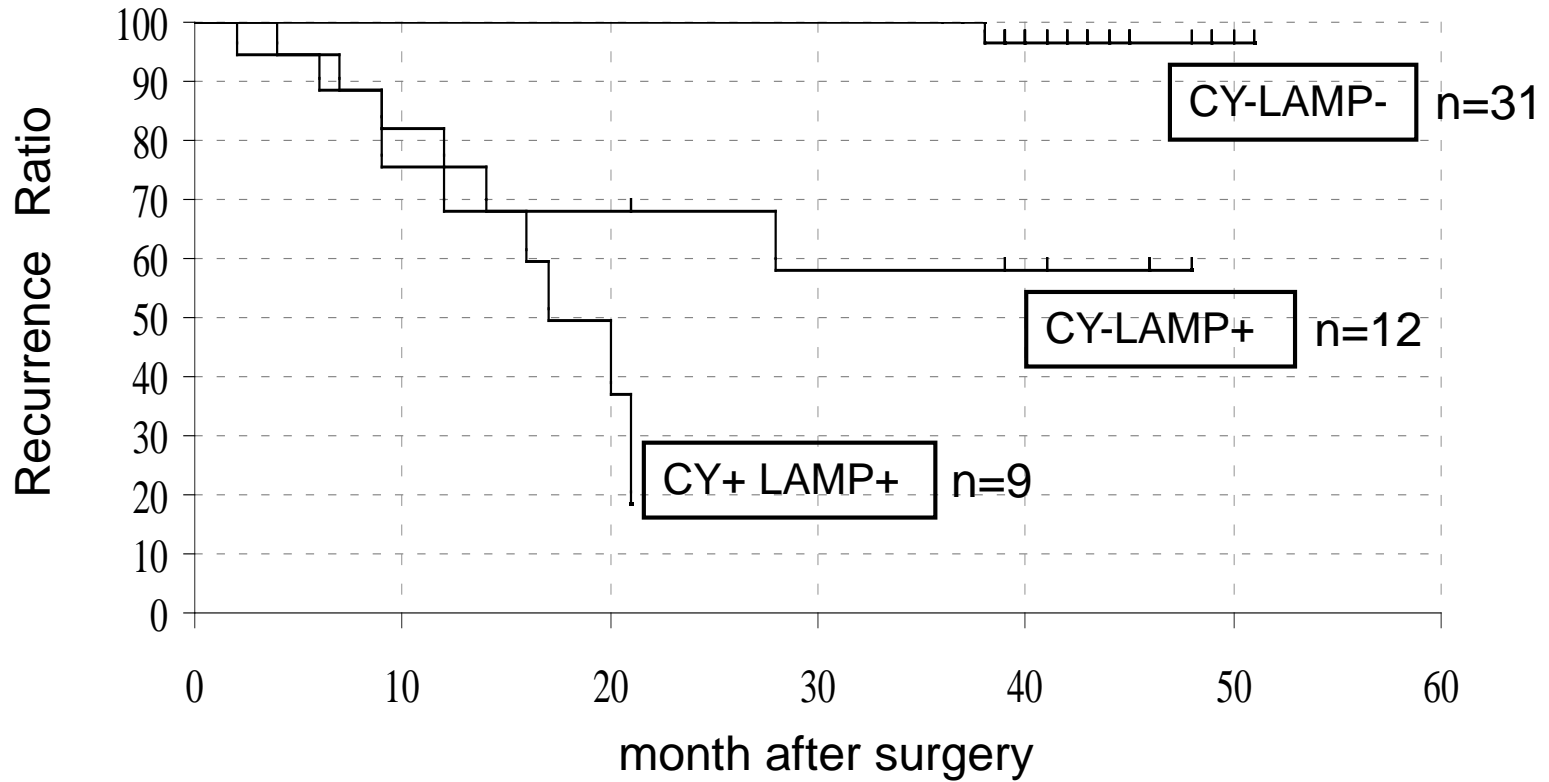
Figure 3C

Peritoneal recurrence-free survival of patients

Table 1: Clinicopathological factors were determined according to the Japanese

Classification of Gastric Carcinoma

		No of patients
Total cases		52
Age(yr)		67.5±2.8
Sex Male/Female		35/17
Depth of tumor invasion	M	11
	SM	13
	MP	6
	SS	9
	SE, SI	13
Lymph node metastasis	N0	22
	N1	13
	N2	13
	N3	4
Peritoneal metastasis	Absent	40
	Present	12
Cytology	Negative	43
	Positive	9
stage		
	Stage IA	18
	Stage IB	7
	Stage II	8
	Stage III	4
	Stage IV	15

M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa;

SE, serosa exposed; SI, serosa infiltrating.

Table 2: Correlation between RT-LAMP diagnosis of peritoneal lavage and clinicopathological parameters and the stage of each gourps.

		Expresson of CK19 mRNA (RT-LAMP)		
		Positive	Negative	P-value
No of patients		21	31	
Sex	Male	14	21	N.S.
	Female	7	10	
Age (yr)		62.45	67.8	N.S.
	M	0	11	
	SM	0	13	
Depth of tumor invasion	MP	3	3	0.00001
	SS	6	3	
	SE,SI	12	1	
Lymph node metastasis	-	3	23	0.00002
	+	18	8	
Lymphatic invasion	-	1	16	0.0091
	+	11	15	
Vessel invasion	-	1	18	0.0033
	+	11	13	

M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa;

SE, serosa exposed; SI, serosa infiltrating.

CY+/LAMP+ (n = 9)　　stage IV; n=9

CY-/LAMP+ (n = 12)　　stage II; n=4, stage III; n=3, stage IV; n=5

CY-/LAMP- (n = 31)　　stage IA; n=19, stage IB; n=6, stage II; n=4, stage III; n=2