

ORIGINAL ARTICLE

## 진행 위암 환자의 복강 세척액에서 CEA와 CK20 전령 RNA 측정

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### The Detection of Messenger RNA for Carcinoembryonic Antigen and Cytokeratin 20 in Peritoneal Washing Fluid in Patients with Advanced Gastric Cancer

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**Background/Aims:** Peritoneal micrometastasis is known to play an important role in the recurrence of gastric cancer. However, its effects remain equivocal. Herein, we examine the messenger RNA (mRNA) as tumor markers, carcinoembryonic antigen (CEA), and cytokeratin 20 (CK20), in peritoneal washing fluid. Moreover, we evaluate whether these results could predict the recurrence of gastric cancer following curative resection.

**Methods:** We prospectively enrolled 132 patients with gastric cancers, who had received an operation, between January 2010 and January 2013. The peritoneal lavage fluid was collected at the operation field and semi-quantitative PCR was performed using the primers for CEA and CK20. We excluded patients with stage IA (n=28) early gastric cancer, positive cytologic examination of peritoneal washings (n=7), and those who were lost during follow up (n=18).

**Results:** A total of 79 patients with gastric cancers were enrolled, and the mean follow-up period was 39.95±19.25 months (range, 5-72 months). According to the multivariate analysis, T4 stage at the initial diagnosis was significantly associated with recurrence. All cases of recurrence were CEA positive and 6 cases were CK20 positive. The positive and negative predictive values of CEA were 32.0% and 100%, respectively, whereas those of CK20 were 37.5% and 71.4%, respectively. Disease free survival of CK20-negative cases was 36.17±20.28 months and that of CK20-positive cases was 32.06±22.95 months (p=0.39).

**Conclusions:** It is unlikely that the real time polymerase chain reaction results of mRNA for CEA and CK20 in peritoneal washing fluid can predict recurrence. However, negative results can convince surgeons to perform curative R0 resection. (**Korean J Gastroenterol 2017;69:220-225**)

**Key Words:** Gastric cancer; Micrometastasis; Carcinoembryonic antigen; Keratin-20

## INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide. Although improvements in diagnostic instruments and therapeutic techniques have increased survival in patients with gastric cancer, it is still the second leading

cause of cancer-related deaths.<sup>1,2</sup> The prognosis of gastric cancer is related to the development of recurrence and metastasis. Gastric cancer frequently disseminates through the hematogenous, lymphatic, or direct peritoneal route. In particular, peritoneal dissemination is the most common mode of metastasis in advanced gastric cancer.<sup>3,4</sup>

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Clinically, peritoneal wash cytology has a low sensitivity rate, and the prediction of recurrence is difficult.<sup>5,6</sup> Recurrent gastric cancers could be complicated by and combined with hematogenous, lymphatic and peritoneal metastases. A highly sensitive and specified marker is necessary for the improvement of therapeutic efficacy. Moreover, to prevent the recurrence of gastric cancer following curative resection, early selection of high-risk patients and aggressive adjuvant therapy are very important. It has previously been reported that recurrence and metastasis were frequent in the upper third tumor location, carcinomas involving the serosa, and neoplasms with an advanced nodal state.<sup>4,7-11</sup> However, it was difficult to assess the overall impact of recurrence.

Micrometastasis originated from free cancer cells, and theoretically, it could be found in blood, lymph nodes, and peritoneal washing fluids. To date, various trials were performed for detecting peritoneal micrometastasis, with the use of various tumor markers (carcinoembryonic antigen [CEA], cytokeratin 20 [CK20], matrix metalloproteinases, survivin and mucin core protein 2); however, sensitivity and specificity of these markers have shown variable results.<sup>12-17</sup> For this reason, several methodologies have been developed. Immunohistochemical detection and molecular biological detection of micrometastasis have been introduced, and efforts have been made to reduce the rate of false-negative and false-positive diagnoses. The effect of peritoneal micrometastasis, which was responsible for the real metastatic disease, remains controversial. A previous study showed that semi-quantitative real time polymerase chain reaction (RT-PCR) analysis of CEA and CK20 in the peritoneal lavage fluid was useful in predicting the recurrence, identifying significant independent prognostic factors of nodal metastasis in patients undergoing a curative resection for gastric cancer.<sup>18</sup> Contrary to the above result, another report asserted that the sensitivity and specificity of RT-PCR analysis of CEA and CK20 were insufficient, and their clinical impact would be small.<sup>19</sup>

We aim to evaluate whether the molecular diagnosis with RT-PCR assay for CEA and CK20 messenger RNA (mRNA) using preoperative peritoneal washing fluid is useful to predict the recurrence in patients with advanced gastric cancer following curative resection.

## SUBJECTS AND METHODS

### 1. Patients

This study was conducted at St. Vincent Hospital, the Catholic University of Korea. We prospectively enrolled 132 patients with gastric cancers who had previously received operations between January 2010 and January 2013. Patients eligible for this study were identified preoperatively as having histological gastric adenocarcinoma. They were offered participation, and were required to provide informed consent. After curative operation, adjuvant combination chemotherapy was administered in patients with stage II and III of the disease classified by the 7th edition of the American Joint Committee on Cancer cancer staging.<sup>20</sup> We treated patients with 3-week cycles of chemotherapy (oral capecitabine 1,000 mg/m<sup>2</sup> twice daily from the evening of day 1 until the morning of day 15 plus intravenous oxaliplatin 130 mg/m<sup>2</sup> on day 1 of each cycle). All patients received hydration and standard prophylactic medications to reduce any toxic effects. Treatment was continued for 6 months until unacceptable toxic effects occurred.

A follow-up of all participants was carried out according to the standard protocol of our institution (every three months for at least 2 years, every 6 months for the next 3 years, and after 5 years, every 12 months). The check-up items included physical examination, tumor marker examination, and computed tomographic scan. Endoscopic examination was carried out regularly (every 6 months for at least 2 years, every 12 months for the next years). Recurrent and mortality events were recorded and disease free survival (DFS) was calculated to assess the prognosis.

### 2. Methods

Before manipulation of the primary tumor, normal saline was introduced into the right upper abdomen, left upper abdomen, and pelvis, and then aspirated after gentle agitation. Samples were collected. One of the samples was sent to the pathology department for cytologic examination with conventional Papanicolaou staining. Additionally, 50 mL of the samples was collected into a specimen cup and transported on ice to the laboratory for RNA isolation.

Negative peritoneal washing was obtained from a patient undergoing laparoscopy for benign condition (tuberculous peritonitis). Positive control was a patient with carcinoma-

tosis peritonei, which was confirmed by a positive cytologic examination of the peritoneal lavage. Semi-quantitative RT-PCR was performed in peritoneal washing samples using the primers for CEA and CK20. Total RNA was extracted, using the guanidinium isothiocyanate-phenol-chloroform method. The extracted total RNA was converted to the first strand complementary DNA, and it was immediately used for PCR



**Fig. 1.** RT-PCR for mRNA of CEA and CK20. RT-PCR, real time polymerase chain reaction; mRNA, messenger RNA; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

amplification, which was performed with a Light Cycler (Roche Diagnostics, Mannheim, Germany). RT-PCR was performed using the single-step method (50 cycles), via hybridization probes. The primers for CEA, CK20, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed. Both CEA and CK20 mRNA levels were normalized to the GAPDH mRNA level, and the ratios of CEA/GAPDH and CK20/GAPDH were calculated (the CEA or CK20 mRNA level divided by GAPDH mRNA  $\times 10^7$ ) (Fig. 1).

### 3. Statistical analysis

For quantitative variables, the mean and its standard deviation were calculated. The Student's t test was used to compare the continuous variables among the groups. For qualitative variables, the percentage and its 95% confidence interval were calculated. Moreover, the  $\chi^2$  test and/or Fisher's exact test were used to investigate the association with other variables. A binary logistic regression model was used for multivariate

**Table 1.** Clinical Features of Recurrence in the Patients with Gastric Cancers

	Recurrence (n=24)	Non-recurrence (n=55)	p-value
Age	62.04 $\pm$ 13.43	62.53 $\pm$ 12.08	0.91
Sex (male : female)	16 : 8	41 : 14	0.47
Smoke	7	15	0.86
Alcohol drinking	13	31	0.86
Body mass index (kg/m <sup>2</sup> )	23.96 $\pm$ 3.16	23.01 $\pm$ 3.56	0.37
Presence of atrophy	10	30	0.29
Lauren classification (Diffuse)	12	24	0.60
Differentiation (Poorly differentiated / Signet ring cell cancer)	15	27	0.08
Lymphatic invasion	19	25	<0.01 <sup>a</sup>
Vascular invasion	12	10	<0.01 <sup>a</sup>
Perineural invasion	15	19	0.02 <sup>a</sup>
Number of dissected lymph nodes at operation	42.75 $\pm$ 13.84	37.15 $\pm$ 15.55	0.28
T stage at diagnosis (T4)	18	8	<0.01 <sup>a</sup>
N stage at diagnosis (N3)	12	7	<0.01 <sup>a</sup>
Chemotherapy	24	42	-
Positive result of mRNA-CEA in peritoneal washing	24	51	-
Positive result of mRNA-CK20 in peritoneal washing	6	10	0.49
Recurrence			
Multiple lesions	10		
Lymph node	11		
Liver	5		
Peritoneum	6		
Colon	3		
Pancreas	3		
Bone	2		
Ovary	2		
Lung	1		
Stoma	2		

mRNA, messenger RNA; CEA, carcinoembryonic antigen; CK20, cytokeratin 20.

<sup>a</sup>Statistically significant.

**Table 2.** Multivariate Analysis of the Factors of Recurrence in the Patients with Gastric Cancers

	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
			Lower	Upper
Positive result of mRNA-CK20 in peritoneal washing	0.22	2.69	0.56	12.88
Differentiation (poorly differentiated and signet ring cell type)	0.28	0.43	0.10	1.96
Lymphatic invasion	0.87	1.15	0.22	6.13
Vascular invasion	0.27	2.51	0.48	13.02
Perineural invasion	0.81	1.19	0.29	4.96
T stage at diagnosis (T4)	<0.01 <sup>a</sup>	11.79	2.23	62.21
N stage at diagnosis (N3)	0.17	2.91	0.64	13.30

mRNA, messenger RNA; CK20, cytokeratin 20.

<sup>a</sup>Statistically significant.

analysis. The Kaplan-Meier method was used to analyze DFS between the CK20 negative and positive groups. SPSS statistical package version 21.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses. Statistical significance was defined as  $p < 0.05$ .

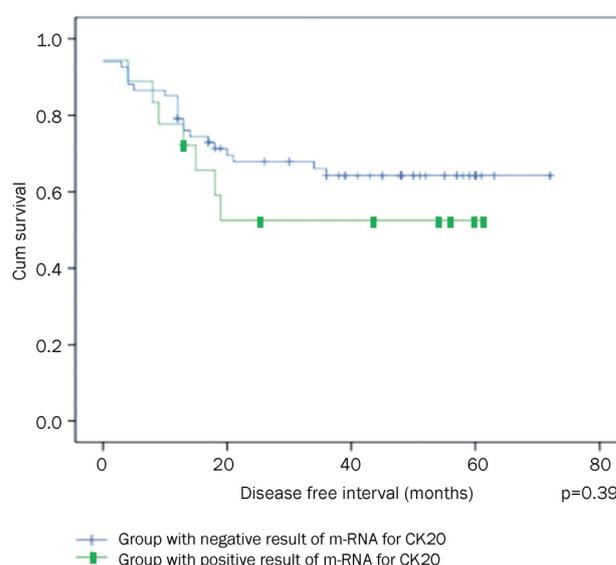
#### 4. Ethics statement

This study was approved by the Institutional Review Board of the Catholic University of Korea (VC09TISI0005).

## RESULTS

We excluded patients with stage IA (n=28) early gastric cancer, positive cytologic examination of peritoneal washings (n=7), and those who were lost to follow up (n=18). A total of 79 patients with gastric cancers (stage IB, 20; stage II, 18; stage III, 41) were enrolled. The mean follow-up period was  $39.95 \pm 19.25$  months (range, 5-72 months). The positive rate of mRNA of CEA in peritoneal washing was 94.9% (75/79), and that of CK20 in peritoneal washing was 20.3% (16/79).

The recurrence rate of gastric cancer was 30.4% (24/79). The basal characteristics are shown in Table 1. Multiple sites of recurrence were found in 41.7% of the cases (10/24), and the most frequent site of recurrence was lymph nodes (45.8%, 11/24). The recurrence rate for the sites (peritoneum, pancreas, colon, and ovary), which imply direct peritoneal seeding of tumor cells, was 58.3% (14/24). Lymphatic invasion, vascular invasion, and perineural invasion were significantly associated with the recurrence of gastric cancer on microscopic examination. T stage (T4) and N stage (N3) at the initial diagnosis showed statistical significance on univariate analysis. On multivariate analysis, only T4 stage was significantly associated with the recurrence of gastric cancer



**Fig. 2.** Kaplan-Meier curve for analyzing DFS between the CK20 negative and positive groups. DFS, disease free survival; CK20, cytokeratin 20; mRNA, messenger RNA.

( $p < 0.01$ : odds ratio, 11.79: 95% confidence interval, 2.23-62.21) (Table 2).

All cases of recurrence were CEA positive, and 6 cases were CK20 positive. The positive and negative predictive values of CEA were 32.0% and 100%, respectively, whereas those of CK20 were 37.5% and 71.4%, respectively. DFS of CK20-negative cases was  $36.17 \pm 20.28$  months and that of CK20-positive cases was  $32.06 \pm 22.95$  months ( $p = 0.39$ ). There was no statistically significant difference between the two groups (Fig. 2).

## DISCUSSION

In advanced gastric cancer patients, about 80% of recurrent events occurred within the first two years postoperation.<sup>4,21</sup>

Direct peritoneal recurrence was the most prevalent pattern, followed by hematogenous metastasis. In general, it has been established that tumors with smaller sizes has greater chemotherapeutic effect. With improved methods for detecting and diagnosing micrometastasis, it is likely for the prognosis of advanced gastric cancer to improve by appropriate adjuvant therapy. Thus, high risk patients with high risk of gastric cancer recurrence will be able to undergo more aggressive therapeutic strategies.

In one Japanese study, relatively high sensitivity and specificity rates of RT-PCR assay for CEA mRNA in peritoneal washing fluid were demonstrated, and the survival of CEA mRNA-positive patients was as poor as that of cytology-positive patients.<sup>14</sup> Other tumor markers have been studied in peritoneal washings of gastric cancer patients. With respect to the RT-PCR assay for CK 20 mRNA alone, sufficient sensitivity was not shown to replace CEA.<sup>19</sup> The RT-PCR assay for matrix metalloproteinase-7 (MMP-7) mRNA was able to detect micrometastatic cancer cells due to the lack of signal of MMP-7 mRNA from normal gastric mucosa, mesothelial cells, fibroblasts, peripheral blood, and lavage fluid. However, the sensitivity for the prediction of peritoneal dissemination by RT-PCR assay for MMP-7 mRNA was only 33%, and that for the combination analysis using cytology and RT-PCR assay for MMP-7 mRNA was 62%.<sup>13</sup> These previous studies had a limitation because they were performed using RT-PCR assay for CEA mRNA combined with cytology results, and the sole efficacy of RT-PCR assay might have been overestimated. Contrastingly, our study was performed with the use of RT-PCR assay only in patients with curative RO resection.

In the studies of CEA and CK20 mRNA in peritoneal washing fluid, false-positive results were reported. When the RT-PCR technique was utilized, the diagnostic specificity was problematic. The main source of such false-positive results is thought to be the amplification of low-level CEA from the peritoneal inflammatory cells.<sup>22</sup> In the case of CK20, false-positive results were attributed to the aberrant expression of mRNA originated from granulocytes.<sup>23</sup> In the present study, RT-PCR assay for CEA mRNA showed a high rate of false-positive results, and the discriminatory power for the prediction of recurrence was low. Disappointingly, the specificity provided by RT-PCR assay for CEA mRNA was only 7.3%, and the sensitivity for CK20 mRNA was no more than 25% in this study. In patients with negative yields on both assays, there

was no recurrence during the follow-up period. The negative results may convince surgeons to perform a curative RO resection.

Locally advanced gastric cancer, defined as T4 in which the tumor perforates serosa (T4a) or invades adjacent structures (T4b), often has a poor prognosis. In previous reports, the overall survival rate for locally advanced gastric cancer patients was under 20% and approximately 30% for those who can undergo surgical resection.<sup>24,25</sup> In our study, only gastric cancer in the T4 stage at the initial diagnosis was an independent risk factor for recurrence, and 30.4% of the patients had recurrent disease. A more potent and aggressive adjuvant therapy is needed after curative resection of locally advanced gastric cancer (T4 gastric cancer).

There are potential limitations to this study. First, this study has a relatively small sample size and may include selection bias. To overcome these issues, we designed a prospective study and pursued a relatively long-term follow-up testing. Second, methodologically, we choose the markers of peritoneal micrometastasis to predict recurrence. Although peritoneal micrometastasis was an important factor for the recurrent disease, there were various routes of recurrence through the blood vessel, lymphatics, and direct peritoneum. Clinically, recurrent diseases are complex and simultaneous multiple metastatic lesions are often found. In this study, there were multiple lesions in more than 40% of the cases. Because the most common pattern of recurrence after curative resection was peritoneal carcinomatosis,<sup>26</sup> the presence of direct peritoneal metastasis was an important factor for the prognosis.

In conclusion, it is unlikely that the RT-PCR results of tumor markers, CEA and CK20, in peritoneal washings can predict recurrence. Negative results can convince surgeons to perform a curative RO resection. For the prediction of recurrence, better markers with higher sensitivity and specificity are necessary.

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