Combination of L-3-phosphoserine Phosphatase and CEA Using Real-time RT-PCR Improves Accuracy in Detection of Peritoneal Micrometastasis of Gastric Cancer

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Abstract. Peritoneal metastasis is the most frequent form of recurrence for advanced gastric cancer. We previously performed a global analysis of the gene expression of gastric cancer cell lines established from peritoneal metastasis with cDNA microarray. One of the up-regulated genes is L-3-phosphoserine phosphatase (L3-PP). We have examined its potential as a novel marker for the detection of peritoneal micrometastasis of gastric cancer. L3-PP mRNA in peritoneal wash from 88 gastric cancer patients was quantified for comparison of carcinoembryonic antigen (CEA) mRNA by means of real-time RT-PCR with a fluorescently-labeled probe to predict peritoneal recurrence. The quantity of L3-PP and CEA correlated with wall penetration. The cut-off value was set at the upper limit of the quantitative value of T1 cases (tumor invades within submucosa) and those above the cut-off value constituted the micrometastasis (MM+) group; eight out of 14 cases with peritoneal dissemination were MM+ L3-PP (57.1% sensitivity) and two out of 57 T1 and T2 cases were MM+ (93% specificity). For two out of 14 cases of peritoneal dissemination only L3-PP could detect micrometastasis of gastric cancer, indicating that L3-PP is superior to CEA especially in poorly-differentiated adenocarcinoma. The combination of CEA and L3-PP improved the accuracy of diagnosis up to 85.7%. Consequently, free cancer cells that cannot be detected by CEA mRNA could be detected using L3-PP mRNA. CEA alone was not sufficient, but L3-PP and CEA in combination can attain a higher accuracy of detection.

Peritoneal dissemination represents 60-70% of the forms of advanced gastric cancer recurrence, however there are few reports of the gene groups involved in the peritoneal dissemination mechanism (1). We previously reported the effects of intraperitoneally administered mitomycin C adsorbed on activated carbon (MMC-CH) for the prevention of relapse of gastric cancer with serosal invasion (2). However, the intraperitoneal dosage of anticancer drugs such as MMC-CH can have some side-effects including ileus and leukocytopenia, and it is necessary to identify intraperitoneal free cancer cells during an operation.

Peritoneal lavage cytology is generally performed to detect floating cancer cells at the beginning of an operation to evaluate the possibility of peritoneal dissemination (3, 4). Intraperitoneal free cancer cells are recognized as one of the most important prognosis-determining factors. However, the

Abbreviations: L3-PP, L-3-phosphoserine phosphatase; CY, cytology; CEA, carcinoembryonic antigen; MM, micrometastasis.

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conventional detection method, Papanicolaou stain, has relatively low sensitivity (5). Another method, conventional RT-PCR, cannot maintain high specificity because false-positives are sometimes obtained due to the weak expression in non-cancerous cells, such as mesothelial cells and lymphocytes (6, 7). Measurement of CEA is useful but cannot maintain high specificity (8). To enhance the sensitivity and specificity, a more specific gene for gastric cancer with peritoneal dissemination needs to be identified, and its expression must be quantifiable. High-density microarray enables the analysis of a large number of genes in one assay, and was suggested to be useful for analyzing gene expression profiles related to the development and progression of tumor. We exhaustively analyzed a gene group with regards to gastric cancer with peritoneal dissemination using a DNA microarray and found over twenty genes that are highly expressed in a cell line originating from peritoneal metastases of gastric cancer (9). One such gene, L3-PP, encodes phosphatase of phosphoserine, an enzyme for biosynthesis of serine, glycine, threonine and cysteine. Since the L3-PP gene is involved in amino acid synthesis, studies have reported that its enzyme activity becomes higher during cell proliferation. Some studies have also reported that L3-PP enzyme activity was higher in lung adenocarcinoma and mesothelioma (10). In the present study, L3-PP expression of intraperitoneal free cells was picked as an index to investigate gastric cancer with peritoneal dissemination. Moreover, L3-PP expression in the cells obtained by peritoneal lavage was quantified by real-time RT-PCR and compared with CEA expression.

Materials and Methods

Cell lines. The mesothelial cell line, MeT5A, was established previously by Roger et al. (11). The gastric cancer cell lines, SNU-1, SNU-5, SNU-6 and SNU-719, were established previously by Park et al. (12-14). KATO-III and GT3TKB were purchased from the Riken Cell Bank (Tsukuba, Japan) (15, 16). All the cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Clinical samples. This study included 88 patients with gastric cancer and 15 patients with non-cancerous diseases, who had undergone surgical treatment in our institute from April 2001 to December 2002. These 88 cases included 40 patients with T1 (tumor invades lamina propria or submucosa), 17 with T2 (tumor invades muscularis propria or submucosa), 18 with T3 (tumor penetrates serosa without invasion of adjacent structures) and 13 with T4 (tumor invades adjacent structures). All tumors were examined microscopically and classified according to the Japanese Classification of Gastric Carcinoma (1993). Written informed consent was obtained from each patient prior to tissue acquisition. Free cells were obtained by peritoneal lavage from all patients at the beginning of laparotomy. One hundred and fifty milliliters saline was poured into the Douglas cavity and aspirated after gentle stirring. These washings were centrifuged at 1,500 rpm for 5 minutes to collect intact cells.

RNA extraction and RT reaction. Total RNA was isolated using the ISOGEN RNA extract kit (Nippon Gene, Inc., Tokyo, Japan) according to the manufacturer’s instructions. cDNA was synthesized from 1 mg of total RNA in a 15 µl reaction mixture containing 5 µl of 5xRT reaction buffer, 200mM dNTP and 100mM solution of random hexadeoxynucleotide mixture. The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min and then chilled on ice.

Real-time quantitative RT-PCR. Quantitative PCR was done using real-time ‘Taqman TM’ technology (17) and analyzed on a Model 5700 Sequence Detector (Applied Biosystems Corp., Foster City, CA, USA).
CEA RT-PCR primers are 5'-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3' and 5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3'.

L3-PP RT-PCR primers are 5'-GATGCTGTGTGTTTTGATGTTGAC-3' and 5'-CTTGACTTGTTGCCTGATCACATT-3'.

Hybridization probes, which were bound to PCR products, were labeled with a reporter dye, FAM on the 5' nucleotide and a quenching dye TAMRA, on the 3' nucleotide. Sequences of hybridization probes are:

CEA: 5'(FAM)CATCTGGAACTTCTCCTGGTCTCTCAGC(TAMRA)3',

L3-PP: 5'(FAM)CCGATTCCTTCTTCACTGATGACCCTG(TAMRA)3'.

Fifty μl reactions contained: 1.25 units Ampli-Taq DNA polymerase, 1xPCR reaction buffer, 180ng of each primer, 200mM dNTP, 400mM dUTP, 100mM Taqman probe and 0.5 U Amplirase (Applied Biosystems Corp.). The Ct value corresponding to the cycle number at which the fluorescent emission monitored in real-time reaches a threshold of ten standard deviations above the mean baseline emission from cycle 1 to 40 was measured as serial dilutions of control cDNA, analyzed for each target, L3-PP and CEA, and served as standard curves from which to determine the rate of change of Ct values (Figure 1). Cycling parameters were: 2 min at 50° C, 10 min at 95° C followed by 40 cycles of 15 s at 95° C and 1 min at 60° C. To minimize the errors arising from the variation in the amount of starting RNA among samples, amplification of β-actin mRNA was performed as an internal reference against which other RNA values could be normalized. The primers and the probe for the β-actin RNA were purchased from Applied Biosystems. Normalized results were expressed as the ratio of copies of each gene to copies of the β-actin gene.

Statistical methods for analysis. Statistical analysis was performed using the NAP system programmed by Aoki (Version 4.0). The various groups of patients were compared by means of either χ2 test or Mann-Whitney U-test. Results with p value of less than 0.05 were considered statistically significant.

Results

Validation of real-time quantitative RT-PCR. Real-time quantitative RT-PCR with the GeneAmp5700 detection system using hybridization probes allowed for rapid and sensitive detection of L3-PP mRNA from the patient samples. With this method, 10² to 10⁷ L3-PP-expressing gastric cancer SNU-16 cells per 10⁷ mesothelial cells could be quantitated (Figure 1). No significant level of L3-PP mRNA was detected in peripheral blood lymphocytes or mesothelial cells from the healthy volunteers.

Quantification of messages with GeneAmp5700 was assessed by determination of the crossover point (Ct), making the cycle when fluorescence of a given sample rose above the background level to yield the maximal slope with respect to log linear amplification. Figure 1B illustrates a standard curve constructed by plotting the log number of 10-fold serially diluted SNU-16 cells against the respective Cts. L3-PP mRNA values for patient samples with unknown concentration were calculated with reference to the calibration curve.

Expression of L3-PP in gastric cancer cell lines and mesothelial cell lines. Expression of L3-PP by gastric cancer cell lines were examined using the conventional RT-PCR technique and were compared with CEA. L3-PP showed poor expression in a mesothelial cell line (MeT5A) and in a cell line derived from a primary cancer (SNU-1), but were highly expressed in cell lines derived from peritoneal metastases of an ascites-causing gastric cancer (SNU-5, SNU-16, SNU-719, KATO-III and GT3TKB). The finding for conventional RT-PCR of L3-PP and CEA in the gastric cancer cell lines is presented in Figure 2.
L3-PP mRNA / β-actin mRNA ratio and the degree of wall invasiveness. To standardize the extracted amount of RNA, β-actin mRNA was used as an internal control. The value for L3-PP extracted was determined as the L3-PP mRNA / β-actin mRNA ratio. The average L3-PP mRNA/β-actin mRNA ratio (x10^5) according to T classification was: T1 3.9x10^5 ± 13x10^3, T2 8.9x10^3 ± 2.3x10^3, T3 2.6x10^5 ± 1.1x10^4 and T4 4.7x10^4 ± 2.5x10^4 (average ± SD). The plot of L3-PP mRNA / β-actin mRNA ratio (x10^5) is shown in Figure 3. Subjects were further classified into patients with serosal invasion (T3 and T4) and patients without serosal invasion (T1 and T2). These results show that there is a correlation between L3-PP mRNA / β-actin mRNA ratio and the degree of wall invasiveness (Figure 3). The L3-PP mRNA / β-actin mRNA ratio has a significantly lower value in T1 and T2 patients than in T3 and T4 patients (Figure 5).

Specificity and sensitivity to detect peritoneal dissemination using L3-PP mRNA / β-actin mRNA ratio. The highest value of L3-PP mRNA / β-actin mRNA ratio determined for the T1 patients undergoing surgery was set as the cut-off value (dashed line on the graph). Any samples of fifteen non-cancerous patients did not exceed the cut-off value. Values greater than this limit were regarded as positive (MM+L3-PP). Among 88 subjects, 17 were determined to be MM+L3-PP. Eight out of 17 MM+L3-PP cases showed positive cytology (CY+) or were subsequently found to show peritoneal dissemination. Fourteen out of 88 subjects were identified as cases of peritoneal metastasis. Eight of these 14 subjects had results above the cut-off value, which was MM+L3-PP (57.1% sensitivity). Moreover, 53 out of 57 T1 and T2 patients were MM-L3-PP (93% specificity). Table I shows the clinico-pathological features and the expression of L3-PP of 88 gastric cancer patients. Depth of invasion, lymph node metastasis and peritoneal dissemination had statistical significance with expression of L3-PP.
Specificity and sensitivity to detect peritoneal dissemination using CEA mRNA / β-actin mRNA ratio. Similarly, CEA mRNA was measured in each clinical sample, the values were divided by β-actin mRNA and the results were presented as the CEA mRNA / β-actin mRNA ratio. Results for CEA mRNA / β-actin mRNA ratio (x10^8) are: T1 5.3x10^5 ± 3.5x10^5, T2 8.4x10^5 ± 6.8x10^5, T3 7.0x10^6 ± 1.6x10^6 and T4 6.4x10^7 ± 3.6x10^7. Correlation was observed between the degree of wall invasion and the CEA mRNA / β-actin mRNA ratio (Figure 4). Moreover, the CEA mRNA / β-actin mRNA ratio had a significantly lower value in T1 and T2 patients than in T3 and T4 patients (Figure 6). The highest value for the CEA mRNA / β-actin mRNA ratio in T1 patients was set as the cut-off value and samples showing a value greater than this were regarded as MM+CEA. Fourteen patients were determined to be MM+CEA. However, 10 out of 14 patients of peritoneal dissemination were above the cut-off value (MM+CEA) (71.4% sensitivity). Incidentally, 55 out of 57 T1 and T2 patients showed MM-CEA (96.4% specificity). Table II shows the clinico-pathological features and the expression of CEA. The depth of invasion and peritoneal dissemination had statistical significance with expression of CEA.

False-negative for CEA. For this assessment, we used a real-time RT-PCR method to assay for CEA. The results were 71.4% sensitivity and 96.4% specificity, which showed as high an accuracy as the previous results. There were two cases diagnosed as peritoneal dissemination but which tested MM-CEA, which is a false-negative for CEA. The method of detecting micrometastases to the peritoneum by measuring

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**statistical significant
*Mann-Whitney test

**Table I. Expression of L3-PPmRNA and clinico-pathological factors in gastric cancer patients.**

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**statistical significant
*Mann-Whitney test

**Table II. Expression of CEAmRNA and clinico-pathological factors in gastric cancer patients.**
the fixed quantity of CEA mRNA using the real-time RT-PCR method could improve the sensitivity. Figure 3 shows the clinico-pathological features of 14 patients with peritoneal dissemination. Though two patients were observed to have peritoneal metastases, they were negative for CEA. But the L3-PP mRNA / β-actin mRNA ratio results for these two patients were positive (MM+L3-PP) (Table III).

Improvement of the accuracy to detect micrometastasis by combination of L3-PP and CEA. The L3-PP assay could detect micrometastasis in 8 out of 14 patients (57.1% sensitivity). The CEA assay could detect them in 10 out of 14 (71.4% sensitivity). In the case of positive for either L3-PP or CEA, the sensitivity to detect micrometastasis increased up to 85.7% (12 out of 14) (Table III). Thus, the combination of L3-PP and CEA provide higher accuracy than either L3-PP or CEA alone to detect micrometastasis.

Discussion

Gastric cancer is a disease that in Japan has a high mortality rate. Disseminated metastasis to the peritoneum is the most common form of recurrence of advanced gastric cancer (3) and is the greatest factor regulating prognosis. The five-year survival rate for gastric carcinoma invading the serosa is still poor at less than 35% (18). Currently, the only effective medical treatment available to patients diagnosed with peritoneal metastases is chemotherapy. Diagnosis of micrometastases in the peritoneum during operations is indispensable for deciding the course of the operation and for selecting suitable chemotherapy. Therefore, disseminated cancer cells in the abdomen have to be detected within a limited time (during an operation). For this purpose, it is necessary to establish a rapid diagnostic method that has the properties of excellent sensitivity and specificity.

In general, cytological examination of peritoneal lavage (CY) is used as the method for diagnosis of micrometastasis to the peritoneum. Although cytology is a comparatively simple method, the reported sensitivity is not very high, being approximately 46% to 56% (19, 20); following that, the more sensitive method of reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to diagnose micrometastases in the peritoneal cavity. Recently, quantitative rapid RT-PCR-based screening methods for the detection of micrometastasis from clinical specimens have become standard procedure (21-23). Detection of micrometastasis of gastric cancer cells to the peritoneum has been reported using many different kinds of gene markers, such as CEA, keratin19, AFP, MMP-7, or mutation of E-cadherin (24-28). Among these markers reported earlier, CEA is the most common for this purpose. However, CEA mRNA is not expressed in all cases of peritoneal metastasis and because CEA is weakly expressed in mesothelial cells and lymphocytes, there are problems with both the sensitivity and specificity using CEA as diagnostic marker in the detection of micrometastasis. To reduce false-negative results, a gene marker with a greater sensitivity than CEA is required. To identify these novel
markers for peritoneal dissemination, we previously performed a global analysis of the gene expression of gastric cancer cell lines established from peritoneal metastasis with cDNA microarray. We identified more than 20 genes that are highly expressed in cell lines established from peritoneal metastases of gastric cancer (9), which are candidates for new markers of peritoneal metastasis of gastric cancer.

One of the up-regulated genes is L3-PP. Hundreds of protein phosphatases are known. Some of them have been reported in association with carcinogenesis and tumor growth, or in the control of growth. L3-PP is an intermediary enzyme in both amino acid biosynthesis and gluconeogenesis, and is an enzyme that acts specifically in the synthesis of serine from 3-phosphoserine (28, 29). It has been reported that activity of the enzyme L3-PP increases when cell multiplication and frequency of mitosis increases (30). Enzyme activity is higher in tumorous tissue than in normal lung tissue and is also higher in highly-differentiated lung tumors than in undifferentiated lung tumors or in mesothelioma (31, 32).

We confirmed L3-PP overexpression in gastric cancer cells from peritoneal metastasis by cDNA microarray as well as RT-PCR, suggesting that overexpression of L3-PP is probably involved in peritoneal metastasis of gastric cancers. L3-PP mRNA in peritoneal wash in 88 gastric cancer patients was quantified for comparison of carcinoembryonic antigen (CEA) mRNA by means of real-time RT-PCR with a fluorescently-labeled probe to predict peritoneal recurrence. Our facilities also performed real-time RT-PCR indexed as CEA mRNA and obtained comparatively satisfactory results (71.4% sensitivity and 96.4% specificity). However, two cases of disseminated peritoneal metastases were experienced at an early stage in cases that had tested CEA negative. For this reason, it was suggested that the method for predicting micrometastasis by measuring only CEA mRNA is inadequate. The L3-PP mRNA assay had 57.1% sensitivity (8 out of 14) and 93.0% specificity (53 out of 57), concluded that there is no significant difference between the sensitivity of L3-PP and CEA assay. However, when diagnosing micrometastases to the peritoneum using real-time RT-PCR indexed to CEA mRNA, there are a few false-negative cases (21). High expression of L3-PP was observed in the two cases of disseminated peritoneal metastases that had tested negative for CEA mRNA. This suggests that it is possible for L3-PP mRNA to indicate intra-peritoneal micrometastasis that was undetectable when only testing with CEA mRNA (false-negative). The combination of CEA and L3-PP improved the sensitivity of diagnosis from 71.4% up to 85.7%.

This study suggests that L3-PP has a specific relationship with the dissemination of gastric cancer and that development of an assay for L3-PP mRNA in peritoneal lavage is effective for detecting free cancer cells. Further, micrometastases, which were undetectable when only using CEA mRNA assay, could diagnose by combination of L3-PP mRNA and CEA mRNA assay. The quantification of L3-PP was studied to determine whether it could be a supportive factor for detecting micrometastasis by CEA quantification. It is necessary to investigate further the effectiveness of the intra-operative diagnosis by studying more cases and performing a long-term analysis.

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