Molecular diagnosis and therapy for occult peritoneal metastasis in gastric cancer patients

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To apply an individualized oncological approach to gastric cancer patients, the accurate diagnosis of disease entities is required. Peritoneal metastasis is the most frequent mode of metastasis in gastric cancer, and the tumor-node-metastasis classification includes cytological detection of intraperitoneal cancer cells as part of the staging process, denoting metastatic disease. The accuracy of cytological diagnosis leaves room for improvement; therefore, highly sensitive molecular diagnostics, such as an enzyme immunoassay, reverse transcription polymerase chain reaction, and virus-guided imaging, have been developed to detect minute cancer cells in the peritoneal cavity. Molecular targeting therapy has also been spun off from basic research in the past decade. Although conventional cytology is still the mainstay, novel approaches could serve as practical complementary diagnostics to cytology in near future.

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Key words: Gastric cancer; Peritoneal lavage; Cytology; Molecular diagnostic techniques; Reverse transcriptase polymerase chain reaction; Carcinoembryonic antigen

Core tip: For patients with gastric cancer, cytological detection of cancer cells in the peritoneal cavity is important to predict future manifestation of peritoneal recurrence. However, its improvement has been a matter of research, because of its low sensitivity and specificity. The new diagnostic modalities have been investigated along with the development of modern molecular biology. The recent innovative challenges regarding molecular diagnosis of intra-peritoneal gastric cancer cells have been thoroughly covered and summarized. The new therapies for gastric cancer with peritoneal spreads were also referred.

Kagawa S, Shigeyasu K, Ishida M, Watanabe M, Tazawa H, Nagasaka T, Shirakawa Y, Fujiwara T. Molecular diagnosis and therapy for occult peritoneal metastasis in gastric cancer patients. World J Gastroenterol 2014; 20(47): 17796-17803 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17796.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17796

INTRODUCTION

Gastric cancer is one the leading causes of death in the world[1], and the most prevalent cancer in Eastern Asia[2]. Although the radical resection of cancerous lesions is the only cure for gastric cancer, multi-disciplinary therapy...
for advanced disease can palliate the disease and even prolong life [3,4]. Therefore, the accurate and appropriate diagnosis of the disease entity is required so that an individualized oncological approach can be used. The tumor-node-metastasis (TNM) staging system is the universally accepted method to describe the degree of cancer advancement [5,6]. As with other cancers, gastric cancer has disease-specific factors in its staging. One of them is the cytology of a peritoneal wash or ascites because peritoneal metastasis is the most frequent mode of distant metastasis and post-surgical recurrence. However, it is often difficult to diagnose peritoneal metastasis by conventional imaging modalities, such as computed tomography and positron emission tomography. The cytological detection of free cancer cells in the peritoneal cavity is a very important finding in gastric cancer. Positive cytology means that peritoneal metastasis exists anywhere in the peritoneal cavity even if it is invisible, so it implies a high probability of future manifestations of peritoneal metastasis [7-12]. Therefore, peritoneal lavage cytology findings as well as peritoneal metastasis are factors in gastric cancer staging in Japan as stage 4 disease [13]. The most recent TNM classification system includes intraperitoneal cancer cell detection as part of the staging process, denoting metastatic disease [3].

Peritoneal carcinomatosis is an incurable disease with poor prognosis. In cases of peritoneal carcinomatosis, although debate about surgical application still remains, palliative chemotherapy would be preferred [14-17]. From this point of view, peritoneal carcinomatosis needs to be precisely diagnosed before surgery or at the beginning of surgery for surgeons to determine the most appropriate therapeutic approach [18]. However, in reality, the uneven shape of the peritoneal cavity makes it impossible for the entire cavity to be thoroughly inspected and difficult for the surgeon to definitively judge whether the peritoneal cavity is completely free of metastatic foci. Consequently, peritoneal lavage cytology is needed for the indirect diagnosis or prediction of peritoneal metastasis, and it must be as accurate as possible. The accuracy in peritoneal lavage cytology depends greatly upon the experience of the cytopathologist; therefore, the diagnosis remains inevitably subjective. In addition, several studies indicate that the sensitivity and specificity of peritoneal lavage cytology is unsatisfactory and that there is still room for improvement [19]. Over the past decade, several new diagnostic approaches have been studied. As an alternative to conventional cytology by Papanicolaou staining, immunocytochemistry or PCR-based genetic detection of epithelial or malignant cells in the peritoneal fluid has emerged (Table 1). There are advantages and shortcomings of each approach [20]. In this review, we examine recent studies, summarize findings on the molecular biology-based diagnosis of peritoneal cancer cell existence, and discuss recent advances in the treatment of peritoneal carcinomatosis.

### CONVENTIONAL CYTOLOGY

Since the method of lavage cytology was described by Moore et al. [21] in 1961, several clinical studies have demonstrated the prognostic significance of intraperitoneal free cancer cells at the time of surgery [22-25]. The Japanese Classification of Gastric Carcinoma (2nd English edition) first included the result of peritoneal cytology as one of the staging parameters in 1999 [26]; since then, the Japanese Gastric Cancer Association includes peritoneal cytology in their staging system [27]. Although the most recent TNM classification has included the detection of intraperitoneal free cancer cells as part of the staging process, denoting M1 disease [8], the application of peritoneal cytology in preoperative staging is still controversial. The European Society for Medical Oncology practice guidelines recommend laparoscopy, but regard cytology as optional, and the current National Comprehensive Cancer Network (NCCN) guidelines also do not include cytology in the treatment algorithm [28]. Nevertheless, peritoneal cytology has important clinical implications in the management of advanced gastric cancer [27].

In gastric cancer surgery, by either laparotomy or laparoscopic approach, about 100-200 mL of saline is usually instilled into the Douglas pouch (and occasionally into the left subphrenic space) and gently stirred. A washing sample is then aspirated and subjected to cytology. Traditionally, Papanicolaou or Giemsa stainings are employed, and specimens are diagnosed by experienced cytopathologists. The accuracy, sensitivity, and specificity of conventional cytology in predicting peritoneal recurrence were 73.0%-91.9%, 11.1%-80.0%, and 86.4%-100.0%, respectively [20]. Thus, sensitivity had a particularly wide range, which indicated the need for further advanced techniques.

### CARCINOEMBRYONIC ANTIGEN IN PERITONEAL LAVAGE

Kanetaka et al. [29] recently reported that the measurement of carcinoembryonic antigen (CEA) level in peritoneal lavage (pCEA) by an enzyme immunoassay can predict poor prognosis and may help to elucidate a cohort who need more intensive adjuvant chemotherapy to improve their prognosis. Since Asao et al. [30] first reported that the CEA antigen level in peritoneal lavage could reflect the presence of peritoneal metastasis more accurately than conventional cytology in 1991, other investigators have demonstrated the clinical significance of pCEA levels [30-33]. Most of these reports showed a significant correlation between pCEA level and survival after surgery, implying that pCEA could be a potential predictor of poor prognosis. However, the pCEA level may reflect both the production of CEA in the peritoneal cavity and the serum CEA level and may not be specific as a marker for the existence of intraperitoneal free cancer cells or occult peritoneal metastasis.
GENETIC DETECTION OF INTRAPERITONEAL GASTRIC CANCER CELLS

Molecular diagnosis with reverse transcriptase-polymerase chain reaction (RT-PCR) has been employed for the detection of minimal cancer cells due to its high sensitivity. Among the messenger RNA (mRNA) specific to cancer cells or epithelial cells, the most common target molecule is CEA mRNA. PCR evaluation of CEA mRNA in peritoneal fluid has increased sensitivity for the detection of peritoneal cancer cells as compared to cytology\(^{[36,37]}\), and positive results have been associated with poor survival. Kodera et al.\(^{[38]}\) demonstrated that CEA PCR-positive patients had significantly worse overall survival and recurrence-free survival as compared to PCR-negative patients, independently of cytology. PCR appears to increase the accuracy of detection of occult disease.

In addition, molecular targets for PCR other than CEA have been investigated and include metalloproteinase-7\(^{[39,40]}\) and cytokeratin 20\(^{[41,42]}\). The expression level of a single gene was heterogeneous, so limited sensitivity hinders its use alone. To further improve the sensitivity and specificity of the mRNA detection approach, multiplex PCR may prove to be more clinically useful in capturing intraperitoneal free cancer cells\(^{[41,43]}\).

Mori et al.\(^{[44]}\) tried to select marker candidates out of tens of thousands of genes with microarray analysis, and they identified the genes specific to cytology-positive samples. They further manufactured a microarray chip containing 10 marker genes as a “MiniChip” and demonstrated that the MiniChip assay has a sensitivity and specificity equal to or better than conventional cytology in detecting minimal free cancer cells in peritoneal fluid\(^{[43]}\).

Recently, a new rapid genetic diagnostic technique to detect minute cancer cells has been developed and applied in the sentinel node navigation surgery as surgical decision making\(^{[45-48]}\). One-step nucleic acid amplification (OSNA) uses reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect mRNA expression of target sequences from crude samples without RNA purification\(^{[49]}\). The reaction can be completed in a single test tube and within 1 h. Kumagui et al.\(^{[50]}\) reported a multicenter study evaluating the clinical performance of the OSNA assay that detects cytokeratin 19 (CK19) mRNA in detecting lymph node (LN) metastases in gastric cancer patients, and this method showed high concordance rate to pathology. Although the OSNA assay is useful in the intraoperative rapid diagnosis of LN metastasis for gastric cancer, it remains unproven if this technique could be ap-

| Ref. | Molecule | Technique | Number of patients | Results |
|------|----------|-----------|--------------------|---------|
| Asao et al.\(^{[44]}\) | CEA | Enzyme immunoassay | 120 | Correlation with 2-yr survival rate |
| Irinoda et al.\(^{[51]}\) | CEA, sialyl-Tn antigen | Enzyme immunoassay | 96 | Correlation with peritoneal metastasis and prognosis |
| Abe et al.\(^{[42]}\) | CEA | Enzyme immunoassay | 56 | Correlation with peritoneal metastasis and overall survival |
| Cetin et al.\(^{[46]}\) | CEA | Enzyme immunoassay | 70 | Correlation with peritoneal metastasis and overall survival |
| Kanetaka et al.\(^{[50]}\) | CEA | Enzyme immunoassay | 597 | Correlation with overall survival and peritoneal recurrence-free survival |
| Yamamoto et al.\(^{[52]}\) | CEA, CA125 | Enzyme immunoassay | 229 | Correlation with overall survival and recurrent cites |
| Li et al.\(^{[53]}\) | CEA | Radioimmunoassay | 64 | Correlation with overall survival |
| Kodera et al.\(^{[54]}\) | CEA | RT-PCR | 189 | Correlation with overall survival and peritoneal recurrence-free survival |
| Wang et al.\(^{[55]}\) | CEA | RT-PCR | 40 | Correlation with peritoneal recurrence |
| Sugita et al.\(^{[56]}\) | CEA, CK20 | RT-PCR | 129 | Correlation with overall survival and peritoneal recurrence-free survival |
| Dalal et al.\(^{[57]}\) | CEA, CK20, survivin, MUC2 | RT-PCR | 40 | CEA had high sensitivity and specificity, while CK20, survivin, and MUC2 showed high false-positive rates |
| Takata et al.\(^{[58]}\) | CEA, CK20 | RT-PCR | 104 | Predict peritoneal recurrence |
| Kodera et al.\(^{[59]}\) | CK20 | RT-PCR | 195 | Not sufficiently sensitive as CEA |
| Yonemura et al.\(^{[60]}\) | MMP-7 | RT-PCR | 152 | Improved the sensitivity for peritoneal dissemination in combination with cytology |
| Mori et al.\(^{[61]}\) | Multiple marker | Microarray | 179 | Correlation with disease-free survival and immunocytochemical cytology |
| Hiraki et al.\(^{[62]}\) | Aberrant gene methylation | Methylation-specific PCR | 107 | Correlation between positive methylation and peritoneal recurrence |
| Mori et al.\(^{[63]}\) | Telomerase activity | TRAP assay | 46 | Some concordance with cytology |
| Da et al.\(^{[64]}\) | Telomerase activity | TRAP assay | 60 | Correlation with high proliferating activity of gastric cancer |
| Wong et al.\(^{[65]}\) | Viral tropism | NDV-GFP imaging | 30 | Higher sensitivity and lower specificity than cytology |
| Kitayama et al.\(^{[66]}\) | EpCAM | Flow cytometry | 195 | Tumor cell/leukocyte ratio reflects peritoneal spread |

CEA: Carcino-embryonic antigen; CA125: Cancer antigen 125; CK20: Cytokeratin 20; TRAP assay: Telomeric repeat amplification protocol assay; NDV-GFP: Newcastle disease virus-green fluorescent protein; MUC2: Mucin 2; RT-PCR: Reverse transcription polymerase chain reaction.
applied to detect intra-peritoneal free cancer cells. It needs to be determined how the different properties of cells in the peritoneal cavity interfere with the reaction and what the minimal number of cancer cells is for detection by this method.

DNA methylation is an important epigenetic change in cancer that leads to the recruitment of transcription repressors and chromatin changes, so methylation analysis has been used as a diagnostic modality for various cancers. Hiraki et al. assessed whether gene methylation in peritoneal fluid from gastric cancer patients is clinically feasible for determining the peritoneal metastasis in gastric cancer. By using quantitative methylation-specific PCR to compare aberrant methylation status in gastric cancer, they isolated 6 genes (BNIP3, CHFR, CYP1B1, MINT25, RASSF2, and SFRP2) as having cancer-specific DNA methylation, and they observed that there was a significant correlation between positive methylation in any of these 6 genes and peritoneal recurrence. Thus, methylation analysis might improve the positive detection of gastric cancer cells in peritoneal lavage.

**TELOMERASE ACTIVITY IN THE PERITONEAL FLUID**

Telomerase activity in cancer cells has been examined as a tag to detect cancer cells in the peritoneal cavity. Telomerase activity is one of the hallmarks of cancer and can be used to discriminate malignant cells from normal ones. Mori et al. analyzed peritoneal lavage fluid employing a TRAP assay that reflects telomerase activity. To improve the efficacy of the assay, they enriched cancer cells with immunomagnetic beads coated with anti-Ber-EP4 antibody. Then, they successfully detected telomerase activity in the samples from gastric cancer patients with serosal or subserosal invasions, and they found some concordance with the results of cytology. Da et al. have also investigated the telomerase activity in peritoneal lavage from gastric cancer patients without enrichment of cancer cells. Although the sample size was relatively small, their data demonstrated that all patients with peritoneal metastasis had detectable telomerase activity in peritoneal lavage fluid, and they found significant correlations between positive rate of telomerase activity and invasion depth, serosa-involved areas, and the presence and extent of peritoneal metastasis. While these methods were unique and appeared to be sensitive, they were not significantly superior to conventional cytology by itself. Nevertheless, telomerase activity analysis in peritoneal lavage fluid might be a helpful adjunct for the cytology in the diagnosis of occult peritoneal metastasis of gastric cancer.

**FLOW CYTOMETRIC ANALYSIS OF FREE CANCER CELLS IN PERITONEAL LAVAGE FLUID**

Kitayama et al. tried to quantify the free cancer cells recovered from ascites or peritoneal lavage fluid from gastric cancer patients by conventional flow cytometry. The peritoneal lavage fluid from gastric cancer patients contains erythrocytes, leukocytes, dissociated peritoneal mesothelium, and a small number of cancer cells. Therefore, molecular detection needs to distinguish cancer cells from normal cells co-existing in the peritoneal cavity. Kitayama et al. stained the cells with monoclonal antibodies to CD45 and CD326 (EpCAM), and CD326-positive and CD45-positive cells were classified as either cancer cell or leukocyte. Instead of using the total number of cancer cells, they calculated the cancer cell/leukocyte ratio and demonstrated that the ratio was significantly higher in the patients with peritoneal metastasis and positive cytology than in those without peritoneal spread. They further showed the ratio to reflect well the effect of intraperitoneal chemotherapy. They thus proposed that the flow cytometry-based measurement of the intraperitoneal CD326(+)/CD45(+) ratio could be a diagnostic marker that reflects the severity of peritoneal metastasis as well as the effectiveness of intraperitoneal chemotherapy.

Besides gastric cancer, ovarian cancer also often forms excess ascites due to peritoneal metastasis, which is routinely drained and discarded for symptomatic relief. Peterson et al. regard the ascites as a source of cancer cells for monitoring the treatment response of ovarian cancer. Miniaturizing and advancing flow cytometric technology, they developed and tested a new microfluidic chip to capture, enrich and analyze ascites tumor cells in ovarian cancer patients. This technology allows the detection of occult cancer cells and enables the molecular profiling of individual cells. The microfluidic chip might be applicable to the diagnostic and molecular analysis of peritoneal fluid from gastric cancer patients.

**DIAGNOSTIC POTENTIAL OF THE VISUAL DETECTION OF CANCER CELLS IN PERITONEAL CYTOLOGY SAMPLES**

As a unique approach, several groups examined virus-mediated fluorescent gene expression to visually detect rare cancer cells in the body fluid or the cytology samples against millions of normal cells. Wong et al. evaluated a novel detection technique for intraperitoneal free cancer cells by using Newcastle disease virus-green fluorescent protein (NDV-GFP), which is genetically modified NDV that expresses the green fluorescent protein gene. Newcastle disease virus has been studied since the 1950s for its ability to infect and replicate specifically in tumors. NDV-GFP targets and infects specifically cancer cells, resulting in specific GFP expression. Wong et al. evaluated peritoneal lavage samples from 30 gastric cancer patients undergoing staging laparoscopy with NDV-GFP. They found that NDV-GFP-mediated detection offers a more sensitive method of identifying free peritoneal gastric cancer cells in peritoneal lavage fluid as compared to conventional Pap staining cytology to dem-
WHAT IS NEXT FOR THE IMPROVEMENT OF INTRAPERITONEAL DIAGNOSIS?

As described above, numerous efforts have been made to improve the detection of intraperitoneal free cancer cells. The purpose of most of these studies appeared to primarily be an improvement of the accuracy in cytology. The secondary purpose will be to make diagnosis more convenient and automatic than subjective conventional cytology. Once the accuracy and procedure is essentially improved over the conventional cytology, what should we do next? The identification of intraperitoneal free cancer cells confers poor prognosis. In patients with positive cytology without macroscopic peritoneal metastasis, the benefit of radical or aggressive surgery is still a matter of debate. While some of these patients are palliated, others may undergo more aggressive therapies. Along with the improved diagnostic modality, the treatment strategy would also have to be a coupled issue.

MULTIMODAL CLINICAL APPROACH FOR PERITONEAL SPREAD OF GASTRIC CANCER

Surgeons have witnessed some patients with peritoneal spread of gastric cancer who underwent radical surgery and experienced cures due to the recent improvements in multimodal treatment. A phase II study of whether gastrectomy with curative intent would be beneficial for patients with positive cytology but absence of macroscopic peritoneal seeding has been conducted[63,64]. The study showed that median overall survival time was 705 d, and the 5-year survival rate was 26% in the patients with positive cytology with no other non-curative factors, suggesting that surgery with curative intent could be indicated even for patients with positive cytology[63,64]. For gastric cancer patients with macroscopic peritoneal metastasis, Yamaguchi et al[65] evaluated intraperitoneal chemotherapy along with systemic chemotherapy as a phase II study. They reported a 1-year survival rate of 77.1%, which is surprisingly high. The same group also reported salvage gastrectomy after intravenous and intraperitoneal chemotherapy for the patients who had peritoneal metastasis but showed apparent shrinkage of their peritoneal nodules as well as negative cytology by the treatment[66]. Those patients who underwent salvage gastrectomy exhibited a 26.4-mo median survival period and 82% of 1-year overall survival. Those results suggested that the more sensitive and specific peritoneal diagnosis with the molecular approach might allow gastric cancer patients to receive more suitable individualized multimodal therapies.

A NEW MOLECULAR-TARGETING THERAPY FOR INTRAPERITONEAL SPREAD OF GASTRIC CANCER

Along with the research for the improvement in detection of intraperitoneal cancer cells, molecular targeting therapies might be derived from the results of basic research. One of the molecular targets is epithelial cell adhesion molecule (EpCAM), a type I transmembrane glycoprotein functioning as a homotypic intercellular adhesion molecule[67]. High-level EpCAM expression was observed in 90.7% of gastric cancer[68]. Catumaxomab is an artificially engineered, tri-functional bispecific monoclonal antibody; Fab binding sites bind to EpCAM on cancer cells and CD3 on T cells, and the Fc region binds and activates accessory immune cells. The tri-cell complex of T-cells, tumor cells and accessory cells induces MHC-unrestricted but specific efficient tumor cell killing. The therapeutic benefit of Catumaxomab for patients with malignant ascites including gastric cancer patients has been reported in a pivotal clinical trial[69], which led to approval of Catumaxomab by the European Medicines Agency (EMA) in 2009. Intraperitoneal Catumaxomab treatment has been shown to trigger the activation of immune effector cells in the peritoneal cavity resulting in the depletion of EpCAM-positive tumor cells[70]. Thus, local strategies with molecular targeting agents might represent the appropriate option for treatment of the peritoneal spread of gastric cancer.

CONCLUSION

In the past decade, enormous strides have been made in the research for molecular detection of intraperitoneal free gastric cancer cells, and many new strategies have been clinically tested in gastric cancer patients. As with the conventional cytology, none of the candidate alternatives to conventional cytology are a perfect modality yet, whereas most of them would potentially be conducive to improve the conventional diagnosis and to predict prognosis. The uncertainty of a definition of positivity in these novel approaches and their clinical relevance remain potential limitations to the practical clinical use of these technologies. Too highly sensitive techniques such as PCR may result in the detection of clinically irrelevant metastatic disease, which could lead to either overtreatment with unnecessary chemotherapy, or worse, the withdrawal of potentially curative surgical treatment. Nevertheless, the development of more sensitive and rapid diagnostics in evaluating minimal peritoneal disease is needed for patients to be properly treated. Since peritoneal lavage cytology has recently been included in the staging criteria of gastric cancer, the cytology diagnosis has been focused on as having an important predictive
role in gastric cancer treatment, and the molecular diagnosis has undergone tremendous challenges. With the accumulated evidence, the molecular diagnosis of peritoneal cytology may be a reality in future gastric cancer practice.

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