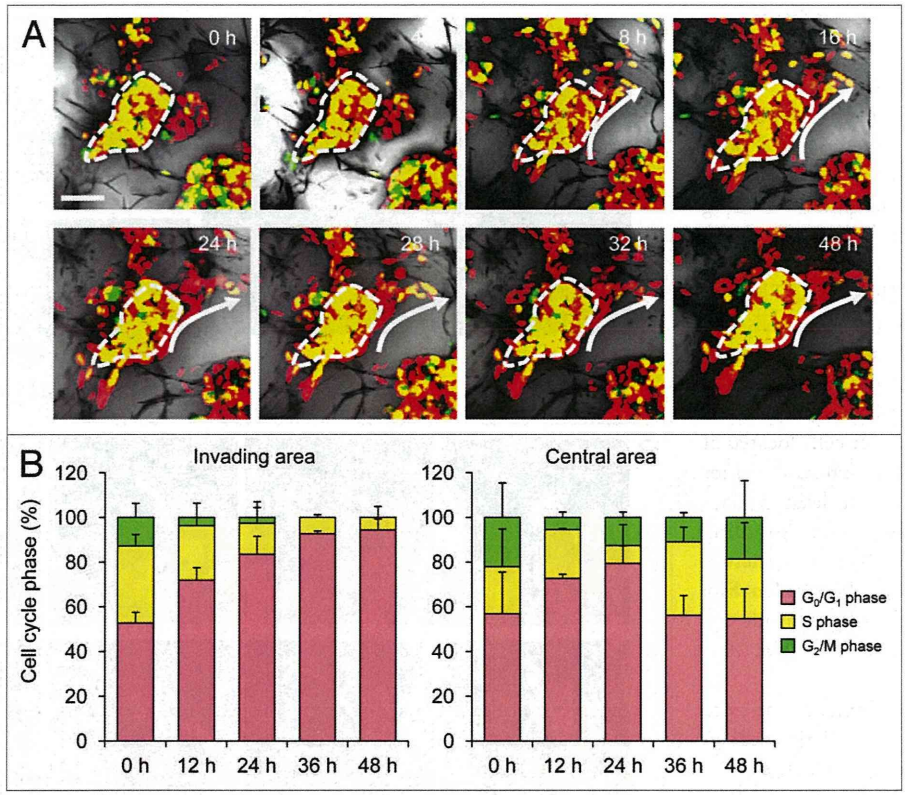

Figure 2. Cell cycle distribution of invasive and noninvasive cancer cells. FUCCI-expressing cancer cells (1×10^7) were placed on Gelfoam® (1 × 1 cm) in RPMI 1640 medium. (**A**) High-magnification real-time images of a small tumor growing on Gelfoam® for 48 h. Arrows show the direction of invading cancer cells. Dashed lines show the non-invading area. (**B**) Histogram of cell cycle distribution of invading area and central non-invading area of a tumor growing on Gelfoam®. Scale bar: 100 μm.

Cancer cells in G_0/G_1 phase migrate faster than cancer cells in $S/G_2/M$ phases in Gelfoam® histoculture

Real-time confocal imaging of single-cell movement from the edge of tumors growing in Gelfoam® was performed. Cancer cells in G_0/G_1 phase migrated more rapidly than cancer cells in $S/G_2/M$ phases. The velocity of G_0/G_1 phase cells was 1.46 ± 0.44 μm/h. In contrast, the velocity of $S/G_2/M$-phase cells was 0.11 ± 0.014 μm/h ($P = 0.006$) (**Fig. 4A–C**).

Cancer cells in G_0/G_1 phase cease migration upon entry in $S/G_2/M$ phases and restart migration after cell division and re-entry in G_0/G_1 in Gelfoam® histoculture

When migrating cancer cells in G_0/G_1 phase subsequently cycled into $S/G_2/M$ phases, they ceased migration (**Fig. 5A; Video S3**). When the cancer cells re-entered G_0/G_1, they began to migrate again (**Fig. 5A; Video S3**).

Thirty G_0/G_1 cells were followed for 66 h. Some of the G_1/G_0 cells cycled into $S/G_2/M$ phases, where they stopped migrating and then divided and cycled into G_0/G_1. These cells were followed for an additional 24 h, during which time they migrated approximately 100 μm (**Fig. 5B**).

Cancer cells in G_0/G_1 phase can attach to Gelfoam® and invade more rapidly than those in S/G_2 phase

Real-time imaging of the behavior of a cell suspension layered on Gelfoam® showed that cancer cells in G_0/G_1 phase attached to Gelfoam® and began invading more rapidly (16.7 ± 8 h) than cancer cells in $S/G_2/M$ phases (30.0 ± 8 h) ($P = 0.0026$) (**Fig. 6A–C; Video S4**).

Chemotherapy does not kill or inhibit the movement of invading G_0/G_1 cancer cells in Gelfoam® histoculture

Cisplatinum (25 μm) effectively killed cancer cells in $S/G_2/M$ phases (85.0 ± 9.1% cells in apoptosis) (**Fig. 7A–E; Video S5**). In contrast, cisplatinum had little efficacy against cancer cells in G_0/G_1 phase (5.0 ± 5.9% cells in apoptosis) (**Fig. 7A–D**) and did not inhibit their movement (**Fig. 7E; Video S5**). These findings indicated that invading cancer cells in G_0/G_1 phase are resistant to cisplatinum.

In the present report, we compared the cell cycle dynamics of invading and non-invading cancer cells in 3-dimensional Gelfoam® histoculture, where cancer cells have in vivo-like behavior. Real-time imaging of cell cycle kinetics was made possible

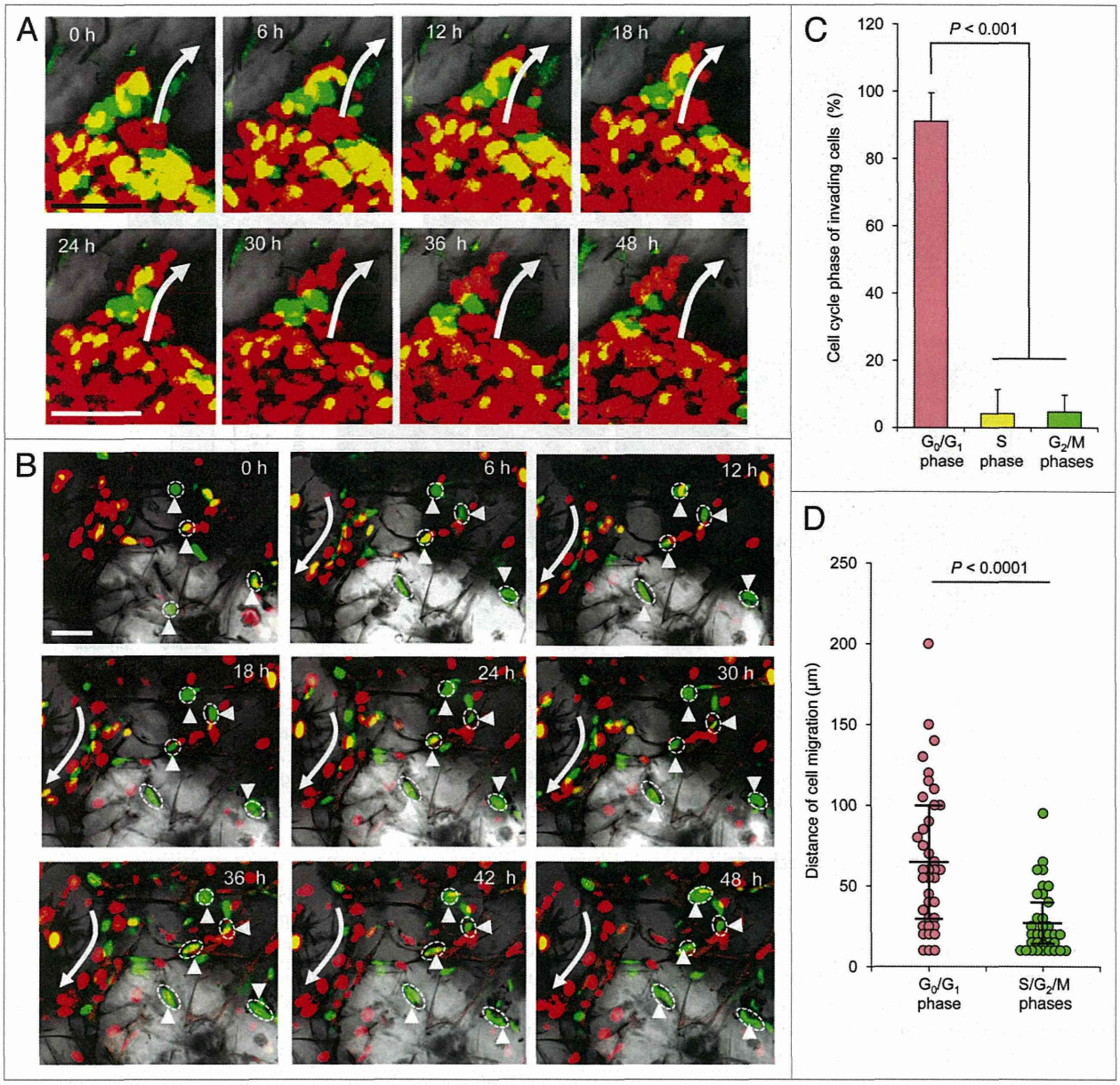

Figure 3. Invasive cancer cells are predominantly in G_0/G_1. FUCCI-expressing cancer cells (5×10^6) were placed on Gelfoam® (1 × 1 cm) in RPMI 1640 medium. (**A**) High-magnification real-time images of invading cancer cells cultured on Gelfoam® for 48 h. Arrows show the direction of invading cancer cells. (**B**) High-magnification real-time images of cancer cells in G_0/G_1 phase and in $S/G_2/M$ phases. Arrows show the direction of invading cancer cells. The cells circled with white dashed lines and pointed by arrowheads are non-invading cells. (**C**) Histogram shows cell cycle phase of invading cancer cells. (**D**) Scatter diagram shows the distance cancer cells migrated in G_0/G_1 phase compared with cancer cells in $S/G_2/M$ phases. Scale bars: 100 μm.

with the use of FUCCI-expressing cancer cells. We demonstrated that cancer cells in G_0/G_1 phase can migrate faster and further than cancer cells in $S/G_2/M$ phases. When cancer cells in G_0/G_1 cycled into $S/G_2/M$ phases, they ceased movement and then only restarted migration after re-entry into G_0/G_1 phase after cell division. Chemotherapy had little effect on G_0/G_1 invading cancer cells. The results of the present report may explain, in part, why cytotoxic chemotherapy has limited efficacy to prevent metastasis.

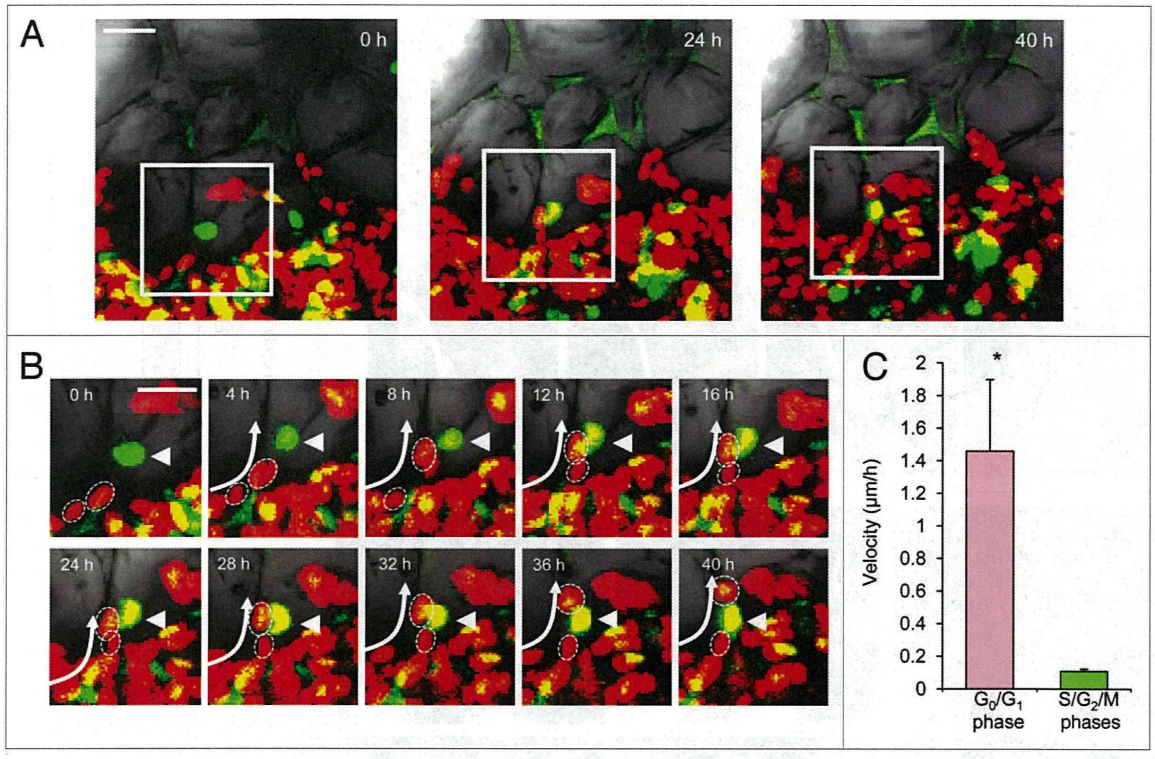

Figure 4. Behavior of individual FUCCI-expressing cancer cells cultured on Gelfoam®. FUCCI-expressing cancer cells (5×10^6) were placed on Gelfoam® (1 × 1 cm) in RPMI 1640 medium. (**A**) Low-magnification image of an overview of cancer cells cultured on Gelfoam® for 0 h, 24 h, or 40 h. (**B**) High-magnification real-time images of cancer cells in G_0/G_1 phase or in $S/G_2/M$ phases cultured on Gelfoam® for 48 h. Arrows show the direction of invading cancer cells. Circles with dashed lines show invading cells in G_0/G_1 phase. Arrowheads show a single non-invading cell. (**C**) Histogram shows the velocity of cancer cells in G_0/G_1 phase and $S/G_2/M$ phases. Scale bars: 50 μm.

Materials and Methods

Cells

MKN45 is a radio-resistant poorly differentiated stomach adenocarcinoma cell line derived from a liver metastasis of a patient.[24] The cells were grown in RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin.

Establishment of MKN45 cells stably transfected with FUCCI plasmids

Plasmids expressing mKO2-hCdt1 (green fluorescent protein) and mAG-hGem (orange fluorescent protein) were obtained from the Medical and Biological Laboratory.[7] Plasmids expressing mKO2-hCdt1 were transfected into MKN45 cells using Lipofectamine™ LTX (Invitrogen). The cells were incubated for 48 h after transfection and were then trypsinized and seeded in 96-well plates at a density of 10 cells/well. In the first step, cells were sorted for green fluorescence (S, G_2, and M phases) using a FACSAria cell sorter (Becton Dickinson). The first-step-sorted green-fluorescent cells were then super-transfected with mAG-hGem (orange) and then further sorted by orange fluorescence.

Cell culture

FUCCI-expressing MKN45 cells were seeded on plastic plates for 2-dimensional culture in RPMI-1640 medium (Mediatech). For 3-dimensional culture, FUCCI-expressing cells were cultured on Gelfoam® in the same medium.

Imaging of MKN45 cells expressing cell cycle-dependent fluorescent proteins

Confocal laser scanning microscopy was performed with the FV1000 confocal laser scanning microscopy (Olympus Corp.) with 2 laser diodes (473 nm and 559 nm). A 4× (0.20 numerical aperture immersion) objective lens and a 20× (0.95 numerical aperture immersion) objective lens (Olympus) were used. Scanning and image acquisition were controlled by Fluoview software (Olympus).[25] The tracing data were imported to Velocity 6.0 version (Perkin Elmer), where all 3D analysis was performed.

Statistical analysis

Data are shown as means ± SD. For comparison between 2 groups; significant differences were determined using the Student *t* test. *P* values of < 0.05 are considered significant.

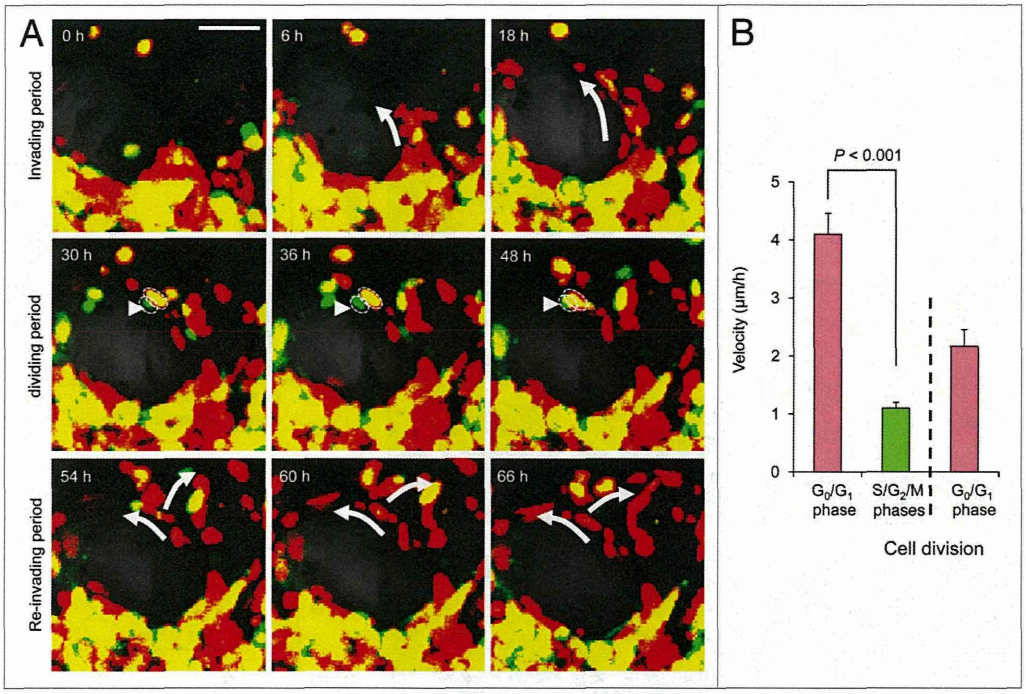

Figure 5. Comparison of migration velocity of cancer cells in G_0/G_1 phase and $S/G_2/M$ phases during cell division. FUCCI-expressing cancer cells (5×10^6) were placed on Gelfoam® (1×1 cm) in RPMI 1640 medium. (**A**) Cancer cells in G_0/G_1 phase migrated and subsequently cycled to $S/G_2/M$ phases. Arrows show the direction of G_0/G_1 phase of cancer-cell migration. Arrowheads show the cancer cells after cycling to $S/G_2/M$ phases. The cells circled with white dashed lines and pointed by arrowheads are non-invading cells. (**B**) Histogram shows the velocity of cancer cells in G_0/G_1 phase and in $S/G_2/M$ phases. Scale bar: 100 μm.

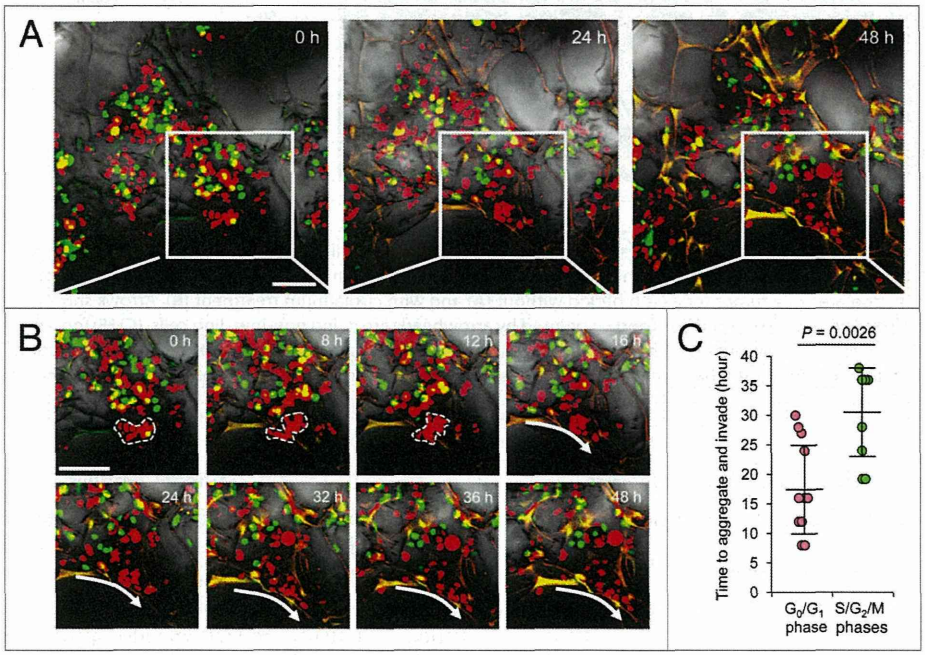

Figure 6. Cell cycle kinetics of cancer cells during seeding on Gelffoam®. FUCCI-expressing cancer cells (5×10^6) were placed on Gelfoam® (1×1 cm) in RPMI 1640 medium. (**A**) Low-magnification image of an overview of cancer cells cultured on Gelfoam® for 0 h, 24 h, or 48 h. (**B**) High-magnification real-time images of cancer cells in G_0/G_1 phase or in $S/G_2/M$ phases cultured on Gelfoam® for 48 h. Dashed lines show aggregating cells. Arrows show the direction of invading cancer cells. (**C**) Scatter diagram shows the number of hours to attach and invade taken by cancer cells in G_0/G_1 phase compared with $S/G_2/M$ phases. Scale bars: 100 μm.

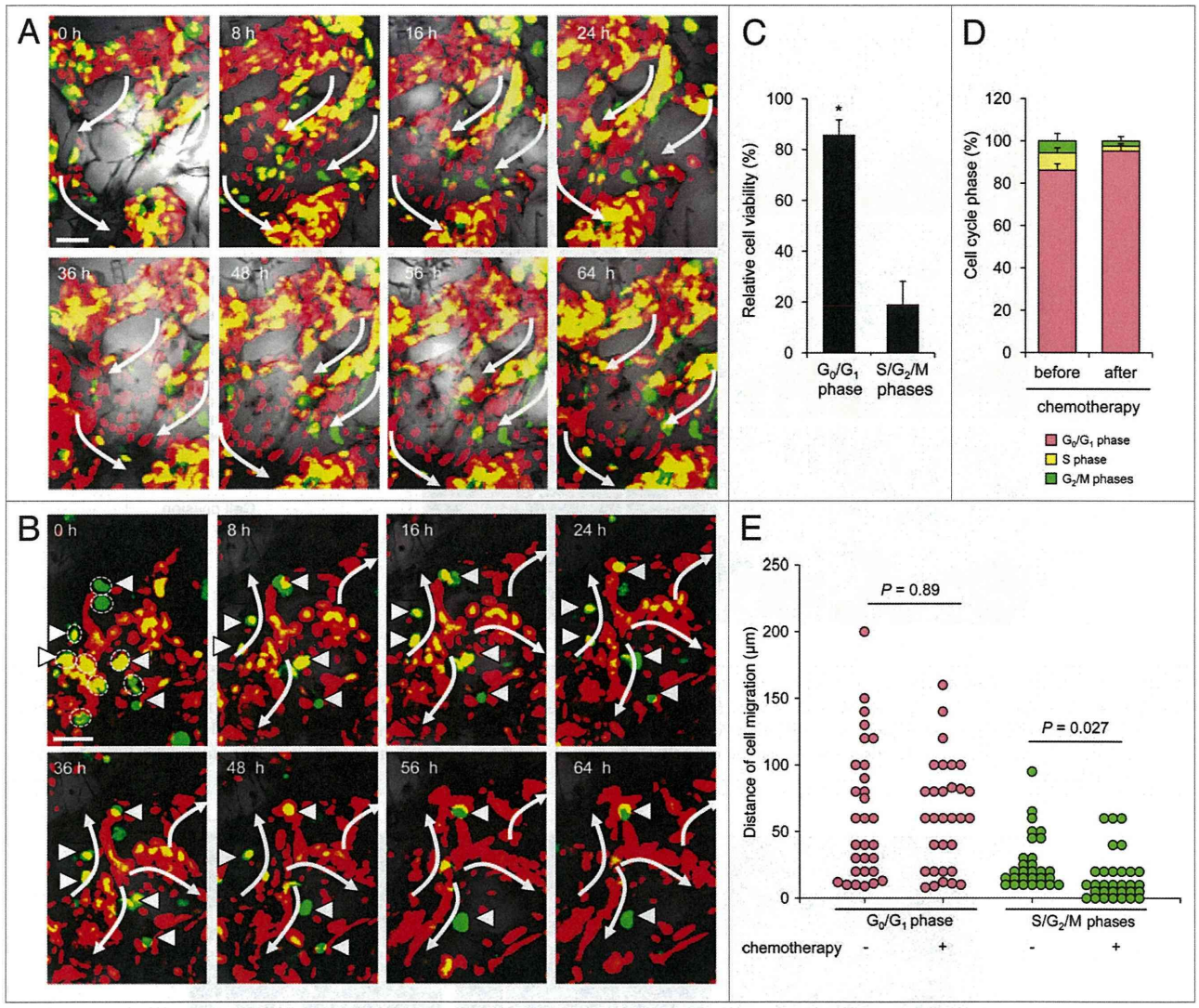

Figure 7. Chemotherapy does not kill or inhibit the movement of single invading cancer cells in G_0/G_1 phase. FUCCI-expressing cancer cells (2×10^6) were placed on Gelfoam® (1×1 cm) in RPMI 1640 medium. Three days after seeding, cisplatinum (25 μM) was added. (**A and B**) High-magnification real-time images of the disseminating area of a tumor for a 64 h period without (**A**) and with cisplatinum treatment (**B**). Arrows show the direction of invading cancer cells. The cells circled with white dashed lines and/or pointed by arrowheads are dying non-invading cells. (**C**) Histogram shows the survival rate of cancer cells in G_0/G_1 phase and $S/G_2/M$ phases 64 h after cisplatinum treatment. (**D**) Histogram shows the cell cycle distribution of cancer cells before and after cisplatinum treatment. (**E**) Scatter diagram shows the distance cancer cells migrated in G_0/G_1 phase and $S/G_2/M$ phases with or without chemotherapy. Scale bars: 100 μm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Dedication

This paper is dedicated to the memory of A.R. Moossa, MD.

Author Contributions

S.Y. and R.M.H. conceived the idea for this project. S.Y. and R.M.H. designed all experiments and wrote the manuscript. S.Y., S.M., S.M., and M.Y. performed all experiments. H.K., H.T., M.B., and T.F. provided crucial ideas and helped with data interpretation. Y.H., F.U., and H.T. provided special technical assistance.

Supplemental Materials

Supplemental materials may be found at:
www.landesbioscience.com/journals/cc/article/27818

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REVIEW

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

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Abstract

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.

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[5].

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[7,10,12,16,17,22-25]. 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[14]. Although the most recent TNM classification has included the detection of intraperitoneal free cancer cells as part of the staging process, denoting M1 disease[5], the application of peritoneal cytology in preoperative staging is still controversial. The European Society for Medical Oncology (ESMO) 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[27]. Nevertheless, peritoneal cytology has important clinical implications in the management of advanced gastric cancer[7,28].

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 was 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[31-35]. 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.

Table 1 List of published studies regarding the molecular diagnosis of peritoneal fluid in gastric cancer

Ref.	Molecule	Technique	Number of patients	Results
Asao *et al*[30]	CEA	Enzyme immunoassay	120	Correlation with 2-yr survival rate
Irinoda *et al*[32]	CEA, sialyl-Tn antigen	Enzyme immunoassay	96	Correlation with peritoneal metastasis and prognosis
Abe *et al*[31]	CEA	Enzyme immunoassay	56	Correlation with peritoneal metastasis and overall survival
Cetin *et al*[34]	CEA	Enzyme immunoassay	70	Correlation with peritoneal metastasis and overall survival
Kanetaka *et al*[29]	CEA	Enzyme immunoassay	597	Correlation with overall survival and peritoneal recurrence free survival
Yamamoto *et al*[33]	CEA, CA125	Enzyme immunoassay	229	Correlation with overall survival and recurrent cites
Li *et al*[35]	CEA	Radioimmunoassay	64	Correlation with overall survival
Kodera *et al*[38]	CEA	RT-PCR	189	Correlation with overall survival and peritoneal recurrence-free survival
Wang *et al*[36]	CEA	RT-PCR	40	Correlation with peritoneal recurrence
Sugita *et al*[41]	CEA, CK20	RT-PCR	129	Correlation with overall survival and peritoneal recurrence-free survival
Dalal *et al*[37]	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*[42]	CEA, CK20	RT-PCR	104	Predict peritoneal recurrence
Kodera *et al*[40]	CK20	RT-PCR	195	Not sufficiently sensitive as CEA
Yonemura *et al*[39]	MMP-7	RT-PCR	152	Improved the sensitivity for peritoneal dissemination in combination with cytology
Mori *et al*[43]	Multiple marker	Microarray	179	Correlation with disease-free survival and immunocytochemical cytology
Hiraki *et al*[52]	Aberrant gene methylation	Methylation-specific PCR	107	Correlation between positive methylation and peritoneal recurrence
Mori *et al*[56]	Telomerase activity	TRAP assay	46	Some concordance with cytology
Da *et al*[57]	Telomerase activity	TRAP assay	60	Correlation with high proliferating activity of gastric cancer
Wong *et al*[62]	Viral tropism	NDV-GFP imaging	30	Higher sensitivity and lower specificity than cytology
Kitayama *et al*[58]	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.

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 metalloprotease-7[39] and cytokeratin 20[40,41]. 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. Kumagai *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-

plied 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[51]. Hiraki *et al*[52,53] 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[52]. 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[54,55]. Mori *et al*[56] 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[56]. Da *et al*[57] 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*[58] 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*[58] 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 leukocytes. 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*[59] 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[55,60,61]. Wong *et al*[62] 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*[62] 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-