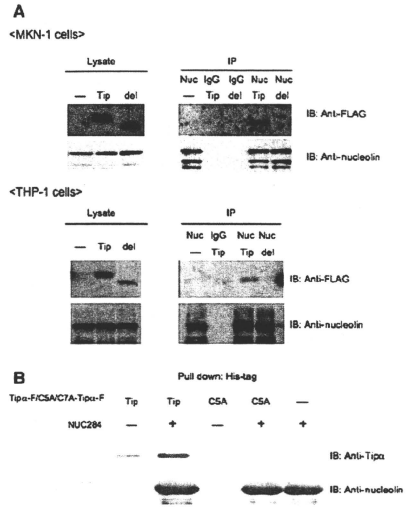

Fig. 2 Direct interaction of nucleolin with Tipα. **a** Tipα was immunoprecipitated with endogenous human nucleolin in MKN-1 and THP-1 cells. MKN-1 and THP-1 cells were treated with 100 μg/ml Tipα-FLAG (Tip) and with del-Tipα-FLAG (del) at 37°C for 1 h. Tipα-FLAG and del-Tipα-FLAG significantly incorporated into the cells (*left panels*). Each cell lysate was immunoprecipitated with anti-nucleolin antibody (NUC) and with rabbit IgG (as a control, IgG). Immunoprecipitates were resolved in 12% SDS-PAGE and immunobotted (IB) with anti-FLAG antibody and anti-nucleolin antibody (*right panels*). **b** Direct interaction of recombinant human nucleolin fragment with Tipα in vitro. His-tag removed Tipα-FLAG (Tip) and His-tag removed C5A/C7A-FLAG (C5A), which were prepared as described in Experimental procedures, were incubated with a 6-His-tag fused recombinant human nucleolin fragment containing 284–710 amino acid residues (NUC284) and then subjected to pull-down assay using Ni^{2+} chelating resins. The precipitates were resolved in 12% SDS-PAGE and analyzed by Western blotting with anti-Tipα antibody and with anti-nucleolin antibody

we first confirmed the sub-cellular localization of nucleolin in MGT-40 cells and THP-1 cells. Although most of the nucleolin was present in the nuclear fraction of MGT-40 cells, significant small amounts of nucleolin were found in membrane and cytosol fractions (Fig. 3a), while THP-1 cells showed large amounts of nucleolin in the membrane fraction (Fig. 3a). Moreover, nucleolin localized on cell surface was further determined by flow cytometry using anti-nucleolin antibody (anti-NUC295). A study of the MGT-40 cells using flow cytometry revealed that the fluorescent peak dramatically shifted to a high fluorescent peak by treatment with anti-NUC295 antibody, and that

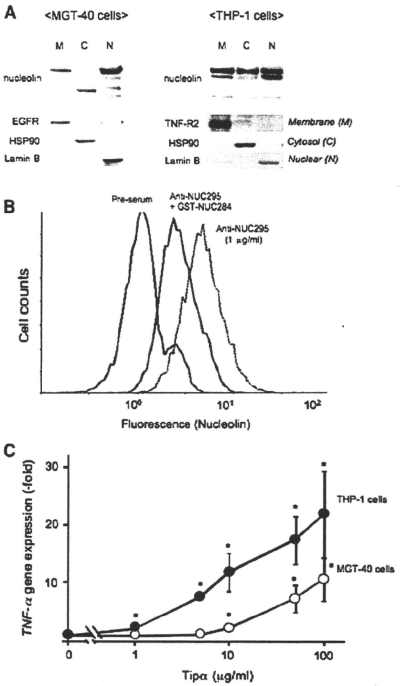

Fig. 3 Localization of nucleolin on cell surface of MGT-40 and THP-1 cells. **a** Subcellular localization of nucleolin analyzed by cell fractionation. MGT-40 and THP-1 cells were fractionated into membrane (M), cytosolic (C) and nuclear (N) fractions, and each fraction was immunoblotted with anti-nucleolin antibody. Each fraction was confirmed by Western blotting with antibodies for fractionation-marker proteins: EGFR for membrane of MGT-40 cells, TNF-R2 for membrane of THP-1 cells, HSP90 for cytosol and lamin B for nuclei. **b** Detection of nucleolin on cell surface shown by flow cytometry. MGT-40 cells were incubated with 1 μg/ml anti-NUC295 (Anti-NUC295) and with pre-immune serum (Pre-serum) as a control in the presence of 10 μg/ml Alexa Fluor 488-conjugated goat rabbit IgG on ice for 30 min. Preincubation of Anti-NUC295 with recombinant nucleolin fragment (Anti-NUC+GST-NUC284) significantly reduced fluorescence. **c** Strong induction of *TNF-α* gene expression with Tipα in THP-1 cells (*filled circle*) and MGT-40 cells (*open circle*). One hour after treatment with Tipα at various concentrations, expression of *TNF-α* and *GAPDH* genes was determined by semi-quantitative RT-PCR. Relative expression of *TNF-α* gene is shown as fold change compared with control after normalization of GAPDH mRNA levels. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical levels between non-treated and Tipα-treated cells were shown to be significant $*P < 0.01$

this fluorescent peak was significantly reduced by preincubation of anti-NUC295 with recombinant GST-nucleolin fragment (GST-NUC284) containing amino acids from 284 to 710 (Fig. 3b). This indicated that nucleolin on the cell surface had interacted with anti-NUC295. Further, we found that Tipα induced dose-dependently *TNF-α* gene expression in MGT-40 and THP-1 cells, based on the results that nucleolin localized on cell surface of both cells (Fig. 3c). The relationship between the amounts of cell surface nucleolin and the potency of Tipα on *TNF-α* gene expression will be reported elsewhere.

Effects of anti-NUC295 on *TNF-α* gene expression induced by Tipα

We studied how anti-NUC295 antibody affects the induction of *TNF-α* gene expression in MGT-40 cells treated with Tipα. First, the treatment with rabbit IgG and anti-nucleolin H-250 antibodies—the latter of which does not recognize nucleolin on cell surface—did not affect the levels of *TNF-α* gene expression induced by Tipα. However, treatment with anti-NUC295 antibody dose-dependently enhanced the *TNF-α* gene expression induced by Tipα up to twofold (Fig. 4a), and treatment with anti-NUC295 antibody dose-dependently enhanced incorporation of Tipα into the cytosol of MGT-40 cells (Fig. 4b). From our results showing that anti-NUC295 antibody internalized into MGT-40 cells as determined by flow cytometry (data not shown), we think that the complex of nucleolin, Tipα and anti-NUC295 internalized into the cells and then induced *TNF-α* gene expression.

Inhibitory effects of down-regulated cell surface nucleolin on biological activity of Tipα

Nucleolin on the cell surface is a glycoprotein containing *N*- and *O*-glycans (Carpentier et al. 2005), and the N-glycosylation of nucleolin is essential for localization on cell surface (Losfeld et al. 2009). We found that treatment of MGT-40 cells with 5 μg/ml tunicamycin, an inhibitor of the N-linked glycosylation of protein, significantly reduced the amounts of nucleolin on the cell surface as determined by flow cytometry (Fig. 5a): The levels of cell surface nucleolin were reduced by approximately 50%. Moreover, pretreatment with tunicamycin inhibited about 50% *TNF-α* gene expression induced by Tipα because tunicamycin reduced the incorporated amounts of Tipα into MGT-40 cells (Fig. 5b, c). The reduced amounts of nucleolin correlated well with reduction of *TNF-α* gene expression. Cell surface nucleolin is thus a functional receptor of Tipα associated with incorporation of Tipα into the cells and subsequent *TNF-α* gene expression.

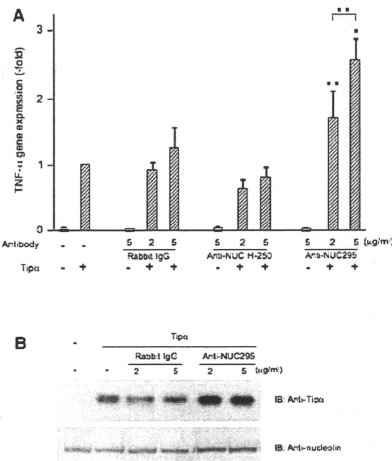

Fig. 4 Significant enhancement of Tipα-induced *TNF-α* gene expression and Tipα incorporation in cells induced by anti-NUC295. **a** MGT-40 cells were previously incubated with rabbit IgG, with anti-NUC H-250 and anti-NUC295 antibodies at 4°C for 1 h, and further treated with 50 μg/ml Tipα at 37°C for 1 h. Relative *TNF-α* gene expression is shown as fold change compared with that of cells treated with 50 μg/ml Tipα after normalization of *GAPDH* gene expression levels. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical significance of effects of anti-NUC295 in TNF-α induction by Tipα compared with non-treated were shown as $*P < 0.01$ and $**P < 0.05$, and the difference between 2 and 5 μg/ml of anti-NUC295 was significant at the level of $**P < 0.05$. **b** Incorporation of Tipα was determined by Western blotting with anti-Tipα antibody. Nucleolin levels were also determined by anti-nucleolin antibody

Discussion

Considering our 1993 discovery that TNF-α is an endogenous tumor promoter in carcinogenesis (Komori et al. 1993), we first cloned a new gene of TNF-α inducing protein from *H. pylori* genome (Suganuma et al. 2005). We also reported that the active form of Tipα is a homo-dimer that induces *TNF-α* gene expression in the cells, resulting in a cancer microenvironment (Suganuma et al. 2006, 2008). Furthermore, Tipα is now widely accepted as a carcinogenic factor of *H. pylori* (Balkwill 2009). This paper reports that Tipα directly binds to nucleolin on the cell surface, and that the complex of Tipα with nucleolin then internalizes into the cells. The results suggest that cell surface nucleolin acts as a receptor of Tipα: nucleolin is mainly localized in the nucleolus, but significant amounts are present on the cell surface, including various cancer

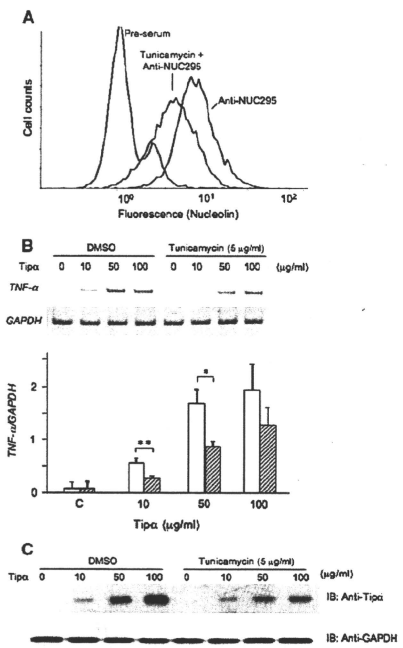

Fig. 5 Inhibition of Tipα-induced *TNF-α* gene expression and Tipα incorporation in cells induced by down-regulation of cell surface nucleolin. **a** MGT-40 cells were treated with or without 5 μg/ml tunicamycin in DMSO at 37°C for 5 h. The cell surface nucleolin on MGT-40 cells was visualized using flow cytometry with anti-NUC295, as described in Materials and methods. **b** Inhibition of *TNF-α* gene expression induced by Tipα after pretreatment with tunicamycin. After pretreatment of MGT-40 cells with or without 5 μg/ml tunicamycin in DMSO at 37°C for 5 h, the cells were treated with various concentrations of Tipα at 37°C for 1 h. The levels of *TNF-α* and *GAPDH* gene expression in MGT-40 cells were determined by semi-quantitative RT-PCR. The results are the averages of three independent experiments. *Bars* indicate standard deviation. Statistical levels were significant $*P < 0.01$ and $**P < 0.05$. **c** Inhibition of Tipα-incorporation into MGT-40 cells by pretreatment with tunicamycin. After treatment with tunicamycin, both MGT-40 cells treated with various concentrations of Tipα and cell lysates were resolved in 12% SDS-PAGE solution and analyzed by Western blotting with anti-Tipα antibody and anti-nucleolin antibody (IB)

cells and proliferating cells (Hirano et al. 2005; Hoja-Lukowicz et al. 2009; Hovanessian et al. 2000; Legrand et al. 2004; Reyes-Reyes and Akiyama 2008). It is well-known that cell surface nucleolin has an important role as a receptor for various extracellular ligands, including human immunodeficiency virus (HIV) particles (Nisole et al. 2002), midkine (Hovanessian 2006; Said et al. 2002), and elongation factor-TU of *Francisella tularensis* (Barel et al. 2008), lactoferrin (Legrand et al. 2004), and endostatin (Shi et al. 2007): Nucleolin acts as a shuttling molecule between cell surface, cytoplasm, and nucleus (Borer et al. 1989). Moreover, it is of interest to note that a specific DNA aptamer of nucleolin, AS1411, is the most well-investigated anti-cancer aptamer, which initially binds to cell surface nucleolin and then internalizes into the cells (Ireson and Kelland 2006). Based on evidence, we think that nucleolin shuttles Tipα, which is supported by the results that some Tipα are present in the nuclei of MGT-40 cells after treatment with Tipα protein. Although the precise function of Tipα in nucleus is not well understood, we found that Tipα directly binds to DNA oligomers in Biacore assay (Kuzuhara et al. 2007). Thus, our understanding on the Tipα function is extended by the several findings, such as nucleolin as the receptor, the translocation of Tipα into the nuclei, and the induction of *TNF-α* gene expression in the cells.

Although it is not well-known how nucleolin translocates across the membrane and how it attaches to the cell surface, glycosylation is assumed to be an essential biochemical modification for nucleolin to localize on the cell surface (Losfeld et al. 2009). In our experiments, tunicamycin significantly reduced the level of cell surface nucleolin in MGT-40 cells—although the nucleolin in nucleoli was not much reduced—and then inhibited both internalization of Tipα and *TNF-α* gene expression. But it is still not clear whether N-glycosylation of nucleolin is involved in Tipα binding because Tipα directly binds to recombinant nucleolin fragment (NUC284) without any glycosylation. Since NUC284 fragment contains four RNA binding domains (RBDs) and a RGG domain—which are well conserved in human, mouse, and rat (Ginisty et al. 1999)—we think that Tipα binds to one of these domains. To understand more precisely the nature of Tipα and nucleolin binding, we used anti-nucleolin antibody (Anti-NUC295) (Hirano et al. 2005): pretreatment with anti-NUC295 unexpectedly enhanced internalization of Tipα and induction of *TNF-α* gene expression by Tipα, indicating that the Tipα binding site of nucleolin is different from epitope of nucleolin and that anti-NUC295 enhances internalizing of nucleolin with Tipα. These findings support the previously reported results with another anti-nucleolin antibody (mAb D3), which recognizes cell surface nucleolin and induces clustering and internalization of nucleolin together with mAb D3 antibody (Hovanessian et al. 2000). Since, we obtained the results that pretreatment with methyl-β-cyclodextrin, which inhibits endocytosis by depletion of cholesterol from membrane, inhibited induction of *TNF-α* gene expression with Tipα (unpublished results), we think that anti-NUC295

enhances endocytosis of Tipα. To prove evidence that nucleolin acts as a specific receptor of Tipα, we conducted a knockdown experiment with shRNA using lentiviral vector, and the growth of THP-1 cells was inhibited, by the complete down-regulation of nucleolin (data not shown).

Several polypeptides co-precipitated with Tipα-FLAG, but did not with del-Tipα-FLAG, in pull-down assay. Although the specificity of the co-precipitation was relatively high, we found that ribosomal protein L4 is an additional binding protein. That is well-known to be a protein interacting with nucleolin. As for interaction of Tipα with nucleolin, the N-terminal portion of Tipα is thought to be an important domain: (1) disulfide bond formation in the N-terminal of Tipα is essential for the interaction, and monomer of del-Tipα does not bind to nucleolin, and (2) we successfully identified nucleolin as the Tipα-binding protein because we used FLAG-tagged at the C-terminal position of Tipα as bait, but did not use the His-tagged at the N-terminal position.

The TNF-α inducing activity of Tipα should be briefly mentioned in connection with *H. pylori*-infection in human stomach cancer development. Tipα-deficient *H. pylori* reduces colonization in mouse gastric mucosa (Godlewska et al. 2008), and vaccinations with Tipα and del-Tipα also effectively prevented colonization of *H. pylori* in the stomach of mice (Inoue et al. 2009). Therefore, we think that targeting molecules, which inhibit the interaction of Tipα and cell surface nucleolin, will be useful tools for the prevention of inflammation induced by *H. pylori* infection and of *H. pylori*-infection itself. For example, lactofferin, which binds to nucleolin (Legrand et al. 2004), is effective in suppression of *H. pylori* colonization (Okuda et al. 2005), which suggests that lactofferin inhibits the binding of Tipα and nucleolin.

How nucleolin is involved in the induction of *TNF-α* gene expression induced by Tipα is an important subject, since TNF-α is a major mediator of cancer-related inflammation in the cancer microenvironment (Balkwill 2009; Komori et al. 1993; Suganuma et al. 1999). A specific DNA aptamer of nucleolin, AS1411 (Ireson and Kelland 2006; Soundararajan et al. 2008) blocks both TNF-α induced- and constitutive-NF-κB activation in human cancer cell lines by forming a complex of nucleolin with an NF-κB essential modulator (NEMO) (Girvan et al. 2006). This indicates that nucleolin regulates NF-κB activation through interaction with NEMO, so it is possible that Tipα incorporated with nucleolin interferes in the interaction of nucleolin with NEMO and affects NF-κB signaling.

Tipα family genes and protein products show carcinogenic activity in combination with v-H-*ras* oncogene: Transfection of *HP-MP1* gene into Bhas 42 cells (v-H-ras transfected BALB/3T3) induces highly malignant transformed cells (Bhas/mp-1): these cells have strong tumorigenicity associated with a high grade of angiogenesis in nude mice (Suganuma et al. 2001). Interestingly, it has been reported that overexpression of nucleolin cooperates with oncogenic mutant Ras in a rat embryonic fibroblast transformation assay (Takagi et al. 2005). And midkine and pleiotrophin, which are ligands of nucleolin, transformed cells (Muramatsu 2002). Nucleolin is specifically expressed on the cell surface in proliferating endothelial cells (Shi et al. 2007), and it is also well-known that nucleolin protein is expressed at high levels on the cell surface of rapid proliferation cells, including cancer cells such as MCF-7 (breast cancer) (Soundararajan et al. 2008), HeLa (cervical cancer) (Li et al. 2009), colo-320 (colon adenocarcinoma) (Reyes-Reyes and Akiyama 2008) and THP-1 cells (Barel et al. 2008; Hirano et al. 2005). We also found that nucleolin is expressed on the surface of mouse and human gastric cancer cell lines (MGT-40 and MKN-1). The study on the expression levels and localization of cell surface nucleolin during development of gastric cancer by *H. pylori*-infection will surely provide a new insight into the identification of high-risk *H. pylori* carriers in more detail. Since 50% of the world population is infected with *H. pylori* (Snaith and El-Omar 2008), our results with Tipα indicate that nucleolin on the cell surface will prove useful as a high-risk biomarker for gastric cancer. This paper is the first report that nucleolin serves as a receptor of Tipα, the carcinogenic factor of *H. pylori*: further results with a complex of Tipα with nucleolin will intensify the understanding of this new carcinogenic mechanism on gastric cancer development in humans.

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CASE REPORT

Eight-year survival after advanced gastric cancer treated with S-1 followed by surgery

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Abstract

We report a case of advanced gastric cancer, with cervical, axillary, and abdominal paraaortic lymph node metastases, that was successfully treated with chemotherapy and surgery. The disease was initially considered unresectable, and the patient was treated with orally administered S-1. Chemotherapy was effective, and all lymph node metastases disappeared after 6 courses. After 27 mo of chemotherapy, the patient underwent curative surgery, with subtotal gastrectomy and lymph node dissection. Histopathological examination revealed many viable poorly differentiated adenocarcinoma cells in the stomach, but no cancer cells in the lymph nodes. The patient is alive, without recurrence, 8 years later. This, therefore, is a case report of an 8-year survivor of advanced gastric cancer with distant lymph node metastasis.

Key words: S-1; Lymph node metastasis; Gastric cancer; Gastrectomy

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INTRODUCTION

Improvements in early diagnosis of gastric cancer (GC) have led to an increase in the number of patients who are able to undergo curative resection[1]. However, the prognosis for patients with unresectable or metastatic disease is very poor, and long-term survival, particularly for more than 5 years, is rare. S-1 is a novel oral fluoropyrimidine that was developed in Japan. It has been available for patients with GC in Japan since 1999 and is currently being investigated worldwide. S-1 consists of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate at a fixed molar ratio of 1:0.4:1. Tegafur is a prodrug of fluorouracil (5-FU), which is the cytotoxic component of this combination. CDHP is a potent reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), the chief catabolic enzyme of 5-FU. Potassium oxonate selectively inhibits orotate phosphoribosyltransferase, the enzyme responsible for 5-FU activation in the gastrointestinal tract, thus reducing the gastrointestinal toxicity of the combination. Many reports have indicated the efficacy of this antitumor agent against gastric cancer.

We report a case of advanced gastric cancer (AGC) with unresectable lymph node metastases treated with S-1.

CASE REPORT

A 71-year-old man visited a local hospital in 2002, complaining of upper abdominal discomfort of one-month duration. Endoscopy revealed a concave lesion in the stomach, and the patient was subsequently referred to our hospital. Endoscopy showed a diffuse concave lesion with ulceration on the lesser curvature in the middle of the stomach. A biopsy specimen showed signet-ring cell carcinoma. Physical examination revealed a few elastic-hard masses in the left supraclavicular area (approximately 7 cm in diameter) and left axillary space (approximately 6 cm in diameter), suggesting lymph node metastases from the GC. Abdominal computed tomography (CT) showed marked swelling of several paraaortic lymph nodes (Figure 1A). We could not find metastasis to the liver, lung, or peritoneum. The patient's diagnosis was AGC with extensive lymph node metastases (stage Ⅳ: cT2 cN3 cP0 cH0 cM1). His general condition was good, with The Eastern Cooperative Oncology Group (ECOG) performance status 0, and biochemical analysis of blood and urine specimens showed no abnormalities. Serum levels of the tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were not elevated (2.1 ng/mL and 12.0 U/mL, respectively). Since curative resection was impossible for this patient, chemotherapy with S-1 was started. As one course, 120 mg (80 mg/m^2) of S-1 was orally administered daily for 28 d, followed by a 14-d rest period; administration occurred primarily at the outpatient clinic. After 2 courses, endoscopy showed no remarkable change in the gastric tumor. However, abdominal CT showed remarkable reductions in the size of regional and paraaortic lymph nodes (Figure 1B), and also in cervical and axillary lymph nodes (Figure 2A and B). This indicated a partial response (PR). The evaluation after 6 courses of S-1 showed that there was no change at the primary site, but CT showed complete disappearance of paraaortic, cervical and axillary lymph nodes. Therefore, this revealed a complete response (CR), and this condition was stable up to the subsequent surgery. The patient did not experience any critical adverse event during S-1 administration, despite developing hematological toxicity; including leucopenia (2100/μL at the minimum), thrombocytopenia, and non-hematological toxicity; including grade 2 hand-foot syndrome and grade 1 stomatitis, according to National Cancer Institute Common Toxicity Criteria Version 2.0. After 19 courses of S-1, the tumor was limited to the primary gastric site, with no metastasis detected from neck to abdomen by CT or positron emission tomography (PET); only the detection of gastric tumor by PET. Therefore, we concluded that curative resection could be achieved. In 2004, distal gastrectomy and lymph node dissection (D2) were performed. We did not find cancer cells in the intraoperative peritoneal lavage, or in the abdominal paraaortic lymph node specimens. Figure 3 shows the resected gastric specimen. A concave lesion of 45 mm × 40 mm with an ulceration scar was located in the lesser curvature of the middle of the stomach (Figure 3). Histopathologi-

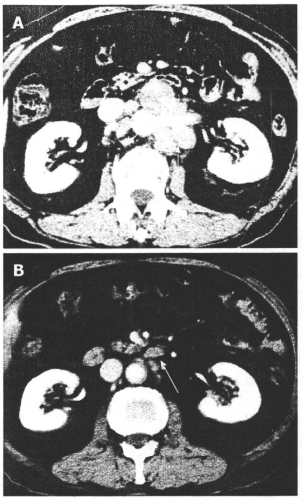

Figure 1 Abdominal computed tomography (CT). A: Marked swelling of paraaortic lymph nodes before treatment (arrow); B: After two courses of S-1, abdominal CT showed remarkable reductions (arrow).

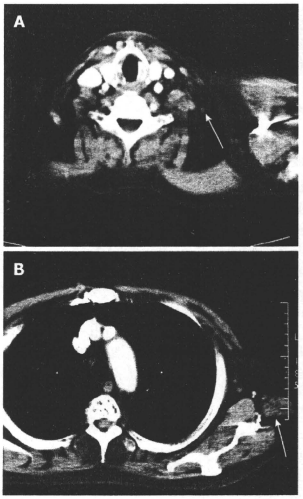

Figure 2 CT shows a Virchow's (A) and an axillary lymph node (B) which were reduced remarkably in size after treatment.

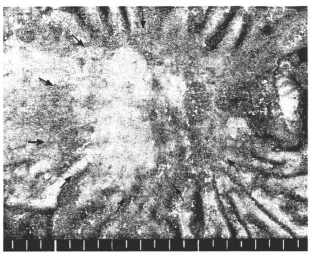

Figure 3 Resected specimen shows a concave lesion of 45 mm × 40 mm (arrows) with an ulceration scar which was located in the lesser curvature in the middle of the stomach.

cal examination showed numerous poorly differentiated adenocarcinoma cells in the mucosal layer (Figure 4A). There were only a small number of poorly differentiated adenocarcinoma cells, but severe fibrosis, in the submucosal layer (Figure 4B). The histological effect of chemotherapy on the primary tumor was limited, and according to the Japanese classification of gastric carcinoma (second English edition)[2], it was classified as grade 1a. The only findings in the lymph nodes were remarkable fibrosis and giant cells, with no cancer cells seen; the histological effect on the lymph nodes metastasis was classified as grade 3 (Figure 4C). Therefore, a radical operation was considered to have been performed, and the final pathological stage assigned as Stage IA (pT1, pN0, sP0, sH0, sM0) according to the Japanese classification of gastric carcinoma (second English edition)[2]. There have been no signs of recurrence since the surgery, and the patient has survived for 8 years after initially receiving chemotherapy.

DISCUSSION

There have been some series that reported high response rates with the use of newer combinations of chemotherapy regimens for unresectable or metastatic GC[3]. However, standard regimens have not yet been established worldwide. S-1-based chemotherapy has shown survival benefits in recent randomized controlled trials. Boku *et al*[4] reported that S-1 monotherapy showed a significant non-inferiority benefit compared to continuously infused 5-FU in unresectable or metastatic GC ($P < 0.001$). Moreover, Koizumi *et al*[5] reported the results of a randomized controlled trial of S-1 *vs* S-1 with CDDP. The overall survival for S-1 with CDDP was superior to S-1 alone. The 1- and 2-year survival rates were 54.1% and 23.6%, respectively, whereas a 2-year survival rate of less than 10% was shown in the 5-FU+CDDP arm of a Japanese randomized controlled trial with a 10-year follow-up[6].

Although recent phase III clinical trials indicated longer survival times[7,8], few patients with unresectable GC have survived longer than 5 years. During the last 10 years at our hospital, only 9 of more than 500 patients treated with chemotherapy for metastatic or recurrent

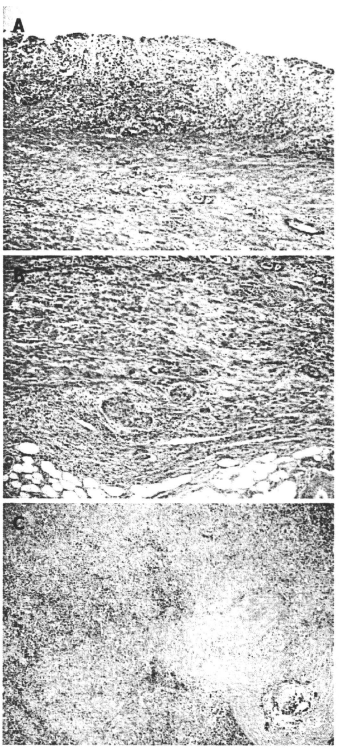

Figure 4 Histopathological examination. A: Histopathological examination showed numerous poorly differentiated adenocarcinoma cells in the mucosa; B: There were only a small number of poorly differentiated adenocarcinoma cells, but severe fibrosis, in the submucosal layer; C: The only findings in the lymph nodes were remarkable fibrosis and giant cells, with no cancer cells seen.

GC have survived for 5 years. Yoshida *et al*[9] reported a 5-year survival rate of 2% for registered Japan Clinical Oncology Group (JCOG) clinical trials and mentioned certain characteristics of these survivors. Most of the 5-year survivors had good performance status, macroscopically non-scirrhous-type tumors, only one metastatic site, a paraaortic node metastasis as the only unresectable factor, and achieved a CR after the initial chemotherapy. However, it is unclear whether such long survivals are because of biological non-aggressiveness or due to a good response to chemotherapy. There have been only five cases reported in the literature of AGC with neck lymph node metastasis in which there was no residual tumor following chemotherapy and surgical resection[10-14]. Ohyama *et al*[10] reported a case of a patient treated with combination chemotherapy, including 5-FU, leucovorin, cisplatin,

and etoposide, in which there was a good response. Chemotherapy was followed by curative resection, including subtotal gastrectomy and inguinal, neck, and abdominal lymph node dissection. Pathological examination showed cancer cells only in the abdominal lymph nodes, but not in the stomach or neck lymph nodes. Iwazawa *et al*[11] reported a case of AGC with cervical lymph node metastasis, in which a CR for cervical lymph nodes was achieved with S-1 alone treatment, and a curative resection of the residual tumor was performed. However, further outcomes of these cases were not described. It remains unknown whether adjuvant surgery for residual tumor after effective chemotherapy is beneficial for GC patients with distant metastasis or not. In our experience, if there is a long-maintained disappearance of inoperable metastasis, adjuvant surgery is considered beneficial.

In our case, there was no confirmation of the neck and axillary lymphadenopathy from imaging examinations before treatment began, and therefore no evidence that this lymphadenopathy was due to metastasis from GC. This is a limitation of our report and we assume responsibility for not obtaining all available evidence of metastasis from GC, although surgeons agreed on the non-operability of this patient in the preoperative conference. However, in patients with histologically diffuse type GC that is greater than 3 cm in size and which has invaded the submucosal layer, 7.5% have lymph node metastasis beyond the perigastric site[15]. Thus, our assumption that cervical and axillary lymphadenopathy was due to GC metastases was probably correct.

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Circulating tumor cells as a surrogate marker for determining response to chemotherapy in patients with advanced gastric cancer

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The purpose of this study was to quantify circulating tumor cells (CTCs) in advanced gastric cancer (AGC) patients, and to demonstrate the role of CTCs in cancer therapy. This study investigates the hypothesis that CTCs can predict clinical outcomes in patients with AGC. From November 2007 to June 2009, 52 patients with AGC were enrolled into a prospective study. The chemotherapy regimen was an S-1-based regimen (S-1 with or without cisplatin) or paclitaxel. CTCs of whole blood at baseline, 2 weeks, and 4 weeks after initiation of chemotherapy, were isolated and enumerated using immunomagnetics. Patients with ≥4 CTCs at 2-week points and 4-week points had a shorter median progression-free survival (PFS) (1.4, 1.4 months, respectively) than those with the median PFS of <4 CTCs (4.9, 5.0 months, respectively) (log-rank test; $P < 0.001$, $P < 0.001$, respectively). Patients with ≥4 CTCs at 2-week points and 4-week points had shorter median overall survival (OS) (3.5, 4.0 months, respectively) than those with the median PFS of <4 CTCs (11.7, 11.4 months, respectively) (log-rank test; $P < 0.001$, $P = 0.001$, respectively). In conclusion, this study demonstrates that CTC measurement may be useful as a surrogate marker for determining response to S-1-based or paclitaxel regimens in AGC. (*Cancer Sci* 2010; 101: 1067–1071)

Gastric cancer is more prevalent in Asia, Eastern Europe, and Central and South America than in other areas. In Japan, this cancer is one of the most common causes of cancer-related mortality, despite dramatic advances in diagnosis and treatment. Outcomes are extremely poor in patients with unresectable gastric cancer, with the median survival ranging from 3 to 5 months with the best supportive care.[1–3] The ability to identify patients with the worst prognoses or those destined to progress quickly could have broad clinical applications.

Circulating tumor cells (CTCs) or disseminated tumor cells (DTCs) in bone marrow and peripheral blood from patients with cancers have been documented.[4–6] Braun *et al.*[7,8] reported that ~30% of women with primary breast cancer have DTCs in bone marrow, and a 10-year follow-up of these patients revealed a significantly decreased disease-free survival and overall survival (OS) when compared with patients without DTCs. However, aspiration of bone marrow is time consuming and, in many cases, uncomfortable for the patients precluding multiple samplings for therapy monitoring studies. Therefore, recent efforts have concentrated on the detection of CTCs in the peripheral blood of cancer patients. Cristofanilli *et al.*[9,10] showed in a prospective study that CTC detection provided significant prognostic information for patients with metastatic breast cancer. Cohen *et al.*[11] showed that the number of CTCs before and during treatment was an independent predictor of PFS and OS in patients with metastatic colorectal cancer. It is not clear whether CTC detection using this system provides prognostic information for patients with advanced gastric cancer. We initiated this study to evaluate whether CTCs could serve as a prognostic and/or predictive marker in patients with AGC.

Materials and Methods

Patients. All patients were enrolled using institutional review board-approved protocols at the Cancer Institute Hospital at the Japanese Foundation for Cancer Research and provided informed consent. The study population consisted of patients aged 18 years or older with histologically proven AGC. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 2; adequate organ function; and S-1-based (S-1 with or without cisplatin) or paclitaxel chemotherapy regimen. The subjects were five patients treated with S-1 (40 mg/m^2, twice daily, days 1–28, repeated every 6 weeks), 26 patients treated with S-1 plus CDDP (S-1 40 mg/m^2, twice daily, days 1–21, CDDP 60 mg/m^2, day 8, repeated every 5 weeks), and 21 patients treated with paclitaxel (80 mg/m^2, weekly).

Sample preparation for isolation of CTCs from blood. Blood was drawn from advanced gastric cancer patients into 10 mL of evacuated blood for CTC in a Cell Save Preservative Tube (Veridex, Raritan, NJ, USA). Blood was always drawn from cancer patients before treatment initiation (baseline), 2 weeks, and 4 weeks after the administration of an S-1-based or paclitaxel regimen. The CellSearch system (Veridex) consists of the CellPrep system, the CellSearch Epithelial Cell Kit (for the measurement of CTC), and the CellSpotter Analyzer. The CellPrep system is a semi-automated sample preparation system, and the CellSearch Epithelial Cell Kit consists of ferrofluids coated with epithelial cell-specific EpCAM antibodies to immunomagnetically enrich epithelial cells; a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratin 8, 18, and 19; an antibody to CD45 conjugated to allophycocyanin; nuclear dye 4′,6- diamidino-2-phenylindole (DAPI) to fluorescently label the cell; and buffers to wash, permeabilize, and resuspend the cells. Sample processing and evaluation were done as described by Allard *et al.* Briefly, 7.5 mL of blood for CTCs were mixed with 6 mL of buffer, centrifuged at 800*g* for 10 min, and then placed on the CellPrep system. After aspiration of the plasma and buffer layer by instrument, ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were then added in conjunction with a permeabilization buffer to fluorescently label the immunomagnetically labeled cells. After incubation in the system, the magnetic separation was repeated, and

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excess staining reagents were aspirated. In the final processing step, the cells were resuspended in the MagNest Cell Presentation Device (Veridex). This device consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis using the CellSpotter Analyzer.

Sample analysis. The MagNest was placed on the CellSpotter Analyzer, a four-color semi-automated fluorescence microscope. Image frames covering the entire surface of the cartridge for each of the four fluorescent filter cubes were captured. The captured images containing objects that met predetermined criteria were automatically presented in a web-enabled browser from which final selection of cells was made by the operator. The criteria for an object to be defined as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratin, and negative staining for CD45. Results of cell enumeration are always expressed as the number of cells per 7.5 mL of blood for CTCs.

Statistical analysis. Progression-free survival (PFS) was defined as the elapsed time from blood collection to progression. Kaplan–Meier survival plots were generated based on CTC levels each time blood was collected, and the curves were compared using a log-rank testing. A P-value <0.05 was considered significant. Cox proportional hazards regression was used to determine univariate and multivariate hazard ratios for selected potential predictors of PFS and OS. The distribution of patients above and below the CTC threshold and clinical response was compared using Fisher's exact test.

Results

Patient characteristics. A total of 52 patients were enrolled. Patients' characteristics at baseline are summarized in Table 1. Patients' characteristics were as follows: median age, 62 years (range, 24–78 years); PS 0/1/2, 39/12/1; primary tumor +/−, 33/19; and regimen S-1/S-1 with cisplatin/paclitaxel, 5/26/21. Thirty-five patients had diffuse-type histology (67.3%). Seventeen patients (32.7%) had intestinal type. Among 52 patients, the best response rates were 28.8% (complete response [CR]/partial response [PR]/stable disease [SD]/progressive disease [PD]: 0/15/19/18). Of 31 patients treated with the S-1-based regimen (S-1 alone or S-1/cisplatin [CDDP]) assessable for response, we observed 14 PR (45.2%), 11 patients (35.5%) with SD, and six patients (19.4%) with PD during treatment. The overall response rate was 45.2%. On the other hand, of 21 patients treated with the weekly paclitaxel regimen assessable for response, we observed one PR (4.8%), eight patients (38.1%) with SD, and 12 patients (57.1%) with progression of disease during treatment, for an overall response rate (RR) of 4.8% (Table 2).

Table 1. Patient demographics

Demographic	Number or median (range)
Median age (range)	62 (24–78)
Male/female	44/8
PS: 0/1/2	39/12/1
S1-based/PAC regimen	31/21
Line: 1st/2nd	34/18
Histopathology: diffuse/intestinal type	35/17
Primary tumor: +/−	33/19
Sites of metastasis: +/−	
Liver	24/28
Lung	3/49
Bone	1/51
Peritoneum	22/30
Lymph node	37/15

Table 2. Objective response

	S1-based regimen (31) S1 alone (5), S1/CDDP (26) 1st line (31)	PAC (21) Weekly PAC (21) 1st line (3), 2nd line (18)
CR	0	0
PR	14	1
SD	11	8
PD	6	12

CDDP, cisplatin; CR, complete response; PAC, paclitaxel; PD, progressive disease; PR, partial response; SD, stable disease.

Stratification according to CTC levels. To select a level of circulating tumor cells that most clearly distinguished patients with a response of chemotherapy, thresholds of 1 to 88 cells for 2-week point were systematically correlated with PFS for 26 of the 30 patients in the training set. The median PFS among patients with levels above or below each threshold differed at the level of one circulating tumor cell per 7.5 mL of blood, and reached a plateau at approximately four cells per 7.5 mL of blood. At the latter level, the Cox proportional hazards ratio signifying the difference between slow and rapid progression of disease also reached a plateau. Thus, a cut-off of four circulating tumor cells per 7.5 mL of blood was chosen to distinguish patients.[12] The Kaplan–Meier circulating tumor-cell counts were available at a 2-week point for 26 of the thirty patients in the training set and for 21 of the 22 patients in the validation set. Neither PFS nor OS was significantly different in the two sets (data not shown). Because the two sets of data were nearly identical, they were combined for the estimation of PFS and OS for the entire population.

CTCs and imaging to assess response to therapy. Thirty-four (65.4%) of 52 patients were classified as having non-progressive disease (non-PD), with 24 of these patients (46.2%) having <4 CTCs and 10 patients (19.2%) having ≥4 CTCs before the initiation of therapy. Ten (19.2%) of 52 patients were classified as having PD, with 11 of these patients (21.2%) having <4 CTCs and seven patients (13.4%) having ≥4 CTCs before the initiation of therapy. The difference between the clinical responses and CTC levels were not significant. In contrast, 33 (64.7%) of 51 patients were classified as having non-PD, with 33 of these patients (64.7%) having <4 CTCs and no patients (0%) having ≥4 CTCs at 2 weeks. Eighteen (35.3%) of 51 patients were classified as having PD, with 11 of these patients (21.6%) having <4 CTCs and seven patients (13.7%) having ≥4 CTCs at 2 weeks. The difference between the clinical responses and CTC levels was highly significant. ($P = 0.001$, Fisher's exact test). Thirty-two (64%) of 48 patients were classified as having non-PD, with 31 of these patients (64.6%) having <4 CTCs and one patient (2.0%) having ≥4 CTCs at 4 weeks. Sixteen (33.3%) of 48 patients were classified as having PD, with eight of these patients (16.7%) having <4 CTCs and eight patients (16.7%) having ≥4 CTCs at 4 weeks. The difference between the clinical responses and CTC levels were highly significant ($P < 0.001$, Fisher's exact test) (Table 3).

Analysis of PFS according to CTC level. Figure 1 shows the Kaplan–Meier plots for prediction of PFS using the baseline CTC counts (Fig. 1a), at 2 weeks (Fig. 1b), and at 4 weeks (Fig. 1c). Seventeen of the patients (32.7%) had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with that of patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.4 months; 95% confidence interval [CI], 1.2–1.6) than the median PFS of <4 CTCs at 2 weeks (4.9 months; 95% CI, 4.0–5.8) ($P < 0.001$) (Fig. 1b). Patients with ≥4 CTCs at the 4-week point had a shorter median

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Table 3. CTCs and correlation with response assessment by imaging

	Non-PD			PD			Fisher's exact P-values
	No. of patients	CTCs <4 (%)	CTCs ≥4 (%)	No. of patients	CTCs <4 (%)	CTCs ≥4 (%)	
Baseline	34	24 (46.2)	10 (19.2)	18	11 (21.2)	7 (13.4)	0.544
2 week	33	33 (64.7)	0 (0)	18	11 (21.6)	7 (13.7)	0.001
4 week	32	31 (64.6)	1 (2.0)	16	8 (16.7)	8 (16.7)	<0.001

CTCs, circulating tumor cells; PD, progressive disease.

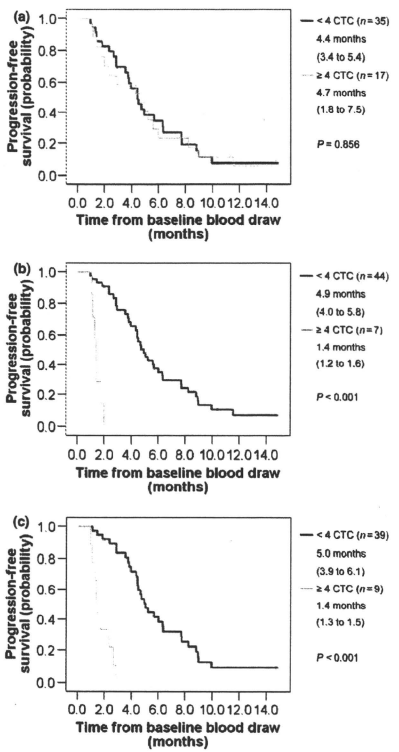

Fig. 1. Kaplan–Meier plots of progression-free survival (PFS) in advanced gastric cancer patients with less than four circulating tumor cells (CTCs) or ≥4 CTCs at baseline (a), 2 weeks (b), and 4 weeks (c).

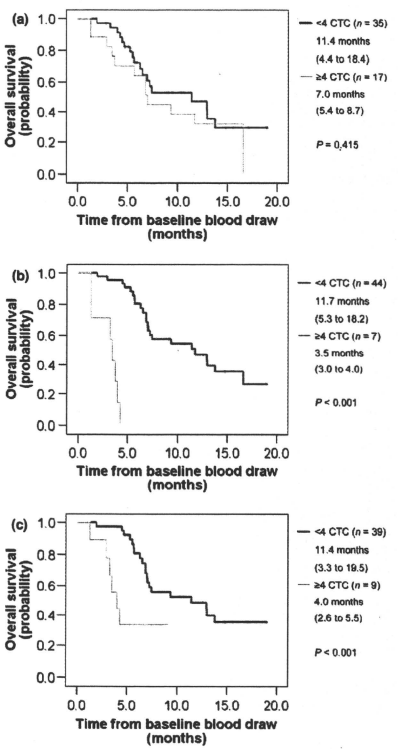

Fig. 2. Kaplan–Meier plots of overall survival (OS) in advanced gastric cancer patients with less than four circulating tumor cells (CTCs) or ≥4 CTCs at baseline (a), 2 weeks (b), and 4 weeks (c).

PFS (1.4 months; 95% CI, 1.3–1.5) than the median PFS of <4 CTCs at 4 weeks (5.0 months; 95% CI, 3.9–6.1) ($P < 0.001$) (Fig. 1c). With the S-1-based regimen, 10 patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with 21 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.2 months) than the median PFS of <4 CTCs at 2 weeks (6.0 months; 95% CI, 4.3–7.7) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median PFS (2.3 months; 95% CI, 0.7–3.9) than the median PFS of <4 CTCs at 4 weeks (6.3 months; 95% CI, 3.0–9.7) ($P < 0.001$). With the paclitaxel regimen, seven patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with 14

patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.4 months; 95% CI, 1.4–1.5) than the median PFS of <4 CTCs at 2 weeks (4.3 months; 95% CI, 3.5–5.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median PFS (1.4 months; 95% CI, 1.0–1.8) than the median PFS of <4 CTCs at 4 weeks (4.4 months; 95% CI, 3.6–5.3) ($P < 0.001$).

Analysis of OS according to CTC level. Figure 2 shows the Kaplan–Meier plots for prediction of OS using baseline CTC counts (Fig. 2a), at 2 weeks (Fig. 2b), and at 4 weeks (Fig. 2c). Seventeen of the patients (32.7%) with ≥4 CTCs per 7.5 mL of blood at baseline had no significant different OS compared with patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (3.5 months; 95% CI, 3.0–4.0) than the median OS of <4 CTCs at 2 weeks (11.7 months; 95% CI, 5.3–18.2) ($P < 0.001$) (Fig. 2b). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (4.0 months; 95% CI, 2.6–5.5) than the median OS of <4 CTCs at 4 weeks (11.4 months; 95% CI, 3.3–19.5) ($P = 0.001$) (Fig. 2c). With the S-1 based regimen, 10 patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significant different OS compared with 21 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (1.3 months) than the median OS of <4 CTCs at 2 weeks (13.8 months; 95% CI, 9.4–18.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (4.0 months; 95% CI, 2.3–5.7) than the median OS of <4 CTCs at 4 weeks (>11.7 months) ($P = 0.031$). With the paclitaxel regimen, seven patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significant different OS compared with 14 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (3.5 months; 95% CI, 3.1–4.0) than the median OS of <4 CTCs at 2 weeks (6.5 months; 95% CI, 5.9–7.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (3.5 months; 95% CI, 2.3–4.7) than the median OS of <4 CTCs at 4 weeks (6.5 months; 95% CI, 5.5–7.5) ($P = 0.013$).

Univariate and multivariate analysis of predictors of PFS and OS. Univariate and multivariate Cox proportional hazards regression was performed to assess the association between factors of interest and PFS or OS. In univariate analysis, PS, treatment regimen, line of chemotherapy, and CTC levels (cut-off, 4) at 2 and 4 weeks predicted PFS and OS (Table 4). In order to evaluate the independent predictive effect of chemotherapy, multivariate Cox regression analysis was carried out (Table 5). CTC levels at 2 and 4 weeks were the strongest predictors.

Discussion

The CellSearch system is designed to enrich and enumerate CTCs from peripheral blood. Furthermore, it is the first system to validate the clinical use of CTCs in patients with advanced gastric cancer. Our results show that the system is a suitable tool for assessment of CTCs in these patients, enabling reliable detection of CTCs in whole blood.

Approaches for isolation of CTCs in a research setting range from enrichment of tumor cells using density-gradient centrifugation[13–15] and flow cytometry.[16,17] CTC number as quantified by the CellSearch methodology[18–21] has been shown to have prognostic significance, and post-therapy decreases and increases in CTC number are associated with a superior and inferior survival, respectively, in patients with breast cancer, prostate cancer, and colorectal cancer. In this study, a finding of <4 CTCs in 7.5 mL of peripheral blood at 2 and 4 weeks after initiation of chemotherapy was associated with significantly longer PFS and OS as compared with these patients with ≥4 CTCs in 7.5 mL of peripheral blood. The results of this analysis demonstrated that the presence of four or more CTCs in 7.5 mL of blood before initiation of chemotherapy is not associated with PFS and OS. But the levels of CTCs at 2 and 4 weeks after initiation of chemotherapy are predictive of treatment efficacy, PFS,

Table 4. Univariate Cox regression analysis of independent parameters for prediction of PFS and OS

Parameter	No. of patients	PFS				OS			
		HR	95% CI	*P*-values	χ^2	HR	95% CI	*P*-values	χ^2
ECOG, 2 *vs* 1 *vs* 0	52	1.817	1.010–3.268	0.046	0.042	2.795	1.416–5.516	0.003	0.002
Treatment regimen	52	0.422	0.225–0.792	0.007	0.006	0.239	0.106–0.538	0.001	<0.001
Line of therapy	52	3.155	1.577–6.311	0.001	0.001	4.527	2.031–10.088	<0.001	<0.001
CTCs at the 2nd week	51	22.633	6.214–82.429	<0.001	<0.001	42.796	8.382–218.515	<0.001	<0.001
CTCs at the 4th week	48	15.947	5.380–47.271	<0.001	<0.001	4.699	1.751–12.609	0.002	0.001

CI, confidence interval; CTCs, circulating tumor cells; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

Table 5. Multivariate Cox regression analysis for prediction of PFS and OS

Parameter	PFS				OS			
	No. of patients	HR	95% CI	*P*-values	No. of patients	HR	95% CI	*P*-values
No. of patients	51				51			
Line of therapy, 1st *vs* 2nd		0.463	0.219–0.977	0.043		0.307	0.129–0.731	0.008
Lymph node metastasis		0.458	0.214–0.980	0.044				
CTCs at the 2nd week		0.049	0.012–0.199	<0.001		0.037	0.007–0.191	<0.001
Model χ^2			<0.001				<0.001	
No. of patients	48				48			
Line of therapy, 1st *vs* 2nd		0.412	0.192–0.880	0.022		0.217	0.089–0.504	<0.001
CTCs at the 4th week		0.082	0.027–0.224	<0.001		0.216	0.077–0.607	0.004
Model χ^2			<0.001				<0.001	

CI, confidence interval; CTCs, circulating tumor cells; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

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and OS. The presence of at least four CTCs at 2 and 4 weeks is a strong independent prognostic factor for inferior PFS and OS. These data demonstrate that CTC measurement may be a useful biomarker for monitoring response to therapy in AGC.

Outcomes are extremely poor in patients with ≥4 CTCs at 2 and 4 weeks, with the median OS ranging from 2 to 5 months. These data suggest the value of this technology in the identification of chemotherapy-resistant patients who could benefit from early treatment change and/or more investigational. Further study should prospectively address whether a change of treatment based on ≥4 CTCs at 2 or 4 weeks after initiation of chemotherapy early in the course of treatment will result in improvement in OS. CTC levels drawn at 2 and 4 weeks, before typical imaging intervals, may have the potential to suggest treatment choices and spare unnecessary toxicity by suggesting that an early change in therapy is warranted. Because the CellSearch system has not been approved in Japan, the price of one sample costs about ¥80 000 as in the case of the extra laboratory in the clinical trial. Several prospective trials led to the FDA approval of CTC counts for monitoring of patients with breast, colorectal, and prostate cancer. We expect CTC counts for monitoring of patients with gastric, breast, colorectal, and prostate cancer to be approved in Japan.

In conclusion, this study demonstrates the independent predictive value of CTCs for patients initiating chemotherapy for AGC. The data obtained in this clinical trial of the CellSearch system were for enumeration of CTCs in AGC. Our study was not designed to assess whether a change in therapy based on ≥4 CTCs is beneficial. However, clinical trials to explore this hypothesis are warranted.

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Disclosure Statement

The authors have no conflict of interest.

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original article

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Bone metastasis and poor performance status are prognostic factors for survival of carcinoma of unknown primary site in patients treated with systematic chemotherapy

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Background: Cancer of unknown primary site (CUP) generally has a poor prognosis, and there is no established standard therapy. There have been no reports of a prognostic model for CUP patients treated with a single regimen of systemic chemotherapy.

Methods: Univariate and multivariate prognostic factor analysis for overall survival (OS) were conducted retrospectively in 58 consecutive CUP patients treated with carboplatin plus paclitaxel (Taxol) therapy as a first-line treatment.

Results: Univariate prognostic factor analysis revealed baseline performance status (PS) of two or more, low serum albumin level, pleural effusion, bone metastasis, and liver metastasis as adverse prognostic factors. Cox proportional hazards analysis showed that poor PS and bone metastasis had the most powerful adverse impact on survival. We developed a prognostic model using those two variables—a good-risk group (PS 0–1 without bone metastasis) and a poor-risk group (PS ≥2 or bone metastasis). The poor-risk group showed significantly poorer OS than the good-risk group (1 year OS 36.8% versus 67.1%, $P = 0.0003$).

Conclusions: Poor PS and bone metastasis were identified as independent adverse prognostic factors in CUP. A simple prognostic model was developed and seems useful for decision making as to whether chemotherapy is indicated for CUP patients.

Key words: cancer of unknown primary site, carboplatin plus paclitaxel, bone metastasis

introduction

Cancer of unknown primary site (CUP) is pathologically diagnosed metastatic carcinoma in which no obvious primary site is identified with a conventional work-up. It is not a rare clinical entity, accounting for 3%–5% of all solid malignancies [1, 2]. The prognosis of CUP is generally considered poor, with median survival ~6–12 months [3]. Briasoulis et al. [4] reported encouraging results from phase II data of carboplatin and paclitaxel combination therapy for patients with CUP. In this study, the overall response rate by an intention-to-treat analysis was 38.7%, and median overall survival (OS) was 13 months at median follow-up time of 28 months. Platinum and taxane combination therapy is now widely used in clinical practice [4–8], but recent multiple-treatment meta-analysis showed that no type of chemotherapy has been proven to prolong survival in patients with CUP [9]. CUP consists of heterogeneous neoplasms with variable biological features, making it difficult to identify clinically useful prognostic survival factors. But several subsets have been identified requiring a specific treatment and having a better prognosis. Women with peritoneal carcinomatosis of serous adenocarcinoma [10], women with adenocarcinoma of axillary lymph nodes [11] or cervical lymph node metastasis of squamous cell carcinoma [12], young adults with poorly differentiated carcinoma of midline distribution [13], and undifferentiated carcinoma with neuroendocrine features [14] are CUP subgroups known to have a better prognosis. But the majority of CUPs have a poor prognosis, as mentioned above. In this article, we report the results of a prognostic factor analysis conducted in a population of 58 patients of CUP treated with carboplatin and paclitaxel as a first-line systemic chemotherapy. We retrospectively investigated baseline characteristics as prognostic factors for survival to identify a subset of patients who would benefit from chemotherapy.

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methods

patient characteristics

The medical and pathological records of 58 consecutive newly diagnosed patients with CUP who received carboplatin and paclitaxel (Taxol, Bristol-Myers Squibb, Tokyo, Japan) combination therapy as first-line therapy at the Cancer Institute Hospital, Japanese Foundation for Cancer Research, from March 2004 to January 2008 were retrospectively reviewed. Patients had pathologically confirmed metastatic cancer and were surveyed for detailed medical history, complete physical examination, blood counts, chemistry profile, chest radiograph, computed tomography (CT) scan of chest and abdomen, and further radiological survey or endoscopy of suspected areas. Serum prostate-specific antigen (PSA) was measured in male patients, and CA 125 was measured in female patients. Women with adenocarcinoma of axillary lymph nodes also received mammography and breast ultrasound. Young adults with poorly differentiated adenocarcinoma involving the mediastinal region were surveyed with α-fetoprotein and β-human chorionic gonadotropin. The gastrointestinal tracts of male and female patients with adenocarcinoma involving abdominal and pelvic lesion were surveyed by upper gastrointestinal endoscopy and colonoscopy. Gynecologic examination was carried out in female patients with abdominal and pelvic disease. Patients with squamous cell carcinoma of cervical lymph nodes also underwent laryngeal endoscopy and upper gastrointestinal endoscopy. Bone metastases were assessed by the combination of bone scintigraphy or positron emission tomography with chest X-ray, CT, or magnetic resonance imaging. Histopathological review including immunohistochemistry (IHC) was carried out to detect primary sites and to exclude other malignancies. Low-molecular cytokeratins (CKs) 7 and 20 were routinely stained for all patients with CUP, and thyroid transcription factor 1, caudal type homeobox transcription factor 2, and PSA were stained for patients with adenocarcinoma of CUP. When a specific origin was suspected by morphological examination and clinical history, distinctive IHC was carried out (chromogranin, synaptophysin, and CD56 for neuroendocrine cell carcinoma; D2-40, placental alkaline phosphatase, human chorionic gonadotropin, and CD30 for germ-cell tumor; and D2-40 and calretinin for mesothelioma). In the case of difficulty in diagnosing epithelial carcinoma, several IHC of S100, vimentin, leukocyte common antigen, and CKs are used for distinguishing melanoma, sarcoma, and lymphoma from the anaplastic cell type of carcinoma.

We excluded patients in favorable subsets that have specific treatments other than carboplatin and paclitaxel—such as women with adenocarcinoma of axillary lymph nodes or cervical lymph node metastasis of squamous cell carcinoma, young adults with poorly differentiated carcinomas of midline distribution, and patients with undifferentiated carcinomas of neuroendocrine features. However, women with peritoneal carcinomatosis of adenocarcinoma who were treated with carboplatin and paclitaxel as first-line treatment were included in this study.

treatment

Carboplatin was administered by a 2-h i.v. infusion, dosed with 6 mg/ml/min target area under the free carboplatin plasma concentration versus time curve and was followed by paclitaxel 200 mg/m^2 in 500 ml of normal saline administered over 3 h. The Calvert formula was used for carboplatin dosing, on the basis of a glomerular filtration rate calculated by the Cockcroft–Gault equation using serum creatinine, body surface area, and age. Chemotherapy cycles were repeated every 3 weeks and responding patients continued the chemotherapy until disease progression or intolerable toxicity. Response to chemotherapy was assessed by Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0). Progression-free survival (PFS) and OS were calculated from day 1 of the first cycle of chemotherapy.

statistical analysis

Survival curves were estimated using the Kaplan and Meier method, compared using the log-rank test, and prognostic factors were identified by univariate analysis. Then the forward stepwise Cox proportional hazards analysis was carried out to identify independent prognostic factors. Statistical analyses were carried out using SPSS software (version 17.0; SPSS Inc., Chicago, IL).

results

patient characteristics

Patient characteristics are shown in Table 1. Fifty-eight CUP patients treated with at least one cycle of carboplatin and paclitaxel combination therapy were retrospectively analyzed. Twenty-eight (48.3%) patients were male, and the median age was 64 years (range 28–79 years). Forty-nine patients (84.5%) had a good performance status (PS) of zero to one. Twenty-six (44.8%) patients had well-differentiated adenocarcinoma, 21 (36.2%) patients had anaplastic or poorly differentiated carcinoma, and 5 patients (8.6%) had squamous cell carcinoma. Another six (10.3%) patients had clear-cell carcinoma, transitional cell carcinoma, or adenosquamous cell carcinoma. Metastatic sites are listed in Table 1. Lymph nodes, lung, bone, and liver were frequently involved sites and cervical, mediastinum, and retroperitonium were common sites for lymph node metastasis.

PSA was measured in 20 male patients (median PSA level 2.04 ng/ml, range 0.34–4.04 ng/ml), and CA 125 was obtained in 26 female patients (median CA 125 level 462 U/ml, range 4.8–50000 U/ml). Five of six male patients with bone metastasis showed a PSA level <4.0 ng/ml, and the PSA value before treatment of one young male patient was not available.

outcome of chemotherapy

A total of 315 cycles were administered, and patients received a median of five cycles of treatment (range 1–21 cycles).

Table 1. Patient characteristics

Number of patients	58
Age, median (range)	64 (28–79)
Sex	
Male	28
Female	30
Performance status	
0–1	49
2–4	9
Pathology	
Adenocarcinoma	26
Squamous cell carcinoma	5
Poorly differentiated/anaplastic carcinoma	21
Other	6
Sites of metastasis	
Lung	15
Bone	13
Liver	11
Pleural effusion	15
Ascites	11
Lymph node	44

The response rates by main histopathological types of adenocarcinoma, squamous cell carcinoma, poorly differentiated carcinoma, or poorly differentiated adenocarcinoma were 42.3%, 60.0%, and 23.8%, respectively (Table 2). For other histology types, one patient with transitional cell carcinoma had partial response. Sixteen patients were treated with second-line chemotherapy. At a median follow-up time of 12 months (range 6–1659 days), median OS and PFS were 16.7 months and 5.9 months, respectively. Six patients had PFS >2 years and one of these patients survived >4 years.

prognostic model of clinical and biological variables

The outcome of univariate analysis of clinical and biological factors is listed in Table 3. Five parameters have prognostic relevance: poor PS (≥2) ($P = 0.01$), low serum albumin level (<3.7 g/dl) ($P = 0.003$), pleural effusion ($P = 0.04$), bone metastasis ($P = 0.02$), and liver metastasis ($P = 0.02$). Multivariate analysis for these five variables was conducted and showed that bone metastasis ($P = 0.002$) and PS of two or more ($P = 0.016$) had significant adverse impact for survival (Table 3). Poor PS was not correlated with presence of bone metastasis.

Table 2. Treatment results

	N	CR (*n*)	PR (*n*)	ORR (%)
Total	58	5	15	34.5
Pathology				
Adenocarcinoma	26	5	6	42.3
Squamous cell carcinoma	6	0	3	50.0
Poorly differentiated anaplastic carcinoma	21	0	5	23.8

CR, complete response; PR, partial response; ORR; overall response rate.

Table 3. Univariate and multivariate analysis of prognostic factors for survival

	Univariate	Multivariate	
	P value	HR (95% CI)	*P* value
PS ≥2	0.01	2.93 (1.22–7.04)	0.016
Age (>65 years)	0.29		
Sex (male)	0.41		
ALP (>UNL)	0.13		
LDH (>UNL)	0.45		
ALB (<3.7 g/dl)	0.003		
Hb (<11.0 g/dl)	0.77		
Pleural effusion	0.04		
Ascites	0.69		
Lung metastasis	0.58		
Bone metastasis	0.02	3.48 (1.56–7.78)	0.002
Liver metastasis	0.02		
Adenocarcinoma	0.81		
Poorly/anaplastic carcinoma	0.32		

HR, hazard ratio; CI, confidence interval; PS, performance status; ALP, alkaline phosphatase; UNL, upper normal limit; LDH, lactate dehydrogenase; ALB, albumin; Hb, hemoglobin.

The incidence of bone metastasis was not significantly different between males and females (6 of 28 males, 5 of 30 females). A prognostic model was developed with those two variables. Nineteen (32.8%) patients were assigned to the good-risk group (defined as PS 0–1 without bone metastasis), and 38 (67.2%) patients were assigned to the poor-risk group (defined as PS ≥2 or bone metastasis). The poor-risk group ($n = 19$) showed significantly poorer OS than good-risk group ($n = 39$) (1 year OS 36.8% versus 67.1%, $P = 0.0003$) (Figure 1).

discussion

To identify a favorable or poor prognostic group of patients with CUP is of great concern when physicians consider whether systemic chemotherapy is indicated. No randomized trial showed better survival with chemotherapy than best supportive care. To our knowledge, the current study is the first that assesses prognostic factors for survival of patients with CUP treated with a single first-line regimen and should give us information as we choose an optimal therapy.

We demonstrated an overall response rate of 34.5% and a median OS of 16.7 months in CUP patients by an intention-to-treat analysis. This result seems similar to the results previously reported by Briasoulis et al. [4] and slightly better than other reports. One reason might be that both studies included female patients with peritoneal carcinomatosis (11 of 58 in ours and 19 of 75 in Briasoulis et al.). In our study, seven women (63.6%) responded to chemotherapy. A second reason might be that our group included a marginally larger number of patients with good PS. Golfinopoulos et al. [9] reported in recent multiple-treatment meta-analysis for CUP that 10 randomized trials assessed in that study included variable rates for patients with poor PS (median 24.5%, interquartile range 12.8%–38.9%). Third, our study included a slightly smaller number of patients

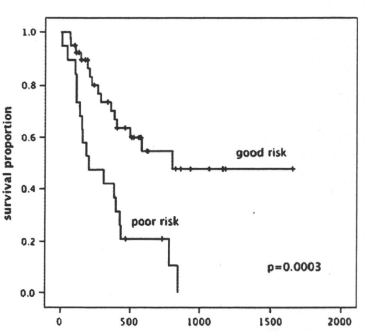

Figure 1. The prognostic model incorporating two variables. The good-risk group ($n = 39$) was defined as performance status (PS) of zero to one without bone metastasis and the poor-risk ($n = 19$) group as PS of two or more, or bone metastasis.

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with liver metastasis, which was reported as an independent poor prognostic factor by Seve et al. [15]. But the rates of liver metastases in previous studies are variable from 16% to 76% [4–8, 16, 17]. The patients in the present study were treatable with combination therapy, so most of them maintained good PS and end organ function. No prospective studies or meta-analysis of prognostic factors for CUP have been published. But several retrospective studies have shown a number of independent adverse factors such as age, male gender, poor PS, adenocarcinoma histology, number of metastatic sites, liver metastasis, bone metastasis, lung metastasis, pleural metastasis, brain metastasis, comorbidity scoring of adult comorbidity evaluation-27 (ACE-27), low serum albumin, high serum lactate dehydrogenase (LDH), high serum alkaline phosphatase, lymphopenia, anemia, thrombocytopenia, high serum carcinoembryonic antigen, and high serum CA 125 [15, 18–20]. Abbruzzese et al. [18] reported adverse prognostic variables from a study of 657 cases of CUP at M. D. Anderson Cancer Center, and multivariate analysis identified male gender, a large number of metastatic sites, adenocarcinomatous histological type, and the presence of liver metastasis as unfavorable indicators. Culine et al. [19] proposed a simple prognostic model using PS and serum LDH levels in a population of 150 CUP patients, excluding favorable subsets, at a French cancer center. More recently, Seve et al. conducted a retrospective study assessing the influence of comorbidities, age, PS, and chemotherapy on survival in a population of 389 patients with CUP in Canada. Multivariate analysis showed that patients who had a PS of two or more and a high overall ACE-27 score had a poor prognosis. They concluded that the impact of comorbidity on survival was limited to patients with low PS [20]. The same author showed in another study that low serum albumin level and liver metastasis were the two most powerful adverse prognostic factors. The prognostic significance of those two factors was validated in another set of 124 patients with CUP [15]. In our study, bone metastases and poor PS (≥2) had a powerful adverse impact on survival. In clinical practice, bone metastases could be the cause of declining PS, but in this study, bone metastases and poor PS were not significantly correlated. Poor PS was also an adverse prognostic factor in studies by Culine et al. and by Seve et al. Bone metastases have been identified as an independent poor prognostic factor for the first time in uniformly treated patients with CUP. Prognostic significance of bone metastases in advanced cancer depends on the primary sites. In breast cancer or prostate cancer, the presence of bone metastases or bone-only metastases indicates a better prognosis [21]. On the other hand, the presence of bone metastases indicates a worse prognosis in lung cancer [22], thyroid cancer [23], or renal cell carcinoma [24]. The worse prognosis of patients with bone metastases in our series might be due to the apparent absence of occult breast cancer or prostate cancer in this set of patients.

Although our study might be small for finding independent prognostic factors retrospectively, it is important to identify clinically useful prognostic factors for CUP patients treated with platinum and taxane combination therapy, which are used frequently in daily practice. It has not been proven that systemic chemotherapy would prolong the survival of unfavorable CUP patients, and the best supportive care is a reasonable choice for patients who have little benefit from systemic chemotherapy.

We designed a new prognostic model that incorporated those two factors, poor PS and bone metastasis. The OS of patients with at least one or more prognostic factor was significantly shorter than those with no adverse prognostic factor. This model might be useful for decision making regarding the use of chemotherapy for CUP patients in daily clinical practice. A validation study of our prognostic model is warranted in the near future.

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disclosure

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