Although the tumor types and the treatment received were heterogeneous and only four patients had C-terminus mutations, the C-terminal deletion mutation seems to be associated with short time to progression and early relapse of disease.

The mean fluorescence intensity results indicate that the C-terminal deletion mutation is strongly associated with decline or disappearance of CD20 expression, and the results of expression studies suggest that C-terminal deletions may mask CD20 expression on the cell surface or affect duration of cell surface exposure to CD20.

Heterogeneity of intensity of CD20 expression in replicate analysis of the same sample is commonly observed with flow cytometric analysis (11). This indicates that subclones expressing lower CD20 levels are present in CD20-positive lymphoma cells and that surviving clones may cause resistance or relapse after rituximab therapy. It is thus vital that these clones are killed to protect patients from the risk of resistance or relapse. Jazirehi et al. (18) have reported that rituximab-resistant lymphoma cells can be chemosensitized following treatment with pharmacologic inhibitors such as bortezomib that target survival/antiapoptotic pathways. Structurally, the C-terminal cytoplasmic domain of CD20 possesses some phosphorylation sites for protein kinases such as casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 (Fig. 4A). S239 is predicted to be phosphorylated by casein kinase 2, and S221 and S225 are potential calcium/calmodulin-dependent protein kinase 2 phosphorylation sites (19, 20); however, the significance of the phosphorylation of these sites remains to be clarified. On the other hand, the cytoplasmic region of CD20 (amino acids 219-225) is known to be required for its redistribution to the detergent-insoluble membrane compartment, which plays an important role in the action of rituximab (21). One of four C-terminal deletion mutants (Fig. 4B) reported here had lost several predicted phosphorylation sites such as casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 in contrast to the other three mutants (Fig. 4C-E). Another feature of the distal region in the C-terminus is the presence of a glutamic acid–rich region (19, 22). The sequence of E233 to E292 is predicted to be a glutamic acid–rich region profile using the Motif Scan program and PROSITE database, and this region may play an important role in retention of calcium ions, analogous to the role of bone sialoprotein (23). It has been reported that B lymphocytes are activated and CD20 is up-regulated by phorbol myristate acetate and ionomycin (24), suggesting that intracellular calcium ions participate in CD20 expression. However, we have shown that the C-terminal deletion mutant CD20 was produced as RNA in the cells but was not detected as a protein on the cell surface. This may be a consequence of the rapid turnover of CD20 mutant molecules between the cell surface and cytoplasm, resulting in exposure at the cell surface that is too brief for detection by immunofluorescence. If so, anti-CD20 antibody linked to anticancer drugs such as ozogamicin could be a useful treatment approach for patients with this mutation.

Two classes of mutations are spontaneous mutations and induced mutations caused by mutagens (25, 26). Spontaneous mutations on the molecular level include tautomerism, depurination, deamination, transition, and transversion, whereas chemicals such as alkylating agents and radiation can cause induced mutations on the molecular level. Alkylating agents such as cyclophosphamide in CHOP therapy can mutate replicating and nonreplicating DNA and has certain effects that then lead to transitions, transversions, or deletions. In this study, 44 patients had received CHOP therapy with rituximab, and three of them (6.9%) had C-terminal deletion mutants when they showed progression disease after R-CHOP therapy. One patient showed C-terminal deletion before R-CHOP therapy. Because Ragg et al. (27) has reported that overexpression and mutant of methylguanine methyltransferase protects mice against effect of alkylators, loss of function of this enzyme may induce gene mutagenesis by alkylating reagents such as cyclophosphamide. Moreover, 4 of 50 cases received radiation therapy during the treatment, and radiation therapy before administration of rituximab was given to two cases, which showed C-terminal deletion mutation after progression disease. Radiation before rituximab administration may also be related to mutagenesis of CD20 gene. Because one patient showed C-terminal deletion mutation before immunochemotherapy, we also need to consider clonal selection of CD20 after R-CHOP therapy. Moreover, microsatellite instability is known to be one of the mechanisms of gene mutation (28). Although microsatellite instability was examined as the cause of CD20 mutation in four patients with the C-terminal deletion mutation, it was not observed in their lymphoma cells (data not shown). Because two of these patients had received radiotherapy before rituximab therapy, radiation may have caused the CD20 mutation before treatment. However, some researchers have found that rituximab-resistant cells with low CD20 levels of rituximab have the same *CD20* gene sequence as that of sensitive cells (29, 30), suggesting that various or other mechanisms may contribute to CD20 down-regulation.

Although we found the C-terminal deletion mutation clones more often in patients with disease progression than at initial diagnosis, C-terminal deletion mutation was also strongly related to a shortening of the drug-free duration. Clinical prognostic factors for B-cell malignant lymphoma are well described and include age, Ann Arbor clinical stage, hemoglobin level, number of affected lymph nodes, and lactate dehydrogenase level (31, 32). Moreover, DNA microarray analysis implicates expression of several genes, including *BCL2*, *BCL6*, and *ZAP70*, as denoting poor prognosis in B-cell malignant lymphoma (33–36). However, there has been no report about gene mutations within molecular markers of lymphoma, such as the *CD20* gene. Here, we have presented the first data showing that a *CD20* gene mutation is related to a decline in CD20 expression and poor patient outcome. Because the mutation was detected in patients with disease progression, a more sensitive assay should be developed to detect CD20 mutations at initial diagnosis.

In conclusion, we found that C-terminal deletion mutations of CD20 were related to relapse/resistance after rituximab therapy, and screening for these mutations should be done in patients with disease progression after partial remission.

Disclosure of Potential Conflicts of Interest

The authors have received a commercial research grant from Chugai and honoraria from the speakers' bureau of Chugai.

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An Imaging-Based Rapid Evaluation Method for Complement-Dependent Cytotoxicity Discriminated Clinical Response to Rituximab-Containing Chemotherapy

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Abstract

Purpose: Rituximab has greatly improved the efficacy of chemotherapy regimens for CD20-positive non-Hodgkin's lymphoma. However, although several mechanisms of action of rituximab have been identified, the exact therapeutic functions of these mechanisms remains to be clarified. In addition, there is no established prognostic marker to predict an individual response. This study verified the validity of *ex vivo* complement-dependent cytotoxicity (CDC) susceptibility as a predictor of pathologic tumor regression in patients undergoing rituximab-containing chemotherapy and examined whether CDC contributes to the mechanism of action of rituximab.

Experimental Design: A rapid assay system was established to evaluate the tumoricidal activity of rituximab using a living cell–imaging technique. We analyzed lymph node biopsies obtained from 234 patients with suspected lymphomas and estimated the association between CDC susceptibility and the response to rituximab-containing chemotherapy in diffuse large B-cell lymphoma and follicular lymphoma.

Results: This study revealed that CDC susceptibility of lymphoma cells freshly obtained from patients was strongly associated with response to rituximab-containing chemotherapy in both diffuse large B-cell lymphoma and follicular lymphoma. This correlation was not apparent in cases that received chemotherapy without rituximab.

Conclusions: The system that we have established allows a successful assessment of rituximab-induced CDC and can distinguish cases refractory to rituximab-containing chemotherapy. The association between CDC susceptibility and therapy response suggests that CDC is pivotal in the ability of chemotherapy including rituximab to induce remission.

Although rituximab can be combined with chemotherapies used in the treatment of non-Hodgkin's lymphoma, efficacy varies from patient to patient. In addition, no prognostic marker to predict individual response has been established to date. Several mechanisms of action have been proposed and tested *in vitro*, mainly in tumor cell lines (1–3). Through its human IgG_1 Fc domain, rituximab can activate cellular effectors for antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis and can recruit serum proteins for complement-dependent cytotoxicity (CDC; ref. 4). Moreover, cross-linking of CD20 molecules on tumor cell lines has been reported to trigger apoptosis, as well as having an antiproliferative effect on some, but not all, cell lines (5, 6). Despite these insights, the mechanisms mediating tumor cell eradication *in vivo* are not well understood. Recently, analyses of FcγRIIIa polymorphisms have clearly shown that ADCC is one of the critical effector functions responsible for the clinical efficacy of therapeutic antibodies (7–9). The FcγRIIIa gene (*FCGR3A*) displays an allelic polymorphism that generates molecules containing either a phenylalanine (F) or a valine (V) at amino acid position 158, which is critical in mediating ADCC. A greater clinical response in patients with the FcγRIIIa allotype (FcgRIIIa-158V), which has a high affinity for human IgG_1, has been observed compared with results obtained from patients with the low-affinity allotype (FcγRIIIa-158F; ref. 10). These reports show the importance of ADCC in clinical outcomes. On the other hand, there are few reports that indicate a contribution to clinical effect in

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Translational Relevance

For the prediction of the therapeutic efficacy of molecularly targeted medicines, it is ideal to evaluate the therapeutic mechanisms in individual patients. Therefore, it is highly significant to evaluate the drug functions in fresh tumor cells near *in vivo* conditions. To date, invasiveness and a great deal of time and effort have been unavoidable in such evaluations. In this study, we developed a rapid evaluation system for complement-dependent cytotoxicity of rituximab by using living cell–imaging technology. The advantages of imaging-based procedures include the need for only a minimal amount of specimen as well as its rapidity. Our system enabled reproducible evaluation even from tiny specimens and distinguished between responsive and refractory groups of rituximab-containing chemotherapy. This evaluation system may be easy to apply to antibody medicines other than rituximab and to other mechanism of actions such as antibody-dependent cellular cytotoxicity. Thus, this study may offer the opportunity to evaluate the effect of various antibody drugs in clinical applications.

the mechanisms, apart from ADCC. Several reports have suggested the role of CDC in the clinical efficacy of rituximab (11-13); however, it is a less convincing argument than that for ADCC. To elucidate this question, it is absolutely critical to evaluate the intrinsic CDC susceptibility of freshly obtained lymphoma cells from patients. To do so, a rapid, reproducible, and sufficiently smaller-scaled assay system is essential. To address this, we established a novel procedure to quantify the susceptibility of patient-derived lymphoma cells to CDC induced by rituximab. We developed a remarkably smaller-scaled assay procedure by using a real-time imaging technique, making it possible to perform multiple measurements with a small portion of the cells derived from lymph node biopsy obtained for diagnosis. In this study, we evaluated the CDC susceptibility of 234 patients with suspected lymphoma, using this new analytic method. Among these cases, as for diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) in which rituximab-containing chemotherapy is frequently chosen, we further evaluated how much CDC is involved in the therapeutic response.

Materials and Methods

Lymphoma cells from patients. Primary malignant lymphocytes were prepared from lymph node biopsy specimens obtained from patients who had given written informed consent. Briefly, an excised lymph node was minced with scissors, and a mononuclear cell fraction was obtained by centrifugation (1,000 × *g* at 25°C for 30 min) through Ficoll histopaque (Sigma). B-lymphoid tumor cells were further purified by immunomagnetic cell sorting (according to the manufacturer's instructions) using an anti-human CD19 antibody conjugated to magnetic beads (MACS System, Miltenyi Biotec) from a small part of the mononuclear cells. The study was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research.

Antibodies. Rituximab was purchased from Chugai Pharmaceutical Co., Ltd., and dialyzed thrice into PBS. For a control study, the $F(ab')_2$ fragment of rituximab was prepared by treating the antibody with immobilized pepsin and separating the resulting $F(ab')_2$ fragments on an immobilized protein A column (Pierce Biotechnology, Inc.). Purified rituximab and its $F(ab')_2$ fragment were labeled with Alexa 488 (Molecular Probes) according to the manufacturer's instructions. FITC-labeled anti-human CD19 mouse monoclonal antibody and PE-labeled anti-CD20 mouse monoclonal antibody were purchased from Becton Dickinson for flow cytometry analysis. Anti-human CD19 Microbeads were purchased from Miltenyi Biotec for immunomagnetic cell sorting.

Live cell imaging-based CDC assay. Forty thousand purified B-lymphoid tumor cells were suspended in 4 μL of RPMI 1640 supplemented with 10% fetal bovine serum, 10 μg/mL of Alexa 488–labeled rituximab, and 5 μg/mL of propidium iodide (PI). The cell suspension was pipetted into a well made of silicon, 2.5 mm in diameter and 2 mm in depth, on a piece of cover glass. Subsequently, the cell suspension was set on the stage of a confocal microscope system (FV-1000; Olympus, Tokyo, Japan) set up in a microscope incubator (Tokken, Chiba, Japan). After 10 min of incubation, 1.0 μL of type AB human serum obtained from a healthy volunteer was added to the well. Cellular alteration immediately after the addition of serum was recorded every 4 s by a time-lapse function at a resolution of 640 × 640 pixels. Using an excitation laser beam with a wavelength of 488 nm, cellular morphology by Nomarski interference contrast imaging, the binding of rituximab at a wavelength of 505 to 525 nm and the PI incorporation into the nuclei of dead cells at a wavelength of 610 to 640 nm were observed. The schema of the principle of this assay is illustrated in Fig. 1. CDC susceptibility index was calculated according to the following formula:

$$\text{CDC susceptibility index (\%)} = \frac{(\text{no. of dead cells at 10 min} - \text{no. of dead cells at 0 min})}{(\text{no. of total cells at 10 min} - \text{no. of dead cells at 0 min})} \times 100$$

Selection of cutoff scores for the CDC susceptibility index. Relevant cutoff scores for predicting therapeutic effect for the CDC susceptibility index were obtained by carrying out receiver-operating characteristic (ROC) curve analysis. In brief, plots of sensitivity and 1– specificity for therapy response were obtained for this index; the closest-to-(0,1) criterion identifies the threshold value.

Statistical procedures. Patients who responded to rituximab-containing chemotherapy were compared with those who were resistant to it. The association of therapy response with the CDC susceptibility index was analyzed using the two-sided Fisher's exact test. $P < 0.05$ was considered significant. A multiple logistic regression analysis was conducted to identify independent predictors of the clinical response to rituximab-containing chemotherapy. A step-down procedure based on a likelihood ratio test was applied, arranged in descending order of importance, with $P = 0.05$ for entry into the model and $P = 0.10$ for removal. Statistical analysis was done using the SPSS software package.

Results

Setting the conditions of the CDC assay. To set the conditions of the assay, CDC susceptibility analysis was done using B-cell NHL cell lines, Daudi, Raji, and Ramos, and by changing several variables. First, to determine the optimal concentration of rituximab, we assessed CDC with various concentrations of rituximab. As a source of complement, human serum at a final concentration of 20% was added to these cell lines suspended in culture medium containing rituximab at a range of 0 to 100 μg/mL. Then, cell death induced during the next 10 min was quantified. CDC reached a plateau at a concentration of >10 μg/mL of rituximab in either cell line (Fig. 2A). To optimize final serum concentration, we next did an assay using various concentrations of human serum with 10 μg/mL of

rituximab. Cell death induced by CDC increased serum concentration dependently, but CDC activity did not reach the plateau at the 0% to 20% level we examined (Fig. 2B). As it was difficult to add more than 20% serum because of the limitations of manipulation, we set the final serum concentration to 20% for subsequent analyses. As to reaction time, cell death in all lines reached a plateau within 10 min after the addition of 20% final concentration of human serum (Fig. 2C). Taking these results into account, we concluded that CDC susceptibility was calculated by measuring the cellular ratio killed in 10 min after the addition of human serum at a final concentration of 20% to the cells suspended in culture medium containing 10 μg/mL of rituximab. To confirm the capability of the assay with clinical specimens, eight cases of freshly obtained lymphoma cells were used to analyze the abovementioned condition. As shown in Fig. 2D, in all eight cases, the proportion of dead cells asymptotically approached a fixed value that varied according to the cases within 10 min. Additionally, under the presence of 10 μg/mL of Alexa 488–labeled human IgG used as a control, a significant increment of cell death was observed in neither case.

The assay system was designed to use a CD19-positive fraction as target cells to reduce the influence of effector cells in the biopsy specimens. To confirm the influence of the effector cells that might not be removed completely in the actual assay, the analysis was carried out using a *F*(ab′)2 fragment of rituximab or heat-inactivated serum. The results obtained from lymphoma cells derived from five patients with non-Hodgkin's lymphoma (two cases of DLBCL and three cases of FL) indicated that cell death was extremely decreased by replacing rituximab with its *F*(ab′)2 fragment or by heat-inactivation of serum. In both cases, a very small percentage of cells underwent necrotic or apoptotic cell death, but the increase of cell death was not statistically significant and was not more than that observed when human IgG was used as a substitute for rituximab (Supplemental Table S1). These results suggested that the cytotoxicity levels induced by effector cells were almost negligible in our assay system.

Patient characteristics. We evaluated the CDC susceptibility of cells derived from lymph node biopsies taken from 234 cases of suspected lymphoma. The diagnosis breakdown was DLBCL, 23.5%; FL, 20.9%; DLBCL with FL, 2.1%; mantle cell lymphoma, 3.4%; mucosa-associated lymphoid tissue, 4.7%; Burkitt's lymphoma, 0.9%; chronic lymphocytic leukemia/small lymphocytic lymphoma, 2.6%; and cases other than B-cell non-Hodgkin's lymphoma, 42.7%. The cases other than B-cell non-Hodgkin's lymphoma included T-cell lymphomas, nonneoplastic lymph proliferative diseases, lymphadenitis, and Hodgkin's disease. The samples evaluated included 51 patients with DLBCL and 45 patients with FL; patient characteristics are shown in Table 1. Cases were excluded in which

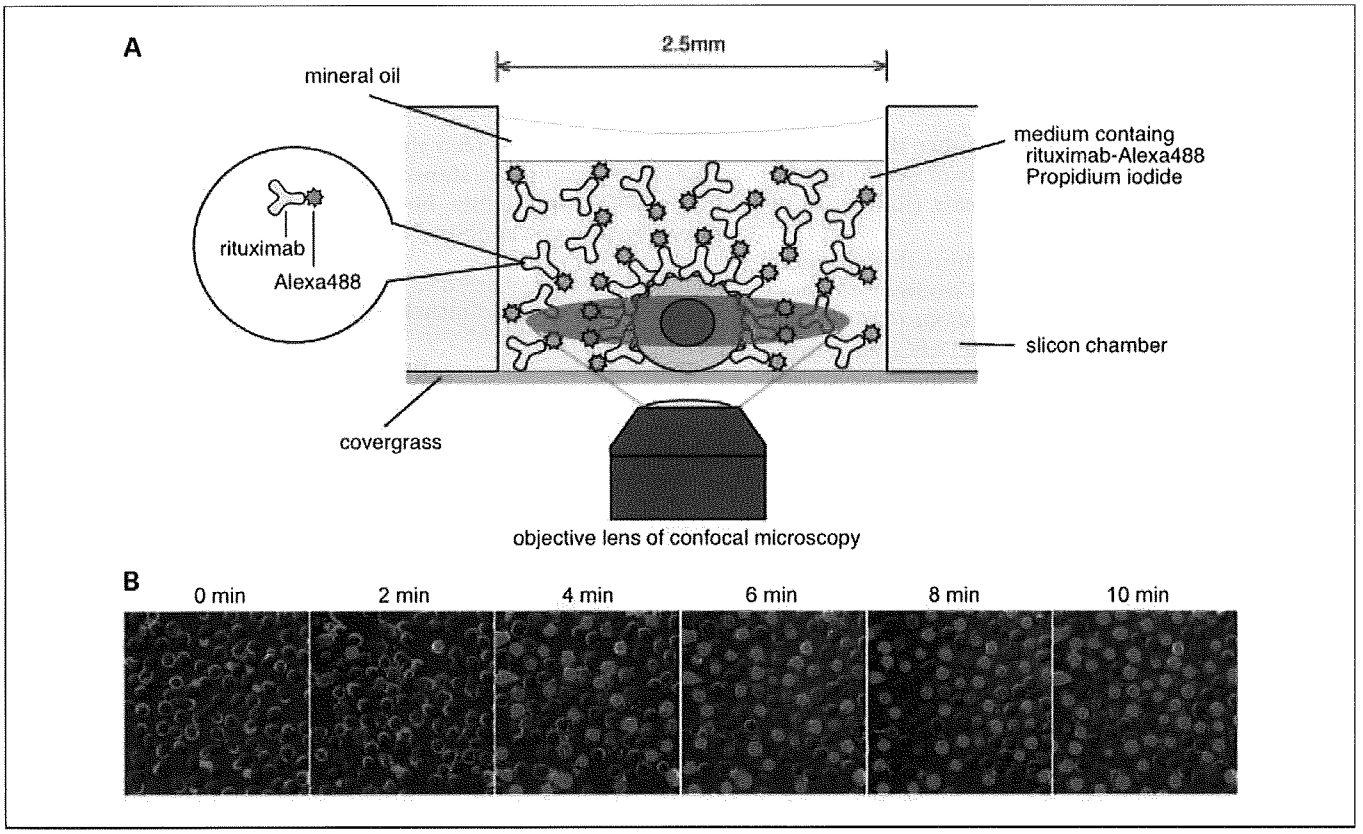

Fig. 1. The principle of imaging-based CDC susceptibility assay. *A*, a schema of CDC susceptibility assay based on living cell imaging. Lymphoma cells were suspended in 4 μL of culture medium containing Alexa 488–labeled rituximab and propidium iodide. The alteration of cellular viability from immediately after the addition of 1 μL of human serum was observed. The CDC-mediated cell killing was distinguished by the incorporation of PI into the nuclei. The background noise from the fluorescent antibody that existed in the culture medium could be excluded by detecting only fluorescent signals around a cellular equatorial plane by using confocal technology. *B*, an example of the assay results from a patient with FL lymphoma. Typical analysis results were shown extracting to images of every 120 s. Green signals, Alexa 488–labeled rituximab; red signals, incorporation of PI into nuclei; bar, 10 μm.

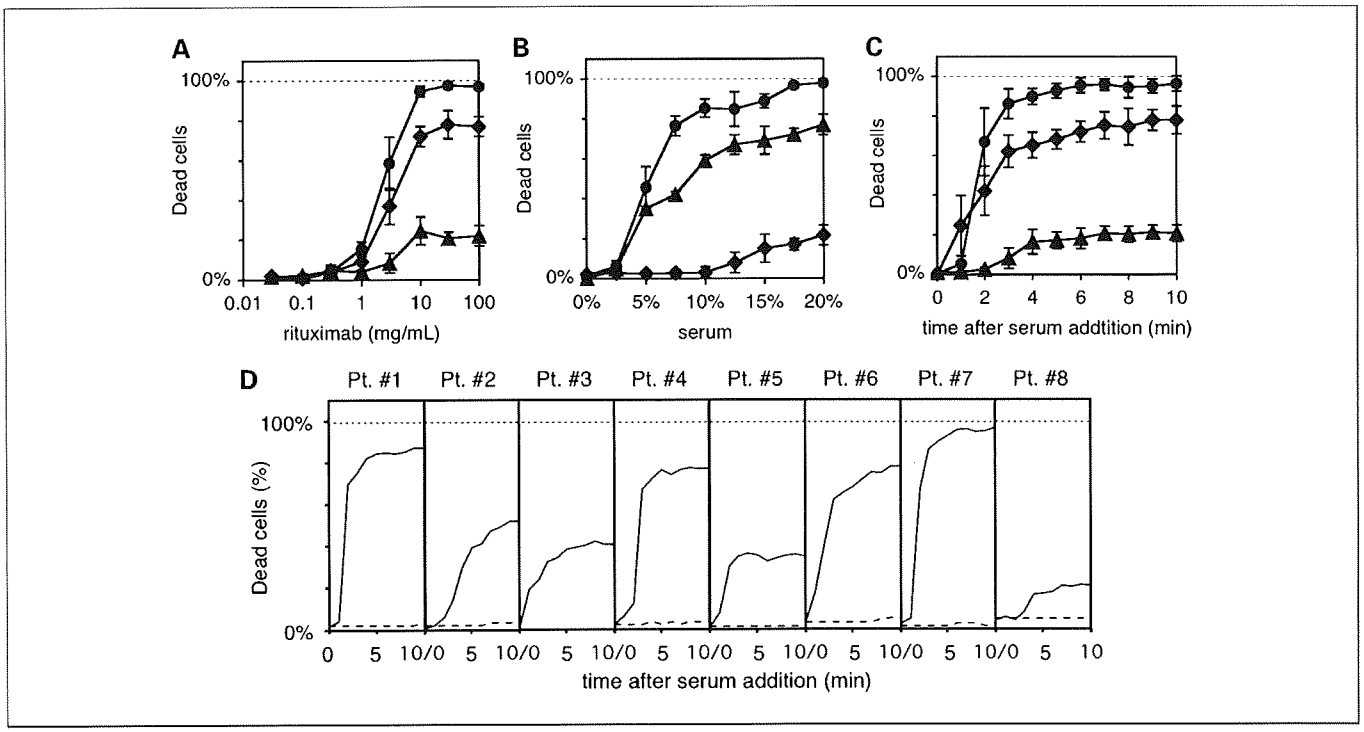

Fig. 2. Rituximab-mediated CDC assays on Burkitt's lymphoma cell lines and primary lymphoma cells. *A*, relationship between the rituximab concentrations and CDC-mediated cell killing in the presence of a final concentration of 20% human serum. Proportions of dead cells were counted and plotted against the rituximab concentrations. Circles, Ramos; diamonds, Raji, triangles, Daudi in *A, B* and *C. B*, relationship between the serum concentration and CDC-mediated cell killing in the presence of 10 μg/mL of rituximab. Proportions of dead cells were counted and plotted against the serum concentrations. *C*, time course of CDC-mediated cell killing in the presence of 10 μg/mL of rituximab and a final concentration of 20% human serum. Proportions of dead cells were counted and plotted against the time after serum addition. *D*, time course of CDC-mediated cell killing in lymphoma cells derived from biopsy of eight patients. Assay was done in the presence of 10 μg/mL of rituximab and at a final concentration of 20% human serum. All experiments were done in triplicate. Points, mean; bars, SD.

treatment was discontinued for reasons other than disease progression, for example, changing hospitals or developing severe (greater than grade 3/4) adverse events (four cases of DLBCL and four cases of FL).

Rituximab-containing chemotherapies. Thirty-three patients diagnosed with DLBCL and 30 patients with FL received cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)–like chemotherapy plus rituximab; 7 patients with DLBCL and 7 with FL received a salvage chemotherapy plus rituximab. The salvage chemotherapy included ifosfamide, carboplatin, and etoposide (ICE) and dexamethasone, cytarabine, and cisplatin (DHAP). Both therapies included rituximab administration at a dose of 375 mg/m^2 i.v. at 1-week intervals of eight cycles. CHOP-like chemotherapy included all regimens with at least standard doses of doxorubicin (50 mg/m^2 i.v., day 1), cyclophosphamide (750 mg/m^2 i.v., day 1), vincristine [1.4 mg/m^2 i.v. (maximum 2 mg), day 1], and prednisone (60 mg/m^2 orally, days 1-5) administered in 3-week cycles.

Measurement of CDC susceptibility of patient-origin lymphoma cells. CD19-positive cells were purified as described in Materials and Methods. The purified cells were mixed with rituximab and human serum on the stage of a confocal microscope, and the CDC susceptibility index was calculated. The CDC susceptibility analysis was carried out by persons unable to access information about diagnosis results or treatment response. Characteristics of CDC susceptibility according to subtype of lymphoma are shown in Fig. 3A. In DLBCL and FL, the CDC susceptibility index had a remarkably wide distribution, from nearly 0% to 100%; means were 54% and 64%, respectively. A comparison with the cases other than B-cell non-Hodgkin's lymphoma showed no significant difference in mean CDC susceptibility; however, it should be noted that relatively low susceptibility cases were included in DLBCL

Table 1. Patient characteristics

Characteristics	DLBCL, *n* = 51	FL, *n* = 45
	n (%)	*n* (%)
Gender		
Female	21 (41)	25 (56)
Male	30 (59)	20 (44)
Age (y)		
<60	14 (27)	23 (51)
≥60	37 (73)	22 (49)
State of disease		
Primary	37 (73)	36 (80)
Relapse	14 (27)	9 (20)
IPI		
Low	31 (61)	20 (44)
Low-intermediate	4 (8)	11 (24)
High-intermediate	8 (16)	11 (24)
High	8 (16)	3 (7)
Treatment		
R-CHOP like	33 (65)	30 (67)
R-ICE	5 (10)	7 (16)
R-DHAP	2 (4)	0 (0)
Without R*	11 (20)	8 (18)

*Chemotherapy regimen without rituximab

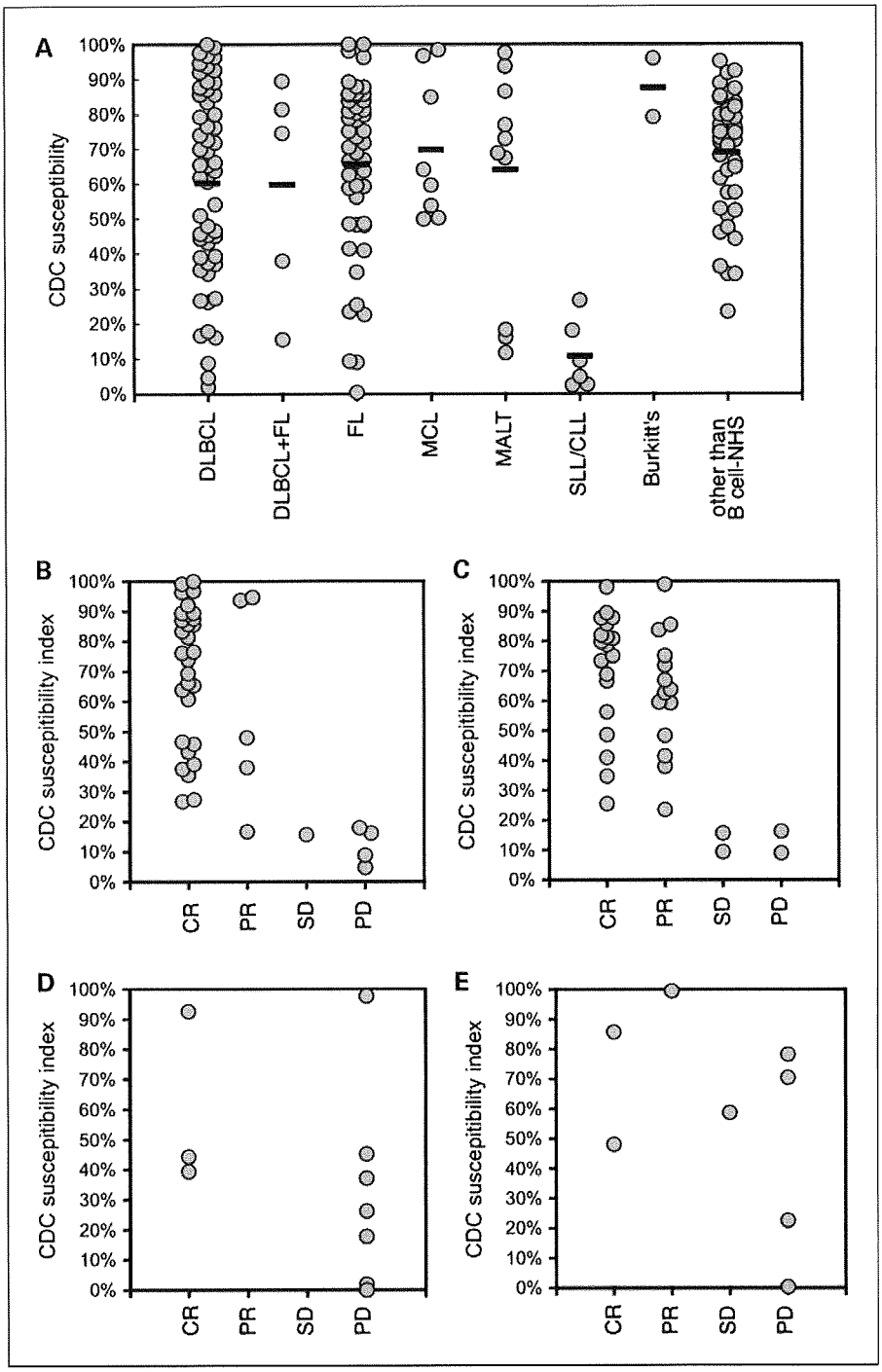

Fig. 3. Profiles of CDC susceptibility index. *A*, a total of 234 cases analyzed for CDC susceptibility were classified in eight categories of disease subtypes. The CDC susceptibility index of each case was plotted. Bar, mean. *B* to *E*, relationship between CDC susceptibility index of lymphoma cells obtained from patients with DLBCL (*B* and *D*) and FL (*C* and *E*) and clinical response of rituximab-containing chemotherapy. Lymphoma cells derived from 40 patients with DLBCL (*B*) and 37 patients with FL (*C*) who received the R+chemotherapy regimen were examined for CDC susceptibility and plotted against prognosis. Similarly, we examined lymphoma cells derived from 10 patients with DLBCL (*D*) and 8 patients with FL (*F*) who received chemotherapy without rituximab.

and FL cases. In mucosa-associated lymphoid tissue and Burkitt's lymphoma, the CDC susceptibility index of all cases we evaluated was >50%, and the means were 69% and 87%, respectively. In contrast, in chronic lymphocytic leukemia/small lymphocytic lymphoma, all cases were <29% with a mean index of 12%. In comparison with a non–B-cell lymphoma case, statistical significant difference was found only in chronic lymphocytic leukemia/small lymphocytic lymphoma cases ($P < 0.001$).

Relationships between CDC susceptibility and response to rituximab-containing chemotherapy. To clarify the association between CDC susceptibility and response to rituximab-containing combination chemotherapy, we did a correlation analysis for DLBCL and FL patients who had undergone CDC susceptibility analysis between January 2005 and December 2007. As for the DLBCL cases that received rituximab combination chemotherapy, 35 patients (87.5%) with complete response (CR) or partial response (PR) were judged effective, whereas 5 patients (12.5%) with stable disease (SD) or progressive disease (PD) were judged not effective. Similarly, for FL cases, 33 patients (89.2%) with CR or PR were judged effective, whereas 4 patients (10.8%) with SD or PD were judged not effective.

To assess the independent contribution of CDC susceptibility to the prediction of clinical response, multiple logistic

regression analysis was done. In the model, we used chemotherapeutic category (induction/salvage) and the International Prognostic Index (IPI) grade as confounding variables. The preceding univariate analysis showed that all these variables were significantly associated with the clinical response ($P < 0.001$, $P = 0.0338$, and $P = 0.0346$ for CDC, IPI, and chemotherapeutic category, respectively). Multiple stepwise logistic regression analysis showed that CDC susceptibility was an independent predictor of clinical response ($P < 0.001$), chemotherapeutic category was also another independent predictor ($P = 0.0473$); on the other hand, the IPI index was not considered to be an independent predictor in this multivariate model.

As CDC susceptibility was reported to be influenced by the target antigen expression levels (14), we then analyzed the relation between rituximab-induced CDC and cell surface CD20 expression levels, and compared their predictive ability. Analysis using Pearson's correlation coefficient indicated a statistically significant correlation between the CDC susceptibility index and CD20 expression level in lymphoma cells derived from patients with both FL and DLBCL (Supplemental Fig. S1; $r = 0.519$, $P < 0.01$, and $r = 0.592$, $P < 0.01$, respectively). ROC curves for both the CDC susceptibility index and CD20 expression levels to distinguish effective (CR + PR) versus noneffective (SD + PD) were produced. The area under the ROC curve for the CDC susceptibility index was significantly larger than that for the CD20 expression level (0.998; 95% confidence interval, 0.992-1.000; and 0.777; 95% confidence interval, 0.578-0.976, respectively; $P = 0.005$), indicating that the CDC susceptibility index was a more powerful discriminator of patients' response than CD20 antigen expression (Fig. 4, *top*).

The ROC curve analysis was further used to calculate the optimal cutoff value of CDC susceptibility for discriminating clinical response. A CDC susceptibility value of 18%, which provided the highest sensitivity and specificity, was selected to categorize lymphoma as high or low CDC susceptibility (Fig. 4, *bottom*). Using this cutoff value, we estimated the prognostic reliability of the CDC susceptibility index in rituximab combination chemotherapy. For DLBCL treated with R-CHOP–like therapy, the response rates were significantly higher in the cases with high CDC susceptibility (96.8% effective) than in those with lower susceptibility (0%; $P < 0.001$; two-sided Fisher's exact test). In addition, in the all-rituximab combination chemotherapy including R-salvage chemotherapy, the cases with high CDC susceptibility exhibited significantly higher response rates (97.2%) than those with a lower CDC index (0%; $P < 0.001$, two-sided Fisher's exact test). Similarly, even in FL, the CDC index showed statistically significant associations with therapy response to both R-CHOP–like therapy and all rituximab combination chemotherapies ($P = 0.0023$ and $P < 0.001$, respectively).

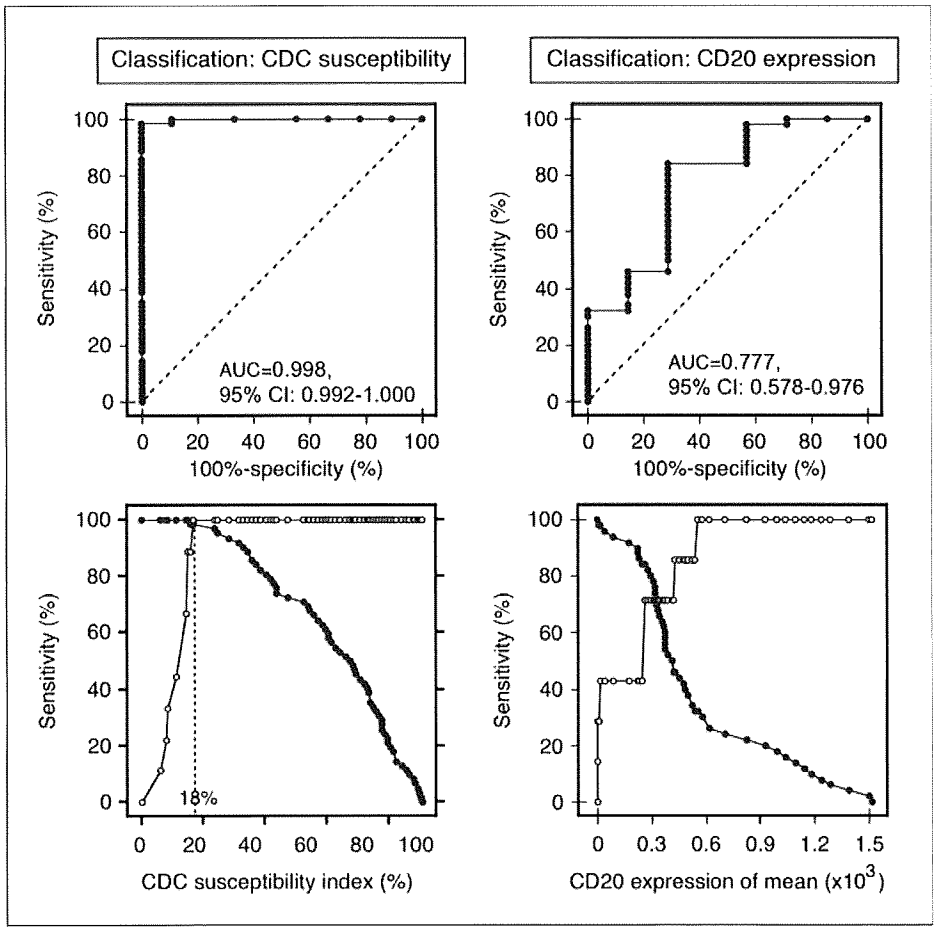

Fig. 4. Comparative analyses between CDC susceptibility and CD20 expression level. ROC curve analysis was done for comparison of predictive power of CDC susceptibility and antigen expression and for optimized threshold value determination. The analyses were conducted on all patients with DLBCL and FL who received the R+chemotherapy regimen, and ROC curves classified by CDC susceptibility index and CD20 expression level were generated (*top*). The areas under the ROC curve (AUC) summarizing the inherent capacity of parameters to discriminate a responder from a nonresponder were calculated. The optimal cutoff value that provides a trade-off between sensitivity (true positives; *solid circles*) and specificity (true negatives; *open circles*) was determined to be 18% for CDC susceptibility from the plot of sensitivity and specificity versus criterion value (*bottom*).

Discussion

Previous studies have suggested that several mechanisms might be involved in the therapeutic efficacy of rituximab, including ADCC (8-10), CDC (4, 11), and the induction of growth arrest or apoptosis (6, 15, 16). Both clinical (17, 18) and experimental (19) studies have offered evidence that ADCC plays an important role. On the other hand, the role of complements in rituximab treatment has been suggested by several preclinical studies. A study using cynomolgus monkeys showed that the ability to deplete CD20-positive cells was almost completely lost when IgG_4, which lacks complement-binding ability, was used in place of IgG_1 (20). In addition, the eradication of syngeneic murine EL4 tumor cells expressing human CD20 by rituximab is dependent on C1q, the first component of the classic pathway, and is independent of natural killer cells (21). Similarly, complement depletion using cobra venom factor markedly reduced the efficacy of rituximab in several lymphoma xenograft models (4). As for clinical evidence, the contribution of CDC to rituximab treatment was indirectly supported by several clinical observations. To give some instances, complement is consumed during rituximab treatment; in some cases, only CD59-positive cells having complement resistance remained after long-term rituximab treatment (11, 22). Thus, several animal models and clinical phenomena suggest the role of complement in rituximab treatment, but no strong, direct evidence of the clinical significance of CDC has yet been shown. It should also be noted that some findings are controversial. Weng and Levy have investigated *in vitro* CDC susceptibility, the expression of CD20, and complement inhibitors CD46, CD55, and CD59 on cryopreserved biopsy specimens from patients with FL, concluding that neither of these variables correlates with the reactivity of rituximab treatment (23).

In the present study, we established a rapid assay method for CDC analysis. Our assay system made it possible to perform an assay with only 40,000 lymphoma cells. In addition, it eliminated the lag-time between incubation and analysis that was unavoidable in other analyses such as flow cytometry methods. These features have contributed highly reproducible multiple measurements, even when only a tiny biopsy specimen could be obtained. Using this system, we evaluated the CDC susceptibility of cells derived from lymphoma patients within 6 hours after the biopsy and analyzed the relationship to clinical response. We succeeded in eliminating most of the influence of effector cells by removing CD19-negative cells from the assay specimen, suggesting that net CDC susceptibility could be estimated. Analysis results on FL and DLBCL suggested a strong correlation between CDC susceptibility and the clinical response to rituximab-containing chemotherapy. This result is inconsistent with the report of Weng and Levy. We believe that the inconsistency was, in part, caused by the method of evaluating CDC susceptibility. Our evaluation method assesses the intrinsic characteristics of the tumor cells by analyzing without cryopreservation after

Table 2. Clinical characteristics in relation to CDC susceptibility in patients with DLBCL and FL

Treatment	CDC susceptibility	Responder	Nonresponder	*P**
DLBCL				
R-CHOP like				
	CDC > 18%	30	0	$P = 0.00067$
	CDC < 18%	1	3	
R+salvage				
	CDC > 18%	5	0	$P = 0.048$
	CDC < 18%	0	2	
R+chemotherapy†				
	CDC > 18%	35	0	$P = 8.0 \times 10^{-6}$
	CDC < 18%	1	5	
Without R‡				
	CDC > 18%	3	4	$P = 0.48$
	CDC < 18%	0	3	
FL				
R-CHOP like				
	CDC > 18%	28	0	$P = 0.0023$
	CDC < 18%	0	2	
R+salvage				
	CDC > 18%	5	0	$P = 0.047$
	CDC < 18%	0	2	
R+chemotherapy†				
	CDC > 18%	33	0	$P = 1.5 \times 10^{-5}$
	CDC < 18%	0	4	
Without R‡				
	CDC > 18%	3	4	$P = 1$
	CDC < 18%	0	1	

*P values were obtained from two-tailed Fisher's exact tests.
†Chemotherapy plus rituximab including R-CHOP–like and R+salvage chemotherapy.
‡Chemotherapy regimen without rituximab.

biopsy and by optimizing assay conditions. Indeed, for some lymphoma cells derived from patients, different results in the CDC susceptibility were observed when the measurement was taken after freeze-thawing versus just after collection (data not shown).

The heterogeneity of the patient backgrounds and of the type of treatment made the data difficult to interpret. Therefore, we did a multivariate analysis to access the independence of CDC susceptibility from the patient background, including the IPI grade and the type of treatment, and found that CDC was a probable independent predictor for effectiveness. In our analysis, the IPI has a significant correlation with clinical response in univariate logistic analysis. However, in multivariate analysis, the IPI did not remain as an independent predictor, perhaps because the data included the salvage therapy. These results suggested that the CDC susceptibility index might be useful in predicting clinical response in both the induction and salvage chemotherapy containing rituximab. In addition, even by being limited to patients who received R-CHOP–like treatment as a first-line chemotherapy, significant correlation was found between CDC and clinical response in both FL and DLBCL (Table 2).

Although a significant correlation between CDC susceptibility index and the cell surface CD20 expression was also found in our analysis, the area under the ROC curve analysis revealed that the CDC susceptibility index was a more powerful discriminator of patients' response than CD20 antigen expression. In the responsive group, the CD20 expression had a wide distribution, including considerably low values (data not shown). These results indicate that cases that will get good therapeutic response in spite of low CD20 expression can be distinguished by CDC susceptibility.

To date, relationships between each mechanism of action, such as ADCC and CDC, have not been clarified completely in the clinical efficacy of therapeutic antibodies. A more detailed mechanism of action in rituximab-containing chemotherapy should be analyzed by evaluating the mutual action of CDC and ADCC. To further understand the relative clinical contribution of both effector mechanisms, we are currently developing an imaging-based reproducible ADCC evaluation system, and we plan to investigate the mutual relationship of ADCC and CDC, as well as the relationship between CDC/ADCC and long-term prognosis such as overall survival or relapse-free survival. These investigations will give us critical information about the exact therapeutic functions of these mechanisms.

The CDC susceptibility distinguished between responsive and refractory groups of rituximab-containing chemotherapy; however, it does not seem to be useful in more detailed classifications, such as complete response or partial response. In addition, the relation to clinical prognosis such as the duration of disease-free survival or overall survival is currently uncertain. In spite of these limitations, our analysis system provides a novel approach to predicting which patients will receive little benefit even if rituximab combination chemotherapy is done. Recently, antibody drugs using different mechanisms for B-cell non-Hodgkin's lymphoma have become increasingly available. Accordingly, it is expected that the prediction of the effects of therapeutic antibodies before the start of therapy will become more and more important in the post-rituximab era, leading to early implementation of optimal therapies, improving clinical outcomes.

In conclusion, we have shown that live cell–imaging is quite useful in improving CDC evaluation methods. The advantages of imaging-based procedures include needing only a minimal amount of specimen as well as rapidity and traceability. All of these features are advantageous to the analysis of clinical specimens. Thus, live cell–imaging may lead to greatly improved clinical evaluations.

Disclosure of Potential Conflicts of Interest

K. Hatake, commercial research support, honoraria, Chugai Pharmaceutical Co., Ltd.

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SURGERY TODAY

Chemotherapy for Small-Bowel Adenocarcinoma at a Single Institution

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Abstract

Purpose. Small-bowel adenocarcinoma (SBA) is rare. No standard chemotherapy for this type of cancer has yet been established. At Cancer Institute Hospital (CIH), the chemotherapy regimen used for colorectal cancer is initially used for patients with SBA, followed by that used for gastric cancer.

Methods. Patients with advanced or recurrent SBA who had been treated with chemotherapy in CIH were retrospectively analyzed. The first-line treatments were fluoropyrimidines used alone or in combination with other drugs, such as 5-fluorouracil plus leucovorin (FL), UFT-E, or TS-1. The second-line treatment was irinotecan (CPT-11) monotherapy.

Results. Fluoropyrimidine-based regimens, mainly FL, were used for 10 patients. Seven patients received the second-line CPT-11 regimen. Disease control was seen in five patients (50%) with the first-line chemotherapy and in three (43%) with the second-line. The median overall survival time was 12 months (range 3–39). The treatments were generally tolerated. Gastrointestinal symptoms were the most common adverse effects.

Conclusions. Fluoropyrimidines as the first-line and CPT-11 as the second-line chemotherapy yielded low response, although the adverse effects were mild. The FOLFOX and FOLFIRI regimens such as those used for metastatic colorectal cancer are potential alternative strategies. Extensive trials are needed to develop standard chemotherapy with new drugs.

Key words Small bowel · Adenocarcinoma · Chemotherapy · 5-Fluorouracil · Irinotecan

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Introduction

Small-bowel adenocarcinoma (SBA) is a rare cancer. Patients suffering from this type of tumor are likely to have a poor prognosis.[1–3] No efficacious standard chemotherapy has been developed that can prolong survival. No aggressive large-scale clinical trial has been undertaken in Japan because of the rarity of this cancer in comparison to other forms of gastrointestinal cancer. In general, empirical chemotherapy regimens established for gastric and colorectal cancer have been used for SBA, with unsatisfactory results.

Patients and Methods

Patients

Patients diagnosed with unresectable or recurrent SBA were treated with chemotherapy between August 2001 and March 2006. The patients' data were retrieved from the tumor registry at Cancer Institute Hospital and the extracted patients' records were reviewed retrospectively.

Chemotherapy

The chemotherapeutic strategy of SBA was discussed and fluoropyrimidine-based chemotherapies were chosen as the first-line, followed by irinotecan (CPT-11) monotherapy as the second-line in the regular Digestive Cancer Board Meeting.

Toxicity and Efficacy Evaluation

Adverse effects were evaluated and graded according to the National Cancer Institute Common Toxicity Criteria.[4] The response was assessed using computed tomography (CT) according to the RECIST criteria,

every 12 weeks. The data of toxicity and tumor evaluation were analyzed retrospectively from the medical records and the examination films of each patient.

The progression-free survival time and overall survival time were defined as the time between the date of treatment initiation and the date of diagnosis of disease progression or death with the date at which the patient was last confirmed to be alive, respectively, using the Kaplan–Meier method.[5]

Results

Study Population

Ten patients with advanced SBA received chemotherapy between August 2001 and March 2006. The characteristics of all evaluated patients are detailed in Table 1. The median age was 60 years (range 37–77). The performance status scores varied from 0 to 2. The locations of the primary small-bowel adenocarcinoma were 7 in the duodenum, 1 in the jejunum, and 2 in the ileum. The metastatic or recurrent sites when chemotherapy for SBA was begun were 4 in the local region, 4 in the liver, 4 in the peritoneum, and 4 in the paraaortic lymph nodes. Six patients underwent a noncurative operation as the primary treatment followed by chemotherapy, and one patient underwent chemotherapy immediately.

Response and Survival

Fluoropyrimidine-based regimens were carried out on 10 patients. A 5-fluorouracil plus leucovorin (FL) regimen was used as the first-line treatment for seven patients: four of those received the Mayo Clinic regimen; 5-fluorouracil (5-FU), 500 mg/m^2 of body-surface area and leucovorin (LV), 20 mg/m^2 for 5 days; three received the Roswell Park Memorial Institute (RPMI) regimen, weekly for 6 weeks followed by a 2-week rest period; D,L-leucovorin (D, L-CF; 500 mg/m^2 in a 2-h infusion) with 5-FU (600 mg/m^2 i.v. bolus) 1 h after the D, L-CF infusion began and the others were treated with oral drugs: UFT-E 300 mg/body, twice daily every day; TS-1 40 mg/m^2, twice daily on days 1 through 28 every 42 days. Seven patients received the second-line CPT-11 regimen, 150 mg/m^2, given biweekly, after a confirmed diagnosis of disease progression during the first-line chemotherapy (Table 2).

The antitumor response to the first-line chemotherapy was partial response (PR) in one patient, stable disease (SD) in four patients, and progressive disease (PD) in four patients. The response to the second-line chemotherapy was three patients in SD and four in PD (Tables 3 and 4). The median progression-free survival following the first-line fluoropyrimidine-based regimen and the second-line CPT-11 was 81 days (range 27–666) and 71 days (range 14–935), respectively. The median overall survival time was 12 months (range 3–39). Six patients succumbed to tumor progression with systemic disease, three are still alive, and one was transferred to another hospital for supportive care (Fig. 1).

Table 1. Patient characteristics (n = 10)

Characteristics	No. of patients
Median age, years (range)	60 (37–77)
Male/female	6/4
ECOG performance status: first-line/second-line	
0	7/3
1	1/1
2	2/3
Primary site	
Duodenum (papilla of Vater)	7 (3)
Jejunum	1
Ileum	2
Metastatic sites	
Liver	4
Nodal (Para-aortic lymph node)	4
Peritoneum	4
Locoregional	4
Histological differentiation	
Adenocarcinoma	10
Well-differentiated	1
Moderately differentiated	1
Poorly differentiated	1
Unknown	7
Operation method (n = 9)	
Bypass	3
Partial resection	4
Pancreatoduodenectomy	2
Tumor size (mm)	
< 40/40–80/unknown	2/2/6
Depth of invasion	
SS/SE/SI/unknown	2/1/1/6
Extent of lymph node metastasis	
N0/N1/N2/N3/N4/Nx	3/1/0/1/4/1
Lymphatic invasion	
Positive/negative/unknown	1/1/8
Venous invasion	
Positive/negative/unknown	1/1/8
Curability of surgery (n = 9)	
A/B/C	2/1/6

ECOG, Eastern Cooperative Oncology Group

Safety

The adverse effects are summarized in Tables 5 and 6. Both treatments were generally tolerated. Gastrointestinal symptoms were the most common in both regimens; two patients had grade 3 nausea. Grade 3 neutropenia was only seen in one patient undergoing the CPT-11 regimen and no other severe hematologic toxicity occurred.

Table 2. Treatment and survival

Patient	Age (years)/Sex	Primary site	Regimens	PFS (days)	Survival time (months)
1	42/M	Jejunum	FL (Mayo)	63	12 (dead)
			CPT-11	52	
2	68/F	Papilla Vater	FL (Mayo)	27	3 (dead)
			CPT-11	14	
3	38/F	Duodenum	FL (Mayo)	34	39 (dead)
			CPT-11	935	
4	37/M	Duodenum	FL (Mayo)	226	12 (dead)
			CPT-11	82	
5	54/M	Papilla Vater	UFT-E	49	7[a]
			CPT-11	216	
6	77/F	Ileum	S-1	666	28 (alive)
7	67/F	Jejunum	FL (RPMI)	164	16 (dead)
			CPT-11	21	
8	66/M	Papilla Vater	FL (RPMI)	248	12 (dead)
9	70/M	Ileum	FL (RPMI)	377	19 (alive)
10	47/M	Duodenum	FL (RPMI)	81	6 (alive)
			CPT-11	71	

[a]This patient was transferred to another hospital for supportive care
PFS, Progression-free survival; FL, 5-fluorouracil plus leucovorin; RPMI, the Roswell Park Memorial Institute

Table 3. Response rates to the fluoropyrimidine-based regimen ($n = 10$)

Status	*N* (%)
Complete response	0 (0)
Partial response	1 (10)
Stable disease	4 (40)
Progressive disease	4 (40)
Not evaluable for response	1 (10)

Table 4. Response rates to the CPT-11 regimen ($n = 7$)

Status	*N* (%)
Complete response	0 (0)
Partial response	0 (0)
Stable disease	3 (42.9)
Progressive disease	4 (57.1)
Not evaluable for response	0 (0)

Table 5. Toxicity profile for the fluoropyrimidine-based regimen

Toxicity	All grades (%)	Grade 3/4 (%) ($n = 9$)
Diarrhea	3 (33.3)	0 (0)
Stomatitis	0 (0)	0 (0)
Alopecia	0 (0)	0 (0)
Nausea	6 (66.7)	1 (11.1)
Infection	0 (0)	0 (0)
Hand-foot syndrome	0 (0)	0 (0)
Fever	0 (0)	0 (0)
Fatigue	3 (33.3)	0 (0)
Anemia	2 (22.2)	0 (0)
Neutropenia	0 (0)	0 (0)
Thrombocytopenia	0 (0)	0 (0)

Table 6. Toxicity profile for the CPT-11 regimen

Toxicity	All grades (%)	Grade 3/4 (%) ($n = 7$)
Diarrhea	3 (42.9)	0 (0)
Stomatitis	0 (0)	0 (0)
Alopecia	2 (28.6)	0 (0)
Nausea	4 (57.1)	1 (14.3)
Infection	0 (0)	0 (0)
Hand-foot syndrome	0 (0)	0 (0)
Fever	0 (0)	0 (0)
Fatigue	2 (28.6)	0 (0)
Anemia	3 (42.9)	0 (0)
Neutropenia	1 (14.3)	1 (14.3)
Thrombocytopenia	0 (0)	0 (0)

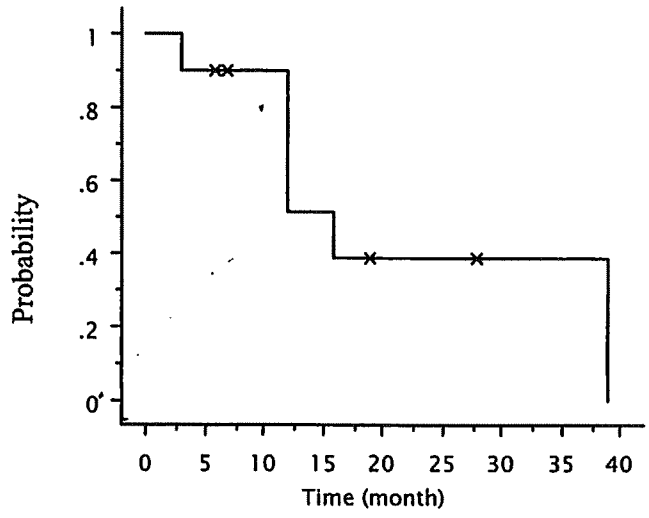

Fig. 1. The overall survival of 10 patients treated with chemotherapy against small-bowel adenocarcinoma

Discussion

Small-bowel tumors are often difficult to diagnose preoperatively. Adenocarcinoma is the most common histology, with a poor prognosis in comparison to carcinoid tumors.[6-8]

Surgery is the usual primary treatment for small-bowel tumors.[6,9,10] For malignancies, standard segmental resections or, if necessary, an extended radical resection including the adjacent organs or as much of the mesentery as is reasonable are recommended. Palliative operations are performed in oncologic emergencies such as gastrointestinal bleeding, obstruction, or perforation. Frost et al. reviewed 30 years of experience with small-bowel adenocarcinoma in their institute and reported the 10-year survival rates of all stages — stage I, II, III and a subgroup of 10 patients (one stage I, seven stage II, two stage III) — undergoing a pancreaticoduodenectomy to be 24%, 75%, 25%, 0%, and 30%, respectively.[9] Talamonti et al. reported their review of 129 surgically treated patients with small-bowel cancer and the prognostic factors for this rare cancer. The 5-year survival rate for an adenocarcinoma was 37%, in which the median survival of patients treated with a curative resection was better than patients with palliative surgery (37 months and 10 months, respectively).[10] According to the reports, late stage was a prognostic factor, while tumor location, size, and patient age were not significant. In addition, aggressive achievement of a sufficient surgical margin and if necessary, extended surgery such as a pancreaticoduodenectomy, are recommended to reduce the risk for local or peritoneal recurrence.

Of the three patients receiving a curative resection in the current study, two underwent a pancreaticoduodenectomy. However, intraoperative extended lymph node metastases were seen in one patient, resulting in para-aortic lymph node metastases. The other patient underwent a partial duodenectomy and the tumor microscopically invaded the serosa, concluding with peritoneal metastases and bilateral ovarian metastases.

In previous reports, few instances of effective chemotherapy and only a small number of large-scale clinical trials have been reported. Gibson et al. administered the FAM regimen (5-FU, mitomycin C, doxorubicin, 5-FU, 600 mg/m^2 on days 1, 8, 29, and 36; mitomycin C, 10 mg/m^2 on day 1; and doxorubicin, 30 mg/m^2 on days 1 and 2) in 38 patients with SBA. In that study, the response rate was 18.4%, including two complete responses; the median survival time was 8 months.[11] Jigyasu et al. also reported their experience using FAM-based regimens at the M.D. Anderson Cancer Center for 14 patients; the MST was 9 months, which was also inadequate.[12] Crawley et al. reported the Royal Marsden experience with protracted venous infusion of 5-FU administration in eight SBA patients with a response rate of 37.5%, including one complete response (CR). The MST and PFS were 13 and 7.8 months, respectively.[13] Polyzos et al. reported the use of irinotecan as salvage chemotherapy for SBA, mentioning irinotecan as a potentially key drug for metastatic SBA similar to metastatic colorectal cancer.[14] Locher et al. assessed the efficacy of 5-FU and either platinum compounds (cisplatin, carboplatin, oxaliplatin) or irinotecan in patients with advanced SBA. Using a combination of 5-FU and platinum compounds, the overall response rate was 21% and median progression-free and overall survival 8 and 14 months, respectively, with tolerable toxicity. The combination of 5-FU and irinotecan as a second-line treatment resulted in 50% disease stabilization with 5 months as the median progression-free survival. But no response was seen in the second-line 5-FU and cisplatin chemotherapy, and the need to try a 5-FU–irinotecan combination chemotherapy as the first-line treatment was indicated.[15] Onodera et al. reported a case of small-bowel adenocarcinoma with extensive lymph node metastases, which showed CR for 10 months after palliative surgery by use of 5-FU and methotrexate sequential chemotherapy.[16] The regimen is generally used for advanced gastric cancer patients who have poor performance status or are unable to receive the current intensive chemotherapy regimens such as S-1 combined regimens.[17-19]

In the current study, an oral fluoropyrimidine agent or bolus 5-FU/LV as the first-line and CPT-11 monotherapy as the second-line, such as the regimen used for metastatic colorectal cancer, were chosen for almost all of the patients. For that reason, no standard chemotherapy against gastric cancer has been established in recent years though both 5-FU and irinotecan were approved and bolus 5-FU/LV had been the standard treatment for first-line metastatic colorectal cancer (mCRC) and CPT-11 monotherapy for the second line until 2004 in Japan, which was applied to SBA patients. This study revealed this strategy to be insufficient against SBA. The FOLFOX or FOLFIRI regimens, which have been the new standard for mCRC in Japan since the approval of infusion of 5-fluorouracil and oxaliplatin in early 2005, are being considered for the treatment of SBA.[20,21] In the present cases, fluoropyrimidines as the first-line chemotherapy produced low response, but S-1 showed some potential, although the treatment was used in only one patient. The efficacy of S-1 or S-1 combined chemotherapy against gastric cancer was demonstrated in 2007, which also provides another indication for application to SBA.[18,19]

Therefore, more intensive chemotherapy is required against this rare malignancy to improve its present poor prognosis. In addition, aggressive surgery to achieve a sufficient surgical margin, followed by adjuvant chemo-

therapy in the later stages, is essential to reduce recurrence. Extensive trials to develop a standard chemotherapy regimen for SBA using capecitabine, oxaliplatin, CPT-11, or S-1 with new drugs such as vascular endothelial growth factor (VEGF) antibodies and epidermal growth factor receptor (EGFR) antibodies, which have been initiated for colorectal cancer, should therefore be started for SBA.[22,23]

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Multicenter safety study of mFOLFOX6 for unresectable advanced/recurrent colorectal cancer in elderly patients

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Abstract

Background: Combination chemotherapy with oxaliplatin plus 5-fluorouracil/leucovorin (FOLFOX) has become a standard regimen for colorectal cancer. An increase of adverse events with combination chemotherapy is predicted in elderly patients, and it remains controversial whether they should receive the same chemotherapy as younger patients. Accordingly, this study of modified FOLFOX6 (mFOLFOX6) therapy was performed to compare its safety between elderly and non-elderly patients.

Methods: We prospectively studies 14 non-elderly patients aged <70 years old and 8 elderly patients aged ≥ 70 years with unresectable advanced/recurrent colorectal cancer who received mFOLFOX6 therapy during the period from March 2006 to March 2007. Adverse events and the response to treatment were compared between the elderly and non-elderly groups.

Results: The main adverse events were neutropenia and peripheral neuropathy, which occurred in 62.5% (≥ grade 3) and 87.5% (≥ grade 1) of elderly patients. The grade and frequency of adverse events were similar in the elderly and non-elderly groups. In some patients with neutropenia, treatment could be continued without reducing the dose of oxaliplatin by deleting bolus 5-fluorouracil. A correlation was found between the cumulative dose of oxaliplatin and the severity of neuropathy, and there were 2 elderly and 3 younger patients in whom discontinuation of treatment was necessary due to peripheral neuropathy. The median number of treatment cycles was 10.0 and 9.5 in the non-elderly and elderly groups, respectively. The response rate was 60.0% in the non-elderly and 50.0% in the elderly group, while the disease control rate was 100% and 83.3% respectively, showing no age-related difference.

Conclusion: mFOLFOX6 therapy was well-tolerated and effective in both non-elderly and elderly patients. However, discontinuation of treatment was sometimes necessary due to peripheral neuropathy, which is dose-limiting toxicity of this therapy.

Background

A high response rate has been reported for FOLFOX therapy that includes oxaliplatin in patients with unresectable advanced/recurrent colorectal cancer, and this therapy is now established as one of the standard treatment option [1,2]. Since the introduction of oxaliplatin to Japan in April 2005, FOLFOX therapy has also become widely used in this country and is recommended as one of the standard treatments [3]. There are a number of versions of FOLFOX therapy among which modified FOLFOX6 (mFOLFOX6) allows more convenient administration and has been adopted by many medical institutions in association with popularization of outpatient chemotherapy. However, there have been few adequate investigations into the safety and efficacy of mFOLFOX6 therapy. A rapid increase in the incidence of colorectal cancer among elderly Japanese persons is anticipated in the future, considering the current long average life span and the increase in the incidence and mortality of colorectal cancer in Japan. However, it remains controversial as to whether the same multi-drug chemotherapy employed for younger patients should also be given to elderly patients, because an increase in the severity of adverse events is likely in the elderly due to the decline of organ function associated with ageing. Accordingly, the present study was performed to examine the safety and efficacy of mFOLFOX6 therapy in patients over 70 years old.

Subjects and methods

Subjects

A multicenter study on the treatment of unresectable advanced/recurrent colorectal cancer was started in 2006 by the Sanin Study Group on colorectal cancer (SSCC). To determine whether mFOLOFX6 could be used safely to treat unresectable advanced/recurrent colorectal cancer in elderly patients, the present study (SSCC-0601) was also performed by the SSCC.

Patients who met the following eligibility criteria and received mFOFOX6 therapy at any of the three participating institutions (Division of Surgical Oncology, Faculty of Medicine, Tottori University; Department of Digestive and General Surgery, Faculty of Medicine, Shimane University; and Department of Surgery, Shimane Prefectural Central Hospital) during the period from March 2006 to March 2007 were enrolled.

The protocol was approved by the institutional ethics committees and this study was carried out according to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines.

The eligibility criteria were histologically proven unresectable colorectal adenocarcinoma; adequate bone marrow, liver, and renal function; Eastern Cooperative Oncology Group (ECOG) performance status (PS) <2; age >20 years at the time of enrolment; and expected survival time >12 weeks. Any previous chemotherapy (only 1 regimen was allowed) must have been completed at least 28 days before enrolment. Postoperative adjuvant therapy was not counted as prior chemotherapy. Patients with multiple malignancies, comorbidities that could influence the outcome, prior radiotherapy, pregnancy or lactation, symptomatic peripheral neuropathy, or a history of serious drug hypersensitivity were excluded. Written informed consent was obtained from all of the subjects.

Treatment schedule

An implantable port and a disposable pump were employed so that chemotherapy could be administered on an outpatient basis. An outline of the administration method for mFOLFOX6 therapy, in which the dose of oxaliplatin was reduced from 100 mg/m^2 to 85 mg/m^2, is shown in Figure 1. A 5-HT_3 antagonist and a steroid were administered as premedication. A 2-hour intravenous infusion of oxaliplatin plus l-leucovorin was followed by bolus intravenous injection of 5-FU, after which 5-FU was administered by continuous infusion for 46 hours. An oral steroid was administered for 3 days from day 2 after the start of therapy. The duration of one cycle was 2 weeks.

With each treatment cycle, administration was only started after confirming that all of the following criteria had been fulfilled.

(1) Hematological toxicity: leukocyte count >3,000/mm^3 and platelet count >75,000/mm^3.

(2) Non-hematological toxicity: Grade 2 or less according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC), and Grade 1 or less for peripheral neuropathy.

(3) Even if these conditions for treatment were met, administration could be postponed at the investigator's discretion (e.g., for a rapid decrease of the leukocyte count/platelet count, occurrence of jaundice, etc).

If any of the criteria were not met, treatment was postponed. The subsequent course could be postponed for up to 21 days (excluding the scheduled day of starting administration). If administration could not be commenced during this period, the study was discontinued.

Discontinuation of therapy

Administration was continued until any of the following criteria for discontinuation were fulfilled.

(1) The patient was judged to have progressive disease (PD), including clinical PD.

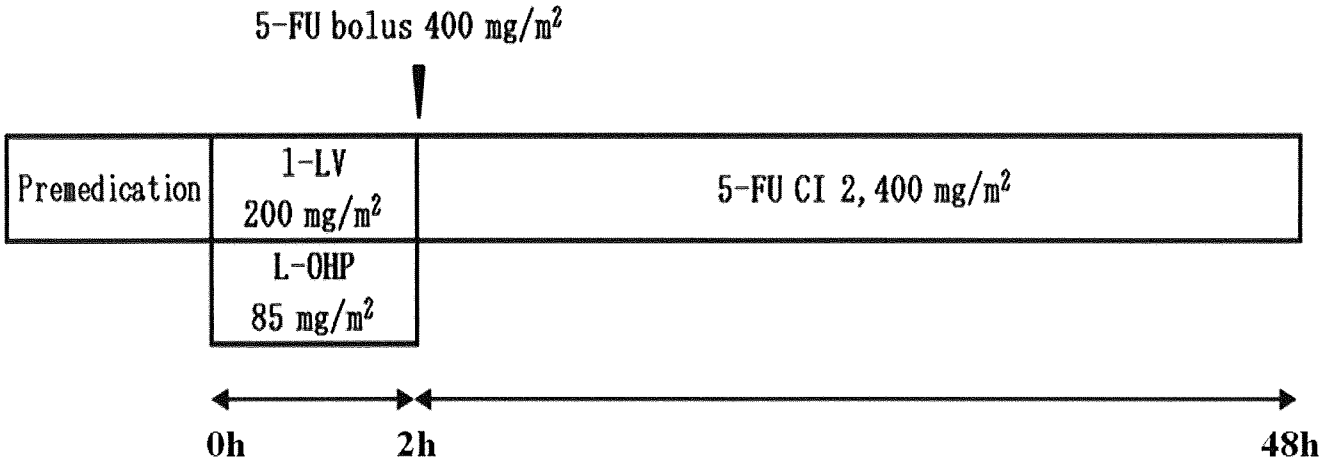

Figure 1
Schedule for mFOLFOX Therapy.

(2) Adverse events occurred that made further administration difficult.

(3) The patient did not fulfill the administration criteria and the next course of treatment could not be started by 21 days after the scheduled day of administration.

(4) A second dose reduction was considered to be necessary (Table 1).

(5) Peripheral neuropathy of grade 3 or 4 occurred.

(6) The PS became 3 or higher.

(7) The patient refused further treatment.

(8) The investigator judged that continuation of the study was difficult for any other reason.

Endpoints

The incidence and severity of adverse events were assessed as the primary endpoints, while the duration of treatment, antitumor effect (response rate, tumor stabilization rate, and duration of response), and the safety and efficacy in elderly patients were assessed as the secondary endpoints. Adverse events and therapeutic efficacy were assessed according to the NCI-CTC (version 3) (Cancer Therapy Evaluation Program, NCI, Bethsada, Md., USA) and the RECIST guidelines (version 3) [4]. Extramural review was performed for judgment of the eligibility and handling of registered patients, as well as for safety and efficacy assessment.

Statistical analysis

The chi-square test for independence, Fisher's exact probability test, and the Mann-Whitney U test were used to compare patient characteristics, treatment status, adverse

Table 1: Dose-Reduction Criteria and Dose to be selected at Dose-Reduction

Item		Oxaliplatin	5-FU (bolus)	5-FU (infusion)
Neutrophil count	**< 500/mm³**	85 → 85	**400 → 0**	2,400 → 2,400
Platelet count	**< 50,000/mm³**	85 → 85	**400 → 0**	2,400 → 2,400
Non-hematological toxicity	**≥ Grade 3**	**85 → 65**	**400 → 300**	**2,400 → 2,000**
Skin symptoms	**≥ Grade 3**	85 → 85	**400 → 300**	**2,400 → 2,000**
Peripheral neuropathy	**Grade 2**	**85 → 65**	400 → 400	2,400 → 2,400
Acute*1 laryngopharyngeal dysesthesia (feeling of difficulty in breathing)		**85 → 85 Infusion time is prolonged to 6 hours*2**	400 → 400	2,400 → 2,400
Peripheral neuropathy	**≥ Grade 3**	**Discontinuation**		
PS	**≥ 3**	**Discontinuation**		

Abbreviation: PS, performance status
*1 During the period from administration of oxaliplatin to 2 hours after completion of administration.
*2 Administration of 5-FU should not be started until the completion of administration of oxaliplatin.

events, and antitumor effect. A probability (P) value of less than 0.05 was considered statistically significant for comparisons between the younger and elderly groups. The Kaplan-Meier method was used to estimate the time to treatment failure (TTF).

Results

Patient profile

All of the 22 patients enrolled in this study were eligible. Their median age was 66 years (range: 39–79 years), including 14 non-elderly patients with a median age of 63.5 years (range: 39–69 years: younger group) and 8 elderly patients with a median age of 74.5 years (range: 71–79 years: elderly group). Although the elderly group had a higher incidence of colon cancer (P = 0.011), there were no marked differences of the other background factors (Table 2).

Treatment status

The total number of cycles administered was 198, with a median of 10.0 cycles per patient in the younger group and 9.5 cycles in the elderly group, showing no difference (P = 0.8912 by the Mann-Whitney U test). Postponement of treatment due to toxicity occurred during 14.4% (18/125) of the treatment cycles in the younger group and 6.8% (5/73) of the cycles in the elderly group (P = 0.1907 by the chi-square test for independence).

Adverse events

Adverse events that showed a high incidence included neutropenia and peripheral neuropathy. The grade and frequency of the other adverse events were similar between the younger and elderly groups (Table 3). In 3 patients (one younger patient and 2 elderly patients) who developed grade 4 neutropenia, treatment could be continued without reducing the dose of oxaliplatin by deleting bolus 5-fluorouracil (Table 1). Peripheral neuropathy of grade 1 or more occurred at an incidence of 86.4% in the younger group and 87.5% in the elderly group (P = 0.7090), while grade 3 neuropathy occurred in 3 patients (14.3%) from the younger group and 1 patient (12.5%) from the elderly group (P = 0.7090) (Table 3). The incidence of neuropathy in relation to the number of treatment cycles is shown in Table 4. There was an increase in the incidence along with the dose of oxaliplatin, and grade 2 or worse neuropathy showed an incidence higher than 50% during the 11th cycle in the younger group and the 10th cycle in the elderly group (Figure 2).

Duration of Treatment

The time to treatment failure (TTF) was 6.2 months in the younger group, and 4.9 months in the elderly group, being slightly shorter in the latter group (Figure 3). The major reasons for discontinuation of treatment were tumor progression in 2 patients (14.3%) and peripheral neuropathy in 3 patients (21.4%) from the younger group versus 4 patients (50.0%) and 2 patients (25.0%), respectively, in the elderly group (P = 0.0963 and 0.6199 by Fisher's exact probability test). In the younger group, there was also 1 case of discontinuation after re-resection and 2 patients discontinued treatment due to hematological toxicity (a second dose reduction was necessary according to the criteria in Table 1).

Response

Nineteen patients (12 from the younger group and 7 from the elderly group) could be evaluated for their response to treatment (Table 5). There were no patients with a complete response. The response rate was 60.0% in the younger group and 50.0% in the elderly group, while the disease control rate (PR+SD) was 100% and 83.3% in the younger and elderly groups, respectively. Thus, there was no difference of the response in relation to age.

Discussion

In 1957, 5-fluorouracil (5-FU) became available clinically, and the advent of 5-FU therapy [5,6] was followed by 5-FU/leucovorin (LV) therapy [7] that has remained standard chemotherapy for colon cancer for a very long time. After irinotecan and oxaliplatin became available, clinical studies including randomized comparative trials [8-10] of concomitant treatment with these agents and 5-

Table 2: Patients Characteristics

	< 70 Years (n = 14)	≥ 70 Years (n = 8)	P values
Age (median)	63.5 [39–69]	74.5 [71–79]	-
Sex (male/female)	11/3	5/3	*0.3695
PS (ECOG) 0/1/2	9/5/0	7/1/0	**0.2505
Primary tumor Colon/rectum/colorectal	4/8/2	7/1/0	*0.011/0.052/0.3939
Target lesions liver/lung/LN/peritoneum/others	4/2/6/0/2	4/1/1/1/1	*0.291/0.709/0.161/0.364/0.709
Previous surgery (+/-)	12/2	8/0	*0.3939
Adjuvant chemotherapy(+/-)	4/10	2/6	*0.6305
Previous treatment (+/-)	1/13	1/7	*0.6060

Abbreviation: PS, performance status; ECOG, Eastern Cooperative Oncology Group; LN, lymph node.
*P values for SEX, primary tumor, target lesions, previous surgery (+/-), adjuvant chemotherapy (+/-) and previous treatment (+/-) were calculated with the use of Fisher's exact probability test. **P values for PS were calculated with the use of Mann-Whitney U test.