Among the eight ESCC cell lines supplied by RIKEN cell bank (Tsukuba, Japan), TE14 cells showed the highest MRP2 mRNA expression and were subsequently transfected with 15 nmol l^{-1} siRNA using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen). After 24 h, the medium was replaced by standard medium, and then 96 h from the siRNA administration, cells were collected for the following growth inhibitory assay as described below.

Growth inhibitory assay

Cells (TE14, 1×10^4 cells per well) were added in triplicate to a 96-well microplate, and after overnight incubation, the medium was replaced with 100 μl of fresh medium containing various concentrations of DXR and CDDP, both of which chemoagents have been reported to be transported by MRP2 in some types of cell lines. The TE14 cells suspended in complete medium were used as a control for cell viability. After 4 h (DXR and CDDP) treatment, the cells were washed with fresh medium. The number of viable cells was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) assay. Briefly, 10 μl (50 μg) of MTT were added to each well after 48 h (DXR and CDDP) from the chemoadministration. The plate was incubated for 4 h at 37°C, followed by removal of medium and the addition of 100 μl of 2-propanol to each well to dissolve the resultant formazan crystals. Plate absorbance was measured in a microplate reader at a wavelength of 650 nm. After a pulsed exposure, the IC_{50} was calculated as percentage of control cultures that were not exposed to chemoagents using an interpolated logarithmic concentration curve. Results were derived from three independent sets of triplicate experiments.

Statistical analysis

Data are expressed as mean ± s.d. Correlations between MRP2 expression and various clinico-pathological parameters were each evaluated by the χ^2 test and Fisher's exact probability test. Differences in continuous parameters between two groups were evaluated by the Mann–Whitney's *U*-test. Prognostic variables were assessed by log-lank test, and OS was analysed by Kaplan–Meier method. These analyses were carried out using SPSS for Windows v10 (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 denoted the presence of statistical significance.

RESULTS

MRP2 protein expression by immunohistochemistry in ESCC and its correlation with clinico-pathological parameters

A total of 81 samples that contained both cancerous and non-cancerous lesions were evaluated for MRP2 protein expression by IHC analysis. As a positive control, liver tissue showed strong MRP2 immunostaining mainly in the hepatocyte plasma membrane (Figure 1A). No normal squamous epithelium showed significant levels of immunostaining (Figure 1B). Of all samples, 14 (17.3%) showed positive MRP2 expression, mainly in the cell membrane and cytoplasm of tumour cells (Figure 1C), whereas the remaining 67 (82.7%) were negative for MRP2 expression (Figure 1D). The positive staining was almost homogeneous in single-cancer nests and among different areas (surface, central, and deepest areas) of the cancer lesion.

Table 1 lists the correlations between MRP2 expression and various clinico-pathological parameters. Of note, MRP2 expression was exceptional in the ESCC patients without NACT (2 out of 37, 5.4%), but was significantly more frequent in patients after NACT (12 out of 44, 27.3%). Women tended to have a higher rate of MRP2 expression than men (44.4 *vs* 13.9%, respectively), although the difference was small. Other clinico-pathological parameters including age, histological type, tumour location, pT, pN, and pStage were not associated with MRP2 expression.

Disease recurrence after curative resection was diagnosed in 35 (46.7%) of 75 patients with curative resection (R0) and the mean

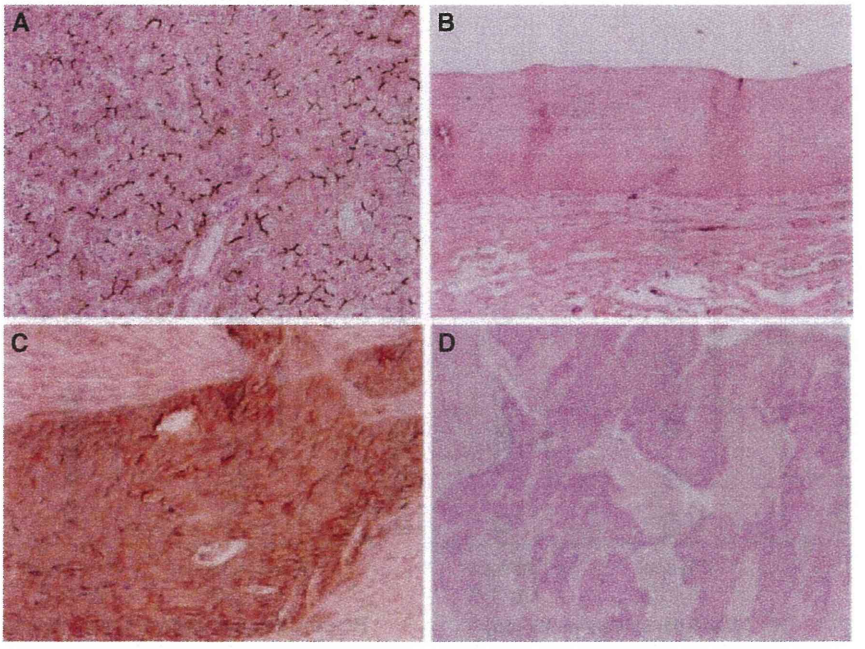

Figure 1 MRP2 expression by immunohistochemistry. (**A**) Strong MRP2 expression in liver tissue as a positive control (magnification, × 400). (**B**) Representative normal squamous epithelium negative for MRP2 expression (magnification, × 200). (**C**) Representative MRP2-positive ESCC showing staining mainly in the membrane and cytoplasm of tumour cells (magnification, × 200). (**D**) Representative MRP2-negative oesophageal squamous cell carcinoma with no appreciable staining of tumour cells (magnification, × 200).

time to recurrence was 10.5 months. A total of 35 (43.2%) patients died and their average survival time from diagnosis to death was 1.4 years (range 0.2–4.2 years). The total 5-year OS rate was 50.9% and MRP2-positive patients showed a significantly poorer prognosis than MRP2-negative patients (5-year OS 55.7 *vs* 25.6%) (Figure 2).

MRP2 mRNA expressions in resected specimens and endoscopy biopsy samples

RT–PCR analysis was performed to quantify the expression of MRP2 mRNA in surgically removed specimens from 26 representative cases, including 16 with NACT and 10 without NACT. MRP2 mRNA expression in tumours with NACT was 2.1-fold higher than in those without NACT, although there was no significant difference in TNM stage and other clinico-pathological parameters between the groups (data not shown) (Figure 3A).

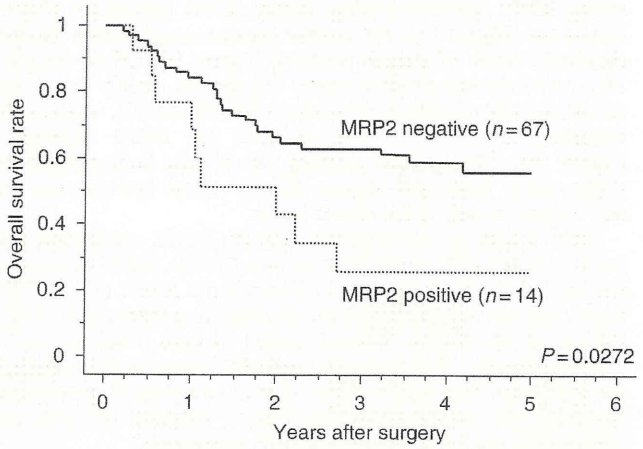

Figure 2 Survival curves according to MRP2 expression. Overall survival curve classified according to MRP2 expression for all patients were plotted by Kaplan–Meier method. Differences between two groups were evaluated by log–rank test. Ordinate: overall survival rate, abscissa: time after surgery (years).

The association between MRP2 mRNA expression and the effect of NACT was investigated in biopsy samples before NACT from 42 patients; the response of these patients to NACT was classified as non-responder in 22 and responder in 20. As shown in Figure 3B, MRP2 mRNA expression in non-responders was 2.9-fold higher than that in responders. Again, although these 42 samples were all advanced tumours with clinically positive lymph node metastases, there was no significant difference in clinical background parameters between the groups (data not shown).

Association between MRP2 mRNA expression and chemoresistance in ESCC cancer lines

To explore whether MRP2 expression functions specifically in chemoresistance to CDDP, we tested for a correlation between MRP2 mRNA expression and CDDP resistance (IC_{50}) in eight ESCC cell lines (Figure 4). Relatively high MRP2 expression was observed in TE14 and TE5 cell lines, both of which displayed strong resistance to CDDP. Regression analysis showed a significant correlation between MRP2 mRNA expression and IC_{50}

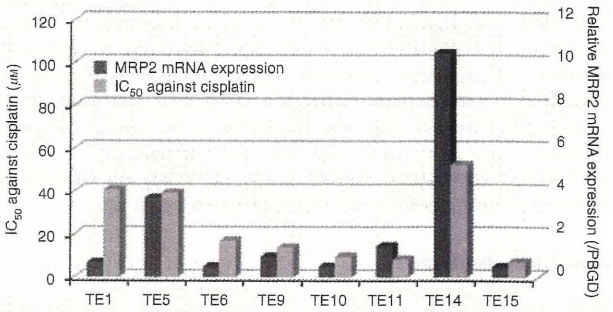

Figure 4 Correlation between MRP2 mRNA expression and CDDP-resistance (IC_{50}) in eight cell lines of ESCC. Relatively high MRP2 expression was observed in TE14 and TE5 cell lines, both of which displayed strong resistance to CDDP. Black bar: the relative ratio of MRP2 mRNA expression, grey bar: IC_{50} values against CDDP.

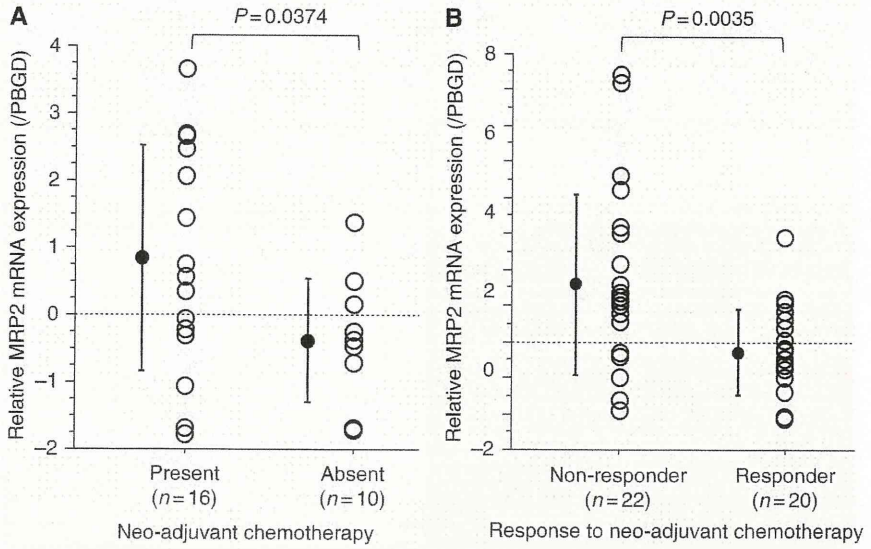

Figure 3 Differences in MRP2 mRNA expression between patients with and without neo-adjuvant chemotherapy in resected specimens (**A**), and between responders and non-responders at biopsy (**B**). (**A**) The relative ratio of MRP2 mRNA expression in resected tumours treated with neo-adjuvant chemotherapy ($n = 16$) was significantly higher than in untreated cancers ($n = 10$). (**B**) In endoscopy biopsy samples, the relative ratios of MRP2 mRNA expression in responders ($n = 22$) were significantly higher than those in non-responders ($n = 20$). Data are shown as mean ± s.d. (log2 values).

Table 2 Modulation of resistance against cisplatin and doxorubicin by MRP2 siRNA

	IC_{50}	
	Cisplatin (μM)	**Doxorubicin (μM)**
TE14 NC	32.4 (± 1.2)	6.2 (± 0.16)
TE14 siRNA-1	20.5 (± 1.4)[a]	5.8 (± 0.47)[b]
TE14 siRNA-2	17.8 (± 1.2)[c]	5.4 (± 0.54)[d]

Abbreviations: NC = negative control; IC_{50} = half maximal inhibitory concentration; siRNA = small-interfering RNA. [a]$P = 0.0003$, compared with NC. [b]$P = 0.2869$, compared with NC. [c]$P = 0.0005$, compared with NC. [d]$P = 0.2285$, compared with NC. Data are shown as mean ± s.d.

against CDDP ($R = 0.741$, $R2 = 0.549$), suggesting that ESCC cell lines with higher MRP2 mRNA expression were more resistant to CDDP compared with those showing lower MRP2 expression.

To confirm these findings by an alternative approach, we transfected MPR2 siRNAs into the TE14 line, which had the highest cellular MRP2 expression. The specific gene silencing started 48 h after the administration of siRNA (two siRNAs for MRP2 with different sequences were used: siRNA-1 and siRNA-2) and continued for 144 h, which was examined by quantitative PCR, resulting in 63.8% (siRNA-1) and 65.9% (siRNA-2) of peak MRP2 downregulation compared with NCs. The knockdown effect was stable during this period. As shown in Table 2, downregulation of MRP2 conferred increased sensitivity to CDDP, but not to DXR. IC_{50} values against CDDP were significantly lower in TE14 cell lines transfected with siRNA-1 and siRNA-2 compared with those transfected with NC (20.5 ± 1.4, 17.8 ± 1.2 *vs* 32.4 ± 1.2 μM, (siRNA-1 *vs* NC); $P = 0.0003$, (siRNA-2 *vs* NC); $P = 0.0005$). On the other hand, IC_{50} values of DXR were almost similar among TE14 cells transfected with siRNA-1, siRNA-2, and NC (5.8 ± 0.47, 5.4 ± 0.54 *vs* 6.2 ± 0.16 μM, (siRNA-1 *vs* NC); $P = 0.2869$, (siRNA-2 *vs* NC); $P = 0.2285$).

DISCUSSION

To our knowledge, this study is the first to identify the clinical significance of MRP2 expression in chemoresistance in ESCC. Such a relationship was strongly suggested by the findings that (1) MRP2 expression in the clinical biopsy samples before NACT was significantly negatively correlated with the effect of NACT, and (2) in the cultured cell line, artificial MRP2 downregulation resulted in increased resistance to the chemotherapy. Furthermore, the clinical samples of patients treated with NACT showed significantly higher expression of MRP2 at both the protein and mRNA levels than those without NACT, and the increased MRP2 expression was associated with poor prognosis. Although complicated, these clinical observations implicated MRP2 in the acquired resistance to chemotherapy commonly encountered in ESCC patients.

Intrinsic or acquired drug resistance is a major factor limiting the effectiveness of chemotherapy in various cancers including ESCC. Drug resistance by tumours occurs not only to a single cytotoxic agent but also in the form of cross-resistance to many agents called MDR. One of the major mechanisms of MDR is an increased ability of tumour cells to actively efflux drugs, decreasing the intracellular drug accumulation. This mechanism is mediated by ATP-dependent drug efflux pumps known as ABC transporters (Leonard *et al*, 2003; Ozben, 2006). To date, at least 48 human ABC transporters have been identified, and they have been divided into seven sub-families, ABC-A through ABC-G. The first ABC transporter identified in this context was P-glycoprotein (PgP, MDR1, ABCB1) (Kartner *et al*, 1983), and in the absence of overexpressed MDR1, the protein MRP1, ABCC1 was discovered because of the MDR phenotype (Cole *et al*, 1992).

Cisplatin resistance is not a feature of MDR phenotypes conferred by either MDR1 or MRP1 (Borst *et al*, 2000). The finding that ABC transporter MRP2 could mediate active efflux of CDDP conjugated to glutathione (Taniguchi *et al*, 1996), supported by evidence that intracellular glutathione levels were related to CDDP toxicity (Ozols, 1985), suggested a possible role for active efflux as a resistance mechanism. In addition, human carcinoma cell line studies showed increased levels of MRP2 mRNA associated with relative CDDP resistance, decreased intracellular accumulation of CDDP, and decreased DNA adduct formation (Kool *et al*, 1997; Liedert *et al*, 2003). In ESCC cell lines (TE2, TE13) Tanaka *et al* (2010), in their analysis of the intracellular localisation of CDDP by using in-air micro-particle induced X-ray emission, recently reported that TE2 cells, which express lower MRP2 than TE13, had higher intracellular CDDP concentrations and sensitivity than TE13 cells. This is also in agreement with our present *in vitro* data regarding CDDP. In human tissue samples, accumulating evidence indicates that MRP2 expression is also associated with intrinsic CDDP resistance in the clinical setting, using tissues obtained from patients with colorectal cancer (Hinoshita *et al*, 2000), small-cell lung carcinoma (Ushijima *et al*, 2007), and ovarian cancer (Surowiak *et al*, 2006). These results are also consistent with our data from cancer tissue samples, although our *in vitro* data involving each single agent could not necessarily be translated directly to a clinical response to combination chemotherapy because of possible synergistic effects. However, in contrast with these data, other studies failed to show a significant association between MRP2 expression and chemosensitivity in patients with ovarian cancer (Arts *et al*, 1999; Materna *et al*, 2004) or lung cancer (Filipits *et al*, 2007; Kim *et al*, 2009). It therefore seems likely that multiple factors such as drug accumulation, DNA repair capacity, and apoptotic sensitivity contribute to clinical tumour chemosensitivity, and a mechanistic relationship could be difficult to detect amongst an unselected patient cohort in which a number of other factors also affect clinical outcome. As clinical significance of MRP2 other than chemosensitivity, Gan *et al* (2010) reported that MRP2 expression was significantly higher in poorly differentiated ESCC tumours compared with moderate or well differentiated ones, which was not observed in our study.

In terms of the contribution to acquired chemoresistance, the present IHC and qRT-PCR data showed higher MRP2 expression in resected tumours with NACT compared with those without NACT, implying residual tumours after NACT acquired the feature of chemoresistance. Unfortunately, we could not compare MRP2 expression levels in cancer tissues from the same patient before and after NACT because no samples were available. Nooter *et al* (1998) reported significantly higher MRP expression, although not specific MRP2 expression, in ESCC tumours from non-responders to CDDP-based chemotherapy when comparing MRP levels in paired tumour samples before and after chemotherapy, suggesting that chemotherapy was selected for drug-resistant cell clones. Furthermore, other *in vitro* analyses by Noma *et al* (2008) established two CDDP-resistant pancreatic cancer cell lines (SUIT-2-CD3 and SUIT-2-CD4) by continuously administering 10 nM CDDP for 3 and 4 months, respectively. Results of RT-PCR indicated that induction of MRP2 mRNA expression was significantly increased by 1.5- and 2.5-fold in SUIT-2-CD3 and SUIT-2-CD4 cells, respectively, compared with parent cells, whereas MRP1 and MRP3 expression remained unchanged, implying a contribution of MRP2 to acquired resistance for CDDP in pancreatic cancer.

An important observation regarding the functional significance of MRP2 expressed in tumour cells could be the sub-cellular localisation. In normal tissues, MRP2 is expressed in functionally

polarised cells in which it specifically localises to the apical membrane of these cells. Apical localisation has also been described in tumours arising from these sites, a feature attributed to a targeting signal in the C-terminus of the MRP2 molecule (Harris *et al*, 2001). Single-nucleotide polymorphisms in MRP2 have been described that result in cytoplasmic localisation of the protein and that may reduce *in vivo* function (Hirouchi *et al*, 2004). Reduced CDDP sensitivity has also been reported in polarised mammalian kidney cells transfected with appropriately localised MRP2 (Cui *et al*, 1999). Furthermore, data of Surowiak *et al* (2006) indicated that MRP2 could confer resistance to CDDP in ovarian carcinoma only when expressed at the nuclear membrane, and this was supported by *in vitro* data (Materna *et al*, 2006). Although our IHC results showed MRP2-positive staining of both cytoplasm and membrane in tumour cells, MRP2 protein located in the cell cytoplasm might not function as an efflux pump (Evers *et al*, 1998). Further analysis focusing on the sub-cellular localisation of MRP2, and on the functional and clinical significance of such cellular location, is needed to elucidate the specific mechanism of chemoresistance induced by MRP2 in ESCC.

In conclusion, MRP2 expression seems to be associated with intrinsic resistance to chemotherapy in patients with ESCC, and is likely to also have a role in acquired chemoresistance. Further studies with larger cohorts are warranted to verify these results prospectively. The findings of this study open the door for exploration of efficacious treatment strategies and development of new therapeutic approaches for ESCC.

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Relationship between circulating cytokine levels and physical or psychological functioning in patients with advanced cancer

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ABSTRACT

Objective: To investigate the relation between functional impairments of cancer patients and circulating cytokines using a multiplex technique.

Design and methods: 50 patients with cancer were assessed using the quality of life (QOL) questionnaire. 27 plasma cytokine levels were determined by using the Bio-Plex array system. The relation to QOL scores was assessed using Chi-square test for categorical variables and univariate linear regression analysis for cytokine levels.

Results: Multivariate analysis showed that interleukin-6 (IL-6) level is a significant independent determinant of physical ($\beta = -0.238$, $P = 0.0126$) and cognitive functioning ($\beta = -0.462$, $P = 0.0006$) and that vascular endothelial growth factor (VEGF) level is a significant independent determinant of emotional functioning ($\beta = -0.414$, $P = 0.039$).

Conclusion: This study, in which 27 cytokines are simultaneously tested with cutting edge technology, demonstrates that plasma IL-6 and VEGF are significant independent determinants of functional impairments in patients with cancer.

Introduction

Many cancer patients have severe and persistent symptoms that can be physical (pain, gastrointestinal symptoms, fatigue, shortness of breath), cognitive (memory problems, impaired concentration), or affective (especially depression and anxiety), many of which co-occur. Interruptions to treatment caused by severe symptoms negatively influence treatment effectiveness [1]. Previous studies of cancer-related symptoms have had several limitations. Symptoms have often been assessed and treated discretely, while they undoubtedly interrelate in complex ways. Furthermore, studies of predictors of cancer-related symptoms have traditionally focused on disease-related variables (e.g., stage of disease), clinical health status (e.g., performance status, comorbid conditions), and sociodemographic characteristics (e.g., age, gender, race, marital status), and have not included mechanism-based assessments.

Recently, several convergent lines of clinical and experimental research have revealed that pro-inflammatory cytokines might be pivotal in the pathophysiology of cancer symptoms. Changes in concentration of serum cytokines such as IL-1, IL-6 and TNF-α were associated with physical, cognitive and affective symptoms [2–5]. The cytokine-induced sickness that occurs in animals after the administration of inflammatory agents or certain pro-inflammatory cytokines has much in common with the symptoms experienced by cancer patients [6,7]. It seems likely that many cytokines are involved in the development of cancer-related psychobehavioral symptoms.

These symptoms collectively cause a symptom burden and have a negative impact on quality of life (QOL) in patients with various types of cancer. Symptom burden can affect patient functioning, and in most patients with cancer, both physical and psychological functions are impaired [4,8]. Although the relationship between cytokines and cancer-related symptoms has been examined in previous studies, the number of cytokines examined was limited, and descriptive studies of the relationship between circulating cytokine levels and functional impairments in patients with cancer remain far from comprehensive. Therefore, we aimed to simultaneously measure the concentrations of 27 cytokines using a multiplex technique and to study their association with functional impairment in patients with cancer.

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Table 1
Clinical characteristics of patients.

Characteristics (n = 50)	Number
Sex	
Male/female	26/24
Age (y)	
Median (range)	63 (23–83)
ECOG performance status	
0/1/2/3/4	16/21/6/7/0
Disease site	
Gastric cancer	9
Esophageal cancer	2
Colorectal cancer	3
Pancreatic cancer	7
Cholangiaodactal cancer	2
Hepatic cell carcinoma	3
Head and neck cancer	4
Lung cancer	7
Breast cancer	1
Uterine cancer, sarcoma	4
Ovarian cancer	4
Others	4
Disease status	
Advanced inoperable state	36
Recurrent	14
Previous chemotherapy	
None	2
One line	19
Two lines or more	29

ECOG, Eastern Cooperative Oncology Group.

Methods

Patients

From November 2008 until May 2009, 50 consecutive patients with solid malignancies who received immunotherapy at Hyakumanben Clinic (Kyoto, Japan) were registered in the study. All diagnoses were verified by histopathological examination. Additionally, as controls thirty-three age-matched healthy subjects ranging from 40 to 80 years of age, were recruited from individuals for routine health checkups. Healthy subjects had no history of cancer, chronic infectious or autoimmune diseases, diabetes mellitus, nephritis or asthma.

The institutional Ethical Committee approved the study, and each patient gave written informed consent.

Blood sampling and assay

Blood samples from all patients were collected using sodium heparin tubes and centrifuged at 1600 g for 10 min. All plasma samples were frozen at −80 °C until analysis. Cytokine levels in the samples were measured using a multiplex cytokine array system (Bio-Plex; Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The Bio-Plex Human Cytokine 27-Plex Panel includes 27 cytokines (IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF). Data acquisition and analysis used Bio-Plex Manager software version 5.0. Based on data from Bio-Rad laboratories, the intra-assay and inter-assay coefficients of variation (CV) were 5–15% and 4–11% respectively in human serum or standards spiked into serum using automatic bead washers. The variations in our results were also within these ranges.

Assessment of physical, emotional and cognitive functioning

Physical and psychological functions were assessed using the QOL questionnaire (QLQ-C30) of the European Organization for Research and Treatment of Cancer (EORTC) [9] (version 3.0, Japanese version) on the day of blood sampling. This questionnaire gives separate scores for multi-item scales (functional scale, symptom scale, a global health status/QOL scale) and six single-item measures. The validity and reliability of EORTC QLQ-C30 and its Japanese version have been reported [10,11]. We analyzed three functional scales (physical, emotional and cognitive functioning) in this study and transferred all scores to continuous scales ranging from 0 to 100 on which a high score represents a high (healthy) level of functioning.

Statistical analysis

Univariate linear regression was used to assess the possible associations between functional scale scores and circulating cytokine levels. To analyze differences in clinical characteristics in relation to functional scale scores, patients were divided into two groups by the median of each functional scale score, and the chi-square test was used. When data were not normally distributed (i.e., IL-2, IL-9, GM-CSF, IFN-γ and VEGF), a logarithmic transformation was performed prior to the statistical analysis.

The prognostic values of cytokine levels in plasma and all clinical variables were first examined with univariate analyses. The independent relationship to each functional scale score of those variables that were possibly associated with the outcomes of interest ($P<0.20$ after univariate analysis) was tested using stepwise multiple linear regression analysis. $P<0.05$ was considered to indicate statistical significance. All statistical analyses were carried out with Stat View software (version 5.0; SAS, Cary, NC).

Results

Patient characteristics

Patients' characteristics are summarized in Table 1.Their median age was 63 years (range: 23 to 83 years) and they suffered from various types of cancer, which was advanced and inoperable or recurrent in all cases. There were 37 patients whose Eastern Cooperative

Table 2
Plasma cytokine concentrations in patients.

	Median (ng/L)	Interquartile range (ng/L)
IL-1β	0.22	BDL–0.42
IL-1RA	37.56	27.42–67.54
IL-2	0.32	BDL–1.46
IL-4	0.24	0.14–0.48
IL-5	0.42	BDL–0.67
IL-6	3.99	2.63–5.97
IL-7	BDL	BDL–0.45
IL-8	8.79	5.53–16.35
IL-9	5.49	2.86–10.17
IL-10	0.79	0.50–1.17
IL-12p70	1.70	1.24–3.15
IL-13	0.90	0.49–2.02
IL-15	3.38	2.29–5.41
IL-17	3.36	2.43–5.28
TNF-α	4.14	BDL–9.68
IFN−γ	11.86	4.43–18.56
bFGF	10.93	BDL–20.04
PDGF-BB	199.30	85.28–438.90
G-CSF	3.04	2.17–4.03
GM-CSF	11.94	7.95–20.40
IP-10	306.02	237.80–474.50
MCP-1	44.1	30.25–62.07
MIP-1α	1.08	BDL–1.70
MIP-1β	74.45	54.85–107.50
Eotaxin	23.98	16.62–40.27
RANTES	747.01	595.30–1018.00
VEGF	21.93	11.70–42.91

BDL; below detection limit.

Table 3
Univariate correlation of functional scale scores with circulating cytokine levels.

	Physical functioning		Emotional functioning		Cognitive functioning	
	r	*p*- value	*r*	*p*- value	*r*	*p*- value
IL-1β	0.063	0.68	0.021	0.89	0.090	0.55
IL-1RA	−0.12	0.42	−0.12	0.42	0.077	0.61
IL-2*	0.17	0.26	−0.053	0.72	−0.038	0.80
IL-4	0.084	0.58	−0.0080	0.96	−0.036	0.81
IL-5	0.12	0.45	0.22	0.14	0.11	0.45
IL-6	−0.40	0.0064	−0.37	0.010	−0.45	0.0017
IL-7	−0.0040	0.98	0.094	0.53	−0.037	0.81
IL-8	−0.16	0.29	0.077	0.61	0.061	0.68
IL-9*	0.097	0.52	0.016	0.92	−0.047	0.76
IL-10	−0.11	0.48	−0.14	0.37	−0.16	0.27
IL-12p70	−0.11	0.47	−0.10	0.50	−0.24	0.11
IL-13	−0.083	0.58	−0.020	0.90	−0.16	0.29
IL-15	0.057	0.70	−0.056	0.71	0.0070	0.96
IL-17	−0.10	0.50	−0.064	0.67	−0.037	0.80
TNF-α	0.043	0.78	−0.11	0.47	−0.036	0.81
IFN−γ*	−0.020	0.89	−0.15	0.33	−0.23	0.13
bFGF	0.0050	0.97	−0.16	0.30	−0.17	0.25
PDGF-BB	−0.11	0.46	−0.25	0.095	−0.25	0.093
G-CSF	−0.24	0.11	−0.35	0.016	−0.31	0.035
GM-CSF*	−0.016	0.92	−0.085	0.57	−0.0092	0.54
IP-10	−0.25	0.099	0.12	0.43	0.0010	0.99
MCP-1	0.092	0.54	−0.047	0.75	0.039	0.79
MIP-1α	−0.0080	0.96	−0.11	0.44	−0.020	0.89
MIP-1β	−0.057	0.71	0.085	0.57	0.033	0.83
Eotaxin	0.28	0.060	0.021	0.89	0.093	0.54
RANTES	0.061	0.69	−0.13	0.39	−0.19	0.20
VEGF*	−0.36	0.015	−0.41	0.0039	−0.38	0.0080

r indicates the linear correlation coefficient. * Logarithmically transformed.

Oncology Group (ECOG) performance status was 0 or 1, and there were 13 with defective general status, whose ECOG performance status was 2 or more. Of the 50 patients, 48 had received one or more chemotherapy lines before registration. Median cytokine values and interquartile range of plasma samples are shown in Table 2. Seven cytokine assays (IL-1β, IL-2, IL-5, IL-7, TNF-α, bFGF and MIP-1α) yielded undetectable values for more than 25% of the samples.

Physical functioning

Univariate analysis showed plasma levels of IL-6 ($r=-0.397$, $P=0.0064$) and VEGF ($r=-0.360$, $P=0.015$) to have a significant negative correlation with physical functioning scales (Table 3). And these cytokine levels of cancer patients were significantly higher than those of 33 healthy volunteers (IL-6: mean 6.93 vs 2.19 ng/L, $P=0.012$, VEGF: mean 29.31 vs 18.72 ng/L, $P=0.047$). G-CSF ($r=-0.240$, $P=0.11$), IP-10 ($r=-0.246$, $P=0.099$) and eotaxin ($r=0.279$, $P=0.060$) levels remained non-significant at the 5% level (Table 3). The group of patients with lower physical functioning cale scores differed significantly from the group with higher scores on ECOG performance status ($P=0.0037$, Table 4). Stepwise multiple linear regression analyses using the candidate variables (i.e., IL-6, G-CSF, IP-10, eotaxin, VEGF and ECOG performance status) indicated that IL-6 and ECOG performance status were significant independent determinants of physical functioning (adjusted $R^2=0.641$, $P<0.0001$, Table 5A).

Table 4
Clinical data in patients with lower functioning scales and higher patients.

	Physical functioning		*p*-value	Emotional functioning		*p*-value	Cognitive functioning		*p*-value
	≤86.7	>86.7		≤83.3	83.3<		≤83.3	83.3<	
Sex (M/F)			0.58			0.055			0.18
M	14 (28.0)	12 (24.0)		14 (29.8)	10 (21.3)		11 (23.4)	13 (27.7)	
F	15 (30.0)	9 (18.0)		7 (14.9)	16 (34.0)		15 (31.9)	8 (17.0)	
ECOG performance status			0.0037			0.27			0.013
0–1	17 (34.0)	20 (40.0)		15 (31.9)	22 (46.8)		17 (36.2)	20 (42.6)	
2–4	12 (24.0)	1 (2.0)		6 (12.8)	4 (8.5)		9 (19.1)	1 (2.1)	
Disease site			0.48			0.92			0.75
Gastro-intestinal	10 (20.0)	4 (8.0)		6 (12.8)	7 (14.9)		8 (17.0)	5 (10.6)	
Pancreato-biliary	4 (8.0)	5 (10.0)		4 (8.5)	5 (10.6)		5 (10.6)	4 (8.5)	
Lung	5 (10.0)	2 (4.0)		3 (6.4)	3 (6.4)		3 (6.4)	3 (6.4)	
Uterine/ovarian	5 (10.0)	2 (4.0)		2 (4.3)	5 (10.6)		5 (10.6)	2 (4.3)	
Others	6 (12.0)	7 (14.0)		6 (12.8)	6 (12.8)		5 (10.6)	7 (14.9)	
Disease status			0.79			0.42			0.15
Advanced	22 (44.0)	14 (28.0)		16 (34.0)	17 (36.2)		16 (34.0)	17 (36.2)	
Reccurent	8 (16.0)	6 (12.0)		5 (10.6)	9 (19.1)		10 (21.3)	4 (8.5)	
Previous chemotherapy			0.82			0.53			0.068
0 or 1	13 (26.0)	8 (16)		10 (21.3)	10 (21.3)		8 (17.0)	12 (25.5)	
≥2	17 (34.0)	12 (24.0)		11 (23.4)	16 (34.0)		18 (38.3)	9 (19.1)	

Values are expressed as numbers (percentages).

Table 5A
Multiple stepwise regression analysis for determinants of physical functioning scales.

Variables	β	SE	*p*- value
IL-6	−0.238	0.214	0.0126
PS	−0.724	5.36	<0.0001
G-CSF	−0.236		NS
Eotaxin	0.154		NS
IP-10	−0.022		NS
VEGF	−0.114		NS

β indicates standardized regression coefficients. SE indicates standard errors. Adjusted R2 = 0.641, p<0.0001.

Emotional functioning

Significant negative correlation was found between plasma levels of IL-6 (r = −0.371, P = 0.010), G-CSF (r = −0.349, P = 0.016), VEGF (r = −0.410, P = 0.0039) and emotional functioning scale score in univariate analysis (Table 3). PDGF level (r = −0.247, P = 0.095) remained non-significant at the 5% level (Table 3). The group of patients with lower emotional functioning scale scores differed, though not significantly, from the group with higher scores on sex (P = 0.055, Table 4). Stepwise multiple linear regression analyses using the candidate variables (i.e., IL-5, IL-6, PDGF, G-CSF, VEGF and sex) indicated that VEGF was a significant independent determinant of emotional functioning (adjusted $R^2 = 0.153$, P = 0.039, Table 5B).

Cognitive functioning

Plasma levels of IL-6 (r = −0.447, P = 0.0017), G-CSF (r = −0.309, P = 0.035) and VEGF (r = −0.380, P = 0.008) were negatively correlated with cognitive functioning scale scores in univariate analysis (Table 3). PDGF level (r = −0.248, P = 0.093) remained non-significant at the 5% level (Table 3). The group of patients with lower cognitive functioning scale scores differed significantly from the group with higher scores on ECOG performance status (P = 0.013). Patients with lower cognitive functioning scale scores tended to have many previous chemotherapy lines (P = 0.068, Table 4). Stepwise multiple linear regression analyses using candidate variables (i.e., IL-6, IL-12, IFN-γ, PDGF, G-CSF, RANTES, VEGF, sex, ECOG performance status, disease status, and number of chemotherapy lines) indicated that IL-6 and number of chemotherapy lines were significant independent determinants of cognitive functioning (adjusted $R^2 = 0.272$, P = 0.004, Table 5C).

Discussion

Our results suggest that the circulating level of IL-6 affects physical and cognitive functions in patients with advanced cancer and that the circulating level of VEGF affects emotional function. Notably, multiple linear regression analysis in which variables such as other cytokines and ECOG performance status (known to be a negative determinant of QOL) are included showed that circulating levels of cytokines are independent determinants of each functional impairment.

Table 5B
Multiple stepwise regression analysis for determinants of emotional functioning scales.

Variables	β	SE	*p*- value
VEGF	−0.414	6.06	0.039
IL-5	0.232		NS
IL-6	−0.282		NS
PDGF-BB	−0.02		NS
G-CSF	−0.253		NS
Sex	0.181		NS

β indicates standardized regression coefficients. SE indicates standard errors. Adjusted R2 = 0.153, p = 0.039.

Table 5C
Multiple stepwise regression analysis for determinants of cognitive functioning scales.

Variables	β	SE	*p*- value
IL-6	−0.462	0.242	0.0006
Chemotherapy line	−0.323	5.044	0.0133
IL-12p70	−0.104		NS
IFN-γ	−0.2		NS
VEGF	−0.21		NS
PDGF-BB	−0.055		NS
G-CSF	−0.192		NS
RANTES	−0.091		NS
Sex	−0.191		NS
PS	−0.217		NS
Disease status	−0.262		NS

β indicates standardized regression coefficients. SE indicates standard errors. Adjusted R2 = 0.272, p = 0.0004.

Previous large, epidemiologic studies have demonstrated that high circulating levels of IL-6 are associated with functional disability of daily living [12–14]. Those patients with advanced ovarian cancer who had higher levels of IL-6 reported greater fatigue and worse physical and functional well-being [4]. Consistent with these previous reports, we found plasma IL-6 level to be a significant independent determinant of physical disability. It has been suggested that IL-6 may influence physical disability by stimulating muscle wasting [12,15].

Moreover, peripheral IL-6 is a likely mediator of cognitive disturbances, penetrating the blood–brain barrier directly through active transport mechanisms [16,17]. In addition to their direct effects on brain function, these cytokines also provoke a stress hormone cascade that can affect mood and cognition as well as having discrete effects on brain neurotransmitter systems [18]. Epidemiologic studies have demonstrated that peripheral IL-6 levels covary inversely with cognitive function in middle-aged to old subjects [19,20]. Numerous reports document an association between Alzheimer's disease and high levels of central and peripheral IL-6 [21,22]. Further support for a relationship between IL-6 and cognitive function comes from transgenic mice that overexpress brain IL-6 and show deficits in memory and learning [23]. Thus, the pathways of communication between the central nervous system and the periphery, including the circulatory system and peripheral nervous system, are well understood [16,17,24] and increased circulating levels of cytokines such as IL-6 are known to be associated with cancer [25–27]. However, we have been able to identify only one published clinical study that showed an association between increased levels of circulating cytokines and cognitive impairment. In that study, five cytokines (TNF-α, IL-6, IL-8, IL-1RA and IL-1β) were examined in patients with acute myeloid leukemia or myelodysplastic syndrome, and it was shown that higher IL-6 levels were associated with poorer executive function, whereas higher IL-8 levels were associated with better memory performance [2]. Here we present the first report to demonstrate, using multiple cytokine analysis, that peripheral IL-6 level is a significant independent determinant of cognitive functioning scales in cancer patients. In this study, ≤2 lines of chemotherapy were another significant independent determinant of cognitive function, so it appears that chemotherapy also has adverse effects on cognitive function.

There is plenty of evidence concerning the involvement of cytokines in emotional function [28,29,3]. For example, cancer patients who are clinically depressed have significantly higher levels of circulating IL-6 than non-depressed patients or healthy controls [3]. In this study, a significant negative correlation was observed between emotional functioning scores and plasma levels of IL-6, G-CSF and

VEGF in univariate linear regression analysis. Multivariate analysis showed only plasma VEGF level to be a significant independent determinant of emotional functioning. Consistent with our results, emotional functions in patients with ovarian and colorectal cancer have been shown to correlate with serum VEGF levels [30,31]. Lutgendorf et al. reported that higher levels of helplessness and worthlessness were significantly associated with higher VEGF levels in patients with ovarian cancer [30]. Sharma et al. showed a significant positive correlation between VEGF levels and depression, and found that VEGF levels were negatively correlated with cancer-related concerns and positive affectivity [31]. The mechanisms of the relationship between VEGF and emotional functions remain unknown. Given that high levels of VEGF in patients with greater tumor burden might result in impairment of emotional functions via uncertain mechanisms, VEGF is a potential treatment target for cancer-related impairment of emotional functions.

Contrary to previous studies [32–34], cytokines such as IL-1β and TNF-α did not have a relation with cancer-related impairment in this study. Although IL-1β or TNF-α have been proposed as markers in the fatigue pathway, neither correlated with fatigue in several studies of cancer-related fatigue [2,35]. The differences in these studies may result, in part, not only from heterogeneity of study populations, but also from a difference in methods for measuring cytokines and/or evaluating cancer-related symptoms.

Conclusion

In conclusion, we have revealed the relationship between multiple circulating cytokines and functional impairment in patients with cancer, and demonstrated that levels of circulating IL-6 and VEGF can be biologic markers of cancer-related functional impairment. Identification of physiologic mechanisms for this impairment will allow the development of interventions that target cytokines and their receptors, highly promising candidates for improving functioning of patients. Multivariate descriptive analysis with larger sample sizes would identify patterns of symptoms and help to clarify the biological mechanisms for cancer-related functional impairment, as would basic scientific studies using animal models.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Diffusion-weighted Magnetic Resonance Imaging for Detecting Lymph Node Metastasis of Rectal Cancer

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Abstract
Background Preoperative diagnosis of lymph node metastasis is important in determining the optimal therapy for rectal cancer. It has been shown that diffusion-weighted magnetic resonance imaging (DWI) is a useful tool for detecting malignant tumors.
Methods One hundred twenty-nine consecutive patients with rectal cancer were examined with DWI + conventional (T1-weighted and T2-weighted) MRI and computed tomography (CT). All 129 patients underwent rectal resection with total mesorectal excision. Findings on DWI + conventional MRI and CT were compared with those from histopathologic examinations.
Results Fifty-nine (46%) patients had metastatic lymph nodes on histopathologic examinations. Two hundred twenty (18%) of 1,250 lymph nodes were pathologically positive for tumor metastasis. The overall patient-based sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of DWI + conventional MRI were 93, 81, 81, 93, and 87%, respectively. Corresponding values of CT were 73, 79, 74, 77, and 76%, respectively. The overall node-based sensitivity, specificity, PPV, NPV, and accuracy of DWI + conventional MRI were 97, 81, 52, 99, and 84%, respectively. Corresponding values of CT were 86, 80, 48, 96, and 81%, respectively.
Conclusion DWI + conventional MRI is effective for the detection of lymph node metastasis and useful for selection of the optimal therapy for rectal cancer.

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Introduction

Depth of invasion in the rectal wall and lymph node involvement have been shown to be major prognostic factors in determining the risk of recurrence in rectal cancer. If these prognostic factors can be assessed accurately, an optimal therapeutic strategy, such as transanal local excision or rectal resection with total mesorectal excision (TME) with or without preoperative chemoradiotherapy, can be selected.

Concerning tumor (T) staging of rectal cancer, the accuracy of diagnosis obtained by computed tomography (CT) or magnetic resonance imaging (MRI) is 65–75% and 55–85%, respectively [1]. In particular, MRI is useful for identifying the extent of rectal cancer that extends beyond the muscularis propria [2], and its findings can predict the circumferential resection margin (CRM) status with 92% agreement [1]. On the other hand, concerning lymph node (N) staging, the accuracy of diagnosis on CT and MRI is 55–65% and 47–89.8%, respectively [1]. It is sometimes difficult to accurately diagnose lymph node involvement because micrometastasis often occurs in normal-sized lymph nodes [3–6].

Diffusion-weighted imaging (DWI) is a new MRI technique and it has been in wide use in the acute stage of cerebral infarction [7–9]. Improvements of MRI techniques, such as echo planar imaging (EPI) and parallel imaging, make it possible to shorten the total image

acquisition time and to reduce specific artifacts. These advancements in MRI techniques enable DWI to be applied to the trunk region of the body; for example, several studies have reported that DWI is useful in the detection of primary rectal cancer [10, 11]. Sumi et al. [12] have reported that DWI is useful to discriminate between metastatic and benign nodes in patients with head and neck cancers; however, the efficacy of DWI on diagnosing lymph node metastasis in rectal cancer has not been examined. As reported by Beets-Tan et al. [2] and others in detail, the accuracy of T staging by conventional MRI has been quite high, because conventional MRI can visualize the layers of the rectal wall and mesorectal fascia at high spatial resolution; however, DWI has no advantage over T staging because the spatial resolution of DWI is poor. Therefore, we focused on studying the accuracy of lymph node staging by DWI + conventional MRI. To our knowledge, this is the first report demonstrating the efficacy of DWI for detecting nodal metastasis in patients with rectal cancer.

Patients and methods

Patients

A total of 129 patients, who were pathologically diagnosed with rectal cancer at Kitano Hospital between January 2004 and June 2010, were enrolled in this study. All patients were examined preoperatively by both enhanced CT and DWI + conventional (T1-weighted and T2-weighted) MRI. No bowel preparation was performed before examination. Sixty patients diagnosed preoperatively with tumor invasion of T3 or T4 or as node positive (N1 or N2) were treated with preoperative long-course chemoradiation consisting of 40-Gy irradiation delivered in fractions of 2 Gy and chemotherapy with 370 mg/m^2 UFT®, an oral 5-FU prodrug. Surgery was performed 4–6 weeks after completing the chemoradiotherapy. Patients who received preoperative chemoradiation were re-examined by CT and DWI + conventional MRI before surgery. More than half of the patients (33 of 60) showed apparent radiological downstaging after neoadjuvant therapy. Because we could not directly compare the radiological diagnosis with the pathological findings before neoadjuvant therapy, we focused on the accuracy of radiological images after neoadjuvant therapy in patients who received neoadjuvant therapy. The other 69 patients underwent surgery without preoperative chemoradiation.

MRI protocol

MRI was performed using a 1.5T system (Philips Medical, Best and Heeren, The Netherlands). Abdominal and pelvic examination consisted of T1-weighted and T2-weighted MRI and DWI, which could be performed in one session. All studies were performed with a four-channel body surface coil using the SENSE parallel imaging protocol (sense reduction factor, 1.5–2.0) under free breathing. Sagittal T1-weighted images (turbospin echo; repetition time/echo time [TR/TE] 500/7.2–10; average 6; bandwidth about 225 Hz) were obtained with a slice thickness of 6 mm, section gap 1–5 mm, field of view (FOV) 250 mm, matrix 256 × 256, and scan time of about 2 min 45 s. Sagittal and axial T2-weighted images (turbospin echo; TR/TE 3672/120; average 6; bandwidth about 200 Hz) were obtained with a slice thickness of 6 mm, section gap 1–5 mm, FOV 250 mm, matrix 288 × 288, and scan time of about 2 min 25 s. DWI was performed in the axial plane using the echo planar imaging (EPI) sequence with the following parameters: TR/TE/inversion time (TI) 6000–6500/60–67/180, FOV 400 mm, acquisition matrix 128 × 128, reconstruction matrix 256 × 256 (with zero-fill interpolation), 5-mm slice thickness, no section gap, and average 6. DWI trace images were acquired with motion-probing gradient (MPG) pulses applied in three directions with b values of 1000. Acquisition time was 2.5–3.5 min. Sagittal and axial parallel MIP reconstruction was performed using the same slice thickness as the center gap of T1- and T2-weighted images for correlative observation. Antispasmodics and cathartic agents were not administered before scanning.

CT imaging protocol

Helical CT was performed (Asteion: four-row multidetector system; Toshiba Medical, Tokyo, Japan) with a maximum continuous scanning time of 20 s. The scanned region included the area from the level of the diaphragm to the pelvic floor. A total of 80–100 ml nonionic contrast medium was administered intravenously with an automatic injector at a flow rate of 1.5–2 ml/s. In all cases, the same scan protocol (4-mm section collimation, helical pitch 5.5 at 120 kV/260 mA electron voltage/current) was employed. Scanning was started 75 s after the initiation of intravenous administration of contrast medium.

Evaluation of DWI and CT images

Two radiologists (SK and RO) evaluated DWI + conventional MRI and CT images independently before surgery. In general, the following criteria were used to detect lymph node involvement. When soft tissue nodules with high signal intensity were detected on DWI and their existence was confirmed on T1-weighted and T2-weighted conventional MRI, they were considered to be positive for lymph node metastasis. When soft tissue nodules were greater than 1 cm or abnormally clustered on CT, they were

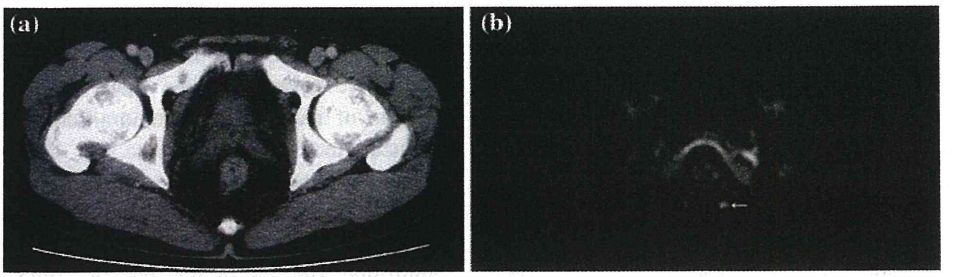

Fig. 1 CT and DWI in a 64-year-old male patient with rectal cancer and lymph node metastasis. **a** CT showed the rectal wall thickness, but no findings of lymph node metastasis. **b** DWI clearly showed a high-intensity nodule (*arrow*) that highly suggested lymph node metastasis. On histopathologic examination, this lymph node was diagnosed as positive for metastasis

considered to be positive for lymph node metastasis. The radiologists were blinded to all clinical information, including the timing of the images and pathological findings. They judged the N stage solely from radiological images. Figure 1 shows an example of lymph node metastasis, which was correctly diagnosed by DWI + conventional MRI but not by CT.

Results

Table 1 gives patient and tumor characteristics. The median age of the group was 66 years old (range = 36–89). The surgical procedures were low anterior resection in 92 patients, abdominoperineal resection in 26, and Hartmann's procedure in 11. The location of tumors was within 5 cm from the anal verge in 60 patients, 5.1–10 cm in 32, and more than 10 cm in 37. On histopathologic examination, 127 and 2 patients were diagnosed with moderately/well-differentiated adenocarcinomas and mucinous carcinoma, respectively. According to the TNM classification, the study group included 32 patients with stage I, 37 patients with stage II, 47 patients with stage III, and 13 patients with stage IV.

Fifty-nine (46%) patients had metastatic lymph nodes on histopathologic examination. As shown in Table 2, the overall patient-based sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of DWI + conventional MRI were 93% (55 of 59), 81% (57 of 70), 81% (55 of 68), 93% (57 of 61), and 87% (112 of 129), respectively. Corresponding values of CT were 73% (43 of 59), 79% (55 of 70), 74% (43 of 58), 77% (55 of 71), and 76% (98 of 129), respectively.

Of the 1,250 lymph nodes sampled from 129 patients, 220 nodes (18%) in 59 patients (46%) were positive for metastasis. A total of 197 lymph nodes (16%) that were false-positive on DWI + conventional MRI revealed nonspecific reactive changes on histopathologic examination. As shown in Table 3, the overall node-based sensitivity, specificity, PPV, NPV, and accuracy of DWI + conventional MRI were 97% (214 of 220), 81% (833 of 1030), 52% (214 of 411), 99% (833 of 839), and 84% (1047 of 1250), respectively. Corresponding values of CT were 86% (190 of 220), 80% (823 of 1030), 48% (190 of 397), 96% (823 of 853), and 81% (1013 of 1250), respectively.

Table 1 Clinical and pathologic characteristics

Age (years)	
Median	66
Range	36–89
Sex	
Male	88
Female	41
Type of resection	
Low anterior resection	92
Abdominoperineal excision	26
Hartmann's procedure	11
Therapy	
CRT + TME	60
TME alone	69
Distance of tumor from anal verge	
<5 cm	60
5.1–10 cm	32
>10 cm	37
Tumor differentiation	
Moderately/well	127
Poorly	0
Mucinous	2
Node stage (pN)	
pN0	70
pN1–2	59
TNM stage	
I	32
II	37
III	47
IV	13

CRT chemoradiotherapy, *TME* total mesorectal excision, *pN* pathological N stage

Table 2 Detectability of lymph node metastasis on DWI and CT (patient-based analysis)

	Histopathologic diagnosis		
	Positive	Negative	Total
DWI[a]			
Positive	55	13	68
Negative	4	57	61
Total	59	70	129
CT[b]			
Positive	43	15	58
Negative	16	55	71
Total	59	70	129

[a] Sensitivity = 55/59 (93%), specificity = 57/70 (81%), positive predictive value (PPV) = 55/68 (81%), negative predictive value (NPV) = 57/61 (93%), accuracy = 112/129 (87%)

[b] Sensitivity = 43/59 (73%), specificity = 55/70 (79%), positive predictive value (PPV) = 43/58 (74%), negative predictive value (NPV) = 55/71 (77%), accuracy = 98/129 (76%)

Table 3 Detectability of lymph node metastasis on DWI and CT (lymph node-based analysis)

	Histopathologic diagnosis		
	Positive	Negative	Total
DWI[a]			
Positive	214	197	411
Negative	6	833	839
Total	220	1030	1250
CT[b]			
Positive	190	207	397
Negative	30	823	853
Total	220	1030	1250

[a] Sensitivity = 214/220 (97%), specificity = 833/1030 (81%), positive predictive value (PPV) = 214/411 (52%), negative predictive value (NPV) = 833/839 (99%), accuracy = 1047/1250 (84%)

[b] Sensitivity = 190/220 (86%), specificity = 823/1030 (80%), positive predictive value (PPV) = 190/397 (48%), negative predictive value (NPV) = 823/853 (96%), accuracy = 1013/1250 (81%)

Discussion

Rectal cancer is a common tumor in Western countries. Early-stage localized diseases can be cured by rectal resection with TME [13, 14]; however, advanced diseases often show local recurrence and/or distant metastases after surgery. Not only the depth or extent of tumor invasion in the rectal wall but also lymph node involvement has been shown to be a major prognostic factor in determining the risk of recurrence; for example, a large Dutch trial demonstrated that nodal disease is a prognostic indicator of local recurrence as well as distant metastases [15]. Patients with stage III (TxN1) disease had a tenfold higher risk for local recurrence than those with stage I (T1-2N0) and a threefold higher risk than those with stage II (T3N0). Recently, several studies have reported the beneficial effects of neoadjuvant chemoradiotherapy in terms of a reduced local recurrence rate [16, 17], and neoadjuvant chemoradiotherapy before rectal resection with TME has been widely accepted as the standard treatment for locally advanced rectal cancer. For example, a recent randomized controlled trial has shown that the combination of neoadjuvant chemoradiotherapy and surgery can reduce local recurrence to 5.6% at 5 years compared to 10.9% with surgery alone [18]. Therefore, it is important to accurately diagnose the major prognostic factors, extent or depth of tumor invasion and lymph node involvement, to select the optimal treatment strategy.

Conventional MRI has been reported to show accuracy and reproducibility for predicting the extent or depth of tumor invasion, because it can visualize the layers of the rectal wall and the mesorectal fascia at high spatial resolution [2]. In contrast, it is sometimes difficult to correctly detect lymph node involvement by conventional MRI and CT. The present study demonstrated that the detectability of lymph node metastasis with DWI + conventional MRI was superior to that with CT. CT is useful in identifying lung or liver metastatic diseases, but it is not reliable for T and N staging in rectal cancer. Because the pelvic region is crowded with the gastrointestinal tract and blood vessels, the contrast between lymph nodes and normal tissues is usually low on CT. In particular, lateral nodes, which are important in rectal cancer treatment, are often difficult to detect on CT. In contrast, DWI has high detectability for lymph node metastasis of rectal cancer because it can depict metastatic lymph nodes, but not normal mesorectal tissues, as hyperintense nodules. Although it has not been clearly elucidated why intensity on DWI differs between tumors and normal tissues, it is likely that the cellularity of the tumor, which is higher than that of the surrounding normal tissues, is a possible reason [19]. Bipat et al. [20] reported in their meta-analysis of literature for a 16-year period that the sensitivity estimates for lymph node involvement of endoluminal ultrasonography (EUS), CT, and MRI were 67, 55, and 66%, respectively. The specificity values were 78% for EUS, 74% for CT, and 76% for MRI. Compared with these results, the accuracy of DWI + conventional MRI estimated in the current study was superior, whereas that of CT was similar. Because EUS is not commonly used for rectal cancer staging in Japan, we cannot demonstrate the comparison with EUS in this study.

The advantage of DWI + conventional MRI is its high sensitivity to detect lymph node metastasis. Indeed, we could appropriately identify patients with metastatic lymph nodes, who were expected to benefit from neoadjuvant

chemoradiotherapy before curative surgery, with high frequency (93%). However, DWI + conventional MRI also detected nonspecific reactive swelling of lymph nodes and exhibited false-positive results in 197 lymph nodes (16%) of 13 patients (10%) who might not need neoadjuvant chemoradiotherapy. Further studies, such as refining MRI techniques or combining with other modalities, will be required to minimize false-positive results. Because the NPV of DWI + conventional MRI is high (93% in patient-based analysis and 99% in lymph node-based analysis), patients with tumor invasion of T1 or T2, who are judged to be negative for lymph node metastasis on DWI + conventional MRI, can be treated by surgery alone without neoadjuvant chemoradiotherapy.

Recently, ^{18}F-fluorodeoxyglucose (FDG)–positron emission tomography (PET) and CT (PET/CT) have been used for staging primary rectal cancer. Gearhart et al. [21] have reported that FDG–PET/CT improved the staging accuracy, especially in low rectal cancers. Because our data on FDG–PET/CT in primary rectal cancers are limited, it is difficult to directly compare the accuracy of FDG–PET/CT with that of DWI + conventional MRI, but it is probable that the combination of FDG–PET/CT with DWI + conventional MRI will increase the accuracy of diagnosis in rectal cancer. DWI + conventional MRI has some advantages over FDG–PET/CT; for example, new facilities are not required because existing MRI facilities are suitable for DWI. In addition, patients have no risk of radiation exposure with DWI + conventional MRI. Therefore, DWI + conventional MRI with the enhanced ability to detect metastatic lymph nodes may replace FDG–PET/CT.

Our results suggest that DWI + conventional MRI is better than the currently available modalities, such as CT, in determining whether patients should have preoperative chemoradiation. There is a possible benefit of this new modality for selecting the optimal treatment for rectal cancer patients.

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ORIGINAL ARTICLE

Phase II trial of combined treatment consisting of preoperative S-1 plus cisplatin followed by gastrectomy and postoperative S-1 for stage IV gastric cancer

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Abstract
Background To improve the poor prognosis in patients with stage IV (StIV) gastric cancer (GC), we conducted a multicenter phase II study of preoperative S-1 plus cisplatin followed by gastrectomy and postoperative S-1 for StIV GC (the protocol is registered at the clinical trial site of the National Cancer Institute; KYUH-UHA-GC03-01, NCT00088816).
Methods Eligibility criteria included histologically proven StIVGC. Patients received S-1 (80 mg/m^2/day, days 1–21) plus cisplatin (60 mg/m^2 on day 8) for 2 courses. After preoperative chemotherapy (CTx), radical gastrectomy was performed. Postoperative S-1 (80 mg/m^2/day, days 1–14) was administered every 3 weeks for 1 year.
Results Fifty-one patients were enrolled and all patients were followed for more than 2 years. The 2-year overall survival and progression-free survival rates were 43.1% (95% confidence interval [CI] 29.4–56.1%) and 33.3% (95% CI 20.9–46.2%), respectively. Preoperative chemotherapy was accomplished in 44 patients (86.3%). These 44 patients underwent surgery and R0 resection was achieved in 26. The rate of R0 resection for GC with a single StIV factor ($n = 24$) was 79.2% and that for GC with multiple StIV factors ($n = 27$) was 25.9%. All patients with cancer cells in peritoneal washings (cytology [Cy] 1) alone ($n = 12$) became Cy0 after preoperative chemotherapy. Postoperative chemotherapy was completed in 11 patients, including 8 with Cy1 alone. No treatment-related death was recorded. Recurrences were observed in 14 patients after R0 resection. The most frequent recurrence site was the peritoneum. Patients who underwent R0 resection and those with Cy1 alone had a better survival.
Conclusions This perioperative treatment was safe and feasible for StIVGC but failed to show a survival benefit. In patients with StIVGC with Cy1 alone this treatment resulted in a better prognosis.

Keywords Gastric cancer · Induction chemotherapy · Surgery · S-1 plus cisplatin

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Introduction

Gastric cancer (GC) is one of the leading causes of cancer death worldwide [1]. It is often diagnosed at an advanced stage. The prognosis of stage IV GC is poor (e.g., the 1-, 2-, and 5-year survival rates of stage IV GC are approximately 40, 20, and <10%, respectively [2, 3]), even if patients are treated surgically.

Recently, several novel regimens of combined chemotherapy, including S-1 plus cisplatin, have produced improved overall survival (OS) rates in patients with unresectable stage IV GC [4–6]. The median survival time in patients treated with S-1 plus cisplatin was 13.0 months, and the response rate with this regimen was 54%. But its

2-year OS rate, 23.6% (95% confidence interval [CI] 16.8–30.4), was still dismal [6].

Combinations of some highly effective chemotherapeutic regimens and surgery have given favorable results in resectable, locally advanced GC [7–11]. Preoperative chemotherapy for advanced GC possesses theoretical benefits. First, this chemotherapy usually shrinks cancerous tissue, increasing the likelihood of R0 resection with extended surgery. Second, more intensive chemotherapy than that used postoperatively is possible, with a higher compliance rate. Third, distant occult metastases can be treated before local therapy has begun. Furthermore, postoperative S-1 alone has been proven beneficial for treating stage II and stage III GC [12]. Hence, one of the most potentially favorable multimodal treatments for stage IV GC would be a combination of preoperative administration of S-1 plus cisplatin, subsequent gastrectomy with D2 nodal dissection, and postoperative S-1 administration. The present study was conducted to evaluate the efficacy, feasibility, and safety of this novel multimodal treatment for stage IV GC.

Patients and methods

This was a prospective multicenter phase II study conducted at eight centers of the Kyoto University Surgical Oncology Group in Japan between May 2003 and March 2008. This protocol is registered at the clinical trial site of the National Cancer Institute (KYUH-UHA-GC03-01, NCT00088816).

Patients

Eligibility criteria included: (1) pretreatment (pre-) histologically proven stage IV GC (pre-N3, pre-T4N2, pre-H1, pre-P1, pre-Cy1, pre-M1) diagnosed by pretreatment helical computed tomography (CT) and staging laparoscopy within 4 weeks before registration, (2) no prior therapy, (3) Eastern Clinical Oncology Group (ECOG) performance status of 0 or 1, and (4) age of 20–80 years. Patients must have had no signs of organ failure, including that of bone marrow, heart, lungs, and kidneys. Acceptable hematologic (white blood cell count $\geq$4,000/μL and $\leq$12,000/μL, neutrophil count $\geq$2,000/μL, platelet count $\geq$100,000/μL, hemoglobin $\geq$9.0 g/μL), renal (creatinine clearance $\geq$ 50 mL/min), hepatic (aspartate aminotransferase and alanine aminotransferase $\leq$2.5 times the upper limit at each institution, serum total bilirubin $\leq$1.5 mg/dL), and respiratory function (arterial partial pressure of oxygen [PaO_2] $\geq$ 70 torr in room air) was required for enrollment in the study.

Exclusion criteria included active gastrointestinal bleeding, infection, diarrhea, simultaneous double cancer, pregnancy, interstitial pneumonia, bowel obstruction, past history of myocardial infarction, massive ascites, severe drug allergy, or severe diabetes. The protocol was approved by the ethics review committees of all participating centers, and all patients gave written informed consent. All patients were registered centrally at the Translational Research Informatics Center, Kobe, Japan, where data management and statistical analysis were performed.

Preoperative chemotherapy

Preoperative chemotherapy was administered for two cycles. Each cycle consisted of daily oral administration of S-1 for 21 days (80 mg/m^2/day, days 1–21) and intravenous cisplatin (60 mg/m^2), with hydration, on day 8; followed by a 2-week recovery period [6]. The toxicity of the induction chemotherapy was assessed on the basis of the National Cancer Institute's common toxicity criteria version 2.0 (NCI-CTC) [13]. If patients had grade 3/4 hematologic or nonhematologic toxicity, including grade 2 or above diarrhea, liver dysfunction, stomatitis, and other toxicities of grade 3 or above, chemotherapy was postponed until recovery. If recovery did not occur within 4 weeks, the chemotherapy was stopped.

Some 7–13 days after each course, resectability and clinical response were evaluated mainly on the basis of CT findings. If curative resection was possible after the second course, the patient underwent surgery 2–4 weeks after completion of the chemotherapy. If patients experienced disease progression or severe adverse events, the protocol was discontinued. Treatment after the discontinuation of preoperative chemotherapy was at the physician's discretion.

Surgery

The surgical criteria included possibility of R0 resection, no evidence of infection, no signs of organ failure, and a neutrophil count of more than 1,500/μL. After laparotomy, resectability was again evaluated. The standard surgical procedure was gastrectomy with D2 nodal dissection. The extent of gastrectomy (total or subtotal) depended on the site and size of the primary tumor. For R0 resection, paraaortic nodal dissection (D3), splenectomy and/or distal pancreatectomy, or partial hepatectomy was attempted if cytologic findings were negative. Palliative gastrectomy was attempted in noncurative cases if the tumor was symptomatic and resectable.

Postoperative chemotherapy

Postoperative S-1 (80 mg/m^2/day, days 1–14) was administered every 3 weeks for 1 year. Eligibility criteria for postoperative chemotherapy included resection of the primary lesion and no definite disease progression at surgery. If gastrectomy was not curative, cessation of the protocol was at the physicians' discretion. Other eligibility criteria were the same as those for preoperative chemotherapy. For 1 year, S-1 was repeatedly administered in a 2-week cycle, followed by 1 week of rest. Criteria for discontinuation and cessation of the protocol were the same as those for preoperative chemotherapy. If recurrence or progressive disease was confirmed, the protocol was stopped. After completion of the protocol without recurrence, no further treatment was administered until tumor recurrence was identified. Treatment for recurrence or progressive disease was at the physicians' discretion.

Endpoints and evaluation

The primary endpoint was the 2-year OS rate, because the prognosis of stage IV GC is poor. The secondary endpoints were the progression-free survival (PFS) rate, objective response rate, pathologic response rate, R0 resection rate, surgical complications, the first recurrence sites, and toxic reactions. R0 resection was defined as pathologically confirmed complete resection of the cancerous lesions with negative peritoneal washing cytology.

The pretreatment stage at registration (pre-stage) was diagnosed according to the Japanese Gastric Cancer Association (JGCA) staging system (13th edition) [14] on the basis of CT and staging laparoscopic findings. If the short-axis diameter of the lymph nodes was greater than 10 mm on a CT scan, the lymph nodes were diagnosed as "metastatic" [15, 16]. The anatomic location of metastatic lymph nodes was also identified on CT scans. According to the JGCA staging system, N stage was defined by the anatomic location of the metastatic lymph nodes. At staging laparoscopy, T stage was diagnosed as pre-T3 (tumor penetration of serosa; SE) if the tumor was definitely exposed on the serosal surface. If cancerous changes on the serosal surface were considered minimal at staging laparoscopy, the T stage was diagnosed as pre-T2 (tumor invasion of muscularis propria; MP, tumor invasion of subserosa; SS). The diagnosis of pre-T4 (tumor invasion of adjacent structures; SI) was assessed by CT and laparoscopic findings. Cytology and peritoneal tumor dissemination were also assessed at pretreatment staging laparoscopy [17–20] and at surgery. The cells collected by peritoneal washing were examined cytopathologically using conventional Papanicolaou and Giemsa staining. When definite cancer cells or clusters were identified, patients were diagnosed as pre-Cy1, according to the *Japanese classification of gastric carcinoma* of the JGCA [14]. Objective tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 on the basis of the CT findings [21]. Postoperative final tumor status (post-stage) was diagnosed by comprehensive findings based on clinical, surgical, and pathological findings according to the JGCA classification [14]. Surgical specimens were evaluated pathologically and graded according to the proportion of tumor affected by degeneration or necrosis [11]: grade 0, no part of tumor affected; grade 1a, less than one-third affected; grade 1b, between one-third and two-thirds affected; grade 2, between two-thirds and the entire tumor affected; and grade 3, no residual tumor. The toxicity of preoperative and postoperative chemotherapy was assessed on the basis of NCI-CTC version 2, as described earlier.

Statistical analyses

All data from eligible patients were based on the intention-to-treat principle. This study was designed as a phase II trial with a single stage of accrual. The threshold 2-year survival probability was estimated to be 35%, determined by examining historical data from the Department of Gastroenterological Surgery, Kyoto University, that were collected from 1996 to 2002 ($n = 43$). The expected 2-year survival rate for this therapy was estimated to be 55%. Assuming that two-sided type I errors were 5% and that the power was 80%, the required sample size was calculated to be 46. The planned sample size was set at 50, with the consideration of approximately 5% of patients being ineligible. OS and PFS were estimated using the Kaplan–Meier

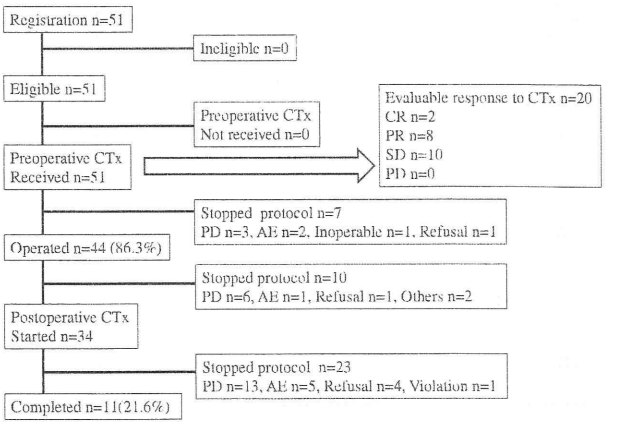

Fig. 1 Flow diagram of 51 eligible patients. *CTx* chemotherapy, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *AE* adverse effects

Table 1 Characteristics of patients and tumor status before and after treatment

	n	%
Age, years, median (range)	63 (35–79)	
Gender		
Male	29	56.9
Female	22	43.1
ECOG performance status		
0	44	86.3
1	7	13.7
Histology		
Intestinal	13	25.5
Diffuse	38	74.5
	Pre- ($n = 51$)	Post- ($n = 44$)
Stage		
IA	0	2
IB	0	6
II	0	5
IIIA	0	3
IIIB	0	3
IV	51	25
T stage		
T1 (M, SM)	0	1
T2 (MP, SS)	7	18
T3 (SE)	38	21
T4 (SI)	6	2
TX	0	2
N stage		
N0	13	11
N1	15	13
N2	10	11
N3	11	7
NX	2	2
Peritoneal cytology		
Cy0	15	32
Cy1	36	10
CyX	0	2
Peritoneal metastasis		
P0	26	29
P1	25	15
Liver metastasis		
H0	46	40
H1	5	4
Distant metastasis		
M0	46	43
M1	5	1
Pathological response (primary lesion)		
Grade 0		6
Grade 1a		15
Grade 1b		6
Grade 2		14
Grade 3		0
Not resected		3

ECOG Eastern Cooperative Oncology Group, *Pre-* pretreatment tumor status at registration, *Post*-postoperative final tumor status diagnosed by comprehensive findings based on clinical, surgical and pathological findings according to the Japanese Gastric Cancer Association (JGCA) classification [14], *M* tumor invasion of mucosa and/or muscularis mucosa, *SM* tumor invasion of submucosa, *MP* tumor invasion of muscularis propria, *SS* tumor invasion of subserosa, *SE* tumor penetration of serosa, *SI* tumor invasion of adjacent structures

method. All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

Between May 2003 and March 2008, 51 patients with stage IV GC were enrolled in this study and underwent preoperative chemotherapy. Disposition of the enrolled patients is shown in Fig. 1. No patients were ineligible, and all were observed for more than 2 years after registration. Table 1 shows the characteristics and tumor status of the 51 eligible patients. The median follow-up period of all patients was 19.2 months (range 4.5–81.5). Patients with a single pre-stage IV factor ($n = 24$) included 12 cases of pre-Cy1, 5 cases of pre-P1, 5 cases of pre-N3, and 2 cases of pre-H1. Simultaneous multiple pre-stage IV factors were observed in the remaining 27 patients. Patients with multiple pre-stage IV factors ($n = 27$) included 1 case of pre-(Cy1, P1, N3, M1), 1 case of pre-(Cy1, P1, N3), 2 cases of pre-(Cy1, P1, T4N2), 1 case of pre-(Cy1, P1, H1), 1 case of pre-(Cy1, H1, M1), 15 cases of pre-(Cy1, P1), 1 case of pre-(Cy1, N3), 1 case of pre-(Cy1, T4N2), 1 case of pre-(Cy1, H1), and 3 cases of pre-(N3, M1).

Survival

The 2-year OS and PFS rates were 43.1% (95% CI 29.4–56.1) and 33.3% (95% CI 20.9–46.2), respectively (Fig. 2a, b). The median OS and PFS were 19.2 and 9.3 months, respectively. There were significant differences in OS in favor of both GC with R0 resection ($p < 0.0001$) and GC with pre-Cy1 alone without any other stage IV factors ($p = 0.0041$), as shown in Fig. 2c, d. The 2-year OS of GC with pre-Cy1 alone was 75.0% (95% CI 40.8–91.2) and that of others was 33.3% (95% CI 19.3–48.0).

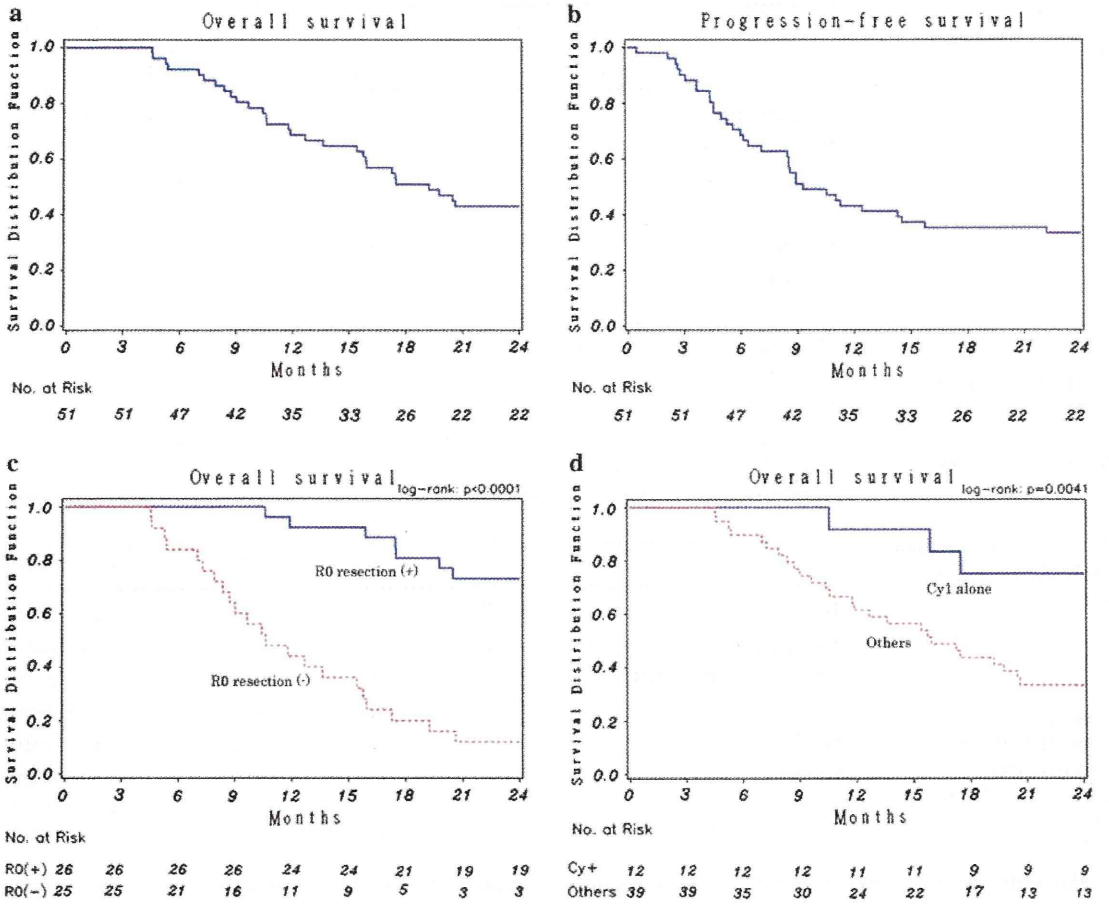

Fig. 2 Kaplan-Meier survival curves for the 51 eligible patients. **a** Overall survival, **b** progression-free survival. **c** Overall survival in patients grouped according to R0 resection or not. **d** Overall survival in patients grouped according to pretreatment Cy1 alone and others

Objective response to preoperative chemotherapy and adverse events

Measurable lesions were confirmed in 20 patients. The objective response rate for these lesions, according to the RECIST, was 50.0% (95% CI 27.2–72.8). Preoperative chemotherapy was completed in 44 patients (86.3%). Reasons for stopping treatment are noted in Fig. 1.

Toxic reactions of grade 3 or above are listed in Table 2. Severe adverse events occurring during preoperative chemotherapy were noted in two patients (3.9%). Grade 4 toxic reactions of leukocytopenia, neutropenia, and anemia were observed in one patient. Cardiopulmonary arrest after diarrhea was observed in another patient, who was resuscitated successfully.

Surgical outcome

Surgery was performed in 44 patients, including total gastrectomy in 30, distal gastrectomy in 11, simple laparotomy in two, and gastrojejunostomy in one patient. The median (range) blood loss and surgery duration were 669 (0–3,228) mL and 286 (50–447) min, respectively. Extended nodal dissection was performed in 36 patients (D2, 32; D3, 4). Palliative gastrectomy with D0/D1 was performed in 5 patients to maintain oral intake. Combined resection was performed in 35 patients, including additional resection of 62 organs; namely, the spleen ($n = 25$), gallbladder ($n = 21$), distal pancreas ($n = 7$), colon ($n = 3$), liver ($n = 2$), mesocolon ($n = 2$), diaphragm ($n = 1$), and adrenal gland ($n = 1$). The surgical complication rate was 18.2%, including three cases of pancreatic fistula and abdominal abscess, and one case of anastomotic leakage.

The postoperative final tumor status (post-stage) of the 44 patients who underwent surgery was determined with comprehensive findings based on clinical, surgical, and pathologic findings according to the JGCA classification (Table 1).

R0 resection rate

According to the pretreatment evaluation, 31 patients were diagnosed as "unresectable". After induction chemotherapy, 44 patients were diagnosed as "resectable" by CT and