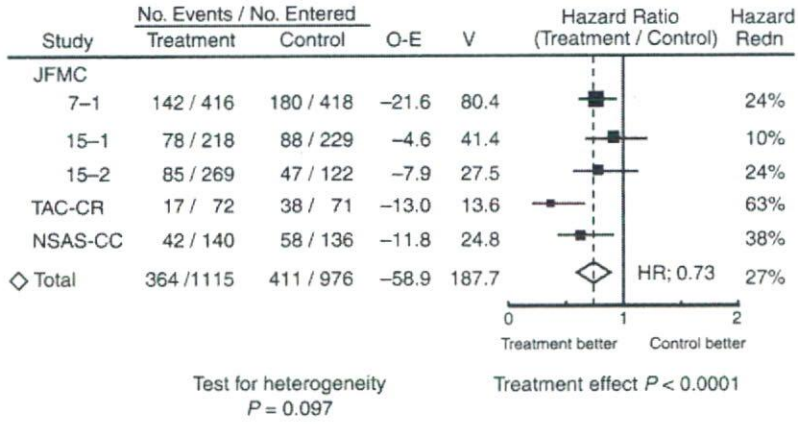

Figure 4 Disease-free survival hazard ratios by individual trial (Abbreviations as in Figure 1).

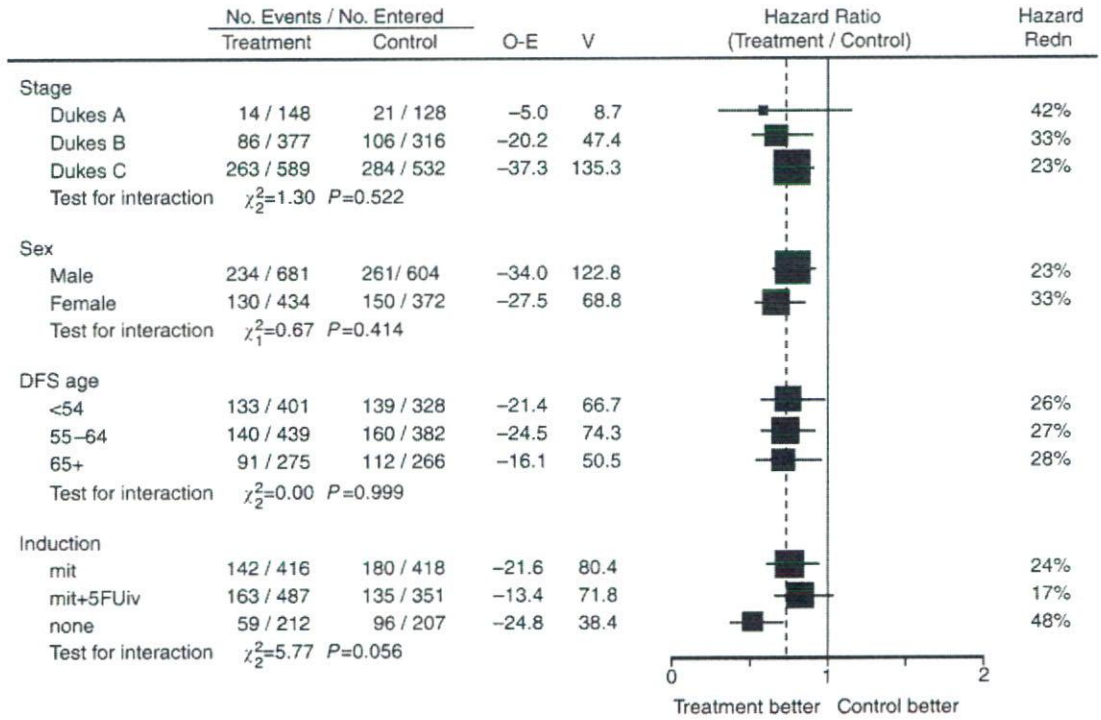

Figure 5 Disease-free survival hazard ratios by patient and treatment characteristics (Abbreviations as in Figure 1).

effects in different trials (χ^2_4 for heterogeneity = 8.82; $P = 0.0658$). UFT also showed significant effect on LRFS of curatively resected rectal cancers.

DISCUSSION

Extensive preclinical and clinical research led to the optimisation of 5-FU administration, with 5-FU bolus in combination with LV as standard therapy both in metastatic disease (Advanced Colorectal Cancer Meta-Analysis Project, 1992) and after curative resection of Stage III (Dukes' C) colon cancer (International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995; O'Connell *et al*, 1997; Wolmark *et al*, 1999). However, the toxicity of bolus 5-FU/LV regimen, especially the risk of haematologic toxicity and mucositis, could not have been negligible.

Continuous-infusion 5-FU modulated by LV, utilised mostly in European countries, showed somewhat better efficacy and definitely better tolerance than bolus 5-FU in advanced diseases (de Gramont *et al*, 1997; Meta-Analysis Group In Cancer, 1998a, b; Schmoll *et al*, 2000). In the adjuvant setting, one of the continuous regimens (LV5-FU2) was shown to have low toxicity than the bolus regimen, but no difference was shown in terms of survival (André *et al*, 2003). Recently, combination of continuous 5-FU/LV and oxaliplatin (FOLFOX 4) was demonstrated to have significant effect on DFS, and is now considered as the standard adjuvant regimen for colon cancer in the Western world.

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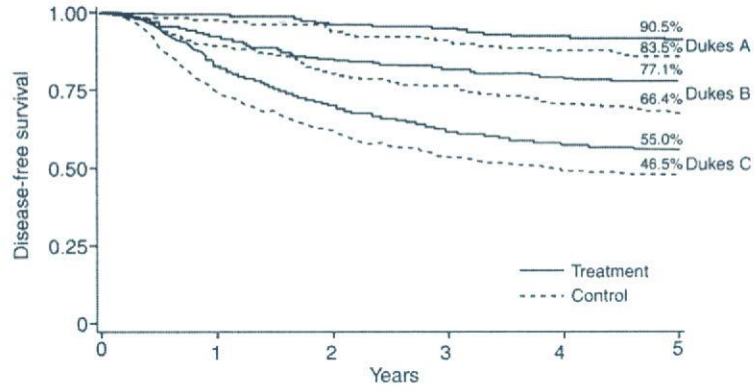

	No. at risk						
Dukes A	Treatment	148	147	143	140	134	132
	Control	128	124	119	115	111	108
Dukes B	Treatment	377	347	315	303	291	282
	Control	316	280	250	237	217	206
Dukes C	Treatment	589	487	403	354	320	298
	Control	532	396	323	277	251	227

Figure 6 Disease-free survival curves by tumour stage and by treatment.

The recent development of O-FPs has therefore opened new perspectives. Oral fluorinated pyrimidines may mimic continuous regimens without its technical inconvenience and deterring patients' quality of life. In patients with advanced colorectal cancer, the efficacy of UFT (typical and most prescribed O-FP) plus oral LV (Carmichael *et al*, 2002; Douillard *et al*, 2002) or of capecitabine alone (Hoff *et al*, 2001; Van Cutsem *et al*, 2001) seems comparable in terms of the efficacy with significantly less significant severe haematologic toxicities and/or stomatitis. The risk of severe hand-foot syndrome is lower in UFT than with capecitabine, but the risk of severe diarrhoea and other gastrointestinal symptoms is higher in UFT and in UFT/oral LV treatment for Western patients.

In Japan, UFT have been administered for many years especially for patients with curatively resected colorectal cancers. For some unknown reason, severe gastrointestinal toxicities are much less frequent in Japanese patients, and patients usually prefer oral chemotherapy especially in an adjuvant setting (Borner *et al*, 2002).

Furthermore, with regard to rectal cancer, it is a difficult objective for a clinical trial to accrue enough patients, compared to colon cancer, and despite the fact that several attempts of determining a standard adjuvant treatment for rectal cancer, almost no clinical trial has succeeded in showing a relevant survival benefit of adjuvant treatment, except one with preoperative radiotherapy (Swedish Rectal Cancer Trial, 1997).

In this context, several Japanese groups conducted randomised clinical trials comparing UFT with surgery alone for curatively resected rectal cancers. Five such trials were identified after a meticulous search, and are included in the present meta-analysis. This meta-analysis was restricted to trials that had been randomised centrally and from which no patient had been excluded for any reason. It represents the largest series of properly randomly assigned patients receiving the single oral adjuvant O-FP agent, that is, UFT, for rectal cancer comparing with patients receiving no therapy after curative tumour resection.

This meta-analysis found a statistically significant benefit of UFT with regard to overall survival (OS) (hazard ratio = 0.82; $P = 0.02$) as well as DFS (hazard ratio = 0.73; $P < 0.0001$), and LRFS (hazard ratio = 0.68; $P = 0.0026$). As can seen by comparing the data in Figures 1 and 4, the data from the NSAS-CC and TAC-CR study show benefits that are, apparently, larger than the others. As shown in Table 1, the dosage and duration of treatment with UFT in the NSAS-CC and TAC-CR trials differed from those in the other three trials; the dose intensity of UFT was higher in the former two trials. Several studies have reported that a high-dose intensity of UFT improves survival in patients given postoperative adjuvant chemotherapy for gastric cancer (Sugimachi *et al*, 1997; Danno *et al*, 2001). The higher dose intensity of UFT in the NSAS-CC and TAC-CR trials may have influenced the outcomes.

Most of the Japanese rectal cancer patients did not receive pre- or postoperative radiotherapy in any of the trials. Although radiotherapy has been considered one of the standard adjuvant treatments in the Western countries, significant survival benefit has not been shown with reproducibility (Wolmark *et al*, 2000; Colorectal Cancer Collaborative Group, 2001). The ostensible advantage of adjuvant radiotherapy is to decrease local recurrence of rectal cancers. As compared with postoperative chemoradiotherapy, preoperative chemoradiotherapy does not improve OS, but inhibits local recurrence and reduces toxicity (Sauer *et al*, 2004). In our study, however, LRFS was also significantly better in the UFT group compared to surgery alone group. As far as our results are concerned, UFT might also be useful in preventing local recurrence in Japanese patients who usually do not receive radiotherapy in an adjuvant setting.

Also, there is still a debate whether adjuvant chemotherapy for early stage rectal cancer is feasible (Buyse and Piedbois, 2001). In terms of numbers needed to treat, these benefits imply that approximately 20 patients need to be treated for one more patient to survive 5 years, and approximately 10 to be treated for one fewer patient to suffer a cancer recurrence within 5 years, regardless of disease stage. Our results show that the therapy is beneficial in Stage II patients not only Stage III patients with nodal involvement (Mamounas *et al*, 1999; Gray *et al*, 2004). As for early stage disease, further investigations are needed to assess potential benefits of treatment because events were infrequent and hazard ratios were small.

Regardless of the disease stage and patient background characteristics, there is a need for further trials involving UFT and new agents that are effective in advanced disease, such as irinotecan, oxaliplatin, and monoclonal antibodies.

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Randomized clinical trial

Randomized controlled trial of adjuvant uracil–tegafur *versus* surgery alone for serosa-negative, locally advanced gastric cancer

T. Nakajima[1], T. Kinoshita[2], A. Nashimoto[3], M. Sairenji[4], T. Yamaguchi[1], J. Sakamoto[5], T. Fujiya[6], T. Inada[7], M. Sasako[8] and Y. Ohashi[9], on behalf of the National Surgical Adjuvant Study of Gastric Cancer Group

[1]Department of Gastrointestinal Surgery, Cancer Institute Hospital, Tokyo, [2]Department of Upper Abdominal Surgical Oncology, National Cancer Centre Hospital East, Chiba, [3]Department of Surgery, Niigata Cancer Centre Hospital, Niigata, [4]Department of Surgery, Kanagawa Cancer Centre, Kanagawa, [5]Department of Surgery, Aichi Cancer Centre, Aichi Hospital, Aichi, [6]Department of Surgery, Miyagi Cancer Centre, Miyagi, [7]Department of Surgery, Tochigi Cancer Centre, Tochigi, [8]Department of Surgical Oncology, National Cancer Centre Hospital, Tokyo, and [9]Department of Biostatistics/Epidemiology and Preventive Health Sciences, University of Tokyo, Tokyo, Japan

Correspondence to: Dr T. Nakajima, Department of Gastrointestinal Surgery, Cancer Institute Hospital, 3-10-6, Ariake, Koto-ku, Tokyo 135-8550, Japan (e-mail: nakajima@jfcr.or.jp)

Background: This prospective randomized study compared the survival of patients with tumour node metastasis (TNM) stage T2 N1–2 gastric cancer treated by gastrectomy alone or gastrectomy followed by uracil–tegafur.

Methods: Patients were randomly assigned to surgery alone or to surgery and postoperative uracil–tegafur 360 mg per m^2 per day orally for 16 months. The primary endpoint was overall survival. Relapse-free survival and site of recurrence were secondary endpoints.

Results: Of 190 registered patients, 95 were randomized to each group; two patients with early cancer were subsequently excluded from the chemotherapy group. The trial was terminated before the target number of patients was reached because accrual was slower than expected. Drug-related adverse effects were mild, with no treatment-related deaths. At a median follow-up of 6·2 years, overall and relapse-free survival rates were significantly higher in the chemotherapy group (hazard ratio for overall survival 0·48, $P = 0·017$; hazard ratio for relapse-free survival 0·44, $P = 0·005$), confirming the survival benefit shown in an interim analysis performed 2 years earlier.

Conclusion: Interim and final analyses revealed a significant survival benefit for postoperative adjuvant chemotherapy with uracil–tegafur in patients with serosa-negative, node-positive gastric cancer. Registration number: NCT00152243 (http://www.clinicaltrials.gov).

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Introduction

Although recent meta-analyses have suggested that adjuvant chemotherapy provides a significant survival benefit after curative gastrectomy in patients with locally advanced gastric cancer[1–8], few individual trials have demonstrated this. Trials of adjuvant chemotherapy have suggested that future studies would require appropriate selection of the target population and intensive dosage regimens based on evidence[9]. After several multicentre clinical trials had produced negative results[10–26], the present authors designed a new dose escalation study with a simple regimen of uracil–tegafur in a well defined target population.

Most previous studies used uracil–tegafur in an adjuvant context in combination with other drugs. The daily dose was generally 300–400 mg (188–250 mg/m^2), lower than

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that recommended as monotherapy, to ensure safety[25]. Studies with multiple drug regimens have generally shown negative or marginal survival benefits, although a trial in patients with moderately locally advanced gastric cancer of tumour node metastasis (TNM) stage T2 N1–2 demonstrated better survival after adjuvant chemotherapy with uracil–tegafur and mitomycin C than surgery alone[25].

In 1997, the National Surgical Adjuvant Study Group decided to perform large, simple clinical trials of uracil–tegafur monotherapy with intensive dosage regimens in breast, colorectal and gastric cancer. In accordance with the standard dose of uracil–tegafur for advanced gastric cancer[27] (response rate 27·5 per cent), 360 mg per m^2 per day was used for 5 days, followed by 2 days of rest, for 16 months. The total dose of uracil–tegafur with this regimen was almost identical to that used for conventional multiple drug regimens (210 mg/m^2 daily for 18 months). In the present study this regimen alone was used in a well defined subset of patients who had undergone curative gastrectomy.

Methods

Eligible patients with T2 N1–2 gastric cancer who had undergone curative gastrectomy and extended lymph node (D2) dissection (complete (R0) resection) were randomly assigned to control or chemotherapy groups within 6 weeks of surgery. A dynamic allocation technique (modified minimization technique) was used for randomization at a central registration centre, with N stage (N1 or N2) and institution as adjustment variables. Random allocation was strictly controlled by an independent National Surgical Adjuvant Study Group Data Centre, and institutional data monitoring was carried out to avoid investigator-related bias.

Within 6 weeks of surgery, patients allocated to the chemotherapy group received an oral daily dose of

Table 1 Characteristics of the 188 patients

	Chemotherapy ($n = 93$)	Control ($n = 95$)
Sex ratio (M : F)	70 : 23	73 : 22
Median age (years)	63	64
Depth of tumour invasion (pT2)		
Muscularis propria	49	46
Subserosa	44	49
Lymph node metastasis*		
n1	69	72
n2	24	23
Type of gastrectomy		
Total	34	26
Distal	59	67
Proximal	0	2
Lymph node dissection*		
D2	80	80
D3	7	8
D4	6	7

*Japanese Classification of Gastric Carcinoma[29].

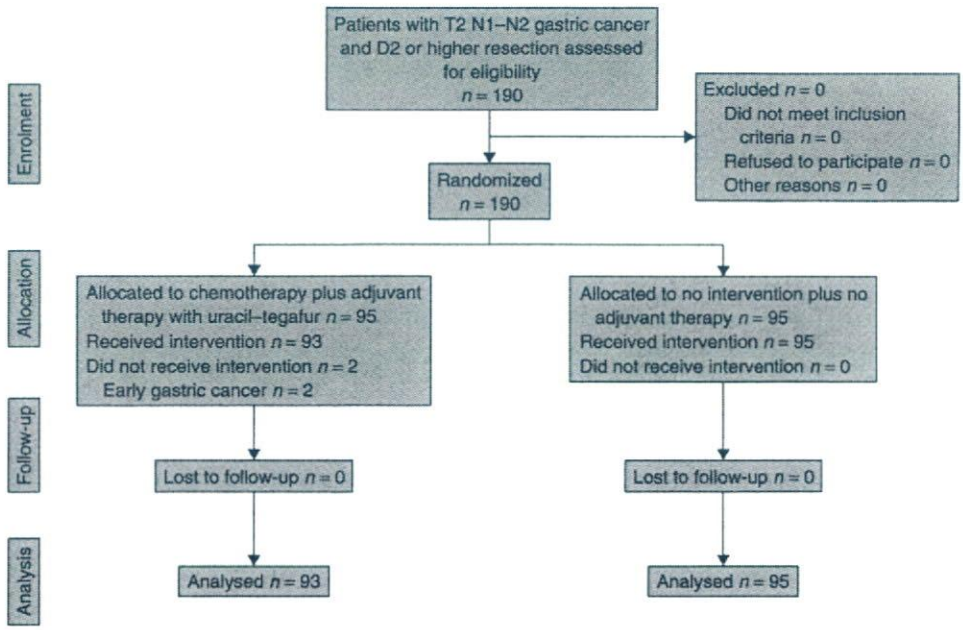

Fig. 1 CONSORT flow chart

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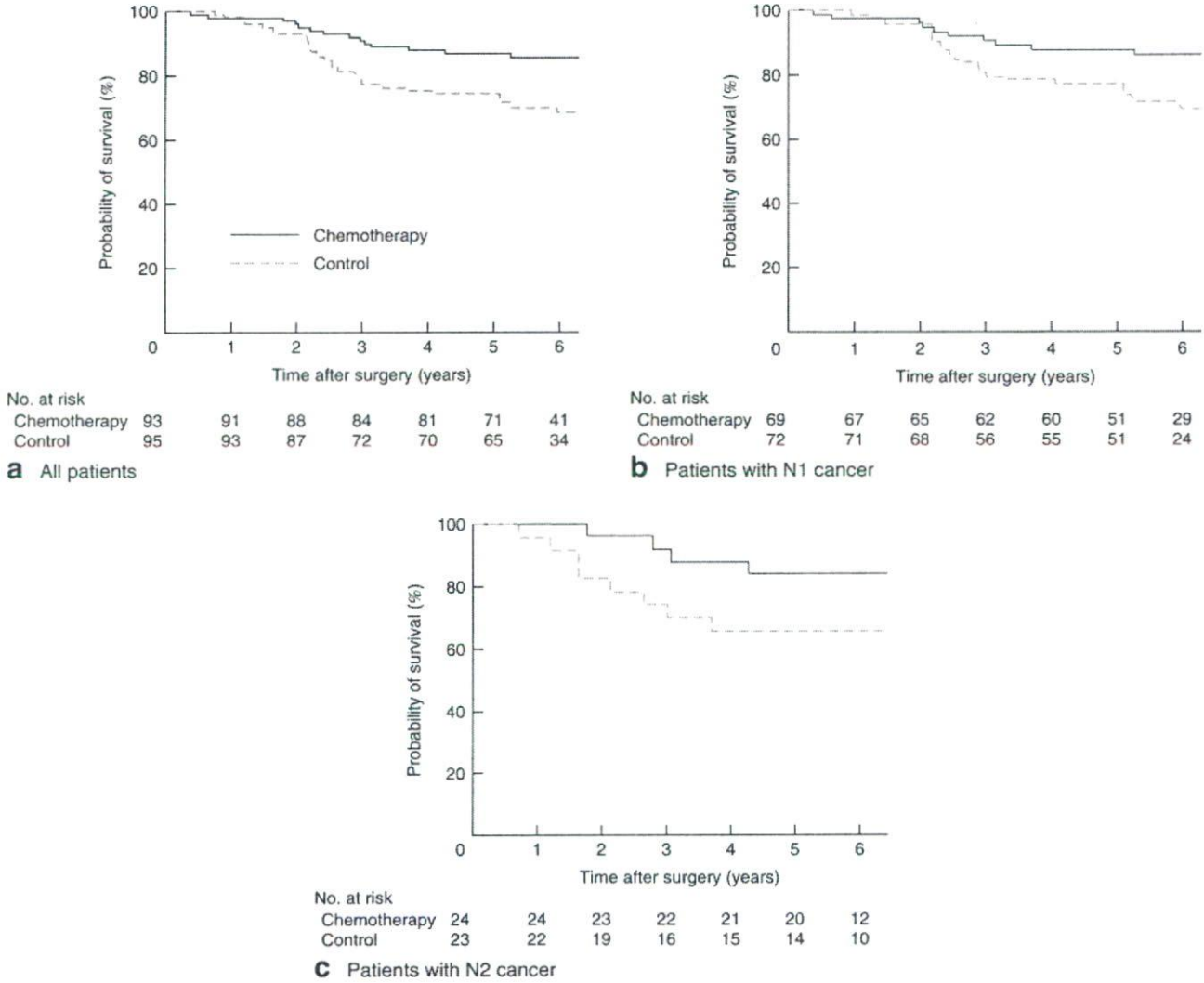

Fig. 2 Overall survival in **a** all 188 eligible patients, **b** 141 patients with N1 cancer and **c** 47 patients with N2 cancer. **a** $P = 0{\cdot}017$, **b** $P = 0{\cdot}061$, **c** $P = 0{\cdot}124$ (stratified log rank test)

uracil–tegafur of 360 mg/m^2 for 5 days every week for 16 months. Patients allocated to the control group were followed up with no adjuvant chemotherapy. Eligibility criteria included histologically proven adenocarcinoma of the stomach, curative gastrectomy with D2 or greater lymph node dissection, pathological T2 N1–2 gastric cancer, an Eastern Cooperative Oncology Group performance status of 0–2, age between 20 and 75 years, no previous chemotherapy and adequate organ function (leucocyte count over 4000 per mm^3, platelet count above 100 000 per mm^3, aspartate and alanine aminotransferase levels lower than twice the upper limit of normal (ULN) at the centre performing the test, total bilirubin concentration less than 1·5 times the ULN, blood urea nitrogen level less than 1·5 times the ULN, and creatinine concentration less than 1·5 times the ULN). Written informed consent was obtained from all patients after approval of the Institutional Review Board at each participating centre.

Statistical analysis

The primary endpoint of the trial was overall survival. Secondary endpoints were relapse-free survival and site of relapse. Overall and relapse-free survival rates were calculated using the Kaplan–Meier method. *P* values were derived with the stratified log rank test according to N stage. Hazard ratios (HRs) were calculated by Cox regression analysis using N stage as a co-variate.

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Table 2 Adverse events

	Chemotherapy ($n = 92$)*		Control ($n = 94$)*	
	Grade 3†	Grade 4†	Grade 3†	Grade 4†
All events	29 of 92 (32)	1 of 92 (1)	4 of 94 (4)	0 of 94 (0)
Neutropenia	11 of 83 (13)	0 of 83 (0)	0 of 78 (0)	0 of 78 (0)
Anaemia	1 of 91 (1)	0 of 91 (0)	0 of 92 (0)	0 of 92 (0)
Raised AST level	1 of 91 (1)	0 of 91 (0)	2 of 92 (2)	0 of 92 (0)
Raised ALT level	2 of 91 (2)	0 of 91 (0)	2 of 92 (2)	0 of 92 (0)
Hyperbilirubinemia‡	8 of 89 (9)	0 of 89 (0)	2 of 90 (2)	0 of 90 (0)
Nausea/vomiting	1 of 92 (1)	0 of 92 (0)	0 of 94 (0)	0 of 94 (0)
Diarrhoea	1 of 92 (1)	1 of 92 (1)	0 of 94 (0)	0 of 94 (0)
Infection	1 of 92 (1)	0 of 92 (0)	0 of 94 (0)	0 of 94 (0)
Anorexia	6 of 92 (7)	0 of 92 (0)	0 of 94 (0)	0 of 94 (0)
Rash	1 of 92 (1)	0 of 92 (0)	0 of 94 (0)	0 of 94 (0)

Values in parentheses are percentages. *One patient excluded from chemotherapy group for refusal of drug administration, and one from control group at patient's request. †Japan Clinical Oncology Group criteria[28]. ‡More than twice the upper limit of normal. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

The 5-year overall survival rate of this patient subset (T2 N1–2) was 70 per cent in a previous study[25], and a 33 per cent reduction in the HR was expected (corresponding to a 5-year overall survival rate of 78·8 per cent). The necessary sample size was 244 patients per group, assuming a 3-year accrual period and 5-year follow-up, with a statistical power of 80 per cent to achieve a one-sided significance level of 0·050. The accrual goal was 500 patients. All analyses were based on intention-to-treat groups.

An Independent Data Monitoring Committee (IDMC) monitored the trial. Two interim analyses were originally planned, 1 and 3 years after all patients had been enrolled. Significance levels were set at 0·005 and 0·020 (one-sided) respectively. After closing the registration, the IDMC decided to undertake a single interim analysis at 2 years, owing to a lower rate of accrual than anticipated. When this interim analysis revealed a difference in survival rates between the two groups, the IDMC did not disclose this finding to investigators. Second interim and final analyses were then undertaken as originally planned at 3 and 5 years. Adverse events were evaluated using the toxicity grading criteria of the Japan Clinical Oncology Group[28].

Multivariable analysis was carried out with a Cox proportional hazards model to identify independent prognostic factors using treatment group, sex, age group, depth of invasion and extent of lymph node metastasis as explanatory variables.

Results

As accrual was slower than expected, recruitment of patients was terminated midway through the trial before the target number of patients was reached. Between June 1997 and March 2001, 190 patients were enrolled in the study, 95 randomized to the chemotherapy group and 95 to the control group. Two patients were ineligible after randomization and were excluded from the analysis because the final pathological report revealed early gastric cancer. Thus, 188 patients, 93 in the chemotherapy and 95 in the control group, were included in the intention-to-treat analysis (*Fig. 1*).

Clinical characteristics of the 188 patients are shown in *Table 1*. All major prognostic factors were similar in the two groups.

Of patients in the chemotherapy group with no recurrence, 80 per cent (73 of 91) received all scheduled doses of uracil–tegafur during the first 3 months, and 51 per cent (44 of 86) did so for 16 months. Two patients were withdrawn from treatment as a result of recurrence during the first 3 months, and seven for recurrence by 16 months.

Adverse events during follow-up are shown in *Table 2*. The main events in the chemotherapy group were bone marrow suppression (grade 3 neutropenia, 13 per cent), liver dysfunction (grade 3 hyperbilirubinaemia, 9 per cent) and gastrointestinal dysfunction (grade 3 anorexia, 7 per cent). Grade 4 diarrhoea occurred in one patient in the chemotherapy group.

At the 2-year interim analysis conducted in December 2003, both overall and relapse-free survival rates were significantly better in the chemotherapy group. The second interim analysis was conducted in November 2004 after a median follow-up of 3·8 years (3 years after registration

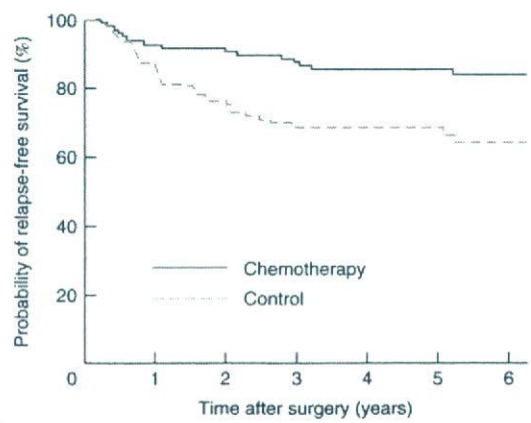

Fig. 3 Relapse-free survival in patients in the chemotherapy group compared with that in the control group. $P = 0·005$ (log rank test)

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Table 3 First site of relapse

	Chemotherapy ($n = 93$)	Control ($n = 95$)	P^*
Peritoneal	4	3	0·680
Local	0	4	0·050
Haematogenous	9	14	0·290
Distant lymph nodes	2	11	0·010
Total no. of relapses	13	28	

Some patients had more than one type of recurrence. *χ^2 test.

was closed). Survival rates remained significantly better in the chemotherapy group (HR 0·46, 13 per cent difference in survival at 4 years).

These survival benefits were confirmed by the final analysis, performed after a median follow-up of 6·2 years after surgery (5 years after registration was closed). The 5-year overall survival rate was 86 per cent in the chemotherapy group and 73 per cent in the control group ($P = 0·017$) (*Fig. 2a*). The HR for overall survival in the chemotherapy group relative to the control group was 0·48 (95 per cent confidence interval (c.i.) 0·26 to 0·89). *Figs 2b* and *2c* show the results of a planned subset analysis of overall survival according to N1 (HR 0·52 (95 per cent c.i. 0·26 to 1·05); $P = 0·061$) and N2 (HR 0·40 (95 per cent c.i. 0·12 to 1·34); $P = 0·124$) status. The results of a similar analysis of 5-year relapse-free survival in chemotherapy and control groups are shown in *Fig. 3* (85 *versus* 68 per cent respectively; HR 0·44 (95 per cent c.i. 0·25 to 0·79); $P = 0·005$).

Multivariable analysis showed that treatment group ($P = 0·021$) and sex ($P = 0·032$) were significant independent prognostic factors, whereas the other three explanatory variables were not (age group, $P = 0·918$; depth of cancer invasion, $P = 0·539$; extent of lymph node metastasis, $P = 0·996$).

All causes of death included 13 recurrences in the chemotherapy group, 28 in the control group, two deaths from other cancers in the chemotherapy group, and one death unrelated to disease (traffic accident) and one for unknown reasons in the control group.

Table 3 shows the first sites of relapse in the two groups. The most common type of relapse was haematogenous metastasis to the liver. Patients in the chemotherapy group had a lower incidence of nodal metastatic recurrence.

Discussion

Both the second interim analysis after a median follow-up of 3·8 years and the final analysis after a median of 6·2 years showed a significant survival benefit for patients with T2 N1–2 gastric cancer following curative D2 gastrectomy and adjuvant chemotherapy with uracil–tegafur. Previous studies of adjuvant chemotherapy have not shown such a significant benefit[30–32].

Kato and colleagues[33] first reported the survival benefit of adjuvant uracil–tegafur alone in non-small cell lung cancer after curative surgery. Uracil–tegafur is widely used in Japan, but not in other countries. This is the first report to document a significant survival benefit for adjuvant uracil–tegafur in patients with gastric cancer.

The unexpectedly large difference in survival between the groups is a cause for concern. Such a significant finding was unexpected because the number of patients was much smaller than planned. Slow accrual might have been due partly to a lack of enthusiasm among investigators for the use of uracil–tegafur, on the basis of earlier trials. Some eligible patients might have been enrolled in other concurrent trials with similar eligibility criteria. Although some institutional selection bias may have been present, this was not reflected in the allocation of registered patients. The interim analysis unexpectedly revealed a HR of 0·46, corresponding to a 13 per cent difference in 4-year overall survival rate, at a median follow-up of 3·8 years, reaching the predefined significance level. The survival difference continued for more than 5 years after surgery and was confirmed at the final analysis, after a median follow-up of 6·2 years.

The large reductions in HR for overall and relapse-free survival may be attributable to several factors. One is the difference in the clinical stage of disease between the patients in this and earlier studies conducted by this group[25,26]. Patients in the present study had T2 N1–2 gastric cancer, whereas the authors' previous study included patients with T1 and T2 N1–2 disease. The exclusion of T1 cancer from the present study resulted in poorer 5-year overall survival in the control group than in the earlier trial, but almost no change in overall survival in the chemotherapy group, resulting in a significant survival difference. The difference in survival may therefore have been attributable to better patient selection, a higher dosage of uracil–tegafur than used in previous regimens[25] and a long duration of treatment.

A second concern was whether the survival difference actually resulted from the chemotherapy. Small numbers of patients per centre might theoretically bias the allocation of patients to treatment, but there was no evidence of this. Treatment allocation was strictly controlled by an independent data centre, minimizing the possibility of bias related to centre or investigator. The clinical characteristics of both chemotherapy and control groups were similar, and only two patients (1·1 per cent) were excluded from

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analysis because of protocol violations (early cancer). The rate of compliance with treatment was 80 per cent during the first 3 months of chemotherapy and 51 per cent at the end of the study, despite the long treatment period. Lower compliance at the end of the study was due to adverse events, patient refusal or loss to follow-up. Compliance rates were consistent with those of other recent trials[33–37].

The cause of death was established in most patients. The incidence of distant lymph node relapse was significantly lower in the chemotherapy group, suggesting that after D2 dissection adjuvant chemotherapy might have inhibited the growth of minimal residual tumour in distant nodes. On subset analysis according to N1 and N2 status, the survivals of patients in the chemotherapy groups were almost identical, and the larger difference, though not statistically significant, in survival rate in patients with N2 disease might have resulted from a higher rate of residual cancer in distant nodes after D2 surgery than in those with N1 disease. No differences were observed in other types of relapse, such as liver or peritoneal metastasis. Multivariable analysis showed that treatment group and sex were significant independent prognostic factors, providing further evidence that the survival benefit was derived from adjuvant chemotherapy.

Although not widely used in Western countries until recently, adjuvant uracil–tegafur treatment appears to be effective in other cancers[34–36]. The survival benefit achieved with oral uracil–tegafur plus leucovorin is similar to that with intravenous 5-fluorouracil and leucovorin, but with less toxicity, in colorectal cancer. Adjuvant chemotherapy with uracil–tegafur alone is effective in patients with non-small cell lung[33] and rectal[38] cancer. Apart from direct cytocidal activity, low-dose chemotherapy with uracil–tegafur has been shown experimentally to have antiangiogenic effects on endothelial cells[39]. This could also influence survival.

In the present trial, the main side-effect associated with uracil–tegafur alone was moderate myelosuppression. Uracil–tegafur alone is associated with milder side-effects than when combined with leucovorin[35,36]. The advantages of survival benefit, mild toxicity and ease of administration on an outpatient basis make this an attractive approach. It was on this basis that a further large-scale clinical trial was recently undertaken in Japan using adjuvant S-1, a successor to uracil–tegafur that is anticipated to be more effective[40].

Patient selection is important in the context of adjuvant chemotherapy trials. It seems unreasonable to assume that a given regimen of adjuvant chemotherapy will be effective for all stages of disease. Conversely, selected groups of patients might benefit in terms of survival. Similarly, the quality of surgery may also be important. D2 gastrectomy for patients in the present trial carried only a small risk of stage misclassification.

Whether the present results can be extrapolated to other countries is important. Provided that D2 gastrectomy can be performed with a high level of reliability and low perioperative mortality, these results should be reproducible, because the outcomes of adjuvant chemotherapy appear to depend largely on the amount of residual tumour and the quality of surgery[41]. Macdonald and colleagues[37] in the USA reported encouraging results for adjuvant chemoradiotherapy in patients who had undergone curative gastrectomy. Their results may be representative as well as reproducible in that country, where D2 lymph node dissection is not performed routinely. Inadequate surgery might have resulted in large amounts of residual tumour in that trial. Adjuvant chemoradiotherapy may have suppressed locoregional relapse, thereby compensating for inadequate lymph node dissection. Although there is no evidence to support the superiority of D2 over D1 (limited lymph node dissection) or D0 (local) resection[42], many Japanese studies, as well as some reports from high-volume centres in Western countries, suggest that extended lymphadenectomy enhances postoperative survival[43,44]. The regimen for adjuvant therapy with uracil–tegafur might produce different outcomes under different surgical resection standards.

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Members of the National Surgical Adjuvant Study of Gastric Cancer were as follows. Trial Chair: T. Nakajima (Cancer Institute Hospital, Tokyo). Statistical Analyst: Y. Ohashi (University of Tokyo, Tokyo). Evaluation Committee: H. Nakazato (Yokoyama Gastrointestinal Hospital, Nagoya). Independent Data Monitoring Committee: N. Saijo (National Cancer Centre Hospital East, Chiba), Y. Ariyoshi (Marumo Hospital, Aichi), S. Ebihara (National Cancer Centre Hospital East, Chiba), H. Origasa (Toyama University, Toyama), M. Fukuoka (Kinki University, Osaka), T. Mitsuishi (Mitsuishi Law and Patent Office, Tokyo), T. Tsuruo (University of Tokyo, Tokyo). Participating Institutions and Principal Investigators: National

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Hospital Organization Sendai Medical Centre, Miyagi (Y. Kunii, T. Saito); Miyagi Cancer Centre, Miyagi (T. Fujiya); Yamagata Prefectural Central Hospital, Yamagata (N. Fukushima); Ibaraki Prefectural Central Hospital, Ibaraki (N. Okazaki, Y. Miyata, M. Ohkuwa, H. Ohkura); Tochigi Cancer Centre, Tochigi (T. Inada); Gunma Prefectural Cancer Centre, Gunma (T. Fukuda, N. Haga); Saitama Cancer Centre, Saitama (Y. Suda, K. Uchida, Y. Kawashima); National Cancer Centre Hospital East, Chiba (T. Kinoshita); Chiba University Hospital, Chiba (T. Suzuki, Y. Gunji, Y. Nabeya); Cancer Institute Hospital, Tokyo (T. Takahashi, K. Ohta, T. Yamaguchi); Keio University Hospital, Tokyo (T. Kubota, Y. Otani, Y. Saikawa); National Cancer Centre Hospital, Tokyo (M. Sasako); Tokyo Metropolitan Komagome Hospital, Tokyo (M. Kitamura, K. Arai); Kanto Medical Centre, Nippon Telegraph and Telephone East Corporation, Tokyo (T. Konishi); Nihon University Itabashi Hospital, Tokyo (M. Fujii, T. Takayama); Kanagawa Cancer Centre, Kanagawa (M. Sairenji, O. Kobayashi); Niigata Cancer Centre Hospital, Niigata (J. Sasaki, A. Nashimoto); Kanazawa University Hospital, Kanazawa (S. Fushida); Aichi Cancer Centre Hospital, Aichi (Y. Yamamura); National Hospital Organization Nagoya Medical Centre, Aichi (K. Kondo, M. Kataoka); Aichi Cancer Centre Aichi Hospital, Aichi (J. Sakamoto, H. Kojima); Osaka Medical Centre for Cancer and Cardiovascular Diseases, Osaka (H. Furukawa, M. Hiratsuka, I. Miyashiro); National Hospital Organization Osaka National Hospital, Osaka (T. Tsujinaka); Sakai Municipal Hospital, Osaka (M. Tatsuta); Hyogo Cancer Centre, Hyogo (S. Nakaya, K. Kawaguchi, Y. Kanbara); National Hospital Organization Kure Medical Centre, Hiroshima (T. Hashimoto, M. Koseki); Yamaguchi Grand Medical Centre, Yamaguchi (Y. Kuroda); Kagawa University Hospital, Kagawa (H. Usuki); National Hospital Organization Shikoku Cancer Centre, Ehime (A. Kurita); National Hospital Organization Kyushu Cancer Centre, Fukuoka (T. Saito, S. Kohnoe, T. Okamura); Kagoshima University Hospital, Kagoshima (T. Aiko).

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Case Report

Clinical and Immunologic Responses to Very Low–Dose Vaccination with WT1 Peptide (5 μg/Body) in a Patient with Chronic Myelomonocytic Leukemia

Manabu Kawakami,[a,c] Yoshihiro Oka,[b] Akihiro Tsuboi,[c] Yukie Harada,[b] Olga A. Elisseeva,[d] Yoshio Furukawa,[e] Machiko Tsukaguchi,[f] Toshiaki Shirakata,[d] Sumiyuki Nishida,[d] Hiroko Nakajima,[d] Satoshi Morita,[g] Junichi Sakamoto,[g] Ichiro Kawase,[b] Yusuke Oji,[h] Haruo Sugiyama[d]

[a]*Department of Medicine, National Hospital Organization, Osaka Minami Medical Center, Osaka, Japan;* [b]*Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka, Japan;* [c]*Department of Cancer Immunotherapy, Osaka University Graduate School of Medicine, Osaka, Japan;* [d]*Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, Osaka, Japan;* [e]*Department of Medicine, Fuchu Hospital, Osaka, Japan;* [f]*Department of Medicine, Sakai Municipal Hospital, Osaka, Japan;* [g]*Young Leaders' Program of Medical Administration & International Affairs, Graduate School of Medicine Nagoya University, Nagoya, Japan;* [h]*Department of Biomedical Informatics, Osaka University Graduate School of Medicine, Osaka, Japan*

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Abstract

The wild-type Wilms tumor gene, WT1, is overexpressed in myelodysplastic syndrome (MDS) as well as acute myeloid leukemia. In a phase I clinical trial of biweekly vaccination with HLA-A*2402–restricted WT1 peptide for these malignancies, 2 patients with MDS developed severe leukocytopenia in association with a reduction in leukemic blast cells and levels of WT1 messenger RNA (mRNA) after only a single vaccination with 0.3 mg of WT1 peptide. These results indicated that the WT1-specific cytotoxic T-lymphocytes (CTLs) elicited by WT1 vaccination eradicated the WT1-expressing transformed stem or progenitor cells and that MDS patients with little normal hematopoiesis required a new strategy of WT1 vaccination to avoid severe leukocytopenia. We describe the first trial for a 57-year-old male patient with chronic myelomonocytic leukemia who was vaccinated biweekly with a small quantity (5 μg/body) of WT1 peptide. After the start of vaccination, the leukocyte and monocyte counts (13,780/μL and 1930/μL, respectively) gradually decreased to within the normal range in association with a reduction in the WT1 mRNA level. Simultaneously, the percentage of WT1-specific CTLs as measured by the HLA-WT1 tetramer assay increased. This case demonstrates for the first time that vaccination with as little as 5 μg of WT1 peptide can induce WT1-specific immune responses and resultant clinical responses.
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1. Introduction

The wild-type Wilms tumor gene, WT1, is overexpressed in acute myeloid leukemia (AML), acute lymphoblastic leukemia, chronic myelogenous leukemia, and myelodysplastic syndrome (MDS), as well as in various types of solid tumors, and plays an essential role in leukemogenesis and tumorigenesis. Our preclinical studies indicated that the WT1 gene product could be a good target antigen for immunotherapy against these malignancies [1-3]. Therefore, we performed a phase I clinical study of WT1 peptide–based immunotherapy for patients with breast or lung cancer, AML, or MDS [4]. The patients were injected intradermally at 2-week intervals with an HLA-A*2402–restricted, natural, or modified 9-mer WT1 peptide (residues 235-243) emulsified with Montanide ISA-51 adjuvant at 0.3, 1.0, or 3.0 mg/ body [5,6]. Twenty-six patients received 1 or more WT1 vaccinations. In all of the patients except the 2 MDS patients included in the clinical study, no toxicity other than local erythema at the WT1

Correspondence and reprint requests: Haruo Sugiyama, Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, 1-7 Yamada-oka, Suita-City, Osaka 565-0871, Japan; 81-6-6879-2597; fax: 81-6-6879-2597 (e-mail: sugiyama@sahs.med.osaka-u.ac.jp).

vaccine-injection sites was observed. In the 2 MDS cases (one was AML transformed from MDS and the other was MDS with myelofibrosis), however, only a single vaccination with 0.3 mg of modified WT1 peptide induced severe leukocytopenia in association with a rapid increase in WT1-specific cytotoxic T-lymphocytes (CTLs). We observed reductions in both the leukemic blast cells and the levels of WT1 messenger RNA (mRNA), which reflected the amount of leukemic blast cells in the bone marrow (BM) [7]. These results indicated that the WT1-specific CTLs elicited by WT1 vaccination eradicated the WT1-expressing transformed stem or progenitor cells and consequently reduced the leukocytes, most of which were derived from the transformed stem or progenitor cells. This severe leukocytopenia indicated that WT1 vaccination had high potential as immunotherapy for MDS but required a new WT1-vaccination strategy that avoids severe leukocytopenia. With the aim of slowly inducing WT1-specific CTLs and thereby avoiding severe leukocytopenia, we are now performing a phase I dose-escalation study of biweekly WT1 vaccination at much reduced doses (5, 15, or 50 μg/body) to be given to 3 MDS patients. It is impossible to optimize the WT1 peptide dose for vaccination in mouse models, because the immunologic sensitivities of tumor-associated antigen (TAA)-derived peptides of mice and humans are quite different. Therefore, we have to optimize the dose directly in clinical trials. For the safety of MDS patients, we considered that a 1- to 2-log reduction of the dose used in the previous trial (0.3 mg) would be suitable as the initial dose; consequently, we decided to vaccinate MDS patients in this clinical trial with peptide at the 3 doses noted above (5, 15, or 50 μg/body).

We present a patient with chronic myelomonocytic leukemia (CMML) who was vaccinated biweekly with a small quantity (5 μg/body) of WT1 peptide and who achieved a gradual reduction in leukocytes.

2. Patients and Methods

2.1. Patients

The phase I clinical study of WT1 vaccination for MDS patients was approved by the ethics review board of the Faculty of Medicine, Osaka University. Patients aged 16 to 80 years with MDS (refractory anemia with excess of blasts (RAEB), CMML, RAEB in transformation, and MDS-AML in the French-American-British classification) were eligible for the study if no other therapy including allogeneic hematopoietic stem cell transplantation was indicated as a standard therapy. Other inclusion criteria were as follows: (1) overexpression of the WT1 gene in BM or peripheral blood (PB) samples as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis; (2) HLA-A*2402 positivity; (3) a performance status of 0 to 1 (Eastern Cooperative Oncology Group); (4) no severe impairment of organ function; (5) a neutrophil count ≥500/μL, a platelet count ≥25,000/μL, and a hemoglobin level ≥6.5 g/dL; (6) <20% blast cells in the BM and PB; (7) no chemotherapy, immunotherapy, immunosuppressive therapy, or radiotherapy administered within 4 weeks before WT1 vaccination; (8) no previous allogeneic stem cell transplantation.

2.2. WT1 Peptide

For immunization, we used a modified 9-mer WT1 peptide (residues 235-243, CYTWNQMNL) with substitution of Y for M at position 2 of the natural 9-mer WT1 235-243 peptide (CMTWNQMNL) [5,6]. The modified WT1 peptide has been shown to induce much stronger CTL activity against WT1-expressing tumor cells than the natural peptide [6]. The WT1 peptide (GMP grade) was purchased as a lyophilized peptide from Multiple Peptide Systems (San Diego, CA, USA).

2.3. Vaccination

After written informed consent was obtained, the patients received a skin test. If the results were negative, we scheduled intradermal injections of WT1 peptide emulsified with Montanide ISA-51 adjuvant at 2-week intervals. We planned to escalate the WT1 peptide doses from 5 μg to 15 μg or 50 μg, each of which was to be given to 3 patients.

2.4. RT-PCR Analysis for Quantitation of WT1 mRNA Levels

WT1 mRNA levels in PB samples were measured by real-time RT-PCR analysis and were expressed relative to the level in K562 leukemia cells, as has previously been described [8].

*2.5. HLA-A*2402/WT1 Peptide Tetramer Assay for WT1-Specific CD8⁺ T-Cells*

PB mononuclear cells (PBMCs) were stained with phycoerythrin (PE)-conjugated HLA-A*2402-WT1 235-243 tetramer (WT1-Tet) (MBL, Tokyo, Japan) in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 2% fetal bovine serum) for 30 minutes at 37°C. Subsequently, the cells were stained for an additional 25 minutes on ice in the dark with 5 additional colors of fluorescently labeled monoclonal antibodies: fluorescein isothiocyanate–labeled anti-CD4, -CD14, -CD16, -CD19, and -CD56; allophycocyanin (APC)/Cyanine 7 (Cy7)-labeled anti-CD8; APC-labeled anti-CD45RA; PE/Cy7-labeled anti-CCR7 (BD Pharmingen, San Diego, CA). The cells were then washed twice with FACS buffer and analyzed with a FACSAria instrument (BD Biosciences, San Jose, CA, USA). WT1-Tet⁺ CD8⁺ T-cells, which were negative for such lineage markers as CD4, CD14, CD16, CD19, and CD56, were considered to represent WT1-specific CD8⁺ T-cells, and the percentage of WT1-Tet⁺ cells among the CD8⁺ T-cells was measured. As a negative control, PBMCs were also stained with PE-labeled irrelevant HLA-A*2402-HIV envelope peptide (RYLRDQQLL) tetramer (Ir-Tet) instead of WT1-Tet and according to the same procedure.

To investigate the differentiation status of WT1-Tet⁺ CD8⁺ T-cells, we also analyzed CD45RA and CCR7 expression in the WT1-Tet⁺ CD8⁺ T-cell fraction.

3. Case Report

A 57-year-old man received a diagnosis of AML M5b in May 1999. The karyotype of the BM cells was 45,X,-Y. This

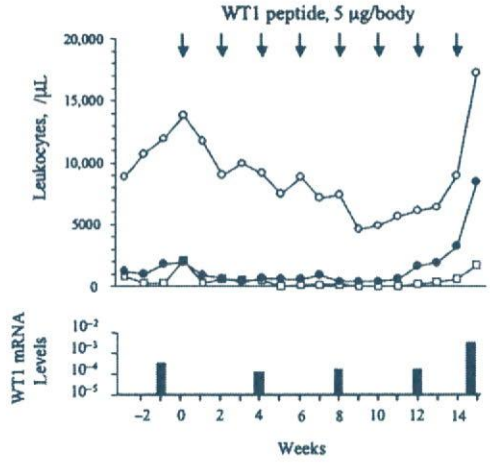

Figure 1. Clinical course of WT1 vaccination. Upper panel, the time courses for counts of white blood cells (open circles), monocytes (closed circles), and myelocytes plus metamyelocytes (open squares). Lower panel, relative levels of WT1 messenger RNA (mRNA) in the peripheral blood.

patient achieved complete remission with the disappearance of the abnormal karyotype by induction therapy with daunorubicin and enocitabine. The patient subsequently underwent 3 courses of consolidation therapy and 4 courses of maintenance therapy. In January 2001, although BM morphologic findings indicated the maintenance of complete remission, complex abnormal karyotypes, including t(3;18)(q25;q21), were detected in 7 of 20 analyzed BM cells, indicating the development of a secondary hematologic malignant disorder. BM cells with abnormal karyotypes, including add(1)(q32), add(3)(q21), and add(18)(q21), appeared and reached 100% in March 2004, despite the administration of 10 courses of chemotherapy with a cytarabine-containing regimen. Thereafter, total white blood cells (WBCs), monocytes, and immature leukocytes (myelocytes and metamyelocytes) gradually increased without chemotherapy. On July 23, 2004, the WBC count reached 13,780/μL, and a WBC analysis showed 10% myelocytes, 5% metamyelocytes, 7% stab neutrophils, 47% segmented neutrophils, 16% lymphocytes, 1% eosinophils, and 14% monocytes (1930/μL). A BM aspirate revealed 1.4% blasts among the nucleated cells. Thus, the patient's diagnosis was secondary CMML, in accordance with this disease's diagnostic criteria. Because the patient satisfied the inclusion criteria (HLA-A*2402$^+$, abnormal levels of WT1 mRNA in the PB or BM, and neutrophil counts >500/μL in the PB) for the vaccine protocol approved by the Institutional Ethics Committee of Osaka University, we started biweekly WT1 vaccination with modified WT1 peptide (5 μg/body) on July 27, 2005 (Figure 1). After the first WT1 vaccination, WBC, monocyte, and immature cell counts gradually decreased to within the normal range. During WT1 vaccination, the percentage of blast cells in the BM stayed at approximately 1.0%, and the karyotypes of BM cells remained abnormal in all 20 cells analyzed. The levels of WT1 mRNA in the PB relative to the level in K562 cells (defined as 1.0; the upper limit of the normal range in PB was 1.0×10^{-4}) decreased from 2.9×10^{-4} before vaccination to 1.1×10^{-4}, 1.7×10^{-4}, and 1.5×10^{-4} on weeks 4, 8, and 12, respectively. On week 15, however, WBC, monocyte, and immature cell counts increased to 17,260/μL, 8460/μL, and 710/μL, respectively, in association with a rapid increase in WT1 mRNA levels in the PB to 2.9×10^{-3}, indicating aggravation of the CMML.

To analyze immune responses to the WT1 vaccination, we measured the percentage of WT1-specific CD8$^+$ T-cells in PB CD8$^+$ T-cells by staining CD8$^+$ T-cells in PB with PE-labeled WT1-Tet. As a negative control, the samples were also stained with Ir-Tet instead of WT1-Tet. Ir-Tet$^-$ cells were negligible (Figure 2A, right). WT1-Tet$^+$ cells were detected at a percentage of 0.04% ± 0.02% (mean ± SD) of the CD8$^+$ T-cells in 5 healthy volunteers (Figure 2A, center). Recently, T-cells have been phenotypically classified into 4 differentiation stages according to their expression of CD45RA and CCR7: the naive stage (CD45RA$^+$CCR7$^+$), the central memory stage (CD45RA$^-$CCR7$^+$), the effector memory stage (CD45RA$^-$CCR7$^-$), and the terminal differentiated effector stage (CD45RA$^+$CCR7$^-$). The majority of WT1-Tet$^+$ CD8$^+$ T-cells (80.0% ± 8.4%) in the 5 healthy volunteers belonged to the naive stage (Figure 2B, center). In the present case, the percentage of WT1-Tet$^+$ CD8$^+$ T-cells was as low as 0.018% before vaccination (Figure 2A, left). In our patient, however, much higher proportions of WT1-Tet$^+$ CD8$^+$ T-cells were found in the central memory, effector memory, and terminal differentiated effector stages (33.0%, 40.0%, and 14.8%,

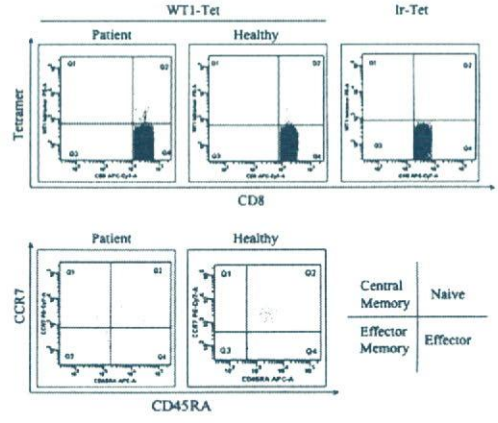

Figure 2. Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) with HLA-WT1 tetramer. A, PBMCs derived from the patient (left) and a healthy volunteer (center) were stained with phycoerythrin (PE)-conjugated HLA-A*2402–WT1 235-243 tetramer (WT1-Tet$^+$). The samples were stained with PE-labeled irrelevant HLA-A*2402-HIV envelope peptide tetramer (Ir-Tet) as a negative control (right), instead of WT1-Tet. Dot plots were gated on CD8$^+$ T-cells, which were negative for CD4, CD14, CD16, CD19, and CD56. The frequencies of WT1-Tet$^+$ cells, which represented WT1-specific T-cells in the gated CD8$^+$ T-cells, were measured. B, WT1-Tet$^+$ CD8$^+$ T-cells derived from the patient (left) and a healthy volunteer (center) were phenotypically classified into 4 differentiation stages according to their expression of CD45RA and CCR7: naive stage cells (CD45RA$^+$CCR7$^+$), central memory stage cells (CD45RA$^-$CCR7$^+$), effector memory stage cells (CD45RA$^-$CCR7$^-$), and terminal differentiated effector stage cells (CD45RA$^+$CCR7$^-$).

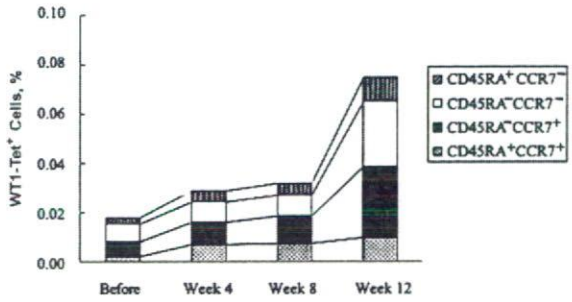

Figure 3. The percentage and differentiation status of WT1-Tet$^+$ CD8$^+$ T-cells before and after WT1 vaccination. The percentage of WT1-Tet$^+$ cells among the CD8$^+$ T-cells in the patient's peripheral blood mononuclear cells and their differentiation status were serially analyzed during the vaccination period.

respectively; Figure 2B, left) than in the healthy volunteers. During the vaccination period, the percentage of WT1-Tet$^+$ cells increased to 0.029%, 0.032%, and 0.075% on weeks 4, 8, and 12, respectively, and their differentiation status did not substantially change from that before vaccination (Figure 3). After aggravation of the disease, we could not obtain blood samples for immunologic analysis because induction chemotherapy began immediately.

4. Discussion

This case demonstrated for the first time that vaccination with as little as 5 μg of WT1 peptide could induce WT1-specific immune responses and resultant clinical responses. Various kinds of TAA-derived peptides have conventionally been administered at doses ranging from 0.1 to 3.0 mg, and the administration dose of the peptide is not considered to correlate with the extent of the TAA-specific immune response elicited by the vaccination [9]. The immune response to WT1 peptide vaccination may be dose dependent, however, especially in MDS, because administration of 0.3 mg and 5 μg of WT1 peptide induced rapid and slow reductions, respectively, in the numbers of abnormal hematopoietic cells.

In the present case, leukocyte counts gradually decreased, and no infectious disease developed, suggesting that vaccination with very low doses of WT1 peptide may become a safe method for use in MDS cases. After the fifth vaccination, leukocyte counts began to increase slowly, and then became rapidly elevated after the eighth vaccination. If the dose of WT1 peptide used for vaccination had been increased when the leukocyte count had begun to increase, aggravation of the disease might have been prevented; however, the protocol prohibited increasing the WT1 peptide dose.

The frequency of WT1-Tet$^+$ CD8$^+$ T-cells before vaccination was much lower in this case (0.018%) than the frequencies for the 2 MDS patients (0.98% and 0.62%) and the 12 patients with de novo AML (0.31% ± 0.25%) who were vaccinated in our former phase I clinical study of WT1 peptide vaccination at doses of 0.3 mg, 1 mg, or 3 mg [4]. Despite such a low frequency of WT1-Tet$^+$ CD8$^+$ T-cells in this case, a higher proportion of them had the central memory, effector memory, or terminal differentiated effector phenotype before and after vaccination, indicating that WT1-Tet$^+$ CD8$^+$ T-cells had already been highly activated and differentiated before vaccination, in contrast to the cells in healthy volunteers. In our previous phase I clinical study, the clinical responses (tumor regressions in 2 breast cancer patients and reductions of tumor markers in 3 lung cancer patients, morphologically detected leukemic blasts in 2 cases, and minimal residual leukemic cells detected with WT1 gene expression analysis in 5 cases) were significantly correlated with a ≥1.5-fold increase in the WT1-Tet$^+$ CD8$^+$ T-cell frequency after the vaccination. Similarly, the percentage of WT1-Tet$^+$ cells in this case increased more than 1.5-fold after the vaccination, suggesting that the vaccination enhanced WT1-specific immune responses and induced a clinical response.

This case suggested that vaccination with very low doses of WT1 peptide might become a safe and effective therapy for MDS patients. This conclusion should be confirmed by further studies with a larger number of patients.

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