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Clinical Studies

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Randomized clinical trial

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

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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 m² 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/m²), 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].

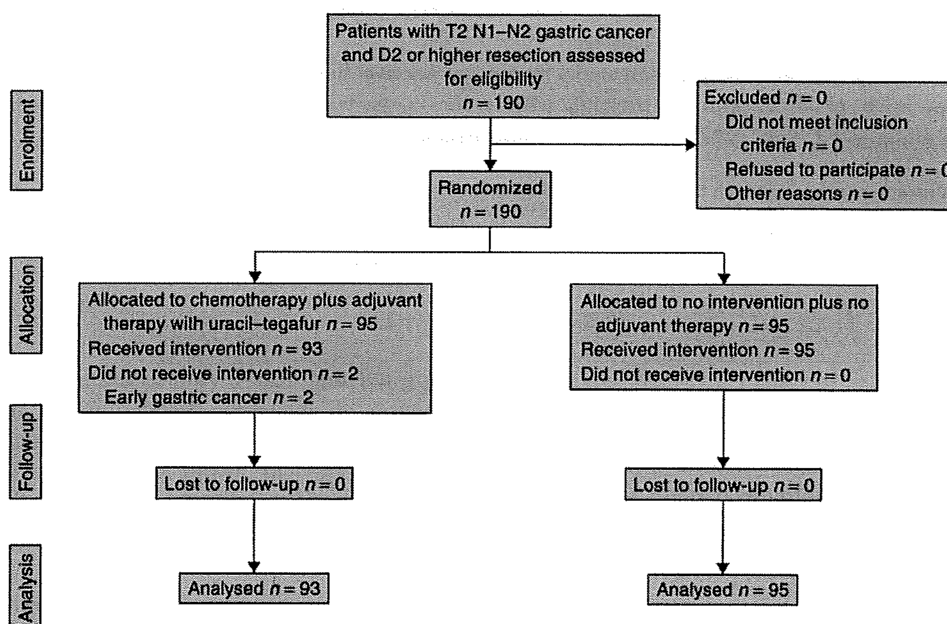

Fig. 1 CONSORT flow chart

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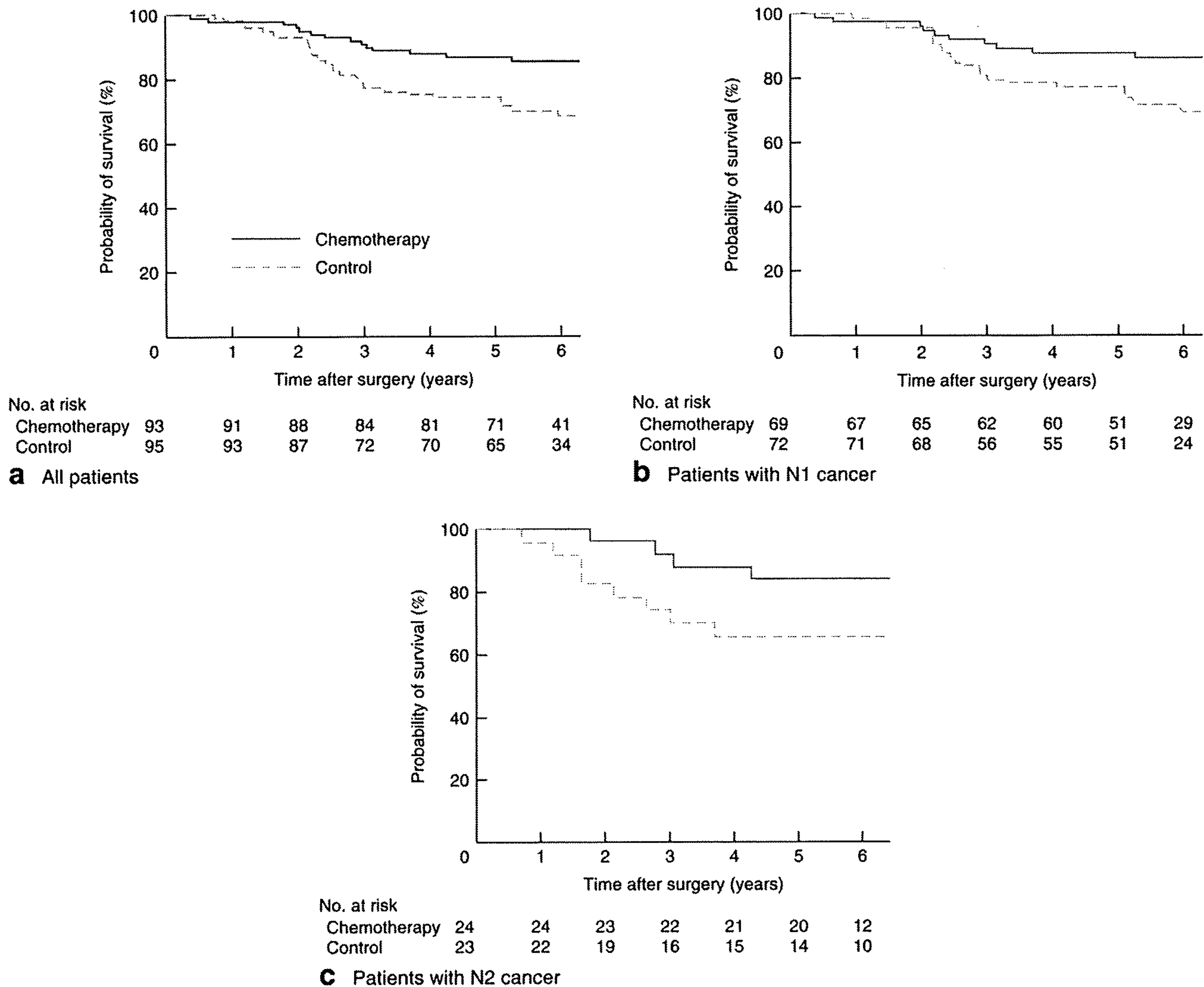

Fig. 2 Overall survival in **a** all 188 eligible patients, **b** 141 patients with N1 cancer and **c** 47 patients with N2 cancer. **a** $P = 0{\cdot}017$, **b** $P = 0{\cdot}061$, **c** $P = 0{\cdot}124$ (stratified log rank test)

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

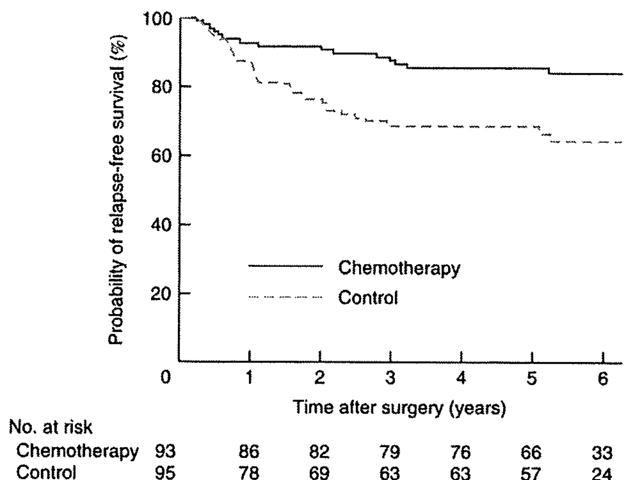

Fig. 3 Relapse-free survival in patients in the chemotherapy group compared with that in the control group. $P = 0{\cdot}005$ (log rank test)

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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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Case Report

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

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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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Key words: Wilms tumor gene; WT1; Cancer vaccine; Myelodysplastic syndrome (chronic myelomonocytic leukemia)

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

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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

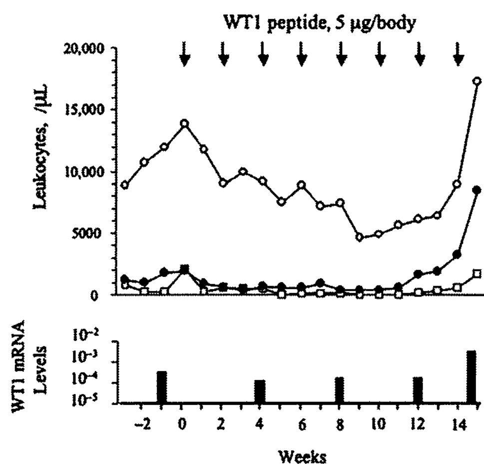

Figure 1. Clinical course of WT1 vaccination. Upper panel, the time courses for counts of white blood cells (open circles), monocytes (closed circles), and myelocytes plus metamyelocytes (open squares). Lower panel, relative levels of WT1 messenger RNA (mRNA) in the peripheral blood.

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%,

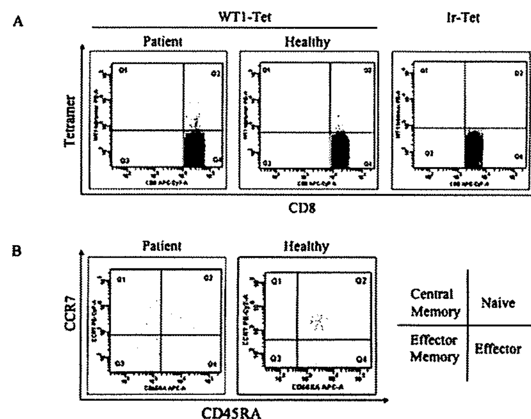

Figure 2. Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) with HLA-WT1 tetramer. A, PBMCs derived from the patient (left) and a healthy volunteer (center) were stained with phycoerythrin (PE)-conjugated HLA-A*2402–WT1 235-243 tetramer (WT1-Tet$^+$). The samples were stained with 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$^-$).

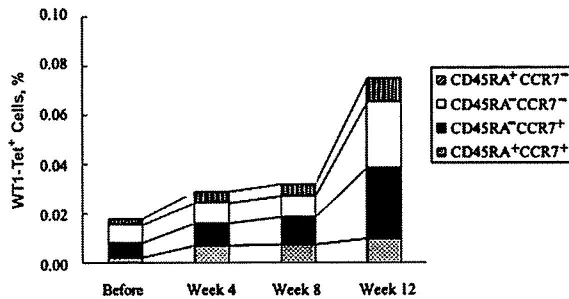

Figure 3. The percentage and differentiation status of WT1-Tet$^+$ CD8$^+$ T-cells before and after WT1 vaccination. The percentage of WT1-Tet$^+$ cells among the CD8$^+$ T-cells in the patient's peripheral blood mononuclear cells and their differentiation status were serially analyzed during the vaccination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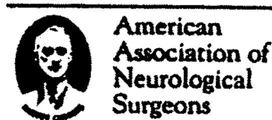

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Object. The object of this study was to investigate the safety and clinical responses of immunotherapy targeting the *WT1* (Wilms tumor 1) gene product in patients with recurrent glioblastoma multiforme (GBM).

Methods. Twenty-one patients with WT1/HLA-A*2402–positive recurrent GBM were included in a Phase II clinical study of WT1 vaccine therapy. In all patients, the tumors were resistant to standard therapy. Patients received intradermal injections of an HLA-A*2402–restricted, modified 9-mer WT1 peptide every week for 12 weeks. Tumor size, which was obtained by measuring the contrast-enhanced area on magnetic resonance images, was determined every 4 weeks. The responses were analyzed according to Response Evaluation Criteria in Solid Tumors (RECIST) 12 weeks after the initial vaccination. Patients who achieved an effective response continued to be vaccinated until tumor progression occurred. Progression-free survival and overall survival after initial WT1 treatment were estimated.

Results. The protocol was well tolerated; only local erythema occurred at the WT1 vaccine injection site. The clinical responses were as follows: partial response in 2 patients, stable disease in 10 patients, and progressive disease in 9 patients. No patient had a complete response. The overall response rate (cases with complete or partial response) was 9.5%, and the disease control rate (cases with complete or partial response as well as those in which disease was stable) was 57.1%. The median progression-free survival (PFS) period was 20.0 weeks, and the 6-month (26-week) PFS rate was 33.3%.

Conclusions. Although a small uncontrolled nonrandomized trial, this study showed that WT1 vaccine therapy for patients with WT1/HLA-A*2402–positive recurrent GBM was safe and produced a clinical response. Based on these results, further clinical studies of WT1 vaccine therapy in patients with malignant glioma are warranted.
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KEY WORDS • **cancer vaccine** • **glioblastoma multiforme** • **glioma** • **immunotherapy** • **Wilms tumor 1**

CURRENTLY, the standard treatment for gliomas is surgery, followed by external radiation and chemotherapy. In patients with newly diagnosed GBM, however, concurrent irradiation and temozolomide therapy, followed by adjuvant temozolomide therapy for at least 6 months, offered a modest benefit, with a median survival of 14.6 months and a 2-year survival rate of 26.5%.[22] To date, therapeutic options for patients with malignant glioma have been limited, and extensive research is ongoing.

Immune therapy against malignant gliomas includes several therapeutic approaches that involve dendritic cell–based immunotherapy and antibody-mediated immunotherapy.[31] Cancer vaccination is another novel form of therapy.[30] Recent advances in molecular biology and tumor immunology have resulted in the identification of a large number of tumor-associated antigens that could be used for cancer vaccination, since their epitopes associated with HLA Class I molecules were recognized by CTLs. One of the identified tumor-associated antigens was the product of the Wilms tumor gene, *WT1*.[17]

Abbreviations used in this paper: CTL = cytotoxic T-lymphocyte; DSMC = Data Safety Monitoring Committee; ECOG = Eastern Cooperative Oncology Group; FDG = fluorodeoxyglucose; GBM = glioblastoma multiforme; HLA = human leukocyte antigen; MR = magnetic resonance; PBMC = peripheral blood mononuclear cell; PET = positron emission tomography; PFS = progression-free survival; RECIST = Response Evaluation Criteria in Solid Tumors; SPECT = single-photon emission computed tomography; WHO = World Health Organization; WT1 = Wilms tumor 1.

The *WT1* gene was isolated as a gene responsible for Wilms tumor. It encodes a zinc finger transcription factor, which is involved in cell proliferation and differentiation, apoptosis, and organ development. Although the *WT1* gene was first categorized as a tumor suppressor gene, it was later proposed that the wild-type *WT1* gene functions as an oncogene rather than as a tumor-suppressor gene. In response to granulocyte colony–stimulation factor, growth promotion and differentiation inhibition were identified in wild-type *WT1* gene–transfected myeloid progenitor cells.[25] In many reports, the wild-type *WT1* gene was shown to be overexpressed in various types of solid tumors. The WT1 protein was found to be an attractive target antigen for immunotherapy against these malignancies.[20]

Recently, we performed a Phase I clinical trial to examine the safety of a WT1-based vaccine, as well as the clinical and immunological responses of patients with a variety of cancer types, including leukemia, lung cancer, and breast cancer.[19] The authors demonstrated that WT1 peptide vaccine emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at 2-week intervals was safe for patients other than those with myelodysplastic syndromes. Furthermore, the vaccination induced peptide-specific CTLs and was associated with clinical response. Very recently, it was confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3 mg per week) with the same adjuvant were also acceptable.[15]

An increasing number of central nervous system studies have reported that systemic immunotherapy is capable of inducing an antitumor response within the immunologically privileged brain.[29–31] These advances suggest the possibility of the development of a new peptide-based cancer immunotherapy. The blood–brain barrier, which was thought to be one of the hurdles hindering the development of therapeutically effective immunotherapy for gliomas, does not always function effectively in cases involving recurrent gliomas.[29]

Like many other solid tumors, gliomas have been found to express WT1 protein.[8] A definite correlation has been observed between WT1 expression and cellular proliferation activity, as indicated by WHO grade.[8] In the present study, we investigated the clinical responses to peptide-based immunotherapy targeting the *WT1* gene product in patients with recurrent GBMs. We also evaluated the correlation between the clinical response and the WT1 expression level in tumor tissues using immunohistochemical staining, as well as WT1-specific CTL frequencies (tetramer assay) in patients' PBMCs.

Clinical Materials and Methods

The WT1 Peptide

The immunization consisted of an HLA-A*2402–restricted, modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL), in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. About 60% of Japanese have HLA-A*2402 which is the most common HLA Class I type in the Japanese population. The modified 9-mer WT1 peptide was shown to induce much stronger CTL activity against WT1-expressing tumor cells than the natural peptide.[26] The WT1 peptide (GMP grade) was purchased from Multiple Peptide Systems as the lyophilized peptide.

Patient Population

Twenty-one patients were enrolled in this study. All patients seen in our unit who had recurrent or progressive GBM were eligible to be enrolled if their disease was resistant to conventional chemotherapy and radiotherapy. Patients who had refused chemotherapy but wanted to receive WT1 vaccine therapy under the auspices of this clinical trial were also eligible. In patients who received stereotactic radiosurgery as part of their initial therapy, true recurrence or progression was distinguished from radiation necrosis by metabolic imaging or histological confirmation.

Additional inclusion criteria were: 1) age between 16 and 80 years, 2) expression of WT1 in the glioma cells determined by immunohistochemical analysis, 3) HLA-A*2402–positivity, 4) estimated survival of more than 3 months, 5) ECOG Performance Status Grade 0–2, 6) no severe organ function impairment, and 7) the written informed consent of the patient. All enrolled patients had histologically proven GBM (Grade 4) based on the WHO criteria. After initial resection of the tumor, patients underwent a course of external radiation therapy and chemotherapy. Magnetic resonance imaging was used to monitor patients for recurrence or progression of their tumor during initial therapy and during maintenance therapy. No patient was treated with chemotherapy or radiotherapy during the 4 weeks prior to WT1 immunotherapy. Registered patients had methionine-PET, FDG-PET, thallium-SPECT, and MR imaging to confirm recurrence or progression and to exclude radiation necrosis. All patients underwent electrocardiography, and blood samples were obtained to confirm that there were no abnormalities.

After informed consent was obtained, it took at least 2 weeks for the immunohistochemical analysis, HLA-typing analysis, image analysis, and other tests to be completed. Therefore, the presence of tumor recurrence or progression was again assessed > 2 weeks after registration for WT1 treatment. The DSMC independently reviewed the eligibility of each enrolled patient. Protocol compliance, safety, and on-schedule study progress were also monitored by the DSMC. The WT1 peptide–based Phase II study was approved by the ethical review boards of the Osaka University Faculty of Medicine.

Vaccine Preparation and Vaccination

Patients received intradermal injections of 3.0 mg of HLA-A*2402–restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. The WT1 vaccinations were scheduled to be given weekly for 12 consecutive weeks. Twelve weeks after the initial vaccination, the response was evaluated on MR imaging. If an effect was observed after the 12 vaccinations, WT1 vaccination was continued at 1-week intervals (with the patients' informed consent) until tumor progression was again noted.

Immunohistochemical Analysis

Immunohistochemical analysis was performed to confirm WT1 protein expression in malignant glioma tissue using a procedure that has been previously described.[8] Brief-

ly, formalin-fixed tissue sections were prepared from the resected tumors. Sections were microwaved in citrate buffer for antigen retrieval and incubated with anti–human WT1 mouse monoclonal antibody 6F-H2 (DAKO; diluted 1:50). The WT1 reaction was visualized with the Vectastain ABC kit (Vector Laboratories) and diaminobenzidine (WAKO). The sections were then counterstained with hematoxylin. Control positive staining was evaluated with Wilms tumor, and control negative staining was evaluated with normal brain. Expression of WT1 seen in the sections was classified on a scale from 0 to 4 based on the staining density and the pattern of the glioma cells according to the following criteria: 0, negative staining; 1, slightly increased staining in some tumor cells compared with that in normal glial cells; 2, staining at intermediate intensity in some tumor cells; 3, strong staining in some tumor cells and intermediate staining in almost all tumor cells; and 4, greatly increased staining in almost all tumor cells compared with that in normal glial cells. Three investigators scored every sample independently; scores agreed upon by at least 2 investigators were accepted.

For MIB-1 immunostaining, antibody against the Ki 67 antigen (DAKO) was diluted 1:50 and used as previously described.[11] In each case, MIB-1 immunostaining was performed on the same serial sections used for WT1 immunohistochemical evaluation. The staining index reflecting each tumor's proliferation activity was determined by calculating the percentage of positively stained tumor cell nuclei out of 1000 evaluated tumor cell nuclei. All assessments were made in areas with the greatest degree of immunostaining.

*The WT1 peptide/HLA-A*2402 Tetramer Assay of WT1-Specific CTLs*

The WT1 (a natural, HLA-A*2402–restricted, 9-mer WT1 peptide)/HLA-A*2402 tetramer was kindly provided by M. Gotoh of Sumitomo Pharmaceuticals. This tetramer stained > 90% of the TAK-1 cells, which were WT1-specific CTLs that could recognize the complex of the natural 9-mer WT1 peptide and HLA-A*2402 molecules. The PBMCs from HLA-A*2402–positive patients were double-stained with PerCP-CD8 antibody (BD Pharmingen) and phycoerythrin tetramer. The cells were analyzed by fluorescence-activated cell sorting. A double-positive fraction was considered to represent WT1-specific CD8-positive CTLs.

Evaluation of Toxicity

Blood samples were evaluated every week. Toxicities were evaluated according to the US National Cancer Institute Common Toxicity Criteria and independently reviewed by the DSMC.

Evaluation of MR Images

Magnetic resonance imaging was performed every 4 weeks. After the WT1 vaccine was administered 12 times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on MR images. The tumor size, corresponding to the contrast-enhanced area on T1-weighted MR images, was measured and analyzed according to RECIST,[23] with results reported as complete response, partial response, stable disease, and progressive disease. The response rate was calculated as the percentage of the number of cases in which there was a complete or partial response divided by the total number of cases. The effective rate was calculated as the percentage of the number of cases in which there was a complete or partial response or stable disease divided by the total number of cases.

Additional Vaccinations and Calculation of the Survival Period

If an effect was observed after 12 vaccinations, further WT1 vaccination at 1-week intervals was given only with the patients' informed consent. The PFS period was calculated from the day of the first WT1 vaccination to the day of the last image prior to the detection of disease progression; this was used as the principal end point. The overall survival period after WT1 vaccination was also calculated, as was the overall survival period after tumor recurrence for WT1-vaccinated patients.

Statistical Analysis

Our main objectives were to evaluate the duration of PFS, the 6-month PFS rate, the overall response rate, the disease control rate, and toxicity based on the WHO criteria. The objective assessments of tumor response were reported using RECIST and were based on major changes in tumor size seen on Gd-enhanced MR images in comparison with the baseline images. Hematological and nonhematological toxicities were assessed using the US National Cancer Institute Common Toxicity Criteria, and the safety and tolerability of the treatment were estimated. Statistical evaluation was performed using Stat View version 4.5 (Abacus Concepts, Inc.). Probability values < 0.05 were considered statistically significant. The Kaplan–Meier method was used to analyze overall survival and PFS. The log-rank test was used to assess the strength of the association between survival time and single variables corresponding to factors that were considered prognostic for survival.

The required sample size for this Phase II trial was estimated to be 20 at 5% Type I and 20% Type II errors, under the assumption of 10 and 30% 6-month PFS rates for the null and alternative hypotheses, respectively. Allowing for the possibility that we might not be able to obtain complete data in all cases, the sample size was set at 21.

Results

Patient Characteristics

During the trial period, 37 patients were assessed for inclusion in the trial. All 37 had WT1-positive GBM, as determined by immunohistochemical analysis. Because we use HLA-A*2402–restricted WT1 peptide, 16 patients with HLA-A*2402–negative type were excluded, and 21 patients (7 women and 14 men) with HLA-A*2402–positive type were enrolled in the study (Table 1). In all the cases involving HLA-A*2402–negative excluded patients, the survival time from recurrence or progression to death was investigated. The median survival time after tumor recurrence in the HLA-A*2402–negative patients was 21 weeks, which was almost the same as that of the historical

TABLE 1

*Characteristics of and clinical results in all enrolled patients**

Case No.	Age (yrs), Sex	RT Dosage (Gy)	Chemo	Add'l Tx	Steroid Tx	KPS Score	Response	PFS (wks)	OS (wks)	WT1 Score
1	63, M	60	CE × 3	IFN	yes	50	SD	28.1	36.1	4
2	33, M	60	—	—	yes	60	PR	23.4	32.4	4
3	45, M	60	CE × 3	IFN	yes	70	PD	5.1	32.6	1
4	29, F	60	CE × 3, ACNU × 2	IFN	—	90	SD	16.0	30.1	2
5	69, M	60	—	IFN	—	80	PD	8.0	36.7	3
6	69, M	60	CE × 3	IFN	—	80	SD	24.4	106.1	3
7	42, M	50	—	—	—	60	SD	32.0	87.1	4
8	46, F	56	—	SRS	yes	60	SD	>96.0	>96.0	3
9	63, M	60	ACNU × 3	SRS	yes	80	PD	0	>87.3	4
10	67, M	60	ACNU × 3	IFN	—	90	PD	4.0	15.0	3
11	40, F	60	ACNU × 3	—	—	80	SD	51.3	69.4	3
12	76, M	60	ACNU × 3	IFN	yes	70	SD	21.1	>79.4	1
13	54, M	50	CE × 3	IFN	yes	50	PD	4.0	18.4	2
14	55, M	60	CE × 3	IFN	—	90	PD	2.0	28.4	2
15	58, F	60	CE × 3	IFN	—	90	SD	42.4	61.7	3
16	20, F	60	ACNU × 2	—	—	90	PR	20.0	29.3	4
17	42, M	60	—	—	—	90	PD	4.3	35.6	3
18	41, M	60	CE × 3, ACNU × 2	SRS	yes	100	SD	>43.6	>43.6	3
19	54, M	60	ACNU × 3	IFN	yes	90	PD	8.0	>41.6	2
20	58, F	50	—	SRS	—	50	SD	>32.1	>32.1	4
21	55, F	60	—	—	yes	100	PD	0	>31.4	4

* ACNU = nimustine hydrochloride; Add'l = additional; CE = carboplatin and etoposide; Chemo = chemotherapy; IFN = β-interferon; KPS = Karnofsky Performance Scale; OS = overall survival; PD = progressive disease; PR = partial response; RT = radiotherapy; SD = stable disease; SRS = stereotactic radiosurgery; Tx = therapy; — = not administered.

control patients at Osaka University Hospital (20 weeks, data not shown). The mean age of the 21 enrolled HLA-A*2402–positive patients was 51.4 years (range 20–76 years). Of the 21 patients, 15 had recurrent disease and 6 had disease progression after initial therapy. All patients had radiotherapy with or without chemotherapy or interferon treatment. All enrolled patients had an ECOG performance status of 0–2 (Karnofsky Performance Scale score > 50), and 10 of them were receiving a maintenance dose (1–4 mg/day betamethasone) of glucocorticoid therapy at the time of vaccination due to local symptoms or symptoms of increased intracranial pressure caused by edema in the area around the tumor. Eight patients underwent surgery after recurrence for mass reduction and confirmed recurrence, and methionine-PET, thallium-SPECT, and FDG-PET were performed in all cases to confirm tumor recurrence.

Clinical Response to Vaccination

All treated patients had a local inflammatory response with erythema at the WT1 vaccine injection site. No Grade 3 or 4 toxicities were observed. Liver dysfunction was detected in Case 9, but improved after the patient's anticonvulsant therapy was changed. This event was considered by the DSMC and was judged to have had no relationship to the WT1 treatment.

A summary of patient responses to WT1 immunotherapy is shown in Table 1. Clinical responses included partial response in 2 patients; stable disease in 10 patients; and progressive disease in 9 patients, including 2 who dropped out of the trial due to tumor progression and poor general condition (Cases 10 and 13). Patients who had an effective response continued to receive vaccinations until tumor progression was demonstrated. All responses were assessed by the DSMC.

The overall response rate was 9.5%. The disease control rate, calculated from the number of patients with complete response, partial response, or stable disease in the initial 3 months (the clinical trial period) was 57.1%. The Kaplan–Meier survival probability curves are shown in Fig. 1. Median PFS in the 21 patients with GBM who were included in the study was 20.0 weeks, and the PFS rate at 6 months (26 weeks) was 33.3%. Median overall survival after initial vaccination was 36.7 weeks. Median overall survival after tumor recurrence in WT1-vaccinated patients was 46 weeks.

Two patients (Case 2 and Case 16) experienced partial response. In both cases, immunohistochemical analysis of the tumor specimens showed high WT1 expression levels, but neither patient survived for a long period (PFS of 23.4 weeks in Case 2 and 20.0 weeks in Case 16). Both patients had disease progression after the 12-week trial period, with leptomeningeal dissemination of the glioma cells and formation of a mass at a different site.

In contrast, in the stable disease group 4 patients (Cases 8, 11, 15, and 18) experienced gradual tumor stabilization; that is, they had a response during the late period of the 3-month WT1 vaccination course. These patients survived for a long time without progression (PFS > 96.0 weeks in Case 8, 51.3 weeks in Case 11, 42.4 weeks in Case 15, and > 43.6 weeks in Case 18).

Relationship Between PFS and WT1-Immunostaining Intensity

In all 21 patients, immunostaining was positive for WT1. The WT1 expression score was 4 in 7 cases, 3 in 8 cases, 2

in 4 cases, and 1 in 2 cases (Table 1). Figure 2 shows representative photomicrographs of Score 2 (Fig. 2A), Score 3 (Fig. 2B), and Score 4 (Fig. 2C) WT1 immunostaining, and Fig. 2D shows MIB-1-immunostaining of a section from the same lesion as Fig. 2C. Both of the patients who had a partial response to vaccination had Score 4 immunostaining. The patients were grouped according to WT1 expression scores, and PFS curves were estimated for each group and then compared. The patients with Score 3 immunostaining tended to have the longest PFS time. The patients with Score 3 or 4 had a statistically longer PFS time than the patients with Score 1 or 2 ($p = 0.0020$, Fig. 3 *right*). Among the patients with high WT1-immunostaining scores (3 and 4), the patients with Score 4 had a shorter PFS time than those with Score 3, although partial response was achieved in 2 patients with Score 4. This might reflect the fact that the patients with Score 4 had high proliferation activity of the GBM cells that was recognized by the high MIB-1 staining index, although they also had the highest amount of target WT1 protein recognized by the induced WT1-specific CTLs.

Relationship Between PFS and MIB-1 Staining Index

The MIB-1 staining index, which reflects each tumor's proliferation activity, was determined by calculating the percentage of positively stained tumor cell nuclei. No statistical difference in PFS was observed between the 2 groups (Fig. 3 *left*). The proliferation activity was found not to directly affect PFS after WT1 vaccination.

Evaluation of WT1-Specific CTL Frequencies in PBMCs

The frequencies of WT1-specific CTLs before WT1 vaccination were significantly higher in patients with GBM than in healthy controls ($p = 0.0019$, Fig. 4). These results indicate that the immune system in patients with WT1-expressing GBM cells responded to the WT1 protein derived from the tumor cells and elicited WT1-specific CTLs that were present before WT1 vaccination; this suggests that the WT1 protein in GBM cells is naturally immunogenic. The existence of the high frequencies of WT1-specific CTLs before WT1 vaccination may have contributed to the favorable clinical responses in patients with GBM. There was no correlation between the induction of a clinical response and WT1-specific CTL frequencies in the PBMCs of the patients prior to vaccination (Fig. 4). Furthermore, the CTL frequencies did not increase after vaccination, even in the patients who responded.

Discussion

The WT1 gene is physiologically expressed in some organs, such as the kidneys, bone marrow, and pleura. Experimental evidence shows that WT1-specific CTLs kill WT1-expressing tumor cells without killing normal cells.[24] Consistent with these data, in the present study, patients with a clinical response had adverse effects of the WT1 vaccination that were limited to local erythema at the injection sites of the WT1 vaccine.

The primary end points of this study were PFS and the PFS rate at 6 months. The objective response rate and the disease control rate with WT1 vaccination, as well as its safety and tolerability, were also estimated.

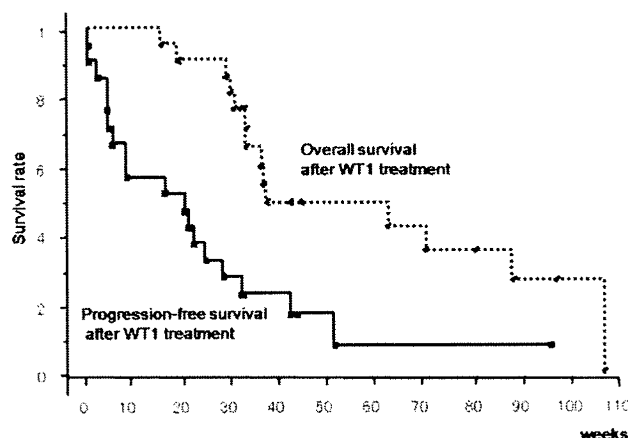

Fig. 1. Kaplan–Meier curves for PFS *(solid line)* and overall survival *(dotted line)* after initial WT1 vaccination for patients with recurrent GBM.

A review of the literature suggested that an agent demonstrating a 6-month progression-free survival rate ≥ 10% would be considered active.[9] A retrospective analysis of 8 Phase II chemotherapy trials conducted from 1986 to 1995 and involving a total of 225 patients with GBM was performed at the M. D. Anderson Cancer Center; a median PFS of 9 weeks and a 6-month PFS rate of 15% were reported.[28] Temozolomide, the most recent drug to be introduced for the treatment of GBM, has been shown to produce results that were not very different from those achieved with carmustine (BCNU). A study that included a series of 112 patients with GBM demonstrated a response rate of 6% with a 6-month PFS rate of 21%;[32] another study, which included a series of 138 patients with GBM, demonstrated a response rate of 8% and a 6-month PFS rate of 18%.[3] The use of BCNU chemotherapy in recurrent GBM was also recently studied; the median time to progression was 13.3 weeks, and the 6-month PFS rate was 17.5%.[4] Following these reports, 6-month PFS rates for the null and alternative hypotheses were assumed to be 10 and 30%, respectively, in this trial, and the sample size was set at 21.

In our study, the median duration of PFS was 20.0 weeks, and the PFS rate at 6 months was 33.3%. The response rate was 9.5%, whereas the disease control rate was 57.1%. The 6-month PFS rate was 33.3% in our patients with GBM—which was higher than the 10% that was set as indicating an active level—and, moreover, was higher than the 30% that was set as the alternative hypothesis before the study was started. Thus, this result suggested that WT1 vaccination was active. The median PFS and median overall survival after WT1 vaccination were 20.0 weeks and 36.7 weeks, respectively; these results are comparable to those reported in the literature for various combination regimens of chemotherapy and/or radiotherapy.

All the treated patients had an inflammatory response with erythema at the WT1 vaccine injection site, but no systemic toxicities were observed. Taken together, these findings allow one to conclude that WT1 vaccination had an anti-GBM effect, it was safe, and the patients tolerated it well.

Although the response rate in our study (9.5%) was not