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Cellular and Humoral Immune Responses Induced by an HLA Class I–restricted Peptide Cancer Vaccine Targeting WT1 Are Associated With Favorable Clinical Outcomes in Advanced Ovarian Cancer

Sumiyuki Nishida, Soyoko Morimoto,† Yusuke Oji,‡ Satoshi Morita,§ Toshiaki Shirakata,‖ Takayuki Enomoto,¶# Akihiro Tsuboi,‖ Yutaka Ueda,¶ Kiyoshi Yoshino,¶** Alzaaqi Shouq,‡ Mizuki Kanegae,‡ Satoshi Ohno,‖†† Fumihiro Fujiki,‡‡ Hiroko Nakajima,‡‡ Yoshiki Nakae,* Jun Nakata,‡ Naoki Hosen,† Atsushi Kumanogoh,*§§‖‖ Yoshihiro Oka,†§§ Tadashi Kimura,¶ and Haruo Sugiyama‡‡*

Summary: The HLA-A*24:02–restricted peptide vaccine targeting Wilms' tumor 1 (WT1) (WT1 vaccine) is a promising therapeutic strategy for ovarian cancer; however, its efficacy varies among patients. In this study, we analyzed WT1-specific immune responses in patients with advanced or recurrent ovarian cancer that was refractory to standard chemotherapies and their associations with clinical outcomes. In 25 patients, the WT1 vaccine was administered subcutaneously weekly for 3 months and biweekly thereafter until disease progression or severe adverse events. We assessed Wilms' tumor 1–specific cytotoxic T lymphocytes (WT1-CTLs) and Wilms' tumor 1 peptide-specific immunoglobulin G ($WT1_{235}$-IgG). After vaccination, the percentage of tetramer high-avidity population of WT1-CTLs among $CD8^+$ T lymphocytes (%tet-hi WT1-CTL) and the $WT1_{235}$-IgG titer increased significantly, although the values were extremely low or below the limit of detection before vaccination (%tet-hi WT1-CTL: 0.003%–0.103%.; $WT1_{235}$-IgG: <0.05–0.077 U/mL). Patients who had %tet-hi WT1-CTL of $\geq$0.25% (n = 6) or $WT1_{235}$-IgG of $\geq$0.10 U/mL (n = 12) had a significantly longer progression-free survival than those of patients in the other groups. In addition, an increase in $WT1_{235}$-IgG corresponded to a significantly longer progression-free survival ($P = 0.0496$). In patients with systemic inflammation, as evidenced by elevated C-reactive protein levels, the induction of tet-hi WT1-CTL or $WT1_{235}$-IgG was insufficient. Decreased serum albumin levels, multiple tumor lesions, poor performance status, and excess ascites negatively influenced the clinical effectiveness of the WT1 vaccine. In conclusion, the WT1 vaccine induced antigen-specific cellular and humoral immunity in patients with refractory ovarian cancer. Both %tet-hi WT1-CTL and $WT1_{235}$-IgG levels are prognostic markers for the WT1 vaccine.

Key Words: ovarian cancer, WT1, immunotherapy, vaccine, immune response

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From the Departments of *Respiratory Medicine and Clinical Immunology; †Cancer Stem Cell Biology; ‡Functional Diagnostic Science; ‖Cancer Immunotherapy; ‡‡Cancer Immunology; ¶Obstetrics and Gynecology, Osaka University Graduates School of Medicine; §§Department of Immunopathology, Immunology Frontier Research Center, Osaka University; ‖‖Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University, Suita, Osaka Prefecture; §Department of Biomedical Statistics and Bioinformatics, Kyoto University Graduate School of Medicine, Kyoto; #Department of Obstetrics and Gynecology, Niigata University Medical School, Niigata; **Department of Obstetrics and Gynecology, University of Occupational and Environmental Health, Kita-Kyushu, Fukuoka Prefecture; and ††Clinical Research Support Center, Shimane University Faculty of Medicine, Izumo, Shimane Prefecture, Japan.

S.N.: helped in conceptualization, methodology, investigation, validation, data curation, formal analysis, visualization, writing—original draft, and funding acquisition. S. Morimoto: helped in methodology, investigation, validation, and writing—original draft. Y. Oji.: helped in methodology, investigation, validation, and writing—review and editing. S. Morita: helped in methodology, formal analysis, and writing—review and editing. T.S., S.O., A.S., M.K., H.N., Y.N., J.N., and N.H.: helped in the investigation. T.E., Y.U., and K.Y.: helped in resources, investigation, and writing—review and editing. A.T.: helped in investigation and writing—review and editing. F.F.: helped in methodology and investigation. A.K.: helped in supervision. Y. Oka: helped in methodology, investigation, and writing—review and editing. T.K.: helped in resources and supervision. H.S.: helped in conceptualization, methodology, and supervision.

Reprints: Sumiyuki Nishida, Department of Respiratory Medicine and Clinical Immunology, Osaka University Graduate School of Medicine, 2-2, Yamada-oka, Suita 565-0871, Osaka Prefecture, Japan (e-mail: sumiyuki-n@imed3.med.osaka-u.ac.jp).

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Active cancer immunotherapy eradicates cancer cells via cytotoxic effects on immune cells. To improve prognosis, improvements in the strategy and the identification of biomarkers to confirm or predict clinical effects are essential.

Cancer immunotherapy is a particularly attractive strategy for ovarian cancer owing to its immunogenicity.[1] Tumor-infiltrating lymphocytes and immunoreactive gene signatures have been detected in ovarian cancer and are associated with prolonged survival.[2,3] Histologically, ovarian cancer cells often express ligands for programmed cell death 1, and $CD8^+$ T cells infiltrate the tumor microenvironment.[2,4] These results indicate that immune checkpoint blockades (ICBs), such as nivolumab, may be beneficial in ovarian cancer; however, the clinical efficacy of these monotherapies range from mild to modest.[5–7] Accordingly, combinations of ICBs and various immunomodulatory

treatments, including cancer vaccines, adoptive cell therapy, and oncolytic virus-based therapy, have been evaluated.[8,9]

Ovarian cancer tissues express tumor-associated antigens (TAAs).[10–12] Wilms' tumor 1 (WT1) is a promising TAA targeted in therapeutic vaccines for ovarian cancer owing to its high expression and roles in tumorigenesis.[13–15] The National Cancer Institute considers WT1 to be the highest priority TAA for cancer immunotherapy.[16] WT1 expression in ovarian cancer is a poor prognostic factor.[17] Therefore, active immunotherapies targeting WT1 for ovarian cancer have been developed.[18–20]

We have previously performed a phase I/II study of an HLA-A*24:02–restricted peptide-based cancer vaccine targeting the *WT1* gene product (WT1 vaccine) for patients with solid malignancies refractory to conventional therapies since 2004.[21] In the phase II part of our study, 40 patients with advanced or recurrent gynecologic malignancies (the gynecologic malignancy cohort), including 24 patients with ovarian cancer[18] were enrolled, wherein delayed-type hypersensitivity (DTH) to the WT1 peptide was used for immune monitoring after vaccination. We found that DTH-positive patients had a longer survival time than that of DTH-negative patients. In patients with ovarian cancer, the DTH positivity rate was around 70%, which was higher than those reported in other cancers (eg, ~50% in a gastrointestinal malignancy cohort, unpublished data). We observed that some of DTH-positive patients continued to receive the WT1 vaccine and survived for a long time, while others did not obtain a clinical benefit from the WT1 vaccine. These results implied that DTH is not an effective predictive marker for the efficacy of the WT1 vaccine, at least in ovarian cancer.

We have recently reported that assessments of Wilms' tumor 1 peptide-specific cytotoxic T lymphocytes (WT1-CTL) and Wilms' tumor 1 peptide-specific immunoglobulin (Ig) G antibodies (WT1-IgG) during WT1 vaccine therapy can help predict clinical outcomes after vaccination in patients with pancreatic cancer or glioblastoma.[22–24] These parameters directly reflect the immune responses specific to the WT1 peptide; the former is an indicator of cellular immunity, and the latter is an indicator of humoral immunity. In particular, WT1-CTL, which is usually analyzed by the HLA class I–restricted peptide tetramer assay, is an indicator of the antigen-specific cytotoxic immune response. The production of WT1-IgGs may indicate antigen-specific helper T-cell activity because isotype class switching from IgM to IgG depends on the helper T-cell response.[25] However, the generalizability of these markers to other cancer types, including ovarian cancer, has not been established to date. Therefore, in this report, we assessed the immune response induced by the WT1 vaccine in patients with advanced ovarian cancer and focused on the association between vaccine effectiveness and these antigen-specific immune responses. We also identified several interesting patient characteristics associated with the clinical effectiveness of the WT1 vaccine and immune responses. Our results could be beneficial for the development of immune-based treatments for ovarian cancer.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from all patients. Patients with advanced or recurrent gynecologic malignancies refractory to standard chemotherapy were recruited for the noncomparative, open-label, phase II study (Trial Registration ID: UMIN000002001), as previously described.[18] Other major eligibility criteria were as follows: HLA-A*24:02 positivity; WT1 expression in tumor cells; measurable disease as defined by Response Evaluation Criteria in Solid Tumor (RECIST) criteria; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; age of 20–79 years; life expectancy of 3 months or longer; and adequate organ function.

Eligible patients with ovarian cancer, fallopian tube cancer, or primary peritoneal cancer, all of which were defined as ovarian cancer, were selected from the gynecologic malignancy cohort who had blood samples taken at least at baseline and 1 month after vaccination. The percentage of the Japanese population with HLA-A*24:02 allele was ~60%.

Treatment Schedule and Sample Collection

The WT1 vaccine was prepared according to a previously reported method.[26] The WT1 vaccine was a water-in-oil emulsion product composed of 3 mg of an HLA-A*24:02–restricted, modified, 9-mer WT1 peptide (CYTWNQMNL; mp235; Peptide Institute Inc., Osaka, Japan) and incomplete Freund's adjuvant (Montanide ISA51VG; Seppic, Paris, France). An mp235 peptide was produced according to the Good Manufacturing Practice guidelines. The WT1 vaccine was intradermally administered at 6 different sites (bilateral upper arms, lower abdomen, and femoral regions) weekly for 3 months, and then biweekly until any of the following occurred: disease progression, unacceptable adverse events, or patient withdrawal of consent (Supplementary Fig. S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A644). Peripheral blood mononuclear cells and serum samples were collected before vaccination and at 1, 2, and 3 months after the start of vaccination and were cryopreserved until use.

Assessment of WT1-CTLs: Tetramer Assay

WT1-CTLs were analyzed by a tetramer assay, as described previously.[22] Frozen peripheral blood mononuclear cells were thawed and incubated at 37°C for 1 hour in X-VIVO 15 medium (Lonza Inc., Walkersville, MD) supplemented with 10% human AB serum (Gemini Bio-Products, West Sacramento, CA). Then, the cells were incubated with Clear Back (MBL Co. Ltd., Nagoya, Japan) in phosphate-buffered saline containing 5% fetal bovine serum and 0.02% sodium azide (FACS buffer) at 25°C for 5 min, stained using PE-labeled HLA-A*24:02/$WT1_{m235}$ peptide tetramer (WT1 tetramer) (MBL) at 4°C for 1 hour, and further stained with anti-CD3, CD8, and CD4 antibodies (BD Biosciences, San Jose, CA) at 4°C for 25 minutes. Next, the cells were washed with phosphate-buffered saline 3 times, resuspended in an appropriate volume of FACS buffer, and incubated with 7-AAD (eBioscience, San Diego, CA) for 5 minutes before analysis. The cells were analyzed on a FACS Aria (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Ashland, OR). The frequency of WT1-CTLs (%WT1-CTLs) was defined as WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T cells/CD3$^+$ CD8$^+$ T cells. The mean fluorescence intensity—high population of WT1-CTLs, was defined as the tetramer high-avidity population of CD8$^+$ T cells (tet-hi WT1-CTLs).[27,28]

Assessment of WT1 Peptide-specific Immunoglobulin

The production of IgG antibody against $WT1_{235}$ peptide ($WT1_{235}$-IgG) was analyzed by an enzyme-linked immunosorbent assay, as described previously.[23] In particular, 0.2 μg of

TABLE 1. Patient Characteristics at Baseline

	Total Number = 25 [n (%)]
Age (y)	
Median (minimum, maximum)	58.0 (35, 76)
PS (ECOG)	
0	14 (56.0)
1	8 (32.0)
2	3 (12.0)
Primary lesion	
Ovary	20 (80.0)
Fallopian tube	3 (12.0)
Peritoneum	2 (8.0)
Histologic type	
High-grade serous carcinoma	17 (68.0)
Clear cell carcinoma	3 (12.0)
Undifferentiated carcinoma	3 (12.0)
Endometrioid carcinoma	1 (4.0)
Others	1 (4.0)
Time from the first diagnosis (mo)	
Median (minimum, maximum)	38 (10, 119)
Maximum tumor diameter	
≤ 50 mm	15 (60.0)
> 50 mm (cystic)	4 (16.0)
> 50 mm (solid)	6 (24.0)
Ascites*	
None	17 (68.0)
Mild	5 (20.0)
Moderate to severe	3 (12.0)
No. tumor lesions or metastatic organs	
1	7 (28.0)
2	9 (36.0)
3	7 (28.0)
4	2 (8.0)
Metastatic organ†	
Liver	9 (36.0)
Spleen	1 (4.0)
Lung	1 (4.0)
Peritoneal dissemination	
No	5 (20.0)
Yes	20 (80.0)
CA125 (U/mL)	
Median (10%, 90%)	532 (14.8, 4670.4)
Albumin, g/dL	
Median (10%, 90%)	4.0 (3.28, 4.60)
CRP (mg/dL)	
Median (10%, 90%)	0.2 (< 0.04, 8.09)
Neutrophils (/μL)	
Median (10%, 90%)	3100 (1610, 6880)
Lymphocytes (/μL)	
Median (10%, 90%)	1310 (760, 1930)
Neutrophil/lymphocyte ratio	
Median (10%, 90%)	2.73 (1.18, 5.06)
$CD3^+CD4^+$ T cells (%)	
Median (10%, 90%)	65.5 (45.7, 77.1)
$CD3^+CD8^+$ T cells (%)	
Median (10%, 90%)	29.9 (16.5, 44.1)
$CD4^+/CD8^+$ ratio (%)	
Median (10%, 90%)	2.16 (1.05, 4.88)

*Severity of ascites is defined as follows: mild, limited to a pelvic cavity or Morrison fossa; moderate, beyond the pelvic cavity; severe, occupy the entire peritoneal cavity.

†Lymph node metastases and peritoneum dissemination are excluded.

CA125 indicates carbohydrate antigen 125; CRP, C-reactive protein; ECOG, Eastern Cooperative Oncology Group; PS, performance status.

$WT1_{235–252}$ peptide (CMTWNQMNLGATLKGVAAPKK), modified by the additional PKK sequence at the C-terminus to increase solubility, was covalently linked to each well of a 96-well plate using a Peptide Coating Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. After blocking, serum diluted at 1:100 was added to peptide-coated or peptide-noncoated wells and incubated overnight at 4°C. Bound $WT1_{235}$-IgG was detected using horseradish peroxidase–conjugated rabbit anti-human IgG antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Dallas, TX) as the second and third antibodies, respectively. Absorbance was measured at 450 nm. All samples were measured in duplicates. We defined $WT1_{235}$-IgG levels as: average absorbance value of peptide-coated wells−average absorbance value of the corresponding uncoated wells.

Assessment of the Clinical Effectiveness of the WT1 Vaccine and Treatment-related Adverse Events (TRAEs)

Computed tomography imaging was performed monthly during the first 3 months of treatment and then at 1-month to 2-month intervals until discontinuation to assess the clinical effectiveness of the WT1 vaccine. Tumor response was defined by an investigator assessment according to RECIST, version 1.0. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE, version 3.0). TRAEs were defined as adverse events that were definitely, probably, or possibly related to the WT1 vaccine. The severity of the local skin reaction at the vaccine injection site was defined as mild (redness and induration), moderate (small vesicle formation), or severe (ulceration or infection).

Statistical Analyses

The nonparametric Wilcoxon signed-rank test was used to calculate the significance of temporal changes in immune parameters. Progression-free survival (PFS) was defined as the time from the start of vaccination to disease progression or death due to any cause. PFS was analyzed using the Kaplan-Meier method, and differences in PFS between 2 groups were compared using the log-rank test. The relative hazard ratios (HRs) were estimated using a Cox proportional-hazards model. A dynamic prediction analysis was used to explore the associations of serial change over time in %tet-hi WT1-CTLs and $WT1_{235}$-IgG levels with PFS.[29] The Fisher extract test was used to calculate *P*-values for the association between the 2 parameters. A *P*-value <0.05 was considered significant. Statistical analyses were performed using JMP Pro, version 13 for Windows (SAS Institute Inc., Cary, NC), and the R package Dynpred (Core Team: R, 2014).

RESULTS

Patient Characteristics

From September 2004 to January 2013, a total of 50 patients with advanced or recurrent gynecologic malignancies refractory to standard chemotherapies were enrolled in the phase II study of the WT1 vaccine (ie, the gynecologic malignancy cohort). These 50 patients consisted of the 40 reported in our previous paper[18]; the remaining 10 were subsequently enrolled. Of the 50 patients, 30 had ovarian cancer, 25 of which were selected to analyze the antigen-specific immune response after vaccination (Supplementary Fig. S2, Supplemental Digital Content 2, http://links.lww.com/JIT/A645). Four of the remaining 5 patients were excluded from the analyses because they discontinued the study treatment without any assessments

TABLE 2. Study Treatment and Response to Therapy

		Histology		
	Total (N = 25)	**Serous Type (n = 17)**	**Nonserous Type (n = 8)**	***P****
No. vaccination				
Median (minimum–maximum)	15 (5–61)	13 (5–61)	15.5 (8–26)	0.5593
Completion of 3-mo vaccination [n (%)]	19 (76.0)	12 (70.6)	7 (87.5)	0.6237
Reason for discontinuation (n)				
Disease progression	6	5	1	—
Adverse events	0	0	0	
Response to therapy [n (%)]				
CR/PR	0 (0.0)	0 (0.0)	0 (0.0)	0.6608
SD	9 (36.0)	7 (41.2)	2 (25.0)†	
PD	16 (64.0)	10 (58.8)	6 (75.0)	

*P-value was calculated using an appropriate test method to compare serous and nonserous types.
†The histology of them was undifferentiated carcinoma.
CR/PR indicates complete response/partial response; PD, progressive disease; SD, stable disease.

of their immune response after vaccination due to early disease progression (n = 3) or adverse events of grade 2 hepatic toxicities unrelated to the WT1 vaccine and rapid exacerbation of cancerous pain (n = 1). Another patient was excluded because no blood samples after vaccination were collected from them. Table 1 summarizes the patient characteristics at baseline.

Treatment Course and Clinical Effects

Table 2 summarizes the treatment course and clinical effects. The median number of vaccinations received was 15. Of the 25 patients, 19 (76.0%) completed the 3-month vaccination schedule, whereas the other 6 patients discontinued the study treatment within 3 months due to disease progression, including the rapid exacerbation of clinical symptoms. Nine patients (36.0%) showed disease stabilization for > 3 months, although no patients achieved a complete or partial response. There were no significant differences in the total number of vaccinations, completion of the 3-month vaccination schedule, or disease control rate between histologic types [eg, high-grade serous carcinoma (HGSC) or non-HGSC].

In all patients, the median PFS was 73 days [95% confidence interval (CI): 55–108 d], and the PFS rates at 3 and 6 months were 48.0% and 21.8%, respectively (Supplementary Fig. S3A, Supplemental Digital Content 3, http://links.lww.com/JIT/A646). An immune assessment using DTH to the WT1 peptide after vaccination was available for 23 of the 25 patients. The median PFS did not differ significantly between DTH-positive patients (n = 18, 82 d) and others (n = 7, 56 d) ($P = 0.4706$) (Supplementary Fig. S3B, Supplemental Digital Content 3, http://links.lww.com/JIT/A646), suggesting that DTH may not be an effective marker for predicting PFS in patients with ovarian cancer.

Analyses of WT1-specific Immune Responses: Induction of WT1-CTLs and Production of $WT1_{235}$-IgG

The induction of WT1-specific immunity is the primary immunologic goal of the WT1 vaccine. We evaluated both (1) WT1-CTL frequencies in peripheral blood and (2) serum $WT1_{235}$-IgG levels as WT1-specific immune responses. We used the WT1 tetramer assay to assess WT1-CTLs in $CD8^+$ T cells (Fig. 1A). The median percentage of WT1-CTLs increased from 0.27% [10th percentile (10%), 90th percentile (90%) (0.09, 1.25)] before vaccination to 0.68% [(10%, 90%) (0.18, 3.50)] after vaccination (Fig. 1B). These differences were statistically significant ($P = 0.0017$). Majority of the patients (14/25; 56.0%) showed a > 2-fold increase in %WT1-CTL 1 month after the start of vaccination, and the median-fold increase in %WT1-CTL was 2.17 [(10%, 90%) (0.43, 10.82)].

We noticed that tet-hi WT1-CTLs emerged after vaccination (Fig. 1A, Supplementary Fig. S4, Supplemental Digital Content 4, http://links.lww.com/JIT/A647). All patients had no or only a few tet-hi WT1-CTLs at baseline. After vaccination, 15 patients showed the induction of tet-hi WT1-CTLs to various degrees, while 10 patients maintained no or few tet-hi WT1-CTLs (Fig. 1C). The median frequency of tet-hi WT1-CTLs increased from 0.003% [(10%, 90%) (0.001, 0.029)] before vaccination to 0.103% [(10%, 90%) (0.013, 0.594)] after vaccination (Fig. 1C). These increases were statistically significant ($P < 0.0001$). The median-fold increase was 19.4 [(10%, 90%) (2.5, 225)]. We further evaluated the time series of tet-hi WT1-CTLs 2 and 3 months after vaccination. In general, %tet-hi WT1-CTL peaked 1 month after the start of vaccination and then declined (Supplementary Fig. S5, Supplemental Digital Content 5, http://links.lww.com/JIT/A648). Patients with %tet-hi WT1-CTL of ≥ 0.25% at 1 month, within the upper quartile, still had higher %tet-hi WT1-CTL values at later time points than those of the patients showing low %tet-hi WT1-CTL (Fig. 1D). The median %tet-hi WT1-CTL in the former and the latter groups were 0.095% and 0.022%, respectively, at 2 months and 0.067% and 0.018%, respectively, at 3 months. These differences were statistically significant (2 mo, $P = 0.0005$; 3 mo, $P = 0.0057$).

We evaluated the production of $WT1_{235}$-IgG by enzyme-linked immunosorbent assay. Before vaccination, the $WT1_{235}$-IgG levels were below the detection sensitivity (< 0.05 U/mL) in all patients except for 1 (Fig. 1E). In 12 patients, the titers became detectable at 2 months, with further increases during the vaccination period (Supplementary Fig. S6, Supplemental Digital Content 6, http://links.lww.com/JIT/A649). The median value for the maximum $WT1_{235}$-IgG after vaccination was 0.077 U/mL [(10%, 90%) (< 0.05, 0.930)] ($P < 0.0001$) (Fig. 1E). In 12 of the 25 patients (48.0%), $WT1_{235}$-IgG levels increased to above 0.10 U/mL, whereas $WT1_{235}$-IgG levels remained below 0.05 U/mL in 9 patients (36.0%) (Fig. 1E).

Association Between the Induction of the WT1-specific Immune Response and Clinical Outcomes

We have reported an association between the induction of immune parameters representing antigen-specific immune

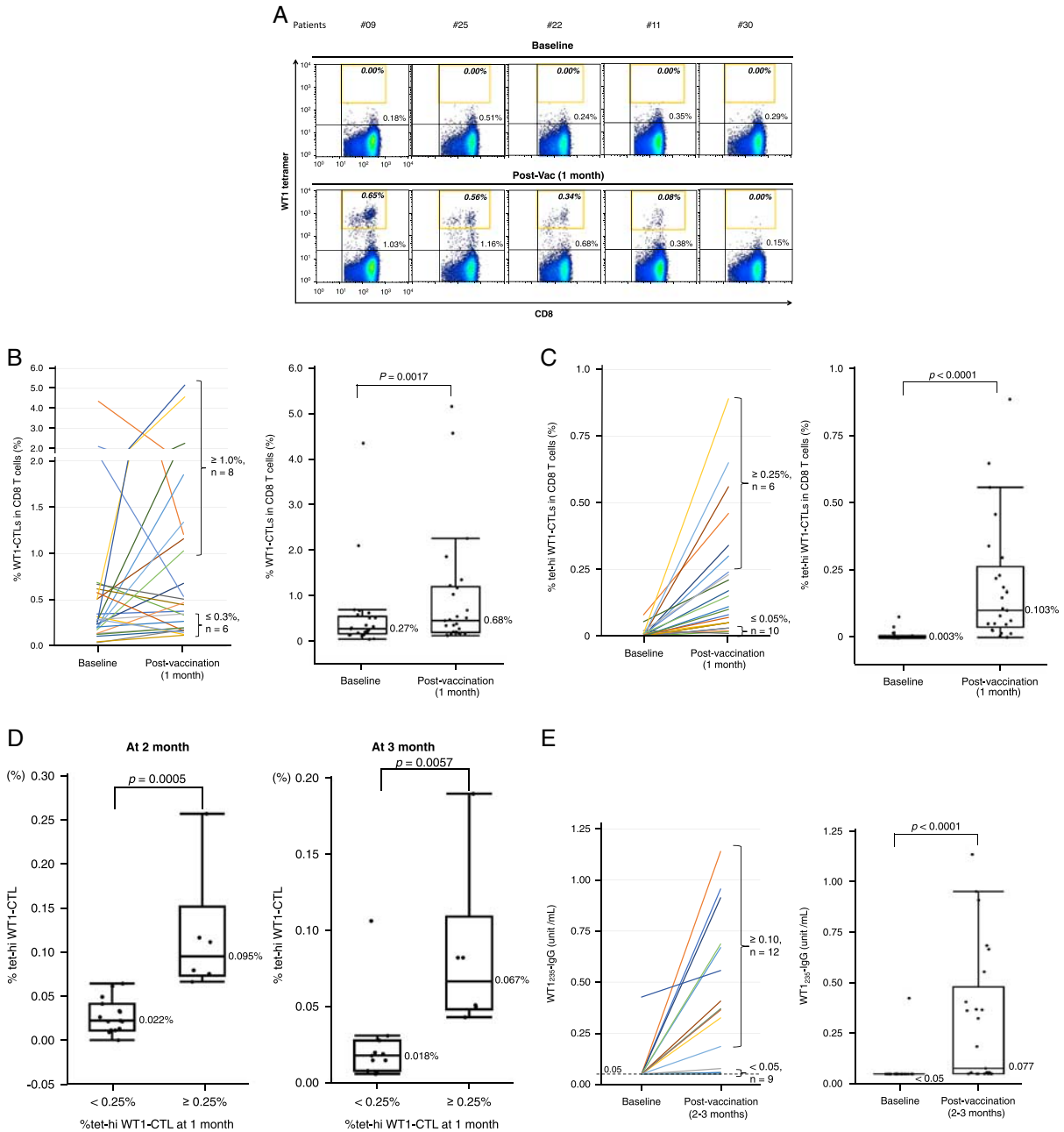

FIGURE 1. Wilms' tumor 1 (WT1)-specific immune responses. A, Wilms' tumor 1–specific cytotoxic T lymphocytes (WT1-CTLs) assessed using a FACS analyzer by the WT1 tetramer assay. WT1-CTLs are defined as WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T cells. The mean fluorescence intensity—high population indicated by a yellow square represents tet-hi WT1-CTL, tetramer high Wilms' tumor 1–specific cytotoxic T lymphocytes (tet-hi WT1-CTLs). Upper, before vaccination; bottom, 1 month after the start of vaccination. Representative data from 5 patients are shown. Patients #09, #25, and #22, #11, and #30 had better (≥ 0.25%), mild (> 0.05, <0.25%), and no or slight (≤ 0.05%) induction of tet-hi WT1-CTLs, respectively. B, Percentage of WT1-CTLs (%WT1-CTL). C, Percentage of tet-hi WT1-CTLs (%tet-hi WT1-CTL). D, Comparison of %tet-hi WT1-CTL 2 months (left) and 3 months (right) after the start of vaccination between patients who had %tet-hi WT1-CTL value of ≥ 0.25% at 1 month (n = 6) and other patients (n = 19). E, Titer of Wilms' tumor 1 peptide-specific immunoglobulin G ($WT1_{235}$-IgG) in individual cases (left) and all patients (right). In the boxplots, black dots represent individual cases. The *P*-value is calculated with the nonparametric Wilcoxon signed-rank test. In the boxplots, black dots represent individual cases. The *P*-value is calculated with the nonparametric Wilcoxon signed-rank test.

responses and favorable clinical outcomes.[22–24] Here, we hypothesized that patients with stronger induction of WT1-specific immunity, including a %tet-hi WT1-CTL value of ≥ 0.25% at 1 month and/or $WT1_{235}$-IgG levels of ≥ 0.10 U/mL during the first 3 months of vaccination, would have better clinical outcomes. The median values for PFS in patients with high (n = 6) and low (n = 19) %tet-hi WT1-CTL were 171.5 and 62 days, respectively, and the HR

for PFS for high %tet-hi WT1-CTL was 0.275 (95% CI: 0.062–0.851; $P = 0.0232$) (Fig. 2A). The median values for PFS in patients with increases in the whole %WT1-CTLs of > 2-fold (n = 14) and <2-fold (n = 11) were 77 and 73 days, respectively, and there was no significant difference between these groups ($P = 0.7455$) (Supplementary Fig. S3C, Supplemental Digital Content 3, http://links.lww.com/JIT/A646). Next, we investigated the effect of $WT1_{235}$-IgG on PFS. The median values for PFS in patients with high (n = 12) and low (n = 13) $WT1_{235}$-IgG levels were 133 and 55 days, respectively, and the HR for PFS for high $WT1_{235}$-IgG levels was 0.238 (95% CI: 0.081–0.621; $P = 0.0031$) (Fig. 2B). These results suggested that both parameters were more useful than DTH for the prediction of clinical outcomes after WT1 vaccination.

We administered the WT1 vaccine to patients repeatedly and observed changes in antigen-specific immune parameters over time. We performed a dynamic prediction analysis to statistically assess the association between PFS and the time series of these immune parameters. The increase in $WT1_{235}$-IgG over time was associated with a significantly longer PFS ($P = 0.0496$), while the temporal change in % tet-hi WT1-CTL was not predictive ($P = 0.8690$).

Patient Status and Background Factors Influenced the Clinical Effectiveness of the WT1 Vaccine

We selected several parameters, including prognostic factors for advanced ovarian cancer[30] and immunologic parameters, for univariate analyses of factors influencing the clinical effectiveness of the WT1 vaccine (defined as the PFS). Decreased serum albumin levels (< 3.5 g/dL), > 2 tumor lesions, worse PS (ECOG PS 1 or 2), or the presence of moderate-to-severe ascites were significantly associated with an unfavorable PFS (log-rank test $P < 0.05$), with HRs for PFS of 9.24, 5.96, 3.19, and 3.99, respectively (Figs. 3A–D). There were no significant differences in PFS curves for other factors (Figs. 3E–L).

Determinants of WT1-specific Immune Responses

Several underlying factors, including factors associated with an unfavorable PFS, could influence WT1-specific immune responses after WT1 vaccination. Univariate analyses were performed to identify factors associated with the insufficient induction of WT1-specific immune responses, such as %tet-hi WT1-CTL of <0.25% at 1 month or $WT1_{235}$-IgG levels of <0.10 U/mL during the first 3 months of vaccination (Table 3). A high C-reactive protein (CRP) level exceeding the standard upper limit (> 0.2 mg/dL) was significantly associated with both the insufficient induction of tet-hi WT1-CTL and insufficient production of $WT1_{235}$-IgG (tet-hi WT1-CTL: Fisher exact test $P = 0.0149$; $WT1_{235}$-IgG: $P = 0.0048$). A low $CD4^+/CD8^+$ ratio (< 2.0) was also significantly associated with the insufficient induction of tet-hi WT1-CTL ($P = 0.0196$). There were no significant associations between insufficient WT1-specific immune responses and other factors, including metastases, histologic types, tumor diameters, and neutrophil/lymphocyte ratio (Table 3).

TRAEs

Lymphopenia, proteinuria, and hematuria were commonly reported (≥ 10%) as systemic TRAEs (Supplementary Table S1, Supplemental Digital Content 7, http://links.lww.com/JIT/A650). No grade 3 or higher TRAEs were observed, although 2 patients died within 30 days after the last vaccination due to disease progression. All patients had local skin reactions at the vaccine injection sites classified as either mild (n = 16), moderate (n = 8), or severe (n = 1).

One patient had severe skin reactions (ie, ulcerations). In particular, the skin reactions at the third, fourth, and fifth vaccine sites developed into ulcers after the sixth vaccination. The patient also experienced fatigue and low-grade fever (< 38°C) for 2 days after vaccination, and these subjective symptoms quickly resolved without any treatment. Notably, she exhibited efficient immune responses: %tet-hi WT1-CTL = 0.46% and $WT1_{235}$-IgG = 1.140 U/mL.

DISCUSSION

Through retrospective analyses, we found that the WT1 vaccine elicited WT1 peptide-specific CTLs in patients with advanced or recurrent ovarian cancer that was refractory to standard chemotherapies and that WT1 peptide-specific cellular

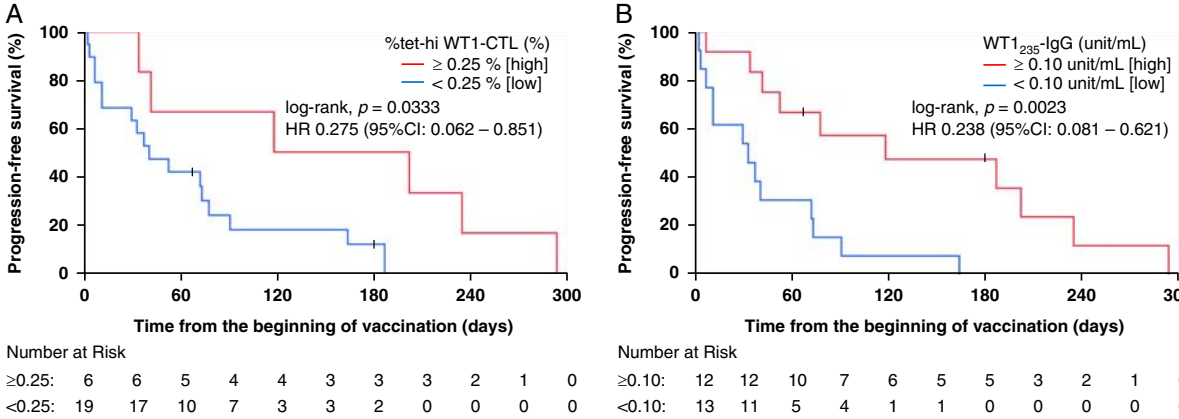

FIGURE 2. Association between WT1-specific immune responses and PFS. Strong induction of WT1-specific immunity is defined as follows: %tet-hi WT1-CTL value of ≥ 0.25% at 1 month and $WT1_{235}$-IgG levels of ≥ 0.10 U/mL during the first 3 months of vaccination. A, PFS in patients who had values of ≥ 0.25% (n = 6) (red line) or <0.25% (n = 19) (blue line) for %tet-hi WT1-CTL. B, PFS in patients who had values of ≥ 0.10 U/mL (n = 12) (red line) or <0.10 U/mL (n = 13) (blue line) for $WT1_{235}$-IgG. PFS among the 2 groups were compared using the log-rank test. Relative HRs are estimated with the use of a Cox proportional-hazards model. CI indicates confidence interval; HR, hazard ratio; PFS, progression-free survival; tet-hi WT1-CTL, tetramer high Wilms' tumor 1–specific cytotoxic T lymphocytes; WT1, Wilms' tumor 1; $WT1_{235}$-IgG, Wilms' tumor 1 peptide-specific immunoglobulin G.

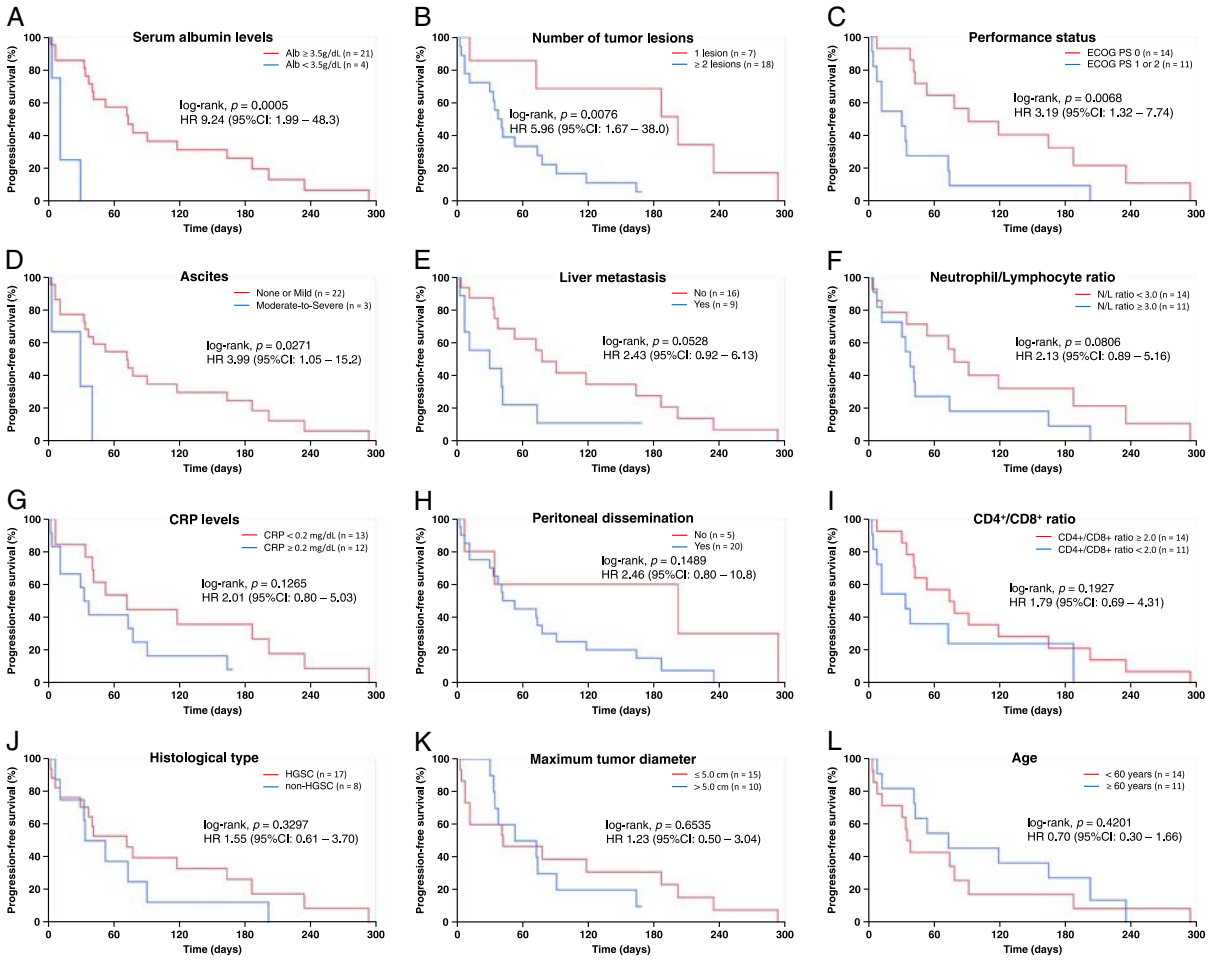

FIGURE 3. Kaplan-Meier survival curves for progression-free survival by several prognostic factors and immunologic factors. A, Serum albumin levels ($\geq$3.5 vs. <3.5 g/dL). B, Number of tumor lesions (1 lesion vs. $\geq$2 lesions). C, Performance status (ECOG PS 0 vs. 1 or 2). D, Presence of ascites (none or mild vs. moderate-to-severe). E, Presence of liver metastasis (no vs. yes). F, N/L ratio (<3.0 vs. $\geq$3.0). G, CRP levels (<0.2 vs. $\geq$0.2 mg/dL). H, Presence of peritoneal dissemination (no vs. yes). I, $CD4^+/CD8^+$ ratio ($\geq$2.0 vs. <2.0). J, Histologic type (HGSC vs. non-HGSC). K, Maximum tumor diameter ($\leq$5.0 cm tumor vs. >5.0 cm cystic or solid tumor). L, Age (<60 vs. $\geq$60 y). The number of tumor lesions represents counts of residual ovarian tumors, local recurrence, or metastatic organs. Multiple lymph node metastases or peritoneal dissemination are counted as a single lesion. The severity of ascites is defined as follows: mild, limited to a pelvic cavity or Morrison fossa; moderate, beyond the pelvic cavity; severe, occupying the entire peritoneal cavity. Differences in progression-free survival among the 2 groups were compared using the log-rank test. Relative HRs were estimated with the use of a Cox proportional-hazards model. CI indicates confidence interval; CRP, C-reactive protein; ECOG PS, Eastern Cooperative Oncology Group performance status; HGSC, high-grade serous carcinoma; HR, hazard ratio; N/L, neutrophils/lymphocytes.

and humoral immunity constituted prognostic markers for the WT1 vaccine. Several patient characteristics and their immunologic status influenced the induction of WT1-specific immunity and the clinical effectiveness of the WT1 vaccine (eg, PFS).

We previously found that DTH to the WT1 peptide after vaccination is associated with prolonged survival and is an independent prognostic marker in a phase II study of the WT1 vaccine in patients with refractory gynecologic malignancies.[18] We noticed a high rate of DTH positivity in patients with ovarian cancer in the retrospective reevaluation of DTH. However, contrary to our expectations, DTH positivity did not consistently reflect the effectiveness of the WT1 vaccine against ovarian cancer. In the present study, we analyzed WT1-specific cellular and humoral immunity.

The WT1 vaccine induced WT1-CTLs. Such primary effector cells attack tumor cells, explaining the observed clinical effectiveness.[31] We observed that 56% of the patients had >2-fold increase in the percentage of whole WT1-CTLs. However, a clear association between these increases and clinical outcomes was not observed, which was similar to the results for DTH positivity and PFS.

Tet-hi WT1-CTLs detected by FACS reflect functional CTLs against the WT1 peptide and may have optimal antitumor activity according to our previous findings and those of other studies.[27,28] In the present study, 60% of the patients exhibited the emergence of tet-hi WT1-CTLs after vaccination to various degrees. Of note, patients that exhibited a high induction of tet-hi WT1-CTLs had a prolonged PFS. These results suggest that an increase in tet-hi WT1-CTLs (but not whole WT1-CTLs) is a valuable predictive marker for the clinical effectiveness of the WT1 vaccine. In our previous report, long-term survivors of pancreatic cancer who had received the WT1 vaccine had a high proportion of WT1-CTLs with the memory phenotype

TABLE 3. Univariate Analysis for Wilms' Tumor 1–specific Immune Responses

		Induction of tet-hi WT1-CTLs		
Variables	**N**	**< 0.25% [n (%)]**	***P* (2-sided Fisher Exact Test)**	**Relative Risk (95% CI)**
All patients	25	19 (76.0)	—	—
CRP (mg/dL)				
≤ 0.2	13	7 (53.9)	0.0149	1
> 0.2	12	12 (100.0)		1.86 (1.12–3.07)
$CD4^+/CD8^+$ ratio				
≥ 2.0	14	8 (57.1)	0.0196	1
< 2.0	11	11 (100.0)		1.75 (1.11–2.76)
Peritoneal dissemination				
No	5	2 (40.0)	0.0698	1
Yes	20	17 (85.0)		2.13 (0.72–6.32)
No. tumor lesions*				
1 lesion	7	4 (57.1)	0.2985	1
≥ 2 lesions	18	15 (83.3)		1.46 (0.74–2.86)
Serum albumin (g/dL)				
≥ 3.5	21	15 (71.4)	0.5404	1
< 3.5	4	4 (100.0)		1.40 (1.07–1.84)
Ascites†				
None or mild	22	16 (72.7)	0.5539	1
Moderate to severe	3	3 (100.0)		1.38 (0.92–4.49)
Maximum tumor diameter				
≤ 5.0 cm	15	10 (66.7)	0.3449	1
> 5.0 cm cystic or solid	10	9 (90.0)		2.04 (1.06–1.78)
Liver metastasis				
No	16	11 (68.8)	0.3644	1
Yes	9	8 (88.9)		1.29 (0.86–1.93)
N/L ratio				
< 3.0	14	10 (71.4)	0.6609	1
≥ 3.0	11	9 (71.8)		1.15 (0.74–1.77)
PS (ECOG)				
0	14	10 (71.4)	0.6609	1
1or 2	11	9 (81.8)		1.15 (0.74–1.77)
Histologic type				
HGSC	17	13 (76.5)	1.0000	1
Non-HGSC	8	6 (75.0)		0.98 (0.61–1.58)
Age (y)				
< 60	14	12 (85.7)	0.3500	1
≥ 60	11	7 (63.6)		0.74 (0.45–1.22)
		Production of $WT1_{235}$-IgG		
		< 0.10 U/mL [n (%)]	***P* (2-sided Fisher Exact Test)**	**Relative Risk (95% CI)**
All patients	25	13 (52.0)	—	—
CRP (mg/dL)				
≤ 0.2	13	3 (23.1)	0.0048	1
> 0.2	12	10 (83.3)		3.61 (1.30–10.1)
Peritoneal dissemination				
No	5	1 (20.0)	0.1602	1
Yes	20	12 (60.0)		3.00 (0.50–18.0)
Serum albumin (g/dL)				
≥ 3.5	21	9 (42.9)	0.0957	1
< 3.5	4	4 (100.0)		2.33 (1.42–3.82)
Ascites†				
None or mild	22	10 (45.5)	0.2200	1
Moderate to severe	3	3 (100.0)		2.20 (1.39–3.48)
No. tumor lesions*				
1 lesion	7	2 (28.6)	0.2016	1
≥ 2 lesions	18	11 (61.1)		2.14 (0.63–7.30)
$CD4^+/CD8^+$ ratio				
≥ 2.0	14	5 (35.7)	0.1107	1
< 2.0	11	8 (72.7)		2.04 (0.92–4.49)
N/L ratio				
< 3.0	14	5 (35.7)	0.1107	1
≥ 3.0	11	8 (72.7)		2.04 (0.92–4.49)

TABLE 3. (*continued*)

Variables	N	Production of $WT1_{235}$-IgG		
		< 0.10 U/mL [n (%)]	*P* (2-sided Fisher Exact Test)	Relative Risk (95% CI)
PS (ECOG)				
0	14	5 (35.7)	0.1107	1
1or 2	11	8 (72.7)		2.04 (0.92–4.49)
Maximum tumor diameter				
≤ 5.0 cm	15	6 (40.0)	0.2262	1
> 5.0 cm cystic or solid	10	7 (70.0)		1.75 (0.83–3.67)
Liver metastasis				
No	16	7 (43.8)	0.4110	1
Yes	9	6 (66.7)		1.52 (0.74–3.14)
Histologic type				
HGSC	17	8 (47.1)	0.6728	1
Non-HGSC	8	5 (62.5)		1.33 (0.64–2.77)
Age (y)				
< 60	14	9 (64.3)	0.2377	1
≥ 60	11	4 (36.4)		0.57 (0.24–1.36)

*The number of tumor lesions represent the number of residual ovarian tumors, local recurrence, or metastatic organs. Multiple lymph node metastases or peritoneal dissemination are counted as a single lesion.

†Severity of ascites is defined as follows: mild, limited to a pelvic cavity or Morrison fossa; moderate, beyond the pelvic cavity; severe, occupy the entire peritoneal cavity.

CI indicates confidence interval; CRP, C-reactive protein; ECOG, Eastern Cooperative Oncology Group; HGSC, high-grade serous carcinoma; N/L, neutrophils/lymphocytes; PS, performance status; tet-hi WT1-CTL, tetramer high Wilms' tumor 1–specific cytotoxic T lymphocytes; $WT1_{235}$-IgG, Wilms' tumor 1 peptide-specific immunoglobulin G.

over a long period.[32] We speculate that the population of tet-hi WT1-CTLs includes both actual effector T cells able to effectively eradicate cancer cells and memory T cells contributing to the long-term anticancer effect. Further fundamental research is necessary to elucidate the cytogenetic and functional characteristics of tet-hi WT1-CTLs induced by the WT1 vaccine.

In the present study, 48% of the patients exhibited WT1 peptide-specific IgG production after receiving the WT1 vaccine. $WT1_{235}$-IgG was undetectable before vaccination and was subsequently produced during the 3 months of WT1 vaccination, indicating that the WT1 vaccine induced the humoral $WT1_{235}$-IgG immune response. The production of $WT1_{235}$-IgG is also associated with a prolonged PFS. These results suggest that an increase in WT1 peptide-specific IgG is also a useful predictive prognostic marker for the clinical effectiveness of the WT1 vaccine. The mechanisms by which $WT1_{235}$-IgG is produced are unclear. We previously reported that a subclass of these WT1-specific IgGs, including $WT1_{235}$-IgG, express IgG1 or IgG3, reflecting the Th1-type response, suggesting that the production of $WT1_{235}$-IgG is a biological marker related to the activation of the cellular immune response.[23,25] Moreover, the biological function of $WT1_{235}$-IgG remains unclear. WT1 is not expressed on the surface of ovarian cancer cells because it is a nuclear transcription factor[13,33]; therefore, theoretically, $WT1_{235}$-IgG does not bind to these cancer cells. A lack of binding of $WT1_{235}$-IgG to the WT1 peptide/MHC class I molecule complex has been reported.[23] These findings suggest that WT1 peptide-specific IgG does not directly inhibit tumor growth. $WT1_{235}$-IgG may function as on opsonin. Cancer antigens opsonized with an antibody promote its uptake by antigen-presenting cells, including dendritic cells.[34] The production of $WT1_{235}$-IgG, which binds to the mp235 WT1 peptide, may promote peptide uptake by dendritic cells, promoting the induction of the WT1-specific immune response. Further investigations are necessary to clarify the biological functions of WT1 peptide-specific IgG.

We used a dynamic prediction analysis to explore the associations of temporal changes in %tet-hi WT1-CTL and $WT1_{235}$-IgG with PFS because these immunologic parameters were not applicable biomarkers before vaccine treatment. A continual increase in $WT1_{235}$-IgG levels during treatment was associated with a longer PFS, further suggesting that $WT1_{235}$-IgG is a useful predictive biomarker for a prolonged PFS in response to the WT1 vaccine. In contrast, we did not detect an association between serial changes in %tet-hi WT1-CTL and a longer PFS. However, the dynamic prediction method may not be suitable for analyzing biomarkers that reach a steep peak early in the treatment period and then rapidly decrease. We presume that WT1-CTLs induced by the WT1 vaccine emerged in the peripheral blood and spread to tumor lesions.[8] Accordingly, %tet-hi WT1-CTL in the peripheral blood peaked 1 month after the beginning of vaccination and then declined, making it difficult to detect differences among patients.

Several well-known prognostic factors in standard chemotherapy for ovarian cancer[30,35] negatively influenced the clinical effectiveness of the WT1 vaccine and the induction of WT1-specific immune responses. We found that lower serum albumin levels, tumor lesions distributed in multiple organs (or total residual disease volume), poor PS, and the presence of moderate-to-severe ascites were associated with an unfavorable PFS. Additionally, high CRP levels and low $CD4^+/CD8^+$ ratios were related to inadequate WT1-specific immune responses. We did not identify factors that commonly influenced both PFS and WT1-specific immune responses. However, most factors were directly or indirectly related to systemic inflammation, a widely recognized hallmark of cancer.[36] The cancer status of a subset of patients enrolled in our study was advanced. Some patients had tumor lesions distributed in multiple

organs, causing tumor-related systemic inflammation. This potentially cancer-related inflammation could further exacerbate the condition of patients, leading to a worse PS. Several previous reports, including meta-analyses, have revealed that the inflammatory status, such as elevated CRP and decreased serum albumin, is associated with inferior survival outcomes in ovarian cancer.[37–39] Systemic inflammation related to advanced cancer sometimes causes cancer cachexia or sarcopenia.[36,40] Cancer cachexia (or sarcopenia), which is generally associated with an inadequate response to cancer treatment, could adversely affect the effectiveness of immunotherapy as the immunosuppressive status (eg, T-cell exhaustion) progresses.[41,42] Previous studies from our group and another group have recently revealed that sarcopenia is a predictor of a worse prognosis in patients with advanced cancer who are receiving immunotherapy.[43,44] Our results suggested that the antitumor effects of the WT1 vaccine are relatively weak under systemic inflammation.

Liver metastasis and peritoneal dissemination often result in decreased serum albumin levels and increased ascites. The neutrophil/lymphocyte ratio is widely used as a systemic inflammatory parameter.[37,45] However, we found no significant associations between these factors and an unfavorable PFS or inferior induction of WT1-specific immune responses. This may be explained by the insufficient sample size for statistical assessments. In fact, PFS curves for patients with liver metastasis, high neutrophil/lymphocyte ratios, or high CRP levels were unfavorable. In addition, no patients with decreased serum albumin or excess ascites at baseline exhibited the effective induction of the WT1-specific immune response. Further investigations are necessary to confirm this hypothesis.

Differences in histologic types and tumor diameters are well-known prognostic factors for chemotherapy in ovarian cancer.[35] We found no differences in the induction of WT1-specific immune responses between patients with HGSC and those with non-HGSC. Consistent with this, there was no difference in PFS between the histologic subgroups. We presume that the cancer vaccine lacks cross-resistance to conventional cytotoxic drugs against ovarian cancer due to the difference in antitumor mechanisms. In addition, in WT1-expressing ovarian cancer, there might not be a difference in susceptibility to cytotoxicity due to WT1-CTLs, regardless of histologic type. Unexpectedly, the tumor diameter did not predict the induction of WT1 immunity. The WT1 vaccine could induce WT1-CTLs resulting in temporary tumor growth inhibition in patients with a good PS and a limited lesion, even if the tumor mass is large.

We hypothesized that induction of WT1-specific immunity by the WT1 vaccine would improve the prognosis of cancer patients. Our results led us to consider that patients with good PS without tumor-related inflammation would particularly benefit from the WT1 vaccine. However, another possibility was that because the factors themselves are prognostic factors for ovarian cancer, the prolongation of PFS in better-conditioned patients might be independent of vaccine-induced immunity. Also, we did not identify any factors that could accelerate antigen-specific immunity. Further investigations are necessary to identify the oncological and immunologic features of patients with better immune induction among those in a good condition without inflammation.

The present study had at least 4 principal limitations. First, it was an exploratory single-arm study. A placebo-controlled randomized study is necessary to confirm the clinical effectiveness of the WT1 vaccine. Second, the sample size was small. We could not perform a multivariate analysis to identify associations between several background factors, immunologic factors, and clinical outcomes. We identified several independent predictors of the clinical or immunologic effectiveness of the WT1 vaccine by univariate analyses; however, we could not distinguish confounding factors. Third, we did not perform bioassays, such as a cell-killing assay and peptide-stimulated cytokine assay, with WT1-CTLs induced by the WT1 vaccine, although we conducted immune monitoring to assess the WT1-specific immune response. Fourth, the WT1 vaccine used in this study contained only a single peptide restricted to the HLA-A*24:02 molecule. Other immune modifications (eg, coadministration with helper peptides and the selection of other immune adjuvants) and combination therapies with immune checkpoint inhibitors are expected to enhance the immune response induced by the HLA class I–restricted peptide vaccine.

In conclusion, the WT1 vaccine induced WT1-CTLs in patients with advanced ovarian cancer. The induction of cellular and humoral immunity targeting WT1 gene products was related to the improvement of PFS, suggesting that these parameters have prognostic value. Our results provide a basis for the design of further clinical studies. When the tumor burden is low, for example, after debulking surgery followed by chemotherapy, the WT1 vaccine is likely to show better clinical effectiveness in maintaining remission. In this scenario, because the PS is good and there is no systemic inflammation related to cancer, WT1-CTLs are effectively induced, a phenomenon that is expected to improve prognosis in ovarian cancer.

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Conflicts of Interest/Financial Disclosures

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All authors have declared that there are no financial conflicts of interest with regard to this work.

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