then incubated overnight with the primary antibody. After binding of relevant peroxidase-conjugated secondary antibodies, the filters were developed with ECL (GE Healthcare).

Competitive enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with GST-WT1 protein (50 ng/well) at 4°C overnight, and then blocked with 0.1% casein in PBS at 4°C overnight. Serial dilutions of WT1 peptide were incubated with anti-WT1 antibody (WT1-R) at a concentration of 3 μg/mL at 4°C overnight. The mixtures were added to GST-WT1-coated plates and incubated at 37°C for 30 min. After incubation with peroxidase-conjugated antirabbit IgG, absorbance was determined at 492 nm with an ELISA reader.

Real-time quantitative-PCR (RT-PCR). For RT-PCR, total RNA was extracted from each cell line and reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Tokyo, Japan). Quantitative PCR was performed using a LightCycler (Roche Diagnostics) with LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. The primers used for PCR were as follows. Human GAPDH forward: 5′-TGAACGGGAAGCTCACTGG-3′, reverse: 5′-TCCACCACCCTGTTGCTGTA-3′; Human WT-1 forward: 5′-CCAGGCTGCAATAAGAGATA-3′, reverse: 5′-TCTTTTGAGCTGGTCTGAA-3′. PCR for human GAPDH was performed for 40 cycles consisting of 95°C for 10 s, 60°C for 10s, and 72°C for 12 s. Human WT-1 was amplified by 40 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 5 s.

Immunohistochemistry. For immunohistochemical staining, cells were fixed in PBS with 10% formaldehyde and then these fixed cells were embedded in agar blocks and sections were then cut using a microtome for immunostaining. Breast cancer and normal gastric mucosa specimens were obtained with informed consent (Pathology Institute Corporation, Toyama, Japan), and antigen retrieval was performed after deparaffinization of the slides by heating the sections in Tris-buffered saline, pH 9.0, in a water bath at 95°C for 40 min. Sections were allowed to cool to room temperature and endogenous peroxidase activity was blocked with 3% H_2O_2. The sections were then incubated with WT1-R or 6F-H2 as the primary antibody at a dilution of 1:500 (WT1-R) or 1:50 (6F-H2) for 30 min, followed by detection using the Dako EnVision/Polymer System (Dako, Ely, Cambridgeshire, UK). Sections were lightly counterstained with hematoxylin.

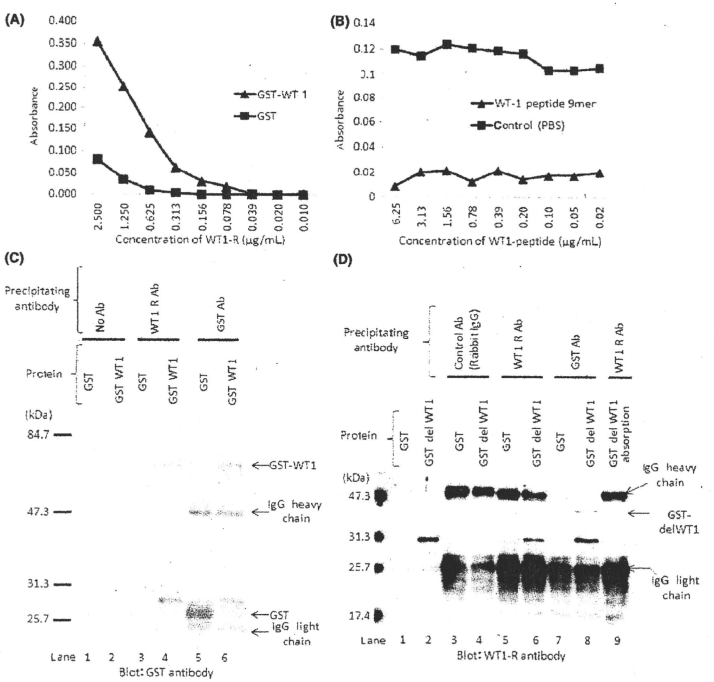

Fig. 1. Reactivity of WT1-R antibody against 9-mer peptide region of Wilms' tumor 1 (WT1) protein targeted by WT1 vaccine. (A) Dilution curves of WT1-R. Plates coated with WT1-GST or GST protein were incubated with serial dilutions of WT1-R antibody and the binding of WT1-R antibody to plates was determined. (B) Results of competitive ELISA. Serially diluted WT1 peptide was incubated with or without 3 mg/mL of WT1-R antibody, and this mixture was then added to GST-WT1-coated plates. (C,D) Western blotting analysis with GST or WT1-R antibody. Proteins were immunoprecipitated with the antibodies indicated above before electrophoresis.

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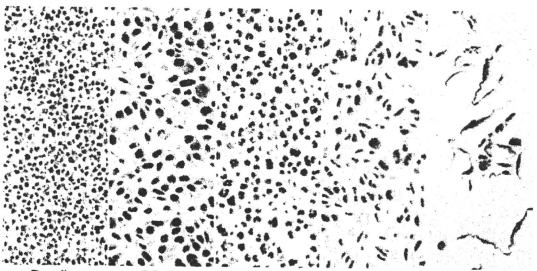

Fig. 2. Comparison between immunohistochemical staining with WT1-R and real-time quantitative PCR (RT-PCR) to detect Wilms' tumor 1 (WT1) expression in five cell lines (Daudi, PC-14, K562, HT1080, and SW480). RT-PCR results are shown beneath the photographs. Magnification, ×400.

Results

Anti-WT1 polyclonal antibody WT1-R binds specifically to GST-WT1 protein and 9-mer peptide corresponding to WT1 vaccine antigen. To assess antibody reactivity, WT1-R was serially diluted and its binding to plates coated with GST-WT1 protein and GST protein was examined. WT1-R bound strongly to GST-WT1-coated plates, whereas no binding was observed on GST-coated plates (Fig. 1A). To evaluate the specificity of WT1-R antibody, we performed competitive ELISA with WT1 peptide. As shown in Figure 1(B), antibody binding to GST-WT1 was markedly decreased by preincubation with WT1 peptide compared to control (PBS). Next, we examined antibody binding by immunoprecipitation and Western blotting analysis. As shown in Figure 1(C), GST-WT1 protein was immunoprecipitated by WT1-R and anti-GST antibodies and detected by anti-GST antibody (lanes 4 and 6). Figure 1(D) shows the results of western blotting analysis using WT1-R as the primary antibody. In this experiment, we used GST-delWT1 protein, which included the amino acid sequence targeted by WT1-R. Purified GST-delWT1 protein (lane 2) was detected by WT1-R, whereas WT1-R did not react with GST protein (lane 1). GST and GST-delWT1 protein were immunoprecipitated with control (rabbit IgG), anti-GST, or anti-GST antibody before western blotting, and WT1-R reacted only with GST-delWT1 immunoprecipitated by WT1-R (lane 6) and anti-GST antibody (lane 8). These bands disappeared when the antibodies were preincubated with 9-mer WT1-peptide before immunoprecipitation (lane 9). These results indicated that WT1-R antibody could detect WT1 protein and the binding was specific for part of WT1 protein corresponding to the 9-mer peptide used for WT1 vaccine.

Immunohistochemical analysis of WT1-R antibody. Immunohistochemical analysis of five cell lines (K562, Daudi, HT-1080, SW480, and PC-14) was performed and the results were

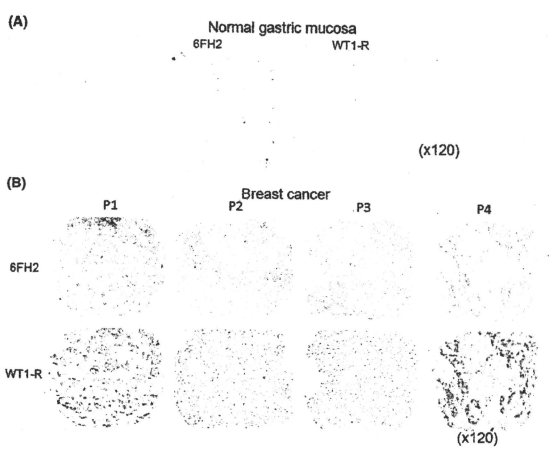

Fig. 3. Immunohistochemical analysis of normal gastric mucosa (A) and breast cancer tissue samples from four different patients (B). In (B), the upper and lower photographs show the results of immunohistochemical staining with 6F-H2 and with WT1-R, respectively. Magnification, ×120.

compared with those of RT-PCR. As shown in Figure 2, K562 (WT1 copy number/GAPDH copy number: 0.048), Daudi (0.061), and SW480 (0.042) showed about 5–6-fold higher levels of expression than HT-1080 (0.01), and the expression level in PC-14 was almost 1000-fold lower (0.000052) than in the other lines. These results indicated that the expression was not equivalent in these cell lines although they expressed WT1. On the other hand, in contrast to the difference in WT1 mRNA expression between cell lines, immunohistochemical analysis with WT1-R showed almost the same binding intensity in these cell lines (Fig. 2).

Next, we compared the reactivity of WT1-R with that of 6F-H2 antibody in normal gastric mucosa tissue and breast cancer tissue samples obtained from four different patients (Fig. 3). In normal gastric mucosa, plasma cells showed nonspecific staining with 6F-H2 antibody, but were completely negative for staining by WT1-R with no nonspecific binding (Fig. 3A). In breast cancer tissues, immunostaining with 6F-H2 showed weak positive reactivity in breast cancer samples, while staining with WT1-R was clearly positive in both the nucleus and cytoplasm of breast cancer cells and the staining intensity was significantly higher with WT1-R than in 6F-H2 (Fig. 3B). These observations indicated that WT1-R antibody is more sensitive for detection of WT1 protein in breast cancer than 6F-H2 antibody.

Discussion

RT-PCR is widely employed for determination of WT1 expression.[11,21] However, the results of RT-PCR are not always correlated with protein expression[22] and this method is not suitable for tumors that consist of cells of various types, including malignant and non-malignant cells, because the results are dependent on the proportion of malignant to normal cells. Therefore, immunohistochemical analysis of WT1 expression in solid tumors may be a better option than RT-PCR. In the present study, the results of immunohistochemical analysis of WT1 expression were not correlated with those of RT-PCR. The level of WT1 mRNA transcript expression in PC-14 was very weak, but protein expression level was almost the same as in the other cell lines. This observation suggested that WT1 mRNA expression is not equivalent to its protein expression. A discrepancy between WT1 mRNA and protein expression was reported previously in childhood leukemia.[22] These results suggested that immunohistochemical analysis is important to predict the efficacy of WT1 vaccines. The WT1-R antibody developed in the present study shows sensitivity for detection of WT1 protein and may be useful for immunohistochemical analysis.

Currently, there is no standard method for immunohistochemical analysis of WT1 because of a lack of appropriate antibodies. It has already been reported that staining results with 6F-H2 and another antibody against WT1 showed marked differences in some types of tumor.[21] The WT1-R antibody developed in the present study showed high sensitivity for detection of WT1 protein in breast cancer samples compared with 6F-H2 antibody, and may be appropriate for immunohistochemical analysis of WT1. Further studies of the sensitivity and specificity of WT1-R antibody in various types of cancer are required.

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Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL ARTICLE

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Prognostic value of WT1 protein expression level and MIB-1 staining index as predictor of response to WT1 immunotherapy in glioblastoma patients

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Abstract The use of Wilms' tumor 1 (WT1) immunotherapy is considered to be an innovative approach for the treatment of malignant gliomas. Because of its novelty, tools that can accurately predict response to this therapy are still lacking. In this article, we investigated the role of WT1 protein expression level (score 1–4) and MIB-1 staining index in predicting survival outcome after therapy in patients with recurrent or progressive glioblastoma multiforme. Tumor samples from 37 patients enrolled in a phase II clinical trial on WT1 immunotherapy were immunohistochemically analyzed for WT1 levels and MIB-1 index. Results showed that median progression-free survival (PFS) was longer in the WT1 high expression group (score 3 and 4) compared with that of the low expression group (score 1 and 2) (20.0 weeks vs. 8.0 weeks; $P = 0.022$), and that the median overall survival (OS) was likewise longer in the former compared to the latter group (54.4 weeks vs. 28.4 weeks; $P = 0.035$). Furthermore, within the WT1 high expression group, tumors with intermediate staining intensity (WT1 score 3) have both the longest median PFS and OS, 24.4 weeks and 69.4 weeks, respectively. On the other hand, no significant correlation was noted between MIB-1 staining index and survival. In conclusion, our study has shown that WT1 protein expression level, not MIB-1 staining index, can be used as a prognostic marker to foretell outcome after immunotherapy, and that patients whose tumors have intermediate WT1 expression have the best survival outcome.

Key words Glioblastoma · Wilms' tumor 1 · Immunotherapy · MIB-1 staining index · Prognostic value

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Introduction

Our understanding of the biological behavior of cancers has expanded through the years. With therapeutic strategies available, both standard and "nonstandard" modalities, finding a suitable treatment for a patient has become increasingly difficult. Oncologists have been constantly on the lookout for tools that can accurately predict response to therapy.

A novel approach for the treatment of glioblastoma multiforme (GBM) is with the use of Wilms' tumor 1 (WT1) immunotherapy. Studies have shown that the WT1 gene is highly expressed in this disease and that it is absent in normal astrocytes.[1,2] Similarly, it has been found to play a significant role in gliomagenesis.[1] A preliminary report on WT1 immunotherapy has demonstrated survival outcome that is equal or superior to chemotherapy in patients suffering from recurrent or progressive GBM.[3]

Consequently, this information has stirred up interest among glioma specialists on finding reliable indicators that can help predict treatment response in this category of patients. One promising marker is the level of tumor expression of WT1. As it is tagged as one of the major culprits in glioma pathogenesis, this choice seemed rational. Another potential one, implicated in the proliferative nature of the disease, is the MIB-1 staining index. Up to the present, there have been no substantial studies that specifically involved patients who underwent WT1 immunotherapy therapy to address this concern.

In this article, we assessed the expression of WT1 levels, as well as MIB-1 staining index, in tumor samples of GBM patients before WT1 immunotherapy and correlated it with survival outcome. As an adjunct, we also analyzed the same parameters in tumor samples of postvaccine patients who failed therapy.

Materials and methods

Patients and treatments

Patients were enrolled in a phase II clinical trial of WT1 immunotherapy for recurrent or progressive GBM. Details of the trial were described previously.[3] Patients who were eligible received intradermal injections of 3.0 mg HLA-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant weekly for 12 consecutive weeks. Response was determined by measuring the change in the size of the target lesions on magnetic resonance (MR) imaging, labeled as either complete response, partial response, stable disease, or progressive disease, based on the RECIST criteria.[4]

If an effect was observed after 12 vaccinations, WT1 immunotherapy was further given at 2-week intervals until disease progression was noted. The progression-free survival (PFS) period was defined as the day of the first WT1 immunotherapy to the day of the last image before the detection of disease progression. The overall survival (OS) period was defined as the time from the day of the first WT1 immunotherapy to death.

Tumor specimens were taken at the time of the initial diagnosis of GBM before WT1 immunotherapy, as well as during the second operation after failed immunotherapy (if available).

All patients provided written informed consent, and the study was approved by the ethics review board of the Osaka University Faculty of Medicine.

Immunohistochemical analysis

WT1 expression level

Formalin-fixed tissue sections were prepared from resected tumors of eligible patients. Sections were microwaved in citrate buffer for antigen retrieval and incubated with anti-human WT1 mouse monoclonal antibody 6F-H2 (diluted 1:50, DAKO North America, Carpinteria, CA, USA). The WT1 reaction was visualized with the Vectastain ABC kit (Vector Laboratories, Burlingume, CA, USA) and diaminobenzidine (WAKO, Osaka, Japan). Sections were then counterstained with hematoxylin. The level of WT1 expression was classified, based on a scale proposed by Izumoto and colleagues,[3] as follows: 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 (Fig. 1).

Samples were scored independently by three competent authors (Y.C., N.H., and N.K.). A score agreed upon by at least two of them was deemed acceptable.

MIB-1 staining index

The same serial sections used for WT1 immunohistochemical evaluation were utilized for MIB-1 staining. Antibody against the Ki-67 antigen (DAKO) was diluted 1:50. The staining index was determined by calculating the percentage of positively stained tumor cell nuclei of 1000 seen in areas with the greatest degree of immunostaining. A cutoff of 20 was arbitrarily chosen to divide the group into a high and a low MIB-1 labeling group.

Statistical analysis

To assess the relationship of WT1 levels and MIB-1 staining index with survival outcome after treatment in the study population, the Kaplan–Meier technique was utilized. PFS and OS were estimated using the Kaplan–Meier curves and were compared using the log-rank test. A P value < 0.05 was considered statistically significant, and all statistical computation was performed using StatMate III software (ATMS, Tokyo, Japan).

Results

Patient population

Table 1 shows the characteristics of the 37 patients (23 men and 14 women; mean age, 49.0 years; range, 20–75 years) included in the study. Of the patients, 51% had right-sided lesions, most of which were in the frontal region, whereas 41% had lesions involving the left lobe.

Table 1. Characteristics of 37 patients

Sex	
Male	23
Female	14
Age (in years)	
Average	49.0
Range	20–75
Tumor location	
Right side	19
Frontal lobe	11
Temporal lobe	4
Parietal lobe	2
Occipital lobe	0
Basal ganglia	2
Left side	15
Frontal lobe	8
Temporal lobe	5
Parietal lobe	0
Occipital lobe	2
Basal ganglia	0
Bilateral frontal	2
Cerebellum	1

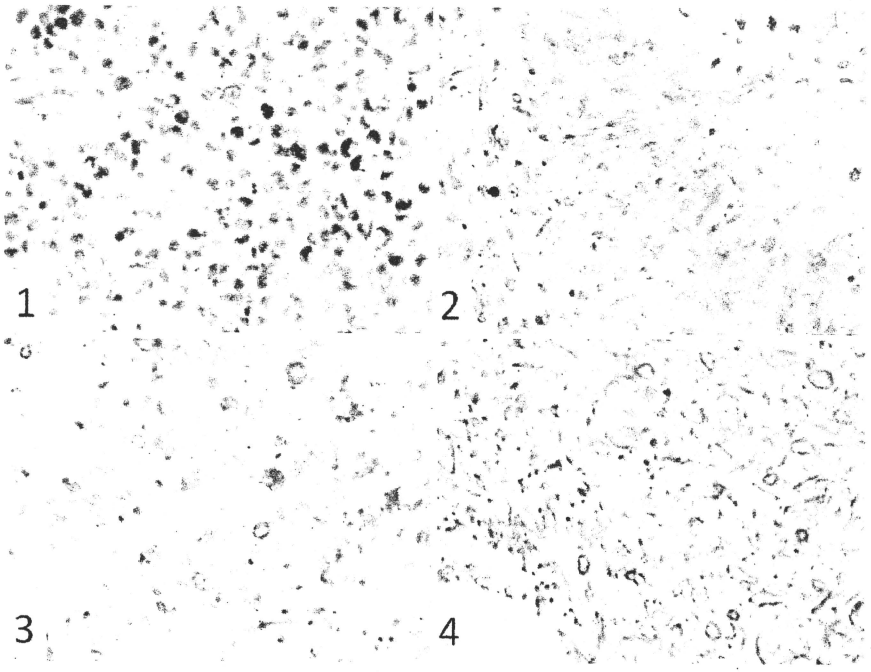

Fig. 1. The level of Wilms' tumor 1 (WT1) expression was classified. Score *1*, slightly increased staining in some tumor cells compared with that in normal glial cells; score *2*, staining at intermediate intensity in some tumor cells; score *3*, strong staining in some tumor cells and intermediate staining in almost all tumor cells; score *4*, greatly increased staining in almost all tumor cells compared with that in normal glial cells

Response to WT1 immunotherapy included partial response in 2 patients; stable disease in 17 patients; and progressive disease in 18 patients. No statistical association between treatment response and WT1 expression level, as well as MIB-1 staining index, was observed (data not shown).

Correlation between level of WT1 expression and survival outcome

Immunohistochemical analysis of WT1 expression was performed on 37 GBM specimens from patients enrolled in the WT1 peptide-based phase II clinical trial. All 37 had WT1-positive GBM. The WT1 scores obtained were as follows: score 1 in 3 specimens; score 2 in 9 specimens; score 3 in 13 specimens; and score 4 in 12 specimens (Table 2).

In terms of survival among patients who received WT1 immunotherapy, the longest median PFS was 24.4 weeks and the longest OS was 69.4, both of which were observed in patients whose tumor specimens were assigned with score 3 (Table 2). On the other end of the spectrum, specimens from patients with score 1 were noted to have the shortest median PFS at 5.1 weeks and those with score 2 the shortest OS at 28.4 weeks. Notwithstanding the fact that specimens with score 4 had shorter PFS and OS compared with score 3, which was not statistically significant ($P = 0.171$ and 0.106, respectively, by log-rank test) (Fig. 2a,c), a tendency of better survival after treatment with higher levels of WT1 expression seemed to emerge in the

Table 2. Effect of the level of Wilms' tumor 1 (WT1) expression on survival

WT1 expression score	Number of patients	Median PFS (weeks)	Median OS (weeks)
Score 1	3	5.1	32.6
Score 2	9	10.0	28.4
Score 3	13	24.4	69.4
Score 4	12	20.0	38.1

PFS, progression-free survival; OS, overall survival

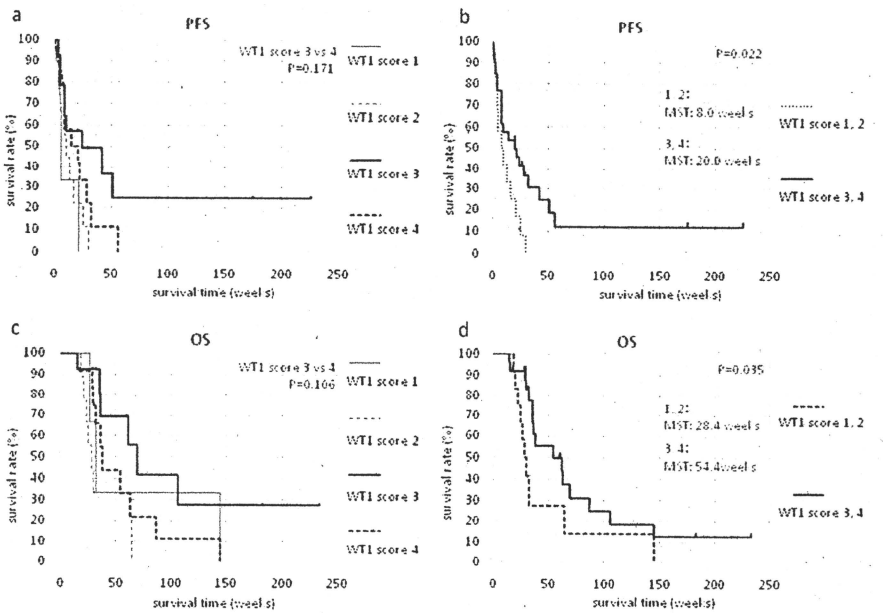

Fig. 2. Kaplan–Meier curve shows correlation between WT1 score and survival outcome in glioblastoma multiforme (GBM): progression-free survival (PFS) estimate for each score (**a**); PFS estimate for low (score 1 and 2) and high (score 3 and 4) expression groups (**b**); overall survival (OS) estimate for each score (**c**); OS estimate for low (score 1 and 2) and high (score 3 and 4) expression groups (**d**)

data gathered. The Kaplan–Meier curve further illustrates this trend (Fig. 2).

To further verify this observed pattern, the levels were reclassified into two groups: low WT1 expression (scores 1 and 2) and high WT1 expression (scores 3 and 4). Median PFS and OS after treatment were noted to be higher in the latter group at 20.0 weeks and 54.4 weeks, respectively. Similarly, Kaplan–Meier estimates revealed significant differences in terms of PFS and OS in the two groups ($P = 0.022$ and 0.035, respectively, by log-rank test) (Fig. 2b,d).

Correlation between MIB-1 staining index and survival outcome

Analyses of MIB-1 labeling were done in only 32 of the 37 specimens because 5 of the specimens were an insufficient sample. Of these specimens, 17 had an MIB-1 staining index <20 whereas 15 had an index ≥20. The median PFS and OS for the former group were noted to be longer at 14.6 weeks and 35.7 weeks, respectively (Table 3). The Kaplan–Meier estimate, however, was not statistically significant for either PFS ($P = 0.860$, by log-rank test) or OS ($P = 0.856$, by log-rank test) (Fig. 3a,b).

Table 3. Relationship of MIB-1 staining index to survival

MIB-1 staining index	Number of patients	Median PFS (weeks)	Median OS (weeks)
Staining index <20	17	14.6	35.7
Staining index ≥20	15	10.0	36.7

Correlation of WT1 score and MIB-1 index before and after WT1 immunotherapy

Only five tumor samples from GBM patients were available for analysis for this part of the study. Data from these specimens are tabulated in Table 4. It was evident that no change in the WT1 score was seen. On the other hand, a mean increase in MIB-1 index was observed, from 17.7 to 23.4, which was not a statistically significant change ($P = 0.17$, by paired t test).

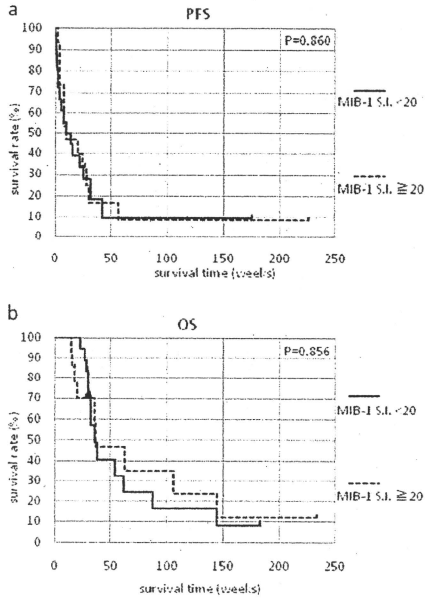

Fig. 3. Kaplan–Meier curve showing (**a**) PFS and (**b**) OS according to MIB-1 staining index

Table 4. WT1 score and MIB-1 index of three patients who failed therapy

Samples	MIB-1 index		WT1 score	
	Before	After	Before	After
1	21.0	20.6	4	4
2	16.0	30.6	3	3
3	5.8	16.5	2	2
4	6.4	2.3	4	3
5	39.1	47.2	3	3
Mean	17.7	23.4	3.2	3.0

Discussion

The WT1 gene is a gene responsible for a childhood renal neoplasm, Wilms' tumor. It comes about secondarily to the inactivation of both its alleles located at chromosome 11p13.[5] Although initially categorized as a tumor suppressor gene, newer studies revealed that WT1 exerts an oncogenic rather than a tumor-suppressive function in various hematological and solid malignancies.[6,7] It was found to be overexpressed in leukemias,[8] breast cancers,[9] lung cancers,[10] renal cell carcinomas,[11] and bone and soft tissue sarcomas.[12] Recently, it was discovered to promote tumor cell proliferation and survival by inhibiting the p53-mediated cell apoptosis,[1] thus making it a suitable target for cancer therapy and an ideal prognostic marker of disease outcome.[7,13] In fact, in an article from the National Cancer Institute that prioritized 75 cancer antigens, WT1 was ranked as the top by predefined criteria.[14]

Studies on gliomas have revealed that WT1 is highly expressed in patients with GBM and that it plays a significant role in gliomagenesis.[1] We have reported that, in 73 glial tumors, almost all high-grade gliomas highly expressed WT1 and a significant correlation was seen between WT1 expression level and MIB-1 index.[15] In light of these findings and the successful development of a WT1 peptide vaccine, a clinical trial utilizing this novel treatment strategy for GBM was started. Preliminary result of the phase II trial, involving patients with progressive or recurrent GBM, showed a favorable outcome compared to that of a standard chemotherapeutic regimen, in patients with WT1/HLA-A*2402 positivity who received WT1 immunotherapy.[3]

With mounting treatment options for GBM nowadays, markers that reliably predict treatment responses are critical for the selection of an appropriate treatment approach for each GBM patient.

The results of our study showed that tumor specimens with high levels of WT1 expression before immunotherapy were associated with a longer survival period after WT1 vaccination. This association was evidenced by an increase in the PFS of 12 weeks and OS of 26 weeks in patients whose tumor had a score of 3 and 4 against those with a score of 1 and 2. This improvement in the survival outcome, when compared with the standard treatment protocol for newly diagnosed GBM patients using a combination of radiotherapy and temozolomide (Stupp's regimen), far exceeded the 10 weeks increase reported by Stupp and colleagues in their patients.[16] Hence, it can be said that WT1 expression level can be used as a gauge to predict survival outcome after immunotherapy.

Another relevant observation, with regard to expression and outcome, is that a linear relationship seems to exist between these two variables, with higher expression leading to longer survival. The rationale behind WT1 immunotherapy is the induction of WT1-specific cytotoxic T lymphocytes (CTL) that will target tumor cells.[17] In the presence of higher WT1 levels, which will serve as tumor antigens, more CTL response will likely be produced and more tumor cells will likely be attacked. This progression subsequently leads to a decrease or cessation of tumor proliferation and, eventually, better survival. Thus, the finding of a linear association between expression and survival seems logical.

However, it is evident that this relationship has its saturation point, as in most other disease models. It could be noted that, in the study population, those with high WT1 expression (score 4) did not fare better compared to those with intermediate expression (score 3), although they did fare significantly better than those with minimal expression (scores 1 and 2). This finding indicates that the upper limit of the linear association may have already been reached and

that beyond this point the immune response is already inadequate to combat the massive tumor burden, leading to "tumor escape."

We also determined the relationship between MIB-1 staining index and outcome after WT1 immunotherapy. Our data showed that, as an independent predictor of treatment response, the initial MIB-1 staining index before immunotherapy did not significantly predict survival outcome after therapy. This finding is in congruence with previous studies on the prognostic value of MIB-1 labeling index that reported no association between treatment outcome and standard treatment protocols.[18,19]

To gain further insight on the value of WT1 expression level and MIB-1 staining index, we analyzed tumor samples from five patients who failed WT1 immunotherapy. It can be observed that the mean WT1 expression levels in these samples did not change much from that observed before treatment. In contrast, the MIB-1 staining index increased after vaccine failure. This finding seems to imply the aforementioned "tumor escape" phenomenon may be related more to the proliferative index of the tumor. Further investigations are warranted to verify this theory.

This is the first article to focus on WT1 expression level as an indicator of outcome after immunotherapy. Based on the results of our study, there is good and solid evidence that WT1 expression levels can be used as a prognostic indicator of survival outcome and that tumors with an intermediate degree of WT1 level have the best survival outcome. Moreover, our study revealed that the MIB-1 staining index does not reliably predict survival outcome after therapy. However, the authors admit that because of study and methodological restrictions some factors that could have some influence in the outcome were not controlled (e.g., co-morbid conditions, immunological status); therefore, attributing the improvement in survival solely to WT1 expression levels may be premature at this time.

Conclusion

In this article, we have demonstrated that only WT1 expression level and not MIB-1 staining index can predict tumor response to WT1 immunotherapy. Furthermore, tumors with intermediate WT1 staining intensity seemed to have the best survival outcome after treatment.

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A Clear Correlation between WT1-specific Th Response and Clinical Response in WT1 CTL Epitope Vaccination

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Abstract. *Clinical studies of WT1-targeted cancer vaccine are being performed. However, WT1-specific Th response in cancer patients remains unclear. Using quantitative real-time RT-PCR, we investigated IFN-γ and IL-10 mRNA expression from Th cells by stimulation with helper peptide $WT1_{332}$. Seventeen patients, of whom 10 had achieved stable disease and the remaining 7 had progressive disease, were weekly vaccinated with WT1 CTL epitope (modified $WT1_{235}$) and examined for $WT1_{332}$-specific Th response. A clear correlation between $WT1_{332}$-specific Th response and clinical response was observed at 4 weeks post-vaccination. In patients who responded, a clear inverse correlation between IL-10-type and IFN-γ-type $WT1_{332}$-specific Th response was detected at pre- and 4 weeks post-vaccination, and the shift of the Th response from IL-10-type dominancy at early phase to IFN-γ-type dominancy at late phase was observed. From this study we concluded that occurrence of $WT1_{332}$-specific Th response could predict good clinical response of WT1 CTL epitope vaccination.*

The Wilms' tumor gene *WT1* was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1, 2). The *WT1* gene encodes a zinc finger transcription factor, and the gene product positively or negatively regulates transcription of various kinds of genes (3). Although the *WT1* gene was first categorized as a tumor-suppressor gene (4), we and others recently demonstrated that the wild-type WT1 gene, which was highly expressed in leukemia and various kinds of solid cancers, performed an oncogenic role rather than a tumor-suppressor function in these malignancies (5-9). These findings led us to consider that the *WT1* gene product could be a promising target antigen for cancer immunotherapy. In fact, cytotoxic T lymphocyte (CTL) epitopes of WT1 protein were already identified (10, 11) and used as peptide vaccines for the treatment of malignancies in the clinical setting (12-17).

Recently, many clinical studies of cancer immunotherapy are being performed and data of tumor-specific CTLs has been accumulating. Although CTLs are the most important effectors in antigen-specific cancer immunotherapy, activation of CTLs alone often leads to only limited clinical responses (18). Activation of other kinds of immune cells, particularly CD4+ helper T (Th) cells, is also needed to obtain stronger immunological responses (19-22). However, information of tumor antigen-specific Th cell was not enough. We previously identified WT1 protein-derived helper peptide $WT1_{332}$ that could promiscuously bind to HLA-DRB1*0405, 1501, 1502 and -DPB1*0901 molecules(23, 24). It was also shown that the $WT1_{332}$ was naturally generated from WT1 protein through processing in cancer cells and dendritic cells (DCs) and preferentially induced Th1 responses *in vitro*. Therefore, this peptide should be useful for co-vaccination with CTL epitopes to induce stronger immunological responses.

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In the present study, to investigate whether or not $WT1_{322}$-specific Th response is involved in clinical outcome of WT1 CTL-epitope vaccination, peripheral blood mononuclear cells (PBMCs) from patients who were treated with CTL epitope peptide vaccine were stimulated by $WT1_{332}$ and IFN-γ and IL-10 mRNA expression in Th cells which were measured using quantitative real-time RT-PCR method. Here we clearly demonstrated an apparent correlation between $WT1_{332}$-specific Th response and clinical response.

Patients and Methods

Patients and clinical study of WT1 CTL epitope peptide vaccination. In "Phase I/II clinical trial of WT1 peptide-based vaccine for the patients with malignant tumors" (ID: UMIN000002001), which was approved by the Ethical Review Boards of Faculty Medicine, Osaka University, patients with various kinds of solid cancers were intradermally injected with a 9-mer modified $WT1_{235}$ peptide (CYTWNQMNL) emulsified with Montanide ISA51 adjuvant at weekly intervals for 3 months and clinical responses were assessed by RECIST criteria. Major entry criteria were as follows: 1) expression of WT1 in cancer tissues determined by RT-PCR and/or immunohistochemistry, 2) HLA-A*2402-positivity, 3) estimated survival of >3 months, 4) performance status from 0 to 1 (Eastern Cooperative Oncology Group), 5) no severe impairment of organ function, and 6) no administration of chemotherapy, immunotherapy, immunosuppressive therapy, or radiotherapy within 4 weeks before WT1 vaccination.

WT1 peptides. A 9-mer modified $WT1_{235}$ peptide (CYTWNQMNL, 235-243) (11), in which M at position 2 (an anchor for HLA-A*2402-binding) in the natural epitope (CMTWNQMNL) was replaced by Y, was used in the phase I/II clinical study of WT1 immunotherapy. This modified peptide had been shown to induce stronger CTL activity against WT1-expressing tumor cells than the natural peptide and purchased from Neo-Multiple Peptide Systems (San Diego, CA).

$WT1_{332}$ helper peptide (KRYFKLSHLQMHSRKH, 332-347) that could induce Th cell response in the context of multiple HLA class II molecules(23) were purchased from Sigma Genosys Japan (Hokkaido, Japan).

Cell preparation and $WT1_{332}$ stimulation. Peripheral blood (PB) samples were obtained from 17 patients at pre- and post-WT1 vaccinations and from 22 healthy donors. PBMCs were separated by density centrifugation using Lymphocyte Separation Solution (nacalai tesque, Japan) and frozen until use was indicated.

For analysis of $WT1_{332}$-specific Th responses, the frozen PBMCs were thawed, washed twice with RPMI medium, suspended in X-VIVO 15 medium supplemented with 10% AB serum in 24-well plate and incubated at 37°C in a humidified 5% CO_2 overnight. On next day, the PBMCs were harvested and washed twice, and suspended in X-VIVO 15 medium supplemented with 1% AB serum and CD28/49d Costimulatory Reagent (BD Bioscience, San Jose, CA) and put in 12×75 mm polystyrene tubes. Ten μl of water containing or non-containing 20 μg of $WT1_{332}$ peptide were added to the culture tubes and then the culture tubes were incubated at a 5° slant at 37°C for 4 h in a humidified 5% CO_2 atmosphere. After the incubation, CD4+ T cells were isolated from PBMCs using BDTM IMag Human CD4 T Lymphocyte Enrichment Set (BD Bioscience) according to the manufacture's protocol. The purity of the isolated CD4+ T cells was confirmed to be more than 93% by flow cytometric analysis.

RNA isolation from CD4+ T cells and cDNA synthesis. RNA isolation from CD4+ T cells was performed with Trizol (Invitrogen, California, USA) according to the manufacturer's instruction. RNA was dissolved in water and stored at –70°C. For cDNA synthesis, Approximately one μg of total RNA from each sample was reverse transcribed into cDNA with Oligo$(dT)_{12-18}$ primer (Invitrogen) using a SuperScript™ III first strand synthesis SuperMix kit (Invitrogen) according to the manufacturer's instructions. cDNA was stored at –80°C until use.

Construction of plasmid cDNA standards. cDNA of IFN-γ, IL-10 and β-actin were obtained from activated lymphocytes that were cultured in the presence of PHA and IL-2 for 3 days. cDNA fractions of the IFN-γ, IL-10 and β-actin were amplified by a PCR reaction using a PCR thermocycler 9700 system (Applied Biosystems, Foster City, CA). The oligonucleotide primers used were as follows: IFN-γ forward primer, 5'-GTC CTT TGG ACC TGA TCA GC-3'; reverse primer, 5'-CCT TGA TGG TCT CCA CAC TC-3'; IL-10 forward primer, 5'-TAA GGG TTA CCT GGG TTG CC-3'; reverse primer, 5'-GCC ACC CTG ATG TCT CAG TT-3'; β-actin forward primer, 5'-CCA TCA TGA AGT GTG ACG TGG-3'; reverse primer, 5'-CGC AAC TAA GTC ATA GTC CGC-3'. The PCR reaction solution contained 1 μl cDNA template (10-100 ng), 200 μM dNTPs, 2 μM of each primer, 2 mM $MgCl_2$, 1.25 U TaqGold polymerase (Applied Biosystems, CA, USA) and 5 μl 10× Taq buffer (Applied Biosystems) and adjusted to the final volume of 25 μl by the addition of water. The PCR conditions were as follows: 9 min at 95°C, 30 cycles of 45 s at 94°C, 45 s at 60°C, 45 s at 72°C, and 7 min at 72°C. Amplification products were purified using QIA quick PCR Purification Kit. The purified PCR amplicons were then ligated into a TA-cloning vector using T4 DNA ligase supplied with the TA Cloning® Kit. The vector-PCR product ratio was 1:1, and the ligation reaction solution consisted of a fresh PCR sample, 50 ng pCR2.1 vector, T4 DNA Ligase (4.0 U) and 10× Ligation Buffer, were adjusted to a final volume of 10 μl by the addition of water and incubated at 14°C overnight. Two microliters of the ligation reaction samples were mixed with competent E. coli Top10 cells for transformation using the heat shock method described in the manufacturer's manual (Invitrogen). Clones containing inserts were identified by PCR analysis. Positive clones were sequenced in a Perkin Elmer ABI-Prism 377 DNA sequencer (Applied Biosystems) using ABI-prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The clones containing the correct IFN-γ, IL-10 and β-actin cDNA inserts were used for standard curve.

Quantitative real-time RT-PCR. Measurement of gene expression was performed utilizing the ABI prism 7900HT Sequence Detection System (Applied Biosystems). Primers and TaqMan probes (Applied Biosystems) were designed to span exon-intron junctions to prevent amplification of genomic DNA and to let amplicon size reduce to <150 bp for enhancement of efficiency of PCR amplification. Primer and probe sequences were as follows: IFN-γ (forward), 5'-TTT TCA GCT CTG CAT CGT TTT G-3'; IFN-γ (reverse), 5'-GCT ACA TCT GAA TGA CCT GCA TTA A-3'; IFN-γ (probe), FAM-TCT TGG CTG TTA CTG CCA GGA CCC A-TAMRA; IL-

Table I. *Patient profile.*

Pt. no.	Age (years)	Gender	Disease	DR‡	$WT1_{332}$-specific response (IFN-γ ratios/IL-10 ratios)§			Clinical response¶
					Pre	4weeks post	8weeks post	(RECIST criteria)
1	54	F	BC	4	0.53/2.62	0.77/2.94	0.68/1.08	SD
2	75	M	GBM	15	0.35/7.4	0.29/7.35	0.94/0.81	SD
3	56	M	Astrocytoma	4	0.56/3.07	0.66/3.52	0.84/1.03	SD
4	66	M	GBM	15	0.46/2.24	1.58/u.e.	n.t.	SD
5	53	M	PC	4	0.81/1.53	0.32/2.75	0.77/1.24	SD
6	40	F	Astrocytoma	4	0.7/0.39	1.69/0.34	1.65/0.09	SD
7	70	M	Renal cancer	15	1.17/0.45	1.16/0.14	1.21/0.48	SD
8	62	F	CC	15	1.12/0.86	1.44/0.3	n.t.	SD
9	52	M	Rectal cancer	15	1.4/u.e.	1.39/1.95	0.6/0.45	SD
10	69	M	GBM	15	1.01/0.83	0.79/0.54	0.93/1.4	SD
11	58	M	CC	4	0.71/0.66	0.72/3.65	n.t.	PD
12	55	M	GBM	15	0.86/1.85	1.37/1.15	1.42/4.54	PD
13	76	M	Mediastinal tumor	4	0.77/0.22	1.02/1.47	0.73/0.81	PD
14	56	F	BC	15	0.95/0.62	0.86/1.78	1.19/0.43	PD
15	54	F	BC	4	1.05/0.6	0.62/0.99	n.t.	PD
16	78	F	PC	15	1.27/0.78	1.49/0.7	1.21/0.81	PD
17	33	M	Astrocytoma	15	0.86/1.6	1.11/0.72	0.88/1.58	PD

GBM, glioblastoma multiforme; astrocytoma, anaplastic astrocytoma; CC, colon cancer; PC, pancreatic cancer; BC, breast cancer; ‡HLA-DR allele. DR4 and DR15 represent DRB1*0405 and DRB1*1502, respectively; §ratio of relevant gene mRNA copies of $WT1_{332}$-stimulated Th cells to those of unstimulated Th cells. ¶clinical response by WT1 peptide (CTL-epitope) vaccination; SD, stable disease; PD, progressive disease; n.t., not tested; u.e., unevaluated.

10 (forward), 5'-CCC CAA GCT GAG AAC CAA GAC-3'; IL-10 (reverse), 5'-GGC CTT GCT CTT GTT TTC ACA-3'; IL-10 (probe), FAM-ATC GAT GAC AGC GCC GTA GCC TCA-TAMRA; β-actin (forward), 5'-TTG CCG ACA GGA TGC AGA A-3'; β-actin (reverse), 5'-GGA CAG CGA GGC CAG GAT-3'; β-actin (probe), FAM-TCA AGA TCA TTG CTC CTC CTG AGC GC-TAMRA. To make standard curves, the plasmid solution containing respective cDNA standards was serially diluted and amplified *via* quantitative real-time RT-PCR. Quantitative real-time RT-PCR reaction was performed in a total volume of 20 μl with TaqMan Master Mix (Applied Biosystems), 900 nM primers and 250 mM probes. Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Standard curves were prepared for each gene and extrapolation of copy number was performed for *IFN-γ*, *IL-10* and *β-actin*. Normalization of samples was performed by dividing the copy number of *IFN-γ* and *IL-10* by that of *β-actin*. All PCR assays were performed in duplicates and reported as the mean.

Statistical analysis. When the expression levels of IL-10 mRNA of CD4+ T-cells increased or decreased more than 2-fold by the stimulation with $WT1_{332}$ helper peptide compared to no stimulation with the peptide, $WT1_{332}$-specific CD4+ T-cell response was defined to be positive and classified into IL-10-Up- and -Down-type response, respectively. The cut-off value of 2-fold was used here, as previously described (25).

The Mann-Whitney *U*-test was used to determine whether there was a statistically significant difference in expression level of IFN-γ and IL-10 mRNA between patients and healthy donors. The Wilcoxon signed-ranks test was used to analyze change of expression level of *IFN-γ* and *IL-10* mRNA among pre-vaccination, 4 and 8 weeks post-vaccination. The Spearman's correlation coefficient by rank test was used to analyze correlation between expression levels of *IFN-γ* and *IL-10* mRNA in patients. The Fisher's exact probability test was used to investigate a correlation between $WT1_{332}$-specific CD4+ T-cell responses and clinical responses.

Results

A clear correlation between $WT1_{332}$ helper peptide-specific Th response and clinical response. PBMCs from WT1 CTL epitope peptide-vaccinated cancer patients were obtained from 17 patients at three time points (pre-, 4 and 8 weeks post-WT1 vaccination) and stimulated with or without $WT1_{332}$ helper peptide in the presence of CD28/49d Co-stimulatory Reagent. Then Th cells were enriched and examined for both the mRNA expression levels of *IFN-γ* and *IL-10* by quantitative real-time RT-PCR methods (Table I).

$WT1_{332}$-specific Th responses were classified into two types. IL-10-Up-type: *IL-10* mRNA expression increased more than 2-fold by $WT1_{332}$ stimulation compared to no peptide stimulation. IL-10-Down-type: *IL-10* mRNA expression decreased more than 2-fold by $WT1_{332}$ stimulation compared to no peptide stimulation. The

Table II. *$WT1_{332}$-specific Th response and clinical response in cancer patients.*

Pt. No.	$WT1_{332}$-specific response			Clinical response‡ (RECIST criteria)
	pre	4w	8w	
1	Up	Up	n.s.	SD
2	Up	Up	n.s.	SD
3	UP	Up	n.s.	SD
4	Up	u.e.	n.t.	SD
5	n.s.	Up	n.s.	SD
6	Down	Down	Down	SD
7	Down	Down	Down	SD
8	n.s.	Down	n.t.	SD
9	u.e.	n.s.	Down	SD
10	n.s.	n.s.	n.s.	SD
11	n.s.	Up	n.t.	PD
12	n.s.	n.s.	Up	PD
13	Down	n.s.	n.s.	PD
14	n.s.	n.s.	Down	PD
15	n.s.	n.s.	n.t.	PD
16	n.s.	n.s.	n.s.	PD
17	n.s.	n.s.	n.s.	PD

Up, IL-10-Up-type response; Down, IL-10-Down-type response; n.s., not significant (an increase or decrease in *IL-10* mRNA expression was below 2-fold); n.t., not tested; u.e., unevaluable. ‡Clinical response by WT1 CTL epitope peptide vaccination: SD, stable disease; PD, progressive disease.

Table III. *A correlation between $WT1_{332}$-specifc Th response and WT1 CTL epitope peptide vaccination-induced clinical response.*

	Clinical response‡ Pre		
$WT1_{332}$-specific Th response†	+	–	p-Value§
+	6	1	0.054
–	3	6	
	9	7	
	4 Weeks post		
	+	–	p-Value
+	7	1	0.02
–	2	6	
	9	7	
	8 Weeks post		
	+	–	p-Value
+	3	2	0.75
–	5	3	
	8	5	

†The number of patients who showed (+) or did not show (–) an IL-10-Up- or IL-10-Down-type response; ‡The number of patients of SD (+) or PD (–) capable of evaluating $WT1_{332}$-specific Th response; §*p*-values were obtained by Fisher's exact probability test.

$WT1_{332}$-specific Th response types and clinical responses were summarized in Table II. Relationship between $WT1_{332}$-specifc Th response and clinical response was investigated (Table III). A clear correlation (p=0.02) between occurrence of $WT1_{332}$-specific Th response of either IL-10-Up- or -Down-type and clinical response was found at 4 weeks post-WT1 vaccination. At pre-WT1 vaccination, such a correlation was also observed although it was not statistically significant (p=0.059).

Thus, these results indicated that monitoring of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination and probably at pre-WT1 vaccination should be useful for prediction of clinical response of WT1 CTL epitope peptide vaccination.

An inverse correlation between $WT1_{332}$-specific IL-10 and IFN-γ responses. In order to dissect the difference in $WT1_{332}$-specific Th response between responders and non-responders, the ratios of IL-10 and IFN-γ mRNA expression in Th cells by $WT1_{332}$ stimulation to those in Th cells by no peptide stimulation were plotted on two axes at three time points (Figure 1). In responders, a clear inverse correlation between *IL-10* and *IFN-γ* mRNA expression ratios was shown at pre- and 4 weeks post-WT1 vaccination (rs=–0.75 and –0.8, respectively) (Figure 1A). In contrast to responders, in non-responders, such an inverse correlation was not observed at any of the three time points (Figure 1B). Importantly, in responders, data were quite scattered for the *IL-10* axis at pre- and 4 weeks post-WT1 vaccination (mean=2.15, SD=2.19 and mean=2.2, SD=2.32, respectively), but they converged on approximately ratio=1.0 at 8 weeks post-WT1 vaccination (mean=0.80, SD=0.44). In association with a decrease in *IL-10* mRNA expression at 8 weeks post-WT1 vaccination, *IFN-γ* mRNA expression (mean=0.98, SD=0.33) slightly increased at that time point, compared to IFN-γ mRNA expression at pre- and 4 weeks post-WT1 vaccination (mean=0.75, SD=0.29 and mean=0.95, SD=0.49, respectively). On the other hand, in non-responders, data converged on nearly ratio=1.0 not only in the *IL-10* axis but also in the *IFN-γ* axis.

These results indicated that $WT1_{332}$-specific Th response was strongly induced in responders compared to non-responders. In responders, IL-10-type of $WT1_{332}$-specific Th response was dominant at pre- and 4 weeks post-WT1

vaccination, but its dominancy became weak at 8 weeks post-WT1 vaccination, leading to the disappearance of the inverse correlation at that time point. Thus, these results suggested that repeated vaccination of WT1 CTL epitope peptide gradually allowed the cytokine production to switch from IL-10 to IFN-γ in the responders, implying the shift to Th1 response by the repeated WT1 CTL epitope peptide vaccination.

Enhancement of spontaneous IFN-γ expression in Th cells in responders. Spontaneous *IFN-γ* and *IL-10* mRNA expression were measured in Th cells in the absence of *in vitro* $WT1_{332}$ stimulation (Figure 2). Spontaneous IFN-γ mRNA expression in Th cells was significantly higher in responders at pre-WT1 vaccination than in healthy donors and the higher expression continued (Figure 2A). In contrast, in non-responders the expression levels were not significantly different from those in healthy donors at any time points (Figure 2A). As for spontaneous *IL-10* mRNA expression in Th cells, no significant difference was found between healthy donors and responders, and healthy donors and non-responders (Figure 2B). These results indicated that spontaneous expression of *IFN-γ* had been increased and continued in responders, suggesting that Th cells in responders were already activated towards pro-immune state before the start of the vaccination.

Discussion

ELISPOT and intracellular cytokine staining assay are considered to be a reliable method for analysis of antigen-specific T-cell responses. However, if the frequencies of the antigen-specific T-cells such as $CD8^+$ CTLs and $CD4^+$ Th cells are extremely low, it is very difficult to measure "quantitatively" the antigen-specific responses by using these assays. In order to circumvent this difficulty, quantitative real-time RT-PCR method was often used for the analysis of cancer antigen-specific T-cell responses in peripheral blood and tumor tissue as previously described (25, 26). In the present study, we adopted this method to measure mRNA of *IFN-γ* and *IL-10* quantitatively in the Th cells for the analysis of $WT1_{332}$-specific Th cells. We demonstrated here that i) occurrence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination correlated with clinical response in responders, and ii) high levels of spontaneous IFN-γ expression were induced in responders prior to and after WT1 vaccination.

To date, there have been no reports demonstrating the correlation between TAA-specific Th response and clinical response. We demonstrate for the first time that occurrence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination clearly correlated with clinical response. These findings suggested that monitoring of $WT1_{332}$-specific Th

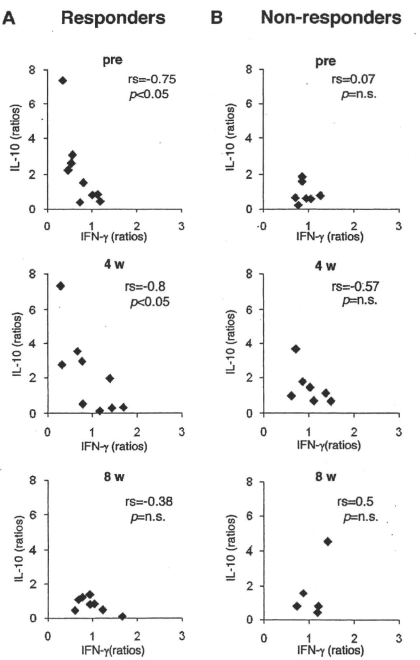

Figure 1. *An inverse correlation between $WT1_{332}$-specific IL-10 and IFN-γ mRNA expression in responders. Ratios of IL-10 and IFN-γ mRNA expression in Th cells by $WT1_{332}$ helper peptide stimulation to those in Th cells by no peptide stimulation were plotted at three time points (pre-, 4 and 8 weeks post-WT1 vaccination) in individual responders (A) and non-responders (B). The Spearman's correlation coefficient by rank test was used to analyze correlation between ratios of $WT1_{332}$-specific IL-10 and IFN-γ mRNA expression in patients.*

response was available for the prediction of clinical response by WT1 CTL epitope peptide vaccination. Unexpectedly, correlation between occurrence of $WT1_{332}$-specific Th response and clinical response disappeared at 8 weeks post-WT1 vaccination. This disappearance seemed to be due to the decrease in IL-10-type response at 8 weeks post-WT1 vaccination. $WT1_{332}$-specific *IFN-γ* mRNA expression slightly increased along with a decrease in *IL-10* mRNA expression at 8 weeks post-WT1 vaccination, indicating that the dominant response type changed from IL-10 response (pre- and 4 weeks post-WT1 vaccination) to IFN-γ response (8 weeks post-WT1 vaccination). Lack of correlation

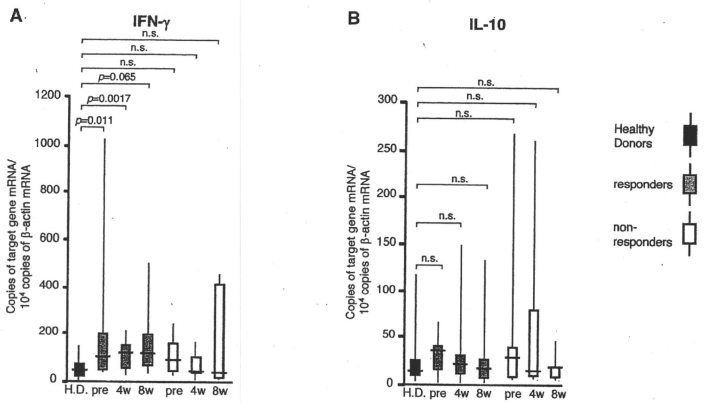

Figure 2. *Increased spontaneous IFN-γ expression in responders. Spontaneous IFN-γ (A) and IL-10 (B) mRNA expression levels in Th cells of responders and non-responders at three time points were shown in comparison with those in Th cells of healthy donors. Horizontal bars indicate median values of the expression levels. The Mann-Whitney U-test was used to determine the statistical significance of difference in the expression levels of IFN-γ and IL-10 mRNA between patients and healthy donors.*

between $WT1_{332}$-specific Th response and clinical response at 8 weeks post-WT1 vaccination should result from the time point still being a transitional stage from an IL-10 response to an IFN-γ one and that thus the IFN-γ response was not strong enough to consider the response as a positive one (it is represented as "n.s." in Table II).

We demonstrated here that IL-10-Up-type and -Down-type responses were an important factor for prediction of clinical response. It is generally thought that IL-10 is mainly produced from Tr-1 and $CD4^+CD25^+Foxp3^+$ Treg cells and that these cells facilitate the suppression of anti-tumor immunity. Recent studies reported an immunosuppressive function of IL-10 that strongly inhibited the activation of antigen-presenting cells, leading to a reduced production of pro-inflammatory mediators and resulting in diminished T-cell stimulation (27, 28). In addition, the inhibitory effects of IL-10 on the pro-inflammatory function of CTLs, Th1-type $CD4^+$ T-cells and NK cells further contribute to its immunosuppressive role. However, in striking contrast with the crucial role of IL-10 in immunosuppression, IL-10 also displays its immunostimulatory function (29, 30). IL-10 can enhance tumor rejection (31, 32), elicit tumor-reactive CTLs (33) and prevent T-cell apoptosis (34). These reports show that IL-10-producing tumor antigen-specific Th cells facilitate anti-tumor immunity in cancer patients. In the present study, the responders had higher IL-10 response, compared to the non-responders at pre- and 4 weeks post-WT1 vaccination. Afterward, the IL-10-type response declined accompanying with enhanced $WT1_{332}$-specific IFN-γ-type response. Therefore, we hypothesized that IL-10 was mainly involved in anti-tumor immunity at early phase (4 weeks post-WT1 vaccination), while at the late phase (8 weeks post-WT1 vaccination), IFN-γ mainly contributed to anti-tumor immunity. Actually, biphasic function of IL-10 has been reported using autoimmune disease model. In this model, early participation of IL-10 in the disease process provoked rapid pathogenic autoimmunity (35, 36). In contrast, the later participation of IL-10 in the disease process protected NOD mice from destructive autoimmunity (37, 38). These paradoxes evidently related to the period in which the immune system was exposed to high levels of IL-10. In human, Mocellin *et al.* previously demonstrated that tumor lesions which expressed high levels of *IL-10* mRNA at pre-vaccination showed tumor regression after the vaccination, and that during the vaccination, *IFN-γ* mRNA expression levels significantly increased in such regressing lesions, compared to non-regressing lesions (39). Taken together, these results indicated that IL-10 functioned as an immunostimulatory cytokine at early phase in WT1 immunotherapy. The immunostimulatory function of IL-10 is needed to be addressed in more detail.

It is very important to establish an immunological monitoring method to predict clinical responses in patients who are being treated with cancer vaccine. Recent report on melanoma patients vaccinated with GP100 peptide showed

that tumor progression occurred despite of induction of high levels of tumor antigen-specific $CD8^+$ T-cells that were detected by tetramer and ELISPOT assay(18). This report indicated that the detection of expanded numbers of vaccine-induced, tumor antigen-specific $CD8^+$ T-cells was not sufficient as a "surrogate marker" for vaccine efficacy. In the present study, the existence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination was predictable for occurrence of clinical response in WT1 CTL epitope peptide vaccination. Thus, Th responses should be valuable as the prediction markers for clinical efficacy of CTL epitope peptide vaccination.

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Conflict of Interest Disclosure

All Authors have no financial conflicts of interest to disclose.

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WT1 Expression Correlates with Angiogenesis in Endometrial Cancer Tissue

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Abstract. *Background: No direct comparison has been made of the relationship between the expression of Wilms' tumor gene WT1 within tumor cells and angiogenesis in vivo. Materials and Methods: A series of 70 endometrial cancer patients who had undergone a curative resection was studied by immunohistochemistry to determine the correlation between WT1 expression, angiogenesis (proliferation of endothelial cell adhesion molecule-1, PECAM-1/ CD31) and angiogenic growth factor (vascular endothelial growth factor, VEGF). Results: A strong association was found between WT1 expression score and mean vascular density ($p<0.001$, $n=70$, $\varrho=0.568$). Immunohistochemistry of serial sections revealed that WT1 and VEGF were co-expressed in the same area of endometrial cancer tissue. Conclusion: Tumor-produced WT1, which may regulate the expression of VEGF, is found to be associated with induction of angiogenesis in endometrial cancer.*

Endometrial cancer is the most common gynecological malignancy in the United States. In Japan, it is the second most common gynecological cancer, but its frequency has dramatically increased in the last decade. Although there are well-established surgical and chemotherapeutic treatments, the need for molecular-target therapy has increased, especially for recurrent disease that has acquired radio- or chemoresistance. A better understanding of the molecular pathways of endometrial carcinogenesis is thus.

The Wilms' tumor gene *WT1* has been isolated and identified as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1-3). This gene encodes a zinc finger transcription factor and plays important roles in cell growth and differentiation (4, 5). Although *WT1* was first categorized as a tumor-suppressing gene, it was recently demonstrated that the wild-type *WT1* possessed an oncogenic rather than tumor-suppressing function in many kinds of malignancies (6). *WT1* is highly expressed in hematological malignancies and solid tumors, including endometrial cancer (7, 8).

In addition, recent studies have reported correlations between WT1 and neovascularization in histogenetics, normal genitourinary development, cardiac malformation and tumor angiogenesis (9-11). Angiogenesis is an important step for tumor growth in the transition from a small cluster of malignant cells to a visible macroscopic tumor capable of spreading to other organs *via* the vasculature (12, 13). The seminal *in vitro* study by Cash *et al.* revealed that the DNA binding domain of WT1 within tumor cells plays an essential role in the transcriptional regulation of vascular epithelial growth factor (VEGF), which is an important factor in inducing tumor angiogenesis (14). Although the potential for angiogenesis associated with tumor-produced WT1 has been suggested, few reports are available on the effect of WT1 as an angiogenic inducer in the intratumoral microenvironment of human solid tumors.

To date, no direct comparison has been made of the relationship of WT1 expression within tumor cells and angiogenesis in terms of the mechanism of tumor progression and metastasis. To address these questions, our work aimed to investigate the role of WT1 within tumor cells in angiogenesis by assessing proliferation of endothelial cell

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Key Words: Wilms' tumor gene, *WT1*, angiogenesis, immunohistochemistry.

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Table I. *Characterization of the antibodies used in this study.*

Antibody (Manufacture)	Species	Dilution	Incubation time (temperature)	Epitope retrieval
WT1 (DakoCytomation Inc. USA)	Mouse IgG	1:100	Overnight (4°C)	Microwave
CD31 (DakoCytomation Inc. USA)	Mouse IgG	1:1	1 hour (room temperature)	Microwave
VEGF (Thermo Fisher Scientific Inc. USA)	Mouse IgG	1:100	2 hours (room temperature)	Microwave

adhesion molecule-1 (PECAM-1, CD31) in surgically-removed endometrial carcinomas.

Materials and Methods

Patients. This study included 70 primary endometrial carcinoma patients consecutively admitted, treated and followed-up by the Department of Obstetrics and Gynecology, Kanazawa University Hospital from January 1995 to December 2002. None of the patients had received any pre-surgical treatment and all had undergone a total abdominal or radical hysterectomy plus bilateral salphingo-oophorectomy. At the time of laparotomy, peritoneal fluid samples were obtained for cytological testing. Systemic pelvic lymphadenectomy was performed in 51 (73%) patients. Paraaortic lymph node sampling was performed in two patients because of visible or palpable enlarged lymph nodes. All patients were classified by the FIGO surgical staging system (1988). No patient had remaining macroscopic tumors or known distant metastasis immediately following surgery. High-risk patients (*e.g.* those with deep myometrial invasion, cervical involvement, special histology, or peritoneal cytology) underwent external radiotherapy and/or six cycles of chemotherapy (paclitaxel: 180 mg/m^2, carboplatin: according to Chatelut's formula [area under the curve, AUC=5 mg min/ml]) as postoperative adjuvant therapy. All treatments and clinical research were conducted with written informed consent.

Immunohistochemistry. WT1, CD31 and VEGF were evaluated in serial sections stained with appropriate antibodies. Table I shows the primary antibodies, their sources, dilutions, incubation times and epitope retrieval. Formalin-fixed, paraffin-embedded slides (5 μm-thick) were deparaffinized and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide for 30 minutes. Staining was performed by avidin-biotin complex (ABC) technique, using SAB-PO kit (Nichirei Co.,Tokyo, Japan). Color development was carried out with the peroxidase substrate 3-amino-9-ethylcarbazole (AEC) against WT1, CD31 and VEGF. All slides were counterstained with Mayer's hematoxylin. Sections without primary antibodies, as well as those with non-immunized mouse serum, served as negative controls.

Evaluation of staining. For evaluation of WT1 expression, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%) according to the percentage of positive staining area in relation to the whole carcinoma area. The sum of the intensity and extent score was used as the final staining score (0-7) for WT1. Tumors having a final staining score of ≥5 were considered to exhibit strong expression.

In assessment of tumor microvessels, the densest vascular areas were identified by scanning tumor sections at low magnification (×40). After identification of the densest vascularization, a vessel count was performed at ×400 magnification. The average vessel number in 5 fields was expressed as the microvessel density (MVD).

Evaluation and counting were conducted by two observers (S.D. and S.O.) who were unaware of any details regarding the patients' background.

Statistical analysis. Chi-square test for 2×2 tables was used to compare the categorical data. The difference in continuous variables between groups was compared using Mann-Whitney *U*-test. Relations between continuous variables were investigated by means of Spearman's rank correlation coefficient test. A p-value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed using the statistical package StatView version 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA).

Results

Characteristics of patients. The patients' average age at time of surgery was 57.3 years (range, 26-78, years). Patients with endometrial cancer included: 22 of premenopausal status, 4 of perimenopausal status and 44 of postmenopausal status. The patients' mean preoperative body mass index (BMI) was 24.0 (range, 16.9-32.9).

WT1 expression. WT1 expression was positive exclusively in cancer cells in 64 cases (91%). The expression of WT1 was strong (final staining score of 5-7) in 31 patients (44%) and weak (final staining score of 0-4) in 39 patients (56%). Typical WT1 expression in endometrial cancer cells is shown in Figure 1A. The majority of positive cases showed diffuse or granular staining in the cytoplasm. Staining of WT1 was heterogeneous in advanced tumors and WT1 was frequently located at the invasion front of the tumor. The association between WT1 expression and clinicopathological variables is shown in Table II.

WT1 overexpression was associated with advanced FIGO stage (p=0.0266), myometrial invasion (p=0.0477) and high-grade histological differentiation (p=0.0049), indicating up-regulation of WT1 expression with tumor progression in this study.

CD31 expression. CD31-positive endothelial cells lining microvessels within tumors were detected in 58 out of 70 patients (Figure 2). MVD varied from 0 to 66.8, with a median of 13.6 and mean of 20.0 (standard deviation, 20.1). MVD was significantly higher in tumors of advanced FIGO

Table II. *WT1 expression and clinicopathological characteristics.*

Variable	WT1 expression		*P*-value (χ^2 test)
	Strong (n=31)	Weak (n=39)	
Age (years)			
<60 (n=43)	16	27	
≥60 (n=27)	15	12	0.1325
FIGO stage			
I (n=52)	19	33	
II, III, IV (n=18)	12	6	0.0266
Lymph node metastasis			
Negative (n=65)	28	37	
Positive (n=5)	3	2	0.4629
Depth (myometrial invasion)			
a (n=17)	4	13	
b, c (b, n=36; c, n=17)	27	26	0.0477
Histopathology, degree of differentiation			
Grade 1 (n=38)	11	27	
Grade 2, 3 (n=32)	20	12	0.0049
Menopause			
Peri, pre (n=26) 8 18			
Post (n=44) 23 21 0.0801			
Body mass index			
<25 (n=45)	19	26	
≥25 (n=25)	12	13	0.6410

Table III. *Microvessel density and clinicopathological characteristics.*

Variable	Microvessel density mean±SD (median)	*P*-value (Mann-Whitney *U*-test)
Age (years)		
<60 (n=43)	19.3±19.9 (13.6)	
≥60 (n=27)	21.3±20.8 (13.6)	0.8135
FIGO stage		
I (n=52)	16.9±18.3 (10.9)	
II, III, IV (n=18)	29.2±23.0 (19.6)	0.0191
Lymph node metastasis		
Negative (n=65)	19.9±20.0 (13.6)	
Positive (n=5)	21.8±24.8 (10.8)	0.8999
Depth (myometrial invasion)		
a (n=17)	9.3±14.5 (4.2)	
b, c (b, n=36; c, n=17)	23.4±20.6 (17.8)	0.0120
Histopathology, degree of differentiation		
Grade 1 (n=38)	18.2±19.2 (13.6)	
Grade 2, 3 (n=32)	23.3±21.8 (13.6)	0.0019
Menopause		
Peri, pre (n=26)	12.6±14.7 (6.0)	
Post (n=44)	28.8±22.3 (19.4)	0.5223
Body mass index		
<25 (n=45)	17.1±18.2 (13.9)	
≥25 (n=25)	21.7±21.2 (12.2)	0.4388

stage (p=0.0191), with myometrial invasion (p=0.0120) and of high-grade histological differentiation (p=0.0019) (Table III). The other clinicopathological characteristics were not associated with MVD.

Association between WT1 expression and CD31. CD31 in the strong-expression WT1 group was significantly higher than in the weak-expression WT1 group, by Mann-Whitney *U*-test (p<0.001) (data not shown). Moreover, considering WT1 expression scores as continuous variables, a strong association was found between WT1 expression and CD31 expression (p<0.001, n=70, ϱ=0.568) using Spearman's rank-correlation coefficient (Figure 3).

VEGF expression in WT1-positive cancer cells. Typical VEGF expression within tumor cells is shown in Figure 1B. Immunohistochemistry of serial sections revealed that VEGF and WT1 were co-expressed in the same area of endometrial cancer tissue.

Discussion

Recent studies showed that tumor-produced WT1 influenced tumor progression in various types of cancer. Yamamoto *et al.* found that WT1 protein might be an accelerator of the progression of ovarian serous adenocarcinoma (15). Sera *et al.* concluded that overexpressed WT1 was associated with tumor growth, and resulted in a worsening prognosis of hepatocellular carcinoma (16). Hylander *et al.* demonstrated that patients with WT1-positive tumors had a higher grade (p=0.006) and advanced stage (p=0.002) of epithelial ovarian cancer (17). Miyoshi *et al.* found that tumors >2 cm also showed a trend toward an increase (p=0.09) in *WT1* mRNA levels as compared with tumors <2 cm in breast cancer (18). In the present study, we found that WT1 overexpression was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation, indicating up-regulation of *WT1* expression with tumor progression. Our results are congruent with previous reports of the other types of cancer.

Tumor development and progression are inherently dependent on the process of angiogenesis. Ozalp *et al.* found that microvessel density (MVD) as assessed by factor VIII antigen was correlated with high surgical stage (p<0.001), cervical involvement (p=0.01), adnexal involvement (p=0.04), lymphovascular space involvement (p=0.02), pelvic and paraaortic lymph node metastasis (p<0.001), and positive peritoneal cytology (p<0.001) in endometrial carcinoma (19). Salvesen *et al.* found that MVD as assessed by CD105/endoglin antigen was significantly correlated with MVD as assessed by factor VIII antigen (linear regression