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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

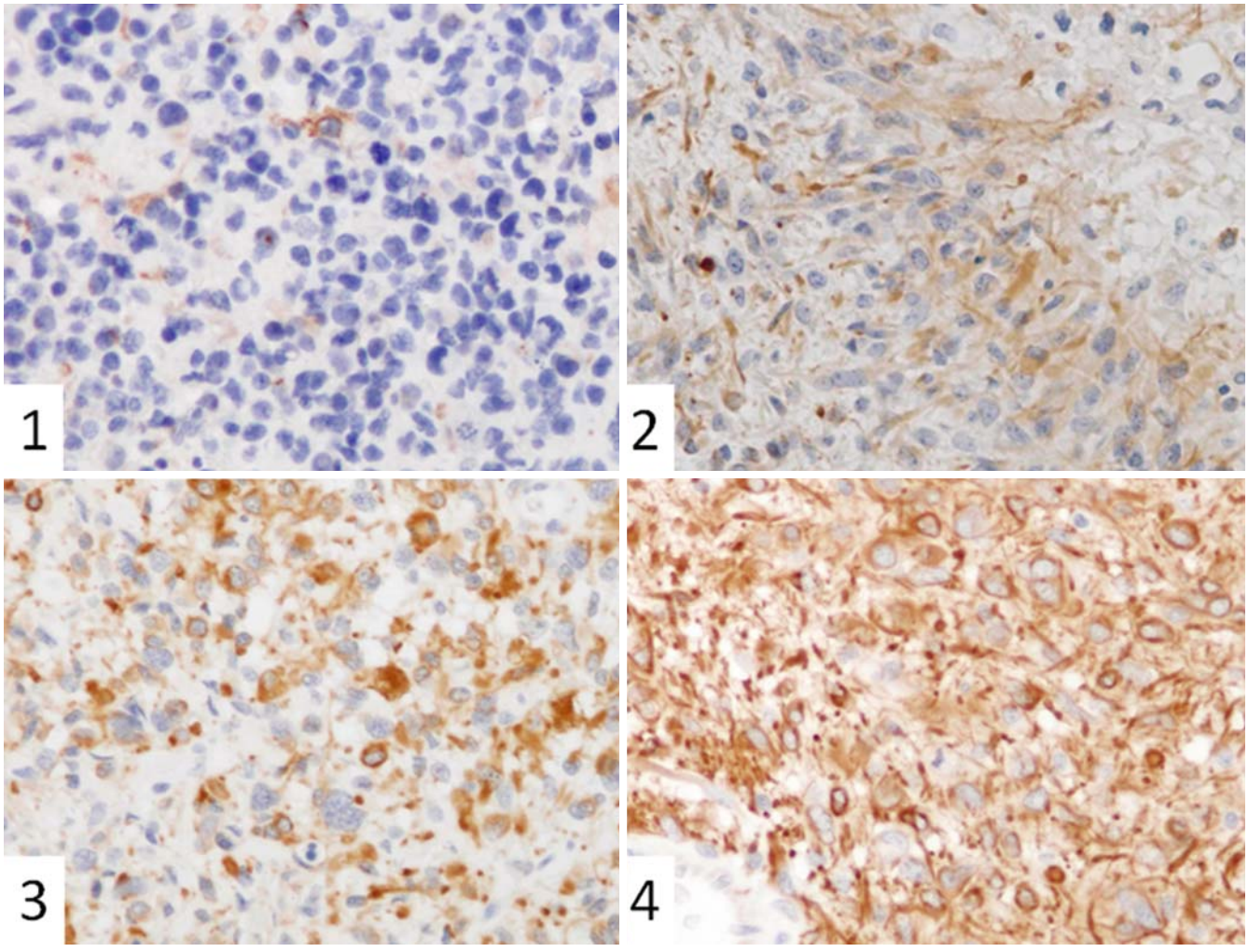

Fig. 1. The level of Wilms' tumor 1 (WT1) expression was classified. Score *1*, slightly increased staining in some tumor cells compared with that in normal glial cells; score *2*, staining at intermediate intensity in some tumor cells; score *3*, strong staining in some tumor cells and intermediate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

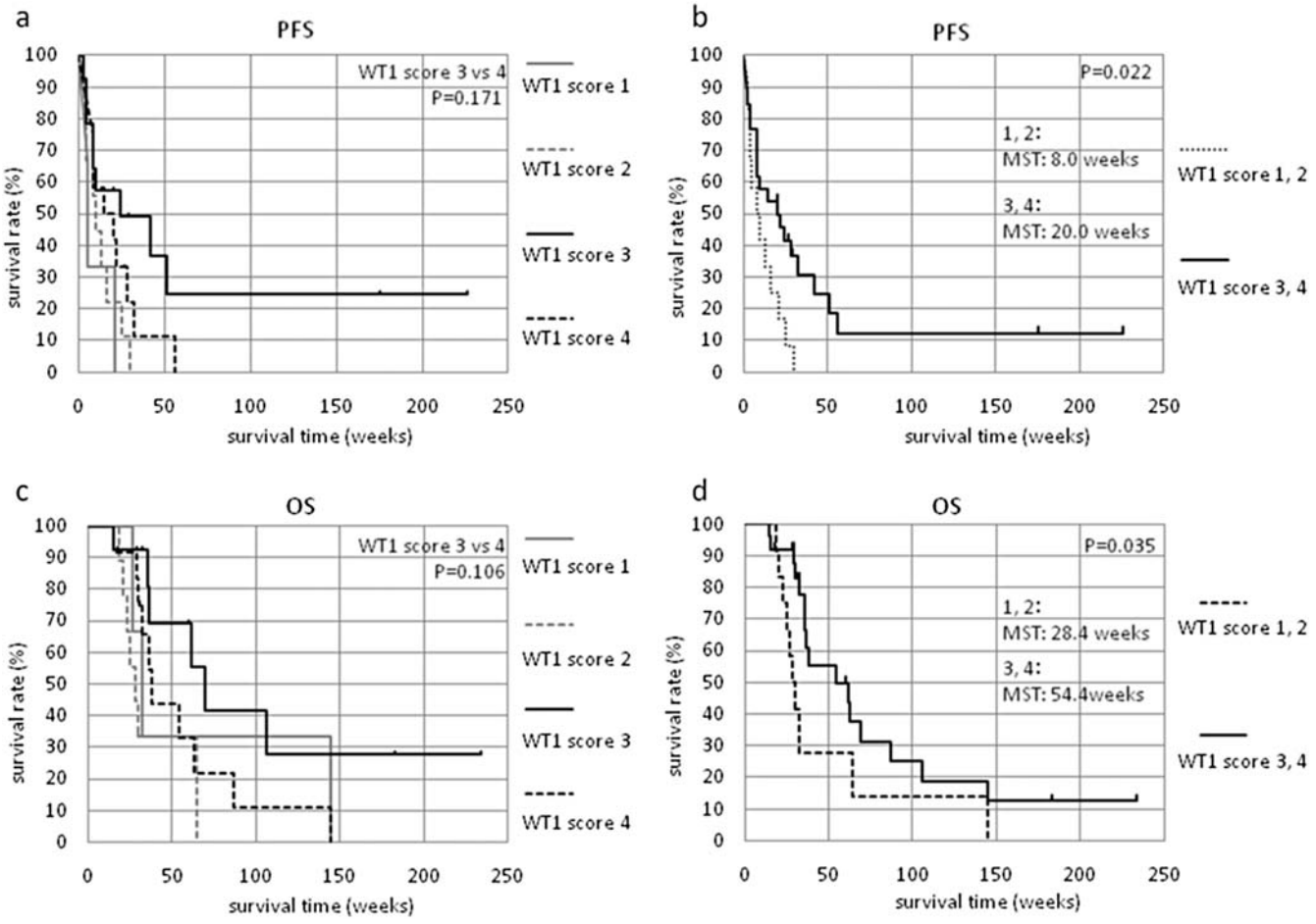

Fig. 2. Kaplan–Meier curve shows correlation between WT1 score and survival outcome in glioblastoma multiforme (GBM): progression-free survival (PFS) estimate for each score (**a**); PFS estimate for low (score 1 and 2) and high (score 3 and 4) expression groups (**b**); overall survival (OS) estimate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

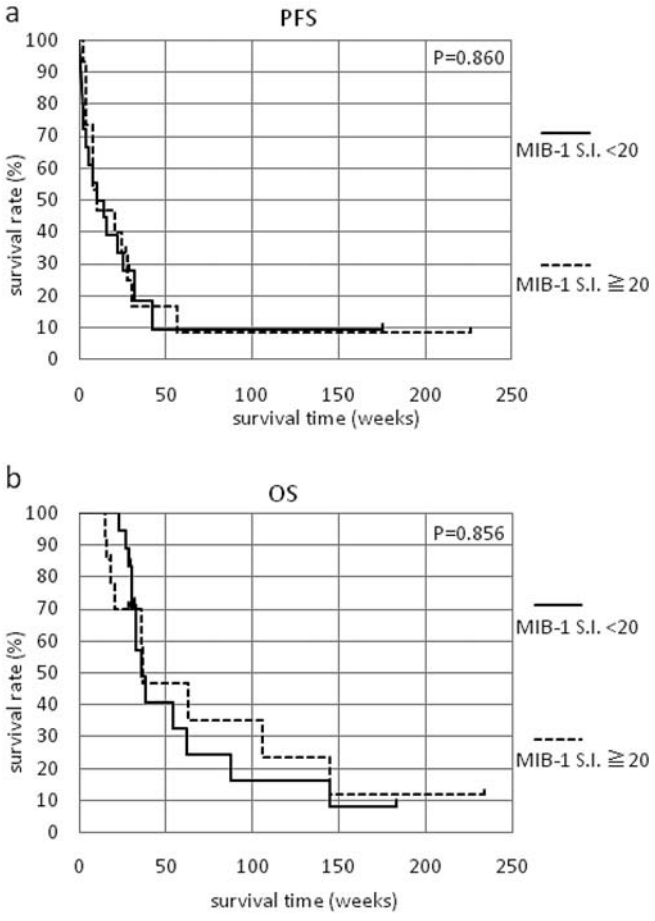

Fig. 3. Kaplan–Meier curve showing (**a**) PFS and (**b**) OS according to MIB-1 staining index

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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