Active Specific Immunotherapy Targeting the Wilms' Tumor Protein 1 (WT1) for Patients with Hematological Malignancies and Solid Tumors: Lessons from Early Clinical Trials

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LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Explain the role of the Wilms' tumor protein 1 (WT1) as a tumor antigen in peptide- and dendritic cell-based cancer immunotherapy trials.
2. Describe the immune responses elicited by WT1-based cancer vaccines and their potential for creating clinical responses in a majority of evaluable cancer patients

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ABSTRACT

There is a growing body of evidence that Wilms' tumor protein 1 (WT1) is a promising tumor antigen for the development of a novel class of universal cancer vaccines. Recently, in a National Cancer Institute prioritization project, WT1 was ranked first in a list of 75 cancer antigens. In this light, we exhaustively reviewed all published cancer vaccine trials reporting on WT1-targeted active specific immunotherapy in patients with hematological malignancies and solid tumors. In all clinical trials, vaccine-induced immunological responses could be detected. Importantly, objective clinical responses (including stable disease) were observed in 46% and 64% of evaluable vaccinated patients with solid tumors and hematological malignancies, respectively. Immunogenicity of WT1-based cancer vaccines was demonstrated by the detection of a specific immunological response in 35% and 68% of evaluable

patients with solid tumors and hematological malignancies, respectively. In order to become part of the armamentarium of the modern oncologist, it will be important to design WT1-based immunotherapies applicable to a large patient population, to standardize vaccination protocols enabling systematic review, and to further optimize the immunostimulatory capacity of the vaccine components. Moreover, improved immunomonitoring tools that reveal clinically relevant T-cell responses will further shape the ideal WT1 immunotherapy strategy. In conclusion, the clinical results obtained so far in WT1-targeted cancer vaccine trials reveal an untapped potential for inducing cancer immunity with minimal side effects and hold promise for a new adjuvant treatment against residual disease and against cancer relapse. *The Oncologist* 2012;17:250–259

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INTRODUCTION

Wilms' tumor protein 1 (WT1) is a promising tumor antigen for the development of a novel class of universal cancer vaccines. In this review, we focus solely on cancer vaccines targeting WT1 as an antigenic target for active specific immunotherapy. In early publications, *WT1* was described as a tumor suppressor gene [1–4], but afterward it became clear that it also can act as an oncogene [5, 6]. WT1 is a transcription factor and is involved in cell proliferation, differentiation, as well as apoptosis and organ development [7–10]. Several features of this gene make it a promising target for immunotherapy. First, it is highly expressed in several types of hematological malignancies as well as in solid tumors (Table 1) [11–50]. Growth inhibition could be shown in leukemic and solid tumor cells using treatment with WT1 antisense oligonucleotides [11, 51–53]. WT1 has a negative influence on differentiation, but promotes proliferation of progenitor cells [53, 54]. Osaka et al. [6] showed that cells with high levels of WT1 had a stronger tendency to develop into leukemias. WT1 protein is an immunogenic target and exhibits high T-cell antigenicity, as shown by several groups [55–59]. WT1-specific T cells as well as IgG anti-WT1 antibodies have been demonstrated in cancer patients [12, 60–67]. Loss of WT1 expression leads to cessation of proliferation or death of the cancer cells. Therefore, the risk for tumor immune escape resulting from emergence of antigen loss variants is believed to be very small [11, 13, 51]. WT1 is also expressed in a small number of normal tissues, like gonads (testis, ovary), kidney, spleen, and bone marrow [14, 68–70]. Despite expression in normal tissues, there are no reports so far indicating autoimmune reactions in mice or humans after WT1-targeted immunotherapy [55, 57–59, 71, 72]. Because WT1 is a self antigen, it is believed that high-affinity T cells against this antigen are deleted from the repertoire by clonal deletion in the thymus (reviewed by Wieggers et al. [73]). Several mechanisms have been proposed to explain the finding that WT1-specific cytotoxic T lymphocytes (CTLs) could kill leukemic cells, but not WT1-expressing normal cells. A first explanation is that the level of WT1 expression in normal cells is too low to be recognized by low-affinity WT1-specific T cells. Hence, only WT1-overexpressing tumor cells could be the target of those low-affinity T cells. However, this hypothesis has been challenged by the data of Hosen et al. [70], who showed that, at the single-cell level, WT1 levels are similar between normal CD34⁺ progenitors and their leukemic counterparts. Other possible mechanisms include a lower major histocompatibility complex (MHC) class I expression level in normal cells than in tumor cells; weak, if any, WT1 processing and presentation on MHC class I molecules in normal cells; and absent or weak expression of costimulatory molecules on normal WT1-expressing cells.

WT1-TARGETED CANCER VACCINE TRIALS: WHAT HAVE WE LEARNED?

In the last decade, several WT1-based vaccines have been tested in early-phase clinical trials. Recently, a working party from the National Cancer Institute (NCI) performed a large-

scale prioritization study of cancer antigens and produced a priority list of 75 promising tumor-associated antigens based on (a) therapeutic function, (b) immunogenicity, (c) role in oncogenicity, (d) specificity, (e) expression levels and percentage of antigen-positive cells, (f) expression in stem cells, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular localization of antigen expression [74]. Interestingly, WT1 headed this list, supporting translational research to further design WT1-based cancer vaccines. Here, we review and discuss a list of 21 published clinical trials that used WT1 as immunogenic target. Each trial has unique features in terms of tumor type, patient inclusion criteria, nature of the WT1-derived immunogen, type of adjuvant, and route and frequency of administration. Nevertheless we have tried to carry out a systematic review of these trials in order to address a set of parameters impacting clinical outcome and immunogenicity. In addition, several important differences among the trials that may have had an impact on both the immune responses and the clinical effects are discussed.

TUMOR TYPE AND ADVERSE EVENTS

Because WT1 expression has been documented in a wide variety of solid and hematological tumors (Table 1), many cancer types have been the subject of early-phase clinical trials using WT1-based vaccines (Table 2) [75–95]. In summary, seven trials included patients with different types of solid tumor, two trials reported on patients with either solid or hematological malignancies, and 12 trials focused on patients with hematological cancers.

No severe adverse events were reported, except for two patients with myelodysplastic syndrome (MDS) who showed severe leukopenia [75]. The explanation for this observation was that MDS originates from CD34⁺ hematopoietic stem cells and that leukopenia is induced following WT1 vaccination targeting myelodysplastic CD34⁺ cells. In one patient with chronic myelomonocytic leukemia, the dose of WT1 peptide was decreased and no leukopenia was observed [76]. Common adverse events are local inflammation reactions at the sites of injection. In one case, there was transient thrombocytopenia that spontaneously resolved after the vaccination cycle [77]. Thus, in general, vaccinations are well tolerated and safe. There were no reports of autoimmune reactions resulting from the expression of WT1 in some normal nonhematopoietic tissues.

ANTIGEN SOURCE AND HUMAN LEUKOCYTE ANTIGEN RESTRICTION

Based on the WT1 antigen source, the clinical trials summarized here can be divided into four groups: (a) human leukocyte antigen (HLA)-restricted peptide vaccines, (b) non-HLA-restricted long peptides vaccines, (c) dendritic cell (DC) vaccines loaded with HLA-restricted peptide, and (d) DC vaccines loaded with mRNA encoding full-length WT1.

The majority of trials have used HLA-restricted WT1 peptides. Given the high prevalence of HLA-A*2402 and HLA-A*0201 in the Japanese and Caucasian populations, respectively, peptide vaccines restricted to those HLA haplo-

Table 1. Overexpression of WT1 in solid tumors and hematological malignancies

Tumor type	Detection method of WT1 overexpression
Solid tumors	
Biliary cancer [15]	Immunohistochemistry
Bone and soft tissue carcinoma [16]	RT-PCR and immunohistochemistry
Brain tumor [15]	Immunohistochemistry
Breast cancer [12, 15, 17, 18]	RT-PCR, Southern blot, and immunohistochemistry
Cervical cancer [15]	Immunohistochemistry
Colon cancer [19]	RT-PCR and Western blot
Colorectal adenocarcinoma [20]	RT-PCR and immunohistochemistry
Colorectal cancer [15]	Immunohistochemistry
Desmoid tumor [21]	RT-PCR and immunohistochemistry
Endometrial cancer [15]	Immunohistochemistry
Esophageal cancer [15, 22]	RT-PCR and immunohistochemistry
Gastric adenocarcinoma [15]	Immunohistochemistry
Glioblastoma multiforme [23]	RT-PCR
Gynecological tumor [24]	Immunohistochemistry
Head and neck squamous cell carcinoma [25]	RT-PCR and immunohistochemistry
Lung cancer [11, 13, 15, 26]	RT-PCR and immunohistochemistry
Malignant melanoma [15]	Immunohistochemistry
Osteosarcoma [15]	Immunohistochemistry
Ovarian cancer [15]	Immunohistochemistry
Pancreatic cancer [15]	Immunohistochemistry
Pancreatic ductal adenocarcinoma [27]	Immunohistochemistry
Primary astrocytic tumor [28]	RT-PCR and immunohistochemistry
Primary thyroid cancer [29]	RT-PCR and immunohistochemistry
Prostate cancer [15, 30, 31]	Immunohistochemistry
Renal cell carcinoma [15, 32]	Northern blot and immunohistochemistry
Rhabdomyosarcoma [33]	RT-PCR and Western blot
Soft tissue sarcoma [15]	Immunohistochemistry
Testicular germ-cell tumor [34]	RT-PCR
Urothelial cancer [15]	Immunohistochemistry
Uterine sarcoma [35]	RT-PCR and immunohistochemistry
Hematological malignancies	
Acute lymphocytic leukemia [14, 36–41]	RT-PCR and immunohistochemistry
Acute myeloid leukemia [14, 36, 38, 42–44]	RT-PCR and immunohistochemistry
Chronic myeloid leukemia [36, 45]	RT-PCR and immunohistochemistry
Myelodysplastic syndrome [38, 42, 46, 47]	RT-PCR
Multiple myeloma [48, 49]	RT-PCR
Chronic eosinophilic leukemia [50]	RT-PCR

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; WT1, Wilms' tumor 1.

types have been the subject of intensive investigation. One trial [75] focused on naturally occurring as well as modified HLA-A*2402-restricted WT1_{235–243} peptides, nine trials [75, 76, 78–80, 83, 86, 87, 89, 91] focused on the modified heteroclitic HLA-A*2402-restricted WT1_{235–243} peptide, one trial [84] focused on the natural HLA-A*2402-restricted WT1_{235–243} pep-

tide, and another trial [85] focused on the natural HLA-A*0201-restricted WT1_{126–134} peptide. Three trials [81, 93, 94] used a combination of two HLA-A*0201-restricted peptides, a proteinase 3-derived peptide, PR1_{169–177}, and a WT1_{126–134} peptide. Two reports [88, 90] described vaccination of cancer patients with four different WT1-derived pep-

Table 2. Overview of clinical trials investigating active specific immunotherapy targeting WT1 as a principal tumor antigen

Reference	Tumor	n	Immunogenic agent	Adjuvant	Results	
					Clinical responses	Immunological responses
Oka et al. [75]	Breast cancer	2	Natural peptide WT1 ₂₃₅₋₂₄₃ or modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	12/20	13/23 ↑ in tetramers or IFN-γ ⁺ T cells
	Lung cancer	10			↓ leukemic blasts	
	Leukemia (AML and MDS)	14			↓ tumor size ↓ tumor markers	
Morita et al. [78]	Glioblastoma	5	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y subst)	Montanide ISA-51	1 PR (glioblastoma), 5 SD	ND
	Breast cancer	2				
	Malignant fibrous histiocytoma	1				
	Primary neuroectodermal tumor	1				
	Rectal cancer	1				
Iiyama et al. [79]	Renal cell carcinoma	2	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	2 SD	2/2 positive DTH
						1 tetramer ⁺ T cells at wk 8 but then ↓ 1 flattening tumor marker (IAP) and ↑ tetramer ⁺ cells
Tsuboi et al. [80]	MM	1	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	↓ % myeloma cells in BM	↑ WT1 tetramer ⁺ T cells
					↓ M protein	↑ CD107a/b ⁺ tetramer ⁺ T cells ↑ CXCR4 ⁺ tetramer ⁺ T cells in BM, but ↓ in PB
Kawakami et al. [76]	CMML	1	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	↓ WBC ↓ WT1 transcripts	↑ WT1 tetramer ⁺ T cells
Rezvani et al. [81]	Myeloid leukemia (AML, CML, and MDS)	8	Peptide PR1 ₁₆₉₋₁₇₇ and peptide WT1 ₁₂₆₋₁₃₄	Montanide ISA-51 and GM-CSF	3/6 ↓ WT1 transcripts	8/8 ↑ tetramer ⁺ T cells: 7/8 ↑ PR1 tetramer ⁺ T cells and 5/8 ↑ WT1 tetramer ⁺ T cells Correlation with IC IFN-γ
Kitawaki et al. [82]	AML	1	DCs pulsed with modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution) Response to WT1: no tetramer ⁺ or IFN-γ ⁺ T cells Response to KLH: IFN-γ ⁺ , perforin ⁺ , and granzyme B ⁺ T cells	KLH	No clinical response	1/1 positive DTH
Izumoto et al. [83]	Glioblastoma multiforme	21	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	2 PR, 10 SD, 9 PD, of which 2 dropped from protocol (poor general condition)	No ↑ in CTL frequencies after vaccination
Yasukawa et al. [84]	AML	1	Peptide WT1 ₂₃₅₋₂₄₃	Montanide ISA-51	↓ myeloblasts	↑ WT1 tetramer ⁺ T cells
	MDS	1			↓ WT1 transcripts	
Keilholz et al. [85]	AML	17	Peptide WT1 ₁₂₆₋₁₃₄	GM-CSF and KLH	10 SD, of which 4 had ↓ >50% blasts and 2 had hematologic improvement	8/18 ↑ WT1 tetramer ⁺ T cells
	MDS-RAEB	2			1 CR and 3 SD after initial progression of WT1 transcripts: 6/16 ↓, 7/16 =, 3/16 ↑	50% showed IFN-γ and/or TNF-α producing T cells
Ohta et al. [86]	Rhabdomyosarcoma	1	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	CR (>22 mos)	↑ WT1 tetramer ⁺ T cells

(continued)

Table 2. (Continued)

Reference	Tumor	n	Immunogenic agent	Adjuvant	Results	
					Clinical responses	Immunological responses
Ohno et al. [87]	Gynecological malignancy	12	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	3 SD 9 PD	ND
Maslak et al. [88]	AML	10	4 WT1 peptides, of which 3 were long peptides and one was a modified 9 AA peptide	Montanide ISA-51 and GM-CSF	5/9 continuous CR	7/8 ↑ CD4 ⁺ T-cell responses 3/3 CD8 ⁺ T-cell responses: ↑ tetramer ⁺ T cells and ↑ IFN-γ secretion 1/1 CTLs killed WT1 ⁺ target cells 3 showed positive DTH
Narita et al. [89]	CML	1	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	<i>BCR-ABL</i> transcripts ↓ and ↑ after 8th vaccine, ↓ after 13th vaccine	↑ WT1 tetramer ⁺ T cells ↑ Tregs, ↓ after cessation of vaccine T cells cytotoxic in MLPC
Van Tendeloo et al. [77]	AML	10	DCs electroporated with WT1 mRNA	KLH	5/10 with normalization of <i>WT1</i> transcripts: 2 CR from PR (1 relapsed after 9 mos), 3 continuous CR (1 relapsed after 47 mos) 5/10 with no normalization of <i>WT1</i> transcripts and PD	↑ Plasma IL-2, DTH ⁺ , ↑ HLA-DR ⁺ CD4 ⁺ T cells ↑ WT1-specific IFN-γ ⁺ T cells ↑ HLA-DR ⁺ NK cells, ↑ tetramer ⁺ T cells
Krug et al. [90]	Mesothelioma	9	4 WT1 peptides, of which 3 were long peptides and one was a modified 9-AA peptide	Montanide ISA-51 and GM-CSF	1 SD (mesothelioma)	6/9 CD4 ⁺ T-cell responses, 5/6 CD8 ⁺ T-cell responses: ↑ tetramer ⁺ T cells and ↑ IFN-γ secretion
	NSCLC	3			10 PD	3/6 CTLs killed WT1 ⁺ target cells 2/7 showed positive DTH
Hashii et al. [91]	Rhabdomyosarcoma	1	Modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Montanide ISA-51	CR	3/5 ↑ WT1 tetramer ⁺ T cells
	Osteosarcoma	1			PD	
	Liposarcoma	1			SD	
	Synovial sarcoma	1			PD	
	ALL	1			PD	
Coosemans et al. [92]	Endometrial carcinoma	1	DCs electroporated with WT1 mRNA	Imiquimod	Transient ↓ CA125 PD	↑ WT1 tetramer ⁺ T cells
Rezvani et al. [93]	MDS	2	Peptide PR1 ₁₆₉₋₁₇₇ and peptide WT1 ₁₂₆₋₁₃₄	Montanide ISA-51 and GM-CSF	1 SD, 1 PD, 2 CR	7/7 ↑ WT1 and PR1 tetramer ⁺ T cells, in 6/6 no tetramer ⁺ T cells after vaccine 6
	AML	6			4 relapse, of which 1 before vaccine and 1 after first vaccine	
Kuball et al. [94]	AML	4	Peptide PR1 ₁₆₉₋₁₇₇ and peptide WT1 ₁₂₆₋₁₃₄	PADRE, CpG7909, Montanide ISA-51	2 SD	No WT1 tetramer ⁺ T cells after vaccine in all patients
					2 PD	No DTH responses
Kitawaki et al. [95]	AML	3	DCs pulsed with modified peptide WT1 ₂₃₅₋₂₄₃ (M2Y substitution)	Zoledronate (one part of DCs) and KLH (other part of DCs)	1 SD	2/3 showed positive DTH
					2 PD	2/3 detection of WT1 tetramer ⁺ T cells after in vitro stimulation

Abbreviations: aa, amino acids; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; CA125, cancer antigen 125; CMML, chronic myelomonocytic leukemia; CR, complete remission; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DTH, delayed type hypersensitivity test; HLA, human leukocyte antigen; IAP, immunosuppressive acidic protein; IC, intracellular; ID, intradermal; IFN, interferon; IL, interleukin; KLH, keyhole limpet hemocyanin; M2Y, peptide in which Y was substituted for M at position 2; MDS, myelodysplastic syndrome; MDS-RAEB, MDS-refractory anemia with excess blasts; MLPC, mixed lymphocyte peptide culture; MM, multiple myeloma; ND, not done; NK, natural killer; NSCLC, non-small cell lung cancer; PADRE, pan HLA-DR T helper cell epitope; PB, peripheral blood; PD, progressive disease; PR, partial response; PR1, peptide of proteinase 3; SD, stable disease; TNF, tumor necrosis factor; Treg, regulatory T cell; WT1, Wilms' tumor 1.

tides, of which one was a modified WT1_{126–134} peptide whereas the others were long WT1 peptides (19 or 22 amino acids) that had the purpose of broadening immunogenicity over several HLA types. Finally, four papers described the use of DCs as antigen-presenting cells to enhance the presentation of WT1 by loading DCs with a modified HLA-A*2402-restricted WT1_{235–243} peptide [82, 95] or by electroporating DCs with mRNA encoding the entire *WT1* gene [77, 92].

Although the use of peptides is straightforward and cost-effective, it has several disadvantages. A major drawback is MHC restriction and thus dependency on the patient's HLA haplotype. By selecting one type of MHC class I molecule (e.g., HLA-A*0201 or HLA-A*2402), the eligible patient population is diminished. In addition, immunodominant peptides for only a limited number of MHC molecules are currently known. Short peptide vaccines are designed to predominantly boost the patient's CD8⁺ cytotoxic peptide-specific T cells, but cannot directly activate cognate CD4⁺ helper T cells, which are believed to be needed to provide help to cytotoxic T cells and to sustain immunity by inducing memory T-cell responses.

Several options are available to enhance and broaden the immunostimulatory capacity of peptide vaccines. For example, the group of Scheinberg [88, 90] addressed this issue by using three longer WT1 peptides (19–22 amino acids) harboring MHC class II epitopes combined with a modified short WT1_{126–134} peptide in which arginine (R) at position 1 was replaced by tyrosine (Y) residue. To enhance the binding affinity for an MHC class I molecule or for subsequent better recognition by the T-cell receptor, synthetically modified peptides, so-called heteroclitic peptides, can be used. These are variants of the naturally occurring peptides with the same MHC class I specificity but with enhanced binding and T-cell stimulatory capacities. As described above, 14 of 21 WT1 clinical trials used at least one modified peptide to stimulate the immune system of cancer patients. Whether or not this observation can be extrapolated to other heteroclitic peptide trials remains to be established, especially in view of the data outside the WT1 vaccination field by Lesterhuis et al. [96], who showed no superiority of the gp100 heteroclitic peptide over the natural peptide in colorectal cancer patients. The inclusion of multiple WT1 epitopes can be obtained by the use of whole WT1 protein, but this strategy is rather cumbersome because of the difficulty of manufacturing WT1 protein of a good manufacturing practice grade level. Moreover, protein vaccination is known to result in humoral rather than cellular immune responses, and loading of DCs with proteins would lead predominantly to a CD4⁺ T-helper response through MHC class II presentation in the absence of CD8⁺ cytotoxic T-cell induction. This method was not addressed in any of the trials summarized here. Other strategies to circumvent HLA restriction were exploited by our group [77] and a collaborating group [92] using DCs transfected with full-length WT1 mRNA either modified or not with an MHC class II-skewing sequence derived from dendritic cell lysosome-associated membrane glycoprotein. The latter strategy is designed to include all epitopes of the WT1 antigen that are tailored by the DCs to the patient's own MHC

molecules, independently of pre-existing knowledge about the patient's HLA type. The use of mRNA has many other advantages, such as its clinical safety profile (i.e., no risk for integration into the host genome) and the possibility of transfecting DCs simultaneously with immune-stimulating molecules [97]. Our group already demonstrated that transfecting DCs using mRNA electroporation is a clinically safe, reproducible, and very efficient procedure resulting in a transient expression, processing, and presentation of the electroporated antigen [98–100].

ADJUVANTS

In general, vaccines, whether prophylactic or therapeutic, are administered together with an adjuvant to boost the innate immune system at the site of injection. Different adjuvants, cell based or not, are currently used in therapeutic WT1-based cancer vaccines to enhance the effect, the potency, and the longevity of the vaccination. Montanide ISA-51, also called incomplete Freund's adjuvant, is widely recognized as an effective and safe adjuvant for vaccination. In some cases, recombinant GM-CSF was added with the aim of obtaining a more profound effect and stronger immune stimulation [81, 85, 88, 90, 93]. In those trials, three patients were reported with allergy [88], hypersensitivity to GM-CSF [88], and transient chest pain [93], probably because of the addition of GM-CSF. Another adjuvant often used in cancer vaccine trials is keyhole limpet hemocyanin (KLH), a xenogenic and highly immunogenic protein. Similarly here, combinations with GM-CSF were used in a WT1 [85] and a tyrosinase peptide [101] vaccine trial. KLH is commonly used as a noncognate CD4⁺ T-helper antigen in peptide or DC vaccines. One group [92] applied an imiquimod cream (Aldara®; Meda AB, Solna, Sweden) at the site of injection to boost the immune system. Imiquimod is a Toll-like receptor 7 ligand and has been shown to promote the survival and maturation of DCs as well as enhance priming of T cells [102, 103]. Kuball et al. [94] used a combination of pan HLA-DR T-helper cell epitope, low-dose CpG7909, and Montanide ISA-51. Finally, zoledronate was used as an adjuvant by Kitawaki et al. [95] to promote the activation of tumor antigen-specific T cells by activation of interferon (IFN)- γ -producing V γ 9V δ 2 T cells and stimulation of interacting DCs.

ROUTE OF ADMINISTRATION

The two most frequent administration routes in active WT1 immunotherapy trials are intradermal (12 trials) and s.c. (seven trials). One trial combined both routes [85] and one group vaccinated one part of the DC vaccine intradermally and another part i.v. [95]. Until now, there has been no consensus on what is the most optimal way to deliver a peptide- or DC-based cancer vaccine. In general, possible cancer vaccine injection routes include intradermal, s.c., i.v., intralymphatic, and intranodal injections. i.v. administration is not commonly used because the cells end up in the lungs, spleen, and liver and can only migrate secondarily to the lymph nodes. Furthermore, it was shown that i.v. delivery of antigen resulted in a humoral rather than a cellular response [104]. A major disadvantage of

Table 3. Overview of clinical and immunological responders in clinical trials investigating active specific immunotherapy targeting Wilms tumor 1 as a principal tumor antigen

	Clinical responders					Immunological responders		
	Total <i>n</i> patients	<i>n</i> responders	<i>n</i> patients with stable disease	<i>n</i> evaluable patients	Total response rate (%) (including stable disease)	<i>n</i> responders	<i>n</i> evaluable patients	Response rate (%)
Solid tumors	75	10	23	72	45.8	17	48	35.4
Hematological malignancies	83	29	18	74	63.5	52	77	67.5

the intranodal route is the complexity and the difficult-to-standardize procedure of the injection in the lymph nodes.

NUMBER AND FREQUENCY OF VACCINATIONS

The frequency of vaccine administration ranged from weekly to monthly. The first clinical trial using WT1 peptide was reported with biweekly vaccinations [75]. Morita et al. [78] investigated the safety of weekly vaccinations regarding grade 3 or 4 toxicities. No severe toxicities were seen. In summary, patients were vaccinated on a weekly basis in eight trials [78–80, 83, 86, 87, 91, 92], whereas 10 other trials reported a biweekly scheme [75–77, 82, 84, 85, 89]. Two studies had an interval of 4 weeks between the first and second injections, followed by biweekly injections thereafter [88, 90]. In a first report, Rezvani et al. [81] injected patients once with peptides. In a subsequent trial [93], they vaccinated patients every 2 weeks for six vaccines with one booster vaccine 12 weeks thereafter. Briefly, the number of vaccines ranged from one to >64. After a first standard vaccination scheme according to the protocol (different in all studies), in many cases additional booster vaccinations were given with the patient's consent. Because there is no clear evidence for a standard booster scheme, every group independently decided on the interval between additional booster vaccinations. As evident from the above, there is no standardized treatment scheme in terms of frequency and number of vaccinations, resulting in a wide variety of vaccine intervals in early-phase trials.

OUTCOME

As expected, the clinical outcomes in the reported trials were diverse. Overall, an objective clinical response was defined as a reduction in the tumor mass (e.g., breast cancer, lung cancer), a decrease in the number of malignant cells (e.g., leukemic cells, myeloma cells), a decrease in a tumor marker, and stable disease for a prolonged period of time [105]. For acute myeloid leukemia (AML) patients in remission, the number of WT1 RNA transcripts in blood or bone marrow can be monitored and used as a minimal residual disease biomarker to assess the effect of vaccination and to predict incipient relapse [106]. In view of this, normalization of WT1 RNA to threshold levels after vaccination points to the induction of molecular remission [77].

Clinical responses were seen in all trials except for one, in

which only a single AML patient was included [82]. In total, 158 patients were included in 21 clinical trials, of whom 75 had solid tumors and 83 had hematological malignancies (Table 3). Clinical responses postvaccination were observed in 78 of 146 evaluable patients, resulting in overall response rates of 45.8% for patients with solid tumors and 63.5% for patients with hematological tumors. In the solid tumor clinical trials, two patients obtained a complete remission, three had a partial remission, and five showed a decrease in tumor marker or tumor size. In patients with hematological malignancies, complete remission was reached in 13 patients and a decrease in tumor marker or tumor size was reached in 16 patients. Importantly, additional responses were seen if patients with stable disease were also included as clinical responders according to the new criteria for the evaluation of cancer immunotherapy recently put forward by Hoos et al. [105]. Twenty-three patients with solid tumors and 18 patients with hematological malignancies showed stable disease after tumor vaccination.

Most trials also documented immunological responses after WT1 vaccination. T-cell responses were demonstrated using WT1–MHC class I tetramer staining, the presence of IFN- γ -producing or tumor necrosis factor α -producing T cells, and the presence of other activation molecules (CD107a, CXCR4). A few reports described the cytotoxicity of WT1-specific T cells in some patients [88–90]. When using one or more defined peptides, it is relatively straightforward to monitor peptide-specific T-cell responses. However, when using DCs loaded with full-length mRNA, it becomes more complex to delineate all the T-cell responses elicited by the vaccine because of the presentation of different epitopes presented by different HLA molecules in an MHC class I- and class II-restricted fashion. Detection of tetramer-positive T cells is also limited to known peptides in combination with the restricting HLA subtype. Thus, in fact, there are still major limitations to standardized immunomonitoring regarding the evaluation of immune responses generated by non-HLA-restricted vaccines. The scarcity of circulating tumor-specific T cells in the blood prompted several groups to investigate other techniques to detect vaccine-induced immune responses. Delayed-type hypersensitivity (DTH) skin reaction tests as a measure of *in vivo* cellular immune response were performed by several groups. In most cases, positive skin reactions were observed and linked to postvaccination immune activation [77, 79, 82,

88, 90, 95]. It would be interesting to further analyze these DTH sites for the presence of DTH-infiltrating lymphocytes and determine their antigen specificity and reactivity [107].

Whereas two clinical trials did not provide immunological monitoring data [78, 87], immune responses were observed in 17 of 19 trials. One trial [83], in glioblastoma multiforme patients, showed no increase in CTL frequencies in any of the 21 vaccinated patients, whereas another trial [94], in AML patients, showed no increase in WT1 tetramer-positive cells and could not demonstrate any reaction in DTH tests. For the 17 other trials, immunological responses could be demonstrated in 69 of 125 evaluable patients (17 patients with solid tumors and 52 hematological patients). Overall immunological response rates of 35.4% for patients with solid tumors and 67.5% for patients with hematological malignancies were obtained throughout the 19 clinical trials reporting on WT1-specific immunomonitoring (Table 3).

CONCLUSIONS AND FUTURE PERSPECTIVES

Altogether, most studies showed some benefit (immunological, clinical, or both) for a sizable number of patients. Surrogate immunological markers for clinical benefit were only sporadically reported [75, 77] and remain to be confirmed in larger randomized controlled trials. Therefore, at present, it is difficult to predict which patients will benefit most from active immunotherapy and how one should discriminate responders from nonresponders before enrolling patients into a vaccine protocol. Recently Busse et al. [108] showed that immune escape in AML patients was not a result of mutation of *WT1*, loss of WT1 expression, or decreased expression of MHC class I molecules on tumor cells. Although the mechanisms behind tumor immune escape after immunotherapy are largely unknown, several hypotheses have been put forward. The short-term beneficial effect often observed with WT1 peptide vaccines might be explained by the induction of T-cell tolerance caused by short peptides [109]. The presence of regulatory T-cell populations or other immune inhibitory pathways could play a role in the outcome of treatment with therapeutic

cancer vaccines. Also, the combination, type, and dose of adjuvants used in immunotherapy trials must be taken into account. Montanide ISA-51 was reported by Rezvani et al. [93] to have a negative effect on the long-term immune response. Moreover, the findings of Kuball et al. [94] demonstrated that certain combinations and doses of adjuvants could have detrimental effects on the activation of tumor-specific T cells and could induce negative effects such as T-cell deletion and anergy.

In conclusion, WT1-based cancer vaccines have been shown to be feasible and safe in patients with multiple tumor types. Furthermore, these vaccines elicited WT1-specific immune responses and showed promising clinical results in a majority of patients. These conclusions run parallel with a recent NCI report [74] on the prioritization of cancer antigens and justify the further development of WT1-targeted immunotherapies. Design of larger phase II trials as well as two-arm trials will establish optimal vaccination strategies and will eventually reveal the true potential of WT1 as a universal cancer vaccine target in the adjuvant setting.

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