Wilms' Tumor Gene 1 (*WT1*) – loaded Dendritic Cell Immunotherapy in Patients with Uterine Tumors: A Phase I/II Clinical Trial

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Abstract. *Aim: Treatment options are limited in uterine cancer, leading to a poor prognosis. Overexpression of Wilms' tumor gene 1 (WT1), the highest ranked tumor antigen, is attractive for immunotherapy. Patients and Methods: Six pre-treated patients with uterine cancer received four weekly vaccines of autologous dendritic cells (DCs) electroporated with WT1 mRNA. Safety, feasibility and immunogenicity were assessed. In cases of response, patients received monthly booster vaccines. Results: The technique was feasible. One patient had a local allergic reaction. Three out of four Human Leucocyte Antigen-A2 (HLA-A2)-positive patients showed an oncological response; an enrichment of WT1-specific T-cells was observed in two of them. Two HLA-A2-negative patients did not show an oncological or an immunological response. Conclusion: A first series of six patients with uterine cancer treated with WT1 mRNA-electroporated DCs is presented herein. Oncological and immunological responses were observed and are supportive for further research.*

Uterine tumors can be sub-divided into endometrial carcinomas and uterine sarcoma. With the standard treatments, prognosis remains poor for those with higher-stage disease with 5-year survival ranging from 5 to 40% in carcinoma (depending on the subtype) and 10% in sarcoma. Therefore, better treatment modalities are needed and should be explored (1, 2).

Wilms' tumor gene 1 (*WT1*), located on chromosome 11p13, is overexpressed in several hematological and solid malignancies (3). The oncogenic role of *WT1* and its correlation with a malignant phenotype has especially been reported for acute myeloid leukemia. Additional studies also indicate a role in the initiation phase of malignant diseases (4). In several studies, WT1 expression in uterine carcinoma was examined and approximately 20% of these tumors were positive for WT1 (5). In addition to WT1 expression in tumor cells, WT1 expression in intratumoral endothelial cells may also serve as a relevant target for immunotherapy. When taking into account WT1 expression in these intratumoral endothelial cells, the percentage of uterine carcinomas with WT1 expression rises to 72% (5). For sarcomas, our group has demonstrated the presence of WT1 in 63% of high-grade sarcomas and we were also able to correlate its overexpression to a deterioration of the prognosis in high-grade uterine sarcoma (6, 7). WT1 has been ranked as the most important tumor-associated antigen (8), making it an attractive target for immunotherapy. Since 2004, several clinical case reports and studies have been published on WT1 immunotherapy. Most commonly, a modified WT1 peptide is injected to elicit a WT1-specific T-cell response. Vaccination with autologous dendritic cells (DCs) loaded with WT1 peptides is another means to establishing such a response. To date, 78 cases of solid tumors [including eight uterine tumors (9-11)] and 82 hematological malignancies have been the subject of a total of 23 studies and case reports, with a clinical response to WT1 in 54% of cases (3). One of the obstacles for the use of synthetic peptides is that patient accrual is limited to patients with the specific Human

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Leucocyte Antigen (HLA) type, for which the peptide is restricted. One means that would allow WT1-targeted immunotherapy for patients regardless of their HLA-type, is transfection of *WT1* mRNA into DCs to achieve transient expression and subsequent presentation of antigenic epitopes. The *in vitro* work of several laboratories has suggested that mRNA transfection is an effective, if not superior, method of generating immunostimulatory DCs (12-15). Our research groups developed the technique of DC immunotherapy in the context of high-grade glioma (16-19), acute myeloid leukemia (20) and endometrial carcinoma (10). In the present phase I/II trial, we wanted to treat the first six uterine cancer patients with *WT1* mRNA-dendritic cell immunotherapy.

Patients and Methods

Study design. Following the first patient with endometrial carcinoma to be treated with *WT1* mRNA-electroporated mature DCs (DCm-*WT1*-RNA) by our group (10), we initiated a phase I/II trial, approved by the Institutional Ethical Commission (EudraCT 2009-016868-37). This was a single-arm, pilot clinical trial in patients with end-stage uterine cancer, carried out in a single institution (University Hospital Leuven, Belgium). DCm-*WT1*-RNA was administered as four weekly intradermal injections in imiquimod-treated skin in the groins of the patients. Imiquimod was applied one night before and two nights after vaccination. Patients with disease stabilization or regression received further individually designed vaccinations with DCm-*WT1*-RNA until disease progression. The primary study end-points were to evaluate the feasibility, toxicity and tumor response rate according to Response Evaluation Criteria in Solid Tumors (RECIST) (21). Since this was an exploratory study, no pre-defined statistical hypothesis was used. Secondary end-points were the assessment of the *in vivo* induced immune response and the molecular response of the tumor marker Cancer Antigen 125 (CA125).

Vaccination protocol. For both the first HLA-A2-positive (Table I) and HLA-A2-negative (Table II) patients, the DCm-*WT1*-RNA vaccine was manufactured as described earlier (10). For the other patients, vaccines were prepared with a closed culture methodology. In brief, after leukapheresis, monocytes were purified by elutriation (Elutra®; Terumo BCT, Lakewood, Co, USA) and cultured in closed cell culture bags (CellGenix, Freiburg, Germany). Cytokines for differentiation and maturation as well as *WT1* mRNA electroporation conditions were unchanged. DCm-*WT1*-RNA were frozen in liquid nitrogen. For each injection, a sample was thawed. Although no autoimmune reactions have been reported so far in literature (22), a clinical examination was performed at each vaccination time point and a questionnaire regarding side-effects was completed by the patients. The grading of adverse events was based upon the National Cancer Institute toxicity scale (http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf).

Immunological response assessment. The immune status and response were assessed at the time of the first vaccine administration (baseline) and at the time of the fourth vaccination (*post* V3). For patients receiving boost vaccines, additional blood samples were collected when feasible. Peripheral blood mononuclear cells (PBMCs) were isolated for further analysis. For HLA-A2-positive patients (except for patient 1), T-cells recognizing the $WT1_{126-134}$ HLA-A*0201-restricted epitope (RMFPNAPYL) were measured using tetramer staining. These T-cells were detected by flow cytometry using a combination of phycoerythrin (PE)-labeled WT1 or influenza tetramers (Glycotope Biotechnology, Heidelberg, Germany), fluorescein isothiocyanate (FITC)-labeled anti-cluster of differentiation 8 (CD8) (BD Biosciences, San Jose, CA, USA) and Peridinin Chlorophyll Protein Complex Cyanine 5.5 (PerCp-Cy5.5)-labeled anti-CD3 (Biolegend, San Diego, CA, USA). At the same time, Natural Killer (NK) cells were measured using PE-conjugated anti-CD161 (Biolegend), PerCp-Cy5.5 conjugated anti-CD16 (BD Biosciences) and PE-Cy7 conjugated anti-CD56 (Biolegend). The determination of WT1-specific T-cells in the first HLA-A2-positive patient was slightly different, as described and published earlier (10). For HLA-A2-negative patients, WT1-specific T-cells were detected using the CD137 assay. In brief, thawed PBMCs were incubated overnight with an overlapping peptide mixture covering WT1 (PepTivator; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. After 24 h incubation, cells were analyzed by flow cytometry using FITC-labeled anti-CD4 (BD Biosciences), PE-labeled anti-CD137 (BD Biosciences), PerCp-Cy5.5-labeled anti-CD3 (Biolegend) and Allophycocyanin-Hilite 7 (APC-H7)-labeled anti-CD8 (BD Biosciences). The culture supernatant was stored at -20°C for cytokine quantification using the BD Cytometric Bead Array (BD Biosciences) according to the manufacturer's instructions.

Oncological outcome assessment. Clinical response was measured by CA125 analysis in case of endometrial carcinoma and by computed tomography (CT) prior to vaccination, after four sequential vaccines and upon clinical indication once booster vaccines were given, according to the RECIST criteria.

Results

Patients' characteristics. Patients' characteristics are presented in Tables I and II. Patients were pre-treated with a mean of four therapies (range two to six). Patient 5 was 76 years old and presented with stage I leiomyosarcoma (LMS). She was operated twice, refused subsequent chemotherapy, but preferred non-invasive immunotherapy because of her age. Patient 4, also with stage I LMS, experienced relapse after surgery and chemotherapy, and specifically requested immunotherapy. The case of the first HLA-A2-positive patient (patient 1) has been published separately (10).

Vaccine characteristics. The numbers of DCs administered *per* vaccine are listed in Tables I and II. Preparation of the DC vaccines was feasible for all patients. Quality of the DCs was comparable to published data (16-19). The fourth HLA-A2-positive patient had a special booster regimen. Since she showed a mixed response after four weekly vaccines (stable disease in most of the metastases but progression of the lung lesions), four subsequent DC vaccines, loaded with *WT1* mRNA and tumor lysate, obtained from a resected growing lung metastasis, were administered. The lysate was prepared as described before (16).

Table I. *Clinical characteristics of HLA-A2-positive patients*

	Patient number									
	1			2			3		4	
General characteristics										
Age, years	46			50			63		48	
Cancer subtype	Serous endometrial carcinoma			Leiomyosarcoma			Serous endometrial carcinoma		Leiomyosarcoma	
Stage	IV			IV			IIIc		I	
Number of previous therapy regimens	5			5			4		2	
Vaccine characteristics										
Number and frequency of vaccines	4 (V1-V4: weekly)			6 (V1-V4: weekly; V5-V6: monthly)			4 (V1-V4: weekly)		8 (V1-V4: weekly; V5-V8: weekly)	
Antigens loaded on DCs	WT1 mRNA			WT1 mRNA			WT1 mRNA		V1-V4: WT1 mRNA; V5-V8: WT1 mRNA + lysate of progressive lung metastasis	
Number of injected DCs	7.6×10^6 (range 6×10^6 – 8.8×10^6)			21.5×10^6 (range 13.6×10^6 – 31.6×10^6)			17.11×10^6 (range 8×10^6 – 21.75×10^6)		8.78×10^6 (range 4.9×10^6 – 20.9×10^6)	
Clinical characteristics	**At V1**	**At V4**	**At V1**	**At V4**	**At V6**	**At V1**	**At V4**	**At V1**	**At V4**	**At V8**
Karnofsky Performance Score	60	50	90	90	80	NAV	60	90	90	90
HQLE	3	3	6	6	5	3	3	6	6	7
Oncologic outcome	**Before V1**	**Post V4**	**Before V1**	**Post V4**	**Post V6**	**Before V1**	**Post V4**	**Before V1**	**Post V4**	**Post V8**
Radiological status (CT scan)	PD	PD	PR	SD	PD	PD	PD	PD	Mixed response	PD
CA125 (kU/l)	4980	4675	NA	NA	NA	2336	3516	NA	NA	NA
Immune response	**At V1**	**At V4**				**At V1**	**At V4**	**At V1**	**At V3**	**At V5**	**At V8**
WT1-specific T-cells (%)	0.09	0.26				2.63	1.19	0.35	0.6	0.19	0.25
NK cells ($CD161^+$ $CD56dim$ $CD16^+$) (%)	NA	NA				5.56	15.7	12.5	6.11	4.08	4.33
Subsequent therapy (after immunotherapy stop)											
	Paclitaxel-carboplatin 18×			Epirubicin-dacarbazine 2×			NA		Radiotherapy lung and Trabectedin	
Survival										
PFS with immunotherapy (months)	0			3			0		2	
Survival after end of immunotherapy (months)	10			4			1		4	
OS (months)	36			43			34		22	

HQLE: Health and Quality of Life Estimation, On a scale ranging from 1 (very poor) to 7 (excellent); PFS: progression-free survival; OS: overall survival; V#: vaccination #; PD: progressive disease; CT: computed tomography; NA: not applicable; NAV: not available.

Toxicity. All patients showed redness at the sites of injection. Patient 5 had a local allergic reaction to the imiquimod cream. For this patient, imiquimod was not used for vaccinations 3 and 4 (V3 and V4) but was replaced by the addition of 20 μg/ml prostaglandin E2 (Pharmacia, Puurs, Belgium) to the maturation mix at day 6 of the DC culture.

Clinical and oncological outcome. The condition (Karnofsky score and general health status) did not deteriorate in any of the patients during the immunotherapy procedure. Out of the HLA-A2-positive patients, patient 1 showed a transient molecular response after four vaccines and patients 2 and 4 showed a radiological response, as indicated in Table I, which was not maintained with booster vaccines. The HLA-A2-negative patients both experienced disease progression (Table II). Out of those with end-stage disease (patients 1, 2, 3 and 6), an oncological response was observed in patients 1 and 2. They both received subsequent chemotherapy leading to survival which was remarkably longer than for the two other patients who did not receive further chemotherapy (patient 1 and 2 vs patient 3 and 6).

Table II. *Clinical characteristics of HLA-A2-negative patients.*

	Patient number					
	5			6		
General characteristics						
Age, years	76			64		
Cancer subtype	Leiomyosarcoma			Serous endometrial carcinoma		
Stage	I			III		
Number of previous therapy regimens	2			6		
Number of injected DCs (V1-V4)	5.9×10^6 (range: 1.12×10^6–12.24×10^6)			10.1×10^6 (range: 7.3×10^6–13.5×10^6)		
Clinical outcome	At V1	At V4		At V1	At V4	
Karnofsky Performance Score	80	90		50	50	
HQLE	5	5		2	2	
Oncologic outcome	**Before V1**	**Post V4**		**Before V1**	**Post V4**	
Radiological status (CT scan)	PD	PD		PD	PD	
Immune response	**At V1**	**At V3**	**Fold increase**	**At V1**	**At V3**	**Fold increase**
NK cells ($CD161^+$ CD56dim $CD16^+$) (%)	19.02	16.35	-	8.29	7.84	-
$CD8^+CD137^+$ T cells (%)	7.0	3.0	-	2.567	1.175	-
$CD4^+CD137^+$ T cells (%)	3.0	1.667	-	1.543	0.3	-
IL-2 , IL-10, IFN-γ, TNF-α (fg/ml)	-°	-°	-°	-°	-°	-°
Survival						
PFS with immunotherapy (months)	0			0		
Survival after end of immunotherapy (months)	3			0		
OS (months)	20			42		

HQLE: Health and Quality of Life Estimation, on a scale ranging from 1 (very poor) to 7 (excellent); PFS: progression-free survival; OS: overall survival; V#: vaccination #; PD: progressive disease; CT: computed tomography; NA: not applicable; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor. °all values below detection levels.

Immunological response analysis. As shown in Table I, three out of four HLA-A2-positive patients showed an immunological response (enrichment of WT1-specific T-cells in patient 1 and 4; increase in NK cells in patient 3) after three vaccinations, which matched an oncological response in two of them. Unfortunately, material was lost for patient 2 due to a technical error. Because patient 4 had a unique immunotherapy regimen, cytokines and the CD137 assay were also analyzed (Table III). When vaccination 4 (V4) was compared to vaccination 1 (V1), the enrichment of $CD8^+CD137^+$ and $CD4^+CD137^+$ T-cells and the increase in Interleukin-2 (IL-2) and IL-10 was in line with the enrichment of WT1-specific T-cells. When patient 4 received four additional vaccinations, no significant immune responses were noted. Tumor in this patient had progressed at that time. The HLA-A2-negative patients showed no immunological response, which matched the absence of any oncological response.

Discussion

We present the first series of six patients with uterine cancer treated with autologous DCs loaded with *WT1* mRNA. This approach was feasible and well-tolerated by the patients. The only side-effect was the allergic reaction to the imiquimod cream in one patient. A transient oncological (patient 1, 2, 4) and immunological (patient 1, 3, 4) reaction was seen in three patients after four vaccinations, all of them HLA-A2-positive. In two of them, the oncological and immunological responses matched. When receiving booster vaccines, the immune response was not maintained, correlating with progressive disease on a CT scan. HLA-A2-negative patients did not show an oncological or immunological response. For comparison, Ohno *et al*. reported two uterine cancer patients receiving WT1 peptide immunotherapy, both showing progressive disease (9). Miyatake *et al*. recently reported five patients with uterine sarcoma receiving WT1 peptide immunotherapy, showing

Table III. *Immune monitoring data for patient 4 (HLA-A2-positive).*

	V1	V4	Fold increase V1 *vs*. V4	V5	V8	Fold increase V5 *vs*. V8	Fold increase V1 *vs*. V8
WT1 tetramer-specific T-cells (%)	0.345	0.598	×1.7	0.190	0.251	×1.32	-
NK cells ($CD161^+$ CD56 dim $CD16^+$) (%)	12.5	6.11	-	4.08	4.33	×1.06	-
$CD8^+CD137^+$ T-cells(%)	0.667	1.867	×2.8	1.116	0.936	-	×1.4
$CD4^+CD137^+$ T-cells(%)	0.924	1.155	×1.25	1.013	0.917	-	-
IL-2 (pg/ml)	2.724	6.419	×2.36	-	1.221	-	-
IL-10 (pg/ml)	5.578	5.819	×1.04	5.106	1.042	-	-
IFN-ψ (pg/ml)	19.577	1.143	-	1.029	0.209	-	-
TNF-α (pg/ml)	15.762	13.789	-	3.328	4.016	×1.21	-

IL: Interleukin; IFN: interferon; TNF: tumor necrosis factor.

disease stabilization in one patient (11). In the two studies, immune response monitoring was limited to a test for delayed-type hypersensitivity reaction specific to the WT1 peptide used for vaccination. No match between immunological and clinical response was found. Taking together all published series and case reports, in 54.5% of cases, the immunological response matched the clinical response (3).

The limited clinical responses in our study can partly be explained by the condition of the included patients. A large tumor burden, present in four patients, creates an immunosuppressive environment, which hampers the efficacy of immunotherapy. Moreover, the immune system was heavily challenged in nearly all patients because of prior chemotherapy and surgery. Therefore, the immune system may not have fully recovered prior to the immunotherapy regimen. The limited clinical responses observed might also be due to the fact that the immunotherapy was stopped once radiological progression was demonstrated. Oncological conclusions may have been drawn too early, since the induction of an immune response needs time and is often accompanied by initial disease progression before regression is apparent. In another study, patients with acute myeloid leukemia and myelodysplastic syndrome received WT1 immunotherapy for at least three months (23). In this period, progressive disease was allowed and immunotherapy was continued if no other treatment options were available. By continuing the vaccination, patients started to show remission.

As in our trial, Miyatake *et al*. recorded longer survival because of the addition of WT1 immunotherapy (11). Comparing the survival of the first HLA-A2-positive patient with that of comparable historical controls in University Hospital of Leuven (Belgium) shows a striking difference in survival from the moment palliation was declared: 11 months *versus* 67 days. The same was true for the second HLA-A2-positive patient with end-stage disease. Four comparable historical cases were available in our database. In this small group, two patients died of progression during chemotherapy, one patient before chemotherapy could be initiated, but one patient had a similar cancer history as our patient. She died five months after she was put on palliative care, whereas our patient survived for 10 months. From these observations, it seems that a combination of immunotherapy and chemotherapy might be beneficial, as shown in other studies (24). Doxorubicin, for example, results in apoptotic tumor cell death, which increases the antigen uptake by the antigen-presenting cells, leading to *in situ* immunization against tumor antigens. Chemotherapy might also lead to direct activation of DCs, previously demonstrated for doxorubicin and paclitaxel, or their effector mechanisms (25). Lower doses of chemotherapy, *e.g*. cyclophosphamide, have been shown in mice to be synergistic with immunotherapy, by depleting regulatory T-cells and retaining memory T-cells (26). In our study population, there was a clear discrepancy between HLA-A2-positive and -negative patients. HLA type is a known prognostic factor in some types of cancer. However, to our knowledge, nothing has been demonstrated regarding the HLA type and the response to immunotherapy, nor of a possible dominance of the A2 epitope of WT1. Larger studies are needed to clarify this aspect. In conclusion, this study presents promising results of the first six patients with uterine cancer to be treated with autologous DCs loaded with *WT1* mRNA. The technique is feasible and safe. All transient oncological reactions were seen in the HLA-A2-positive patients and matched the immunological response in two out of three patients.

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