Immunological Response after Therapeutic Vaccination with *WT1* mRNA-loaded Dendritic Cells in End-stage Endometrial Carcinoma

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Abstract. *Background: Wilms' tumour gene 1 (WT1), a highly ranked immunotherapeutic target, is expressed in uterine cancer and therefore WT1 immunotherapy may present an attractive treatment option. Patient and Methods: An HLA-A2.1-positive 46-year-old woman with end-stage serous endometrial cancer received 4 weekly injections of WT1-RNA-loaded dendritic cells. Response was measured clinically (CT scan), biochemically (CA125) and immunologically (WT1-specific T cells). Results: The patient showed WT1 positivity in 10% of tumour cells and diffusely in the intratumoural endothelial cells of the recurrent disease. After 2 injections, CA125 started to decrease and WT1-specific T-cells increased 2.5-fold. The treatment was feasible and there were no treatment-related side-effects. However, the patient, suffering from diffuse disease which became progressive again, died 8 months later. Conclusion: This is the first patient with a WT1-positive endometrial carcinoma, to receive immunotherapy with WT1-RNA-loaded dendritic cells, resulting in a vaccine-specific T cell response.*

Uterine tumours can be subdivided into endometrial carcinomas (epithelial tumours) and uterine sarcomas (mesenchymal tumours). Standard treatment in this patients group is optimal cytoreductive surgery, followed by chemotherapy (1). Carboplatin and paclitaxel or cisplatin and adriamycin have been demonstrated to be the most efficient chemotherapy combinations. As long as radical surgery is possible, the overall prognosis in the carcinoma group is fair (90% 5-year survival for stage I), but in cases of systemic relapse, the disease course is invariably fatal. For systemic relapse, better treatment modalities are needed and should be explored (2).

Wilms' tumour gene 1 (*WT1*) is a gene located on chromosome 11p13. It was first cloned in 1990 in Wilms' tumour, a paediatric kidney cancer. More recently, *WT1* overexpression was detected in several haematological and solid malignancies (3). The oncogenic role of *WT1* and its correlation with a malignant phenotype has been reported especially for acute myeloid leukaemias. Additional studies also indicate a role in the initiation phase of the malignant diseases (4).

WT1 expression in uterine carcinoma has been examined in several studies and roughly 20% of these tumours were positive (a review is given in 5). In addition to WT1-expression in tumour cells, WT1 expression in intratumoural endothelial cells may also serve as a relevant target for immunotherapy. When taking into account WT1 expression in these intratumoural endothelial cells, the percentage of uterine carcinomas with a positive WT1 expression rises to 72% (5).

Since WT1 is believed to be relevant in the maintenance of the malignant phenotype of the tumour cells and is mostly restricted to malignant tissues, it is an attractive target for immunotherapy (6). Vaccination with autologous dendritic cells (DCs) loaded with antigens restricted to the tumour is one way to establish an efficient T-cell response in the patient. Immunotherapy for several types of cancer targeting WT1 has been applied using a WT1 peptide, thereby achieving limited clinical benefit in small series of patients (7-11). One of the obstacles for the use of synthetic peptides is that patient accrual is limited to patients with the specific

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HLA type, able to bind the administered peptides onto their own MHC class I molecules. One way that would allow WT1-targeted immunotherapy to be applied to 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 (12-17). Clinical studies in prostate cancer and renal cell carcinoma with RNA-transfected DCs have been conducted, demonstrating at least immunological responses (12). DC immunotherapy based on RNA electroporation is also being studied in an ongoing clinical trial for stage IV melanoma patients (NCT00126685). Recently, feasibility and safety of intradermal administration of *WT1* RNA-electroporated DCs was shown in patients with acute myeloid leukaemia in remission (18).

The technique of DC immunotherapy was developed in the laboratories of UZ Gasthuisberg in the context of high-grade glioma patients (19-21). By treating patients with relapsed glioblastoma multiforme by means of mature DCs loaded with tumour antigen, progression-free and overall survival rates improved (22-25).

This report describes a patient with end-stage disease of metastasised relapsed uterine carcinoma who was treated with autologous mature DCs loaded with *WT1* RNA (DCm-WT1 RNA) in order to explore and document the feasibility and toxicity of this approach.

Patient and Methods

Case Report. A 45-year-old woman was diagnosed with stage IV serous endometrial cancer, with pericardial and pleural effusions, in December 2006. She received 3 cycles of chemotherapy (paclitaxel and carboplatin) and underwent interval debulking surgery with no residual tumour. She received 3 more cycles of paclitaxel and carboplatin. Four months later, in November 2007, she relapsed abdominally with pericardial effusion. She received single-agent doxorubicin once every 3 weeks. After 6 cycles, she showed progression and medroxyprogesterone was given. Her disease progressed further and paclitaxel and carboplatin infusions were administered on a weekly basis for 18 cycles. A whole-body computed tomography (CT) scan in October 2008 showed partial remission. She had progressive disease again in January 2009 (rise of CA125, increase of pleural effusion and ascites). At that time, the patient consented to the start of WT1 immunotherapy.

WT1 vaccination. An open laparoscopy was performed to take a biopsy from the recurrent disease. Immunohistochemical staining, which was performed as described before (26), showed WT1 positivity in 10% of the tumour cells and the intratumoural endothelial cells showed widely distributed WT1 positivity (5) (Figure 1).

The patient underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMCs), which were stored in liquid nitrogen. For each vaccine, PBMCs were thawed and seeded in culture flasks. On average, 1×10^9 PBMCs were cultured for every vaccination. After adherence, monocytes were differentiated to immature dendritic cells (DCi) in the presence of recombinant IL-4 (20 ng/ml) (Peprotech, London, UK) and rGM-CSF (850 IU/ml) (Gentaur, Brussels, Belgium) (19). The yield of DCi ranged between 3% and 5%. On day 6, DCi were electroporated (300 V, 150 μF, exponential decay pulse (16)) with *WT1* RNA (10 μg/4×10^6 DCi). *WT1* RNA was manufactured by CureVac (Tübingen, Germany) from a *WT1* plasmid (pGEM4Z-WT1-A64). Electroporated DCs were put back in culture in the presence of TNF-α (0.24 μg/ml) (Sanquin, Amsterdam, the Netherlands) and IL-1β (0.24 μg/ml) (Gentaur). Of all DCi, 20% (range 12%-34%) survived electroporation and maturation. On RT-PCR, Western blot and immunohistochemistry, electroporation with *WT1* mRNA was demonstrated to be effective and WT1 protein positivity was shown in the maturated fraction (data not shown). On day 7, DCm-*WT1*-RNA was harvested, resuspended in DPBS with 2.5% human serum albumin and injected intradermally in the patient's groin. On the day before the injection, and for the next two days, the patient applied imiquimod cream (Aldara; Meda AB, Sweden) at the site of injection, similar to the protocol for high-grade glioblastoma vaccination (25). Imiquimod, exerting its function *via* Toll-like receptor 7, was used to augment the maturation status of the dendritic cells. Four weekly DC cultures and injections were performed.

Table I. *Characteristics of the four vaccines.*

Vaccine	Starting PBMC number	Number of DCi	Number of DCm-WT1-RNA	Yield (*versus* input PBMC)
V1	9.9×10^8	48.6×10^6	6.0×10^6	0.6%
V2	9.2×10^8	25.6×10^6	8.8×10^6	1.0%
V3	1.1×10^9	31.4×10^6	8.6×10^6	0.8%
V4	1.0×10^9	43.7×10^6	6.5×10^6	0.7%

V1-V4: Vaccination 1 to 4; PBMC: frozen/thawed peripheral blood mononuclear cells; DCi: immature dendritic cells; DCm-WT1-RNA: WT1-mRNA electroporated mature dendritic cells

Immune monitoring. APC-labelled MHC-multimers (Proimmune, Oxford, UK) for WT1 and $gp100_{209\text{-}217}$ were used for MHC-multimer-staining. 10^6 T-cells were stained for 20 min at room temperature, using a 1:200 dilution of the respective pentamer, followed by staining with the following antibodies: CD8-FITC, CD19-PE and CD56-PE (all from BD Biosciences, Heidelberg, Germany). Samples were washed twice and resuspended in 200 μl PBS/1% human serum. Ten minutes prior to data acquisition, 10 μl of 7-AAD (BD Biosciences) were added to the samples. Each sample was stained in triplicate to correct for intra-assay variability. Measurements were performed with a FACS Calibur flow cytometer (BD Biosciences).

Analysis was performed using a hierarchical gating strategy: lymphocytes were gated according to light scatter properties. Next, $CD19^+$ or $CD56^+$ cells as well as dead cells were excluded. Among the remaining cells, $CD8^+$ cells were selected and analysed for MHC-multimer+ events. A minimum of 3×10^4 $CD8^+$ T-cells were acquired for analysis.

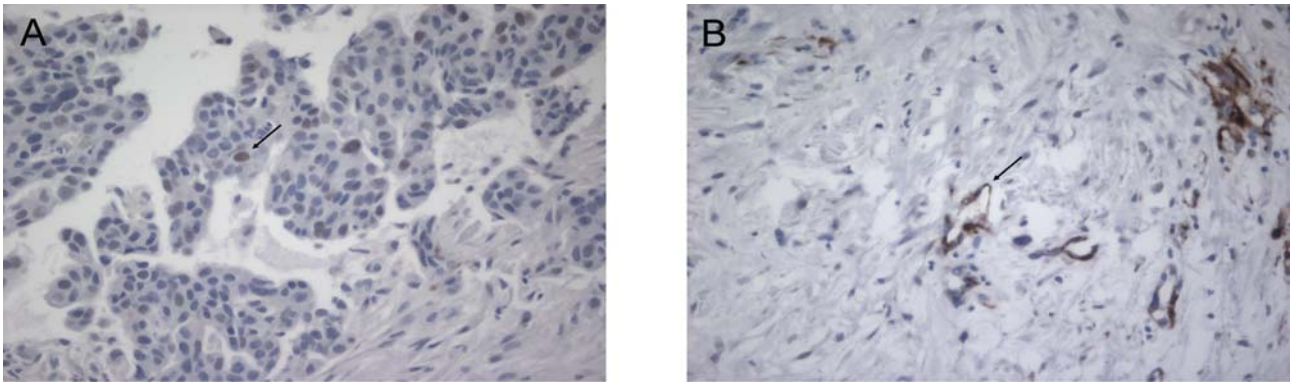

Figure 1. *WT1 immunohistochemical staining of the recurrent disease (peritoneum). A: Tumour cells were WT1 positive in 10% (weak nuclear positivity, -an example is indicated by the arrow). B: Intratumoural endothelial cells were diffusely WT1 positive (cytoplasmic staining, -an example is indicated by the arrow).*

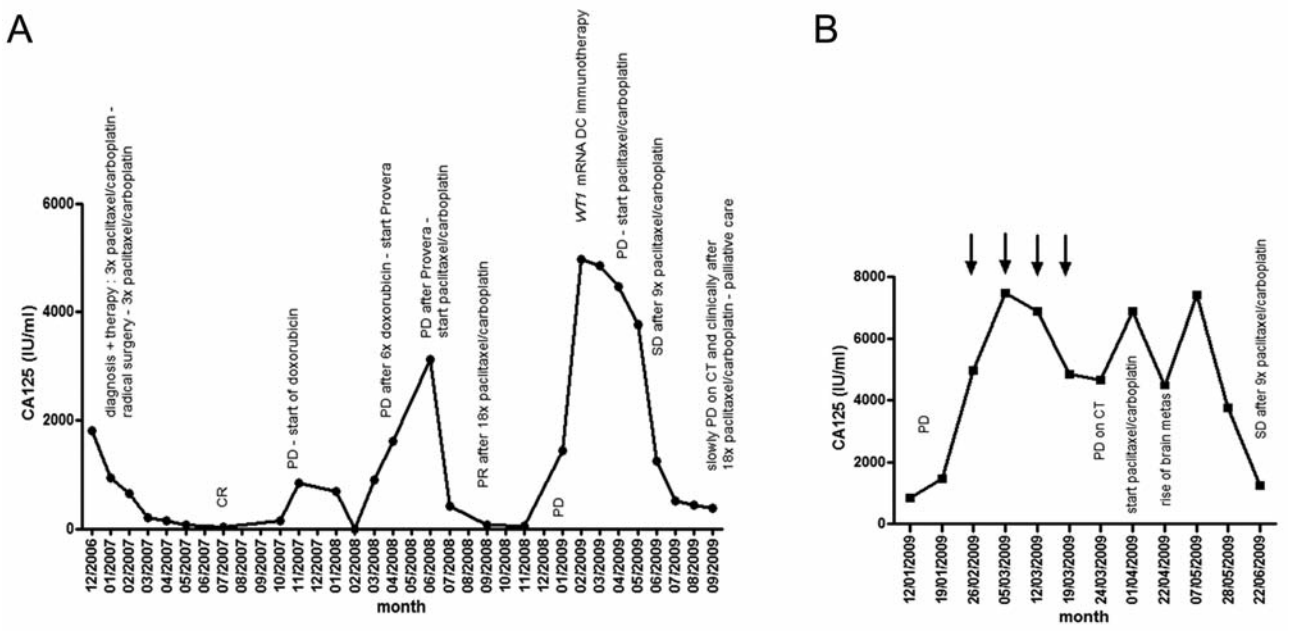

Figure 2. *A: Evolution of CA125 during the course of the disease. B: Evolution of CA125 round the vaccination period. The arrows indicate the administration of the four vaccines. CR: Complete remission; PR: partial remission; SD: stable disease; PD: progressive disease.*

Results

Feasibility. An overview with technical specifications of the produced vaccines is shown in Table I. The production of the vaccines was feasible. Of all PBMCs, 4% (range 3%-5%) developed into DCi, of which 20% (range 12%-34%) survived electroporation and maturation. The injections of the vaccines were performed on an ambulatory basis in the day care centre. The patient was observed for one hour after injection. The Fertigkeitenskala Münster-Heidelberg (FMH-2000b) ability scale was used for quality of life assessment.

Clinical evolution. The patient was followed on a weekly basis. At the start of the vaccination, she was without complaint. Her Karnofsky Performance Scale was 60 with a general health and quality of life estimation of 3 at a scale ranging from 1 (very bad) to 7 (excellent). CA125, a non-specific tumour marker (Figure 2), was 4980 kU/l. At the start of the second vaccination, she felt subjectively better, indicating a general health and quality of life estimation of 4. CA125 further increased till 7455 kU/l. At the third vaccination, she showed more dyspnoea. Karnofsky Performance Scale was 50 and general health and quality of life estimation was again 3. A

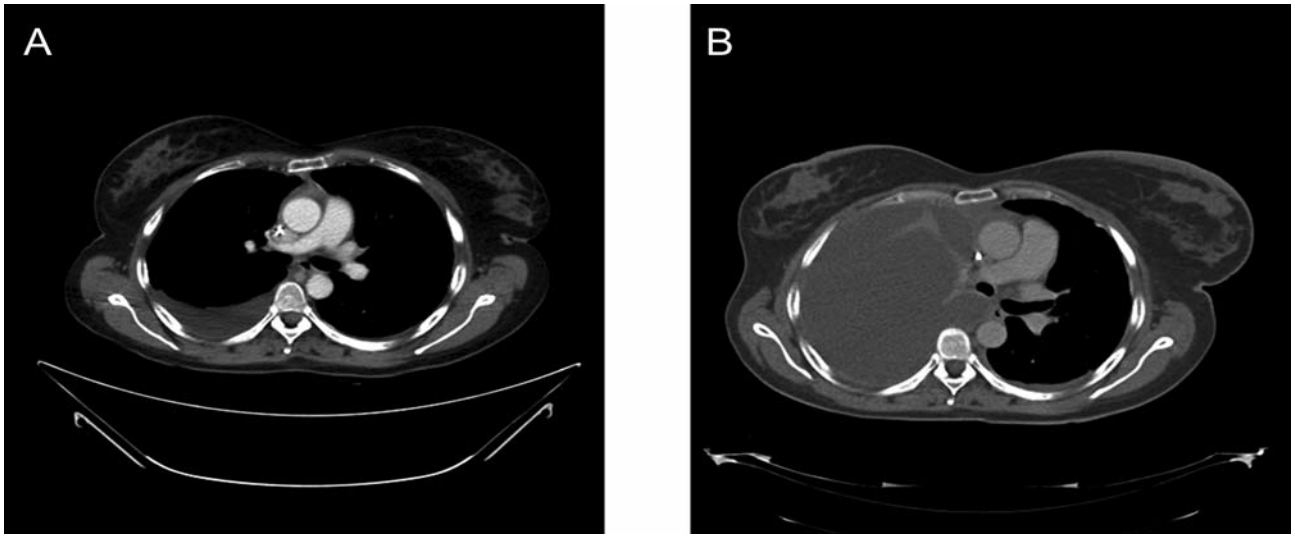

Figure 3. *A: CT scan 2 weeks prior to vaccination, showing minimal pleural effusion in the right lung. B: CT scan 1 week after V4 showing progressive disease: increase of pleural effusion in the right lung and rise of pleural metastasis in the left lung.*

chest X-ray showed pleural effusion. An evacuating puncture was performed. In contrast, CA125 started to decrease. This continued until one week after the cessation of vaccinations (4675 kU/l). She complained again of dyspnoea and, again, a puncture was performed. CA125 started to increase again. During the vaccination period of 4 weekly vaccines, no vaccine-related toxicities were observed.

A CT scan was performed 2 weeks prior to vaccination and 1 week after V4. The latter scan showed an increase of pleural and abdominal effusion, and progression of peritoneal metastasis (Figure 3). In light of the progression, DC vaccination was terminated. The patient received 17 new cycles of paclitaxel and carboplatin but developed brain metastasis after 2 cycles and showed clinical progression after 17 cycles. At that stage, a decision for palliative treatment was taken. She died 4 months after cessation of therapy.

Immune response. At leukapheresis and V4 (=after 3 vaccinations), a heparinised blood sample was taken. PBMCs were isolated using a ficoll gradient and were kept frozen for analysis of both samples together. In order to compare the frequency of antigen-specific T-cells with healthy individuals, previously frozen PBMCs from 3 HLA-A2$^+$ volunteers were used.

Baseline frequency for WT1-specific CD8$^+$ T-cells prior to vaccination was in the same range as in all three healthy donors. After 3 weekly vaccinations, the number of WT1-specific CD8$^+$ T-cells increased 2.5-fold. In contrast, the frequency of gp100-specific T-cells, which served as irrelevant control, did not increase and remained in the same range as that in healthy donors (Figure 4).

Discussion

This report is the first to describe a patient with advanced stage of serous endometrial carcinoma who was treated with autologous dendritic cells loaded with *WT1* RNA and demonstrate the feasibility of this treatment approach without induction of toxicity. Moreover, an increase of circulating WT1-specific CD8$^+$ T-cells was demonstrated upon vaccination.

In a recently published study of Ohno *et al*., one patient with an endometrioid type of endometrial cancer was treated with WT1 peptide immunotherapy (27). She showed progression after 3 months of vaccination treatment (1 injection/week). Enrichment of specific cytotoxic T lymphocytes (CTL) was not measured. Only delayed type hypersensitivity (DTH) after 48 hours was evaluated.

Only two blood samples, one prior to vaccination and one after vaccination, were available. After 3 vaccinations, a 2.5-fold increase in WT1-specific T-cells was noted. In order to increase sensitivity, 2 dump channels were used to exclude B cells, NK cells and dead cells, which otherwise may have given false positive results. Furthermore, to control for intra-assay variation, each staining was performed in triplicate, demonstrating a narrow variation range. Therefore, the increase in MHC-multimer$^+$ CD8$^+$ T-cells was considered to be a true increase in WT1-specific CD8$^+$ T-cells, suggestive of a vaccination-induced immune response. Because of the advanced stage of the patient, it was not possible to take larger blood volumes to measure circulating cytotoxic T-cell activity against WT1-loaded target cells. The induction of a WT1-specific T-cell response after active WT1-targeted immunotherapy has been described previously in

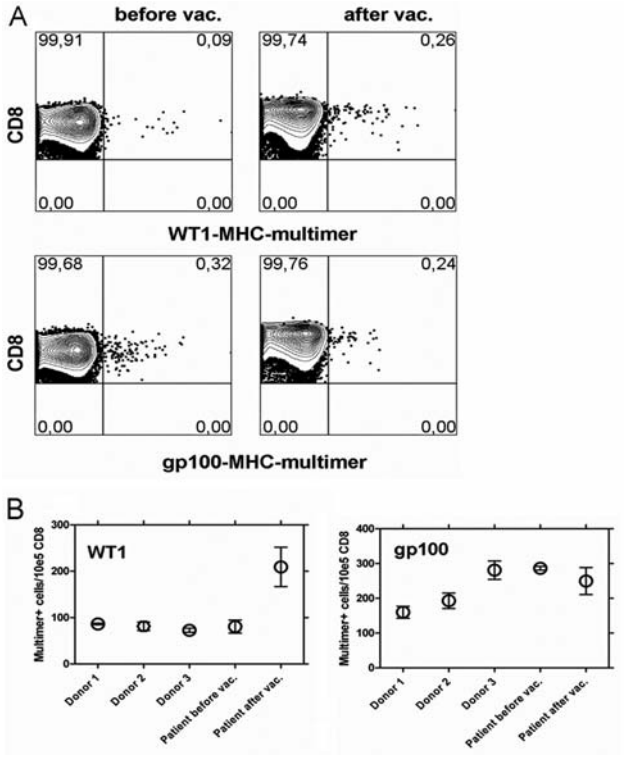

Figure 4. *A: Presence of WT1-specific T cells by HLA-A2 pentamer staining before and after vaccination. B: At baseline, the amount of specific T-cells of the patient were comparable to the average of 3 healthy controls (donor 1-3) . After 3 vaccinations (this is at V4), there was a 2.5-fold increase in WT1-specific T-cells. In contrast, the frequency of gp100-specific T-cells, which served as irrelevant control, did not increase and remained in the same range as in healthy donors.*

haematological malignancies, breast and lung cancer. A recent paper on WT1 immunotherapy in 18 patients with acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) noted a more than 2-fold increase in WT1-specific T-cells in 44% (28). Oka *et al*. showed that measurable clinical responses in breast and lung carcinoma correlated with an increase in circulating WT1-tetramer$^+$ T-cells (10). However, the same group found no enrichment of WT1-specific T-cells in glioblastoma patients (8).

A clinical benefit of the vaccination could not be established in this patient with a large tumour burden. The high CA125 levels at the beginning of the treatment reflected this high tumour burden. However, CA125 remained stable (transient decrease by 7%) during the time of vaccination. It is interesting to notice that the course of CA125 is comparable to a DC-mRNA immunotherapy treated metastatic ovarian carcinoma patient in 2007 (29). In general, the clinical efficacy of cancer immunotherapy is believed to be optimal at the time of minimal residual disease (30). It is also possible that the DC vaccination was ceased too early. In another study, AML and MDS patients received WT1 immunotherapy at least for 3 months (28). If the patient showed progression in this period and no other treatment options were available, WT1 immunotherapy was continued. . Upon continuation of the vaccination, remission was achieved in some patients. Moreover, WT1 in the present patient was expressed in only 10% of the tumour cells, but in 40% of the endothelial cells. Hence the biological effect of DCm-WT1-RNA may have been more anti-angiogenic rather than directly against the tumour cells. Targeted therapies directed against angiogenesis in endometrial cancer have been and are also currently being explored. A comprehensive overview was recently reported by Gehrig *et al*. (31). A recent phase II trial with bevacizumab (Avastin; Genentech), a humanised mAb to VEGF-A, has demonstrated promising activity in recurrent endometrial carcinoma.

These observations open new perspectives for patients with relapsed and/or metastasised WT1-positive uterine tumours and form the basis of a phase I clinical trial which will be initiated at the host centre.

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