Wilms' Tumour gene 1 (WT1) as an immunotherapeutic target in uterine cancer

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Abstract

High grade uterine sarcoma and recurrent endometrial carcinoma are aggressive cancers with limited treatment options, resulting in a poor prognosis. In this research we focused on the detection of a highly immunogenic tumour-associated antigen Wilms’ tumour gene 1 (WT1) in uterine tumours. We were able to reveal its overexpression in the tumour cells of high grade sarcomas and carcinosarcomas. Moreover, patients with WT1 positive tumours had a significantly worse prognosis than patients who were WT1 negative.

For carcinomas, WT1 was present in only a minority of tumour cells, but in the majority of intratumoural blood vessels. Small blood vessels in the normal tissue surrounding the carcinoma were also WT1 positive, suggesting a role for WT1 in angiogenesis. WT1 was hardly expressed or absent in the non-tumour or benign tumoural uterus (myoma, polyp).

The next step was to develop a targeted treatment against WT1. We opted for dendritic cell (DC) based immunotherapy. Nevertheless a basal expression of WT1 in monocytes and in vitro cultured unloaded DC was observed, the electroporation of in vitro cultured DC with WT1-mRNA resulted in a higher expression of WT1 by the DC. WT1-mRNA loaded DC were used for in vivo stimulations of T cells, resulting in the rise of WT1-specific T cells and a transient molecular response (decrease of CA125) in an end stage endometrial carcinoma patient. No toxic side effects were reported. Future in vivo research, carried out in a phase I clinical trial in our center, will reveal the ability of this new therapy to induce an immunological and possible clinical response in WT1 positive uterine cancer patients.

Key words: Dendritic cells, immunotherapy, uterine tumour, Wilms’ tumour gene 1, WT1.

Introduction

Uterine tumours

Worldwide, endometrial cancer is the seventh most common malignant disorder (Parkin et al., 1999). Each year, in Europe, an estimated 9000 women die of endometrial cancer. Endometrial cancer occurs mainly in postmenopausal women with a mean age of 65 years. Uterine sarcoma is a rare entity and constitutes 2 to 5% of all uterine malignancies worldwide. An increased incidence of uterine sarcoma has been associated with the use of tamoxifen in the treatment of breast cancer.

Endometrial carcinoma has an epithelial origin as it arises from the epithelium that lines the uterine cavity, the endometrium. There are 2 major pathogenetic groups in endometrial carcinoma to be defined. Type 1 endometrial carcinoma is the largest group (90%), consists of the endometrioid type of endometrial cancer and develops under estrogen production. Type 2 endometrial carcinomas (serous and clear cell carcinoma) are less frequent (10%) but more high grade, independent from estrogen
production (Kurman et al., 2002; Amant et al., 2005). Historically, carcinosarcoma (CS) or malignant mixed mullerian tumour (MMMT) used to be classified as uterine sarcoma. However, the biphasic cellular population of CSs had alerted many researchers to reveal if either one of two cell populations were responsible for the origin of this tumour. Studies demonstrated a monoclonal epithelial origin, determining their spreading pattern. Therefore, they are treated as poorly differentiated carcinomas with a prognosis comparable to the group of the sarcomas (Kurman et al., 2002). However, classifications do not always follow these conclusions (Tavassoli et al., 2003). Uterine sarcoma has a mesenchymal origin. There are 3 major pure mesenchymal histological subtypes (leiomyosarcoma, undifferentiated sarcoma and endometrial stromal sarcoma) and one small entity, the smooth muscle tumour of uncertain malignant potential.

Since vaginal bleeding is the most important symptom, endometrial cancer is often detected in an early stage. Early diagnosis and early stage have a clear impact on the prognosis. More than 73% of the patients with endometrioid carcinoma presents in stage I where the tumour is limited to the uterus, resulting in a 5-year survival of 85 to 90%. However, serous and clear cell carcinoma display a more aggressive behavior and thus often present in more advanced stages, with 38% in stage III and IV at diagnosis. Their 5-year survival at stage I is also lower than for the endometrioid subgroup, being 60%. The overall 5-year survival at later stages decreases gradually to 40% and 15% for stage III and IV endometrioid carcinoma respectively and 20% and 5% for serous and clear cell carcinoma (Amant et al., 2005). In contrast to carcinomas, sarcomas are often detected at higher stages since they do not always cause visible symptoms and have the potential for early hematogeneous metastasizing. The intramural component explains the diagnostic delay when compared to endometrial cancer. Prognosis is poor, with 50% 5-year survival at stage I and II of the high grade sarcomas and 10% at more advanced stages (Mutch et al., 2009). ESSs on the other hand have a 5-year survival of 92% in early stages and still 66% in the more advanced stage. However, since they have an indolent growth pattern, their 10-year survival is worse. The chance on recurrent disease is 36% at early stages and 76% at advanced stages (Amant et al., 2009).

Surgery – if disease is limited – is the corner stone for both carcinomas and sarcomas of the uterus. Adjuvant therapy is often questionable but probably favourable in carcinoma. Adjuvant radiotherapy only has a place in lymph node positive carcinomas. In cases of recurrent disease or diagnosis of an advanced stage of the disease, chemotherapeutical options are limited and often shortlasting. Radiotherapy can be of help in local recurrent endometrial carcinoma. The mean survival for recurrent endometrial cancer is approximately 12 months and even less for uterine sarcoma. It is clear that there is an urgent need for new and better drugs, preferably with a targeted profile, thus limiting the toxicities.

**Wilms’ tumour gene 1 (WT1)**

Wilms’ tumour gene 1 (WT1) is a gene located on chromosome 11p13. It was first cloned in 1990 in Wilms’ tumour, a pediatric kidney cancer. WT1 is required for kidney development and male sex determination (Guo et al., 2002; Hastie, 2001). WT1 is also involved in the development of the heart, central nervous system and blood (Ariyaratana et al., 2007; Scholz and Kirschner, 2005; Wagner et al., 2005; Wagner et al., 2005). After birth, the expression is limited to the urogenital system, the central nervous system and in tissues involved in hematopoiesis (Yang et al., 2007). During the last decade, WT1 wild type overexpression has been detected in several hematological and solid malignancies (Oji et al., 2003; Oji et al., 2002; Silberstein et al., 1997): acute myeloid leukemia, myelodysplastic syndrome, brain tumours, breast cancer, colorectal adenocarcinoma, desmoids tumours, thyroid cancer, … This overexpression has been suggested to be involved in the tumourigenesis of several malignancies, but has been proven to play a tumourigenic role in breast cancer and acute myeloid leukemia. Moreover, WT1 has recently been defined as the most important cancer antigen (Cheever et al., 2009), resulting from a ranking based on specificity, oncogenicity, immunogenicity and therapeutic function. WT1 has been proven to be an oncofetal antigen, associated with oncogenic processes, immunogenic in clinical trials also showing fair clinical responses. WT1 is also expressed in a large variety of tumour blood vessels (Wagner et al., 2003). Furthermore, WT1 is involved in endothelial cell proliferation, vascular formation and migration, indicating that it might be a general marker for angiogenesis (Wagner et al., 2008).

**Immunotherapy**

Cancer immunotherapy can be defined as the treatment of cancer by inducing, enhancing or suppressing an immune response. Active immunotherapy implies cancer vaccines, where the immune response has to be elicited by the human body. Passive immunotherapy implies the administration of preformed specific immunotherapeutical agents.
Dendritic cell immunotherapy is an example of active immunotherapy.

Dendritic cells (DC) are a small (< 1%) subset of the white blood cell population. They reside at an immature state (DCi). DCi can capture antigens, a process that is mediated through the Fc receptors. In the presence of danger signals, DC then start to mature, allowing them to migrate to the lymph nodes. In the lymph nodes, antigens are presented to T cells in the context of both MHC class I and II. Thus, both CD4+ T helper cells and CD8+ cytotoxic T cells can be stimulated (Banchereau et al., 2000). Since DC are professional antigen-presenting cells, they are explored as a carrier for tumour antigens in immunotherapy. Usually in vitro differentiated DCs are used since isolation of circulating DCs would be inadequately due to their rarity. Figure 1 gives a schematic overview of this production process in UZ Leuven.

Immunothenapy in uterine cancer is in its preliminary phase. A total of 16 patients have been treated with 7 different approaches on immunotherapy. So far, no conclusions can be drawn.

**Methods**

The need for new therapies in uterine cancer is urgent. WT1 is a highly ranked TAA (tumor associated antigen) and there is evidence in literature that WT1 is present in the tumoural uterus. Therefore, we

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**Fig. 1.** — Dendritic cell based immunotherapy:
Dendritic cells (DCs) can be cultured starting from monocytes. The latter can be obtained by plastic adherence of peripheral blood mononuclear cells (PBMCs), by CD14 selection or by counterflow centrifugal elutriation. PBMCs are obtained via blood sampling, mostly leukapheresis to achieve large amounts. These cells are differentiated in the presence of cytokines into immature DCs (DCi). The cytokines and their amount vary among published studies, though IL4 and GM-CSF (granulocyte macrophage colony-stimulating factor) are mostly used. DCi need maturation to become mature DCs (DCm). Several methods to mature DCi have been described, using all one or a combination of factors mimicking the in vivo danger signal: tumor necrosis factor α (TNF-α), PGE2, IL-1β, IL-6, bacterial lipopolysaccharides (LPS), … Before or after maturation, DCs are loaded with antigens. They can be divided into 2 groups: whole tumour cell approaches and tumour-associated antigens (TAA). Although working with whole tumour cell products (obtained by tumour lysates, total tumoural RNA, …) might theoretically cause auto-immune problems, several tumours, like for example glioblastoma, do not have a well defined TAA. Whole tumour cell approaches are then the only option. The use of specific TAA on the other hand (like WT1, Melan-A, Her2-neu, MUC-1, MAGEA3, bcr-abl, …) risks to select escape variants. TAA can be offered to DCs as synthetic peptides or defined mRNA. Loading of RNA can be performed by electroporation. One major advantage of the RNA approach is its independence of HLA haplotyping. If the DCm are injected into a patient, this immunotherapy injection is often called “vaccine”. 

Based on the experimental and clinical experiences of the group of Prof Dr Van Golld in glioblastoma
asked ourselves the following two questions. First, is WT1 present in uterine tumours and the tumoural environment. If yes, is its presence tumour specific and if so, what is its importance for uterine tumours? This question solved in 4 separate studies on uterine sarcomas, endometrial carcinomas and the normal tissue surrounding tumours.

Second, if we could prove that WT1 was indeed necessary in the tumourigenesis of uterine tumours, would it then be feasible to design dendritic cell immunotherapy against this WT1? If so, would it be able to result in an immune response and – more important – would it be safe and applicable in clinical practice? This question was solved in 2 subsequent studies.

Results

WT1 in uterine sarcoma (Coosemans et al., 2007; Coosemans et al., 2011)

In the literature, only a few small studies can be found studying WT1 expressing, mostly at the protein level, not including all subtypes of uterine sarcomas, endometrial carcinomas and the normal tissue surrounding tumours. We noticed the same or reduced staining pattern. WT1-mRNA expression could be measured in all-but-one sample. Western blot results showed the presence of WT1 protein in all 12 samples. The majority (75%) of samples had more cytoplasmic expression of WT1. No mutations or deletions were detected.

Comparing the immunohistochemical staining in 87 sarcomas with 40 myomas and 10 normal myometrium samples, we noticed that the expression in myomas was weak or absent and nearly absent in normal myometrium, whereas WT1 was overexpressed in most sarcomas. This raised the possibility of a possible involvement of WT1 in the tumourigenesis of uterine sarcoma.

Of 71 patients with high grade uterine sarcoma, we had a clinical follow up of at least 12 months and a known WT1 status (WT1 positive tumours n = 49, WT1 negative tumours n = 22). Univariate analysis showed a worse progression free survival (PFS) and overall survival (OS) for patients with a WT1 positive uterine sarcoma (Fig. 3). PFS and OS were 7 and 16 months respectively in patients with a WT1 positive sarcoma compared to 29 and 64 months in patients with a WT1 negative sarcoma. This difference remained significant in a multivariate analysis including age, subtype, stage and size.

In conclusion, WT1 is overexpressed in the majority of uterine sarcomas and the presence of WT1 worsens their prognosis, suggesting a role for WT1 in the tumourigenesis of uterine sarcoma.

WT1 in endometrial carcinoma (Coosemans et al., 2008; Coosemans et al., 2009)

In endometrial carcinoma, WT1 staining using IHC ranges from 0% up to 79%, according to a total of 11 studies including 423 patients. According to a meta-analysis in 2005 by Heatley (Heatley, 2005), including 7 immunohistochemical studies, the detection rate of WT1 in endometrial carcinoma was 29.1% (20.5–39.4%). So far, only 1 study was conducted using PCR to determine WT1 expression.

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Table I. — Overview on WT1 positive uterine sarcoma samples by immunohistochemistry and RT-PCR.

| Immunochemistry | RT-PCR |
|-----------------|--------|
| CS              | ESS    | LMS    | US    | LMS | CS | ESS | US |
| Agoff SN et al., 2001 | 9/10   |        |       |      |    |    |    |
| Dupont J et al., 2004 | 7/10   |        |       |      |    |    |    |
| Sumathi VP et al., 2004 | 13/14  |        |       |      |    |    |    |
| Sotobori T et al., 2006 | 5/5    |        |       |      |    |    |    |
| Coosemans A et al., 2007 | 12/27  | 7/15   | 29/38 | 4/7  | 6/6 | 4/4 | 6/6 |

CS : carcinosarcoma; ESS : endometrial stromal sarcoma; LMS : leiomyosarcoma; US : undifferentiated sarcoma; RT-PCR : real-time polymerase chain reaction.
at RNA level (Rackley et al., 1995). In total, 6/14 samples (subtype not specified) were WT1 positive. Because of this often low detection level at protein level and the large variation in the amount of WT1 positive samples, the paucity of information at the RNA level and the conflicting results in benign endometrium, we set up a study in 38 samples to analyse WT1 expression at the protein level by immunohistochemistry in 3 different biopsies per tumour and at the RNA level by RT-PCR. Results were also compared with normal endometrium, hyperplasia and polyps, in total 27 samples.

In the 38 endometrial carcinoma samples, WT1 positivity was observed in the cytoplasm of endothelial cells lining intratumoural blood vessels and in the cytoplasm of endometrial tumour cells (Fig. 4). Comparing the three biopic sites immunohistochemically, heterogeneity in WT1 positivity could be observed either in the tumours cells and/or the endothelial cells. Pooled data (mean results) revealed WT1 positivity in 7/36 samples (19%) in the tumour cells, while 22/35 samples (63%) were positive if only WT1 positivity in the blood vessels was taken into account. Finally, 24/32 samples (75%) showed WT1 positivity with RT-PCR. Adding together the mean of immunohistochemical results of tumour and endothelial cell positivity (of 3 biopic sites), 72% of all tumours were WT1 positive (26/36).

When normal endometrium was examined, the cells were negative for WT1. As is the case for the sarcomas compared to myomas and normal myometrium, malignancy involving the endometrium also upregulated WT1. However, the blood vessels were 100% positive if the endometrium was triggered (by hormones (spontaneous reproductive cycle or hormonal replacement therapy during menopause) or by benign tumoural growth (atypic hyperplasia or benign, atypic polyps) (Fig. 5A). If, on the other hand, the endometrium was atrophic blood vessel positivity remarkably reduced (Fig. 5B). Comparing the blood vessel positivity between benign and ma-
Lignant endometrium, the percentage of positive endothelial cells was remarkably higher in triggered endometrium, benign tumoural endometrium and endometrial carcinoma type I, suggesting a possible role for estrogens in the WT1 positivity of intra-tumoural blood vessels.

Since some WT1 positivity could be noted in endothelial cells, it was worth looking at the surrounding environment of the tumour. Therefore, paraffin embedded tissue samples of 89 patients with different primary tumours were collected. Additionally, 7 human myocardial infarction samples (2 acute, 5 old) were analysed. In malignant tissue, detailed analysis of the distribution pattern of the WT1 positive blood vessels in the tissue surrounding the tumour revealed that the blood vessels were also positive for WT1 in the close periphery of the tumour and became negative with increasing distance from the tumour. This 'gradient'-phenomenon was observed in 67% of all epithelial malignant tumours. It was absent in almost all (93%) uterine sarcomata. Benign tumours (polyps and myomata) behaved similarly as their malignant counterparts. A comparison of endothelial cell staining in normal tissue surrounding normal versus tumoural tissue of colon and uterus in the same specimen clearly showed that

**Fig. 3.** — Survival curves of WT1 positive versus WT1 negative high grade uterine sarcoma:
A. Progression free survival.
B. Overall survival. [WT1 positive tumours (n = 49), WT1 negative tumours (n = 22)].

**Fig. 4.** — WT1 expression in endometrial carcinoma:
A. Cytoplasmic expression of WT1 in tumour cells of an endometrial carcinoma.
B. Cytoplasmic expression of WT1 in endothelial cells of the same tumour.
the amount of WT1 positive blood vessels was much less in tissue surrounding normal colonic mucosa and endometrium compared to the tissue surrounding colorectal and endometrial carcinoma, respectively. Staining of both acute and old human myocardial infarction specimens showed WT1 positive blood vessels in the periphery of the lesion in all-but-one of the samples.

A mechanism controlling WT1 expression has to be supposed. It might be that these blood vessels are neo-angiogenic or that WT1 expression is switched-on in pre-existing blood vessels, derived from WT1 expressing endothelial progenitor cells by hypoxia (Kanato K et al., 2005). This mechanism was already shown by Wagner et al. in tubular cells of kidneys of hypoxic rats (Wagner et al., 2003).

In conclusion, endometrial carcinomas are WT1 positive in the majority of cases if both tumour cells and intratumoural endothelial cells are taken into account, though more than one biopsy has to be analysed since the tumour is heterogeneous. Moreover, blood vessels in normal myometrium surrounding the endometrial carcinoma are positive close to the tumour and negative with growing distance from the tumour. This gradient-phenomenon might be hypoxia driven.

**WT1 immunotherapy (Coosemans et al., 2010)**

Developing WT1-mRNA dendritic cell immunotherapy

We developed the technique of dendritic cells (DC) immunotherapy in our laboratory in the context of high grade glioma patients (De Vleeschouwer et al., 2005, 2007; Maes et al., 2009). By treating patients with relapsed glioblastoma multiforme by means of mature DC loaded with total tumour antigen, we were able to improve progression-free and overall survival (De Vleeschouwer et al., 2004; De Vleeschouwer et al., 2008; Rutkowski et al., 2004, Van Gool et al., 2009) (Fig. 1). Based on this experience we set up a series of experiments to insert WT1 antigen into dendritic cells (Fig. 1).

However, in control experiments with healthy volunteers, we noticed that a certain (small) percentage of the monocytes, the *in vitro* cultured immature dendritic cell (DCi) and the *in vitro* cultured mature unloaded dendritic cell (DCm unloaded) showed already some WT1 expression. This finding was true on both protein and RNA level. This was unexpected. The phenomenon is not observed on freshly isolated *in vivo* circulating DC. Since the culturing conditions seemed to influence the amount of WT1 expression, the adherence of the monocytes on specific structures of the culture recipient possibly plays a crucial role for induction of and/or up-regulation of WT1 in the monocytes during the differentiation culture towards DC.

The finding that WT1 is expressed as RNA and protein not only in hematopoietic stem cells (Maurer U. et al., 1997), but also in a small percentage of circulating monocytes in the blood of a healthy individual could rises questions on the suitability of WT1 as an immunotherapeutic target in case of cancer. However, there have been no reports of autoimmune adverse effects in clinical trials. The most important consequence of this finding is that patients...
who are treated with tumour antigen-loaded DCm might be stimulated for an anti-WT1 immune response as well. Our finding could also explain why anti-tumour responses were observed upon vaccination with unloaded DC (Nesselhut et al., 2004).

However, when we loaded the tumour specific antigen WT1 onto the DCi, the amount of WT1 was much higher than in the unloaded DCm. We choose to load WT1 in its RNA form because of its main advantage, being its independence of the HLA-status to load WT1 in its RNA form because of its main advantage, being its independence of the HLA-status of the patient. WT1-mRNA was inserted into DCi by means of electroporation (electroshock of 300V to open the cell membrane to insert the mRNA). The WT1 immunotherapy experiments were further conducted with WT1-mRNA loaded DCm.

Effectiveness of the WT1-mRNA dendritic cell immunotherapy

To evaluate the effectiveness of immunotherapy, it is mandatory that it is tested in an in vivo human setting, because immunotherapy has to interact with an existing immune system - partially conditioned by the existing tumour – in the human body, a condition that can hardly be mimicked in vitro. A humanised mouse model could definitely solve some critical questions and pave the way for optimisation of the immunotherapy protocol, however, it takes a very long time to develop such a model. Nevertheless, steps have been made in this direction by our team and we already created mice with uterine sarcomas.

But the real proof of effectiveness remains the clinical setting. A 45-year-old woman was diagnosed with a 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 3 weekly. After 6 cycles, she showed progression and medroxyprogesterone was given. She progressed further and paclitaxel and carboplatin infusions were administered on weekly basis during 18 cycles. Total body scan in October 2008 showed partial remission. She was progressive again in January 2009 (rise of CA125, increase of pleural effusion and of ascites). At that time, the patient consented to the start of WT1 immunotherapy. An open laparoscopy was performed to take a biopsy from the recurrent disease. Immunohistochemical staining, which was performed as described before (Coosemans et al., 2007), showed WT1 positivity in 10% of the tumour cells and the intratumoural endothelial cells showed widely distributed WT1 positivity (Coosemans et al., 2008). She underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMCs). At day 7 of the cultures, DCm-WT1-RNA were harvested, resuspended in DPBS with 2.5% human serum albumin and injected intradermally in the groins of the patient. The day before, the day of and the day after the injection, the patient applied imiquimod cream (Aldara, Meda AB, Sweden) at the site of injection, similar to the protocol of high grade glioblastoma vaccination (Van Gool et al., 2009). 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 on an ambulatory basis in the day care centre. During the vaccination period of 4 weekly vaccines, no vaccine-related toxicities could be observed.

The response to this new type of treatment was followed at 3 levels : the immune response in the blood (rise of WT1 specific T cells), the molecular response (changes in the tumour marker CA125) and the clinical response (by CT scan). After the second vaccine, CA125 started to decrease (Fig. 6). This molecular response was perfectly correlated with an increase in WT1 specific T cells in the blood of the patient. The decrease in CA125 continued until one week after the stop of vaccination. Then, the patient had more complaints of dyspnea. CA125 started to increase again and CT scan showed an increase of pleural and abdominal effusion, and progression of peritoneal metastasis. In light of the progression, DC vaccination was terminated. The patient received 17 new cycles of paclitaxel + 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 therapy stop.

We reported the first patient with advanced stage of serous endometrial carcinoma who was treated with autologous dendritic cells loaded with WT1 RNA, and demonstrated feasibility of this treatment approach without induction of toxicity. Moreover, we could demonstrate an increase of circulating WT1-specific CD8+ T cells upon vaccination.

A clinical benefit of vaccination could not be established in this patient with large tumour burden. The high CA125 levels at the beginning of the treatment reflected this high tumour burden. However, we were a little bit surprised that a patient, who was declared palliative (no further classical treatment options left since she relapsed < 6 months after the last platin-based chemotherapy) at the start of immunotherapy, responded to Taxol-Carboplatin after the immunotherapy and lived for 10 more months. Her overall survival was 36 months. We
analysed a reference population from the Leuven oncology patient group. Eleven cases were available with a comparable cancer history as our patient, including the use of chemotherapy and surgery as primary treatment. Overall survival was 21 months (range 12-29 months). When treatment was stopped and no further cytotoxic treatment was planned, mean survival was 67 days (range 0-11 months). It has been proposed that the combination of immunotherapy with standard therapy might result in a synergistic effect (Kandalaft et al., 2010, Antonia et al., 2006).

Clinical study

The encouraging clinical results in this index patient stimulated us to develop a phase I clinical trial. The ideal population for immunotherapy is the one with minimal residual disease, since at that timepoint effector/target ratio is high and the immune-suppressive environment induced by the cancer cells is low (Steinman and Banchereau, 2007). However, new cancer therapies are explored in end stage patients. Therefore, the intended population for the phase I/II clinical trial WT1-2009 includes patients with advanced refractory uterine sarcoma or carcinoma.

The trial was approved by the ethical committee of the UZ Leuven (WT1-2009, EudraCT 2009-016868-37) and started recruiting in February 2010. So far, seven patients are included.

In conclusion, we have been able to develop dendritic cell immunotherapy against WT1. It is feasible to create dendritic cells and to load them with WT1-mRNA. They can induce a WT1-specific T-cell response \textit{in vivo} and applied in patients, they are safe without side-effects.

Conclusions

Uterine high grade sarcoma recur in approximately 50% of cases. Also the prognosis of recurrent endometrial carcinoma is dismal with a mean survival of only one year. Classical treatment options are limited and often with shortlasting results, resulting in short survival. Therefore, the search for new and more targeted treatments is urgent and highly needed.

Based on previous genetic findings of our group (Amant et al., 2001; Amant et al., 2002; Amant F, 2002), we started a research program to target one of the most attractive tumour-associated antigens, WT1 (Cheever et al., 2009). Expression of WT1 had been investigated largely in literature in uterine carcinoma on protein level (IHC), demonstrating that 20% of tumours were positive (Heatley et al., 2005). Its expression on RNA level had not been studied. Data on WT1 expression (protein and RNA level) in uterine sarcoma were sparse (Agoff et al., 2001; Sumathi et al., 2004; Sotobori et al., 2006). Also, there were no consistent data on the presence or absence of WT1 in the non-tumoural uterus, which is an important issue in developing targeted treatment (side-effects) (Agoff et al., 2001; Sumathi et al., 2004; Dupont et al., 2004; Riedlinger et al., 2005).
We found overexpression of WT1 in the majority of the tumour cells of high grade sarcomas and CS. This was the case on both protein and RNA level. For low grade sarcomas (ESS), the presence of WT1 was much lower. Interestingly, for high grade sarcomas and CS, we were able to define WT1 overexpression as an independent prognostic factor, suggesting a substantial role of WT1 in the tumourgenesis of uterine sarcoma.

In uterine carcinoma, we confirmed literature findings in tumour cells, but we discovered, by determining the amount of WT1-RNA, that WT1 in the intra-tumoural blood vessels accounted for a substantial amount of WT1, which was not reported before. Also in the normal tissue surrounding the tumour, we found WT1 positivity in blood vessels close to the tumour. This effect disappeared with growing distance from the tumour. The finding of WT1 in tumour-related endothelial cells was relatively new and suggested a relation to tumour-induced blood vessel formation. However, the mechanism for this over expression is yet unclear.

Studies on the normal uterus and the benign tumoural uterus (polyps and myomas), revealed that WT1 expression augmented with the grade of malignancy. It was weakly expressed or absent in benign tumoural tissue (myoma) and absent in myometrium, endometrium and endometrial polyps.

All these findings strengthened our hypothesis that WT1 in malignant uterine tumours was a good candidate for targeting through the immune system.

Because of the experiences with DC immunotherapy in our group (De Vleeschouwer et al., 2005; De Vleeschouwer et al., 2008), we decided to develop DC immunotherapy based on WT1 loaded DC. Preclinical experiences of our group with RNA electroporation of DCs (Maes et al., 2009) and the theoretical advantages of the use of RNA in comparison to peptides have led us to develop WT1-mRNA loaded DC as vaccine, which is a new approach. We detected the presence, though limited, of WT1 RNA and protein in monocytes and in in vitro cultured unloaded DCs, which could as a consequence imply that patients who are treated with tumour antigen-loaded DCm might be stimulated for an anti-WT1 immune response as well. Electroporation of DCs with WT1-RNA resulted in an increase of WT1 in the DCs compared to the unloaded state. DCm-WT1-RNA were used for further experiments.

In a palliative stage IV patient with WT1 intra-tumoural blood vessel positive endometrial carcinoma, we were able to demonstrate a 2.5-fold enrichment of WT1-specific T cells upon vaccination with DCm-WT1-RNA. Moreover, she showed a transient decrease of the tumour marker CA125. The encouraging clinical results in this index patient stimulated us to develop a phase I clinical trial.

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What is already known?

Wilms’ tumor gene originally defined as a tumor suppressor gene plays an important role in tumorigenesis in various cancers including uterine and breast cancer. WT1 gene expression is a known prognostic factor in almost all types of solid tumours; this is confirmed in this study. WT1 antigen is a top ranked target for immunotherapy based on its therapeutic function, its immunogenicity, and number of patients with antigen-positive cancers. Phase I clinical trials have shown WT1 targeted immunotherapy to be safe and to show clinical efficacy.

What is new from this research?

The demonstration of WT1 gene expression at mRNA level: 72% of uterine cancers are WT1-positive at mRNA level as detected by reverse transcriptase PCR.

Substantial WT1-mRNA expression in blood vessels surrounding the uterine cancer, levels that decrease the further the blood vessels are remote from the uterine cancer suggesting a role of WT1 in angiogenesis.

A new technique of immunotherapy in uterine tumors using dendritic cells loaded with tumour-specific WT1 mRNA.

WT1 immunotherapy using autologous dendritic cells loaded with tumour-specific WT1-mRNA showed a clinical and CA125 response in a women with advanced stage serous endometrial cancer treated with third-line chemotherapy.

Start of a Phase I/II trial on WT1-mRNA vaccine in women with refractory uterine sarcoma and carcinoma.

Which questions will these new findings arise?

Determining the role and timing of immunotherapy as a modality amongst surgery, radiotherapy and chemotherapy in the strategy of cancer treatment.

E. de Jonge