METHODS

Study design

This study was designed as a single-arm, phase I/II, monocentric clinical trial to evaluate the feasibility, safety, immunogenicity, and clinical activity in patients with solid tumors of adjuvant vaccination with autologous dendritic cells electroporated with mRNA encoding the Wilms' tumor protein WT1 (*WT1*-mRNA/DC). The study was approved by the Ethics Committee of the Antwerp University Hospital/University of Antwerp (Antwerp, Belgium) and registered under the identifiers NCT01291420 (ClinicalTrials.gov) and 2011-000547-24 (EudraCT).

Patients

Patients with glioblastoma multiforme (GBM), metastatic breast cancer (MBC) or locally advanced breast cancer, malignant pleural mesothelioma (MPM), sarcomas, or a rare tumor (brain stem astrocytoma) were included in the trial. All had metastatic or locally advanced disease and had received at least one prior treatment according to available gold standards.

Vaccine manufacturing

Good manufacturing practice (GMP)-grade production of *WT1*-mRNA/DC vaccines was performed as described previously [1–3]. Briefly, patients underwent leukapheresis to enable the collection of peripheral blood mononuclear cells (PBMCs). $CD14^+$ monocytes were isolated from PBMCs via positive immunomagnetic selection and cultured into immature dendritic cells (DCs) in the presence of granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4. After maturation/activation with tumor necrosis factor (TNF)-α, prostaglandin E_2, and the cognate helper antigen keyhole limpet hemocyanin (KLH), DCs were electroporated with *WT1* mRNA, using either a full-length construct or a codon-optimized construct lacking the 5' and 3' untranslated regions and the nuclear localization signal of *WT1*

and incorporating a major histocompatibility complex class II-skewing signal (*DC-LAMP-OPT*) [1].

Vaccine administration

Cryopreserved vaccine aliquots (10×10^6 DCs intended per vaccination) were thawed and administered via the intradermal route into the ventromedial part of the upper arm, approximately 5–10 cm from the axillary lymph nodes. Lot release criteria were specified previously [2]. Vaccines were administered on an intended biweekly basis for an intended period of 6 months. Optional continuation of the biweekly vaccinations was considered depending on the clinical situation and vaccine availability.

Immune response evaluation

To assess cell-mediated immunity *in vivo*, delayed-type hypersensitivity (DTH) skin reactions were determined at T1, which occurred after an intended period of 8 weeks following V1 (first dose of *WT1*-mRNA/DCs) [1,3]. Unless stated otherwise, immune monitoring was performed at baseline ('PRE' time point, usually at V1), 8 weeks after V1 ('POST' time point, usually the same day as the DTH test), and at completion of the intended treatment phase ('FIN' time point).

PBMCs were frozen viably in fetal bovine serum (Thermo Fisher Scientific) supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich) to allow batch analysis at a later time point. Direct *ex vivo* analysis of lymphocyte subsets was performed using flow cytometry as described previously [1, 3], and WT1-specific $CD8^+$ T-lymphocytes were enumerated using peptide-HLA-A*02:01 tetramers generated in-house [1, 3, 4] or obtained from the Fred Hutchinson Cancer Research Center. Intracellular cytokine staining was used to detect antigen-specific $CD4^+$ and $CD8^+$ T-lymphocytes expressing the type 1 cytokines interferon (IFN)-γ and TNF-α and/or the type 2 cytokine IL-5, as described previously [1, 3]. In these assays, PBMCs were left unstimulated or stimulated for 6 hr with pooled 15-mer peptides overlapping by 11 amino acids

spanning the entire protein sequence of WT1 (PepTivator WT1, Miltenyi Biotec), non-KLH-exposed *WT1* mRNA-electroporated DCs, or KLH (Immucothel) in the presence of brefeldin A (BD Biosciences). Data were acquired using a CyFlow ML flow cytometer (Partec) and analyzed using FlowJo software (FlowJo LLC).

Plasma WT1 IgG concentrations were quantified using an ELISA as described previously [5]. Four WT1 peptides were used as capture antigens: SQASSGQARMFPNAPYLP (WT1-118), CMTWNQMNLGAPKK (WT235-11PKK), CAYPGCNKRYFKLSHLQM (WT1 325-18), and YESDNHTTPILCGAQYRI (WT1-271). WT1-118 and WT235-11PKK peptides were used to detect antibodies against the WT1 cytotoxic T-lymphocyte epitopes WT1-126 and WT1-235, respectively [6]. WT1 325-18 and WT1-271 peptides were used to detect antibodies against the WT1 helper T-lymphocyte epitope WT1-332 and another representative WT1 epitope, respectively [7]. Cutoff levels for each antibody positivity were set at an absorbance value of 0.15 based on the mean + 2 standard deviations obtained from independent assays performed using negative samples (N = 5).

Clinical response and overall survival

Disease status was assessed before or at the start of *WT1*-mRNA/DC vaccination (V1), at T1 (intended at 8 weeks after V1), and at T2 (intended at 6 months after V1) using disease-appropriate imaging studies, specifically magnetic resonance imaging (MRI), computed tomography (CT), or ^{18}F-FDG positron emission tomography (PET)-CT. Clinical responses were determined according to the (modified) RECIST 1.1 criteria. The overall response rate was defined as the percentage of patients achieving complete remission or partial response. The disease control rate was defined as the percentage of patients achieving complete remission, partial response, or stable disease. Median overall survival (OS) was calculated from the initial diagnosis for patients with GBM and MPM and from the diagnosis of metastatic disease for patients with MBC. Median OS was compared with the expected OS reported in the literature.

Statistical considerations

Associations were examined among immune parameters and between specific measures of immune reactivity and disease control or OS. Significance was evaluated using a 2 x 2 contingency analysis with Fisher's exact test [1, 3]. *P* values <0.05 were considered significant. All statistical analyses were performed using Prism version 5.0 (GraphPad).

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