Cancer Immunol Immunother (2014) 63:737–747
DOI 10.1007/s00262-014-1552-9

FOCUSSED RESEARCH REVIEW

Pilot study of a novel combination of two therapeutic vaccines in advanced non-small-cell lung cancer patients

Zaima Mazorra Herrera · Tania Crombet Ramos

Received: 24 September 2013 / Accepted: 11 April 2014 / Published online: 29 April 2014

Abstract Cancer vaccines contain tumor antigens in a pro-inflammatory context with the purpose to generate potent antitumor immune responses. However, tumor cells develop different immunosuppressive mechanisms that limit the effectiveness of an anticancer immune response. Therefore, therapeutic vaccine treatment alone is usually not sufficient to generate tumor regression or survival improvement, especially in the advanced disease scenario in which most clinical studies have been conducted. Combining cancer vaccines with different anticancer therapies such as chemotherapy, radiotherapy and other immunotherapeutic agents has had different levels of success. However, the combination of cancer vaccines with different mechanisms of action has not been explored in clinical trials. To address this issue, the current review summarizes the main clinical and immunological results obtained with two different therapeutic vaccines used in advanced non-small-cell lung cancer patients, inducing an immune response against epidermal growth factor (CIMAvax-EGF) and NGcGM3 ganglioside (racotumomab). We also discuss preliminary findings obtained in a trial of combination of these two vaccines and future challenges with these therapies.

Keywords CIMT 2013 · Cancer vaccines · NSCLC · Combination · NGcGM3 ganglioside · Epidermal growth factor

This paper is a Focussed Research Review based on a presentation given at the Eleventh Annual Meeting of the Association for Cancer Immunotherapy (CIMT), held in Mainz, Germany, 14th-16th May, 2013. It is part of a CII series of Focussed Research Reviews and meeting report.

Z. M. Herrera (✉) · T. C. Ramos
Clinical Immunology Department at Clinical Direction, Center of Molecular Immunology, Street 216 Corner 15, PO box 16040, Havana, Cuba
e-mail: zaima@cim.sld.cu

Abbreviations

AEs	Adverse events
AL	Aluminum hydroxide
APCs	Antigen-presenting cells
BSC	Best supportive care
CEA	Carcinoembryonic antigen
CIM	Center of Molecular Immunology
CRC	Colorectal cancer
Cy	Cyclophosphamide
DCs	Dendritic cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
GAR	Good antibody responders
GM3	NANAα2-3Galβ1-4Glcβ1-Cer
MAb	Monoclonal antibody
NGcGM3	NGNAα2-3Galβ1-4Glcβ1-Cer
NSCLC	Non-small-cell lung cancer
OS	Overall survival
PAR	Poor antibody responders
PS	Performance status
RFS	Recurrence-free survival
STKIs	Small tyrosine kinase inhibitors
TGFα	Transforming growth factor α
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

Introduction

Lung cancer is a main cause of cancer-related mortality in Cuba and worldwide. Most patients present with unresectable disease and conventional therapies provide only a small advantage in survival benefit compared with best supportive care (BSC) [1].

Between 75 and 85 % of non-small-cell lung cancers (NSCLC) overexpress the epidermal growth factor receptor (EGFR) and some of its ligands, i.e., epidermal growth factor (EGF) and transforming growth factor α (TGF-α). Overexpression of EGFR is often involved in the process of malignant transformation by promoting cell proliferation, cell survival, invasion and angiogenesis [2]. Another membrane component, NGNAα2-3Galβ1-4Glcβ1-Cer (NGcGM3) ganglioside is highly expressed in various tumors including NSCLC and not in normal tissues [3, 4]. This glycosphingolipid is considered an important molecule for tumor progression since it can be shed into the tumor microenvironment in which it impairs the anti-tumor immune response [4, 5].

The Center of Molecular Immunology (CIM) placed in Havana, Cuba, has developed two therapeutic vaccines against the two aforementioned tumor targets: NGcGM3 ganglioside and EGF. The first vaccine named racotumomab is an anti-idiotypic antibody able to generate an effective humoral immune response against NGcGM3 ganglioside. These antibodies are able to kill NGcGM3-expressing tumor cells by a complement-independent mechanism. The second one is CIMAvax-EGF vaccine, which generates specific anti-EGF antibodies able to immunodeprive the tumor from this growth factor. Even though the mechanisms of action of these vaccines are not completely elucidated, the results obtained so far point toward an important role for specific antibodies contrasting with "classical cancer vaccines" focused on induction of a T-cell response.

Notably, both vaccines have had promising results as monotherapies in patients with advanced NSCLC. Based on the complementary mode of action of racotumomab and CIMAvax-EGF, a synergistic effect could be expected if they are concomitantly administered. This review summarizes the results of an exploratory study combining these two vaccines in advanced NSCLC patients. This unprecedented combination showed a potentiation of the immune response and encouraging clinical results.

Racotumomab: an anti-idiotype vaccine related to Nglycolyl-containing ganglioside

Based on Jerne´s idiotypic network theory [6], anti-idiotypic antibodies bearing an "internal image" of the antigens are able to mimic and generate an effective antigen-specific response, even against non-immunogenic tumor-associated antigens like gangliosides [7].

Clinical studies have demonstrated the capacity of anti-idiotype antibodies to mimic different antigens in cancer patients. For example, the anti-idiotype monoclonal antibody (MAb) 3H1 mimics a specific epitope on the carcinoembryonic antigen (CEA), widely expressed on gastrointestinal tumors. In the last Phase III clinical trial performed with 3H1, advanced colorectal cancer patients received 5-fluorouracil and leucovorin combined with 3H1 or placebo. Even though, the addition of 3H1 did not improve the clinical response, a strong anti-CEA response was associated with longer survival times [8].

Abagovomab, an anti-idiotype MAb directed against the tumor-associated antigen CA-125, has been used to treat ovarian cancer. In early clinical trials performed in advanced patients, the vaccination was able to induce significant immune response against CA-125. In addition, patients who developed anti–anti-idiotype (Ab3) response had significantly longer overall survival (OS) [9]. In spite of these encouraging results, a recent Phase II/III study (MIMOSA) to evaluate abagovomab as a maintenance therapy in ovarian cancer patients with no residual disease after frontline therapy was a clinical failure since vaccination did not improve recurrence-free survival (RFS) or OS [10].

Anti-idiotype antibodies have also been developed that mimic gangliosides. Bec2 MAb is directed against GD3 ganglioside that is overexpressed in tumors from neuroectodermal origin. A Phase III trial was conducted in limited disease, small-cell lung cancer (SCLC) patients after a major response to chemotherapy and chest radiation. Only one-third of the patients elicited an anti-GD3 response. Although this trial failed to show significant survival advantage for vaccinated patients, a trend toward prolonged survival was observed in those patients who developed a humoral response [11].

Racotumomab, our anti-idiotype vaccine, targets the NGcGM3 tumor-associated ganglioside. This molecule is an attractive target for immunotherapy, since it is not present in the cytoplasmic membrane of normal humans cells [12]. In contrast, it has been detected in several human tumors [3, 4, 13, 14]. In addition, NGcGM3 is suggested to play a suppressive role against immunological antitumor activity [5]. The rationale of the vaccine was to use racotumomab as a surrogate of NGcGM3 ganglioside intending to induce a specific immune response not only against the Ab2 idiotype (racotumomab) but also against the nominal antigen (NGcGM3), via the idiotypic network theory [6].

In preclinical studies, vaccination with racotumomab showed a significant antitumor effect. In the highly metastatic 3LL-D122 Lewis lung carcinoma model, administration of racotumomab MAb promoted significant reduction in spontaneous lung metastasis. The antitumor effect was associated with an increase in the number of tumor-infiltrating T cells, a reduction in tumor angiogenesis and an increase in apoptotic tumor cells in lung nodules [15].

Based on these positive preclinical data and toxicology studies, several Phase I clinical trials have been performed against several different tumor types: advanced melanoma

[16], breast cancer [17–19] and lung cancer [8, 20]. In most of these clinical trials, patients received intradermal vaccinations with 10 doses of 1 or 2 mg of racotumomab-alum: the first four or five doses biweekly (induction phase) and the remaining six administrations every 28 days (maintenance phase). Re-immunizations were maintained if the patients had a favorable performance status (PS $\leq$ 2). In all of these trials, racotumomab was well tolerated. The main adverse events (AEs) were grade I/II according to NCI-CTC criteria and consisted mainly of local reaction at the injection site with erythema and induration lasting a few days. Notably, in most patients, a very strong and specific anti-idiotype antibody (Ab3) response against NGc-containing gangliosides was generated. This antigen-specific response was long-lasting, and re-immunization in some patients induced an increase in antibody titers. An interesting finding was observed in advanced breast cancer patients using a long-lasting racotumomab vaccination schedule. In this case, 5 out 13 evaluable patients developed an NGcGM3-specific T cell response measured by ELISPOT assay [18].

Clinical and immunological data from racotumomab in advanced NSCLC patients

Two clinical trials in advanced NSCLC patients have been performed using racotumomab-alum vaccine. The first was a compassionate-use-based study performed in 71 stage IIIb/IV NSCLC patients. After receiving standard chemo–radio therapy, patients were injected with five biweekly doses of vaccine (induction phase) and over a maintenance period received one vaccination monthly for 10 months. After this period, patients who maintained good performance status were re-immunized every 28 days. The vaccination stopped in the cases of worsening PS (PS > 2), patient refusal or unacceptable toxicity. Although patients received the vaccine over a long period of time, no unexpected or serious adverse effects were reported. The OS of vaccinated patients was significantly higher in comparison with a historical control group of advanced NSCLC patients receiving onco-specific treatment in the same hospital (9.93 vs. 4.53 months). In a subgroup of immunized patients with PS1 and at least disease stabilization after standard therapy, the OS was significantly superior compared with a subset of control patients with similar characteristics (11.50 vs. 5.70 months). Thus, these results suggested that racotumomab-alum vaccine was able to benefit this group of patients [21].

Antibody response in a subset of 20 racotumomab-immunized patients was assessed. Most patients showed a strong specific antibody response of both IgM and IgG isotypes against NGcGM3 ganglioside. Hyperimmune sera not only recognized ganglioside-positive tumor cells, but also directly killed the cells by a complement- independent mechanism. This type of cell death was characterized by cellular swelling and the formation of large lesions on cell membranes resembling a necrotic oncosis process [9]. Regarding to immunological surrogates of clinical benefit, an association between the induction of NGcGM3 antibody titers and longer survival was found (median survival time for responders was 14.26 and 6.35 months for non-responders, $p < 0.01$, log-rank test) [22].

Looking for the proof of concept, a Phase II/III randomized, multicentre, double-blind clinical trial of racotumomab was conducted against advanced NSCLC (manuscript submitted). Racotumomab vaccine was used as switch maintenance therapy after platinum-based chemotherapy in patients with at least stable disease and PS $\leq$ 1. Intent-to-treat analysis showed that vaccinated patients had significantly increased OS ($n = 87$, median OS: 8.23 months), compared with a placebo group ($n = 89$, median OS: 6.80 months, $p = 0{,}004$, log-rank test). Notably, the cytotoxic properties of the hyperimmune sera were associated with longer survival. Based on these encouraging results, racotumomab received conditional approval by the Cuban regulatory authority for the treatment of advanced NSCLC patients.

CIMAvax-EGF: an epidermal growth factor-based vaccine

EGF is a potent growth factor that binds with high affinity to the EGFR. This interaction activates a signal transduction cascade which results in cellular proliferation, differentiation and survival. High expression of EGFR is often observed in epithelial tumors. Simultaneous expression of EGFR and its ligands in lung tumors and adjacent tissues is associated with a higher recurrence rate and lower OS in patients [23–25].

Several anti-EGFR therapies have been developed. These targeted therapies include anti-EGFR MAbs which inhibit receptor autophosphorylation and dimerization [26] and small tyrosine kinase inhibitors (STKIs) which block the receptor pathway by interfering with ATP binding to the EGFR [27].

In the case of MAbs, cetuximab is the most widely used anti-EGFR antibody for the treatment of malignant diseases. In spite of the high number of clinical trials that have evaluated the efficacy of cetuximab, it has been approved for locally advanced and metastatic/recurrent head and neck carcinoma and for K-ras wild-type advanced colorectal cancer (CRC). In the case of advanced lung cancer, cetuximab has been evaluated without success. This was the case for the FLEX study, which compared platinum-based first-line chemotherapy plus cetuximab with chemotherapy

alone in 1,125 patients with EGFR-expressing advanced NSCLC. The study showed that adding cetuximab to chemotherapy improved OS by only 1.2 months [28].

Among STKIs, erlotinib has been indicated for maintenance after platinum-based first-line chemotherapy in patients with at least stable disease [29], for treatment of advanced NSCLC, as first-line treatment, in patients with EGFR mutations [30] and after one unsuccessful chemotherapy regimen [31], and as first-line treatment, combined with gemcitabine for locally advanced pancreatic cancer [32]. In spite of the marketing approvals, the clinical benefit has been limited. This was demonstrated in advanced NSCLC patients after one or two chemotherapy regimens. The OS of the erlotinib-treated group was 6.7 versus 4.7 months for the placebo arm [31].

Although the success of MAbs and STKIs against advanced NSCLC is quite limited, the use of predictive biomarkers may significantly improve the efficacy of these targeted therapies. The FLEX study demonstrated that using cetuximab in patients with high EGFR expression significantly improved OS compared with the control group (HR = 0.73, $p = 0.011$) [28]. In the case of erlotinib, activating mutations in the tyrosine kinase domain of EGFR increase sensitivity to the inhibitor in advanced NSCLC patients in terms of response rate and progression-free survival [29, 33].

The rationale of CIMAvax-EGF is to induce anti-EGF antibodies able to inhibit the EGF binding to EGFR, preventing tumor cell proliferation. CIMAvax-EGF is a first-in-class therapeutic vaccine for lung cancer. It consists of human recombinant EGF, coupled to a carrier protein, P64 from *Neisseria meningitides* and Montanide ISA 51 as adjuvant.

Twenty years ago, preclinical studies were undertaken to select the most effective vaccine formulation for inducing EGF immunogenicity [34]. With the aim to induce a potent and prolonged anti-EGF Ab response, mice were immunized with CIMAvax-EGF vaccine (EGF/P64 k/Montanide ISA 51) and immune pharmacological variables were assessed during both the induction phase (priming) and the re-immunization (boosting) period. The optimal schedule resulted in the administration of the vaccine in high, but fractioned doses in multiple anatomical sites that increased the immune response [35]. Antitumor experiments demonstrated the effectiveness of CIMAvax-EGF in Ehrlich ascites tumor, in which mice with antibody titers against self-EGF had better survival than the control group [34].

Clinical and immunological data from CIMAvax-EGF in advanced NSCLC

EGFR is overexpressed in lung tissues during development and progression of tumors. The magnitude of EGFR expression has been appointed as a predictive biomarker of clinical response to biotherapy in NSCLC patients [36, 37]. Several exploratory trials with CIMAvax-EGF in advanced NSCLC patients have been performed to optimize the vaccine formulation in terms of treatment schedule, carrier proteins and adjuvants [38].

The first controlled trial to evaluate efficacy of CIMAvax-EGF recruited 80 stage IIIB and IV NSCLC patients previously treated with established first-line chemotherapy (ChTVV: chemotherapy-vaccine schedule). Patients with at least stable disease were randomized 1:1 to receive 4-week induction doses of vaccine and then monthly re-immunizations. Vaccinated patients received an immunomodulatory cyclophosphamide (Cy) dose (200 mg/m^2 of body surface) three days before starting CIMAvax-EGF treatment. The safety profile reported in earlier Phase I/II vaccine trials was confirmed in this study. The vaccine was very well tolerated. The most frequent AEs were fever, headache, chills and pain at the injection site. Intent-to-treat analysis showed a non-significant trend toward survival advantage in vaccinated patients compared with randomized untreated controls. However, OS was significantly longer in the set of patients that were 60 years or younger ($n = 22$, mean = 18.53 months; median = 11.47 months) compared with age-matched controls ($n = 28$, mean = 7.55 months; median = 5.33 months, $p = 0.012$, log-rank test) [39].

In the above clinical trial, antibody titers against human EGF were measured in a group of 42 patients. Based on the magnitude of antibody titers, patients were classified as good antibody responders (GAR) if anti-EGF antibodies reached titers equal to or higher than 1:4,000 and at least four times higher than the pre-immunization levels. Conversely, patients were considered poor antibody responders (PAR) if titers were below 1:4,000. This antibody response criterion was arbitrarily established during the first clinical trial and has been used since then for optimizing vaccine composition and schedule. Most patients developed high and long-lasting antibody titers. Seventy-three percent of vaccinated patients developed a good antibody response. An inverse correlation was found between higher anti-EGF antibody titers and lower serum EGF concentrations. In addition, an association between higher anti-EGF Ab titers and longer survival times was also confirmed in this trial. The functional capacity of anti-EGF antibodies was also studied. In vitro experiments with an EGFR-positive cell line confirmed the ability of patient sera to inhibit EGF/EGFR binding. Moreover, the property of hyperimmune sera to inhibit EGFR phosphorylation was established for the first time. A significant positive association was found between high inhibition of EGF/EGFR binding and longer survival times of vaccinated patients [40].

To confirm vaccine efficacy, a Phase III randomized, placebo-control clinical trial was recently concluded. The

same ChTVV therapeutic schedule from the Phase II clinical trial was used, but the vaccine was distributed in four injection sites. The study recruited 405 patients with a 1:2 randomization (1 control for each 2 vaccinated patients). Preliminary results of 351 patients showed a statistically significant difference in OS between vaccinated and control patients (11.2 vs. 7.7 months, $p = 0.016$ log-rank test). Most vaccinated patients developed a high anti-EGF antibody response. In a set of 45 evaluated patients, there was a significant reduction in circulating EGF at month 3 after vaccination. As an immunological surrogate, high antibody titers at month 4 after treatment beginning correlated with longer survival (manuscript in preparation). Preliminary, retrospective analysis of the Phase III trial pointed toward the prognostic and predictive value of the EGF concentration in patients. Advanced NSCLC patients with high serum EGF concentration after platinum chemotherapy had a worse prognosis compared with non-vaccinated patients with low EGF serum levels. In contrast, patients with high serum levels of EGF had a better survival if vaccinated with CIMAvax-EGF (manuscript in preparation). Although the clinical and immunological final analysis is still pending, CIMAvax-EGF has marketing approval in Cuba by the Cuban regulatory agency (CECMED) for the treatment of advanced NSCLC patients, which have at least stable disease after first-line platinum-based chemotherapy. CECMED agency granted CIMAvax-EGF approval after evaluating the interim analysis results. However, the trial continues enrolling patients at the moment of submission and 54 advanced NSCLC patients were recruited up to CECMED decision. The final analysis including all recruited patients is currently ongoing.

Additionally, the largest clinical trial using CIMAvax-EGF was recently performed in primary care units. This Phase IV clinical trial was launched to evaluate the safety and effectiveness of vaccinating advanced NSCLC that exhausted their treatment options. In total, 1,084 patients referred by their oncologists were enrolled in the trial alongside the whole country. CIMAvax-EGF was very safe, but, moreover, the feasibility and convenience of vaccination at primary care units were demonstrated. Effectiveness evaluation is ongoing (Table 1).

In total, more than 3,000 advanced cancer patients have been treated with this vaccine thereby demonstrating it to be safe, immunogenic and able to increase survival of the advanced NSCLC patients with a good quality of life.

Combining two vaccines against different targets

The purpose of therapeutic vaccines in cancer is to generate a strong and effective immune response against tumor cells which will be able to reduce tumor burden or control tumor progression. This immune system manipulation will ultimately produce a long-term control of disease, prolonging patients' survival [41]. The combined use of vaccines with cytotoxic treatments, immune checkpoints inhibitors, small-molecule-targeted therapies and cytokines has had different levels of success. The combination of FDA-approved anti-CTLA-4 MAb Ipilimumab with cancer vaccines seems to significantly increase the antitumor efficacy of several therapeutic vaccines in preclinical models [42–44]. However, the combination of vaccines targeting different molecules is unexplored clinically and it seems to be an appealing strategy not only for improving survival but also quality of life of advanced cancer patients, taking into account the low toxicity profile of vaccines.

Based on our clinical results with individual racotumomab and CIMAvax-EGF vaccines in advanced NSCLC patients, an exploratory study of a combination vaccine in treatment-refractory patients after first-line chemotherapy was performed. Twenty NSCLC patients, who completed first-line chemotherapy with no evidences of disease stabilization, received intradermal injections of racotumomab, 5 doses biweekly. A week after initiating this regimen, a second program of immunization was begun with intramuscular injections of CIMAvax-EGF, 4 doses every 14 days. Three days before CIMAvax-EGF vaccination, a low dose of cyclophosphamide (200 mg/m^2) was administered intravenously. After this induction phase, patients received maintenance treatment with one dose of each vaccines monthly until patients' clinical condition worsened (PS = 3) or they developed unmanageable toxicity. Both vaccines were very well tolerated. Only mild adverse events, mainly characterized by injections site reactions, were reported.

Anti-EGF antibody titers and inhibition of EGFR phosphorylation by sera were measured in a set of 12 patients. Notably, significantly higher antibody titers against EGF were obtained in comparison with previous CIMAvax-EGF clinical trials ($p < 0.000$, generalized linear model). All patients were classified as GAR, and 7 of 12 developed antibody titers higher than 1:64,000. Complete reduction in circulating EGF was obtained 2 months after vaccination. In addition, a non-significantly higher inhibition of EGFR phosphorylation was obtained at month 5 compared with inhibition capacity of sera from Phase III trial (80 vs. 62 %).

Immunogenicity of racotumomab was measured as well. Anti-idiotypic response was significantly higher compared with the maximum titers obtained in previous racotumomab lung cancer trials ($p < 0.000$, generalized linear model). The magnitude of anti-NGcGM3 antibody response and the capacity to kill ganglioside-positive tumor cell lines were equivalent to the data reported with this vaccine in previous clinical trials [22].

Table 1 Brief description of CIMAvax-EGF clinical trials in advanced NSCLC patients

Clinical trial	Vaccine formulation/schedule/number of patients	Main results	References
Phase I/II controlled randomized	EGF/P64 k/aluminum hydroxide (AL) versus EGF/P64 k/Montanide ISA 51 ChVV Schedule 20 patients, 10 per arm	Montanide ISA 51 confirmed as adjuvant. PAR and GAR classification of vaccinated patients.	Ann Oncol. 2003 Mar; 14(3):461–6
Phase I/II controlled randomized	Cyclophosphamide immunomodulation pretreatment (200 mg/m^2 SC) EGF/P64 k/AL versus EGF/P64 k/Montanide ISA 51 ChVV Schedule 20 patients,10 per arm	Higher, but not statistically significant, anti-EGF antibody titers with cyclophosphamide immunomodulatory dose	Ann Oncol. 2003 Mar; 14(3):461–6
Phase I/II controlled randomized	EGF/P64 k/AL (1 deltoid) versus EGF/P64 k/AL (2 deltoids) ChVV Schedule 43 patients (21 and 22 per arm)	Increased immunogenicity with higher dose divided in 2 deltoids. Correlation among antibody titers, serum EGF concentration and survival.	Cancer Biol Ther. 2006 Feb; 5(2):130–40
Phase I/II	EGF/P64 k/Montanide ISA 51 administered in 4 sites (2 deltoids, 2 gluteus) Vaccine-chemotherapy-vaccine schedule (VChV) 20 patients	Longer survival in vaccinated patients as compared to non-randomized controls. Increased frequency of GAR patients	J Immunother. 2009 Jan;32(1):92–9
Phase II controlled randomized	EGF/P64 k/Montanide ISA 51 administered in 4 sites ChVV schedule 80 patients, 40 per arm	Significant longer survival in vaccinated patients ≤60 years as compared to randomized controls. Correlations between antibody titers/functionality and survival times	Clin Cancer Res. 2008 Feb 1; 14(3):840–6. JCO 2008 Mar 20;26(9):1452–8
Phase III controlled randomized	EGF/P64 k/Montanide ISA 51 administered in 4 sites ChVV 405 patients, 270 vaccinated, 135 controls	Significant longer survival times in vaccinated patients as compared to control group (cohort of 351 included patients). Final analysis is ongoing Confirmation of correlation between anti-EGF antibodies titers and clinical benefit Circulating EGF as potential predictive biomarker	Manuscript in preparation
Phase IV in primary care units	EGF/P64 k/Montanide ISA 51 administered in 4 sites ChVV 1,084 patients	The vaccine was safe. Clinical data analysis is ongoing	Manuscript in preparation

Promising clinical results in this advanced set of patients were obtained. The survival rate at 1 year of treatment was 40 %, comparable with the data reported for second-line chemotherapy, but with significantly less toxicity. Remarkably, 30 % of patients were alive at 18 months after treatment. Final analysis of data and preparation of the manuscript are currently ongoing.

Hypothesizing the mechanism of action of vaccines combination

The mechanisms by which the combination of these two vaccines potentiates immune and clinical response are not elucidated. We hypothesize that the induction of humoral effective immune response against an immunosuppressive molecule like NGcGM3 and the immunodeprivation of an important ligand of the EGFR at the same time not only inhibit tumor cell proliferation but also leave tumor cells vulnerable for immune system attack.

Hayashi and collaborators published recently the expression of NGcGM3 ganglioside in 86 of 93 biopsies from NSCLC patients. Moreover, patients with high expression of this ganglioside showed lower OS and a significantly lower progression-free survival rate. Normal human tissues contain little or undetectable amounts of NGcGM3. The enzyme, which catalyzes the conversion of N-acetyl to N-glycolyl sialic acid, is inactive in both normal and tumor tissues [45]. This suggests the existence of an exogenous pathway to acquire the NGc-containing gangliosides by tumor cells. It was reported that tumor hypoxia induces the transcription of the sialic acid transporter, sialin, promoting the incorporation of NGc-type sialic acid from dietary sources [20]. This might be the cause of NGc-containing ganglioside expression in NSCLC tissues transforming this molecule into a tumor-specific target.

Most non-small-cell lung cancers overexpress the EGFR and its ligands. Overactivation of this pathway promotes cell proliferation, and blood vessels formation, increases tumor cell invasion and metastasis, and inhibits apoptosis. EGFR signaling and its transduction pathway can be efficiently interrupted by EGF deprivation, with negative repercussion on cell proliferation and, consequently, on tumor development [38].

An interesting association has been previously reported between EGFR-signaling pathway and GM3 expression depending of urokinase plasminogen activator (uPA). The uPA system, consisting of the serine protease uPA, its two endogenous inhibitors and the uPA receptor (uPAR), has been implicated in accelerated cell growth, cancer invasion and metastasis [46]. Increased expression and/or activation of uPA/uPAR have been found in melanoma and tumors of the breast, lung and colon and have been correlated with poor prognosis [47]. uPA is able to stimulate cell proliferation via either ERK-dependent or ERK-independent pathways. GM3 overexpression, in the presence of uPA, increases uPAR signaling-induced proliferation by an ERK-independent mechanism (EGFR-independent) that involves p70S6 kinase activation able to stimulate cell proliferation and survival. In contrast, GM3 depletion allows the association of the complex uPAR/integrinα5β1 with EGFR, promoting EGFR-signaling and ERK-dependent cell proliferation [48]. These studies provide a rationale to combine therapies that target both GM3- and EGFR-signaling pathways to strongly inhibit tumor cell proliferation.

In addition to promoting tumor cell proliferation, NGcGM3 is considered an immunosuppressive molecule. This ganglioside is able to downmodulate CD4 expression on human and murine T helper cells [5] and to inhibit dendritic cells (DCs) differentiation from bone marrow precursors and LPS-induced maturation [49].

Based on the important roles of the EGFR and NGcGM3 for tumor cell survival and immune evasion, a combination therapy against these two targets may be a rational way to attack tumor cells. Additionally, a single immunomodulatory dose of cyclophosphamide could potentiate vaccine combination efficacy. Several groups have demonstrated that the administration of a single low dose of this cytotoxic drug before vaccine immunizations could act as vaccine immunomodulator and enhance tumor-specific T cells through depletion of Tregs or inhibition of Tregs proliferation [50–52]. This effect could reverse tumor-induced immunosuppression and promote the T helper type I response. We hypothesize that immune restoration of the tumor microenviroment both through circulating NGcGM3 neutralization by racotumomab-induced antibodies and Treg inhibition by an immunomodulatory dose of cyclophosphamide could prevent tumor escape and potentiate humoral response induced by CIMAvax-EGF vaccine. In this case, higher anti-EGF antibody titers may strongly deprive the tumor from an important ligand to not only to stop tumor proliferation but also to induce cell death. In addition, anti-NGcGM3 antibodies are able to kill ganglioside-positive tumor cells. Both cytotoxic mechanisms could promote the capture and processing of different tumor antigens by antigen-presenting cells (APCs) and activation of tumor-associated antigen-specific T cells. Consequently, tumor cells could be eliminated by humoral and cellular immune response (Fig. 1).

So far, the vaccines combination effectiveness seems to be based on the specifically induced antibodies. Although we did not measure T-cell activation, we cannot rule out the involvement of the cellular immune system on the vaccines' mechanism of action. Further studies are planned to elucidate the role of effector and regulatory T cells in the

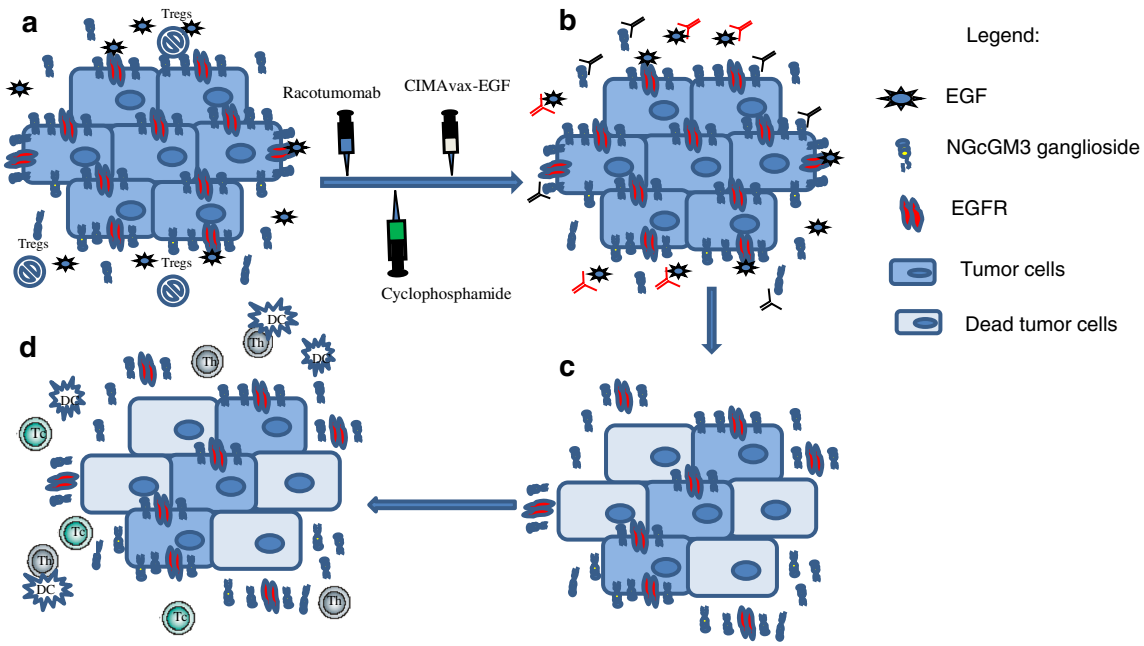

Fig. 1 Combination of racotumomab and CIMAvax-EGF induced effective anti-NGcGM3- and EGF-specific antibodies and might recruit tumor-specific T cells into the tumor microenvironment. **a** Tumor cells overexpress poor prognosis markers such as EGFR, EGF and NGcGM3 ganglioside. Infiltrating Tregs inhibit immune system attack. **b** and **c** Single dose Cy could potentiate vaccines efficacy through Treg inhibition. Racotumomab-induced anti-NGcGM3 antibodies prevent tumor proliferation and immune escape by neutralizing shed NGcGM3, which is immunosuppressive for DCs and helper T cells. Anti-NGcGM3 antibodies also recognize membrane-anchored NGcGM3 ganglioside and kill ganglioside-positive tumor cells. High anti-EGF antibody response induced by CIMAvax-EGF "deprives" tumor cells from a proliferating ligand and might stop proliferation but also induce tumor cell death. **d** The immune restoration together with cytotoxic mechanism can recruit APCs to present tumor antigens from dying cells and activate T cells against different tumor antigens. Hence, combining vaccines against different tumor targets may potentiate the effectiveness of cancer vaccine monotherapy

mechanism of action of these two vaccines, as well as the potential predictive biomarkers of clinical benefit.

Perspectives

Currently, CIMAvax-EGF is being evaluated in a multinational, open label, multi-center, controlled Phase III trial in inoperable late stage (IIIB/IV) NSCLC patients, which is ongoing in Europe and other Asian countries. In this study, patients are receiving vaccination together with first-line chemotherapy. In total, 438 patients (1:1 ratio) will be randomized into the study. Patients in the active treatment cohorts are receiving CIMAvax-EGF plus first-line chemotherapy as per the standard of care, while subjects in the non-vaccinated cohort are receiving only chemotherapy. The primary endpoint is to assess OS of the EGF cancer vaccine compared with the control, while the secondary endpoints include the assessment of progression-free survival, time to progression, response rate and quality of life.

In our country, this vaccine is also being evaluated in other clinical scenarios including earlier stage NSCLC patients (adjuvant setting) and castration-refractory prostate cancer patients.

The next step in the clinical setting will be the prospective validation of a predictive biomarker of greatest clinical benefit (in terms of survival) for vaccinated patients. An EGF concentration cutoff for a larger benefit was established after the Phase III analysis. Vaccinated patients with a pre-vaccination circulating EGF concentration $\geq$900 pg/ml had longer survival times ($n = 68$, median OS: 13.6 months) compared with controls ($n = 26$, median OS: 6.9 months, $p = 0.0001$, log-rank test) with the same EGF levels. A new trial enrolling only stage IIIb/IV patients with serum EGF levels above the pre-specified cutoff concentration will be launched.

Two additional Phase III studies using racotumomab are ongoing. The first is a non-inferiority study performed in our country that compares racotumomab versus docetaxel as switch maintenance or second-line therapy for stage IIIb/IV NSCLC that have received platinum-based therapy. Before randomization, patients are being stratified according the response to first-line chemotherapy. The primary endpoint is the assessment of the comparative overall survival.

The second is a prospective, randomized, multicenter, open label Phase III study of racotumomab plus BSC treatment versus BSC treatment alone in patients with advanced NSCLC. Patients from Cuba, Brazil, Argentina, Indonesia and Philippines among others have been randomized in a 1:1 ratio and stratified with respect to five variables: center, smoking history, histological type, stage of disease and response status to prior first-line therapy. Subjects randomized to receive vaccine will continue vaccination until death or deterioration of PS to grade $\geq$3 or up to unacceptable toxicity. After discontinuation, patients will be followed for survival assessment. Vaccination may be continued during administration of post-study therapies. The primary goal was to compare OS of patients with newly diagnosed or recurrent stage IIIA (unresectable), IIIB or IV NSCLC who are free of progression after first-line therapy, who receive racotumomab or BSC. BSC may include the use of subsequent onco-specific therapy. The estimated sample size is 1,082 patients.

Three Phase II clinical trials are also ongoing in Cuba with racotumomab vaccine for the treatment of advanced colorectal, breast and pediatric neuroectodermal cancers.

Finally, based on the encouraging results of the vaccines combination, a Phase II/III trial that evaluates the efficacy of combining racotumomab and CIMAvax-EGF versus each independent vaccine is in the approval process. The study will include inoperable NSCLC patients classified as unsuitable to receive chemotherapy. In total, 255 patients will be recruited. Primary endpoint will be the evaluation of OS while safety, objective response, progression-free survival and immune response will be assessed as secondary endpoints. The elucidation of the mechanisms of action of vaccines and possible predictive biomarkers of clinical efficacy will be important features in this trial.

Combining each vaccine with other innovative immunomodulators, targeted therapy or classical T-cell response vaccines is a very appealing approach. Limitations are mainly associated with the availability of these drugs in Cuba, due to the commercial restrictions imposed by the US embargo. The Center of Molecular Immunology has a research track devoted to developing novel-proprietary immunomodulatory drugs, which will be combined in the future with CIM vaccines.

Acknowledgments We thank Dr. Peter J. Ferguson; from Department of Oncology, Western University, London, Canada for his valuable English assistance in the edition of this manuscript. We also would like to thank all the patients, medical doctors and researchers involved in these clinical trials.

Conflict of interest The authors declare that they have no conflict of interest.

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