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Précis. After years of unmet expectations, tumor immunotherapy is finally experiencing a renaissance. The second Gene Vaccination in Cancer conference, recently held in Ascoli Piceno (Italy), highlighted how gene vaccines are part of this movement.

Abbreviations list. ALK, Anaplastic Lymphoma Kinase; CMM, canine malignant melanoma; CSPG4, Chondroitin Sulfate Proteoglycan-4; CSC, cancer stem cells , DAA, disease-associated antigens; ECT, electrochemotherapy; EP, electro-permeabilization; GVC, Gene vaccination in Cancer; HPV, human papillomavirus; MDSC, myeloid derived suppressor cells; MUC1, Mucin1; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cells; PDA, ductal pancreatic adenocarcinoma; TAA, tumor associated antigens.
Introduction

The GVC (acronym for Gene Vaccination in Cancer) meeting offered the opportunity to continue and strengthen the existing national and international collaborations between basic scientists, clinical researchers and scientists in industry to overcome obstacles in gene vaccination against cancer. The 2013 GVC meeting covered the following topics: (1) New technologies for gene vaccination; (2) Emerging targets for cancer vaccines; (3) Therapeutic vaccination against HPV-dependent diseases; (4) Pre-clinical challenges; (5) Veterinary applications of gene vaccination in cancer; (6) *In vivo* gene-electro-transfer: from technology development to clinical applications.

To provide the broad background for the topics covered by the meeting, two important lectures, one on the discovery of tumor associated antigens (TAA) and the other on the importance of tumor-induced immunoregulatory networks were given by Olivera J. Finn and Vincenzo Bronte, respectively. **Olivera J. Finn** (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA) emphasized the critical role of the disease-associated antigens (DAA) that are TAA expressed in premalignant lesions or during infections. She pointed to the fact that the efficacy of immunotherapy may be greatly improved by vaccinating patients with very early lesions, boosting the immunological memory developed against DAA during previous infections. It is thus necessary to focus on DAA in order to boost pre-existing responses generated earlier in life. Mucin1 (MUC1) is a typical DAA overexpressed in an abnormal hypoglycosylated form in preneoplastic and cancerous lesions of various histotypes. A recent clinical study in patients with advanced adenoma of the colon showed that anti-MUC1 vaccination was able to elicit anti-MUC1 IgG and long-term memory in about 50% of vaccinated patients. Peripheral blood mononuclear cells (PBMC) of non-responders contained increased levels of myeloid derived suppressor cells (MDSC). The importance of modulating MDSC differentiation and activity in the tumor microenvironment in order to obtain a good vaccination-induced immune response was further discussed by **Vincenzo Bronte** (University Hospital and Department of Pathology, Verona, Italy) who identified miR-142-3p as a master regulator of the expression of the signalling elements of the IL6 receptor and showed that a decrease in this miRNA expression is essential in allowing myeloid cells to acquire the suppressive phenotype. Moreover, he demonstrated that miR-142-3p can be considered a new target for cancer therapy since mice with miR-142-3p constitutive expression in the bone marrow have altered tumor-induced macrophage differentiation and display a marked increase in survival following the adoptive transfer of tumor-specific T cells.

**New technologies for gene vaccination**

In this session, attention was given to the efforts that many companies have done in the design of carriers, adjuvants, viral vectors, self amplifying mRNA vaccines and prime and boost strategies to be used for anticancer immunotherapy. **Ennio De Gregorio** (Novartis, Siena, Italy) reported on the development of new synthetic compounds optimized to stimulate specific immune cells without inducing side effects. A significant increase in vaccine potency and a reduced local and systemic reactogenicity was shown by a synthetic compound specific for Toll-like Receptor 7. New mRNA-based vaccines have also been developed. Of particular note are a non-viral delivery system for self-amplifying RNA from Novartis that has been successfully tested in pre-clinical models, and the RNAActive® vaccines reported by **Karl-Josef Kallen** (CureVac, Tübingen, Germany). RNAActive® vaccines are two component vaccines engineered in a way that maximises the antigen encoding...
properties of mRNA and bestows self-adjuvanticity by activation of TLR7, causing a balanced activation of the adaptive and innate immune system. Initial phase I/IIa studies in patients with prostate and non-small cell lung cancer (NSCLC), showed that RNAActive® vaccines are safe and effective in inducing a long lasting, humoral and cellular immune response. Currently, a multicenter controlled phase IIb study is performed in castrate-resistant prostate cancer in 8 European countries to investigate clinical efficacy of RNAActive® vaccines and a phase Ib study tests the combination of radiotherapy and RNAActive® vaccines in metastatic NSCLC.

Emerging targets for cancer vaccines
Besides the development of more effective gene vaccination technologies, one of the main issues in anti-cancer vaccines is still the identification of proper tumor antigen targets. A number of different strategies have been used to identify new targets, which include protein kinases, metabolic enzymes, chaperone proteins, transporters and angiogenesis related receptors. Anaplastic Lymphoma Kinase (ALK) is known to be a good therapeutic target for the treatment of anaplastic large cell lymphoma. Claudia Voena (University of Torino, Torino, Italy) and coworkers had previously demonstrated that anti-ALK DNA vaccination through electro-permeabilization (EP) is a good strategy to be used in the treatment of anaplastic large cell lymphoma. They reported similar results in the treatment of NSCLC in two independent transgenic mouse models of ALK-driven lung cancers that express two different fusions of ALK (EML4-ALK and TFG-ALK). It is worth noting that anti-ALK DNA vaccination was also effective against tumors harboring ALK mutations that abrogate the efficacy of the ALK inhibitor crisotinib. Voena and coworkers also tested the combined administration of anti-ALK DNA EP and crizotinib, showing that ALK vaccine could prevent the growth of crizotinib resistant tumors in combined therapy.

The glycolytic enzyme α-enolase is a promising target for the immunotherapy of patients affected by pancreatic cancer. It is involved in both growth and metastasis of ductal pancreatic adenocarcinoma (PDA). Francesco Novelli (University of Torino, Torino, Italy) and coworkers used serological proteomic analysis to discover that patients with PDAC produce antibodies against two isoforms of α-enolase, which are also recognized by T lymphocytes. A good correlation between these immune responses and prognosis has also been demonstrated. For this reason, a DNA vaccine targeting α-enolase has been developed and its administration by EP tested in two genetically engineered mouse strains (Kras^{G12D}/Cre mice and Kras^{G12D}/Trp53^{R172H}/Cre mice) that develop autochthonous lethal PDA with different kinetics. A specific anti α-enolase antibody and cellular immune response were both elicited in vaccinated mice and correlated with an increase in their survival times.

Serenella Pupa (Istituto Nazionale dei Tumori, Milan, Italy) discussed an approach for identifying new targets for B-cell non-Hodgkin lymphoma (NHL) immunotherapy. Using sera from autologous tumor-loaded dendritic cell from vaccinated NHL responder patients, Pupa and coworkers identified the chaperon protein HSP105 as a candidate target for NHL immunotherapy. While in vitro administration of a commercial polyclonal anti-HSP105 antibody failed to provide any direct antitumor effect, it significantly reduced tumor burden through antibody-dependent cellular cytotoxicity in vivo. Therefore, HSP105 can be added to the list of non-oncogenes that can be exploited as a new target for anti-B-cell lymphoma therapy.
A growing body of evidence points to cancer tissues being hierarchical systems, where cellular heterogeneity is the result of multi-lineage differentiation processes, and tumor progression, metastasis, recurrence and resistance to therapy are sustained by a subset of cells with stem cell properties, named cancer stem cells (CSC). The identification of molecules involved in CSC self-renewal is a necessary step towards the development of effective therapies. To this aim, Ronald Rooke (Transgene S.A., Illkirch Graffenstaden, France) and Stefania Lanzardo (University of Torino, Torino, Italy) presented two different approaches to identifying specific CSC targets in glioblastoma and breast cancer, respectively. Rooke used proteomic analysis to identify markers overexpressed in neurospheres generated from human samples, while using transcription profile analysis of RNA from mammospheres generated from murine breast cancer cells, Lanzardo identified cystine/glutamate exchange transporter xCT as overexpressed in breast CSC. EP of a plasmid that codes for xCT prevents lung metastasis formation and decreases cancer growth in a preclinical model of HER2+ breast cancer.

Andrea Facciabene (University of Pennsylvania, Philadelphia, PA, USA) reported on the efficacy of targeting tumor vasculature as a promising strategy for the treatment of cancer. He showed that the EP of a DNA vaccine targeting TEM1 (endosialin, CD284), one of the most abundantly expressed tumor endothelial or stromal antigen in humans, is safe and do not affect physiological angiogenesis. Moreover, it results in the induction of TEM1-specific T cell-mediated immunity, in the disruption of the tumor vasculature and in the induction of tumor necrosis/apoptosis and TAA cross-presentation. Combination of TEM1 vaccination with the administration of regulatory T cell inhibitors resulted in an increased vaccine efficiency.

**Therapeutic vaccination against HPV-dependent diseases**

Two human papillomavirus (HPV) vaccines, which are effective in the prevention but not in the treatment of established tumors, are currently on the market. Furthermore, they are expensive and not suitable for the use in developing countries. Hence, the development of new vaccines and more informative preclinical models are needed. Aldo Venuti (Regina Elena National Cancer Institute, Rome, Italy) reported on the new orthotopic mouse models of HPV-associated cancer that can be used for testing therapeutic vaccines. By using a combination of a DNA vaccine and a fusion protein vaccine produced in plants, he obtained a complete protection against HPV+ head and neck orthotropic tumors in vaccinated mice.

Mark Bagarazzi (Inovio Pharmaceuticals, Blue Bell, PA, USA) reported on the efficacy of, VGX-3100, a new therapeutic DNA vaccine against HPV-related diseases. This EP administered DNA vaccine led to the induction of antigen specific antibodies and T cells in the majority of vaccinated patients. In order to significantly increase T cell response, Bagarazzi’s group proposes to combine DNA vaccination against HPV with IL-12 administration or with a vaccination against telomerase (hTERT), an antigen that is known to be immunogenic, as demonstrated by the presence of existing naturally occurring T cell responses in cancer patients.

**Pre-clinical challenges**

The improvement of preclinical models of cancer is one of the issues that needs to be dealt with in immunotherapy since many of those currently used do not exactly recapitulate human disease. As far as breast cancer, emerging evidence suggests that the real transforming form of HER2 is its splice variant, the Δ16HER2. Cristina Marchini (University of Camerino, Camerino, Italy)
described a new preclinical breast cancer model, the Δ16HER2 transgenic mice that develop mammary carcinomas with 100% penetrance. Vaccination of these mice with a plasmid that encodes a chimeric human/rat HER2 protein resulted in an effective antitumor response that was higher than that induced by vaccination with a plasmid coding for a non chimeric human HER2 protein.

Further evidence of the great potential that lies in DNA EP in the field of tumor immunotherapy came from data presented by Maarten Ligtenberg (Stockholm, Sweden), who showed that Cripto-1, a protein involved in cell fate regulation during embryogenesis and in cell proliferation and epithelial-mesenchymal transition in several cancers, would present a prime target for DNA vaccines. Immunization with mouse and human Cripto-1 generates in vivo protective immune responses against Cripto-1⁺ murine tumors. In order to improve the efficacy of anti-Cripto-1 vaccination, Ligtenberg pointed out the possibility of modulating the signalling pathways involved in T cell activation through the use of shRNA targeting the NF-kB signalling pathway.

Preclinical evaluation of the safety of therapeutic vaccines to cancer is a relevant issue. Isabella Andreini (RTC S.p.A., Pomezia, Italy) emphasized the need of critically interpreting preclinical data to obtain correct and useful information for clinical studies and find a compromise between regulatory requirements and scientific logic. Indeed, preclinical models have several limitations: few animal species are available and animals are young, healthy and inbred, while patients are older, sick and genetically heterogeneous. Moreover, the required number of animals is impossible to achieve when using primates and there are inter-species differences that may affect results.

Veterinary applications of gene vaccination in cancer

The use of naturally occurring cancer in pet animals as models of human cancer has gained a relevant role in translational medicine over the last decade, and several companion species, such as cat, dogs and horses have successfully contributed to this effort. This is due to the striking similarity in the histological appearance, tumor genetics, environmental influence, biological behaviour and response to therapy between spontaneous tumors diagnosed in pet animal and human tumors. Wei-Zen Wei (Karmanos Cancer Institute, Detroit, MI, USA) reported on the numerous studies in which DNA EP against HER2 was used successfully in murine models but did not lead to effective results in human clinical practice. She displayed a comparative study on feline mammary tumors showing that HER2 is expressed in 40-85% of them, but it is not amplified. It is very similar in its amino acid sequence to the human ortholog and is recognized by antibodies against human HER2. A vigorous anti-HER2 immune response after DNA EP with a plasmid coding for a mutated form of feline HER2 sequence can be obtained.

Luigi Aurisicchio (Takis s.r.l., Rome, Italy) reported a double-arm cancer vaccine study based on targeting canine telomerase in dogs affected by TERT⁺ B-cell lymphosarcoma. The combination of standard-of-care chemotherapy and heterologous prime/boost, i.e. adenovirus/DNA EP-based vaccination, gave a good clinical efficacy in terms of survival of B-cell lymphosarcoma canine patients. These data confirm support the evaluation of this approach for other cancer types in veterinary field as well as the translation of this approach to human clinical trials. Joe Impellizzeri (Veterinary Oncology Services, Vassar College, New York, NY, USA) reported additional data about an increased survival in dogs affected by B-cell malignant lymphosarcoma when standard of care chemotherapy is combined with a genetic vaccine against canine TERT developed by Aurisicchio and coworkers. He also reported HER2 immunotherapy trials ongoing with feline
mammary cancers and canine osteosarcoma using the same immunotherapy platform (adenovirus followed with DNA plasmid via EP).

**Soldano Ferrone** (Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA) described the use of Chondroitin Sulfate Proteoglycan-4 (CSPG4) as a target of antibody-based immunotherapy for melanoma patients. Several patients with advanced melanoma have been immunized with a CSPG4 mimic and those who developed CSPG4-specific humoral immunity had a statistically significant survival prolongation. Human CSPG4 displays more than 80% homology with its canine counterpart. This has provided the rationale to use human CSPG4 as immunogen to implement active specific immunotherapy in dogs with melanoma, as discussed by **Federica Riccardo** (University of Torino, Torino, Italy). She showed that CSPG4 is expressed by most canine malignant melanoma (MM) and can thus be considered a new diagnostic marker and a target for cancer immunotherapy. EP of dogs with surgically resected stage II-III CSPG4+ oral MM with a plasmid that coded for human CSPG4 resulted in the induction of anti human and canine CSPG4 antibodies and in a significantly longer overall and disease-free survival time. Therefore, xenogeneic vaccination against CSPG4 is able to overcome host unresponsiveness to the self-antigen and appears to be effective for the treatment of canine MM.

**Philip Bergman** (Katonah-Bedford Veterinary Center, Bedford Hills, NY, USA) emphasized that the evaluation of genetic cancer vaccines and delivery technologies in pet dogs is a predictive model for human clinical trials and a tool to provide novel therapeutic opportunities in veterinary oncology. He described the promising results obtained by vaccinating dogs with surgically resected stage II-III CMM with the FDA-approved ONCEPT (Merial) vaccine, a plasmid that codes for human tyrosinase that is administered intramuscularly with a bio-injector.

**In vivo gene-electro-transfer: from technology development to clinical applications**

Several pre-clinical and clinical trials have demonstrated that EP for both gene and chemo transfer is safe and well tolerated. **Mattia Ronchetti** (IGEA S.p.A., Carpi, Italy) reported the efficacy of electrochemotherapy in patients with cutaneous cancer manifestation and the ongoing clinical validation of a new medical device by IGEA, the Cliniporator™ VITAE, for the treatment of deep seated tumor nodules such as bone, liver and soft tissue metastasis. He highlighted the importance of using a single EP technology from pre-clinical experiments to clinical trials to reduce the regulatory requirements needed to be satisfied for translation to clinical practice.

**Julie Gehl** (Copenhagen University Hospital, Herlev, Denmark) discussed that EP conditions must be differently optimized for each molecule and for each tissue. A list of the protocols of ECT, muscle and skin gene EP for the treatment of several cancers developed by the Center for Experimental Drug and Gene Electrotransfer, in the Copenhagen University Hospital, is available at [http://www.herlevhospital.dk](http://www.herlevhospital.dk). Interestingly, a clinical brain electrode for ECT has been recently developed and approved for trial, and a clinical study for patients suffering from brain metastases has commenced.

The second part of this session was focused on the discussion of new strategies that may enhance DNA vaccination efficacy. **Emanuela Signori** (Institute of Translational Pharmacology, Rome, Italy) reported that muscular fiber transfection and consequent antigen expression is significantly enhanced by the combination of hyaluronidase administration and DNA EP. These effects rest on the ability of the coupled treatment to induce in the vaccinated muscle an early release of inflammatory cytokines favouring regeneration of muscle fibers and allowing lower levels of
electric fields to be applied. Another important strategy for enhancing vaccination efficacy is the use of DNA fusion vaccines. Christian Ottensmeier (University of Southampton, Southampton, United Kingdom) reported that DNA vaccines containing tumor antigen sequences fused to microbial genes are safe and close to becoming licensed for clinical use. Anti CEA EP vaccinated cancer patients showed response induction with preliminary indications of tumor growth suppression and evidence for clinically manageable concomitant autoimmunity. A direct correlation between the density of the induced effector T tumor infiltrating lymphocytes and a better outcome was found in these patients. This suggests that T cells induced by vaccination have an effector phenotype. However, often these cells are not able to kill the tumor because they express PD1 on their surface. For this reason, the use of anti cancer vaccines in combination with immuno-stimulant monoclonal antibodies, are needed. A new promising way to increase antigen immunogenicity is to combine peptides of interest with viral particles. These chimeric products can be produced in plants, which further increases their immunogenicity. Preclinical testing of these new vaccine products resulted in the induction of an effective T cell response and will soon be brought into clinical testing.

Special report: the three best abstracts awardees
A prize was given to three young scientists who contributed to the GVC meeting with a relevant poster presentation. The first prize was given to Moitza Principe (University of Torino, Torino, Italy), who demonstrated the involvement of the glycolytic enzyme α-enolase in PDA metastasis. The second prize was awarded to Giuseppina Barutello (University of Torino, Torino, Italy), who demonstrated that maternal immunization against a TAA could offer effective protection to the offspring genetically predestined to develop carcinomas and the third prize was given to Caterina Bartolacci (University of Camerino, Camerino, Italy) who showed the ability of anti-HER2 vaccines to inhibit tumor growth in Δ16HER2 transgenic mice.

Conclusions
The closing lecture delivered by Gennaro Ciliberto (Istituto Nazionale Tumori, Fondazione G. Pascale, Naples, Italy) provided an overview of possible future trends in the development of an easily scalable vaccination platform technology. Several important points have to be taken into account when starting a new cancer vaccine clinical trial. Firstly, the identification of reliable preclinical animal models to test vaccine efficacy is crucial and to this extent the use of pet animals as models of human cancer promises to be a successful strategy in translational medicine. Secondly, since it is difficult to eradicate a tumor, especially if metastatic, the technology used to treat cancer patients should be compatible with prolonged treatment. Thirdly, it is important to combine vaccines with other drugs and it is mandatory to find new biomarkers. Ultimately, the best vaccination strategy to use depends on the kind of immune response is needed: to elicit an antibody response, it is better to use a xenogeneic antigen while to activate T lymphocytes, it is better to use a polypeptide vaccine different from the whole antigen. To get both responses a heterologous prime-boost strategy might be needed.

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