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Trial watch
DNA vaccines for cancer therapy

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Abbreviations: ADA, adenosine deaminase; AFP, α fetoprotein; APC, antigen-presenting cell; CD40L, CD40 ligand; CEA, carcinoembryonic antigen; CIN, cervical intraepithelial neoplasia; CRT, calreticulin; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ERBB2, *v-erb-b2* erythroblastic leukemia viral oncogene homolog 2; GM-CSF, granulocyte-macrophage colony-stimulating factor; HNC, head and neck cancer; HPV, human papillomavirus; HSP70, heat shock 70 KDa protein; IFNγ, interferon γ; IGFBP-2, insulin-like growth factor binding protein 2; IHNV, infectious hematopoietic necrosis virus; IL, interleukin; i.m., intra musculum; i.t., intra tumorem; i.v., intra venam; LPS, lipopolysaccharide; MAGE, melanoma-associated antigen; MUC1, mucin 1; MVA, Modified Vaccinia Ankara; NSCLC, non-small cell lung carcinoma; PAP, prostate acid phosphatase; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; p.o., per os; s.c., sub cutem; TAA, tumor-associated antigen; TLR, Toll-like receptor; TRP2, tyrosinase-related protein 2; VEGFR2, vascular endothelial growth factor receptor 2

The foundation of modern vaccinology dates back to the 1790s, when the English physician Edward Jenner uncovered the tremendous medical potential of prophylactic vaccination. Jenner's work ignited a wave of nationwide vaccination campaigns abating the incidence of multiple life-threatening infectious diseases and culminating with the eradication of natural smallpox virus, which was definitively certified by the WHO in 1980. The possibility of using vaccines against cancer was first proposed at the end of the 19th century by Paul Ehrlich and William Coley. However, it was not until the 1990s that such a hypothesis began to be intensively investigated, following the realization that the immune system is not completely unresponsive to tumors and that neoplastic cells express immunogenic tumor-associated antigens (TAAs). Nowadays, anticancer vaccines are rapidly moving from the bench to the bedside, and a few prophylactic and therapeutic preparations have already been approved by FDA for use in humans. In this setting, one interesting approach is constituted by DNA vaccines, i.e., TAA-encoding circularized DNA constructs, often of bacterial origin, that are delivered to patients as such or by means of specific vectors, including (but not limited to) liposomal preparations, nanoparticles, bacteria and viruses. The administration of DNA vaccines is most often performed via the intramuscular or subcutaneous route and is expected to cause (1) the endogenous synthesis of the TAA by myocytes and/or resident antigen-presenting cells; (2) the presentation of TAA-derived peptides on the cell surface, in association with MHC Class I molecules; and (3) the activation of potentially therapeutic tumor-specific immune responses. In this Trial Watch, we will summarize the results of recent clinical trials that have evaluated/are evaluating DNA vaccines as therapeutic interventions against cancer.

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Introduction

Historical perspective. In 1980, the WHO officially certified the eradication of natural smallpox infection,[1] representing one of the major medical triumphs of history. Such an achievement de facto originated from a series of nationwide vaccination campaigns that were launched throughout the 18th and 19th centuries following the pioneering work of the English physician Edward Anthony Jenner (1749–1823).[2,3] In the 1790s, Jenner demonstrated indeed that a sublethal smallpox (or cowpox) infection can confer complete protection against subsequent, potentially lethal, exposures,[2,3] establishing the foundations of modern vaccinology.

In fact, the term "vaccination" (derived from the Latin adjective *vaccinae*, which means "pertaining to cows, from cow") was coined by Jenner himself for the procedure he had conceived to prevent smallpox, and was given a more general meaning by the French microbiologist Louis Pasteur (1822–1895), another central figure in the history of vaccination, only 50 years later.[4,5] The development and widespread administration of efficient prophylactic vaccines not only has resulted in the eradication of natural smallpox,[1] but also has strikingly abated the incidence of a large panel of life-threatening infectious diseases including (but not limited to) rabies, typhoid, cholera, measles, plague, chickenpox, mumps, poliomyelitis and hepatitis B.[4]

One century after Jenner's work, the German physician Paul Ehrlich (1854–1915) and the American surgeon William Bradley Coley (1862–1936) were the first to propose that vaccination might be successfully employed against cancer.[4] In fact, Ehrlich (who is best known for the concept of a "magic bullet" that would specifically kill malignant cells) failed in his attempts to formally demonstrate that weakened cancer cells may generate antitumor immunity.[4] Conversely, Coley developed a mixture of heat-killed bacteria (best known as the Coley toxin) that mediates potent antitumor effects,[6,7] although it does so by operating as an adjuvant, hence stimulating the maturation of dendritic cells (DCs) via Toll-like receptor (TLR)-transduced signals,[8] rather than as a bona fide vaccine. Of note, the Coley toxin has been commercially available and administered to cancer patients until the early 1960s, when its use was discontinued following concerns raised by the thalidomide case.[9]

Unfortunately, the hypotheses of Ehrlich and Coley have been disregarded for about one century and have generated renovated enthusiasm only recently.[10] One of the major theoretical hurdles against the development of anticancer vaccines (and, more in general, against the affirmation of tumor immunology as a self-standing discipline) was represented by the "self/non-self" dichotomy, as originally theorized by the Australian virologist Sir Frank Macfarlane Burnet (1899–1985) in 1949.[11] According to this model, tumors—as they constitute self tissues—are non-immunogenic and hence completely insensitive to immunotherapeutic interventions.[11] It took more than 45 years for an alternative model that globally explains the modus operandi of the immune system to be formulated. Indeed, in 1994, the American scientist Polly Matzinger proposed that the immune system would not simply recognize and react to non-self constituents but would rather be activated by situations of danger, be them of exogenous (non-self) or endogenous (self) origin.[12] Thus, conditions that have long been viewed as immunologically silent, including trauma and cancer, are de facto capable of activating the immune system, a concept that is nowadays widely accepted.[13–15] Approximately in the same years, (1) the gene coding for MZ2-E, a protein expressed by malignant cells of diverse histological origin but not by a series of normal tissues, was cloned;[16] and (2) cytotoxic T lymphocytes (CTLs) specifically recognizing neoplastic cells in vitro were isolated from patients bearing a variety of tumors,[16,17] lending further support to the notions that (1) malignant cells express immunogenic tumor-associated antigens (TAAs), whereby they can be discriminated from their normal counterparts, and that (2) at least under selected circumstances, the immune system de facto reacts against neoplastic cells; though in the vast majority of cases such responses are unable to control tumor growth.[18]

Anticancer vaccines. Within the conceptual framework provided by Polly Matzinger's danger theory,[12] the discovery of MZ2-E, nowadays known as melanoma-associated antigen (MAGE)-A1, ignited an intense experimental effort, not only resulting in the identification and characterization of hundreds of additional TAAs, but also generating further insights into the mechanisms whereby TAAs, at least in some settings, can break tolerance and elicit an adaptive immune response.[19–21] For didactic purposes, TAAs can be classified into four distinct classes: (1) truly exogenous, non-self TAAs (which are invariably of viral origin); (2) unique, mutated TAAs (stemming from cancer cell-specific genetic alterations); (3) idiotypic TAAs (reflecting the unique way whereby the B-cell receptor expressed by some clonal hematopoietic malignancies is rearranged); and (4) shared TAAs (which are also expressed by normal cells, though often to lower levels). A detailed discussion of the properties of these four groups of TAAs largely exceeds the scope of this Trial Watch and can be found in ref. 22.

As soon as the first TAAs were characterized, great efforts have been dedicated to the development of anticancer vaccines, resulting in a wealth of different approaches including cell-based strategies (most often involving the loading of autologous DCs with tumor material ex vivo, followed by their re-administration to patients),[23] recombinant vaccines (entailing the direct administration of purified TAAs or TAA-derived peptides)[22] and DNA vaccines. The results of such an intense wave of research and development have been very encouraging. However, to date only three vaccines have been approved by FDA for use in humans: Cervarix® and Gardasil®, de facto constituting preventive measures against infection by human papillomavirus (HPV)-16 and HPV-18 and the consequent development of cervical carcinoma,[24,25] and sipuleucel-T (also known as Provenge®), a cellular preparation for the therapy of asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer.[26] This is in stark contrast with the huge number of vaccines that have been developed and commercialized during the last century for the prophylaxis of infectious diseases, and may stem from several reasons including (but not limited to): (1) the antigenic properties of malignant cells, (2) the fact that anticancer vaccines must operate in the vast majority of settings as therapeutic—rather than prophylactic—interventions and (3) the existence of multiple immunosuppressive mechanisms that are activated by malignant cells, both in the tumor microenvironment and systemically. A detailed discussion of these points exceeds the scope of this Trial Watch and can be found in ref. 22.

Anticancer gene therapy. Along with the recognition of the potential of recombinant DNA technologies, great efforts have been dedicated to the development of constructs that would drive the whole-body or tissue-specific expression of therapeutic genes, as well as of vectors and administration protocols that would allow for the efficient delivery of such constructs to patients.[27] Starting in the late 1990s, this intense wave of investigation generated a

considerable number of Phase I–II clinical trials testing whether preclinical observations could be safely and efficacy translated from the bench to the bedside.[28–30] Indeed, especially in the case of monogenic diseases affecting a relatively accessible cell compartment, such as severe immunodeficiency syndromes caused by the lack of adenosine deaminase (ADA) or the γ chain common to multiple cytokine receptors, gene therapy initially appeared to constitute a relatively safe and highly efficient therapeutic option.[31,32] Unfortunately, a few years later the use of retroviral vectors for gene therapy was associated with an increased risk for insertional mutagenesis, de facto abating the general enthusiasm about this therapeutic approach.[33] In the same period, the first clinical trials investigating the possibility to employ gene therapy as an anticancer intervention were concluded.[34–40] These studies were based on at least three distinct approaches, which continue to be actively investigated nowadays: (1) the selective delivery to malignant cells of genes coding for self-sufficient cytotoxic factors, such as the oncosuppressor protein p53,[35,41–43] a cytocydal variant of cyclin G1,[44] the adenovirus 5 E1A protein (which de facto functions as an oncosuppressor in breast cancer cells)[36,40,45,46] and the diphtheria toxin,[47,48] or enzymes that convert inactive drug precursors into poisonous chemicals, like the herpes simplex virus thymidine kinase (which can transform gangiclovir into a lethal triphosphate derivative)[49–53] and cytosine deaminase (which can convert 5-fluorocytosine into 5-fluorouracil);[54] (2) the (most often intratumoral) administration of plasmids coding for relatively unspecific immunostimulatory factors, including, but not limited to, interleukin (IL)-2,[44,55–58] IL-12,[59–63] interferon γ (IFNγ),[64–66] granulocyte-macrophage colony-stimulating factor (GM-CSF),[67] CD40 ligand (CD40L)[68,69] and the MHC Class I molecule HLA-B7;[38,39,70–76] and (3) bona fide DNA vaccines. Of note, none of these gene therapy-based approaches is currently approved by US. FDA for use in cancer patients, yet gendicine, a recombinant adenovirus engineered to express wild-type p53, has been licensed for the treatment of subjects affected by head and neck squamous cell carcinoma in China as early as in 2003.[77,78]

DNA vaccines. DNA vaccines consist in circular DNA constructs (near-to-invariably derived from bacterial plasmids) that encode one or more TAA(s),[79–81] and their use in humans de facto represents a particular case of gene therapy. These vaccines are administered subcutaneously or intramuscularly in the form of naked DNA or within appropriate delivery vectors, resulting in their uptake by resident antigen-presenting cells (APCs), mainly DCs and/or myocytes and local TAA expression. In both scenarios, intracellular TAAs are processed and presented on MHC Class I molecules to TAA-specific T cells (direct presentation). However, whereas professional APCs are very efficient at direct presentation, myocytes generally are not, as they express detectable yet rather low levels of MHC Class I and co-stimulatory molecules.[81,82] Thus, the induction of robust antitumor immunity following the expression of TAAs by myocytes must proceed via cross-presentation, the process whereby APCs take up exogenous material (most often apoptotic debris), process it and eventually present it in association with MHC Class I (rather than Class II) molecules, eventually resulting in the elicitation of $CD8^+$ T-cell responses.[81,83,84] Of note, cross-presentation has been proposed to constitute a major route for the activation of immune responses by DNA vaccines even in settings in which direct presentation can occur, for instance upon the direct delivery of naked DNA to Langerhans cells by gene gun.[85]

As compared with cell-based and recombinant preparations, DNA vaccines are advantageous in that (1) they can be generated in large amounts and with clinical grade purity in a relatively inexpensive and rapid fashion;[79–81,86] (2) they are highly stable (that is, they are relatively insensitive to temperature and have a long shelf life);[79–81,86] (3) they are safe, based on experience accumulated in more than one hundred clinical trials completed to date;[79–81,86] (4) the presence of bacterial sequences, notably unmethylated CpG islands, in the DNA backbone operates per se as an adjuvant, stimulating the activation of TLR9;[87] (5) they can be engineered either for the expression of TAAs fused to non-self proteins that exert adjuvant effects, such as the fragment C of the tetanus toxin,[88] *Pseudomonas aeruginosa* exotoxin,[89] the potato virus X coat protein[90] and green fluorescent protein,[91] or for the co-expression of other immunostimulatory factors, such as the heat shock 70 KDa protein (HSP70)[92,93] and various cytokines, including IL-2, IL-12 and GM-CSF;[93–95] (6) they can be engineered so to alter the intracellular routing of TAAs, resulting in the preferential activation of humoral (when TAAs are targeted to the endoplasmic reticulum) or cellular (if TAAs are targeted to the cytosol or—even more specifically—to the proteasome) immunity;[96,97] and (7) they can induce very robust T-cell responses (leading to the elimination of APCs at boosting) even if the amounts of TAA produced in situ is minimal.[79] However, the efficacy of DNA vaccines is influenced—at least in part—by the achievement of high transfection rates in vivo, raising the need of efficient vectors and administration protocols.

Vectors. Although the use of naked DNA constructs (at least in some circumstances) has been associated with acceptable transfection rates and the elicitation of TAA-specific immune responses, great efforts have recently been dedicated to the optimization of specific vectors for DNA vaccines.[79–81,86] The delivery of TAA-coding genes by lentiviral, adenoviral, retroviral and adeno-associated vectors perhaps constitutes the most investigated approach in this sense, offering high levels of transduction efficiency as well as a relatively stable and protracted TAA production.[98,99] However, these advantages are largely overcome by the facts that (1) viral packaging proteins are immunogenic and elicit potent anti-vector immune responses, de facto precluding the possibility of efficient boosting in prime-boosting settings, and (2) viral vectors are expensive, cannot host large transgenes, have been associated with toxic side effects and are potentially at risk for insertional mutagenesis.[33,98,99] Bacterial and eukaryotic vehicles have been proposed as an alternative to viral vectors, including genetically modified, attenuated strains of *Salmonella typhimurium*, *Pichia pastoris* and *Saccharomyces cerevisiae*.[100–104] In general, these systems are advantageous as they are compatible with oral administration, resulting in TAA expression by splenic APCs[104,105] or in the induction of potent mucosal immune responses,[101] and as multiple bacterial products like lipopolysaccharide (LPS), diacyl lipopeptides, flagellin and bacterial DNA —at least potentially—operate as adjuvants by

activating various TLRs.[6,7,87,106] This said and in spite of promising preclinical results, currently available bacterial and eukaryotic vectors are generally perceived as insufficiently mature for clinical applications;[79–81,86] although a few clinical trials to test their anticancer potential have been launched (see below). Other vectors including liposomes, microparticles, nanoparticles and peculiar polymers are under investigation as a means to increase the transfection rate of DNA vaccines and their immunogenicity, with encouraging results.[107,108] Nevertheless, the vast majority of clinical trials ever launched to date for evaluating the antineoplastic potential of DNA vaccines has been based on naked DNA.[79–81,86]

Delivery methods. Preclinical and clinical data collected during the last two decades demonstrate that the administration route constitutes a critical determinant for the efficacy of DNA vaccines.[79,81,86,109,110] Intramuscular injections were commonly employed during early tests with large animals and humans, resulting in relatively poor efficacy. In retrospective, this could have been predicted, as the efficacy of DNA vaccines administered i.m. strictly depends on the injected volume.[79,81] Thus, while the intramuscular administration of a DNA vaccine in 50 μL vehicle results in the elicitation of robust immune responses in mice, efficacy is gradually lost along with the decrease in injection volumes.[111] Presumably, this stems from the fact that a high hydrostatic pressure not only augments the uptake of the DNA vaccine by myocytes and resident APCs (de facto increasing transfection efficacy) but also promotes (a limited degree of) tissue damage, resulting in the release of danger signals that (1) attract additional APCs and other immune cells to the injection sites and (2) provide immunostimulatory signals via TLRs and other pattern recognition receptors.[6,7,15,112–115] Unfortunately, scaling this volume up for the intramuscular administration of DNA vaccines to humans is unfeasible, raising the need for alternative delivery routes. In this sense, several options have been investigated during the last two decades, including (but not limited to) gene gun-mediated delivery,[108,116] jet injection[117,118] and tattooing,[119] all of which involve the skin route, oral delivery[120–122] and electroporation.[116,123–125] Of note, although most (if not all) of these strategies have already entered the clinical phase of development, nowadays electroporation has emerged as a preferred and efficient delivery method.[126]

Electroporation consists in the electrical stimulation of a skeletal muscle immediately after the intramuscular delivery of naked DNA.[127–129] De facto, electroporation is associated with (1) a consistent increase in transfection efficiency and (2) local tissue injury, resulting in the release of danger signals by dying myocytes, the recruitment of immune cells and the establishment of a pro-inflammatory milieu that stimulates robust humoral and cellular immune responses.[124,130–132] Of note, the efficacy of DNA vaccines administered via electroporation is not compromised by the use of low injection volumes.[111,125] Moreover, although generally perceived as uncomfortable, repeated electroporation appears to cause no major side effects and is accepted by patients with no need for anesthetic procedures.[79,81] Finally, although increased transfection efficiencies as achieved with electroporation elevate the risk of (potentially oncogenic) integration, this appears to remain within acceptable levels.[133] As it stands, electroporation constitutes the delivery method for DNA vaccines best suited for clinical applications; though ever more encouraging results are being obtained with preparation that exploit the oral route, including bacterial and eukaryotic vectors.[121,134]

Along the lines of our monthly Trial Watch series,[6,7,22,23,135–142] here we will briefly discuss the results of recent clinical trials that have investigated/are investigating the antineoplastic potential of DNA vaccines. As mentioned above, no DNA-based preparation is approved by FDA for use in cancer patients as a prophylactic or immunotherapeutic intervention to date (source www.fda.gov). Conversely, three distinct DNA vaccines have been licensed for veterinary use, including one for the prophylaxis of West Nile virus in horses,[143] one for the prophylaxis of infectious hematopoietic necrosis virus (IHNV) in salmonid fish[144,145] and one for the therapy of malignant melanoma in dogs.[146] Intriguingly, the latter relies on the expression of a xenogenous TAA (i.e., human tyrosinase), resulting in the breakdown of tolerance against the endogenous protein and hence in the development of an efficient humoral response that significantly prolongs the overall survival of melanoma-bearing dogs.[147]

Naked DNA-Based Anticancer Vaccines

So far, the safety and efficacy of naked DNA vaccines have been evaluated in a relatively restricted number of clinical settings. In particular, constructs coding for autogenic TAAs or allogeneic factors that would exert cross-immunizing functions have been tested in cohorts of B-cell lymphoma patients (TAA: idiotypic B-cell receptor regions),[148] head and neck cancer (HNC) patients (immunogen: *Mycobacterium leprae* HSP65),[149] melanoma patients (TAAs: gp100, MART-1-derived peptides, tyrosinase or tyrosinase-derived peptides),[150–156] colorectal carcinoma patients (TAA: carcinoembryonic antigen, CEA),[157] HPV-16$^+$ cervical intraepithelial neoplasia (CIN) patients (TAA: HPV-16 E6)[92] and individuals affected by prostate carcinoma (TAA: prostate-specific antigen, PSA).[158,159] The results of these studies (all of which were conducted in a Phase I clinical setting) suggest that the intramuscular, intratumoral and intranodal administration of naked DNA vaccines to cancer patients is safe and can elicit TAA-specific immune responses that—at in least in a fraction of patients—exert bona fide therapeutic effects.

Nowadays (January 2013), official sources list 15 recent (started after January 1, 2008), ongoing (not withdrawn, terminated or completed at the day of submission) clinical trials assessing the safety and efficacy of naked DNA-based vaccines as therapeutic interventions against cancer (**Table 1**). Five of these studies are investigating the therapeutic potential of constructs encoding the E6 and/or E7 proteins of HPV variants that are associated with an increased risk for HNC, cervical cancer and anal carcinoma (i.e., HPV-16 and HPV-18)[24,160] either (1) as a plasmid co-encoding the immunostimulatory protein FLT3 ligand, administered i.m. via electroporation, in patients affected by grade 3 CIN (NCT01634503); (2) as a construct co-encoding the immunostimulatory protein calreticulin (CRT),[112,161,162] administered as a standalone agent i.m., s.c. or i.t., in subjects

Table 1. Clinical trials testing naked DNA-based vaccines as therapeutic interventions against cancer*

Vector	Indication	Phase	Status	TAA	Co-encoded molecule(s)	Co-therapy	Delivery route	Ref.
Mixed	CIN	I	Recruiting	HPV-16 E6/E7	HSP70	E6/E7-coding virus Imiquimod	i.m.	NCT00788164
	HCC	I-II	Recruiting	AFP	–	AFP-coding virus GM-CSF-coding plasmid	i.m.	NCT00669136
Naked DNA	Breast cancer	I	Recruiting	SCGB2A2	–	–	i.m.	NCT00807781
	CIN	I	Recruiting	HPV-16 E6/ E7	FLT3L	–	i.m. + EP	NCT01634503
		II	Recruiting	HPV-16 E6/ E7 HPV-18 E6/ E7	–	–	i.m. + EP	NCT01304524
		n.a.	Recruiting	HPV-16 E7	CRT	–	i.m. s.c. i.t.	NCT00988559
	CRC	I-II	Active, not recruiting	CEA	–	CPA rGM-CSF	s.c. + EP	NCT01064375
	HNC	I	Recruiting	HPV-16 E7	CRT	CPA	i.m. + EP	NCT01493154
	Lymphoma	I	Not yet recruiting	Idiotype	Chemokine (fusion)	–	i.m.	NCT01209871
	Melanoma	I-II	Recruiting	TRP2	Antibody (fusion)	–	i.m. + EP	NCT01138410
	Ovarian cancer	I	Recruiting	IGFBP-2	–	–	s.c.	NCT01322802
	Prostate cancer	I-II	Unknown	PSA	–	–	s.c. + EP	NCT00859729
		II	Active, not recruiting	PAP	–	rGM-CSF	s.c.	NCT00849121
		II	Recruiting	PAP	–	rGM-CSF	s.c.	NCT01341652
		II	Recruiting	PAP	–	rGM-CSF sipuleucel-T	s.c.	NCT01706458

AFP, α fetoprotein; CEA, carcinoembryonic antigen; CIN, cervical intraepithelial neoplasia; CRC, colorectal carcinoma; CRT, calreticulin; CPA, cyclophosphamide; EP, electroporation; FLT3L, FLT3 ligand; GM-CSF, granulocyte-macrophage colony stimulating factor; HCC, hepatocellular carcinoma; HNC, head and neck cancer; HPV, human papillomavirus; HSP70, heat shock 70 KDa protein; IGFBP-2, insulin-like growth factor binding protein 2; i.m., intra musculum; i.t., intra tumorem; n.a., not available; SCGB2A2, mammaglobin A; PAP, prostate acid phosphatase; PSA, prostate-specific antigen; r, recombinant; s.c., sub cutem; TAA, tumor-associated antigen; TRP2, tyrosinase-related protein 2. *Started after January 1, 2008 and not withdrawn, terminated or completed at the day of submission.

affected by grade 2/3 CIN (NCT00988559), or delivered i.m. via electroporation in combination with the immunostimulatory drug cyclophosphamide i.v.[13,112,136,142] to HNC patients (NCT01493154); (3) as a plasmid co-encoding the immunostimulatory factor HSP70,[112,163] administered i.m. together with a viral vector coding for the same TAAs and topical imiquimod[6,7,164] to women bearing grade 3 CIN (NCT00788164); or (4) delivered i.m. via electroporation to patients affected by grade 2/3 CIN (NCT01304524). Of the remaining 10 studies, (1) three are evaluating the safety and efficacy of a construct coding for prostate acid phosphatase (PAP),[165,166] administered s.c. in combination with sipuleucel-T and/or GM-CSF to prostate cancer patients (NCT00849121; NCT01341652; NCT01706458); (2) one is investigating the clinical profile of a plasmid coding for mammaglobin A (a secretoglobin that is often overexpressed by breast carcinoma cells),[167] administered i.m. as a standalone intervention, in women affected by metastatic breast carcinoma (NCT00807781); (3) one is testing a CEA-coding plasmid,[168,169] delivered s.c. via electroporation as a standalone agent or combined with GM-CSF s.c. and cyclophosphamide i.v., in colorectal carcinoma patients (NCT01064375); (4) one is evaluating the therapeutic profile of a prime-boost strategy based on a construct encoding the common TAA α fetoprotein (AFP),[170] administered i.m. together with a GM-CSF-coding plasmid (prime) and an AFP-expressing adenoviral vector given i.m. (boost), in hepatocellular carcinoma patients (NCT00669136); (5) one is assessing the safety and efficacy of a plasmid coding for patient-specific, lymphoma-derived single-chain variable fragments (idiotypic vaccination)[22,171] fused to a not-better specified chemokine, administered i.m. as a standalone intervention, in subjects

affected by lymphoplasmacytic lymphoma (NCT01209871); (6) one is investigating the therapeutic potential of a construct that encodes a tyrosinase-related protein 2 (TRP2) epitope fused to a modified monoclonal antibody targeting the chimera to DCs,[172,173] delivered i.m. via electroporation as a standalone intervention to melanoma patients (NCT01138410); (7) one is testing a plasmid coding for residues 1–163 of insulin-like growth factor binding protein 2 (IGFBP-2),[174,175] administered s.c. as a single agent, in patients affected by Stage III-IV ovarian cancer (NCT01322802); and (8) one is assessing the safety and efficacy of a construct coding for *Macaca mulatta* PSA, which is highly homologous to its human counterpart,[176–178] delivered s.c. via electroporation to patients bearing relapsed prostate cancer (NCT00859729). Of note, all these naked DNA-based vaccination strategies are currently being tested in Phase I-II clinical settings (**Table 1**).

Vector-Based Anticancer Vaccines

Similar to the case of naked DNA vaccines, the safety and therapeutic potential of vector-based anticancer vaccines have been investigated in a relatively low number of clinical scenarios. In particular, the oral administration of bacterial vectors has only been tested in a cohort of pancreatic cancer patients (TAA: vascular endothelial growth factor receptor 2, VEGFR2);[121,179] adenoviral or poxviral vectors (given i.m. or s.c.) have been evaluated in cohorts of non-small cell lung carcinoma (NSCLC) patients (TAA: L523S),[180] melanoma patients (TAA: multiple epitopes from distinct melanoma antigens)[150,181] and prostate carcinoma patients (TAAs: prostate-specific membrane antigen, PSMA);[182,183] and biodegradable polymeric materials have been tested in cohorts of anal dysplasia patients (TAA: HPV-16 E7),[184] CIN patients (TAAs: HPV-16 E6/E7)[185] and individuals bearing advanced solid tumors (TAA: cytochrome P450 1B1).[186] Cumulatively, these clinical trials reported a very low incidence of (near-to-invariably) mild side effects, as well as the development of TAA-specific immune responses that, at least in a subset of patients, translated into a clinical benefit.

Today (January 2013), official sources list 17 recent, ongoing clinical trials investigating the therapeutic potential of vector-based DNA vaccines in cancer patients (**Table 2**). Five of these studies are based on bacterial vectors, as (1) four are testing a live attenuated variant of *Listeria monocytogenes* engineered to express E7 from HPV-16 (ADXS11-001),[187] delivered i.v. either as a standalone intervention to individuals affected by grade 2/3 CIN (NCT01116245), persistent/recurrent cervical carcinoma (NCT01266460) and oropharyngeal cancer (NCT01598792), or in combination with 5-fluorouracil, mitomycin and intensity-modulated radiation therapy to anal carcinoma patients (NCT01671488); and (2) one is assessing the safety and efficacy of an attenuated strain of *Salmonella typhimurium* encoding VEGFR2 (VXM01),[121,179] administered p.o. to patients affected by locally advanced, inoperable Stage IV pancreatic cancer (NCT01486329). Of the remaining 12 studies, all involving (at least in part) viral delivery systems, six are testing Vaccinia virus- or Modified Vaccinia Ankara (MVA) virus-derived vectors (1) either expressing HPV-16 E6 and E7 and co-administered i.m. with a naked plasmid coding for the same TAAs plus HSP70[112,163] and topical imiquimod[6,7,164] to women affected by grade 3 CIN (NCT00788164);[92] (2) either coding for the breast cancer-associated TAA *v-erb-b2* erythroblastic leukemia viral oncogene homolog 2 (ERBB2, best known as HER2)[188–191] and delivered s.c. as a standalone intervention following adjuvant chemotherapy to individuals affected by ERBB2$^+$ breast cancer (NCT01152398); (3) either encoding both PAP[165,166] and PSA[178] and administered s.c. to androgen-insensitive prostate cancer patients (NCT00629057); (4) either coding for two antigens of the Epstein-Barr virus (i.e., EBNA1, LMP2), which is associated with a fraction of HNC cases,[192,193] and delivered s.c. to nasopharyngeal cancer patients with residual viral load after conventional therapy (NCT01094405); (5) either encoding p53,[41,42,194] which is frequently overexpressed by a wide variety of neoplasms as a result of inactivating *TP53* mutations,[195–199] and administered s.c. to subjects affected by gastric, pancreatic or colorectal carcinoma (NCT01191684); (6) or coding for mucin 1 (MUC1)[200] plus IL-2 (TG4010)[201] and delivered s.c. in combination with conventional chemotherapeutic regimens to Stage IV NSCLC patients (NCT01383148). In addition, (1) three studies are assessing the therapeutic profile of the co-administration of fowlpox virus- and vaccinia virus-derived vectors, either coding for PSA[178] plus three T-cell co-stimulatory molecules (TRICOM)[202,203] and delivered s.c. in association with the microtubular poison docetaxel plus prednisone to metastatic, hormone-resistant prostate cancer patients (NCT01145508), either coding for PSA[178] plus TRICOM[202,203] and delivered together with GM-CSF to subjects affected by metastatic, castration-resistant prostate cancer (NCT01322490), or coding for CEA[168,169] plus MUC1[200] and delivered i.t. and s.c. in combination with GM-CSF to individuals bearing unresectable pancreatic carcinoma (NCT00669734); (2) two trials are testing adenoviral vectors, either encoding AFP[170] and delivered i.m. as a boosting strategy following the intramuscular co-administration of AFP- and GM-CSF-coding plasmids (prime) to hepatocellular carcinoma patients (NCT00669136), or coding for CEA[168,169] (ETBX-011) and administered s.c. as a standalone intervention to patients affected by advanced CEA-expressing breast, lung and colorectal carcinoma (NCT01147965); and (3) one study is investigating the therapeutic potential of a live attenuated strain of the Measles virus (Attenuvax®) delivered s.c. as a single agent to Stage IIIB/IV, Measles virus-positive NSCLC patients[204,205] (NCT00828022). Of note, only two of these approaches are in a relatively advanced stage of clinical development and are tested in Phase III settings (NCT01322490; NCT01383148), i.e., (1) the subcutaneous co-administration of fowlpox virus- and vaccinia virus-derived vectors coding for PSA plus TRICOM in combination with recombinant GM-CSF (for the treatment of prostate cancer); and (2) the subcutaneous delivery of an MVA-derived vector encoding MUC1 plus IL-2 in combination with conventional chemotherapy (for the treatment of NSCLC) (**Table 2**). Future will tell whether either of these strategies will become the first therapeutic DNA vaccine to be approved by FDA for use in cancer patients.

Table 2. Clinical trials testing vector-based DNA vaccines as therapeutic interventions against cancer*

Vector	Indication(s)	Phase	Status	TAA	Co-encoded molecule(s)	Co-therapy	Delivery route	Ref.
Adenovirus	Breast cancer CRC Lung cancer	I-II	Active, not recruiting	CEA	–	–	s.c.	NCT01147965
Fowlpox virus Vaccinia virus	Pancreatic cancer	I	Recruiting	CEA MUC1	–	rGM-CSF	s.c. i.t.	NCT00669734
	Prostate cancer	II	Active, not recruiting	PSA	TRICOM	Docetaxel Prednisone	s.c.	NCT01145508
		III	Recruiting	PSA	TRICOM	rGM-CSF	n.a.	NCT01322490
Listeria monocytogenes	Anal cancer	I-II	Not yet recruiting	HPV-16 E7	–	5-FU Mitomycin C IMRT	i.v.	NCT01671488
	Cervical cancer	II	Recruiting	HPV-16 E7	–	–	n.a.	NCT01266460
	CIN	II	Recruiting	HPV-16 E7	–	–	i.v.	NCT01116245
	Oropharyngeal cancer	I	Recruiting	HPV-16 E7	–	–	n.a.	NCT01598792
Measles virus	NSCLC	I-II	Unknown	Measles-virus encoded proteins	–	–	s.c.	NCT00828022
Mixed	CIN	I	Recruiting	HPV-16 E6/E7	–	E6/E7-coding plasmid Imiquimod	i.m.	NCT00788164
	HCC	I-II	Recruiting	AFP	–	AFP- and GM-CSF-coding plasmids	i.m.	NCT00669136
MVA virus	Breast cancer	I	Recruiting	ERBB2	–	–	s.c.	NCT01152398
	CRC Gastric cancer Pancreatic cancer	I	Recruiting	p53	–	–	s.c.	NCT01191684
	Nasopharyngeal cancer	II	Recruiting	EBNA1 LMP2	–	–	s.c.	NCT01094405
	NSCLC	II-III	Recruiting	MUC1	IL-2	Conventional chemotherapy	s.c.	NCT01383148
	Prostate cancer	I	Active, not recruiting	PAP PSA	–	–	s.c.	NCT00629057
Salmonella typhimurium	Pancreatic cancer	I	Recruiting	VEGFR2	–	–	p.o.	NCT01486329

5-FU, 5-fluorouracil; AFP, α fetoprotein; CEA, carcinoembryonic antigen; CIN, cervical intraepithelial neoplasia; CRC, colorectal carcinoma; EBNA1, Epstein-Barr nuclear antigen 1; ERBB2, *v-erb-b2* erythroblastic leukemia viral oncogene homolog 2; GM-CSF, granulocyte-macrophage colony stimulating factor; HCC, hepatocellular carcinoma; HPV, human papillomavirus; IL-2, interleukin-2; i.m., intra musculum; IMRT, intensity-modulated radiation therapy; i.t., intra tumorem; i.v., intra venam; LMP2, latent membrane protein 2; MUC1, mucin 1; MVA, Modified Vaccinia Ankara; n.a., not available; NSCLC, non-small cell lung carcinoma; PAP, prostate acid phosphatase; PSA, prostate-specific antigen; r, recombinant; p.o., per os; s.c., sub cutem; TAA, tumor-associated antigen; VEGFR2, vascular endothelial growth factor receptor 2. *Started after January 1, 2008, and not withdrawn, terminated or completed at the day of submission.

Concluding Remarks

Preclinical and clinical evidence accumulated during the last two decades indicates that DNA vaccines have the potential to induce tumor-specific immune responses that—at least in a fraction of patients—may translate into a therapeutic benefit.[79–81,86] Thus, although no DNA vaccines are currently approved by FDA for use in cancer patients, great expectations are reposited on this technology, also linked to the fact that three distinct DNA-based preparations (of which one is employed in a therapeutic—as opposed to prophylactic—setting) have already been licensed for veterinary use.[80]

DNA vaccines offer great possibilities in that they can be engineered (1) so to express not only the TAA(s) of choice but also immunostimulatory molecules, including cytokines and xenogenous proteins that operate as adjuvants;[88–95] and (2) so that the

intracellular routing of the TAA(s) of choice is pre-determined, resulting in the preferential elicitation of humoral or cellular immune responses.[96,97]

The progress of anticancer DNA vaccines toward clinical applications is confronted with the very same issues that complicate the development of other vaccination strategies.[22,23] These include the limited availability of clinical grade TLR agonists for use adjuvants[6,7] as well as the problems posed by the immunosuppressive tumor microenvironment, raising the need for the delivery of co-stimulatory signals, such as those elicited by CD40 agonists,[206,207] or immune checkpoint inhibitors, such as anti-CTLA4 or anti-PD1 antibodies.[135,141] In addition, the ability of DNA-based preparations to elicit TAA-specific immunity is dramatically influenced by transfection efficacy and delivery route, as these two factors dictate not only the amount of TAA that is available for (direct of cross-) presentation, but also the type and intensity of immunostimulatory signals that are released in situ to promote immune responses.[79,81] Nowadays, the electroporation of naked DNA is perceived as the approach with a more straightforward path to clinical applications,[79,81] whereas the efficacy of viral vectors is limited by the development of anti-vector immune responses that de facto preclude boosting.[98,99] Promising results have been also obtained with bacterial and eukaryotic vectors,[100–104] yet these tools appear to require a consistent degree of refinement before entering the clinical routine.

Only future will tell whether DNA vaccines will ever make their way from the bench to the bedside and transform from a promising investigational approach into a brilliant clinical reality.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

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