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Biomarkers for the Development of Cancer Vaccines

Current Status

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Abstract

Significant improvements in our knowledge of tumor immunology have resulted in more sophisticated vaccine approaches for the treatment of cancer. However, research into biomarkers that correlate with the clinical outcome of immunotherapy has lagged behind vaccine development. To this extent, very few immunological or other markers exist that can be used in clinical trials for immunotherapy. In this review, we discuss the current status of biomarker development specifically for the monitoring and development of cancer vaccines. This includes immunological biomarkers (measurement of T-cell and cytokine responses), autoimmunity as a correlate for treatment outcome, and the possible development of multiple biomarkers using high-throughput proteomics technologies. The generation of such biomarkers will allow us to make clinical decisions about patient treatment at an earlier stage and should aid in shortening the development time for vaccines.

1. Introduction

There is a pressing clinical need for the discovery of effective biomarkers for cancer. In particular, they are useful in the following ways: to diagnose a disease (particularly for early detection or diagnosis of occult tumors); to aid in staging; to predict the outcome of a disease; to act as a surrogate for clinical progression; or, to monitor responses to treatment. Such biomarkers are useful where patient stratification prior to treatment can considerably improve the outcome of clinical trials.

Despite this, very few markers have been approved for clinical use, and efforts to find new markers and to develop techniques for their discovery are ongoing. The lack of approved clinical biomarkers is a result of problems associated with their development and validation. One major difficulty is that markers expressed in tumors may also be seen in normal/healthy tissue or in other disease states, albeit at different levels of expression. An example of this is the acute-phase protein serum amyloid A. High levels of amyloid A are associated with a number of cancers, but levels are also elevated in other inflammatory diseases.[1-3] Furthermore, biomarkers must be validated in clinical trials with

sufficient numbers of patients/samples for statistical significance to be reached, and must be repeatable by multiple groups using standardized methodologies. Consequently, although many prospective biomarkers now exist, few have been validated for use in cancer. A huge volume of literature exists relating to the identification of novel cancer biomarkers, which is beyond the scope of this review. Here, we will concentrate on those biomarkers related specifically to prediction of outcome, monitoring of efficacy, or those which may ultimately be used as endpoints in a cancer vaccine/immunotherapy setting (see table I and table II).

2. Cancer Vaccines and Measurement of Immunological Responses

The range of cancer vaccine treatments that have been, or are being, tested in clinical trials has been extensively reviewed elsewhere and a detailed description of these strategies is beyond the scope of this article. Briefly, cancer vaccines fall into the following three broad categories: (i) protein/peptide vaccines; (ii) DNA vaccines; and (iii) cellular vaccines.

Protein/peptide vaccines are the most widely tested of all cancer vaccines and are composed of a protein or peptide that is tumor specific or over expressed in tumor tissue, relative to normal tissue.[13] Typically, in the case of peptide vaccines, these are restricted only to patients of a particular HLA type.

DNA vaccines are composed of cDNA sequences, again representing tumor-specific epitopes, engineered into plasmid or viral vectors under the control of strong transcriptional promoters.[14,15] These express tumor proteins when injected into tissue (usually muscle).

Cellular vaccines encompass a range of available techniques, including injection with whole irradiated tumor cells[16] or with autologous dendritic cells.[17] Whole tumor cells may either be syngeneic or allogeneic, and both cases rely on the generation of immunity to antigens shared between the vaccine and the patient's own tumor. Dendritic cells take up antigens in different forms and present them to T cells. This often involves pulsing the dendritic cells *ex vivo* with peptide, protein, or with tumor lysate or mRNA taken from the patient.

All of these approaches may elicit cellular and/or humoral immune responses, although dendritic cell therapy is biased towards the generation of a cellular response. Although responses to immunotherapy are optimally measured in terms of clinical response, immunological responses are often used as surrogates. The precise nature of the immunological assay used is dependant upon the type of vaccine administered. Where the patient is likely to respond to a specific epitope, a number of techniques are available to measure T-cell responses to vaccination. Tetramer analysis[18] detects the presence of a T-cell receptor specific for a defined MHC-peptide complex using fluorescence-activated cell scanning (FACS)-based technology. T-cell proliferation assays may be used to assess the capacity of T-cell populations to expand in response to a known antigen and cytotoxic T lymphocyte (CTL) assays[19] measure direct killing of tumor cells or peptide pulsed surrogates. Most clinical trials also incorporate the measurement of cytokine profiles (by ELISA, ELISPOT, intracellular FACS, and multiplex cytokine assays), which give an indication of the pro- or anti-inflammatory nature of the response.[20] In particular, multiplex cytokine assays have expanded the capacity to measure multiple cytokines simultaneously from small samples.[21,22] Finally, antibody responses to defined antigens can be measured, generally by ELISA. Often, there is no consensus about which techniques provide the best readout for vaccines and, to date, little evidence exists to directly correlate immunological response with clinical outcome. A further complication is that little is known about the

Table I. Biomarkers based on immunological responses to vaccination that are correlated with clinical outcome

Response measured	Cancer	Nature of vaccine	Reference
DTH	Melanoma	DC + allogeneic tumor lysate	4
	Melanoma	DC + autologous tumor lysate	5
	Melanoma	Autologous tumor	6
DTH infiltrating lymphocytes	Melanoma	DC + peptides (gp100 + tyrosinase)	7
Tumor infiltrating lymphocytes	Melanoma	Autologous tumor	6
	Melanoma	Autologous tumor	8
Cytokines			
IFNγ (from *in vitro* stimulated T cells)	Various	Peptides (p53 or K-ras)	9
T_h1 cytokines + positive PSAV	Prostate	Allogeneic tumor	10
Autoimmunity	Melanoma	Peptide (gp100) + anti-CTLA4 monoclonal	11

CTLA4 = cytotoxic T-lymphocyte antigen 4; **DC** = dendritic cell; **DTH** = delayed-type hypersensitivity response; **IFN** = interferon; **PSAV** = prostate specific antigen velocity; **T_h1** = T-helper type 1.

Table II. Predictors of clinical response to vaccination

Predicitive phenotype	Cancer	Nature of vaccine	Reference
Lack of MHC expression on tumor	Melanoma	Autologous tumor	8
Survivin expression	Melanoma	Canvaxin®[a]	12

a Canvaxin was tested in a phase III trial, which was recently terminated.

MHC = major histocompatibility complex.

immunological status of patients before treatment, which may affect their ability to respond to vaccination. Here, we set out immunological parameters that have been shown to relate to clinical response and which may prove to be important biomarkers for the development of effective vaccine strategies.

3. Immunological Biomarkers for Cancer Vaccines

One major obstacle to the development of immunological biomarkers is that it is not clear what constitutes an effective anti-tumor immune response. Therefore, in most clinical trials a wide range of immunological parameters (see section 2) are measured in the hope that some will correlate with clinical outcome.

3.1 T-Cell Responses

There is compelling evidence that T-cell responses play an important role in mounting an effective anti-tumor response. Consequently, several approaches are used to measure the specificity and magnitude of the response of T cells circulating in the peripheral blood after vaccination.[20] The very nature of the techniques for measuring T-cell responses must assume some knowledge of the peptide or MHC specificity of the responding cells. However, in cases where the immunological readout depends on peptide specificity, the effect of epitope spread (i.e. spreading of the T-cell response beyond the immunizing epitope) will not be accounted for.[23]

Loss of antigenicity/MHC class I expression by the tumor may make the measurement of specific T-cell responses an unreliable biomarker since the tumors become resistant to T-cell attack. However, the antigenic changes that make the measurement of T cells redundant may themselves provide predictive biomarkers. Recently, attempts to define the nature of antigen escape by colorectal tumors led to the suggestion that low MHC class I expression was associated with reduced disease-specific survival in patients with early stage disease.[24] Furthermore, in melanoma it was shown that loss of antigenicity in metastases was correlated with poor clinical responses, despite high levels of circulating antigen-specific T cells.[25] Interestingly, van Houdt et al.[8] showed that in metastatic melanoma patients treated with autologous tumor plus Bacille Calmette-Guerin, those individuals lacking MHC on their tumor had longer overall survival times than patients whose MHC was intact. Lack of MHC is likely to stimulate innate natural killer-cell activity and it is therefore possible that there are differences between the types of treatment in which a response skewed in this manner is more effective in MHC-negative tumors.

In many trials, delayed-type hypersensitivity (DTH) is measured as a means of monitoring immune responses to vaccines. A number of recent papers support the idea that the presence of a DTH response is correlated with a positive clinical outcome in metastatic melanoma (table I).[4-6] However, such correlations are not always observed and the relationship between DTH and an effective anti-tumor response still requires clarification. In one such study, de Vries et al.,[7] although unable to correlate DTH with outcome, were able to show that biopsies of DTH sites contained tumor-antigen specific CD8+ T cells and that the presence of such antigen-specific DTH infiltrating T cells correlated with increased survival. The use of DTH to monitor anti-tumor responses is controversial, and more data are required to clarify why some studies show positive correlations with clinical outcome while others do not.

A refinement of crude DTH activity is to examine the presence of tumor infiltrating lymphocytes (TILs), which may be of prognostic value. Studies in stage III/IV melanoma,[8] ovarian cancer,[26,27] and glioblastoma multiforme[28] demonstrated better overall survival in patients whose tumors contained a strong T-cell infiltrate. In two of these studies, the infiltrating cells were CD8+ T cells, suggesting that cytolytic cells may be important in anti-tumor responses. Haanen et al.[6] correlated improved overall survival of metastatic melanoma patients with the presence of antigen-specific TILs detected by MHC class I tetramers.

T-regulatory cells (T_{reg}) are also found as infiltrates in tumors. Ablation of these cells, which are known to suppress immune responses to tumors, is associated with improved anti-tumor CTL responses.[29] Increased proportions of T_{reg} TILs have been found in non-small cell lung cancer,[30] pancreatic and breast cancer,[31] and ovarian cancer.[27,32] Moreover, the presence of high levels of T_{reg} in the tumor was correlated with poor survival,[32] while a high CD8+/T_{reg} ratio was associated with improved survival.[27] These cells are likely to help maintain an immunosuppressive environment in the tumor and consequently their presence in tumor biopsies may be predictors of poor response to vaccination. To date, the screening of TILs has been limited to biopsies before treatment and therefore may be of prognostic significance. Further work will be needed to determine whether such TILs can be monitored through the course of the disease, for example through fine-needle aspirates of metastatic lesions.

3.2 Cytokine Profiles

It is postulated, based largely on data from murine tumor models, that a proinflammatory, T-helper type 1 (T_h1) response is most effective against tumors and is associated with tumor regression.[33-36] Proinflammatory responses are associated with a skewed expression of cytokines; typical T_h1 cytokines are interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor-α, whereas T_h2 responses are characterized by IL-4, IL-5, IL-10, and IL-13. Cytokines can be measured either in serum/plasma (where they represent the combined cytokine expression of tissues, lymphocytes, and the tumor itself) or as a secretory product of T cells. The latter is usually measured *ex vivo* and in response to either an antigen corresponding to that administered in the vaccine, or to a polyclonal stimulus such as phorbol myristic acetate.

Serum cytokines, in particular IL-6, have been shown to correlate with poor prognosis in a number of different cancers.[34,37-39] However, although cytokines are often measured in the sera of vaccinated patients, there have been few general correlations between treatment outcome and changes in serum cytokine levels in response to vaccination. The measurement of cytokines produced by T cells has also been a poor biomarker for outcome in clinical trials, since patients who produce T_h1 type responses to *in vitro* stimulation do not necessarily have better anti-tumor responses.[9] A recent notable exception is an article by Carbone et al.,[9] who showed that peptide vaccinations tailored to patient-specific mutations in tumor protein p53 or k-ras gave rise to CTL responses and that IFNγ production by *in vitro*-stimulated T cells correlated with increased median survival times. It is noteworthy that many clinical trials do not attempt such correlations and that negative correlations often go unreported.

In one study,[10] the lack of an obvious correlation between clinical response and immunological response was overcome by using an artificial neural network (ANN) [see section 6] to discern patterns within their data that were not immediately apparent using classical statistical analysis. In this study[10] of prostate cancer patients vaccinated with an allogeneic tumor cell vaccine, positive prostate-specific antigen velocity responses correlated with a T_h1 profile, whereas non-responding patients had a mixed T_h1/T_h2 profile. Such multiparametric analysis may be of use in extracting relevant information from complex datasets in order to demonstrate the use of cytokine profiling for immunological monitoring.

Improvements in high-throughput technology are likely to change this situation. Multiplex cytokine assays have been developed as a fusion of ELISA and FACS technologies.[21,22] These assays, which use beads to capture the analyte instead of binding to a fixed plastic substrate (as for ELISA), allow simultaneous measurement of a wide range of cytokines in one assay using a single sample. It is therefore possible that accurate, simultaneous measurement of a range of cytokines will generate profiles indicating the nature of the immune response, and which can be correlated with clinical responses to vaccination.

4. Autoimmunity

Patients in clinical trials for immunotherapy are monitored for autoimmune toxicity associated with the therapy. Generally, autoimmunity has been considered to be a downside of such therapeutic regimens, although in most cases cancer vaccines have few worrying adverse effects. Paradoxically, in many cancers an effective anti-tumor response requires the therapy to break tolerance and risk the induction of autoimmunity. In three recent papers,[11,40,41] the induction of autoimmunity through the administration of immunotherapeutic regimens correlated with clinical responses in metastatic melanoma. One study used gp100 peptide vaccination in combination with anti-CTLA4 monoclonal antibody therapy for 56 patients, 14 of whom experienced grade 3–4 autoimmune toxicity (affecting gastrointestinal tract, eyes, skin, liver, and pituitary).[11] Five of these 14 patients showed evidence of tumor regression, compared with only 2 of the 42 patients in whom autoimmune toxicity was not seen ($p = 0.008$). There was no correlation between the magnitude of the immune response and the clinical response. Toxicity was managed successfully and all patients in this study remained well. Given the recent experience gained from the TGN1412 trial,[42] induction of autoimmunity should be regarded with caution. However, with careful monitoring and management, autoimmunity may not be as undesirable as previously thought and may be a credible biomarker for monitoring the effects of immunotherapeutic intervention.

5. Predictors of Response to Immunotherapy

Although many biomarkers exist that may be used to predict the outcome of therapy, to date, very few have been clinically validated. Outside the use of immunological monitoring for T-cell and cytokine responses, very little research appears to be available to determine biomarkers that specifically predict the response to immunotherapy (see table II). One exception to this is a recent study where patients with metastatic melanoma were treated with the cancer vaccine Canvaxin®[1] (a mixture of irradiated melanoma cell lines).[12] In this study, low expression levels of the apoptosis inhibitor survivin in tumour tissue (as assessed by quantitative real-time PCR) were found to correlate with overall survival in a

1 The use of tradenames is for identification purposes only and does not imply endorsement.

group of patients who received Canvaxin® post-operatively. Phase I and II trials of Canvaxin® showed a great deal of promise. However, in phase III trials Canvaxin® was ultimately unsuccessful in showing a difference between treatment and control arms of the study. It is interesting to speculate whether the use of survivin to stratify patients in the phase III setting might have made a difference to these data. There is great need for such markers to stratify patients into responder and non-responder groups prior to vaccination.

6. Multiple Biomarkers: The Way of the Future?

There are many good biomarkers for cancer that are used by clinicians, but that have not necessarily been validated for use in clinical trials. These include α-feto protein and human chorionic gonadotropin (chorionic and testicular cancers);[43] CA-125 (ovarian cancer);[44] CA19.9 (pancreatic cancer);[45] carcinoembryonal antigen (colorectal cancer);[46] and prostate-specific antigen (prostate cancer).[47] However, the sensitivity and specificity of tests using such biomarkers can often be improved by combination with other prognostic variables. For example, CA-125 is not approved for diagnostic screening in early ovarian cancer. The combination of three other protein markers (transthyretin, apolipoprotein A1, and a cleavage fragment of inter-α-trypsin inhibitor heavy chain) with CA-125 improved the sensitivity of screening compared with CA-125 alone.[48]

Recent advances in high-throughput analysis of tissue or serum has allowed the identification of protein or gene expression 'profiles', which have greater prognostic value than single biomarkers alone. Although a comprehensive review of high-throughput methods is beyond the scope of this review, a couple of techniques that are now in common use are worthy of mention. Probably the two most widely used high-throughput techniques used in biomarker discovery are DNA/RNA microarray analysis and mass spectrometry.

Microarray analysis is based on the binding of RNA extracted from tumor tissue to a known nucleotide sequence library (DNA or oligonucleotide). It can be used to identify genes whose expression increases or decreases in a population of tumor samples compared with a reference sample (usually normal tissue).[49] This type of analysis has been used to generate expression profiles that differentiate between cancers of different lineage (e.g. between T- and B-cell acute lymphoblastic leukemia),[50] and to identify tumor 'proliferation' signatures[51] or gene expression signatures that can be correlated with patient outcome.[52] This technology was recently used to identify an expression profile of metastatic melanoma lesions, taken before therapy, which predicted patient responses to IL-2 immunotherapy.[53] The wider implication of this work is that it should be possible to use microarray profiling of tumors to identify profiles useful for stratification of patients according to their capacity to respond to vaccination.

A number of proteomic techniques (e.g. surface-enhanced laser desorption ionization [SELDI] and matrix-assisted laser desorption ionization [MALDI]-time of flight [TOF] mass spectrometry) are available, which, like microarray technology, generate molecular profiles of cancers (see review by Stroncek et al.[54]). SELDI-TOF uses the sequential binding of proteins to a microarray, according to biochemical characteristics (e.g. cation or anion exchange), and subsequent elution of whole proteins for mass spectrometry. In a similar way, MALDI-TOF involves binding of proteins to a matrix and subsequent enzymatic digest and mass spectrometry. Generally, such techniques take protein from accessible tumor tissue or from serum. The latter is a good medium in which to look for biomarkers as its constitution is influenced by the tissues through which it passes and there is evidence that tumors influence the proteomic profile of blood. Although proteomics has been used extensively to look for biomarkers in cancer, there is a paucity of data on changes in protein profiles in response to immunotherapy. However, it should be possible to detect changes in tumor markers in response to therapy. As this technology becomes more widespread the application to an immunotherapeutic setting is inevitable.

Analysis of complex data derived from either immunological analysis of samples from clinical trials or of mass spectrometry and protein expression data from tumor tissue can be achieved using ANN.[55] ANN has been devised to enable decisions, based on experience, to be collated and thus allow a computer to discern patterns in very complex sets of data. To achieve this, the network must go through the following three stages: (i) training; (ii) validation; and (iii) pattern recognition. In order to train a network, data from at least two patient groups are taken and the ANN is allowed to examine any correlation that may exist between the immunological data corresponding to each group and the disease phenotype. The network can then be validated, using known clinical outcomes, before finally allowing the ANN to examine raw data. At this point, the outcome is a ranking of variables, ranging from the most to the least predictive of a particular disease state. Using complex algorithms, it is then possible to define a subset of variables, which, when used in combination, may be predictive of a particular clinical outcome. Clearly, this should have benefit in clinical trials, since it may be possible to identify at an early stage those patients who will ultimately benefit from a particular therapy. It should be noted that the ANN only makes decisions based on data which it has previously seen and, therefore, as datasets increase in size so does the predictive power of the ANN. At no

stage does manual intervention lead to possible skewing of the data.

7. Conclusion

There are still no surrogate endpoints specifically validated for immunotherapy, but many biomarkers have been discovered that are known to relate to prognosis and may ultimately be useful for selection of patients according to their capacity to respond to vaccination. Currently, immunological monitoring and clinical response are used as the most significant biomarkers to assess response to vaccines. Although immunological monitoring has not yielded results in terms of prediction of response to therapy, new technologies such as multiplex cytokine assays (to broaden the range of data acquired) and ANN (to extract relevant information from data) hold the promise that use of immunological monitoring as a biomarker will improve. Standard (non-immunological) biomarkers may also be used to monitor the progress of therapy, but are unlikely to contribute to the understanding of underlying mechanisms in anti-tumor responses. Ultimately, it is hoped that improved biomarkers for cancer vaccines will allow accurate endpoints and may improve stratification of patients and result in more individualized approaches to immunotherapy. Advances in high-throughput technology for both measurement and discovery of biomarkers mean that new strategies for monitoring clinical trials may be on the horizon.

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