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The repertoire of human tumor-associated epitopes — identification and selection of antigens and their application in clinical trials

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In cancer patients, active immunotherapy has gained significant importance in recent years by implementation of novel substances into standard clinical care. These new drugs represent strategies which either use defined cancer associated antigens as vaccines or induce tumor-directed immune responses through generation of a general inflammatory state which has extensive autoinflammatory side effects by induction of autoreactive immune cells. Hence, the definition of suitable target antigens for immunotherapy remains a major challenge. These antigens should ideally be specific markers for individual tumors or should be at least structures overexpressed on the tumor as compared to normal cells. Recent approaches have defined algorithms and refined analytical methods for antigen identification and immunological validation that have already been evaluated in clinical studies. This article summarizes recent developments in tissue analysis on genome, transcriptome and HLA-ligandome levels and of antigen application in recent clinical vaccination trials.

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Introduction

Cancer cells vary from non-malignant cells in multiple alterations of gene expression and protein metabolism leading to changes in cell cycle, proliferation and growth control. This altered metabolism can on the one hand lead to overexpression of antigens (tumor associated antigens, TAA) whose expression on non-malignant tissue is limited, but can also lead to expression of tumor-specific antigens (TSA, also referred to as neo-antigens) which are derived from mutated proteins constituting biomarkers for an individual tumor [1–4]. Both, TAA and TSA can be therapeutically targeted with limited autoinflammatory adverse reactions. Since tumor cell metabolism can be altered at all stages, from mutations in DNA sequence to alternate protein expression, vaccination antigens can also be identified at all stages ranging from DNA sequencing to direct identification by analyses of the MHC ligandome.

Cancer immunotherapy has gained significant importance in recent years. Even in advanced stages, therapeutic modification of the immune system has improved clinical outcome in selected patients [5••,6,7••]. Moreover, with Ipilimumab and Sipuleucel-T the first two active immunotherapeutic agents have been legally approved for the treatment of cancer. While Sipuleucel-T represents a vaccination with a native tumor associated antigen (TAA) coupled to an adjuvant and loaded onto autologeous dendritic cells (DC), Ipilimumab nonspecifically activates the patient's immune system by blockade of the cytotoxic T lymphocyte antigen 4 (CTLA-4). This blockade probably results in tumor-directed as well as autoinflammatory reactions [8], with severe toxicities on the one hand but impressive and sustained tumor regression and prolonged survival in a subfraction of patients on the other hand [5••]. In this study, additional application of two anchor-modified peptides (gp100:209–217 (210M), peptide sequence IMDQVPFSV, and gp100:280–288 (288V), peptide sequence YLEPGPVTV) did not further improve clinical outcome. This might be partially due to the reason that the induced T-cells against anchor-modified peptides exhibit only very limited cross-reactivity against the naturally expressed native TAA and, hence, are not capable to effectively lyse tumor cells [9]. In contrast, vaccination with a synthetic multipeptide cocktail combined with low-dose immunomodulating chemotherapy improved overall survival in some renal cell carcinoma (RCC) patients (possibly favourable survival as compared to therapy with thyrosine kinase inhibitors) in the absence of severe side effects [7••]. Thus, the selection of further suitable antigens for cancer vaccination, and additional improvement of immunomodulating adjuvant therapy holds great promise for the development of better immunotherapies.

Definition of target antigens for immunotherapy requires three analytical steps: first, identification of antigens that are expressed by cancer cells. Second, specificity analysis by comparison of antigen expression on tumor tissue as

compared to corresponding non-malignant tissue to prevent or minimize self-reactivity. Third, validation: immunological testing whether identified antigens are immunogenic and can be recognized by the immune system. Immunological validation processes will be reviewed in another article in this issue.

Genomic identification

Recent advances in genome sequencing technology have allowed systematic screening of coding exons and flanking splice junctions for mutations in cancer cells as compared to normal tissue genomes of respective patients. Genetic mutations vary in extent and number from single point mutations [4] to chromothripsis where whole chromosomes are shattered and rearranged [10]. All these mutations could lead — if they are translated — to expression of mutated proteins whose fragments could be loaded onto MHC molecules. Besides their tumor-specificity, these neo-antigens could be exceptionally suitable for cancer vaccination because this technique could also identify mutations that occur early in cancerogenesis and then remain expressed even in advanced disease while other tumor antigen categories might be downregulated [11]. Next generation sequencing has led to reverse identification (see below) of several neo-antigens whose immune recognition is sufficient to eradicate established tumors. In a murine sarcoma model, massively parallel sequencing was used to determine tumor-specific mutations in nascent tumor cells to evaluate the immunogenicity of early tumors. Mutated antigens were crucial for immunoediting of cancer cells, and loss of expression led to tumor growth [12]. In a murine melanoma model, comparison of wild type and melanoma cells revealed point mutations leading to potential aberrant expression of tumor-specific peptides which were predicted on the basis of peptide binding motifs. These peptides could also be validated as immunogenic epitopes in tumor-challenge models [13••]. Of note, only about one third of the identified mutated peptides could be validated as epitopes. This might be due to the reason, that mere genome sequencing is insufficient to determine whether mutated sequences are translated and then result in mutated — and then potentially immunogenic — proteins or peptides [14]. To exclude this, analyses of the proteome and in particular a subfraction of the proteome, the HLA ligandome, would be required for direct antigen identification and selection (see below).

Reverse identification

With the knowledge of disparate protein sequences on the basis of DNA sequencing or known genetic variants between tumor and non-malignant tissue, candidate peptide epitopes for the respective HLA-types of patients can be predicted using Internet based databases like SYFPEITHI [15] or EpiToolKit [16]. These databases calculate epitope scores on the basis of HLA anchor amino acids and polarity of peptides. This *reverse* identification of epitopes can also be done for single protein sequences, then predicting potential epitopes from these proteins (for a recent publication see [17]). The identified candidate epitopes have to be validated for immunogenicity (*in vitro* priming of T cells, HLA-binding assays) [18], and in particular for their relevance, which is usually being done by testing epitope specific T cells for their ability to recognize and eradicate tumor cells expressing the respective antigen [19]. For clinical application, it mostly remains unclear whether these epitopes are expressed on individual tumors. Hence, induced immune cells against these epitopes should be not capable of sufficient tumor recognition when the tumor is lacking an antigen. Immunological validated predicted epitopes have been administered in multiple clinical studies (please refer to Table 1 for an overview of predicted antigens in recent clinical application).

Direct identification

Direct identification of TAA is performed by mass-spectrometry on peptides eluted from HLA molecules derived from target cells. Antigenic peptide sequences are determined by reversed phase HPLC fractionation followed by mass spectrometry. The recorded spectra are matched against spectra generated from publicly available protein databases (e.g. [20–22]). Since common protein databases cannot give information on a particular patient's individual protein sequences and the specific single nucleotide polymorphisms, individual germline mutations without prognostic relevance or alternative reading frames herein [23], the patient's tumor and the corresponding normal cell genome and transcriptome sequences are required for this (see above). The sensitivity of mass spectrometry for detection of HLA ligands has improved tremendously during the last years [21,22] but a full coverage of the entire HLA ligandome is still not possible. To our knowledge, no mutated neo-antigens have been identified by this method so far.

For an integrated identification of overexpressed (not mutated) tumor antigens, including selection and validation of antigens suitable for vaccination, the XPRESIDENT (e**X**pression profiling and analysis of peptide **PRES**entation by HLA molecules for **IDE**ntification of **N**ew tumor antigens in combination with **T** cell screening) platform was developed by our group [18,24]. The first multipeptide vaccination resulting from XPRESIDENT, IMA 901, is currently being evaluated in a phase III clinical study in advanced renal cell carcinoma patients [7••]. An extended version of this platform including mutation analysis is shown as a schematic overview in Figure 1.

Clinical application

Multiple clinical studies have applied defined tumor antigens as therapeutic vaccine. The selection of the applied antigens was performed by different approaches