An Integrative Computational Framework for Personalized Detection of Tumor Epitopes in Melanoma Immunotherapy

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ABSTRACT

In aggressive solid tumors like melanoma, a strategy for therapy personalization can be achieved by combining high-throughput data on the patient's specific tumor mutation and expression profiles. A remarkable case is dendritic cell-based immunotherapy, where tumor epitopes identified from the patient's specific mutation profiles are loaded on patient-derived mature dendritic cells to stimulate cytotoxic T cell mediated anticancer immunity. Here we present a personalized computational pipeline for the selection of tumor-specific epitopes based on 1) patient specific haplotype; 2) cancer associated mutations; and 3) expression profiles of mutation carrying genes. We applied our workflow to one melanoma patient. Specifically, we analyzed tumor whole exome sequencing and RNA sequencing data to first detect tumor-specific mutations followed by epitope prediction based on the patient's HLA haplotype and filtering of epitopes using expression profile and binding affinity. We performed docking studies to predict the best set of epitopes targeting the patient's alleles. The proposed workflow enables us to find personalized tumor-specific epitopes for stimulating cytotoxic T-cell responses.

Keywords: Personalized anti-cancer immunotherapy, NGS data, epitope prediction, docking studies, dendritic cells

INTRODUCTION

Anticancer immunotherapy is a therapeutic approach aiming at triggering or boosting an immune response against malignant cells (Topalian et al., 2011). In most of the cases, the natural immune system has the ability to detect and destroy malignant cells, and therefore a large number of micro-tumors are regularly detected and destroyed by the immune system (Poland et al., 2008). However cancer cells can eventually evolve mechanisms to circumvent the immune response, for example by: a) blocking immune cell activation; b) modulating the surface expression of tumor antigens; or c) creating a tumor microenvironment that inhibits the immune cell infiltration and response; and promotes tumor cell survival (Hanahan and Weinberg, 2000; Quail and Joyce, 2013).

Dendritic cells are a class of immune cells, playing a major role in triggering of the immune response. Dendritic cells migrate to the site of the microtumor, digest tumor debris, thereby processing and presenting tumor antigens to naïve T cells in the lymph nodes and other immune organs (Zitvogel et al., 1998). Naïve T cells differentiate into cytotoxic T cells and proliferate, thereby migrating to the tumor site and promoting tumor-cell killing. Thus, stimulating an immune response by modulating the dendritic cell-mediated response can be a way of boosting the systemic immune response against localized, but also disseminated tumors (Palucka and Banchereau, 2012; Mosca et al., 2007).

In the dendritic cell-based anti-cancer immunotherapy considered here, patient's precursor monocytes cells are taken from patient's blood sample, differentiated into dendritic cells, matured, and loaded with tumor peptides as antigens ex-vivo. Furthermore, these cells are administered to the patient intravenously by injection (See Figure 1). Tumor epitopes are small 8-11 amino acids long peptides from tumor-mutated or overexpressed proteins that are bound to HLA-molecules on the

cell surface. Active cytotoxic T cells recognize specific epitopes presented in the context of HLA molecules at the surface of tumor cells, and trigger the cytolysis of the peptide presenting tumor cells (Florea et al., 2003; Ossevoort et al., 1995).

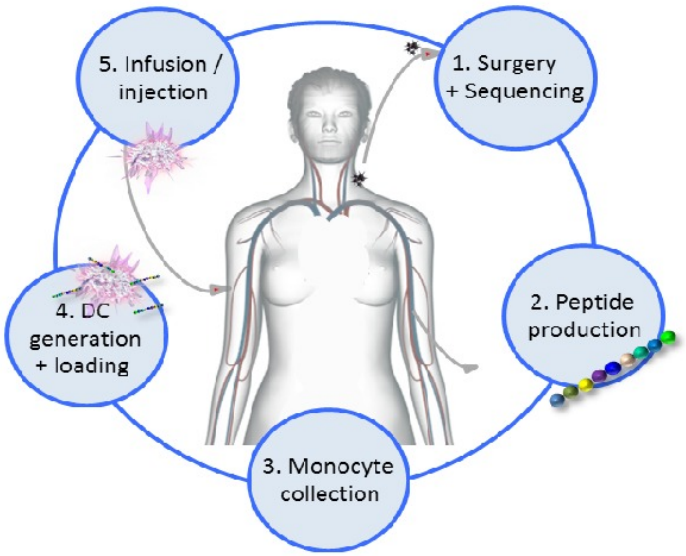

Figure 1. Epitope based dendritic cell vaccine. Immature dendritic cells are matured with a maturation cocktail containing IL-1beta, IL-6, TNF, and PGE2. Matured dendritic cells are loaded with peptides and are further injected into patient's body to induce T-cells that fight tumor cells.

This therapy has proved clinically to induce, in many cases, effective anti-tumor immunity by promoting activation of cytotoxic T cells targeting tumor cells for killing (Timmerman and Levy, 1999). In addition, this anticancer immunotherapy can be personalized (Gupta et al., 2015). To this end, patient's tumor and normal tissue DNA samples are obtained and sequenced to identify tumor-specific mutations. This information is employed to personalize the immunotherapy by selecting epitopes containing tumor-specific mutations for direct administration or loading in dendritic cells (Gilboa et al., 1998; Poland et al., 2008). With this aim, we hereby present a computational pipeline to predict tumor-specific epitopes in a personalized manner. The novelty of our workflow is that it integrates tools from NGS data analysis, epitope detection, and molecular simulations.

METHODS

Data: We analyzed whole-exome sequences (WES), RNA sequences (RNAseq), and the HLA profile from a patient with metastatic melanoma. The samples were obtained from the Department of Dermatology at the Universitätsklinikum Erlangen (Germany) and sequenced by CeGat GmbH (Tuebingen, Germany).

Exome sequencing and RNA Sequencing: Exomes of both tumor and blood samples were sequenced paired–end, with 99 percent of targeted bases covered with at least 10 reads. Exome capturing was performed with Agilent SureSelect Human All Exon Kit version 5(Agilent id S04380110). Only tumor sample was sequenced for transcriptome analysis. Illumina CASAVA was used for base calling and demultiplexing for both exome and RNA sequencing data.

Genotype calling: The data were preprocessed using FastQC (Andrews et al., 2010) and Trimmomatic (version: 0.36) (Bolger et al., 2014) for removal of adaptor sequences and low quality bases. Reads were aligned using the BWA algorithm with default parameters (version: 0.7.12) (Li and Durbin, 2009) against the hg19 assembly from the UCSC Genome browser (Kent et al., 2002). Approximately 97 percent of reads were mapped to the reference genome. Genome analysis toolkit (version: 3.4) (McKenna et al., 2010) was used for SNP/InDel detection. A gaussian mixture model was used to remove false positives due to shallow coverage across targeted exons. Somatic variants

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were detected using VarScan (version: 2.3.9) (Koboldt et al., 2012). Non-synonymous variants were identified using Annovar (Wang et al., 2010).

Gene Expression Analysis: Transcriptomic reads were aligned against the hg19 assembly using Tophat (version: 2.1.0) with bowtie algorithm (Kim et al., 2013) and their expression level was predicted using Cufflinks (version: 2.2.1) (Trapnell et al., 2010) to identify correlation between exome and transcriptomic data to avoid false positives structural variants.

Epitope Prediction: For epitope prediction, we first combined transcriptomic and genomic data to identify non-synonymous variants that were transcribed at significantly high levels. We calculated median to be used as threshold for expression level of transcriptomics data. Epitopes were predicted for all the corresponding protein variants by considering patient's HLA haplotype. To this end, IEDB offline was run with python script using artificial neural network algorithm (Lundegaard et al., 2008). Immunogenicity score for each epitope was calculated using IEDB T-cell epitope - immunogenicity predictor (Calis et al., 2013).

Epitope prioritization: The obtained epitopes were further prioritized and filtered using R script based on: 1) the number of patient alleles they target; 2) the tumor sample expression level of the mRNA for the gene with which the epitopes are associated; and 3) their binding affinity to their targets. For cases in which multiple epitopes were associated with an allele, we further used molecular docking and simulation approaches to predict the best epitopes binding to the HLA alleles. Overall, our aim was to find the minimal set of epitopes targeting all the HLA alleles present in the patient to induce the immune response in case of immune therapy. Three-dimensional structures of the alleles were obtained from the Protein Data Bank (www.rcsb.org) (Heinz et al., 1993) or modelled using the Modeller software (version 9.16) with the homology modeling approach (Webb and Sali, 2014), otherwise. We used Accelrys Discovery Studio 4.0 software suite (DS4.0) to design the 3D structure of the epitopes. We used our above filtering criteria, in order to identify an optimal set of epitopes having best binding affinity with patient specific HLA alleles. Docking of the epitopes in binding cavity of the HLA molecule was performed using CDOCKER protocol of DS4.0, which is a CHARMm-based molecular dynamics simulation method. CDOCKER uses soft-core potentials and an optional grid representation to dock ligands into the receptor active site. All-atom force field (CHARMm) family of force fields, were applied to both HLA allele and epitopes. This force field provide a wide coverage for proteins, nucleic acids and general organic molecules. A total of 10 orientations for each epitope were generated inside the binding cavity of the allele, which were further refined by simulated annealing method using Refined Pose Minimization protocol of DS4.0. All the interaction poses were arranged in the decreasing order of CDOCKER energy score (more negative CDOCKER energy, better the binding), which is the sum of interaction energy and the ligand strain in the binding cavity. After refinement, the top scoring pose for each of the epitope was used to compare and to find the best epitopes for a given HLA molecule.

RESULTS AND DISCUSSION

In many cases, advanced and metastatic solid tumors like melanoma have acquired a large number of somatic mutation and therefore it becomes difficult to manually select tumor–specific mutation-associated epitopes. To this end, we established a computational framework for the detection of an optimal, patient-specific cocktail of tumor peptides. The workflow proposed is shown in Figure 2.

The workflow is about first computing non-synonymous variants using whole exome sequences. By combing this information with expression data of tumor RNA sequences and docking studies, we present herein a computational workflow for epitope prediction. The workflow is designed to yield highly expressed and immunogenic tumor epitopes in a personalized manner. The workflow includes five steps, namely:

1. Preprocessing of the patient next generation data. Approximately 88 million reads were detected.

2. Detection of somatic tumor variants. In this case, the search was reduced to tumor associated single nucleotide variants (SNVs). To this end, WES data from the control and tumor samples were aligned to the human reference genome. Next, we computed the coverage and the SNP/INDELs in the samples using GATK tools. Somatic and germline SNVs were computed using VarScan. Annovar was utilized to obtain a list of non-synonymous ("missense") SNVs and their protein sequences for both control and tumor samples. Approximately 218 non-synonymous SNVs out of 4007 somatic varaints were detected.

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3. Prediction of SNV-associated epitopes for the HLA haplotype of the patient. We used IEDB to predict epitopes derived from non-synonymous tumor SNVs based on the patient's HLA haplotype. We predicted 1020 epitopes containing mutational variants.

4. Ranking of epitopes. This ranking was based on: a) the expression level of the tumor mRNA to which the epitope is associated, and some immune-related features of the epitope and b) the IC50 binding constant of the peptide to the HLA below 500nM. At this step, we were left with 251 mutational epitopes.

5. HLA-epitope docking and simulation studies for the top-ranked SNV-associated epitopes to predict an optimal set of epitopes.

Using the workflow with the case study of patient data, we computed 218 non-synonymous SNVs. IC50 binding affinity for each epitope was predicted only for SNVs involved in melanoma using an artificial neural network algorithm for HLA class I alleles. The total number of epitopes containing somatic non-synonymous SNVs obtained was 251. We generated a ranking of the epitopes based on the tumor expression of their associated mRNA and their immunogenic properties in Figure 3.

The patient's HLA haplotype was HLA-A*31:01, HLA-A*3201, HLA-B*18:01, HLA-B*56:01, HLA-C*01:02 and HLA-C*12:03. Unfortunately, we could not predict epitopes for HLA-B*56:01 and HLA-C*12:03 because MHC class I epitope prediction tools currently do not have these alleles. Table 1 shows the top seven epitopes based on our analysis targeting 4 different HLA alleles.

Epitope	HLA	Binding Affinity(nM)	Expression(FPKM)	Gene
VSHVLVPGGR	HLA-A*31:01	107	405.44	ECE2
KLMSVQLCPPV	HLA-A*32:01	140	256.58	GPT
HERMHSGEKPY	HLA-B*18:01	433	143.05	ZNF343
HALKRVSDDM	HLA-C*12:03	293	140.26	CAP1
DIFDAMFLVSF	HLA-A*32:01	152	119.54	PRKAR1A
AAQDFFSMCR	HLA-A*31:01	27	97.12	UROD
SLKRKNKRR	HLA-A*31:01	56	76.32	ZNF106

Table 1. List of potential epitopes binding to the patient specific HLA profile.

To predict the best set of optimal epitopes for each targeted allele, we compared the MHC-epitope complexes through docking studies. Among the four HLA alleles for which we could predict the epitopes, we found that only the 3D structure available was for the HLA-B*18:01 allele (PDB ID: 4JQV and 4XXC). Structures for other HLA alleles were thus designed using an homology modeling approach by selecting a best template available in the Protein Data Bank. We observed that our filtering criteria resulted in only one epitope for HLA-B*18:01 and HLA-C*01:02. However for HLA-A*31:01 and HLA-A*32:01, we found 3 and 2 epitopes respectively. To further predict the best binding epitopes for these two alleles we used molecular docking and simulation studies. We here present the results for HLA-A*31:01 only. In CDOCKER protocol, we observed that all the three epitopes were successfully bound to the defined receptor binding cavity. We further compared the best binding pose of all the 3 epitopes using CDOCKER energy and CDOCKER interaction energy, where more negative score indicate more favorable binding. The best poses for all the three epitopes along with their CDOCKER energy and CDOCKER interaction energy score are shown in Figure 4. Epitope "SLKRKNKRR" has the best interaction energy in comparison to other potential epitopes predicted for HLA-A*31:01 allele. Thus, indicating that docking studies can be used to filter out and select in a personalized manner the putative tumor mutated epitopes used for dendritic cell anticancer vaccination.

The experimental testing of the predicted epitopes can be performed in cell culture with primary material from healthy donors, which are partially HLA-matched. Dendritic cells can be transfected with mRNAs encoding the epitopes, and used for priming and expansion of autologous T cells. For read-out, again the epitope-encoding mRNAs can be used. Alternatively, PBMCs can be stimulated with the predicted peptides for several rounds, after which they can be electroporated with mRNA encoding the corresponding epitope and IFNgamma secretion can be measured (Prommersberger et al., 2015). This will indicate proper intracellular processing and presentation. To determine MHC-class II responses, long peptides can be utilized. If MHC-monomers are available for the respective haplotype, MHC-peptide tetramers can be generated, and binding to induced T cells can be investigated. These in vitro results will show whether the predicted epitopes are indeed immunogenic, and this information can in turn be used to optimize the in silico workflow.

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We presented a pipeline for patient specific tumor epitopes for personalized vaccines. Our aim was to get the minimal set of potential epitopes targeting to HLA allele. Therefore, we used several epitope filtering criteria and docking studies to detect optimal set of epitopes. Without using any filtering criteria we get a list of list of 1020 epitopes. We tried to select few epitopes that were targeting multiple HLA alleles but unfortunately, the ones that we could find have very low expression value. So, we selected top 10 epitopes out of 251 having high transcriptomic mRNA expression, and binding constant to show our proof of concept. We discarded 3 epitopes because of they showed low binding constant compared to others. There are several reviews discussing about NGS data analysis for epitope prediction but so far very few have been applied on patient data. The novelty in our work is the combination of exomic and transcriptomic data with specific epitope filtering steps followed by docking and simulation studies to predict an optimal set of epitopes. Gubin and colleagues (Gubin et al., 2015) and Schumacher and colleagues (Schumacher and Schreiber, 2015) presented how patient-specific neoantigens can enhance T cell reactivity against HLA alleles. Robbins and colleagues (Robbins et al., 2013) and Van and colleagues (van Rooij et al., 2013) analyzed whole exome sequences data to predict mutated antigens without considering HLA allele of patient's nor any epitope filtering criteria and docking studies. Brown and colleagues (Brown et al., 2014) showed epitope prediction on RNA-Seq analysis. All these contributions exemplify the independent use of several of the methods included in our workflow. However, to date and to the best of our knowledge nobody has explored in a real case study the integration of all this tools in a single computational workflow.

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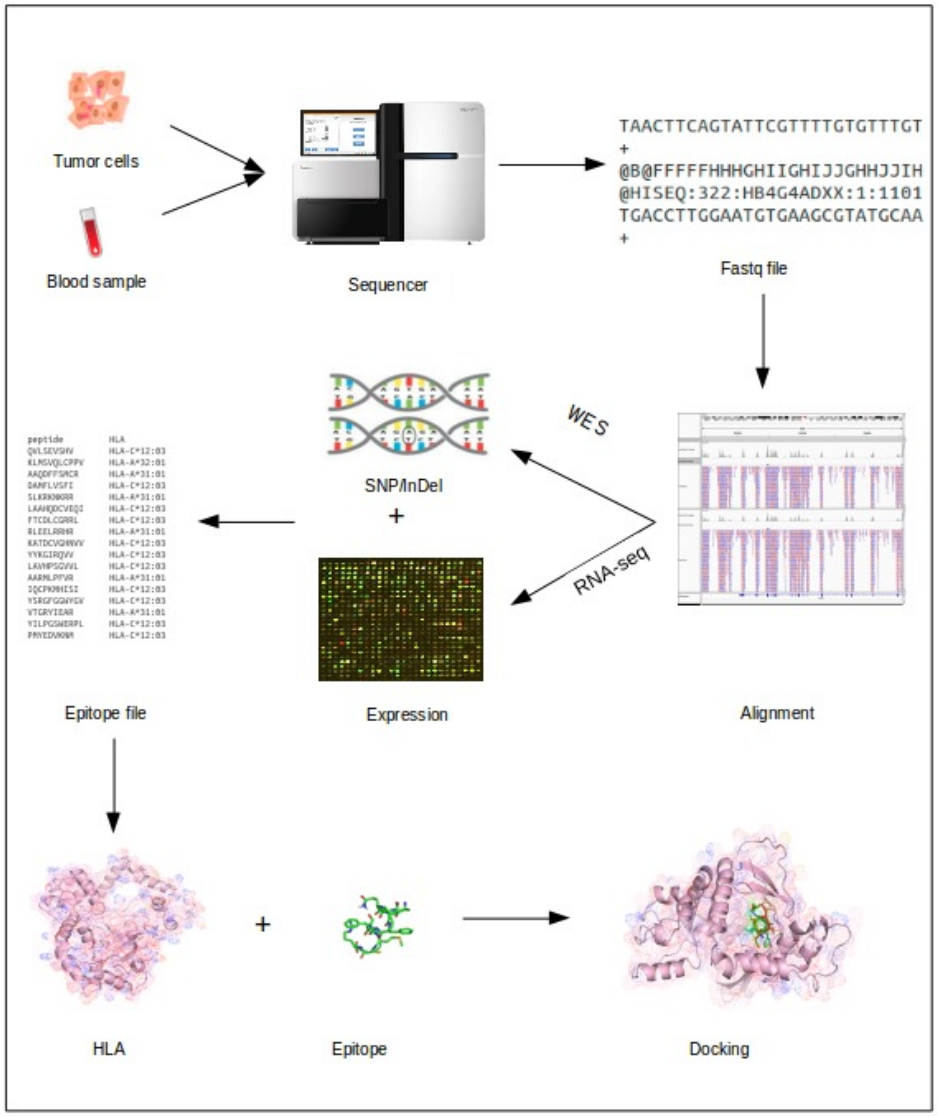

Figure 2. Computational pipeline for detection of patient specific tumor non-synonymous SNVs epitopes.

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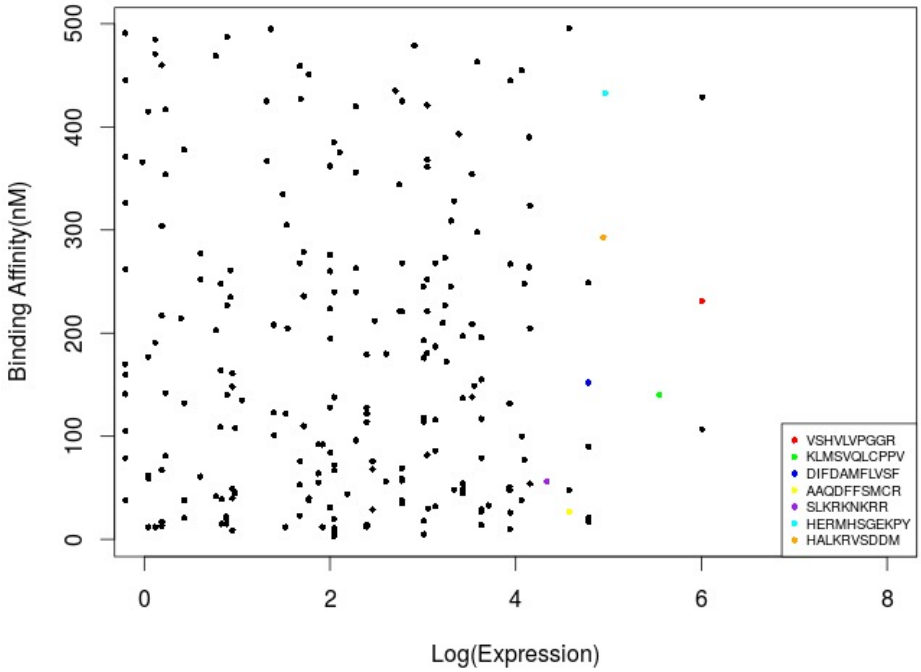

Figure 3. Selection of epitopes based on the expression profile of its gene and their IC50 binding affinity with patient specific HLA alleles.

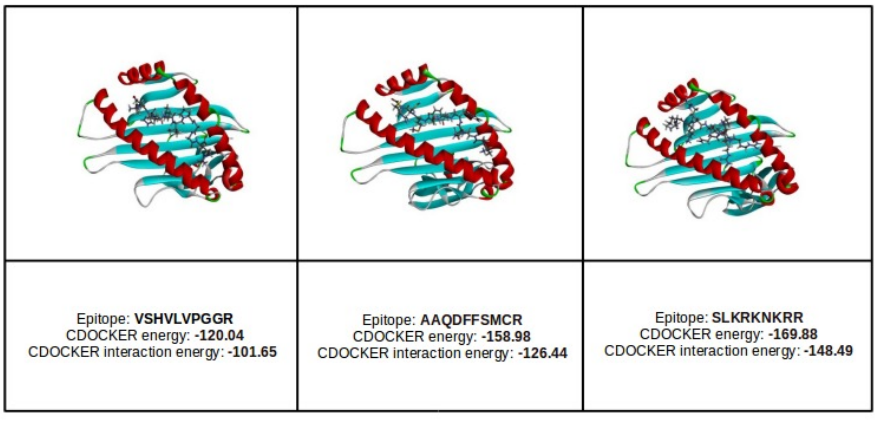

Figure 4. Top interaction of three potential epitopes (VSHVLVPGGR, AAQDFFSMCR and SLKRKNKRR) in the binding cavity of the HLA A*31:01 allele. The CDOCKER energy and CDOCKER interaction energy is mentioned for each of the epitope. HLA molecule is represented as solid ribbon colored according to secondary structure confirmation i.e. helices in red, beta sheets in cyan, turns in green and coils in white color. Epitope is shown as line diagram.

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