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A web platform for the network analysis of high-throughput data in melanoma and its use to investigate mechanisms of resistance to anti-PD1 immunotherapy

Florian S. Dreyer, Martina Cantone, Martin Eberhardt, Tanushree Jaitly, Lisa Walter, Jürgen Wittmann, Shailendra K. Gupta, Faiz M. Khan, Olaf Wolkenhauer, Brigitte M. Pützer, Hans-Martin Jäck, Lucie Heinzerling, Julio Vera*

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ABSTRACT

Cellular phenotypes are established and controlled by complex and precisely orchestrated molecular networks. In cancer, mutations and dysregulations of multiple molecular factors perturb the regulation of these networks and lead to malignant transformation. High-throughput technologies are a valuable source of information to establish the complex molecular relationships behind the emergence of malignancy, but full exploitation of this massive amount of data requires bioinformatics tools that rely on network-based analyses.

In this report we present the Virtual Melanoma Cell, an online tool developed to facilitate the mining and interpretation of high-throughput data on melanoma by biomedical researches. The platform is based on a comprehensive, manually generated and expert-validated regulatory map composed of signaling pathways important in malignant melanoma. The Virtual Melanoma Cell is a tool designed to accept, visualize and analyze user-generated datasets. It is available at: https://www.vcells.net/melanoma. To illustrate the utilization of the web platform and the regulatory map, we have analyzed a large publicly available dataset accounting for anti-PD1 immunotherapy treatment of malignant melanoma patients.

1. Introduction

The application of advanced high-throughput technologies in cancer research and other biomedical disciplines has generated a vast amount of clinical data. Typically, these technologies produce large datasets that are a valuable source of information to establish the complex regulatory molecular relationships behind the emergence of diseases. However, this large amount of data exceeds the processing and analysis capabilities of experimental researchers. Thus, the meaningful, context-specific and biologically relevant interpretation of high-throughput datasets (HTD) is a difficult task. Nevertheless, our current understanding of the complexity behind diseases like cancer makes the application of advanced high-throughput data generation technologies and their companion bioinformatics methods indispensable in biomedical research. Cellular functions and phenotypes are regulated by a multitude of molecular factors that exert their influence through complex signal transduction pathways. Rather than isolated, these signaling pathways are highly interconnected, they crosstalk and can extensively influence each other on the molecular level. Hence, they are integrated in large, complex and precisely orchestrated molecular networks through which cellular phenotypes are established, controlled and maintained. In pathophysiological conditions like cancer, this fine-tuned network orchestration is substantially disturbed. Mutations in and dysregulations of a multitude of individual molecular factors perturb the molecular networks and eventually lead to the transformation of healthy cells and the establishment of malignant cell phenotypes.

The development of malignant melanoma (MM), the most aggressive form of all skin cancers, is a consequence of the critical disturbance of precisely concerted molecular networks in melanocytes. The most common molecular lesions governing the development and progression of MM include, for example, mutations in BRAF or NRAS [1] that render the mitogen-activated protein kinase (MAPK) signaling pathway constitutively active [2,3]. Moreover, inactivating mutations in or the loss of phosphatase and tensin homolog (PTEN) [4,5] lead to an aberrant AKT signaling [6]. In addition, mutations, promoter methylations and deletion events in the cyclin-dependent inhibitor kinase 2A (CDKN2A) gene [7–9], leading to the suppression of $p16^{INK4A}$ expression, alter cell cycle signaling through the loss of negative regulation of cyclin-dependent kinases (CDK) 4/6 and hyperphosphorylation of the retinoblastoma protein (pRB) [10]. Consequently, this leads to

Abbreviations: MM, Malignant melanoma; HTD, high-throughput data; TF, Transcription factor

* Corresponding author at: Department of Dermatology, FAU Erlangen-Nürnberg, Hartmannstrasse 14, Erlangen, Germany.
E-mail address: julio.vera-gonzalez@uk-erlangen.de (J. Vera).

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DNA hyperreplication and aberrant cellular proliferation of melanocytes [10], likely in a E2F dependent manner given that activated MAPK signaling also positively regulates Cyclin D1 expression [11]. During tumorigenesis, the combination of a multitude of these and other mutations and deregulations cause oncogenic signaling events in tightly entangled regulatory pathways that can induce malignant cell growth behavior, tumor progression, and eventually the formation of metastasis.

In addition, these networks are also significantly disturbed when anti-cancer therapies are applied or when therapy-resistant tumor cell phenotypes emerge. In recent years, cancer research has focused on the development of targeted and personalized treatment approaches. These treatments take into account the individual molecular and mutational profile of a tumor in a particular cancer patient to select therapies that specifically interfere with factors whose oncogenic alteration drives tumor growth or progression. For example, vemurafenib is a targeted drug approved for MM therapy that is applied in the framework of personalized treatment regimens [12,13]. This drug selectively inhibits the most common mutation in MM, BRAFV600E [14,15], and it increases the survival of BRAFV600E-positive melanoma patients compared to conventional chemotherapy using dacarbazine [16,17].

However, the high frequency of mutations [18,19] occurring in MM enable the tumor to counteract targeted therapies through the development of resistance mechanisms [20–26], leading to quick disease progression [27]. These resistance mechanisms are often mediated via compensatory pathways and therefore this effect can only be understood in the context of deregulated pathways that crosstalk. Thus, the help of network biology tools is needed.

The high mutation rate of MM increases the mutational load of melanoma cells and the generation of tumor-specific antigens. This makes MM a very immunogenic tumor that should in principle be subject to suppression by the immune system. However, in the melanoma microenvironment, tumor-infiltrating immune cells, like cytotoxic T cells, can hardly execute their effector functions due to the emergence of tumor immune escape mechanisms. These mechanisms include, for example, the upregulation of immune-inhibitory factors on melanoma cells like programmed cell death ligand-1 (PD-L1), an immune checkpoint molecule [28] and antagonist of effector T cell function. This situation generates immunosuppressive conditions where T cell proliferation is inhibited [29] and tumor antigen-specific T cells undergo apoptosis [30].

In order to interfere with these immunosuppressive conditions and to unleash the cytotoxic capacities of tumor-reactive T cells present in melanoma patients [31], immune checkpoint inhibitors have been developed and are currently applied and investigated in clinical settings to treat several cancer types including MM. In recent clinical trials, the response rates to anti-PD1 therapies in metastatic melanoma ranged from 20 to 40%, a significant clinical benefit for melanoma patients [32]. However, a majority of tumor patients do not respond to this immune checkpoint inhibitor. A recent study suggests that mesenchymal melanoma cell phenotypes represent a cause for their resistance to anti-PD1 therapy [33]. Interestingly, the emergence of a mesenchymal phenotype in cancer cells relies on the deregulation of large and complex molecular networks [34]. Hence, the identification of the molecular compounds of this network and the analysis of its mechanism of deregulation in MM represents a suitable approach to detect central molecular factors perpetuating immunotherapy resistant phenotypes.

In this report we present the Virtual Melanoma Cell, an online tool developed to facilitate the mining and interpretation of HTD by biomedical researches. It is based on a comprehensive map of signaling pathways important in malignant melanoma. This map was manually reconstructed based on literature, is expert-validated and compatible with tools like Cytoscape. The Virtual Melanoma Cell is a tool designed to accept, visualize and analyze user-generated datasets. HTD can be mined on a molecular level, in the context of interconnected pathways and in a disease-specific manner. Furthermore, user-supplied datasets can be directly compared to each other or to a multitude of pre-loaded datasets derived from melanoma patient material or melanoma cell lines, selectable from the menu. To illustrate the utilization of our web platform and our melanoma map, we have analyzed a publicly available dataset accounting for anti-PD1 immunotherapy treatment of MM patients [33]. Our results reveal a core network that is differentially regulated in pre-treatment tumors of melanoma patients showing complete or partial response to anti-PD1 immunotherapy in comparison to non-responding patients. Interestingly, this network contains common and new factors known to contribute to the process of epithelial mesenchymal transition (EMT).

2. Material and methods

2.1. Reconstruction and annotation of the melanoma signal transduction map

The melanoma signal transduction map was developed based on current knowledge of melanoma biology available from the NCBI PubMed database. To reconstruct the map we collected and evaluated for suitability and quality published literature on MM, including original articles and reviews. Using this literature, we selected molecular factors and pathways described to be of importance in melanoma development and progression. We paid special attention to signaling pathways and molecular factors described to be mutated, deregulated or druggable in the context of MM. Furthermore, we interconnected the aforementioned pathways to other pathways known to play a role in key cancer phenotypes, like the growth factor receptor tyrosine kinase signaling pathways. We also incorporated miRNAs described in the literature to be deregulated in MM, as well as their experimentally validated targets. We translated the selected scientific knowledge into a map of interconnected signal transduction pathways using CellDesigner (v4.3 and v4.4) [35]. In a subsequent step, we manually queried the databases ClinicalTrials.gov and DrugBank [36] to identify therapeutic targets, and integrated the respective drugs and their mode of action in the network. Utilizing CellDesigner's Minimum Information Requested In the Annotation of Models (MIRIAM) [37] support, we annotated molecular interactions with PubMed IDs, genes with Ensembl [38,39] IDs, proteins with UniProt [40] IDs, miRNAs with miRBase [41] IDs, drugs and their emanating reactions with DrugBank [36] or ClinicalTrials.gov IDs, and small molecules with ChEBI [42] IDs. Beside this general annotation, we annotated all tumor-specific factors present in the map (e.g. amplified or deleted genes, mutated genes or proteins, miRNAs or proteins deregulated in expression) with PubMed IDs referring to reports in which the respective molecular alteration is described. Further we added text notes to the map in order to provide additional information and/or in case it was not possible to graphically reconstruct or annotate relevant molecular knowledge. When possible, we avoided redundancy of individual molecular factors. The pathways depicted in the map were constructed based on reports in which different experimental systems (e.g. cell lines, tissues) were used in *in vitro* or *in vivo* studies.

2.2. Expert validation of the developed and curated map

We recruited two postdoctoral researchers with several years of experience in molecular oncology or molecular immunology. They independently evaluated the accuracy of the reconstructed signal transduction pathways. To this end, we randomly selected from the map 50 reactions (approx. 5% of all reactions) and verified their correspondence to their annotated literature. The examiners were asked to focus on inconsistencies with the annotated literature. Based on this procedure, the findings were classified into one of five categories: 1) Correct, when both the molecules and the interaction are consistent with the referenced literature; 2) Incomplete, i.e. the reaction lacks some biochemical details from the annotated literature; 3) Weak evidence, i.e.