CSIR DAAD Exchange Program Research Project:

Identification of mechanisms of immunotherapy resistance in hepatocellular carcinoma

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Abstract

Liver cancer is the fourth leading cause of cancer-associated deaths worldwide. Hepatocellular carcinoma (HCC), the cancer of hepatocytes, accounts for over 80% of liver cancers. These tumors are treated with systemic chemotherapy, radiation or liver transplantation, but are rarely curable [1]. Currently, up to 50-60% of HCC patients receive systemic therapies such as tyrosine kinase inhibitors and immune checkpoint blockade (ICB), which prolongs patient survival by a few months [2]. Success of ICB in many solid tumors can be predicted by markers such as PD-L1 tumor expression or MMR/MSI status, which is not clinically indicative in HCC. Given the high costs of immunotherapy, the identification of powerful predictors indicating vulnerability is not only of clinical need but also high economic need [6]. Large scale analysis of patient samples as well as studies in mice models have identified several candidate genes that may play a role in predicting and driving response to immunotherapy. However, to the best of our knowledge, very few studies have functionally validated the role of these candidates in driving immune resistance. Furthermore, it is increasingly appreciated that functional validation of target genes in primary patient-derived organoids or stem cell models can have high clinical predictive value [7,8]. Additionally, the development of sophisticated patient derived organoids that can model the tumor microenvironment (TME) can serve as a welcome alternative to animal modelling of cancer.

In this study, we propose to establish immune competent autologous patient-derived organoid (PDO) model systems of HCC and corresponding healthy tissue control. Secondly, we will utilize a list of putative candidate genes that are clinically associated with immune resistance in HCC. Clinical informatics will be performed in published HCC patient datasets as well as biomaterial obtained from HCC patients in University of Magdeburg Medical Hospital to assess if altered expression of these genes is clinically associated with disease prognosis, response to immune checkpoint blockade and patient survival. We will then functionally interrogate the role of these genes in immune resistance through pooled CRISPR screening in HCC PDOs. Upon identification of new pathways mediating immune resistance, pharmacological inhibition of these pathways will be assessed for its ability to boost ICB response in HCC. We anticipate that this study will establish robust PDO models to study tumor-TME interaction in HCC and identify novel therapeutic avenues to reverse immune resistance in HCC.

Background

Liver cancer is an aggressive tumor type whose incidence is increasing every year worldwide, with 1 million cases expected by the year 2025. Hepatocellular carcinoma, the cancer of hepatocytes represents ~80-90% of all liver cancers. The incidence of HCC is on the rise in India, with reported incidence rates of 50-80,000 cases annually [1]. Risk factors for HCC include Hepatitis B and C virus infection (HBV, HBC), alcohol use, cirrhosis, smoking, diabetes and obesity. A majority of HCC cases in India are diagnosed in advanced stages, with patients displaying multi-focal hepatic tumor nodules and intra-hepatic metastasis. A lack of targeted therapies against advanced HCC portends poor survival of HCC patients in India, with a high ratio of mortality to incidence (0.95) [1].

Genomic analyses of HCC patient tumor tissues have been a powerful approach to identify new therapies in HCC. These studies have identified important driver mutations such as *MYC, TP53, CCND1, CTNNB1*, but few are translated to the bedside as they are either not druggable or present at lower frequencies [2,3,4]. This has limited the options in targeted therapy. However, a

breakthrough in HCC management is the advent of Immune checkpoint inhibitors or ICB [5,6]. ICB therapy in cancer typically involves monoclonal antibodies targeting CTLA-4 or Programmed Death Ligand 1 (PD-L1)/PD-1 pathway. PD-L1 is a cell surface protein frequently overexpressed on tumor cells and antigen presenting cells which signals to its receptor PD-1 on T-cells to suppress T-cell growth and activity (**Fig.1A**). Anti-PD-L1/PD-1 antibodies disrupt the ligand-receptor interaction resulting in restored T-cell activity, proliferation and effective tumor killing. Indeed, the development of ICB has been a groundbreaking advancement in the treatment of multiple tumor types such as blood cancers, melanoma, lung cancer and HCC. Particularly, PD-L1/PD-1 immune checkpoint inhibitors such as Nivolumab and Atezolizumab are now prescribed as first line therapy for majority of advanced HCC patients [5,6]. However, only ~30% of patients respond to ICB in HCC [5,6]. In some solid tumors such as colon cancer, MSI status has been shown to be a strong predictor of response to ICB [7]. Similarly, in lung cancer, tumor mutational burden is associated with response to ICB [8]. However, these are not clinically indicative of ICB response in HCC. *Our lack of understanding as to which HCC patient will respond to ICB is a clinically unmet problem as immunotherapy has high economic costs associated with it.* Thus, it is imperative to understand the mechanisms by which HCC display resistance to ICB.

In HCC as well as other cancers, an immunosuppressive 'cold' tumor microenvironment (TME) is associated with poor response to ICB whereas a 'hot' inflamed TME is associated with better responses to ICB and effective tumor killing (**Fig.1B, adapted from [9])**. Consistent with this, in HCC, several studies have identified that an 'inflamed' signature in tumors often predicts patient survival and response to ICB, whereas an 'excluded' signature predicted worse prognosis and poor response to ICB [10,11]. Functionally, mutations in *CTNNB1* and *P53* have been shown to drive ICB resistance in HCC [12]. But besides this, while several signatures that are predictive of ICB have been identified, to our knowledge, the functional role of these genes in driving immune resistance has not been fully elucidated.

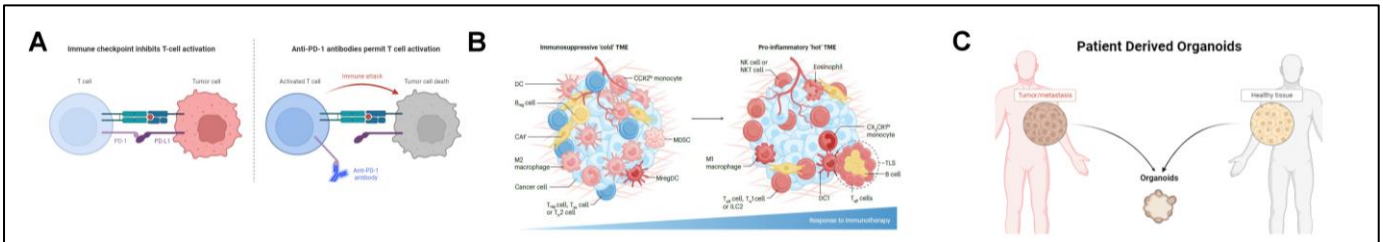

Figure 1. Patient derived organoids to study the tumor-TME. A.The PD-L1/PD-1 immune checkpoint in tumor immune evasion. B. Overview of 'hot' and 'cold' tumors. C. Patient derived organoids as a model to study the tumor- TME

Biological research undertaken to interrogate genes involved in ICB resistance has often utilized genetic mouse models of cancer or syngeneic transplants to dissect the role of genes in immune evasion *in vivo*. Complementary to this, patient derived organoids (PDOs) serve as a clinically relevant biological model system to study mechanisms involved in tumor progression (**Fig.1C**) [9.13]. PDOs recapitulate human HCC progression, including tumor-TME interaction and crosstalk. Unlike 2D cell culture, PDO are patient derived and represent disease progression in its pathological and physiological state. PDO use can also reduce the use of animals in research, while still providing very meaningful insights into both disease phenotypes and deeper molecular mechanisms. PDOs have been increasingly used to perform drug screening for precision medicine as well as test the efficacy of ICB in patient specific manner [9, 13]. Organoid technology advancement now allows for modelling PDO co-cultured with immune cells isolated from the same patient to model tumor-immune interactions in a 3D setting. Additionally, when integrated with approaches such as single-cell sequencing or pooled CRISPR screening, this model system has the potential to identify new mechanisms of immune resistance in HCC.

Key Research Questions and Methodology.

In this proposal, we will combine the power of PDOs, pooled CRISPR screening and the clinical data from the MES Biobank to identify new therapeutic targets that will boost ICB responses in HCC. Intra-clonal heterogeneity is widely prevalent in HCC with different clones demonstrating different patterns of immune infiltration as per patient tissue sample analysis [4,10]. Studies have shown that an 'inflamed' signature is correlated with better immune mediated killing, whereas an 'excluded' gene signature is associated with immune evasion and worse prognosis in patients [10,11,12]. While gene signatures that predict ICB response has been documented, the role of these genes in immune evasion is not well elucidated.

The key questions we propose to answer are:

1. *Are there gene signatures that predict ICB response and patient survival in HCC?*
2. *Are these genes functionally important in ICB resistance?*
3. *Can these genes be pharmacologically targeted to reverse ICB resistance in HCC?*

The Biobank at University of Magdeburg has a documented biobank of tumor tissue samples and matched normal tissues from HCC patients as well as other GI cancers [14, https://mes.ulf-kahlert.com/biobank/]. Magdeburg biobank and databank provides the relevant samples and data for this study; thus, no prospective collection is required. This dataset will be harnessed along with other published datasets such as TCGA HCC to understand the role of gene signatures correlated with ICB responses in HCC. We anticipate that our study will identify new mechanisms by which HCC mediates resistance to ICB and pave the way for therapeutically targeting pathways that will boost ICB responses in HCC patients.

Aim1: Develop HCC PDO-immune co-culture system that recapitulates the tumor immune microenvironment

1a. Set up a PDO-immune co-culture assay using MES HCC patient samples
1b. Quantify the extent of immune infiltration in these PDO models and correlate to ICB responses

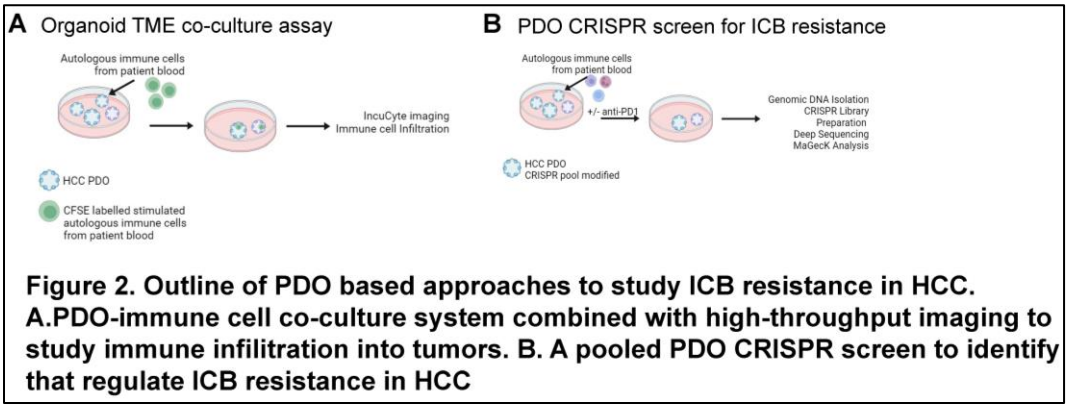

Figure 2. Outline of PDO based approaches to study ICB resistance in HCC. A.PDO-immune cell co-culture system combined with high-throughput imaging to study immune infilitration into tumors. B. A pooled PDO CRISPR screen to identify that regulate ICB resistance in HCC

Aim 1a. In order to better understand immune evasion mechanisms in HCC, we will harness the power of PDOs and generate a PDO-immune co-culture system to recapitulate the physiological state of a tumor inside a human patient. Tumor cells will be dissociated from patient tumors and cultured in Matrigel with appropriate factors to generate PDOs (**Fig.2A**). At the same time, patient blood will be utilized to isolate immune cells and stimulated to induce tumor killing activity. Stimulated immune cells will be labelled with CFSE, a cell permeable dye that allows to track cell division through fluorescence imaging/flow cytometry and co-cultured with PDOs (**Fig.2A**).

Aim 1b. As proof of principle, we will use MES's IncuCyte imaging system to visualize the infiltration of immune cells into PDO domes. Following this, we will systematically quantify the amount of immune infiltration into PDOs and correlate this with the histopathological analysis looking at immune infiltrates in matched tumor sections as well as clinical correlates such as treatment response. Despite the autologous, and patient-individual setup, we are aware of the

limitation of this approach as immune cells stem from systemic circulation and do not represent the tissue invaded immune cells. However, since this approach is scalable and can be done in a high-throughput fashion, we plan to pursue this over tissue slices/micro tumors which are not amenable for long-term manipulation or CRISPR based editing. These initial studies will help standardize the pipeline for subsequent studies.

Aim 2. Identify new mechanisms of ICB resistance in HCC.

2a. Characterize a gene signature predictive of ICB resistance in HCC.
2b. Perform a pooled CRISPR screen in PDOs to identify new genes that promote resistance to ICB in HCC.

Aim 2a. In order to identify mechanisms of ICB resistance in HCC, we will start with a powerful approach by looking at publicly available human HCC patient data [11,15] and generate a gene signature (~10-20 genes) that is predictive of ICB resistance in HCC. We will then use MES Biobank data as well as independent datasets such as TCGA to ask the question – *Is this signature associated with clinical outcomes such as patient survival, treatment responses and immune infiltration*. **A similar approach in human pancreatic cancer was recently demonstrated successfully by Dr. Ulf at MES [14], which highlights the feasibility of the study in this proposal.**

In order to identify which among these genes mediate immune evasion, we will undertake several different experiments. First, we will measure the expression of these genes in PDOs and matched normal liver tissue samples by western blotting and immunofluorescence to ask the question: *Does the expression of these genes at the protein level correlate with ICB response?* The MES Biobank and its databank allows us to ask such specific, clear, clinically relevant scientific questions. Next, we will measure the expression of these genes in PDOs and matched normal liver tissue samples by western blotting and immunofluorescence +/- co-culture with immune cells to address the question- *Does tumor-immune interactions alter the expression of these genes?* Finally, we will measure the expression of these genes in PDOs and matched normal liver tissue samples by western blotting and immunofluorescence +/- anti-PD-1 therapy to assess if ICB alters expression of these genes in HCC. These set of experiments will test if tumor cells regulate the expression of these genes upon encountering immune cells and if ICB further dysregulates the expression of these genes. Additionally, this will serve as validation at the protein level as to whether these genes are clinically correlated with response to ICB therapy.

Aim 2b. In order to functionally interrogate the role of these genes in regulating immune evasion, 10-12 sgRNAs per gene will be cloned into a lentiviral CRISPR plasmid to generate a targeted CRISPR library as performed before [16]. PDOs will be suspended into single cell adherent cultures and transduced with the LentiCRISPR library and transduced clones will be selected in Puromycin for 2-5 days in culture. Following this, PDOs will be co-cultured with activated immune cells +/- anti-PD-1 therapy to promote tumor cell killing by immune cells (**Fig.2B**). After therapy, the resistant clones will be deep sequenced and analyzed using the MaGecK algorithm to quantify sgRNA enrichment in the resistant PDO clones. This will help us identify genes whose loss mediates immune resistance. **Dr. Shruthy has previously successfully performed a genome-wide CRISPR screen in lung cancer for regulators of PD-L1, highlighting the feasibility of this study [17].**

Future directions: We anticipate that our study will establish a PDO based model to study tumor immune interactions and responses to ICB, which is applicable not only to HCC but to other tumor types. Our study will identify new pathways resulting in immunotherapy resistance. These findings will pave the way for subsequent studies to assess if pharmacological inhibition of genes/pathways that drive resistance can boost ICB responses in HCC PDOs.

Timeline and Feasibility: The proposed duration of Indian scientist visit to Germany is 3-4 weeks for this project. During this duration, we anticipate that Dr. Shruthy will be able to setup the PDO-immune co-culture system and perform experiments in Aim 1a and 1b. Aim 2a will be executed by Dr. Ulf's group. The pooled CRISPR screen proposed in Aim 2b will be performed in CSIR-IGIB by Dr. Shruthy during Dr. Ulf's visit where he will get a chance to learn about our molecular biology pipelines, CRISPR based

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