

Immune profiling of NSCLC tumors and matching normal lung samples by multicolor flow cytometry.

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With the recent advancements in cancer immunotherapy, understanding of tumor microenvironment becomes crucial to progressing oncology research. Standard method for tumor immune characterization today is traditional IHC technique with 1-2 color staining which presents certain limitations. In order to simultaneously identify immune infiltrate cellular components and parallel it with the analysis of expression of activation/suppression markers in various subpopulations, we developed a multicolor flow cytometry pipeline for freshly resected tumors.

Thirty one individual biomarkers were used in several antibody staining panels with sufficient redundancy to allow crossover and address intra-experiment variability. Aliquots of the samples were frozen and stained on separate days to test for inter-assay variability.

We performed flow cytometry analysis of 23 NSCLC tumors (17 adenocarcinoma, 1 adenosquamous and 5 squamous carcinoma subtypes) and matched normal lung tissue. Unsupervised clustering of 70 parameters, including percentages of cellular subtypes as well as modulator marker expression, showed distinct segregation of normal and tumor tissue samples. The immune infiltrate in resected tumors exhibited significantly decreased CD66b+ granulocytes and CD56+ NK cells (especially cytotoxic CD56+CD16+ NK cells) and highly increased CD3+ T and CD19+ B cells when compared to flow analysis of normal lung tissue. Adeno and squamous carcinomas were not segregated into separate groups by clustering analysis but rather joined together to form 3 subgroups defined by exhaustion/activation marker expression.

Grouping the tumor samples by the expression of the clinically relevant PD-L1 marker in immune cells indicated that “high PD-L1” tumors tend to have highly activated T cells populations. This finding correlates with the latest views on high PD-1/PD-L1 expressing immune cells being “negatively regulated” rather than “exhausted” as the result of their extensive interaction with cancer cells.

As we continue to characterize the immune profile of NSCLC tumors and paired normal lung, in conjunction with genetic and clinical information, we aim to further understanding molecular and clinical correlates that influence the tumor microenvironment and to ultimately overcome both innate and acquired resistance to immune therapy agents.

An epigenetic-focused CRISPR/Cas9 screen to identify regulators of IFN γ -induced PD-L1 expression

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PD-L1 (B7-H1, CD274) is a clinically validated immuno-oncology target, which is often over-expressed on the surface of tumor cells. PD-L1 binds to PD-1 expressed on T cells generating an immunosuppressive signaling response that limits T cell activation and facilitates immune evasion. The tumor microenvironment often recruits immune cells that produce a number of secreted factors, including IFN γ , a potent inducer of PD-L1 expression on tumor cells. Blocking IFN γ -induced PD-L1 expression with small molecules could be a potential alternative to antibody-based PD-L1/PD-1 blockade. Recent studies on human patient samples indicate that the level of PD-L1 expression on tumor cells is inversely related to the level of DNA methylation at the PD-L1 promoter (Gettinger et al, 2015), suggesting that PD-L1 expression is epigenetically silenced in tumor cells with low PD-L1 expression. Therefore, cytokines that induce PD-L1 expression on tumor cells, such as IFN γ , may regulate the activity of epigenetic silencing factors at the PD-L1 promoter, and identification of these epigenetic factors could provide novel therapeutic targets to block PD-L1 expression on tumor cells.

CRISPR/Cas9 gene editing has recently emerged as a powerful technology for phenotypic screening. To identify potential epigenetic regulators of IFN γ -induced PD-L1 on tumor cells, several murine tumor cell lines were treated with IFN γ and PD-L1 expression was monitored by flow cytometry. The ovarian cancer cell line ID8 demonstrated high PD-L1 expression following IFN γ stimulation, and stable Cas9 expressing clones were generated. A high expressing Cas9 clone was selected for follow-up transduction with a sgRNA library targeting >350 known epigenetic factors, plus numerous positive and negative controls. Library transduced cells were evaluated in two separate screening streams: i) an enrichment screen to identify genes regulating IFN γ -induced PD-L1 expression, and ii) a depletion screen to identify genes essential for the growth of ID8 tumor cells. For the enrichment screen, sgRNA transduced cells were treated with IFN γ , and FACS was performed to collect cells with low PD-L1 expression i.e. cells refractory to IFN γ -induced PD-L1 expression. Genomic DNA was then isolated from the sorted cells and sgRNA sequences were quantified by next-generation sequencing (NGS). As an important validation of the FACS-based screening format, the most highly enriched sgRNAs in the low PD-L1 population were PD-L1 itself, and the canonical mediators of IFN γ signaling JAK1/2 and STAT1. For the depletion screen, sgRNA transduced cells were cultured for up to 14 days, with cell pellets collected on day 0, 3, 7 and 14 for NGS quantification of sgRNAs. All positive and negative controls scored as expected and several epigenetic factors were strongly depleted indicating an

essential role for ID8 cell growth. In summary, CRISPR/Cas9 gene editing is a powerful screening technology for the identification of factors essential for cell growth, and when paired with FACS, is a useful methodology to identify factors regulating expression of immune checkpoint molecules.

Credentialing the concept of "co-clinical trials": Utility of lung cancer PDX models derived from patients on AZD9291 clinical trials.

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Abstract:

Background: The EGFR T790M mutation is the most common mechanism of acquired drug resistance to currently approved EGFR inhibitors gefitinib, erlotinib, and afatinib. AZD9291 is a mutant-selective EGFR inhibitor effective against both EGFR activating and T790M mutations while sparing wild type EGFR. AZD9291 is highly active in patients with lung cancer with the EGFR T790M mutation, with a response rate of 61% and progression free survival of ~10 months. However, as resistance to AZD9291 is beginning to emerge, we aimed to develop patient derived xenografts (PDXs) using pre-treatment biopsies obtained from patients with acquired resistance to first-line EGFR inhibitors enrolling on clinical trials with AZD9291. These clinically annotated PDX models allow direct correlation with the clinical efficacy of AZD9291 in patients and may be useful in studying mechanisms of acquired resistance to AZD9291 and refine strategies for treatments.

Methods: Pre-AZD9291 treatment tumor biopsies (core needle biopsies or pleural effusions) were implanted into the flank or sub-renal capsule of NSG mice. Tumors were serially passed in NSG mice for up to 3 generations. Successfully established models were expanded and treated with AZD9291. The efficacy in the PDX model was compared to the clinical efficacy of AZD9291 in the patient from whom the model was derived. **Results:** 33 patients underwent a pre-AZD9291 treatment biopsy (26 core needle; 7 pleural effusions). 26/33 patients enrolled in the AURA AZD9291 clinical trial for patients with acquired resistance to first-line EGFR inhibitors. From these patients, 10 PDX models have been successfully developed so far and confirmed by ddPCR to maintain fidelity to the original patient tumor's EGFR mutation status. These models include 6 with EGFR T790M and 4 with EGFR non-T790M mechanisms of resistance to erlotinib. Among the 10 patients used to develop PDX models, the best clinical response to AZD9291 included 5 partial responses, 3 progressive diseases, 1 stable disease and 1 acquired resistance. A subset of these PDX models were further tested for their sensitivity to AZD9291 and the data was consistent with the clinical responses of the

patients. Two models, DFCI 243 and DFCI 217 (both with EGFR T790M; both patients with PR > 9 months) were treated with gefitinib (6.25mg/kg) or AZD9291 (25 mg/kg). Both models were confirmed to maintain EGFR T790M and had a dramatic response to AZD9291 but not to gefitinib treatment. However, tumors rapidly regrew upon cessation of AZD9291 treatment. DFCI 306 PDX model established from a patient with acquired resistance to monotherapy with AZD9291, showed AZD9291 resistance in vivo. AZD9291 treatment of DFCI 284, a model with EGFR T790M derived from a patient with primary resistance to AZD9291, is underway.

Conclusion: We have developed PDXs from patients with erlotinib resistance who were treated with AZD9291. The PDX platform is currently being utilized in studies to refine strategies to: a) improve durability of responses to AZD9291 in EGFR T790M mutation positive patients and b) identify combinations in EGFR T790M mutation positive or negative patients who have de novo or acquired resistance to AZD9291.