

Title: Validation of a novel microfluidic device for screening of immune checkpoint inhibitors using 3D organotypic tumor spheroids

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Immune checkpoint blockade, including anti-PD-1 and PD-L1 therapies, is revolutionizing cancer care for melanoma, lung and other malignancies. However, pre-clinical models in which to rapidly and robustly evaluate the efficacy of these treatment approaches, including the development of combination therapies and/or relevant biomarkers, are lacking.

We have developed a novel approach for evaluating *ex vivo* response to immune checkpoint blockade using murine- and patient derived organotypic tumor spheroids (mDOTS/pDOTS) cultured in a new 3D microfluidic system. We have demonstrated that spheroids isolated from fresh mouse and human tumor samples retain autologous lymphoid and myeloid cell populations.

Methods: Using MC38 and B16F10 syngeneic mouse cancer models with reported responsiveness or resistance to PD-1 blockade respectively, we treated mDOTS in the 3D microfluidic device with either control IgG or increasing concentrations of anti-PD-1 for up to 6 days and quantified cell viability by dual labeling fluorescence microscopy. To confirm tumor cell-specific death and survival of mDOTS immune component we applied multi-color immunofluorescent staining of CD8 as well as CD11b+ and CD11c+ myeloid cells in parallel with viability dye calcein AM. Tumor cell-specific death was assessed by absence of calcein AM and pyknotic nuclear staining in CD45 negative mDOTS component.

Results: We demonstrated dose-dependent killing following treatment with anti-PD-1 in MC38 spheroids. We showed presence of viable cytotoxic lymphocytes as well as other immune cells for up to 5 days of *ex vivo* growth, when we observed the beginning of tumor but not immune cell killing. In contrast, mDOTS derived from B16F10 melanoma, which is reported to be minimally sensitive to single agent PD-1 blockade, exhibited little cell death despite identical treatment and comparable immune profiles (at highest anti-PD-1 dose of 10ug/mL viability was $98.2\% \pm 0.04$ versus $29.7\% \pm 1.68$ in MC38). No significant cell death (viability $88.2\% \pm 2.18$) was also noted in spheroids generated from MC38 cell line alone, conceivably due to the absence of autologous cytotoxic T cells. Additional analysis of immune dynamics will be discussed.

Conclusions: These data demonstrate the ability to recapitulate sensitivity and innate resistance to PD-1 blockade *ex vivo*. By being able to study the impact of immunotherapy and targeted therapy in a rapid *ex vivo* system the development of drug combinations and their associated biomarkers can be significantly enhanced. The preclinical studies can be used to develop and guide the next generation clinical trials for lung cancer patients, and hopefully improving their success rate.

Title: Cross-platform detection and quantification of actionable mutations in cell-free DNA shows high concordance and correlation between next-generation sequencing and droplet digital PCR

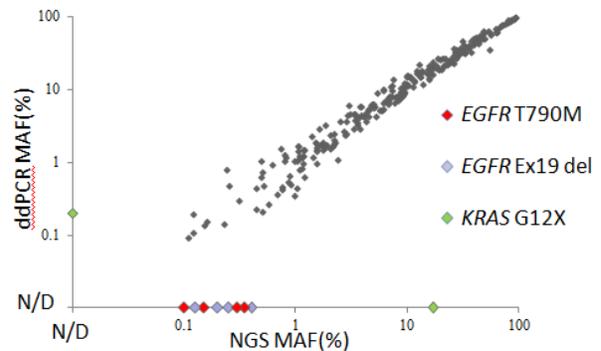
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Background: Non-invasive genotyping of cell-free DNA (cfDNA) provides physicians with the ability to identify genomic alterations relevant to cancer, evaluate response to treatment, and uncover potential mechanisms of resistance. Here, we analyze the concordance of next-generation sequencing (NGS) and droplet digital PCR (ddPCR) assays in detecting and quantifying actionable mutations from cfDNA of lung, colorectal, and breast cancer patients.

Methods: We studied a cohort of 221 advanced cancer cfDNA samples with completed Guardant360 digital sequencing results. Variant allele frequencies (% AFs) ranged from 0.1% to greater than 90% for common driver and resistance mutations. Blinded to plasma NGS results, we performed ddPCR using our validated assays for *EGFR* L858R, exon19 del, T790M (113 presumed lung cases); *BRAF* V600E, *KRAS* G12X (77 colorectal cases); *ESR1* D538G, Y537C and *PIK3CA* H1047R (31 breast cases). Following unblinding, qualitative and quantitative accuracy were compared.

Results: Results (see below) show a high concordance between NGS and ddPCR, with 95.5% (211/221) concordance. 9 of the 10 discordant samples had an AF below 0.5%, while only one sample above 0.5% AF was discordant. Interestingly, the one discordant sample above 0.5% was shown to have a *KRAS* double mutation (c.33T>C A11A and c.34G>T G12C) that abrogated ddPCR probe binding, leading to a false negative call by ddPCR. AFs calculated by NGS and ddPCR showed strong correlation by linear regression (R between 0.951 and 0.999).

Total Concordance	95.5% (211/221)
Concordance greater than 5% AF	99.1% (109/110)
Concordance between 1%-5% AF	100% (71/71)
Concordance between 0.5%-1% AF	100% (15/15)
Concordance less than 0.5% AF	64% (16/25)



Conclusions: This interlaboratory comparison of actionable mutations in cfDNA from lung, colorectal, and breast cancers by orthogonal genotyping platforms demonstrates a substantial concordance in detection and quantification between NGS and ddPCR. Our cross-platform study establishes the feasibility of standardization of qualitative and quantitative measurements of cfDNA-detected alterations. Validation at allele frequencies near the limit of detection (LOD) of liquid biopsy assays remains a challenge, which warrants further investigation into validation near LOD.

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Inhibition of IDO1 with epacadostat enhances anti-tumor efficacy of PD-1 blockade in a syngeneic glioblastoma (GBM) model

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Purpose: To determine if epacadostat, an oral indoleamine 2,3-dioxygenase (IDO1) inhibitor has therapeutic benefit against GBM when administered as single agent and with PD-1 blocking antibody.

Methods: An initial survival experiment was performed to assess efficacy and was followed by an identical repeat experiment for validation. 1×10^5 luciferized GL261 cells, a murine GBM tumor line derived from intracerebral methylcholanthrene implantation, were stereotactically implanted intracranially in albino syngeneic C57BL/6 mice. Mice with increasing bioluminescence on days 3 and 6 were randomized (n=8/group) to receive treatment beginning on day 6: anti-PD-1 (332.8H3, mouse IgG1; 500 μ g intraperitoneal (IP) on day 6, 250 μ g q 3 days X 7); epacadostat (Incyte Corporation, orally dosed at 300 mg/kg/day for 5 days on/2days off for 3 weeks); anti-PD-1 + epacadostat; and control therapy (isotype IgG antibody IP and 0.5% methocel in water). Tumor response assessments were performed by quantifying bioluminescence and survival. A re-challenge experiment was performed in long-term survivors to assess for tumor immune responses capable of preventing relapse. All long-term surviving mice (defined as ≥ 100 days) from the efficacy experiment were injected with 1×10^5 GL261 cells in the contralateral hemisphere and followed for survival.

Results: In both preclinical efficacy experiments, median survival in the epacadostat monotherapy group did not differ from controls (approximately 30 days). Four of 8 mice (50%) treated with anti-PD-1 were long-term survivors in both efficacy experiments. In the epacadostat plus anti-PD-1 combination group, 81% of the mice were long-term survivors (7 of 8 in experiment 1 and 6 of 8 in experiment 2). Of note, none of the long-term surviving mice developed evidence of tumor; thus the median survival among the anti-PD-1 and epacadostat plus anti-PD-1 combination groups were both > 100 days. In the re-challenge study, all of the mice who underwent GL261 re-inoculation survived > 100 more days with no evidence of tumor recurrence.

Conclusions: IDO1 inhibition with epacadostat increased the eradication rate of anti-PD-1 therapy in an orthotopic syngeneic GBM model and long term survivors rejected tumor following orthotopic re-challenge. Further combinatorial studies incorporating IDO inhibitor therapy for GBM, including mechanistic studies, are warranted.