Assessment of RAS-RAF-MAPK Pathway Perturbations and PD-L1 Expression in an Isogenic 3D Tissue-Culture Model of Drug-Resistant Melanoma

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AACR Abstract #5021

Introduction

Development of the next generation of anti-cancer drugs, biologics, and immunotherapies is currently hampered by extremely poor success rates of seemingly promising experimental therapies in human clinical trials. These poor success rates are partially due to a lack of biologically relevant cancer model systems. The 2D culture system, while relatively inexpensive to use and easy to analyze, may not always be adequately representative of the tumor microenvironment. Conversely, the use of animal models is costly and time consuming. The 3D culture system is able to represent biologically relevant complexities such as cell-cell communication, differential proliferation rates, and compound penetration. We use CRISPR/Cas9 genome editing technology to generate carefully designed and precisely edited cell-based 3D model systems with both physiological relevance and well-controlled genetic and drug-susceptibility profiles. These new models will enable the investigation of specific molecular mechanisms, biofunctional outcomes of newly identified genetic alternations, and targeted therapeutic drug responses within a more biologically intricate context.

In this study, we use the CRISPR/Cas9 system[1] to generate isogenic drug-resistant melanoma models that can be used as either 2D or 3D cancer models, or used in studies designed to further our understanding of the mechanisms of acquired drug resistance. Two different models were generated from the BRAF inhibitor sensitive A375 melanoma cell line. We introduced either the NRAS Q61K or the KRAS G13D point mutations, both of which are known to confer BRAF inhibitor resistance and are commonly encountered in BRAF resistant tumor samples.[2] We then assessed the susceptibility of these new isogenic cell lines to traditional BRAF inhibitors in both 2D and 3D model systems. Using the parental A375 line as a control, we also determined the specific effect of these point mutations on the RAS-RAF-MAPK signaling pathway, a key component of cell-cycle escape and tumor proliferation. Furthermore, we assessed the impact of these mutations on the expression of programmed death-ligand 1 (PD-L1), which recent advances in cancer immunology have directly linked to cancer immune evasion and poor clinical outcomes.[3]

Our results show that A375 melanoma isogenic cells carrying KRAS G13D have dramatically increased EGFR expression levels, while isogenic cells carrying NRAS Q61K have constant activation of the MEK-ERK pathway. We also found a significant increase in PD-L1 expression in KRAS G13D, but not in NRAS Q61K A375 cells, indicating that expression of PD-L1 is directly linked to KRAS mutation or the downstream effects thereof, rather than to a general increase in RAS-RAF-MAPK signaling. These results have direct implications for cancer immune checkpoint molecule studies, and highlight the utility of these isogenic melanoma models in both 2D and 3D applications in research and development of novel anti-cancer drugs and combination therapies.

Background

CRISPR Genome Editing Technology

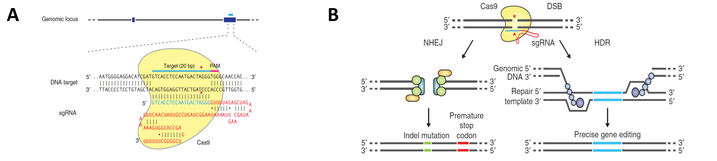


Figure 1. CRISPR/Cas9 genome editing technology. (A) The Cas9 enzyme is an RNA-guided DNA endonuclease that uses a bound single guide RNA (sgRNA) to interrogate the genome for stretches of sequence complementary to the guide sequence. When a match is found Cas9 creates a double-stranded break (DSB) in the genome at that location. (B) DSBs in the genome can be repaired either by the error-prone non-homologous end joining (NHEJ) pathway, or via homology-directed repair (HDR) from a donor sequence to generate a precise edit.

RAS-mediated Resistance to BRAF Inhibitor in Melanoma

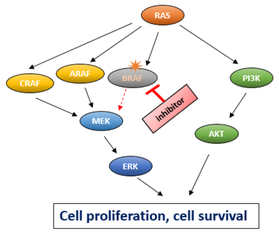


Figure 2. Mechanism of aquired drug resistance in BRAF V600E melanomas. BRAF V600E melanoma cells chronically treated with BRAF inhibitors aquire drug resistance by switching between the three isoformas of RAF (ARAF, BRAF, and CRAF) to activate the MAPK pathway for further cell proliferation and survival. Increasing growth factor receptor signaling or reactivation of the MAPK pathway may thus allow for drug resistance. This can occur following aquisition of activating mutations such as NRAS Q61K and MEK C121S, as well as elevated basal expression of other key components of the pathway.

Results

CRISPR Editing Strategy and Sequencing of NRAS Q61K and KRAS G13D Isogenic Lines

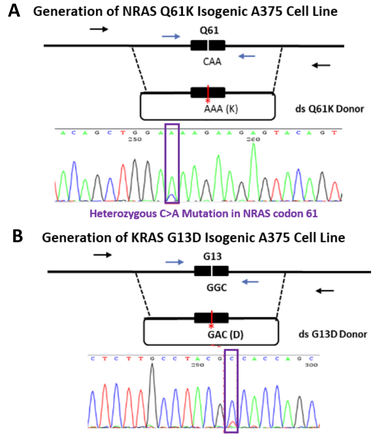


Figure 3. Editing strategy and sequence confirmation of RAS isogenic A375 lines. (A) Two guide RNAs were transiently expressed in ATCC® CRL-1619™ A375 cells from Cas9/sgRNA all-in-one plasmids to make cuts on either side of the target exon. The target exon was then replaced by donor sequence from a plasmid carrying the desired AAA>CAA Q61K point mutation, yielding the heterozygous NRAS WT/Q61K A375 cell line (ATCC® CRL-1619IG-2™). (B) The same genome editing strategy was used for the generation of the heterozygous KRAS WT/G13D A375 cell line (ATCC® CRL-1619IG-1™). Black and blue arrows indicate the location of genotyping and sequencing primers, respectively.

Impact of 3D Culture Format on RAS-RAF-MAPK Signaling in A375 Isogenic RAS Mutant Lines

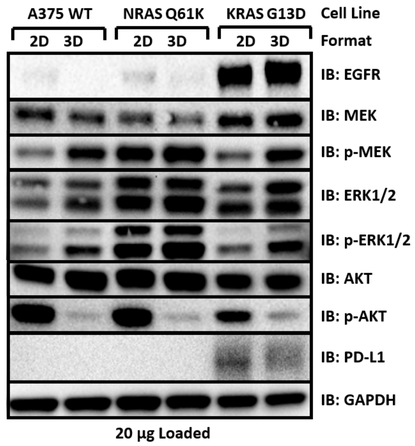
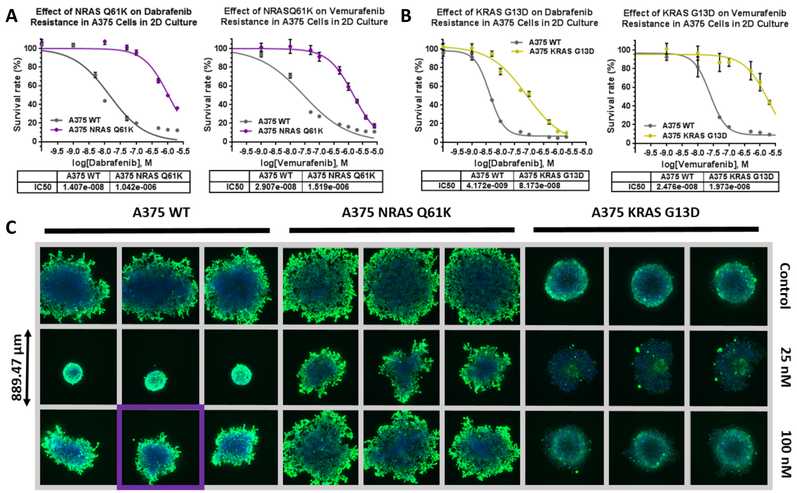


Figure 4. RAS-RAF-MAPK signaling in A375 RAS isogenic lines in 2D and 3D tissue culture. Total cellular protein was harvested from A375 wild-type and isogenic cells grown either in a 2D mono-layer or in a 3D culture format for seven days. The resulting protein was then analyzed by immunoblotting with antibodies against EGFR, MEK, pMEK, ERK1/2, pERK1/2, AKT, pAKT, PD-L1, and GAPDH.

Analysis of BRAF Inhibitor Resistance in NRAS Q61K and KRAS G13D Isogenic Cell Lines in 2D and 3D Tissue Culture



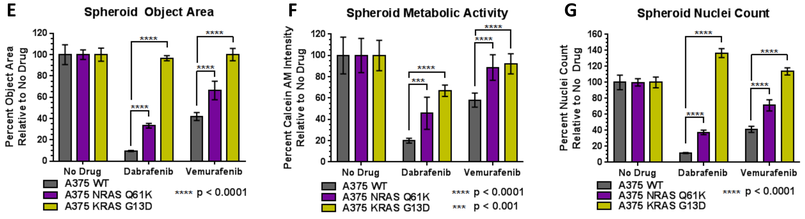


Figure 5. BRAF inhibitor resistance of A375 RAS isogenic lines in 2D and 3D tissue culture. (A) Dose-response curves for the BRAF inhibitors dabrafenib and vemurafenib in A375 WT and NRAS Q61K isogenic cell lines grown in 2D cell culture and treated with the indicated drug for 72h. (B) Dose-response curves for dabrafenib and vemurafenib in A375 WT and KRAS G13D isogenic lines grown in 2D culture and treated with drug for 72h. (C) BRAF inhibitor resistance of NRAS Q61K and KRAS G13D cell lines in 3D cell culture. 500 cells were seeded in each well of a 96-well ULA Spheroid Microplate and grown for 72 hours in the absence of drugs to allow for spheroid formation. Spheroids were then treated with the indicated concentration of BRAF inhibitor for an additional 72 hours. Finally, spheroids were stained with 2.0 µM calcein AM green and live-cell nuclear stain for three hours and imaged by confocal microscopy on a high-content platform. Images were aquired using a 10x objective and each image represents a confocal Z-stack projection of 20 15-micron Z-slices for a total depth-of-field of 300 microns. (D)The resulting composite spheroid images were then analyzed in the green and blue channels for spheroid size/viability and nuclei count, respectively. Left: enlarged dual channel image of purple-boxed A375 WT spheroid from (C). Center: each spheroid was identified and segmented as a single primary object in the green channel (pink outline), and the spheroid object area and total calcein AM green signal intensity were calculated. Right: each nucleus within the spheroid was then identified segmented in the blue channel (green outlines) and the number of nuclei visible in each spheroid was reported. (E) Results from (D) were then normalized to the undrugged condition for each cell line and plotted. Spheroid Object Area represents the total area of the spheroid calculated as the primary object in the green channel. (F) Spheroid Metabolic Activity represents the total signal intensity detected in the green channel within the primary object. (G) Spheroid Nuclei Count represents the number of objects segmented in the blue channel that also fell within the primary object segmented in the green channel. Statistical comparisons were performed by two-way ANOVA with N = 3 or more spheroids for each sample.

Effect of KRAS G13D Mutation on Basal PD-L1 Expression in A375 Cells

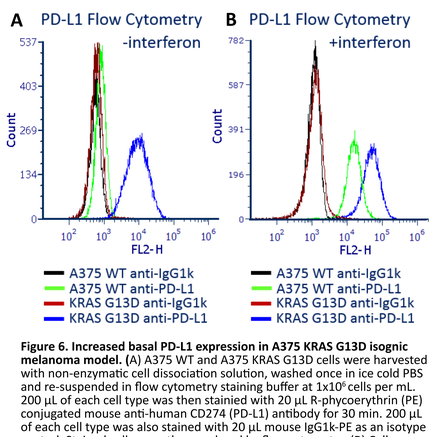


Figure 6. Increased basal PD-L1 expression in A375 KRAS G13D isogenic melanoma model. (A) A375 WT and A375 KRAS G13D cells were harvested with non-enzymatic cell dissociation solution, washed once in ice cold PBS and re-suspended in flow cytometry staining buffer at $1x10^6$ cells per mL. 200 µL of each cell type was then stained with 20 µL R-phycoerythrin (PE) conjugated mouse anti-human CD274 (PD-L1) antibody for 30 min. 200 µL of each cell type was also stained with 20 µL mouse IgG1k-PE as an isotype control. Stained cells were then analyzed by flow cytometry. (B) Cells were stimulated overnight with 200 ng/mL recombinant human interferon gamma then staining and flow cytometry was performed as in (A).

Discussion

In this study, we used CRISPR/Cas9 genome engineering to create precisely defined isogenic cellular models of drug-resistant melanoma via the introduction of specific point mutations known to confer drug resistance. We then determined the effect of these mutations on the basal expression and phosphorylation levels of other key components of the RAS-RAF-MAPK signaling pathway in both 2D and 3D culture, demonstrating perturbations specific to both the engineered RAS mutation and to the cell culture format. Furthermore, using high-content imaging in combination with live-cell staining, we have developed a method for performing high-throughput imaging and analysis of A375 WT and isogenic Ras-mutant spheroids and demonstrated that A375 NRAS Q61K and KRAS G13D spheroids are resistant to the the BRAF inhibitors Dabrafenib and Vemurafenib.

Taken together, these data show that these new cell lines are ideal pre-clinical model systems for the development and screening of the next generation of chemotherapeutic compounds and combination therapies. Furthermore, the finding of constitutive elevated PD-L1 expression in KRAS G13D both with and without interferon stimulation makes this cell line of further use in the screening of new small molecule compounds and biologics targeting the PD-1/PD-L1 system.

References

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