Development of a Novel MEK Mutation Driven Drug Resistant Melanoma Model by CRISPR/Cas9 Technology

Metewo Selase Enuameh [1], Lysa-Anne Volpe [2], Luping Chen [2], Elizabeth Turner [2], John Foulke [2], and Fang Tian [2]
[1]ATCC Cell Systems, Gaithersburg, MD 20877, USA; [2]ATCC, Manassas, VA 20110, VA

AACR Abstract # 2153

Introduction

Melanoma remains the most lethal form of skin cancer, exhibiting high mortality rates due to a high likelihood of developing metastases and acquiring drug resistance. Approximately 40-50% of melanomas contain oncogenic BRAF mutations, of which 75-90% harbor the $BRAF^{V600E}$ mutation. This mutation constitutively activates the mitogen-activating protein kinase (MAPK) signaling pathway, leading to uncontrolled cell growth and oncogenesis. Recent combination therapies of BRAF- and MEK-specific inhibitors have shown improved progression-free patient response in phase II clinical trials. However, in the majority of patients, acquired resistance to MAPK pathway inhibitor therapies develops after approximately 12 months of treatment. Preclinical studies have suggested that MEK1 mutations confer resistance to BRAF and MEK inhibitors. In this study, we used the CRISPR/Cas9 genome-editing technology to generate a drug resistant $MEK1^{Q56P}$ knock-in mutation within the A375 melanoma cell line which naturally harbors the $BRAF^{V600E}$ mutation. We validated this new isogenic cell model using both molecular and biofunctional approaches.

Method and results: Single guide RNAs (sgRNAs) were designed and built into Cas9 plasmids to bind and cut desired regions in the MEK1 target region. The melanoma cell line A375 was co-transfected with single guide-containing Cas9 plasmids and donor plasmids. Single cells were cloned and expanded for subsequent screening of the desired gene mutation events. The introduction of the $MEK1^{Q56P}$ mutation in the cells was then confirmed at the genetic level via Sanger sequencing and next-generation sequencing (NGS). Validation of the mutation in mRNA transcripts was carried out by RT-PCR followed by sequencing. The cell line maintains permanent and genetically stable resistance characteristics during cell culture expansion without the use of selective pressure.

Drug responses to BRAF- and MEK1-specific inhibitors and non-specific chemotherapy drugs were compared between the A375 $MEK1^{Q56P}$ isogenic cell line and the parental cell line in 2D and 3D culture environments. Results demonstrated that the isogenic $MEK1^{Q56P}$ cell line showed significant and specific resistance to BRAF inhibitors in comparison to the parental A375 line.

Using CRISPR/Cas9 gene-editing technology, we have targeted an endogenous loci within a melanoma cell line, creating a novel *in vitro* model to aid in basic and translational melanoma research as well as drug screening efforts. This new approach to cell line development provides direct, *in vitro,* bio-functional evidence of a drug-resistant gene that drives tumor cell survival under targeted anti-cancer treatments. Furthermore, this A375 $MEK1^{Q56P}$ isogenic cell line represents a new type of drug resistance model that contains a defined genetic resistance mechanism. This model provides an invaluable tool for developing next generation therapeutics that can overcome drug resistance in melanoma.

Background

Role of mutant BRAF in preventing apoptosis and driving melanoma cell survival[1]

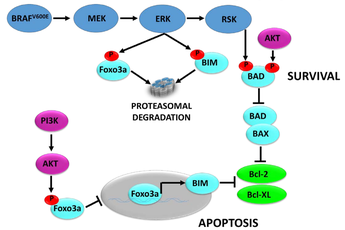

Figure 1. Mutant $BRAF^{V600E}$ drives the phosphorylation and subsequent proteasomal degradation of BIM and Foxo3a via the MEK/ERK pathway. In the absence of BIM, the anti-apoptotic proteins Bcl-2 and Bcl-XL are not inhibited, leading to increased cell survival. When the MAPK pathway is blocked through the use of BRAF inhibitors, the converse occurs.

MEK-mediated resistance to BRAF inhibitor in melanoma[2]

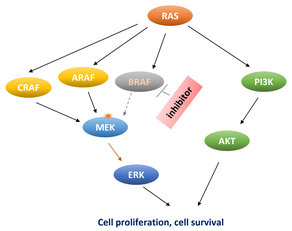

Figure 2. $BRAF^{V600E}$ melanoma cells treated with BRAF inhibitors acquire drug resistance by switching between ARAF, BRAF, and CRAF (isoforms of RAF), leading to the activation of the MAPK pathway. Drug resistance can also occur via the activation of the MAPK pathway following the acquisition of secondary mutations in NRAS (Q61K) and MEK (Q56P). Furthermore, activation of the PI3K/AKT pathway could give rise additional resistance from increased IGFR1 and PDGFR signaling.

Results

Design and construct CRISPR/Cas9 regents to generate the $MEK1^{Q56P}$ mutation in the human melanoma A375 cell line

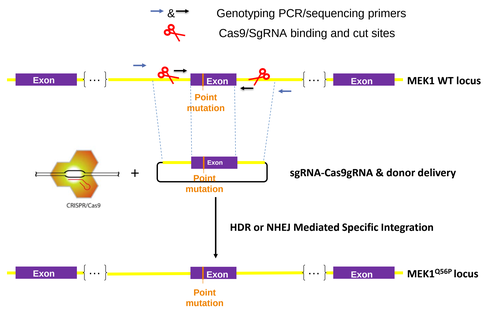

Figure 3. Schematic of the target region for integration of the knock-in DNA bearing the $MEK1^{Q56P}$ point mutation. The sgRNA-Cas9–mediated double stranded break (DSB) of the WT MEK1 locus is depicted as red scissors. Also shown here is the coding region of the $MEK1^{Q56P}$ locus (purple) and the untranslated region (yellow line). The $MEK1^{Q56P}$ donor (2^{nd} construct from the top) is designed to enable integration of the $MEK1^{Q56P}$ sequence by either homology-directed repair (HDR) or non-homologous end joining (NHEJ) at the two sgRNA-Cas9 cut sites.

PCR and sequencing to confirm the MEK1Q56P knock-in mutation

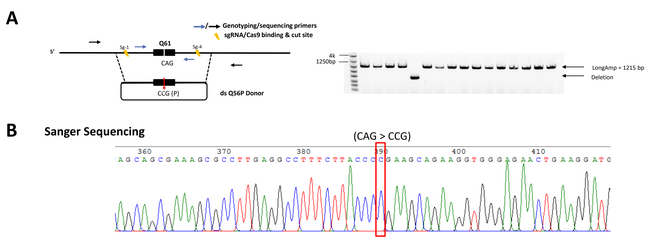

Figure 4. Confirmation of the $MEK1^{Q56P}$ knock-in mutation. (A) Screening for the $MEK1^{Q56P}$ point mutation integration in recovered clones was carried out using PCR primers as shown left, with the gel image of PCR products on the right. (B) Introduction of the $MEK1^{Q56P}$ point mutation into the cell line was confirmed via sequencing primers shown in (A). Boxed in red is the expected A>C mutation.

Confirmation of $MEK1^{Q56P}$ mRNA expression in single cell clones

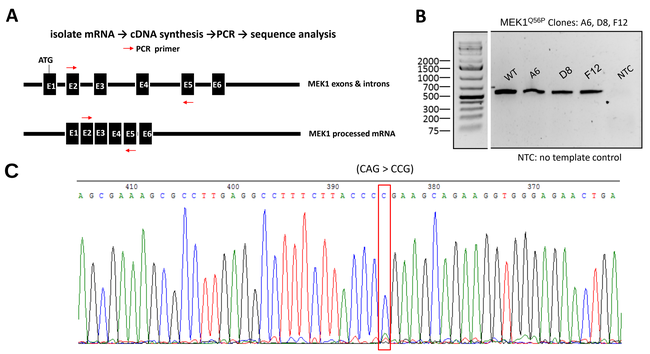

Figure 5. MEK1 mRNA in cell clones carrying the Q56P mutation. (A) Screening for the $MEK1^{Q56P}$ point mutation in recovered clones was carried out via cDNA generation from cells and performing PCR (red arrows). (B) Gel image of the PCR products. (C) The introduction of the $MEK1^{Q56P}$ point mutation into the cell line was confirmed via Sanger sequencing on the right. The expected point mutation is boxed in red.

$MEK1^{Q56P}$ isogenic line is resistant to BRAF & MEK1 inhibitors

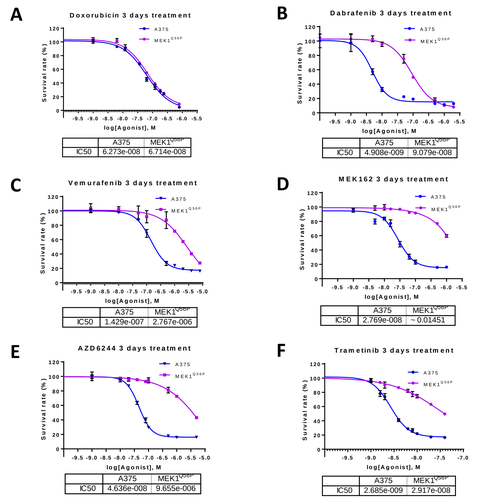

Figure 6. Drug response of the A375 parental line and $MEK1^{Q56P}$ isogenic line. CellTiter-Glo™ luminescent cell viability assay was used to evaluate cell line drug response to BRAF inhibitors (vemurafenib and dabrafenib), MEK1 inhibitors (trametinib, MEK162 and AZD6244), as well as the non-specific chemotherapy drug doxorubicin. $MEK1^{Q56P}$ isogenic exhibits drug resistance to the BRAF and MEK1 inhibitors relative to the parental A375 cell line (B, C and D, E,F respectively). The responses of the A375 and $MEK1^{Q56P}$ isogenic lines to non-specific chemotherapy drugs were similar (A). Data are expressed as mean ± SD (n=3).

$MEK1^{Q56P}$ isogenic line as a cell line model for drug combination therapies

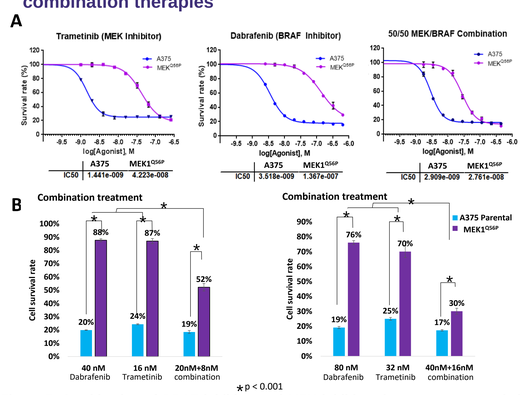

Figure 7. Combination of BRAF inhibitor and MEK inhibitor demonstrate synergistic effect on the $MEK1^{Q56P}$ isogenic lines. CellTiter-Glo luminescent cell viability assay was used to evaluate cell line drug responses to trametinib, dabrafenib, or combination of dabrafinib and trametinib. MEK1 inhibitors (trametinib, MEK162 and AZD6244), as well as the non-specific chemotherapy drug doxorubicin. $MEK1^{Q56P}$ isogenic clone was resistant to the BRAF inhibitor or MEK inhibitor treatment alone. However, it responded to the combination treatment and demonstrated the synergistic effect of BRAF and MEK inhibitors, which was indicated by the narrowing of the separation between the parental and MEK^{Q56P} dose response curves (A). This observation was further validated in (B) when specific concentration of each drug and combinations were tested. Data are expressed as mean ± SD (n=3).

The $MEK1^{Q56P}$ isogenic line has a similar morphology and growth profile as the parental A375 cell line

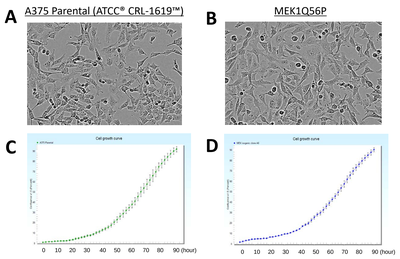

Figure 8. The cell morphology and growth profile of the A375 parental line and the $MEK1^{Q56P}$ isogenic line are similar. The cell morphology images were captured by microscope at 100X magnification (A and B). The cell growth kinetics over 4 days were measured (n=3) by live cell analysis system (C and D).

ERK and AKT activation is enhanced in the $MEK1^{Q56P}$ isogenic line

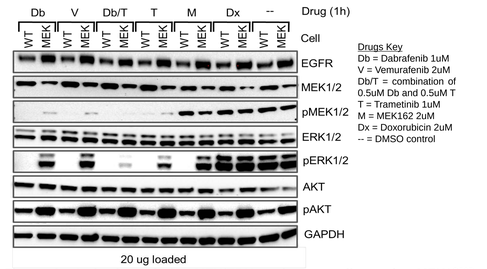

Figure 9. Activation of the MEK/ERK pathway mediates the $MEK1^{Q56P}$ isogenic line's resistance to BRAF inhibitors. Parental A375 cells and $MEK1^{Q56P}$ isogenic cells were treated with dabrafinib, vemurafenib, trametinib, MEK162, a combination of dabrafinib and trametinib, or a chemotherapy drug as indicated for 1 hour. DMSO treatment was used as control. Cells were then harvested, lysed, and analyzed via immunoblotting with antibodies against total EGFR, MEK1/2, phosphor-MEK1/2, AKT, phospho-AKT, ERK, phospho-ERK, and GAPDH (loading control).

Conclusion

- The ATCC $MEK1^{Q56P}$ isogenic A375 cell line (ATCC® CRL-1619IG-3™), derived from the A375 melanoma cell line (ATCC® CRL-1619™), contains the $MEK1^{Q56P}$ mutation. This have been validated at the genomic and transcript levels.
- The $MEK1^{Q56P}$ isogenic A375 cell line is significantly more resistant to BRAF inhibitors (vemurafenib and dabrafenib) and MEK1 inhibitors (trametinib, MEK162, and AZD6244) when compared to the A375 parental line.
- Additionally, the $MEK1^{Q56P}$ isogenic A375 cell line shows sensitivity to a combined dabrafinib and trametinib drug treatment relative to the parental A375 cell line.
- This CRISPR/Cas9-generated isogenic cell line can serve as a useful model system to screen for novel BRAF & MEK1 inhibitors in anti-cancer drug discovery and development.

References

1. Ran F, *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11): 2281-308, 2013.
2. Emery C. M, *et al.* MEK1 mutations confer resistance to MEK and B-RAF inhibition. *PNAS* 106(48): 20411–20416, 2009.