Simultaneous Analysis of DNA, RNA, and Protein Expression in FFPE Melanoma Samples

Introduction

The lifetime risk of developing melanoma of the skin is 2.4%.[1] The latest data demonstrate an increasing incidence of this malignancy in the US[2] with more than 76,000 cases expected to be diagnosed in 2016.[3] Survival is highly dependent on the cancer stage, ranging from 97% (Stage IA) to just 15% (Stage IV).[3] Improved treatments, driven by precision medicine and personalized medicine efforts, have been approved in recent years. For example, in 2011 Zelboraf (Vemurafenib, Roche) was introduced as a treatment for melanomas with the BRAF V600E mutation. This mutant-specific inhibitor down-regulates the proliferative signal through the MAP kinase pathway caused by the activated kinase domain of BRAF V600E. While successful, resistance mechanisms have emerged.

As a consequence, combined BRAF and MEK inhibition treatments have been introduced that prevent mitogen-activated protein kinase (MAPK) reactivation[4] as a mechanism of resistance to monotherapy with a BRAF inhibitor. This approach is now considered the standard of care treatment for patients with BRAF V600E mutant metastatic melanoma. In addition, there is interest to combine MAPK-targeted therapy and cancer immunotherapy with the goal of achieving higher response rates with prolonged duration. A growing body of evidence supports combinatorial approaches that merge the significant response rate of BRAF inhibitor-based targeted therapy with long-term durable responses of immunotherapy in patients with advanced melanoma.[5,6]

NanoString's 3D Biology™ technology enables analysis of DNA, RNA, and protein simultaneously on the NanoString nCounter® system. We have previously demonstrated, using the 3D Biology approach, that drug response to Vemurafenib (+/- Trametinib) was most pronounced in the homozygous BRAF V600E cell line yielding significant changes in gene and protein expression, including the tumor-survival promoting 5-ectonucleotidase CD73, a candidate target to improve melanoma therapy and intracellular phosphorylation state changes consistent with the mechanism of action of therapeutic kinase inhibitors (Figure 1). In this study, we extend those findings and demonstrate how the nCounter platform, a digital single-molecule detection technology using optical barcodes, can be utilized with the nCounter Vantage 3D Solid Tumor Assay to rapidly detect single nucleotide variants (SNV) and measure gene, protein, and phospho-protein expression simultaneously from FFPE samples to potentially monitor treatment effectiveness or identify new biomarkers for melanoma, in the case of BRAF inhibitor resistance.

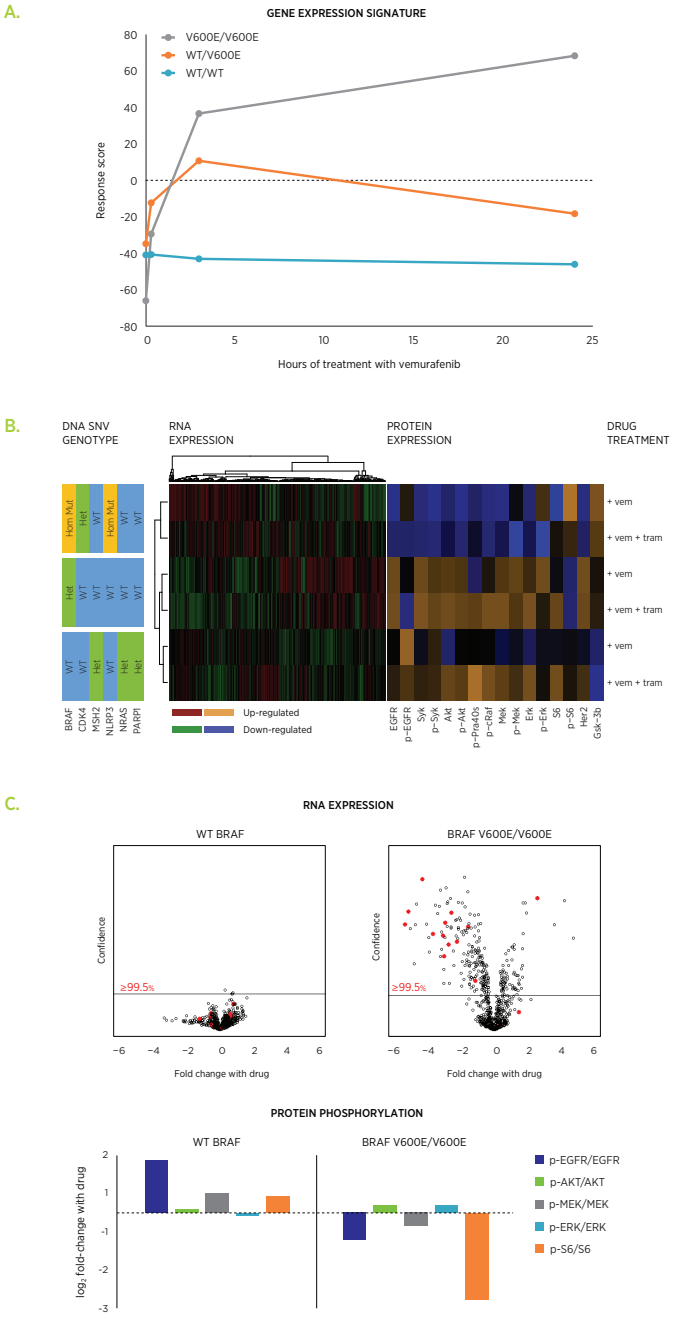

Figure 1 Drug response by BRAF genotype. **A.** A BRAF genotype-specific, 16-gene signature of drug response[7] was profiled in cell lines on the nCounter platform. **B.** DNA, RNA, and protein were quantified simultaneously. Heatmaps represent average levels from biological triplicate samples after normalization to controls. For 8 hours, single and combination drug treatment was measured against treatment with vehicle (DMSO) alone. **C.** Vemurafenib shows remarkably few off-target gene expression effects in cells with WT BRAF V600 alleles. After 8 hours, inhibition of the Ras/Raf/Mek/Erk pathway by Vemurafenib reduces the degree of downstream substrate S6 phosphorylation in BRAF V600E mutant cells 4.8-fold.

Material & Methods

In this this study, 6 melanoma FFPE samples were profiled according to the workflow shown in Figure 2.

Briefly, RNA and DNA were extracted using the Qiagen AllPrep kit according to manufacturer's recommendations. An additional slide was incubated with NanoString barcoded antibodies according to the nCounter Vantage 3D Protein FFPE protocol. Hybridization and quantification of DNA and RNA:Protein was carried out according to NanoString protocols on the nCounter platform. The resulting raw data were analyzed using NanoString's nSolver™ Advanced Analysis.

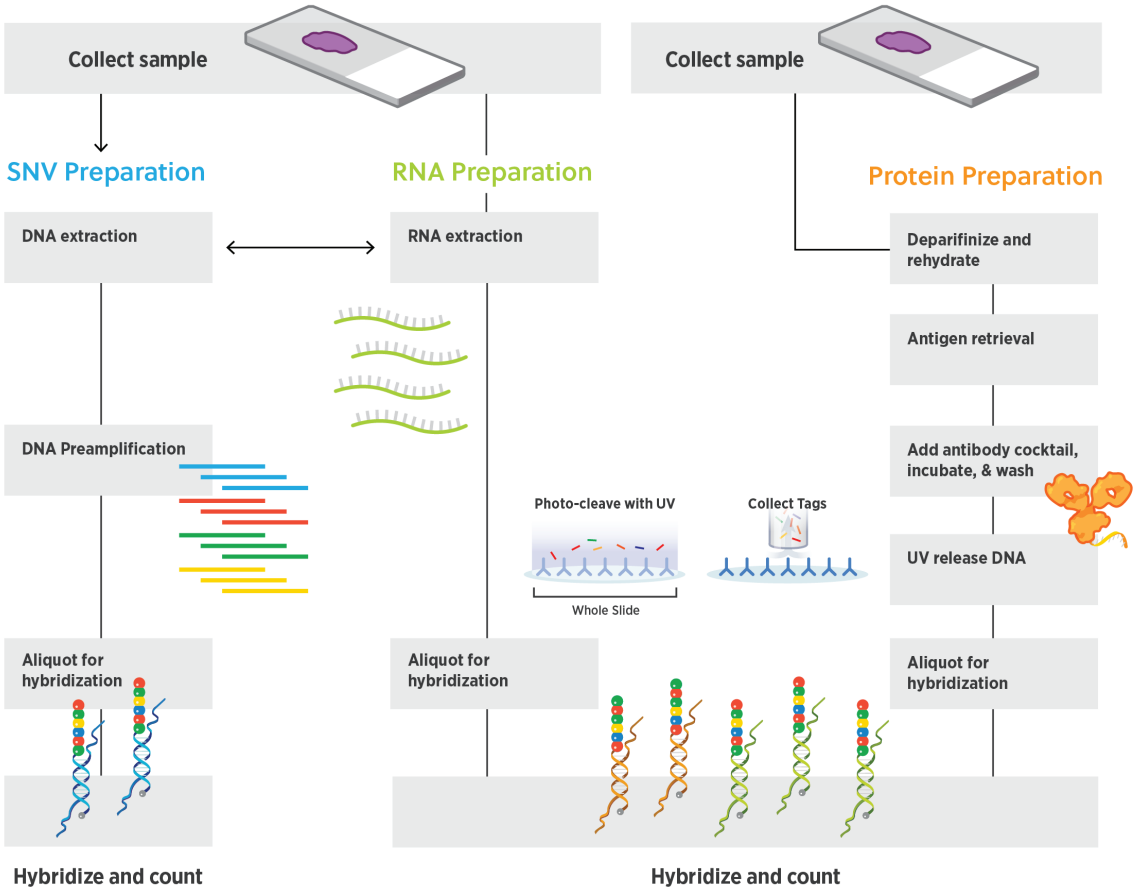

Figure 2 nCounter® Vantage 3D™ FFPE workflow

Results

Based on the built-in process controls, all of our samples passed internal QC metrics and met or exceeded the requirements for background, sensitivity, and assay linearity. For SNV detection, we analyzed 104 polymorphic sites in 25 cancer-related genes using the Vantage 3D DNA SNV Solid Tumor panel. In 3 out of 6 (50%) of our samples, we detected the V600E variant (Figure 3) in homozygous configuration. Analysis of the variants for the gDNA also identified the presence of cancer-related homozygous APC R876 or NRAS Q61K mutations either in combination with or without BRAF V600E, respectively.

The addition of RNA and protein expression profiles generated using the Vantage 3D RNA:Protein Solid Tumor assay revealed that the presence of NRAS Q61K was associated with a substantial increase in the phosphorylation of ERK protein (Figure 4), and therefore activation of the MAPK pathway. However, total ERK protein levels did not change significantly across samples nor did the RNA expression of MAPK3 (ERK) (Figure 5).

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Discussion

This 3D Biology proof-of-principle study shows the simple and fast identification of the mutational load for cancer-associated genes and their impact on the expression of mRNA in 13 key cancer driver pathways, as well as changes in the downstream expression and activation of key regulatory proteins. We identified distinct molecular profiles in melanoma samples based on their BRAF mutational status and profiled the impact of additional mutations in other oncogenes on the RNA and protein level.

Notably, the observed changes in gene and protein expression in the NRAS Q61K sample may suggest a different mechanism of malignant transformation in these tumor cells as compared to BRAF V600E samples. Activation of ERK in the NRAS Q61K sample may be exploited by treatment with an ERK dimerization inhibitor to block extra-nuclear signaling of ERK.[8]

In summary, we demonstrate the successful, simultaneous identification of cancer-related variants (mutations) and associated mRNA and protein expression changes in melanoma FFPE samples using minimal sample input. Our 3D Biology approach, in principal, could be used in research studies to determine the cancer's molecular makeup to better understand the mechanisms of disease.

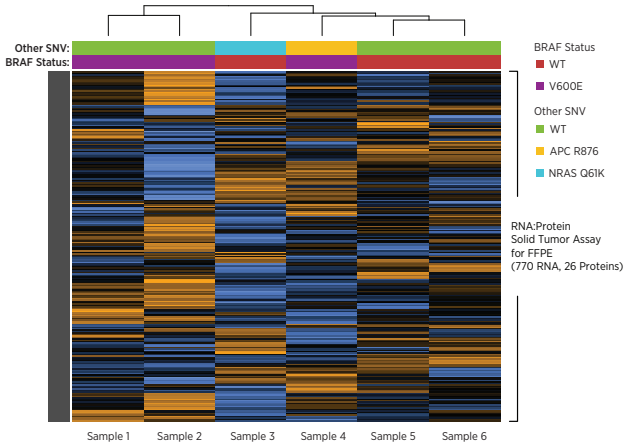

Figure 3 Genotype and RNA and protein expression profile for 6 melanoma FFPE samples. Heatmap generated using nSolver Advanced Analysis.

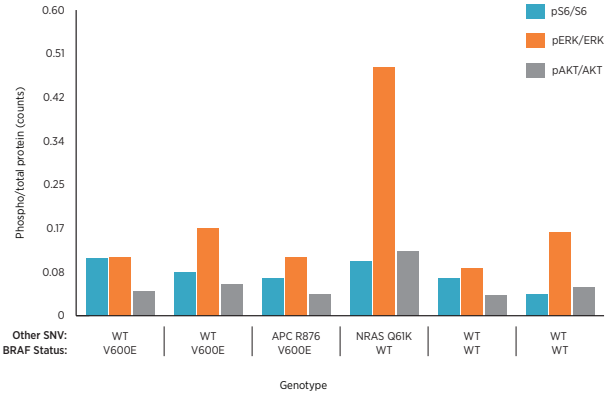

Figure 4 Protein expression profile for a subset of total and phospho-protein targets.

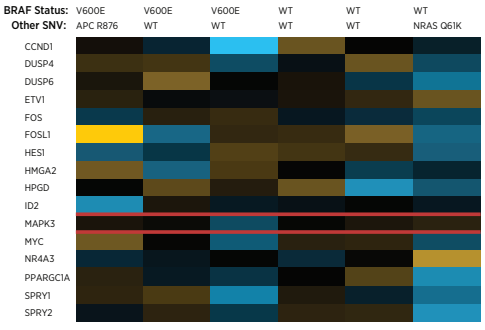

Figure 5 Subset of RNA targets identified previously as a 16 gene signature that is BRAF genotype specific and predictive of drug response (Figure 1)[7] shown at baseline in this sample subset.

References

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