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J Clin Invest. 2019. <https://doi.org/10.1172/JCI123089>.

Concise Communication In-Press Preview Genetics Oncology

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Profound MEK inhibitor response in a cutaneous melanoma harboring a *GOLGA4-RAF1* fusion

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Key words: *RAF1*, Fusion, MEK inhibitor, Immunotherapy, Melanoma

ABSTRACT

BRAF and CRAF are critical components of the MAPK signaling pathway which is activated in many cancer types. In approximately 1% of melanomas, BRAF or CRAF are activated through structural arrangements. We describe here a metastatic melanoma with a *GOLGA4-RAF1* fusion and pathogenic variants in *CTNNB1* and *CDKN2A*. Anti-CTLA4/anti-PD1 combination immunotherapy failed to control tumor progression. In the absence of other actionable variants the patient was administered MEK inhibitor therapy on the basis of its potential action against *RAF1* fusions. This resulted in a profound and clinically significant response. We demonstrated that *GOLGA4-RAF1* expression was associated with ERK activation, elevated expression of the RAS/RAF downstream co-effector ETV5, and a high Ki67 index. These findings provide a rationale for the dramatic response to targeted therapy. This study shows that thorough molecular characterization of treatment-resistant cancers can identify therapeutic targets and personalize management, leading to improved patient outcomes.

Introduction

Mitogen-Activated Protein Kinase (MAPK) pathway activation is a hallmark of many cancers. Many genes in this pathway, including *RAS*, *RAF* (encoding the serine/threonine kinases BRAF and CRAF) and *MEK* can contain activating mutations. *BRAF* mutations are found in 37-50% of melanomas (1, 2). CRAF, encoded by *RAF1*, forms homodimers as well as heterodimers with wild-type BRAF, thereby functioning as an alternative mitogenic signalling mechanism (3).

RAF1 structural variants have been reported in several malignancies, including melanoma, pilocytic glioma and acinar pancreatic cancers (1, 4-11). These variants fuse the *RAF1* kinase domain to diverse 5' partners, but despite potential clinical actionability the majority of

cases have not been acted upon. *In vitro* studies have demonstrated that *RAF1* fusions are sensitive to the multi-kinase inhibitor sorafenib and are partially responsive to the MEK inhibitors (MEKi) selumetinib and trametinib (5, 11). A clinical response to trametinib was also reported in a metastatic melanoma with an *ANO10-RAF1* fusion (10).

We describe a case of *BRAF/NRAS* wild-type metastatic melanoma that failed to respond to immune checkpoint therapy. Molecular analysis identified a *GOLGA4-RAF1* fusion. The patient subsequently showed a profound, albeit transient, clinically significant response to MEKi therapy.

Results and Discussion

A 70 year-old man presented with bulky right inguinal lymph nodes, with biopsy indicating metastatic melanoma. A fluorodeoxyglucose-positron emission tomography (FDG-PET/CT) and MRI brain scan did not locate a primary malignancy or additional metastases. A right inguinal lymphadenectomy retrieved 8 lymph nodes. Histology showed near complete replacement of 3 of these nodes by metastatic melanoma, up to 45mm in maximal dimension, with 10mm of extranodal extension. Tumor cells were mixed spindle and epithelioid. Immunohistochemistry (IHC) showed strongly expressed S100, Melan-A, SOX10 and HMB-45 (Figure 1A-E). *BRAF* (codons 469, 594, 597 and 600) and *NRAS* (exons 2, 3 and 4) were wild-type by Sequenom MassARRAY (Agena Bioscience™).

Immune checkpoint therapy is standard of care for patients with metastatic melanoma without an activating *BRAF* mutation (see Supplemental Material). The patient was administered 3 cycles of treatment of the anti-PD1 inhibitor pembrolizumab but experienced rapid clinical decline. Imaging confirmed marked interval disease progression with FDG-avid nodal, soft tissue, pulmonary, liver, splenic and cardiac involvement. He was then administered anti-CTLA4 (ipilimumab)/anti-PD1 (nivolumab) combination immunotherapy.

Extensive molecular testing was performed to identify an actionable target. This detected a *GOLGA4-RAF1* gene fusion (Figure 1F). Subsequent tests using RT-PCR/Sanger sequencing and the Archer FusionPlex™ Oncology Research Panel both showed an in-frame chimeric mRNA of *GOLGA4* exons 1-21 fused to *RAF1* exons 8-17 (Figure 1G). The entire coding regions of other MAPK pathway genes (*BRAF*, *NRAS*, *KIT*, *GNAS*, *GNAQ*, *KRAS*, *MAP2K1/2*, *MAPK1/2* and *NF1*) were wild-type.

RAF1 and *BRAF* gene fusions are rare but potentially clinically significant (1, 4-11). They fuse a novel N-terminal sequence to the C-terminal kinase domain of CRAF. *GOLGA4* encodes Golgin-245 and is a recurrent 5' partner in *RAF1* fusions, previously described in melanoma (1), breast carcinoma (8) and prostate cancer (9) (Figure 1H). In this case the fusion included the entire CRAF kinase domain but lacked the CRAF N-terminal Ras-binding (RBD) and C1 auto-inhibitory domains (Figure 1F). It also lacked the GRIP domain of Golgin-245 that is required for recruitment to the trans-Golgi network.

Gains of chromosomal regions 5q, 8q, 13q and 17q21.31-qter, focal amplification (6x) of 12p11.21-12.2 (which harbors *KRAS*) and losses of chromosome arms 6q, 9p (which harbors *CDKN2A*), 10p and 10q, were also observed. Mutational signatures of exposure to high UV and alkylating agents were present. Two somatic pathogenic variants were also detected, a missense mutation in *CTNNB1* (NM_001098210.1) c.110C>G p.(Ser37Cys) and a truncating mutation in *CDKN2A* (NM_000077.4) c.148C>T p.(Gln50*).

At the time of returning the genetic results the patient had developed further disease progression with new onset of brain metastatic disease despite the use of combination immunotherapy (Figure 2). His case was reviewed at our institute's Molecular Tumor Board. In view of experimental evidence that tumor cells with *BRAF/CRAF* fusion proteins are potentially sensitive to multi-kinase inhibitors (5-7, 12) or MEKi (10), the decision was made

to proceed with the single agent trametinib, a reversible, highly selective allosteric inhibitor of MEK1/MEK2 (see Supplemental Material). An FDG-PET undertaken at 17 days post-treatment commencement showed a marked response, with both metabolic and structural responses on extra-cranial imaging (Figure 2). Eastern Cooperative Oncology Group (ECOG) performance status also improved from 3 to 1. However, progressive intracranial disease was observed. This is a well-documented phenomenon in melanoma treatment, with both immunotherapy and targeted agents demonstrating lower rates of response in the brain (13, 14). The patient was subsequently treated with whole brain radiotherapy and nivolumab was added. Following 6 weeks of therapy treatment was changed to another potent and highly selective MEK1/2 inhibitor, cobimetinib, due to issues with trametinib access (see Supplemental Material). Treatment continued for 5 months during which time the patient continued to have good extracranial control of disease (Figure 2). Imaging showed improvement in subcutaneous and visceral disease, with a rapid decline in blood LDH levels. Unfortunately, tumor progressed within the brain. The patient ceased MEKi/nivolumab therapy 10 days prior to his death.

We investigated the tumor biology in order to better understand the consequences of the *RAF1* fusion and other genomic alterations. Expression of *RAF1* and *BRAF* RNA were not significantly elevated (Table 1). However, IHC with an anti-CRAF antibody showed intense paranuclear and moderate diffuse cytoplasmic staining in the peripheral tumor cells (Figure 3B), but only weak paranuclear and cytoplasmic staining in the center of the tumor (Figure 3C). This pattern resembles that of wild-type GOLGA4 which localizes to the Golgi and cytosol in human tissue (15). Benign tissue either adjacent to or within the tumor (vasculature and adipose stroma) showed weak cytoplasmic or no staining (Figure 3D). Diffuse cytoplasmic staining without paranuclear expression was seen in our on-slide positive control for the CRAF antibody (a colorectal adenocarcinoma, Figure 3E). Strong nuclear phospho-ERK (pERK) staining was restricted to the same peripheral regions that showed intense CRAF expression (Figure 3F). The ETS (E twenty-six) family of transcription

factors (ETV proteins) are critical downstream co-effectors of MAPK signaling and modulate MEK and RAF inhibitor responses (16). The expression of ETV5 in particular has been found to correlate with BRAF V600E activation in melanoma (17). RNA sequencing using Archer FusionPlex™ showed markedly elevated expression of *ETV5*, but not *ETV1*, 4 or 6 (Figure 3L), consistent with activated MAPK signaling. We predicted that activation of MAPK signalling would lead to increased cell proliferation. Ki67 labelling for actively proliferating tumor cells showed marked regional variation (Figure 3G). It was highest within the same peripheral foci that also showed strong expression of CRAF and pERK (Figure 3A-C, F, performed on adjacent serial sections). The central parts of the tumor and the peripheral regions with low CRAF/pERK expression had a low Ki67 labelling index (Figure 3G).

CTNNB1 activation and CDKN2A inactivation are known melanoma drivers (18, 19). CTNNB1 Ser37Cys impairs a critical GSK-3- β phosphorylation site, leading to aberrant β -catenin accumulation in the nucleus (20) where it functions as a co-activator of TCF4/LEF1, resulting in transcription of genes that include *CCND1* (cyclin D1), thereby promoting cell proliferation (21). Nanostring analysis revealed markedly up-regulated *CCND1* (Table 1). IHC showed strong nuclear and moderate cytoplasmic expression of β -catenin and *CCND1* throughout the nodal metastasis (Figure 3H, I). Nanostring analysis also showed elevated *CCND3* (cyclin D3) and *KRAS* expression (Table 1), the latter consistent with the *KRAS* amplification. *KIT* expression was moderately elevated by Nanostring analysis and anti-KIT IHC showed moderately intense cytoplasmic staining throughout the nodal tumor (not shown). Loss of functional p16/INK4A, primarily through deletion, occurs in ~80-90% of metastatic melanomas (22). p16 is an inhibitor of cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), which regulate progression through the G1 phase of the cell cycle (23). IHC showed no expression of p16 in tumor cells, consistent with a “double hit” of *CDKN2A*, *i.e.* chromosome 9p loss and an inactivating c.148C>T p.(Gln50*) mutation (Figure 3J).

Overall, the strong spatial correlation between Ki67 labelling and elevated expression of CRAF and pERK and the significant extra-cranial response to the MEKi supported GOLGA4-CRAF signaling through pERK to be the critical oncogenic driver. The heterogeneity of GOLGA4-CRAF expression was intriguing and prompted us to perform *RAF1* break-apart FISH to determine if all tumor cells carried *GOLGA4-RAF1*. We detected split *RAF1* 5' and 3' signals in all tumor cells, *i.e.* both in the periphery and center of the tumor (Figure 3K). We speculate that the differences in CRAF expression are due to differences in GOLGA4-CRAF protein stability between the periphery and center, possibly because peripheral tumor cells may more readily access nutrients and oxygen than central tumor cells. Alternatively, since the periphery only represented a minority of total tumor input in the Nanostring analysis, *GOLGA4-RAF1* mRNA expression may be elevated at the periphery of the tumor. The *RAF1* FISH analysis also showed that *GOLGA4-RAF1* was amplified in all tumor cells. There were 5-10 rearranged *RAF1* copies per tumor nucleus (Figure 3K), with a *RAF1*/chromosome 3 ratio of ~4. *GOLGA4-RAF1* amplification further supports activated CRAF as a critical driver. A dermal metastasis was excised from the patient on day 190, 28 days after the commencement of the MEKi. It had a low tumor cell proliferative activity and morphological features of tumor regression, both likely due to the MEKi treatment (see Supplementary Material).

The GOLGA4-CRAF fusion is likely to have high CRAF kinase activity since the RBD and C1 domains which suppress CRAF activity (24) are absent and Golgin proteins can spontaneously multimerize the kinase domains of their fusion partners, leading to increased kinase activity (25). The likely Golgi expression of GOLGA4-CRAF is intriguing since the GRIP domain of GOLGA4, which is sufficient for Golgi-localization, was absent. Nevertheless, it is possible that GOLGA4-CRAF promotes tumor cell proliferation by aiding Golgi complex fragmentation at cell division because CRAF and MEK1 are both implicated in this process (26).

Biomarker analysis may partly explain the lack of response to immune checkpoint therapy. We found no increase in the expression of PD-1, PD-L1 and PD-L2 compared to pan-cancer and melanoma-specific reference datasets (Table 1). Expression of cytotoxic T-cell markers, including granzyme B and perforin, which are typically associated with cytotoxic T-cell killing (27), were low compared to both reference datasets. The lack of tumor infiltrating lymphocytes in the tumor may partly account for low expression of these T-cell markers.

In summary, we describe a metastatic melanoma with a *GOLGA4-RAF1* fusion that demonstrated a profound extracranial response to MEK inhibition. This adds to a growing body of evidence demonstrating success in the therapeutic targeting of RAF structural variants. Correlation of activated RAS/RAF/MEK/ERK signaling and cell proliferation provided a rationale for the therapeutic response. We also identified aberrant WNT activation and likely CDK4/6 activation mediated through both increased cyclin D1/D3 expression and p16 loss. Whilst inhibitors of WNT signaling remain at an early stage of development, CDK4/6 inhibitors targeting cell-cycle dysregulation have been approved by the FDA for specific breast cancer indications. Therefore, combined anti-MEK and anti-CDK4/6 therapy may be a potential therapeutic option in *RAF* fusion-driven tumors.

Methods

A complete description of the methods is provided in the Supplemental Material.

Study approval. All experiments were performed under protocols approved and reviewed by the iPredict (approval HREC/13/MH/326) and SUPER studies (approval 11/117).

Author Contributions

CRM, HX and OWJP coordinated the project and wrote the manuscript. KS analyzed and wrote clinical data. CRM and DCY performed molecular analysis. HX, HSL and APF analyzed sequencing results. DE performed Nanostring experiments and analysis. DJB and

JB performed IHC. VN and BMB performed FISH. OWJP analyzed tumor morphology, IHC and FISH. AI analyzed patient imaging. CK was the treating clinician. SB, JD, DDB and LM coordinated patient recruitment and human ethics. RWT, AHB, SBF and all other authors provided intellectual input.

Acknowledgements

We thank Melbourne Genomic Health Alliances (MGHA) and the Australian Genomic Health Alliance (NHMRC grant 1113531) for funding the iPredict study. We also thank Richard Lupat for assistance in GRIDSS analysis, Jason Li for setting up the analysis pipeline for the nCounter dataset, Jenna Stewart and David Yoannidis for technical assistance, Kelly Waldek for advice on IHC, Chung-Yan Ma for discussions on immunomarkers and Michael McKay and Glen Gurra for critical discussions of the manuscript.

Disclosures

The authors have declared that no conflicts of interest exist.

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Table 1. Changes in the expression of critical genes in the RAS/RAF/MEK/ERK, WNT and cell cycle pathways, and immune response.

Pathway	Gene Symbol	Z score (pan-tumor reference)	Z Score (melanoma-specific reference)	Expression change (pan-tumor/melanoma-specific)
RAS/RAF/MEK/ERK	<i>EGFR</i>	-2.76	-1.49	--/-
	<i>KRAS</i>	1.52	2.81	+/>++
	<i>BRAF</i>	0.29	0.38	NC/NC
	<i>RAF1</i>	0.77	0.67	NC/NC
WNT/Cell cycle	<i>CCND1</i>	3.01	3.16	++/>++
Cell Cycle	<i>CCND3</i>	2.35	3.45	++/>++
Immune response	<i>CD274 (PD-L1)</i>	-0.27	-0.16	NC/NC
	<i>PDCD1LG2 (PD-L2)</i>	0.01	-0.15	NC/NC
	<i>PDCD1 (PD-1)</i>	-1.51	-1.61	-/>-
	<i>FOXP3</i>	-0.92	-1.12	NC/-
	<i>CTLA4</i>	1.02	0.94	+/>NC
	<i>CXCL9</i>	-1.10	-1.74	-/>-
	<i>CD19</i>	-0.49	-0.19	NC/NC
	<i>CD80 (B7.1)</i>	-0.50	-0.55	NC/NC
	<i>CD8A</i>	-1.26	-1.26	-/>-
	<i>CD8B</i>	-1.57	-1.55	-/>-
	<i>IFNG</i>	-0.82	-1.30	NC/-
	<i>GZMA</i>	-0.70	-0.91	NC/NC
	<i>GZMB</i>	-1.90	-3.16	-/>--
<i>PRF1</i>	-1.51	-1.49	-/>-	

Gene expression was determined by Nanostring against two in-house derived reference datasets, a pan-tumor dataset derived from expression of 148 tumors, and a melanoma-

specific dataset derived from 11 melanoma specimens. Expression was grouped into 5 categories: Z-scores of ≥ 2 or ≤ -2 , representing more than 2 standard deviations from the mean, were considered as highly over- or underexpressed (labeled **++**, **--**), while Z scores of between 1 and 2 or -1 and -2 were considered as moderately over- or underexpressed (labeled **+**, **-**). Z scores between 1 and -1 were considered as no change (NC). Gene symbols and names are as approved names by HUGO gene nomenclature committee (HGNC).

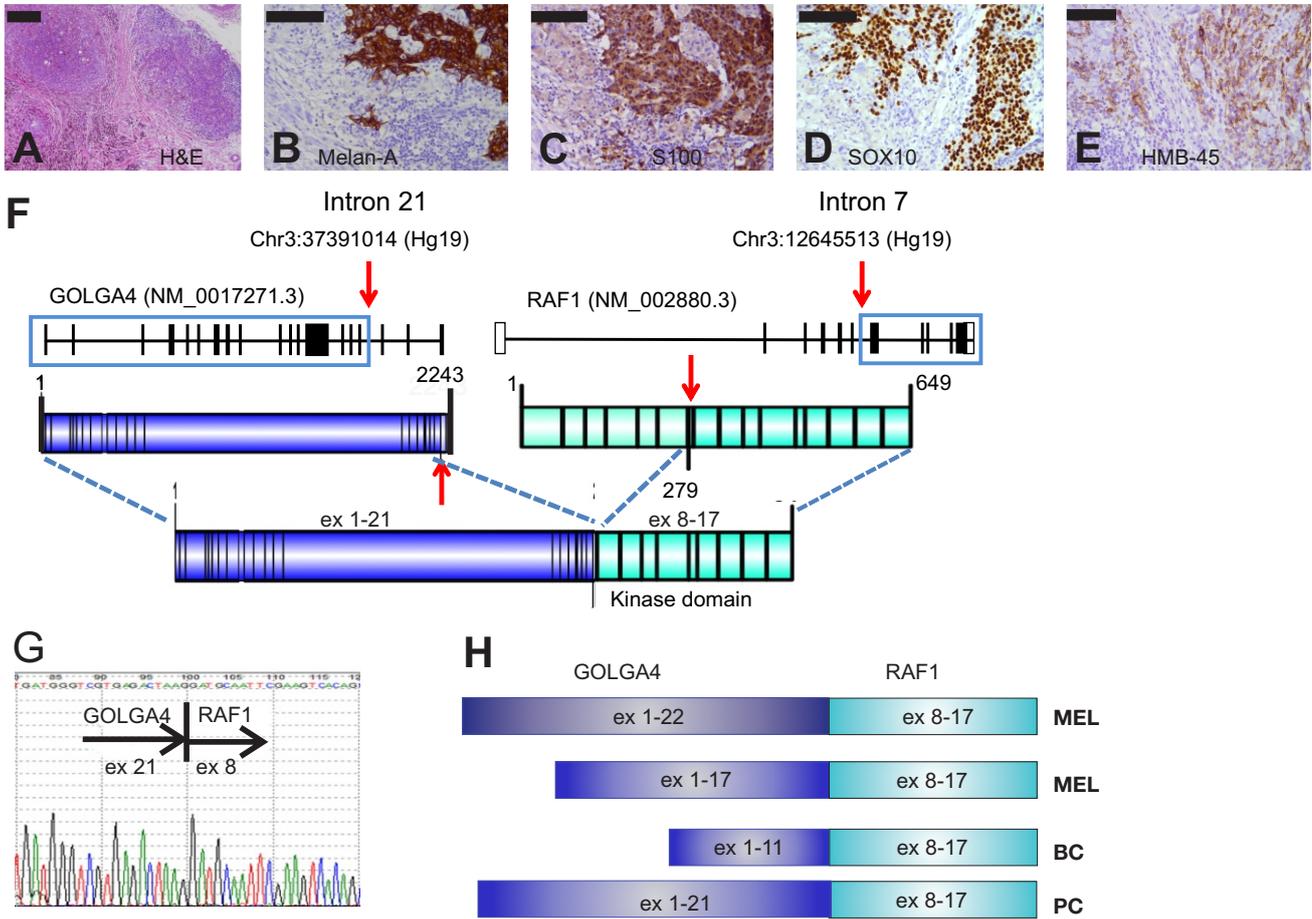


Figure 1. Metastatic melanoma with a GOLGA4-RAF1 fusion. (A) Hematoxylin and Eosin (H&E) stain of lymph node metastasis. (B-E) Immunohistochemistry (IHC) for Melan-A (B); S100 (C); SOX10 (D); HMB-45 (E). (F) Schematic illustration of the GOLGA4-RAF1 fusion. (G) Sanger sequence of RT-PCR product. (H) Illustration of previously described GOLGA4-RAF1 fusions showing consistent retention of the RAF1 kinase domain (exons 8-17). Scale bar = 100 μ m. MEL: Melanoma; BC: Breast Cancer; PC: Prostate Cancer; ex: exon

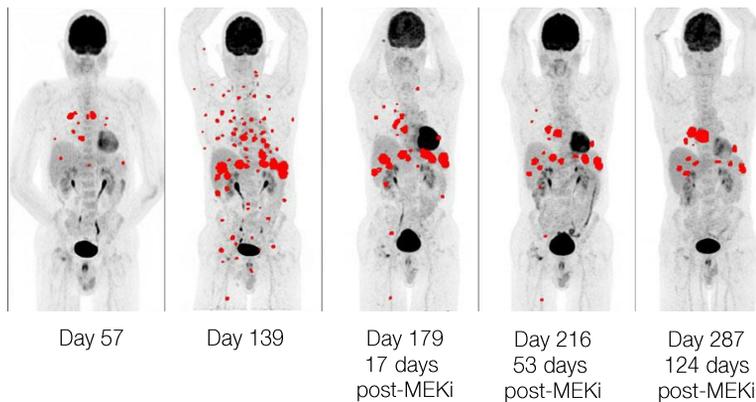
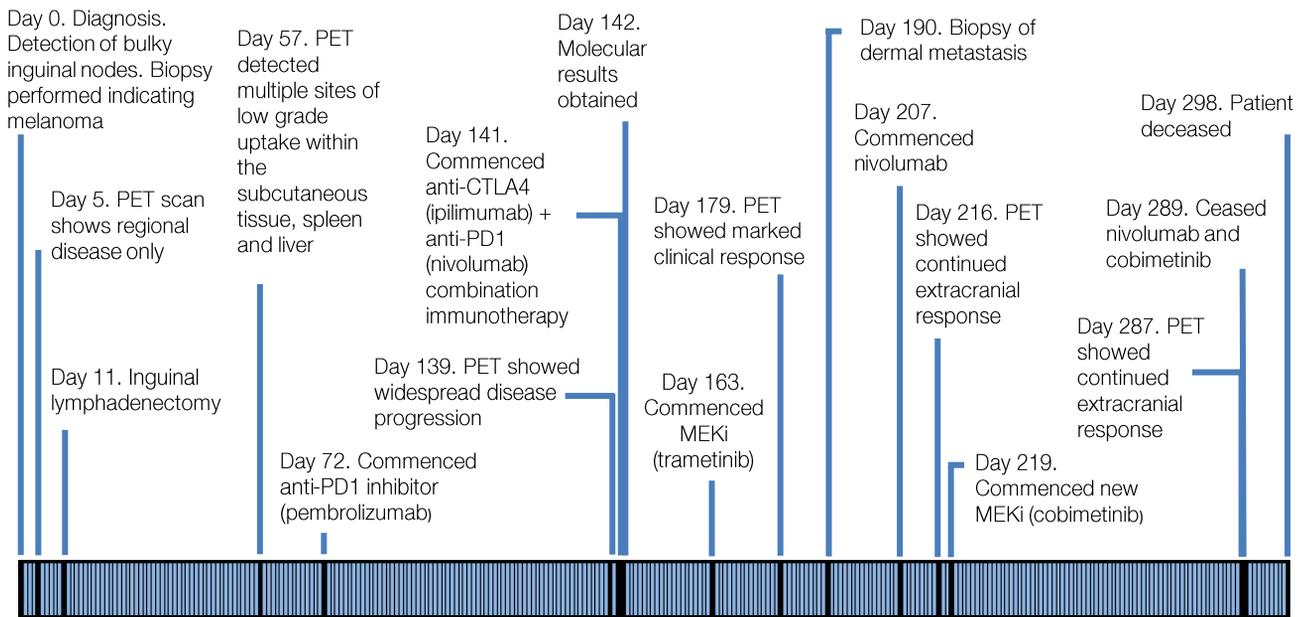


Figure 2. Clinical timeline of patient with Maximum Intensity Projection (MIP) FDG-PET (MIP PET) imaging times noted. Images show profound clinical response post-MEKi treatment. **Day 57:** Image demonstrates baseline metastatic disease pre-MEKi. **Day 139:** Disease progression on follow-up study. **Day 179 (17 days post-MEKi):** Significant metabolic response. **Day 216 (53 days post MEKi)** and **Day 287 (124 days post-MEKi):** Continued metabolic response. The maximum standardised uptake value (SUVmax) of a representative target lesion declined from 9.0 at **Day 139** (pre-MEKi) to 6.0 (**Day 179**), 4.2 (**Day 216**) and 1.8 (**Day 287**). All FDG-avid metastatic sites are colored in red.

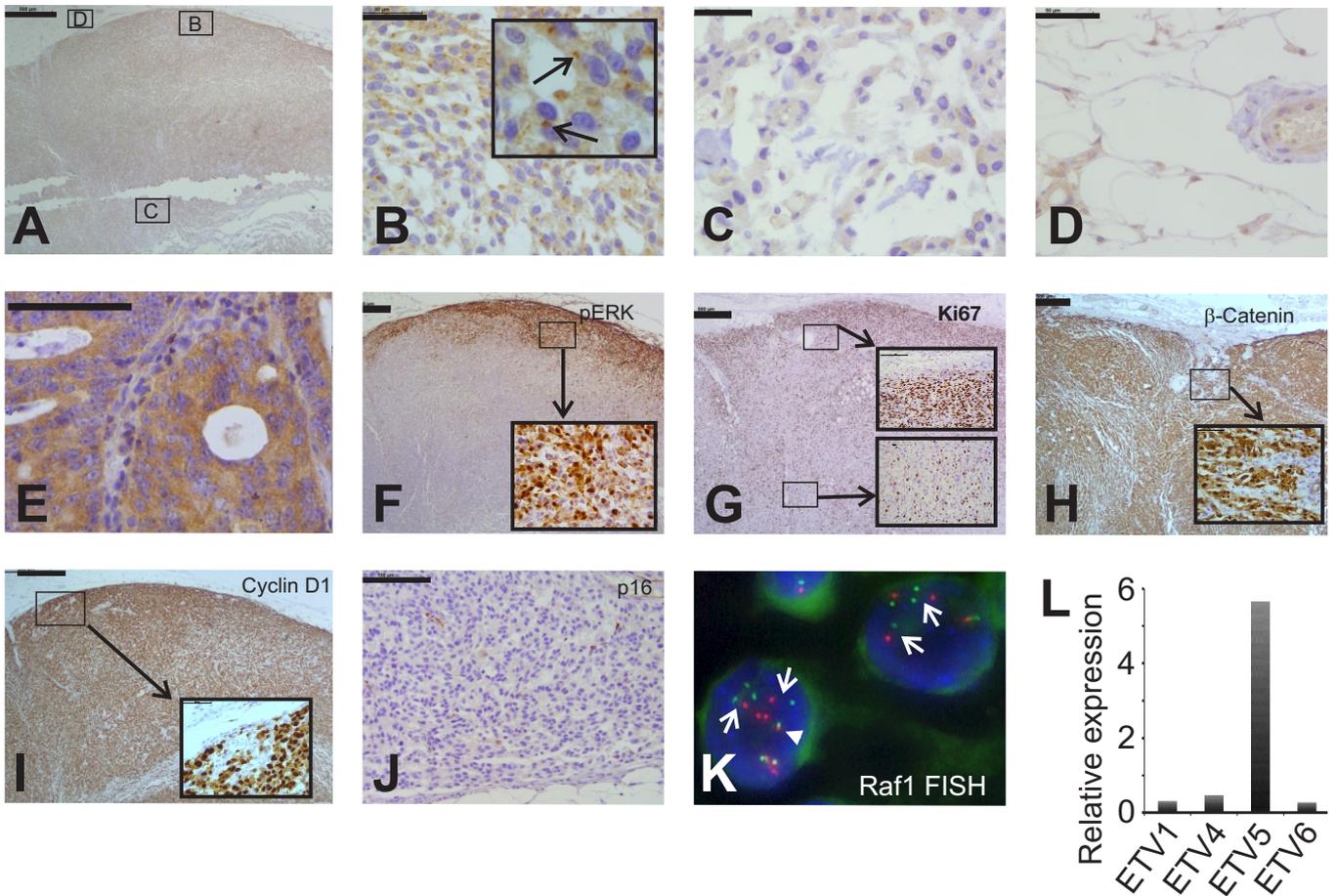


Figure 3. Expression of key MAPK, WNT and cell cycle proteins. (A-E) Immunohistochemistry (IHC) for CRAF in the lymph node metastasis (A-D) and in an on-slide positive control (colorectal adenocarcinoma, E). The locations of the images in B-D (boxes) are shown in A. Arrows show strong paranuclear staining in peripheral tumor cells in the lymph node metastasis (insert B). There is weak cytoplasmic staining in the central tumor cells in the lymph node metastasis (C) and in the perinodal stromal cells (D) and strong diffuse cytoplasmic staining without paranuclear accentuation in the colorectal adenocarcinoma (E). (F) IHC for pERK showing strong nuclear staining and weak in peripheral tumour cells in the lymph node metastasis (high power image in insert). (G) IHC for Ki67 showing high percentage of labelled tumour cells in periphery but not center of the lymph node metastasis. Note the similar expression pattern to pERK. (H,I) IHC for β -catenin (H) and cyclin D1 (I) showing strong nuclear and moderate cytoplasmic staining in both peripheral and central tumor cells (high power image in inserts). (J) IHC for p16 showing no staining in tumor cells (the occasional positive staining is in stromal cells). (K) Breakapart RAF1 FISH in tumor cells in the lymph node metastasis. Note the multiple widely separated 5' (green, centromeric) and 3' (red, telomeric) signals in tumor cells (arrows). A fused 5' and 3' signal is shown for comparison (arrowhead). (L) Archer Dx RNA analysis of relative expression of ETS family members showing elevated expression of the RAS/RAF downstream co effector ETV5. Scale bar = 500 μ m (A, F, G, H, J); 100 μ m (E, I); 50 μ m (B, C, D).