RASopathy Gene Mutations in Melanoma

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Next-generation sequencing of melanomas has unraveled critical driver genes and genomic abnormalities, mostly defined as occurring at high frequency. In addition, less abundant mutations are present that link melanoma to a set of disorders, commonly called RASopathies. These disorders, which include neurofibromatosis and Noonan and Legius syndromes, harbor germline mutations in various RAS/mitogen-activated protein kinase signaling pathway genes. We highlight shared amino acid substitutions between this set of RASopathy mutations and those observed in large-scale melanoma sequencing data, uncovering a significant overlap. We review the evidence that these mutations activate the RAS/mitogen-activated protein kinase pathway in melanoma and are involved in melanomagenesis. Furthermore, we discuss the observations that two or more RASopathy mutations often co-occur in melanoma and may act synergistically on activating the pathway.

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Exome and genome sequencing have unraveled a large number of genetic and genomic changes in melanoma (Hodis et al., 2012; Krauthammer et al., 2012; Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). The results confirmed the presence of frequent activating mutations in *BRAF* and *NRAS* and inactivating mutations in *CDKN2A* and *TP53*, and they unraveled additional lower frequency "drivers," including the recurrent $RAC1^{P29S}$ and IDH^{R132C} and the frequently modified *PPP6C*, *ARID1*, and *ARID2*. The most recent findings highlight the numerous *NF1* (neurofibromin 1) mutations affecting up to approximately 12% of all melanomas, with higher frequency (45%) in melanomas that are wild type (WT) for *BRAF* and *RAS*, with abundant inactivating mutations, such as early termination, insertions/deletions, and splice variants (Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). Consequently, the consensus is that melanomas can be subdivided into four categories: $BRAF^{mut}$, RAS^{mut}, $NF1^{mut}$, and triple WT (Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). Other cancers with large number of *NF1* mutations include glioblastoma (14%) (The Cancer Genome Atlas Network, 2008) and squamous cell carcinoma (11%) (The Cancer Genome Atlas Network, 2012).

The "NF1 discovery" draws attention to the autosomal-dominant genetic disorder neurofibromatosis type 1 (NF1), caused by haploinsufficiency of neurofibromin, a RAS guanosine triphosphate (GTP)ase-activating protein that affects 1 in 2,500 to 1 in 3,500 individuals (Aoki et al., 2016; Ratner and Miller, 2015; Smpokou et al., 2015;). The classic manifestations of NF1 include café-au-lait macules (observed in 95% of patients), skinfold freckling, neurofibromas, brain tumors, iris hamartomas, and characteristic bony lesions. *NF1* early-termination mutations in patients' germlines are frequent (~80%), leading to release of constraints on RAS, followed by mitogen-activated protein kinase (MAPK) activation (Ratner and Miller, 2015), recapitulating the observations in melanoma (Krauthammer et al., 2015).

Neurofibromatosis is one of many autosomal-dominant genetic disorders with overlapping sets of symptoms, currently termed RASopathies (including Noonan and Legius syndromes), that have germline nonsynonymous mutations in genes encoding proteins in the RAS/MAPK signaling cascade. In addition to *NF1*, the list includes *BRAF*, *RAF1*, *NRAS*, *KRAS*, *HRAS*, *RASA2*, *PTPN11*, *SPRED1*, *SOS1*, *CBL*, *SHOC2*, *MAP2K1*, *MAP2K2*, and *RIT1* (Ratner and Miller, 2015; Aoki et al., 2016) (Figure 1 and Table 1). Somatic mutations in these genes are also observed in cancer, where they may be functionally relevant, as assessed by their ability to activate the RAS/MAPK pathway and/or enhance cell proliferation. Often, these somatic mutations alter the very same amino acid present in the germline of RASopathy patients (Table 1). In melanoma, this relationship and functional consequences are most clearly established for changes in *BRAF, NRAS, MAP2K1,* and *RASA2*.

COMPARISONS OF SPECIFIC GENES

BRAF

The canonical V600E/K substitutions lead to *BRAF*-kinase activation, the first to be targeted by specific inhibitors (Bollag et al., 2012). Other changes in *BRAF* (L245F, F468S, G469R, L485F, N581S/T, K601E) are shared between melanoma and the RASopathies cardio-facio-cutaneous and Noonan syndromes (Rodriguez-Viciana and Rauen, 2008) (Table 1). Many of these noncanonical alterations are located within the kinase domain (amino acids 457–713) and are activating mutations that lead to increased kinase activity over $BRAF^{WT}$ and extracellular signal-regulated kinase (ERK) activation in transfected COS cells (Rodriguez-Viciana and

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Abbreviations: ERK, extracellular signal-regulated kinase; GTP, guanosine triphosphate; MAPK, mitogen-activated protein kinase; NF1, neurofibromatosis type 1; WT, wild type

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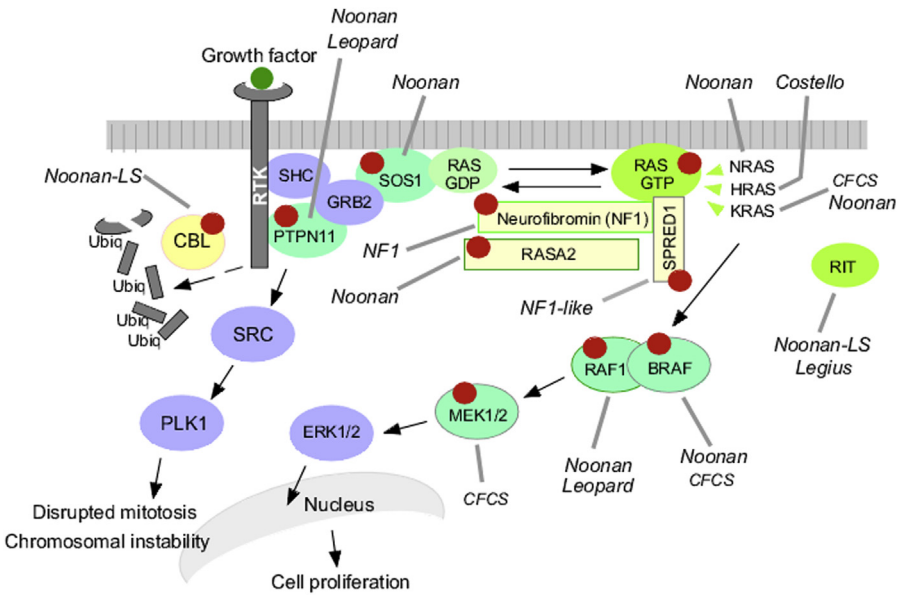

Figure 1. Mitogen-activated protein kinase pathway indicating RASopathy mutant genes and those shared with melanoma. The green and yellow bars are RASopathy genes with (presumably) activating and inactivating mutations, respectively. Those with amino acid changes shared with melanoma are marked with a red dot. CFCS, cardio-facio-cutaneous syndrome; GDP, guanosine diphosphate; GTP, guanosine triphosphate Noonan-LS, Noonan-like syndrome; RTK, receptor tyrosine kinase.

Rauen, 2008; Wan et al., 2004). Furthermore, *BRAF* G469E, D594G, and K601E mutant melanomas display increased ERK phosphorylation over nonmutant control cell lines (Smalley et al., 2009).

Table 1. Melanoma and RASopathy Shared Gene Mutations[1]

Gene Symbol	Shared Amino Acid Change	RASopathy Syndrome Type
NF1	*R1241*, R1362*, R1870Q,* and other nonsense mutations causing premature truncation	Neurofibromatosis 1
BRAF	L245F, F468S, G469R, L485F, N581H (K/D), V600G	Cardio-facio-cutaneous syndrome, Noonan syndrome
NRAS	G12D/R/V, G13D, T50I	Noonan syndrome
KRAS	G12A/I/D/R (S), Q22K (E/R/L), Q61R	Cardio-facio-cutaneous syndrome, Noonan syndrome
HRAS	G13R/D (C), Q61K (R)	Costello syndrome
RAF1	S257L, P261L (H/T/A/S), T491I (R)	Noonan syndrome, LEOPARD syndrome
MAP2K1	P124L (D)	Cardio-facio-cutaneous syndrome
MAP2K2	F57L/V (C)	Cardio-facio-cutaneous syndrome, Noonan syndrome
RASA2	R511C	Noonan syndrome
SPRED1	R117Q (*) and other nonsense mutations causing premature truncation	Neurofibromatosis 1-like syndrome, Legius syndrome
PTPN11	F71L, Y279C, A461T, T468M, P491L, Q506P, Q510H	Noonan syndrome, LEOPARD syndrome
SOS1	P102S (R), M269K (T/R), G434R, R552K (T/S/M/G), D1200E	Noonan syndrome
CBL	L493F	Noonan-like syndrome

[1]The melanoma mutations are from Yale, Broad Institute, and The Cancer Genome Atlas data; the RASopathy syndrome mutations are from the Human Gene Mutation Database (Stenson et al., 2012) and ClinVar (Landrum et al., 2014). The additional alternative amino acid substitutions in RASopathy genes not shared with melanomas are indicated in parenthesis. An asterisk indicates early termination.

NRAS

Melanomas typically harbor changes in the Q61 position of *NRAS* and, to a much lesser degree, in G12 and G13. Mice knock-in studies showed that expression of $Nras^{Q61R}$ but not $Nras^{G12D}$ promoted melanoma formation in vivo in *p16INK4A*-deficient mice (Burd et al., 2014). Functional studies showed that the basis for these differences is $Nras^{Q61R}$ enhanced GTP binding, decreased intrinsic GTPase activity, and increased stability when compared with $Nras^{G12D}$ (Burd et al., 2014). Germline mutations in Q61 were not reported, but Noonan syndrome patients and those with melanomas share the very same G12 and G13 *NRAS* amino acid substitutions (Table 1).

MAP2K1

Recurrent $MAP2K1^{P124L/S}$ mutations are present in melanoma tumors (Krauthammer et al., 2015; Nikolaev et al., 2012), and $MAP2K1^{P124L}$ is also observed in the RASopathy cardio-facio-cutaneous syndrome. The mutation confers increased kinase activity (Carlino et al., 2015; Emery et al., 2009). The effect of the mutation on drug response is likely to be cell specific. The $MAP2K1^{P124L}$ appeared in the tumor of patient who relapsed after treatment with the MEK inhibitor selumetinib (Emery et al., 2009). In addition, pre-existing $MAP2K1^{P124L}$ diminished, but did not preclude, the clinical response to *BRAF* inhibitors of $BRAF^{mut}$ melanomas (Carlino et al., 2015; Johnson et al., 2015). In culture, two double mutant melanoma cells lines showed intermediate sensitivity to dabrafenib but were exquisitely sensitive to the downstream MAPK/ERK kinsase and ERK inhibitors trametinib and VX-11e (Carlino et al., 2015). Likewise, in our studies, treatments with the MAPK/ERK kinsase inhibitor selumetinib showed that one patient-derived melanoma cell line carrying both $BRAF^{V600K}$ and $MAP2K1^{P124L}$ mutations was relatively resistant (YUKSI melanoma line, half maximal inhibitory concentration = 374 nmol/L), whereas another one with $BRAF^{V600R}$ and $MAP2K1^{P124L}$ was highly sensitive (YUZEAL melanoma line, half maximal inhibitory concentration = 15 nmol/L) (Krauthammer et al., 2015).