Histol Histopathol (2015) 30: 763-770
DOI: 10.14670/HH-11-584

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Mutation stability in primary and metastatic melanoma: what we know and what we don't

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Summary. Despite the efficacy and success of targeted therapies, a significant number of patients with melanoma exhibit either intrinsic or acquired resistance to these drugs. Numerous mechanisms for the development of resistance have been postulated, but the precise reason for this is not known. In this review, we examine the incidence of mutations in select genes (*BRAF, NRAS, C-KIT,* and *GNAQ*) known to occur in melanoma, specifically in primary tumors and their paired metastases, to understand the significance of intratumoral heterogeneity by assessing how changes in mutation status alters the process of metastatic spread.

Our data revealed a small yet consistent degree of discordance of mutations in the MAPK pathway commonly occurring in melanoma indicating that failed targeted therapy may be a consequence of this.

Key words: Melanoma, Mutations, Stability, BRAF, NRAS

Introduction

Among skin cancers, melanoma has the greatest potential for metastasis and the worst for prognosis. Localized cutaneous melanoma may be effectively treated with complete excision, but metastatic melanoma is far more difficult to manage. Systemic therapies for melanoma have limited efficacy and are often associated with significant toxicities (Eggermont and Schadendorf, 2009). However, a recent understanding of the molecular pathways underlying melanomagenesis has led to the development of targeted therapies, which inhibit the key molecular drivers in select genotypes of metastatic melanoma. Through a more individualized approach to melanoma treatment, targeted therapies exhibit improved tumor response rates and more favorable side effect profiles compared to conventional therapies (Finn et al., 2012).

The development of targeted therapies was a consequence of the discovery of numerous oncogenes associated with melanoma. Proteins of the mitogen-activated protein kinase (MAPK) signal transduction pathway have been found to be aberrant in at least 70% of tumors (Homet and Ribas, 2014). Their mutation leads to the constitutive activation of signaling proteins, cell proliferation, and escape of the cell from apoptosis. This cascade of signaling proteins includes the receptor tyrosine kinase C-KIT (mutated in 2-3% of melanomas), the G-protein neuroblastoma RAS viral oncogene homolog (NRAS) (mutated in 15-20% of melanomas), the serine/threonine-protein kinase B-Raf (BRAF) (mutated in 40-50% of melanomas), the mitogen-activated protein kinases (MEK), and the extracellular-signal-regulated kinases (ERK) (Homet and Rivas, 2014). The genes *GNAQ* and *GNA11* (guanine nucleotide-binding proteins) encode proteins that activate the MAPK pathway, and are frequently mutated in uveal melanomas (Tsao et al., 2012). Deactivating mutations in the tumor-suppressor gene *PTEN* have often been found to occur concurrently with mutations in the MAPK pathway, leading to constitutive activation of the P13K and AKT proteins of an alternative signaling

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pathway (Haluska et al., 2006). An important inherited melanoma susceptibility gene locus, *CDKN2A*, encodes the proteins $p16^{INK4A}$ and $p14^{ARF}$, which function as both tumor-suppressors and regulators of the cell-cycle and apoptosis. Mutations of these genes as well as in *CDK4* (cyclin-dependent kinase 4) and *RB1* (retinoblastoma 1) occur frequently in melanoma-prone families and result in impaired tumor suppression and dysregulation of the cell-cycle (Hodis et al., 2012; Tsao et al., 2012).

The 2002 discovery of the high prevalence of BRAF activating mutations in melanomas by Davies et al. first prompted investigation into targeted therapy (Davies et al., 2002). This eventually led to the development and Food and Drug Administration (FDA) approval of the first successful BRAF inhibitor, Vemurafenib. Vemurafenib and the newer BRAF inhibitor Dabrafenib have both demonstrated the ability to rapidly shrink tumors possessing the *BRAFV600E* mutation in clinical trials (Jang and Atkins, 2014). Since the development of the BRAF inhibitors, other proteins in the MAPK such as MEK pathway have been selected as targets. Of the MEK inhibitors, Trametenib has so far shown the most promising results, although newer agents are currently in development (Johnson and Sosman, 2013; Kudchadkar et al., 2013).

Despite the efficacy and success of targeted therapies, a significant number of melanoma patients exhibit either intrinsic or acquired resistance to these drugs (Van Allen et al., 2014). For instance, resistance to Vemurafenib often develops within eight months (Chapman et al., 2011). Numerous mechanisms for the development of resistance have been postulated, but the precise reason for this is not known. One explanation is intratumoral heterogeneity (Somasundaram et al., 2012), i.e. the existence of subpopulations of tumor cells that do not exhibit the molecular target. These genetically distinct subpopulations may survive initial treatment and give rise to metastases that are resistant to targeted therapy.

In this review, we examine the incidence of mutations in select genes (*BRAF, NRAS, C-KIT,* and *GNAQ*) known to occur in melanoma, specifically in primary tumors and their paired metastases, to understand the significance of intratumoral heterogeneity by assessing how changes in mutation status alters the process of metastatic spread.

Methods

A retrospective literature search was performed in PubMed using various combinations of the search terms: melanoma, primary, metastatic, corresponding, paired, matched, heterogeneous, BRAF, NRAS, C-KIT, and GNAQ. A total of 16 original research articles published between June 2001 and December 2013 met criteria for inclusion in the review.

Each report was examined to obtain the incidence of a particular mutation within primary melanomas and their corresponding metastases. A primary tumor's corresponding or paired metastasis was defined as a metastasis that had occurred in the same patient and was known to disseminate from that primary tumor. From each report the following pieces of information were collected: the total number of analyzed paired primary tumors, the proportions of mutated and wild-type primary tumors, the number of analyzed paired metastatic tumors, the proportion of concordant metastatic tumors (those that matched the primary in mutation status) and the proportion of discordant metastatic tumors (those that did not match the primary). An effort was made to only include data from tumors that fit the following inclusion and exclusion criteria:

Inclusion criteria

1. Primary tumors with genotypic data as obtained by DNA extraction or immunohistochemistry
2. Metastatic tumors with genotypic data as obtained by DNA extraction or immunohistochemistry
3. Paired primary and metastatic tumors, i.e. both tumors occurred in the same patient and the metastasis was known to have disseminated from the primary tumor

Exclusion criteria

1. Primary tumors that did not give rise to any metastases
2. Primary tumors that gave rise to metastases for which genotypic data was unavailable
3. Metastatic tumors that originated from primary tumors for which genotypic data was unavailable

Three studies (two by Colombino et al. (2012, 2013) and one by Boursalt et al. (2013)) reported the proportions of mutated and wild-type noncorresponding metastases in their studied tumors, but did not indicate which proportions of these were mutated or wild-type for BRAF or NRAS. Thus, for the purposes of calculation, these numbers were extrapolated from the available data for these studies. For example, if the study reported that 55% of total metastases were mutated for BRAF, an assumption was made that 55% of the metastases with matched primaries were also mutated for BRAF (even though this number was not specifically reported).

Results

BRAF mutation analyses

A total of 10 studies (Omholt et al., 2003; Shinozaki et al., 2004; Akslen et al., 2005; Jung et al., 2010; Colombino et al., 2012, 2013; Yancovitz et al., 2012; Boursault et al., 2013; Heinzerling et al., 2013; Zebary et al., 2013; Choi et al., 2014) were identified in which the incidence of BRAF mutations in the primary and corresponding metastases were detailed (Table 1). These

reports included a total of 485 specimens of primary tumor and 666 specimens of paired metastases. Of the primary tumors, 229 (47.2%) exhibited a mutation in BRAF (the majority were *BRAFV600E* mutations, but *BRAFV599E, BRAFV600L, BRAFV600K, BRAFV600D,* and *BRAFV600R* were also present in 8-12% of the

Table 1. Incidence of BRAF mutation in primary and metastatic melanomas.

Reference		Cases studied		Cases analyzed		Methodology	Comments	Limitations
		Primary	Metastases	Primary	Metastases			
Omholt et al., 2003	Total	70	88	70	88		Method: DNA extraction Analyzed exons: 11, 15	Makes no comment on concordance between metastases
	Mutated	40	43	40	43	Mutated		
					0	WT (disc.)		
	WT	30	45	30	4	Mutated (disc.)		
					41	WT		
Shinozaki et al., 2004	Total	59	68	13	13		Method: DNA extraction Analyzed exons: 11, 15	Studied only single primary-metastatic pairs
	Mutated	18	48	4	4	Mutated		
					0	WT (disc.)		
	WT	41	20	9	5	Mutated (disc.)		
					4	WT		
Akslen et al., 2005	Total	51	18	17	18		Studied nodular melanomas only Method: DNA extraction Analyzed exons: 11, 15	Makes no comment on concordance between metastases
	Mutated	15	8	6	6	Mutated		
					0	WT (disc.)		
	WT	33	10	11	2	Mutated (disc.)		
					9	WT		
Jung et al., 2010	Total	96	15	15	15		Method: DNA extraction Analyzed exons: 15	Studied only single primary-metastatic pairs
	Mutated	37	6	6	6	Mutated		
					0	WT (disc.)		
	WT	59	9	9	0	Mutated (disc.)		
					9	WT		
Colombino et al., 2012	Total	102	165	102	165		Method: DNA extraction Analyzed exons: 11, 15 Includes 2 BRAF positive tumors that gave rise to NRAS positive metastases	
	Mutated	44	9	44	NA (70)**	Mutated		
					9*	WT (disc.)		
	WT	58	6	58	6	Mutated (disc.)		
					NA (80)**	WT		
Yancovitz et al., 2012	Total	18	94	18	18		Method: DNA extraction Analyzed exons: 11, 15	Studied only single primary-metastatic pairs
	Mutated	12	29	12	10	Mutated		
					2	WT (disc.)		
	WT	6	65	6	6	Mutated (disc.)		
					0	WT		
Colombino et al., 2013	Total	451	298	138	236		Method: DNA extraction Analyzed exons: 11, 15	Makes no comment on concordance between metastases
	Mutated	221	152	68	NA (116)**	Mutated		
					13	WT (disc.)		
	WT	230	146	70	14	Mutated (disc.)		
					NA (93)**	WT		
Choi, 2014	Total	22	18	8	9		Method: DNA extraction Analyzed exons: 15 Notes concordance in patient with multiple mets	Studied acral and mucosal melanomas only
	Mutated	6	4	2	2	Mutated		
					0	WT (disc.)		
	WT	16	14	6	0	Mutated (disc.)		
					7	WT		
Zebary et al., 2013	Total	88	16	16	16		Method: DNA extraction Analyzed exons: 11, 15	Studied only single primary-metastatic pairs
	Mutated	15	5	5	5	Mutated		
					0	WT (disc.)		
	WT	73	11	11	0	Mutated (disc.)		
					11	WT		
Boursalt et al., 2013	Total	88	142	88	88		Method: DNA extraction Analyzed exons: 15	Studied only single primary-metastatic pairs
	Mutated	42	63	42	NA (40)**	Mutated		
					2	WT (disc.)		
	WT	46	79	46	2	Mutated (disc.)		
					NA (42)**	WT		
Heinzerling et al., 2013	Total	90	210	16	37		Method: DNA extraction Analyzed exons: not mentioned	Notes concordance in patient with multiple mets
	Mutated	41	108	11	13	Mutated		
					16	WT (disc.)		
	WT	49	102	5	0	Mutated (disc.)		
					8	WT		

** extrapolated data. disc, discordant

studied tumors) and 256 (52.8%) exhibited *BRAFWT*. The 229 mutated primary tumors gave rise to 328 metastases, of which 26 (7.9%) were *BRAFWT* and thus discordant with the mutational status of the primary tumor. Similarly, the 256 *BRAFWT* melanomas gave rise to 338 metastases of which 39 (11.5%) were mutated for BRAF and thus also discordant. In total, of 666 metastases, 65 (9.8%) exhibited a discordant BRAF status.

NRAS mutation analyses

A total of 6 studies were identified in which incidence of NRAS mutations in the primary and corresponding metastases were detailed (Table 2). These reports included a total of 360 specimens of primary tumor and 556 specimens of paired metastases. Of the primary tumors, 71 (19.7%) exhibited a mutation in *NRAS* (these included *Q61R, Q61L, Q61K, G12A,* and *G13D* mutations) and 289 (80.3%) exhibited *NRASWT*. The 71 mutated primary tumors gave rise to 112 metastases, of which 4 (3.6%) were *NRASWT* and thus discordant with the mutational status of the primary tumor from which they originated. Similarly, the 289 NRASWT melanomas gave rise to 444 metastases of which 10 (2.3%) were mutated for NRAS and thus discordant with their primary tumors. In total, among 556 metastases, 14 (2.5%) exhibited a discordant NRAS status.

C-KIT mutation analyses

Two studies described the incidence of C-KIT mutation in primary and corresponding metastatic melanomas (Table 3). These reports included a total of 39 specimens of primary tumor and 39 specimens of paired metastases. Of the primary tumors, 11 (28.2%) exhibited a mutation in C-KIT and 28 (71.8%) were wild-type for *C-KIT*. The 11 mutated primary tumors gave rise to 11 metastases, of which 3 (27.3%) were wild-type for *C-KIT* and thus discordant with the primary tumors. Similarly, the 28 wild-type tumors gave

Table 2. Incidence of NRAS mutation in primary and metastatic melanomas.

Reference		Cases studied		Cases analyzed		Methodology	Comments	Limitations
		Primary	Metastases	Primary	Metastases			
Colombino et al., 2012	Total	102	165	102	165		Method: DNA extraction Analyzed exons: 2 and 3 Includes 2 BRAF positive tumors that gave rise to NRAS positive metastases	No comment on concordance between metastases
	Mutated	15	25	15	NA (24)**	Mutated		
					1	WT (disc.)		
	WT	87	140	87	6*	Mutated (disc.)		
					NA (134)**	WT		
Colombino et al., 2013	Total	451	298	138	236		Method: DNA extraction Analyzed exons: 2 and 3	No comment on concordance between metastases
	Mutated	68	48	21	NA (37)**	Mutated		
					1	WT (disc.)		
	WT	383	250	117	4	Mutated (disc.)		
					NA (194)**	WT		
Omholt et al., 2002	Total	74	89	54	89		Method: DNA extraction Analyzed exons: 2	No comment on concordance between metastases
	Mutated	21	34	20	33	Mutated		
					1	WT (disc.)		
	WT	53	55	34	0	Mutated (disc.)		
					55	WT		
Akslen et al., 2005	Total	51	18	15	15		Nodular melanomas only Method: DNA extraction Analyzed exons: 1, 2	Studied only single primary-metastatic pairs
	Mutated	14	4	3	3	Mutated		
					0	WT (disc.)		
	WT	37	14	12	0	Mutated (disc.)		
					12	WT		
Zebary et al., 2013	Total	88	16	16	16		Method: DNA extraction Analyzed exons: 1, 2	Studied only single primary-metastatic pairs
	Mutated	13	3	3	3	Mutated		
					0	WT (disc.)		
	WT	75	13	13	0	Mutated (disc.)		
					13	WT		
Uhara, 2013	Total	67	35	35	35		Method: DNA extraction Analyzed exons: 1, 2	Studied only single primary-metastatic pairs
	Mutated	13	9	9	8	Mutated		
					1	WT (disc.)		
	WT	54	26	26	0	Mutated (disc.)		
					26	WT		

** extrapolated data. disc, discordant

rise to 28 metastases, of which 0 (0.0%) were disocordant with their primary tumors. In total, among 39 metastases, 3 (7.7%) exhibited a discordant *C-KIT* status.

Notably, one of the two studies utilized immunohistochemistry to identify *C-KIT* mutation, while the other used DNA extraction and sequenced exons 9, 11, 13, 17, 18 of the *C-KIT* gene.

GNAQ mutation analyses

A single study analyzed the incidence of GNAQ mutation in primary and corresponding melanomas (Table 4). The study reported a total of 11 primary tumors which gave rise to 11 paired metastases. Of the 11 primary tumors, 3 exhibited the mutant GNAQ while 8 exhibited the wild-type GNAQ. None (0.0%) of the 11 metastases that originated from these tumors were discordant, i.e. the metastases exhibited the same GNAQ mutational status as their primaries.

Discussion

Metastatic melanoma is often challenging to treat as patients often exhibit drug resistance and experience tumor recurrence (Flaherty et al., 2010a,b). While a precise cause is not known, several theories have attempted to explain why patients acquire resistance to therapy (Bradbury and Middleton, 2004; Kauffmann et al., 2008; Sarasin and Kauffmann, 2008; Villaneuva et al., 2010; Somasundaram et al., 2012; Sullivan and Flaherty, 2013). Briefly, resistance may result from changes in the tumor cells or from changes in the tumor microenvironment. Cellular changes that may be selected for by drug therapy include increased drug efflux activity (Schadendorf et al., 1995; Luo et al., 2012), increased DNA repair activity (Bradbury and Middleton, 2004; Kauffmann et al., 2008; Sarasin and Kauffmann, 2008), and the activation of alternate signaling mechanisms (e.g. the activation of alternative RAF isoforms to sustain the MAPK signaling pathway) (Villanueva et al., 2010; Sullivan and Flaherty, 2013). Changes in the tumor microenvironment (TME) include the accumulation of stromal-derived fibroblasts and tumor suppressing immune cells such as macrophages, lymphocytes, dendritic cells, and natural killer cells which secrete chemokines and growth factors to sustain tumor growth and alter treatment outcome (Somasundaram et al., 2012; Sounni and Noel, 2013).

Importantly, resistance of tumors to drug therapy, particularly targeted therapy, may also be explained by intratumoral heterogeneity, or the existence of multiple subclones of tumor cells with varying genotypes (Hiley et al., 2014). These subclones of tumor cells are believed

Table 3. Incidence of C-KIT mutation in primary and metastatic melanomas.

Reference		Cases studied		Cases analyzed		Methodology	Comments	Limitations
		Primary	Metastases	Primary	Metastases			
Dai et al., 2013	Total	39	23	23	23		Acral melanomas only Method: immunohistochemistry	Studied only single primary-metastatic pairs
	Mutated	9	6	6	3	Mutated		
					3	WT (disc.)		
	WT	30	17	17	0	Mutated (disc.)		
					17	WT		
Zebary et al., 2013	Total	16	16	16	16		Method: DNA extraction Analyzed exons: 9, 11, 13, 17, 18	Studied only single primary-metastatic pairs
	Mutated	5	5	5	5	Mutated		
					0	WT (disc.)		
	WT	11	11	11	0	Mutated (disc.)		
					11	WT		

disc, discordant

Table 4. Incidence of GNAQ mutation in primary and metastatic melanomas.

Reference		Cases studied		Cases analyzed		Methodology	Comments	Limitations
		Primary	Metastases	Primary	Metastases			
Dratviman-Storobinsky, 2010	Total	27	11	11	11		Method: DNA extraction Analyzed exons: 5	Studied only single primary-metastatic pairs
	Mutated	12	3	3	3	Mutated		
					0	WT (disc.)		
	WT	15	8	8	0	Mutated (disc.)		
					8	WT		

disc, discordant

to arise either from random genetic drift or from the selection of cells that have a phenotypic advantage within a particular environment. Selective pressures such as hypoxia (Widmer et al., 2013) and even drug therapy (Shi et al., 2014) may influence the development of tumor subclones. The phenomenon of intratumoral heterogeneity has been observed in numerous malignancies including breast, ovarian, prostate, pancreatic, bladder, chronic lymphocytic leukemia, acute myelocytic leukemia, glioma, and clear cell renal cell carcinoma (Hiley et al., 2014). In melanoma, the concept of tumor heterogeneity was described as early as 1820 when a dissected melanoma metastasis was discovered to comprise of sections of differently colored tissue. More recently, Yancovitz et al used laser-capture microdissection to demonstrate substantial genetic variability between different regions in primary melanomas (Yancovitz et al., 2012), and Fusi et al. used a novel sequencing technique to demonstrate *BRAFV600E* mutated circulating melanoma cells in patient whose primary tumor exhibited *BRAFWT* (Fusi et al., 2011). Wilmott et al describe a patient whose melanoma metastasis initially exhibited the *BRAFV600E* mutation, but following 7 months of treatment with Vemurafenib, developed a subclone of tumor cells with a *NRASG13R* mutation resistant to Vemurafenib therapy (Wilmott et al., 2012).

Patients with metastatic melanoma often undergo mutational analysis of a single lesion and an appropriate targeted therapy is selected. If, for instance, mutational analysis reveals a patient's tumor to be positive for BRAF but fails to detect small subpopulations of BRAF negative tumor cells that then form BRAF negative metastases, a BRAF inhibitor would eventually fail in this patient. The objective of our literature review was to understand the significance of this phenomenon of intratumoral heterogeneity by studying the mutational status of primary melanomas and their corresponding metastases. A higher genotypic discordance for a particular mutation between primary and metastatic tumor suggests a greater heterogeneity of that mutation within the primary tumor, and a greater need for additional mutational testing.

Limitations

The foremost limitation of this review is the lack of uniformity between the analyzed literature reports, as many used different methodologies with varied sensitivity and accuracy in detecting mutations. For example, while Zebary et al. (2013) assessed for the presence of a C-KIT mutation by DNA extraction and sequencing, Dai et al. (2013) analyzed 23 primary tumors and 23 paired metastatic tumors by immunohistochemical staining for C-KIT. Furthermore, the studies which extracted and sequenced DNA used techniques and kits of varying sensitivity. Importantly, not all studies sequenced all exons known to exhibit mutations of a particular gene, for example Choi et al. (2014) and Boursalt et al. (2013) analyzed only exon 15 of the BRAF gene, and Omholt et al. (2003) only analyzed 2 of the NRAS gene. These analyses are thus further limited in sensitivity. Thus, the usage of multiple methods to obtain genotypic information limits the reliability of the collected data.

The differences in the types of melanomas studied is another limiting factor of this analysis, as certain mutations are known to exhibit a predilection for melanomas of a certain area of the body. Studies such as the one conducted by Choi et al. (2014) analyzed a disproportionately high number of acral and mucosal melanomas, which are more likely to exhibit the *BRAFWT* than other melanomas, thus diluting the numbers of *BRAF* mutated primary melanomas in our analysis. Similarly, Akslen et al. studied only nodular melanomas known to harbor mutations in both BRAF and NRAS.

Another major limitation of our review is the small sample size; while many reports found in the literature comment on the general incidence of *BRAF, NRAS, C-KIT,* and *GNAQ* mutation in melanomas, few describe that incidence specifically in the context of corresponding (paired) tumors. Thus, we were only able to include 16 studies in this analysis, limiting the sample size of the assessed tumors. Also, many studies did not have access to or chose not to analyze more than one metastasis per primary tumor. Thus, the number of sets of multiple metastases in our review is very small. Of note, in two instances, numbers had to be extrapolated for calculation as they were not reported in the original study.

Conclusion

We found no instances in which multiple metastases that had arisen from the same primary tumor exhibited a different genotype, suggesting that different metastases have a clonal relationship. Of note, our data revealed a small yet consistent degree of discordance of mutations in the MAPK pathway commonly occurring in melanoma indicating that failed targeted therapy may be a consequence of this. Additional studies are required to confirm our findings.

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Accepted January 13, 2015