Title

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Journal

Journal of Investigative Dermatology, 140(8)

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Publication Date

2020-08-01

DOI

10.1016/j.jid.2020.01.027

Peer reviewed

HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2021 August 01.

Published in final edited form as:

J Invest Dermatol. 2020 August ; 140(8): 1609–1618.e7. doi:10.1016/j.jid.2020.01.027.

TERT, BRAF, and *NRAS* mutational heterogeneity between paired primary and metastatic melanoma tumors

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Abstract

Mutational heterogeneity can contribute to therapeutic resistance in solid cancers. In melanoma, the frequency of inter- and intra-tumoral heterogeneity is controversial. We examined mutational

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AUTHOR CONTRIBUTIONS

Conceptualization: GC, BC, JW, YS, CS, DP; Data Curation: GC, BC, JW, JT, SB, NF; Formal Analysis: GC, BC, JW; Funding Acquisition: DP, IO, YS, CS, RS; Investigation: GC, BC, JW, MS, JT, SB, NF, FD; Methodology: GC, BC, JW, JT, SB, DP; Project Administration: DP, IO; Resources: AP, RB, RS, IO, GKN; Supervision: DP, IO; Validation: GC, BC, JW, SB, DP; Visualization: GC, BC, JW, IO, DP; Writing: GC, BC, JW, DP; Writing - Review and Editing: GC, BC, JW, MS, JT, SB, NF, FD, AP, RB, RS, YS, GKN, CS, IO, DP

CONFLICT OF INTEREST STATEMENT

David Polsky was a consultant for Molecular MD until October 2019.

Shria Blake and Cindy Spittle are employees of Molecular MD.

George Karlin-Neumann is an employee of Bio-Rad Laboratories, Inc.

heterogeneity within individual melanoma patients using multi-platform analysis of commonly mutated driver and non-passenger genes.

We analyzed paired primary and metastatic tumors from 60 patients, and multiple metastatic tumors from 39 patients whose primary tumors were unavailable (n=271 tumors). We used a combination of multiplex SNaPshot assays, Sanger Sequencing, Mutation-specific PCR, or droplet digital PCR to determine the presence of $BRAF^{V600}$, $NRAS^{Q61}$, and $TERT^{-124C>T}$ and $TERT^{-146C>T}$ mutations.

Mutations were detected in *BRAF* (39%), *NRAS* (21%) and/or *TERT* (78%). Thirteen patients had $TERT^{mutant}$ discordant tumors; seven of these had a single tumor with both $TERT^{-124C>T}$ and $TERT^{-146C>T}$ mutations present at different allele frequencies. Two patients had both *BRAF* and *NRAS* mutations; one in different tumors and the other had a single tumor with both mutations. One patient with a $BRAF^{mutant}$ primary lacked mutant *BRAF* in least one of their metastases. Overall, we identified mutational heterogeneity in 18/99 (18%) patients.

These results suggest that some primary melanomas may be comprised of subclones with differing mutational profiles. Such heterogeneity may be relevant to treatment responses and survival outcomes.

INTRODUCTION

Inter and intra-tumor heterogeneity have been described in many solid cancers, and may be a source of therapeutic resistance (McGranahan and Swanton, 2017). Some subclones may be naturally resistant to a given treatment, while others arise in response to therapy. Thus, identification of tumor heterogeneity may be relevant when investigating treatment efficacy. Based on recent exome sequencing studies, melanoma and lung cancer are considered to have the least amount of mutational heterogeneity among non-synonymous (i.e. non-silent) gene mutations (McGranahan and Swanton, 2017). However, heterogeneous responses to BRAFMEKi therapy appear fairly common in melanoma, and are associated with reduced survivals (Carlino et al., 2013, Menzies et al., 2014a).

Although a meta-analysis of *BRAF* heterogeneity that only included studies with >10 melanoma patients concluded that *BRAF* mutational discrepancies between tumors occur at rates between 7% - 13% (Valachis and Ullenhag, 2017), some authors have questioned the underlying results. They cite either technical issues, or the existence of an undiagnosed second primary tumor to explain the reported mutational heterogeneity. (Menzies et al., 2014b, Riveiro-Falkenbach et al., 2017, Uguen et al., 2016).

In this study we explored melanoma mutational heterogeneity using multiple, longitudinally collected samples from patients prospectively enrolled in a melanoma biorepository program. We selected patients with at least two tumors available for analysis to assess the frequency of inter-tumor *TERT,* the most frequently mutated genes in melanoma. Their mutation rates are 52%, 28% and 64%, respectively (Akbani et al., 2015). This strategy allowed us to examine individual patients for inter-tumor heterogeneity between the primary and subsequent metastases, as well as between metastatic tumors. We undertook this

investigation using multiple mutation-detection platforms to reduce the possibility of technical errors confounding the results.

RESULTS

Patient and sample characteristics

We studied 99 patients: 60 males and 39 females. Overall we analyzed 63 primary tumors from 60 patients and 208 metastases across all patients. Three patients had two primary tumors each. Thirty-nine patients had multiple metastatic tumors, but no primary tumor available. Patient demographics and clinical characteristics are summarized in Table 1. All patient tumors were assessed for the presence of *BRAF*, *NRAS* and *TERT* mutations using multiplex SNaPshot assays.

Multiplatform mutational analysis

We conducted quality control and validation of *BRAF* and *NRAS* mutations using more than one analytical platform on 248/271 (91.5%) samples based on DNA availability after SNaPshot analysis (Figure 1a). We used Mutation-specific PCR (MS-PCR), Sanger sequencing and/or droplet digital PCR (ddPCR) as orthogonal methods. SNaPshot and MS-PCR were 95% concordant (101/106 samples tested). All five discordant samples were resolved by a third analytical platform. SNaPshot was concordant with Sanger in 190/205 (93%) cases, and with ddPCR in 18/27 (67%) cases (Table S1).

To safeguard against false heterogeneity due to a potential lack of sensitivity of the SNaPshot platform, we tested 18 tumors labeled as wild-type based on SNaPshot analysis and/or MS-PCR or Sanger with ddPCR (a more sensitive method than SNaPshot). These tumors were from patients who had other tumors that tested positive for *BRAF* or *NRAS* mutations (and had sufficient remaining DNA). Unexpectedly we identified mutations in 10/18 (56%) of these tumors, thereby refining the number of patients with *BRAF* or *NRAS* inter-tumor heterogeneity. Of note, 10 samples classified as wild-type by SNaPshot, were not confirmed by another method. Only one of these 10 samples was from a patient with other *BRAF* or *NRAS* positive tumors. Recognizing the potential for SNaPshot to deliver a false negative mutation call and our inability to employ a second platform to confirm this result, we took a conservative approach and removed this patient from the heterogeneity group. The results from this patient (03–085) are described in Table S2a. The remaining nine tumors were from patients in which the all of their other tumors were wild-type.

With respect to *TERT* promoter mutations, the initial SNaPshot analysis yielded 31 patients with inter-tumor heterogeneity (Table S2). Given the high GC content and nucleotide repeats in this region, we re-tested all 89 tumors from these patients using *TERT-mutation* specific ddPCR assays (Corless et al., 2019) after uracil DNA glycosylase (UDG) treatment to reduce the likelihood of artifactual C-T changes (Do and Dobrovic, 2015). Using ddPCR we confirmed 45 mutated and nine wild-type tumors. We also identified previously undetected mutations in 27 tumors, as well as two SNaPshot false positives (i.e. ddPCR wild-type) likely due to C-T artifacts. Among five tumors that failed SNaPshot, ddPCR identified three mutants and one wild-type. One tumor failed ddPCR (Figure 1b). In total, UDG treatment

followed by ddPCR reduced the number of patients with *TERT* inter-tumor heterogeneity from 31 to 13. Of note, 56 samples from 22 patients lacking *TERT* mutations in any of their tumors were labeled as wild-type by SNaPshot only. One other wild-type tumor was from a patient who had *TERT* promoter heterogeneity based on their other tumors, so the wild-type call on this tumor (P1) did not change the heterogeneity status of the patient (05–137) (Table S2).

After completion of the multiplatform analysis and quality control checks, our final analysis identified at least one *BRAF, NRAS* or *TERT* mutation in tumors from 87/99 (88%) patients. Thirty-nine (39%) had a detectable $BRAF^{V600}$ mutation in at least one of their tumors and 21 (21%) patients had at least one $NRAS^{Q61}$ mutant tumor. *TERT* promoter mutations were identified in 77/99 (78%) patients with 35/99 (35%) having at least one −124 [C>T] mutant tumor, 35/99 (35%) having at least one −146 [C>T] mutant tumor, and 7/99 (7%) having at least one tumor with both mutations. The frequency of mutations by anatomic site are shown in Table S3. Overall, we observed mutational heterogeneity in 18 out of 99 patients (18%) (Table 2). Seven out of 99 (7%) displayed *BRAF* tumor heterogeneity, 3/99 (3%) had *NRAS* tumor heterogeneity, and the majority 13/99 (13%) displayed heterogeneity for *TERT*.

Mutational heterogeneity between patients' primary and metastatic tumors

Among patients who had available primary tumors, 12 out of 60 displayed mutational heterogeneity between their primary and metastatic tumors (Table 2). Eight of 12 patients (67%) had mutations detected in their metastatic tumors that were not detected in their primary tumors. Five of the eight patients had a newly detected *TERT* mutation in their metastases which was not found in their primary tumors. Interestingly, four patients had detectable *BRAF* (n=3) or *NRAS* (n=1) driver mutations in one of their metastases which was undetectable in their primary tumors (Table 3a).

Unexpectedly, six of the 12 patients (50%) had mutations in their primary tumor that were undetectable in at least one of their metastases (Table 3b). Five of these six patients had *TERT* promoter mutations in the primary that were undetectable in one of their metastases. Similarly, one of these six patients (06–001) had a *BRAF* mutation in their primary that was undetectable in their metastatic tumors. Specifically, they had a $BRAF^{V600E}/TERT^{wild\text{-}type}$ 1.3 mm thick primary tumor excised from their leg and developed a subsequent regional soft tissue metastasis within one year that lacked a *BRAF* mutation. Interestingly it possessed $NRAS^{Q61K}$ and $TERT^{-124C>T}$ mutations, despite not being treated with any BRAF targeted therapy. Subsequently, the patient developed two additional regional soft tissue metastases over 12 years with the same *NRAS/TERT* mutational profile as the initial regional recurrence (Figure 2a). The mutations detected by SNaPshot were confirmed by Sanger sequencing, and one of the *NRAS* mutations was identified solely by ddPCR. Interestingly, the patient had no evidence of another melanoma in their medical record, nor was BRAF-targeted therapy administered at any time. The $TERT^{wild\text{-}type}$ result could not be confirmed by ddPCR due to insufficient DNA, so this patient was not included in the *TERT* heterogeneity group.

Mutational heterogeneity between patients' metastatic tumors

To examine inter-tumor heterogeneity between metastatic tumors from individual patients, we analyzed 70 patients with ≥2 available metastases. We found nine patients with different *BRAF*, *NRAS* or *TERT* genotypic profiles between their metastatic tumors (Table 2). Of these nine patients, five displayed additional mutations in metastases that developed at later time points. In seven patients mutations were absent in later metastases. In particular, patient 06–075 had two uniquely different metastatic samples (Figure 2b). Surprisingly, this patient had a $BRAF^{V600E}/NRAS^{Q61K}$ metastatic tumor in the left axilla 39 days after diagnosis of their primary melanoma; a $BRAF^{wild\text{-}type}/NRAS^{Q61K}$ metastatic tumor in the left flank 398 days after diagnosis; and a $BRAF^{wild\text{-}type}/NRAS^{Q61K}$ metastatic tumor in the left flank 445 days after diagnosis. All three metastases lacked *TERT* mutations (Table 2).

Intra-tumor heterogeneity in melanomas

Using the ddPCR assays we identified one tumor with both *BRAF* and *NRAS* mutations (noted above). The mutant allele frequencies were 0.78% *(BRAF)* and 1.79% *(NRAS)* (Figure 2b). In addition, we identified both *TERT* promoter mutations within seven tumors from seven individual patients (Table 2). Four of the seven samples were primary tumors, the other three were lymphatic metastases. For example patient 04–050 had a primary tumor with $TERT^{-124\ [C>T]}$ (allele frequency: 21.9%) and $TERT^{-146\ [C>T]}$ (allele frequency: 0.97%), suggesting the presence of both a dominant and minor clone (Figure 3). Overall, the allele frequencies for these mutations ranged from 0.08% to 22.3% and varied in each of the seven tumors, suggesting the presence of different subclones (Table 4). Of note, theses assays do not exhibit any cross-reactivity for the other mutation (Corless et al., 2019).

DISCUSSION

In this study, we investigated the frequency of tumor mutational heterogeneity among genes frequently mutated in melanoma, *BRAF, NRAS* and *TERT*. We identified inter-tumor mutational heterogeneity in 18% of nearly 100 patients. This included several cases where a new mutation was found in one or more metastatic tumors, consistent with tumor evolution and the emergence of more highly mutated tumor genotypes over time (Caswell and Swanton, 2017, Maley et al., 2017, Shain et al., 2015). We also had cases in which mutations identified in primary tumors were undetectable in one or more of their metastatic tumors, suggestive of polyclonality in the primary tumor. Among the strengths of this analysis was that we used at least two methods to confirm the mutational status of 91.5% of tumors, and in all 18 patients in which tumor heterogeneity was observed, at least two methods were used to confirm heterogeneity. In particular, we used highly sensitive and specific ddPCR assays (when they became available in the lab – See Supplementary Methods) to assess cases with presumptive inter-tumor mutational heterogeneity initially identified using the less sensitive platforms. We also used UDG treatment of the FFPE tumor-derived DNAs to reduce the possibility of artifactual mutations in the GC-rich *TERT* promoter. These rigorous analyses resulted in fewer patients with tumor heterogeneity based on *TERT* mutations than initially identified using SNaPshot analysis alone. We also examined a large number of tumors (n=271) for this type of analysis.

Mutational heterogeneity in melanoma has been explored by many investigators for over 10 years, focusing primarily on *BRAF* mutations (Colombino et al., 2012, Lin et al., 2011, Sensi et al., 2006). A meta-analysis of all studies with >10 patients concluded that *BRAF* tumor heterogeneity was found at a rate of 13.4% between the primary and metastatic lesions and 7.3% between metastatic tumors (Valachis and Ullenhag, 2017). Although some authors have questioned these studies, citing either technical issues to explain the lack of detection of mutations in some tumors, or the existence of another primary to explain the presence of mutations in metastatic lesions not detected in the initial primary (Menzies et al., 2014b, Riveiro-Falkenbach et al., 2017, Uguen et al., 2016), our data are in general agreement with the meta-analysis. To address potential technical concerns, we used multiple platforms to confirm mutational heterogeneity, including high sensitivity methods such as ddPCR. To address the concern regarding undiagnosed second-order primary melanomas, we obtained the tumors from our prospective clinical-pathological biorepository that includes complete clinical data and protocol-driven follow up, and included second primary tumors in the few patients in which they occurred. We found *BRAF*, *NRAS* and *TERT* mutational heterogeneity among 7%, 3% and 13% of patients respectively. We are aware of two studies of eight patients each that used whole exome sequencing to assess either for heterogeneity between primary and metastatic lesions (Sanborn et al., 2015) or for intra-tumor heterogeneity (Harbst et al., 2016). Although they did observe a divergence in passenger or late occurring mutations, no heterogeneity in *BRAF*, *NRAS* or *TERT* was found. With only eight patients each, these studies appear underpowered to detect mutational heterogeneity that may occur at rates of 18% or less.

Inter-tumor mutational heterogeneity among patients undergoing targeted therapy is much less controversial, as it has been reported by multiple investigators and is believed to stem from either a polyclonal tumor being subject to selective pressure, or the acquisition of *de novo* mutations in line with clonal evolution (Maley et al., 2017, Raaijmakers et al., 2016, Shi et al., 2014, Venkatesan et al., 2017, Wilmott et al., 2012). The pre-existence of subclones carrying resistance mutations prior to treatment is directly supported by Kemper at al. who demonstrated that resistance to vemurafenib developed due to the presence of a pre-existing *MEK* mutation in one of several metastatic tumors analyzed from a single patient (Kemper et al., 2015). In our study, there were two patients who presented with both *BRAF* and *NRAS* mutations. One patient developed the *NRAS* mutation later in their disease course, while the other patient had both mutations in the same metastasis, but only the *NRAS* mutation persisted among later metastases. *NRAS* mutations are known to confer resistance to BRAF-targeted therapies; however, neither patient received BRAF targeted therapy at any time during their treatment. Similarly, Sensi et al., in a report prior to the advent of BRAF targeted therapy, detected both $BRAF^{mutant}$ and $NRAS^{mutant}$ cells in short term cultures from a single lymph node from a melanoma patient (Sensi et al., 2006). These findings, albeit rare, raise the possibility that the intrinsic nature of melanoma may be the development of multiple subclones throughout tumor evolution, even in the absence of exogenous therapeutic pressure.

Similar to other types of cancer, branched evolution is the current biological model in melanoma (Harbst et al., 2016, McGranahan and Swanton, 2015). In this model, clones stem from a truncal or driver mutation, such as *BRAF*, and subclones (i.e. branches) are defined

by the acquisition of subsequent mutations, such as *TERT* (Davis et al., 2017, Shain et al., 2015). Additional findings from the current study and published reports support the concept of subclones within melanoma tumors, although they raise questions regarding the truncal nature of *BRAF* mutations in all cases. For example, several authors, including us, have described many patients in which *BRAF* mutations appeared to be acquired after the development of metastases, as they were undetected in the paired primary tumor from the same patients. While initially considered to be consistent with a tumor evolution model characterized by the acquisition of mutations, or due to lack of sampling of mutant cells in the primary tumor, this finding alone raises the possibility that *BRAF* mutations are not necessarily truncal mutations in all cases. A truncal mutation should be present in all cells and rarely missed if tumor cells were sampled from primary tumors of patients in which $BRAF^{mutant}$ metastases arose. In support of the polyclonal hypothesis, Lin et al., performed single cell sequencing on a small number of melanoma primary tumors and detected the presence of both $BRAF^{mutant}$ and $BRAF^{wild\text{-}type}$ subclones within the same primary tumor (Lin et al., 2011). Additionally, previous work from our group using laser microdissection identified both $BRAF^{mutant}$ and $BRAF^{wild\text{-}type}$ regions within 6/9 primary melanoma tumors (Yancovitz et al., 2012). If primary tumors are comprised of genetically distinct subclones, each capable of metastatic spread, it could explain the absence of a presumed truncal driver mutation among one or more metastatic tumors arising from a primary tumor with a detectable mutation. Alternatively, polyclonality in primary tumors could explain the apparent gain of a truncal driver mutation in a metastatic tumor derived from a primary tumor in which the driver mutation was present in a small, undetectable subclone. Overall we observed these situations in four patients who had *BRAF* or *NRAS* driver mutations (Table 3). This observation suggests that in rare cases, truncal driver mutations may only be present in a fraction of the cells in the primary tumor that give rise to metastases.

The current study provides direct evidence to support the presence of melanoma subclones within primary and metastatic tumors. We identified *TERT* intra-tumoral heterogeneity within four primary and three metastatic tumors. We also identified one metastatic tumor with both *BRAF* and *NRAS* mutations. It is generally accepted that *TERT* promoter mutations, and *BRAF* and *NRAS* mutations, exist in a mutually exclusive fashion at the cellular level (Akbani et al., 2015, Sensi et al., 2006). Identifying these mutually exclusive mutations in the same tumor, and at differing allele fractions using validated ddPCR assays (Corless et al., 2019), is consistent with the presence of subclones. Of note, a previous study also identified more than one *TERT* mutation in the same recurrent melanoma tumor (Walton et al., 2019), and the presence of $BRAF^{mutant}$ and $NRAS^{mutant}$ melanoma cells from patient derived short term cultures (Raaijmakers et al., 2016, Sensi et al., 2006).

There are several limitations to this analysis. We restricted our assessment to hot spot mutations within three genes. Whole exome analysis would likely uncover additional heterogeneity and could potentially be associated with patient prognosis, as observed in lung cancer (Jamal-Hanjani et al., 2017). In addition, we were not able to analyze all tumors using ddPCR, our most sensitive method. Taken together, we may be underestimating the rate of heterogeneity. In contrast, we could be overestimating the rate of heterogeneity due to selection bias. Most of the patients with heterogeneity had multiple metastatic tumors. Finally, to more directly support the presence of subclones within the primary tumor,

mutational analysis of multiple regions within a tumor (i.e. by laser microdissection) or single-cell analysis would provide further confirmation of the results.

In conclusion, we observed tumor mutational heterogeneity in the three most commonly mutated genes in melanoma. These results suggest that known driver mutations may be subclonal in primary melanomas, albeit in a minority of patients, and that the development of subclones may be inherent to melanoma even in the absence of therapeutic pressure. Our study contributes to the growing evidence of clonal heterogeneity in melanoma. This biological characteristic may be relevant for patient prognosis, could be a source of therapeutic resistance, and thus may be highly relevant for treatment design (McGranahan and Swanton, 2017).

MATERIALS AND METHODS

The detailed protocols are described in Supplemental Materials and Methods online.

Patients and tumors

We studied 271 FFPE tumors from 99 patients with advanced melanoma enrolled in the IRB-approved NYU Interdisciplinary Melanoma Cooperative Group prospective clinical database and biorepository program. All participants provided their written informed consent before enrollment (IRB#10362) (Wich et al., 2009). Only patients with two or more available tumor specimens were included in the study. Tumor content was determined by pathologist's review of H&E stained slides (n=258) or on the basis of the pathology report when H&E was unavailable (n=13).

Mutational Analysis

Multiplex SNaPshot assays (Applied Biosystems, Foster City, CA) were used to detect *BRAFV600E/K*, *NRASQ61K/L/R*, *TERT*−124 [C>T] (C228T), *TERT*-146 [C>T] (C250T) mutations. Amplifications were carried out using the GeneAmp PCR System 9700 (Applied Biosystems) with 10ng of genomic DNA according to manufacturer's instructions.

Quality control multiplatform analysis for BRAF and NRAS hot spot mutations—As a quality control measure for the detection of *BRAF* and *NRAS* mutations, 248/271 (91.5%) samples were retested with an orthogonal method (Figure 1a). One hundred seven randomly selected tumor samples were sent to Molecular MD for analysis using Mutation-specific PCR assays (MS-PCR). Two hundred nine samples with sufficient DNA were analyzed via Sanger Sequencing (Genewiz, South Plainfield, NJ). When droplet digital PCR (ddPCR) became available in the lab we tested tumors based on DNA availability to: resolve mutational discordances between other methods (n=2) and explore the sensitivity of the methods with respect to allele fraction as measured by ddPCR (n=28). ddPCR assays were performed according to the manufacturer's instructions (BioRad Laboratories, Hercules, CA) and as previously described (Syeda et al., 2020).

Quality control multiplatform analysis for TERT promoter mutations—Due to the potential for C>T mutational artifacts to be created by long term formal-infixation (Do and Dobrovic, 2015) all tumor samples from patients initially identified as having tumor

heterogeneity based on *TERT* mutation SNaPshot results were reanalyzed using ddPCR (Corless et al., 2017) following treatment with Uracil DNA Glycosylase (UDG) (n=89 tumors) (Figure 1b). All ddPCR analyses were conducted using a fractional abundance of 0.05% as a threshold for all positive mutation calls *(manuscript in preparation)*.

No datasets were generated or analyzed during the current study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING SOURCES

Study supported by NIH/NCI Grants: R21 CA154786 (DP), R21 CA198495 (DP), P50 CA225450 (IO), P30 CA016087 (NYU Center for Biospecimen Research and Development); FDA Grant: UO1 FD004203 (DP).

Abbreviations:

ddPCR	droplet digital PCR
WT	wild-type

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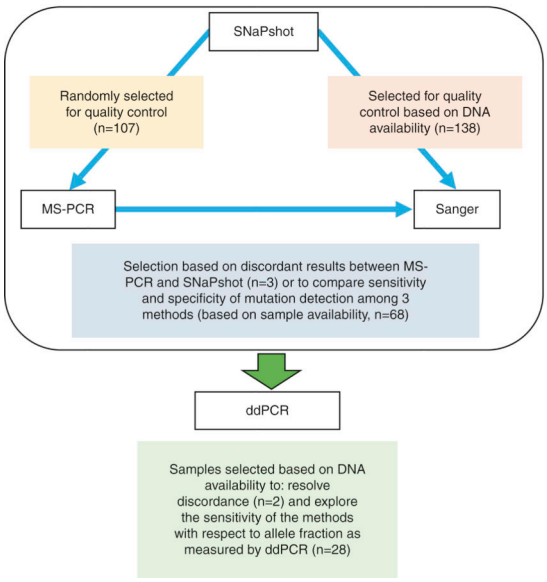
a

		Number of methods used for *BRAF* and *NRAS* analysis							
		1	2	2	2	3	3	3	4
Analytical Platforms used to identify *BRAF* and *NRAS* mutants	ddPCR (n=30)				ddPCR		ddPCR	ddPCR	ddPCR
	Sanger (n=209)		Sanger			Sanger	Sanger		Sanger
	MS-PCR (n=107)			MS-PCR		MS-PCR		MS-PCR	MS-PCR
	SNaPshot (n=275)	SNaPshot	SNaPshot	SNaPshot	SNaPshot	SNaPshot	SNaPshot	SNaPshot	SNaPshot
Number of tumors* (n=271)		23	123	32	3	67	19	4	4

*Number of tumors analyzed by each set of platforms



b

	Number of methods used for *TERT* analysis		
	1	1	2
Analytical Platforms used to identify *TERT* mutants		ddPCR	ddPCR
	SNaPshot		SNaPshot
Number of tumors* (n=269)	179	1	89

*Number of tumors analyzed by each set of platforms

Number of samples	*TERT* mutation results by method		
	SNaPshot	ddPCR	Classification
45	Mutant	Mutant	True Positive
9	Wild-type	Wild-type	True Negative
2	Mutant	Wild-type	SNaPshot False Positive
27	Wild-type	Mutant	SNaPshot False Negative
3	Failed	Mutant	N/A
2	Failed	Wild-type	N/A
1	Failed	Failed	N/A
1	Wild-type	Failed	N/A
1	N/A	Wild-type	N/A

Figure 1: Multiplatform analysis for quality control.

Mutational profiles were determined using a combination of multiplex SNaPshot, Sanger sequencing, MS-PCR, or ddPCR. (a) Summary of *BRAF* and *NRAS* mutation detection methods and flow chart depicting the sequence of sample analysis. All 271 samples were tested by SNaPshot. One hundred and seven samples underwent MS-PCR validation. Samples with sufficient DNA were sent for Sanger Sequencing, including 138 analyzed by SNaPshot and 71 analyzed by both SNaPshot and MS-PCR. Thirty samples with sufficient DNA remaining were tested by ddPCR.

(b) Summary of *TERT* promoter mutation detection methods and results by method. Samples with sufficient DNA were tested by SNaPshot (268/271). Samples which presented with *TERT* promoter heterogeneity by SNaPshot (n=89) were tested using ddPCR. One sample was tested by ddPCR only.

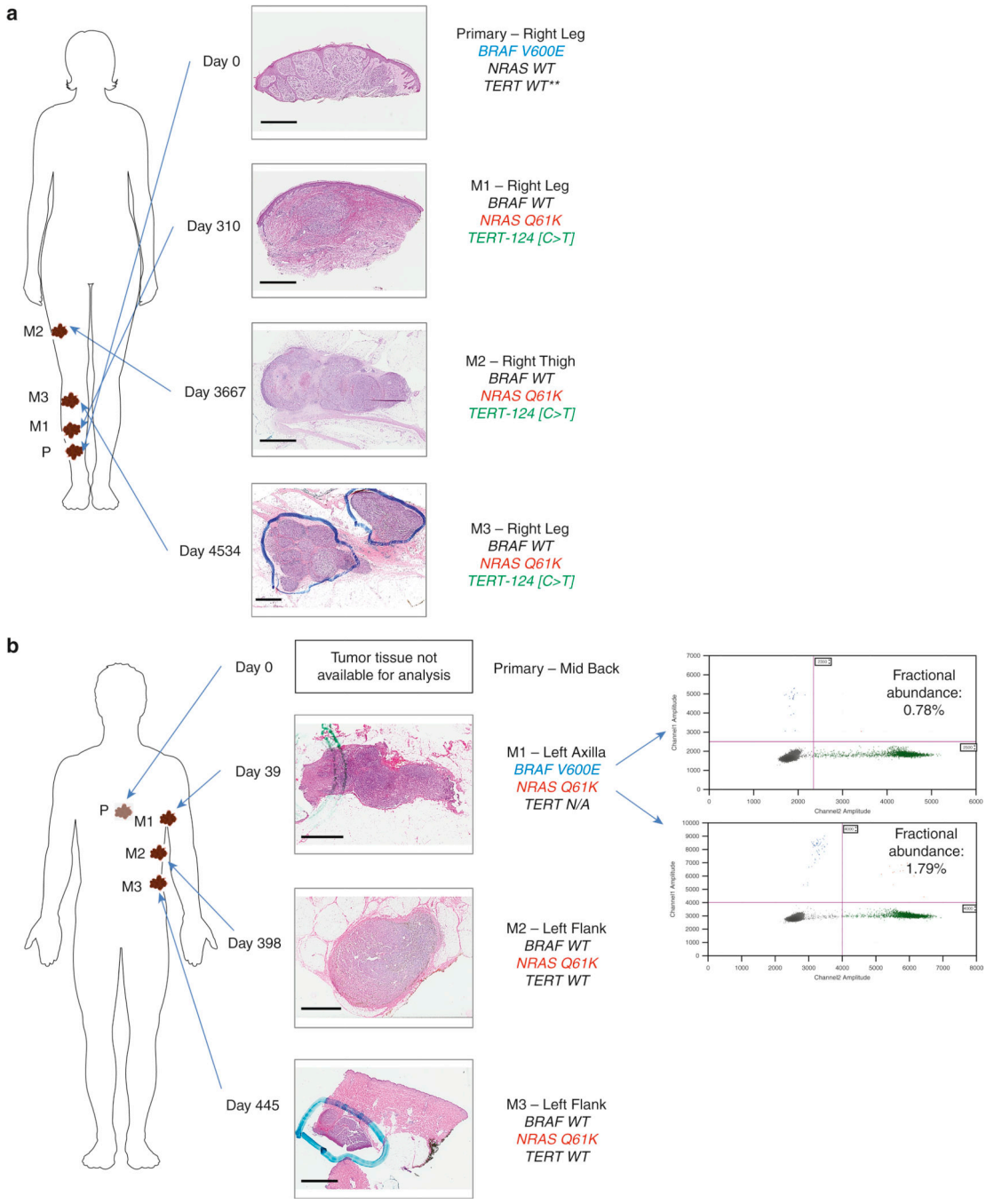


Figure 2: Representative patients exhibiting inter-tumor heterogeneity.

(a) Patient 06–001 was diagnosed with a $BRAF^{V600E}/NRAS^{wild\text{-}type}/TERT^{wild\text{-}type**}$ primary tumor (P) and developed a $BRAF^{wild\text{-}type}/NRAS^{Q61K}/TERT^{-124[C>T]}$ satellite metastasis (M1), 310 days after primary resection. The second metastasis was an in-transit lesion diagnosed 3667 days after initial diagnosis and was $BRAF^{wild\text{-}type}/NRAS^{Q61K}/TERT^{-124[C>T]}$. The patient subsequently developed a regional lymphatic metastasis of the same genotype. Sample marked as ** was wild-type by SNaPshot but failed ddPCR analysis (this

patient was not included in the *TERT* heterogeneity group). Scale bars = 800 μm in (P) and (M1), 4 mm in (M2) and 3 mm in (M3).

(b) Patient 06–075 had three metastatic tumors which presented with two unique genotypic profiles, M1: $BRAF^{V600E}/NRAS^{Q61K}/TERT^{wild\text{-}type}$, M2: $BRAF^{wild\text{-}type}/NRAS^{Q61K}/TERT^{wild\text{-}type}$ and M3: $BRAF^{wild\text{-}type}/NRAS^{Q61K}/TERT^{wild\text{-}type}$. The patient's primary tumor was located on the trunk, in the mid back region, but tissue was not available for mutational analysis. Scale bars = 2 mm in (M1), 5 mm in (M2) and 4 mm in (M3).

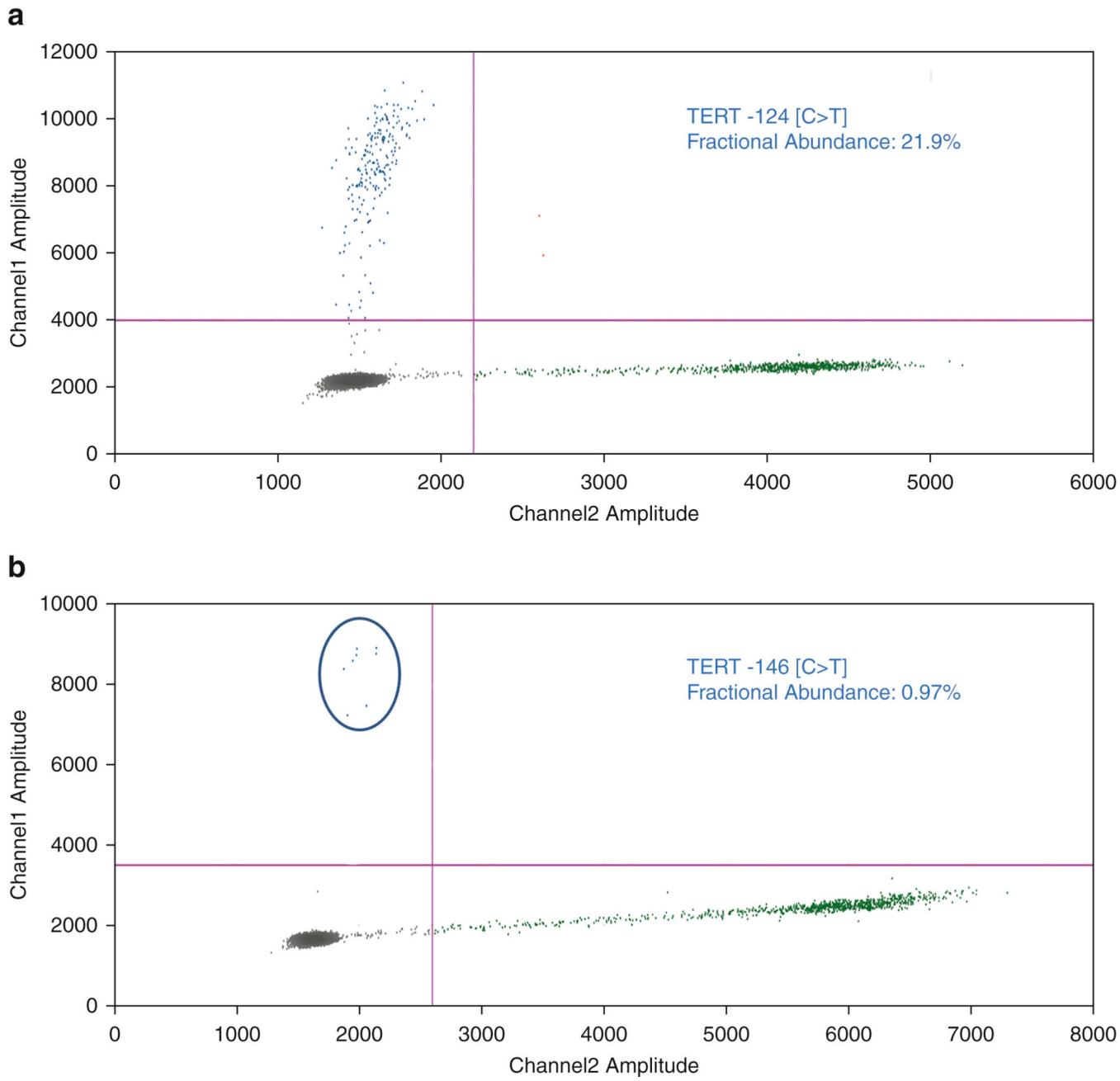


Figure 3: Representative droplet digital PCR two dimensional plots of TERT promoter mutations from primary tumor DNA (patient 04–050).

(a) Two dimensional plot of *TERT* mutation *−124[C>T]*, fractional abundance of 21.9%. (b) Two dimensional plot of *TERT* mutation *−146 [C>T]*, fractional abundance of 0.97%. Y-axis: mutant allele. X-axis: WT allele. Droplets containing different fragments of DNA are displayed as follows: Single positive mutant allele: upper left quadrant (blue); single positive wild-type allele: lower right quadrant (green); double positive mutant/wild-type alleles: upper right quadrant (orange) and droplets not containing either allele: lower left quadrant (grey).

Table 1:

Summary of patient demographics and clinical characteristics.

Patients (n=99)	
Age (mean, median)	56.8, 56
Gender	
Male (mean, median)	60 (58, 59)
Female (mean, median)	39 (54.3, 54)
Primary tumors (n=63)	
Head/Neck	18
Trunk	22
Extremities	22
Unclassified	1
Primary tumor thickness (mm)	
Range	*in situ* – *30mm*
in situ ≤ 0.9	10
1 ≤ 1.9	17
2 ≤ 3.9	19
≥ 4	17
Metastases (n=208)	
Lymphatic	131
Hematogenous	77
Time to First Recurrence (Days) (n=90*)	
Range	51 – 4207
Median	624

*Patients lacking date of primary diagnosis were excluded

Table 2:
Inter-tumor mutational heterogeneity per patient.

(a) Heterogeneity among patients with available primary (P) and metastatic tumors (M1, M2, etc.). (b) Heterogeneity among patients with no available primary (metastases only). Tumors are numbered based on order of chronological appearance. Tumors lacking a detectable mutation are denoted WT. Tumors with a detectable mutation are denoted MUT. Samples that failed to yield a clear result and are marked accordingly. Samples marked by * had low DNA concentrations. Sample marked as ** was wild-type by SNaPshot but failed ddPCR analysis (this patient was not included in the *TERT* heterogeneity group

a.

				Genes			
Patient ID	**Tumor**	**Time from initial diagnosis (days)**	**Anatomical Location**	***BRAF***	***NRAS***	***TERT*$^{-124\ [C>T]}$**	***TERT*$^{-146\ [c>T]}$**
03–036	P	0	R Foot	WT	WT	WT	MUT
	M1	673	R Inguinal	MUT	WT	WT	MUT
	M2	1136	Brain	MUT	WT	WT	MUT
06–001	P	0	R Leg (lower front)	MUT	WT	WT**	WT**
	M1	310	R Leg (lower front)	WT	MUT	MUT	WT
	M2	3667	R Thigh	WT	MUT	MUT	WT
	M3	4534	R Leg (upper front)	WT	MUT	MUT	WT
04–050	P1	0	L Ear	WT	WT	MUT	WT*
	P2	2406	L Ear	WT	WT	MUT	MUT
	M1	3855	L Ear	MUT	WT	WT	MUT
	M2	4275	Front Neck	WT	WT	WT	MUT
08–090	P	0	Back	WT	WT	WT	WT
	M1	0	R Axillary	MUT	WT	WT	MUT
	M2	167	R Upper Back	MUT	WT	WT	MUT
03–092	P	0	R Upper Back	WT	MUT	WT	WT
	M1	1954	Bone - Sternum	WT	MUT	WT	MUT
03–132	P	0	Back	WT	WT	WT	WT
	M1	4315	L Supraclavivular	WT	WT	MUT	WT
	M2	4402	N/A	WT	WT	MUT	WT
04–111	P	0	L Arm (upper ext)	WT	WT	MUT	WT
	M2	785	L Arm	WT	WT	MUT	WT
	M3	836	L Lower Back	WT	WT	WT	WT
	M4	1015	L Arm	WT	WT	MUT	WT
	M5	2152	L Arm	WT	WT	MUT	WT
04–168	P	0	L Arm	MUT	WT	WT	WT
	M1	2625	L Axillary	MUT	WT	MUT	WT
05–137	P1	0	Chest	WT	WT	WT	WT

a.

Patient ID	Tumor	Time from initial diagnosis (days)	Anatomical Location	Genes			
				BRAF	*NRAS*	*TERT*$^{-124\ [C>T]}$	*TERT*$^{-146\ [c>T]}$
	P2	5076	Scalp R Parietal	WT	WT	MUT	MUT
	M1	5706	L Arm Deltoid	WT	WT	MUT	WT
06–002	P	0	Scalp	MUT	WT	WT	MUT
	M1	8	R Post. Occipital	MUT	WT	WT	MUT
	M2	8	R Post. Auricular	MUT	WT	MUT	MUT
	M3	240	Brain	MUT	WT	WT	MUT
07–050	P	0	L Arm (upper front)	MUT	WT	MUT	MUT
	M1	405	Brain	MUT	WT	MUT	WT
10–018	P	0	R Ear	WT	WT	MUT	MUT
	M1	860	R Paratracheal	WT	WT	WT	WT
	M2	860	Lung	WT	WT	WT	WT

b.

Patient ID	Tumor	Time from initial diagnosis (days)	Anatomical Location	Genes			
				BRAF	*NRAS*	*TERT*$^{-124\ [C>T]}$	*TERT*$^{-146\ [c>T]}$
02–074	M1	0	Supraomohyoid	WT	WT	MUT	WT
	M2	778	R Neck	WT	WT	MUT	MUT
03–103	M1	35	R Axillary	WT	MUT	MUT	MUT
	M2	2439	Mediastinum	WT	MUT	WT	MUT
06–004	M1	0	R Upper Back	MUT	WT	WT	MUT
	M2	693	Lung	WT	WT	WT	MUT
	M3	1440	R Axillary	WT	WT	WT	MUT
06–040	M1	235	R Axillary	WT	WT	MUT	WT
	M2	672	Brain	MUT	WT	MUT	WT
	M3	836	Spine C1-C2	MUT	WT	MUT	WT
06–075	M1	39	L Axillary	MUT	MUT	WT	WT
	M2	398	L Flank	WT	MUT	WT	WT
	M3	445	L Flank	WT	MUT	WT	WT
07–080	M1	265	R Calf	WT	MUT	WT	WT
	M2	1318	Spleen	WT	WT	MUT	WT
	M3	1318	Stomach	WT	WT	MUT	WT

Table 3:
Patients with discordant mutations between primary and metastatic tumors.

Twelve patients had discordant mutations between their primary and metastatic tumors. Mutations which were gained (a), or were undetected (b) in at least one of their metastatic tumors are displayed by patient. Specific details for each patient are displayed in Table 2.

a.	
Discordance between Primary and Metastatic Tumors	
Patient #	**Mutations Gained in Metastasis**
03–036	BRAF
03–092	TERT -146
03–132	TERT -124
04–050	BRAF
04–168	TERT -124
06–001	NRAS
06–002	TERT -124
08–090	TERT -146, BRAF

b.	
Discordance between Primary and Metastatic Tumors	
Patient #	**Mutations Absent in Metastasis**
04–050	TERT -124
04–111	TERT -124
05–137	TERT -146
06–001	BRAF
07–050	TERT -146
10–018	TERT -124,-146

Table 4:

Allele frequencies of *TERT* promoter mutations in tumors where both mutations were detected using ddPCR.

Allele frequencies for *TERT −124[C>T]* and *TERT −146 [C>T]* are listed for each of the seven tumors that displayed a positive result for both assays.

	Allele Frequencies (%)	
Patient (tumor)	***TERT -124 [C>T]***	***TERT -146 [C>T]***
02–074 (M2)	22.3	0.29
03–103 (M1)	14.5	3.11
04–050 (P2)	21.9	0.97
05–137 (P2)	2.24	0.79
06–002 (M2)	0.08	0.13
07–050 (P)	3.53	2.41
10–018 (P)	4.62	1.67