

BRAF Mutation Heterogeneity in Melanoma Lesions

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The advent of mutated *BRAF*-targeted therapy has revolutionized the treatment landscape of melanoma, but the clinical benefits of BRAF inhibitors are limited by intrinsic and acquired resistance. Tumour heterogeneity, the presence of both *BRAF*-mutated cells and *BRAF*-wild-type cells, may contribute to acquisition of resistance to targeted treatment (1, 2). This study aims to elucidate the heterogeneous nature of *BRAF* genotypes of primary tumours and metastatic lesions.

CASE REPORT, METHODS AND RESULTS

A 49-year-old Japanese woman presented with a dark-brown nodule on her toenail and inguinal lymph node metastases (Fig. 1A). Melanoma was diagnosed and she was treated by surgical resection. Shortly thereafter, metastases appeared in the lung, brain, bone and skin. Sanger sequencing revealed differences in *BRAF* genotypes: *BRAF*^{V600E} in the primary tumour and *BRAF*^{wild} in a skin metastatic lesion (Case 1, Table I). However, the *BRAF*^{V600E} allele was rare in the primary tumour, as reflected by the small size of the mutation peak in the sequence chromatogram (Fig. 1B). Importantly, because Sanger sequencing does not detect genotypes present in less than 15–20% of cells (3), the *BRAF*^{V600E} allele may have been present at trace levels in the skin metastatic lesion. Therefore, the *BRAF*^{V600E} allele frequency (AF) was measured quantitatively by droplet digital PCR (ddPCR) (QX200 ddPCR system, Bio-Rad, Hercules, CA, USA) (4). The detection limit of ddPCR was 0.01% *BRAF*^{V600E} per 100 ng of DNA; moreover, the assay was linear over the entire test range (Fig. 1C).

The presence of stromal and inflammatory cells can affect the AF in a sample. To minimize this potential confounder, we chose 2 sites containing many melanoma cells and few non-melanoma cells, and then dissected them using a laser-capture microdissection system (Fig. 1D). For the ddPCR, all specimens were run in duplicate. The mean *BRAF*^{V600E} AF at the 2 sites was 17.6% and 19.3%, respectively (Fig. 1E and Table I). By contrast, MART-1 (a melanoma marker) positivity was 92.1% and 89.2%, respectively, as determined by immunohistochemistry. Contamination by *BRAF*^{wild} derived from non-melanoma cells cannot explain the discrepancy between the low *BRAF*^{V600E} AF and the high proportion of melanoma cells at the 2 sites. Furthermore, the metastatic skin lesion had a *BRAF*^{V600E} AF of 0.02% (Table I). Thus, the *BRAF*^{V600E} allele was very rare, but present, in the metastatic lesion, a finding consistent with the heterogeneity of melanoma.

Next, to analyse the heterogeneity of *BRAF* genotypes, we investigated whether there were cases in which the *BRAF*^{wild} genotype was detected in metastatic lesions even when *BRAF*^{V600E} was detected in primary tumours. Using Sanger sequencing, we analysed *BRAF* genotypes in metastatic lesions of another 8 cases with *BRAF*^{V600E}-primary tumours (cases 2–9). Two of these (cases 3 and 8) were characterized as harbouring *BRAF*^{wild} in metastatic lesions (Table I). Because Sanger sequencing is not very sensitive (3), ddPCR was used to analyse the *BRAF*^{V600E} AF in primary and metastatic lesions from both cases. As in case 1, we then performed microdissection to isolate melanoma lesions containing few non-melanoma cells. In cases 3 and 8, MART-1 positivity was 82.9–88.9% and 75.8–90.1%, respectively. The *BRAF*^{V600E} AF in primary tumours was high (case 3, 68.9%, 74.9%, 78.6% at 3 sites; case 8, 31.0%, 34.9%, 37.3% at 3 sites), but low in metastatic lesions: 1.28% in case 3 and 0.19% in case 8 (Table I). Thus, although the *BRAF*^{V600E} allele was abundant in the primary tumours, it was rare in metastatic lesions. These results can be explained by *BRAF* heterogeneity, but not by contamination by non-melanoma cells.

DISCUSSION

Due to genomic instability, melanoma cells frequently show *BRAF* mutations and *BRAF* amplification (5).

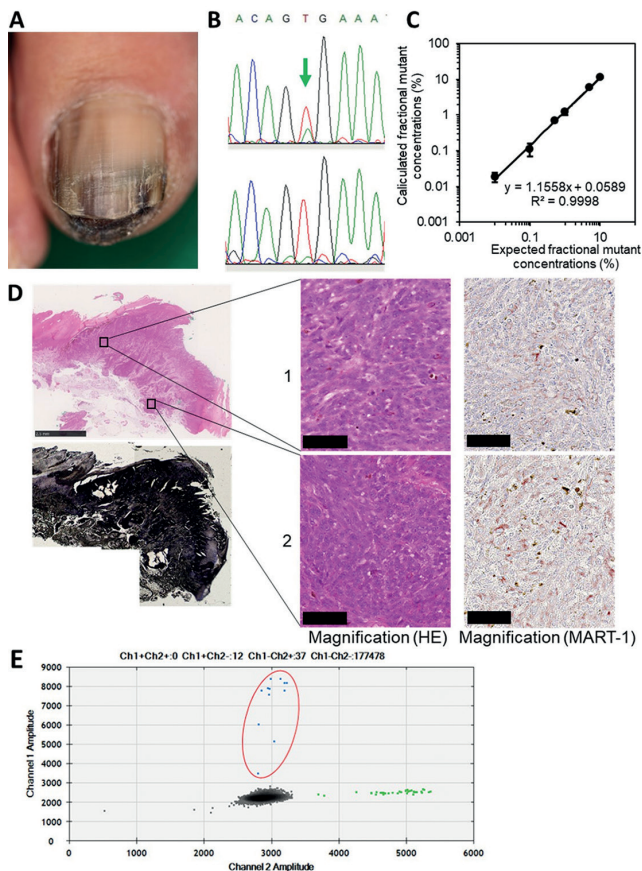


Fig. 1. (A) Clinical features of the primary tumour. (B) Sequence chromatogram of DNA from the primary tumour (upper) and skin metastasis (lower). The arrow shows the *BRAF*^{V600E} peak. (C) Sensitivity of the ddPCR assay of *BRAF*^{V600E}. *BRAF*^{V600E} DNA was diluted in *BRAF*^{wild} DNA (total DNA=100 ng). To validate the limits of detection, *BRAF*^{wild} DNA isolated from Mel-2 cells (*BRAF*^{wild} homozygote) was spiked with *BRAF*^{V600E} DNA isolated from A375 human melanoma cells (*BRAF*^{V600E} homozygote) and then the percentage of *BRAF*^{V600E} DNA measured. Assays were performed in triplicate. Spearman's rank correlation coefficient: $R^2 = 0.9998$, $p < 0.01$. (D) Histopathological and immunohistochemical images of the primary tumour in case 1. Upper left, haematoxylin/eosin stain; lower left, tissue after microdissection; right, microdissected tissues. Scale bars: 2.5 mm (upper left) and 50 μ m (right). (E) ddPCR assay of *BRAF* mutation in primary tumour. *BRAF*^{wild}, green dots; *BRAF*^{V600E}, blue dots; no DNA, grey dots. Dots surrounded by red ellipse indicate *BRAF*^{V600E}.

Table I. Clinical characteristics and *BRAF* mutations in 9 patients with melanoma

Case Age, years/ Sex	Primary site	TT, mm	<i>BRAF</i> status by Sanger sequencing		<i>BRAF</i> ^{V600E} mutation rate by ddPCR (%)		MART-1 ⁺ ratio (%)
			Primary	Meta- stasis	Primary	Meta- stasis	
1. 49/F	Nail apparatus	4.0	V600E	Wild	17.6, 19.3	0.02	92.1, 89.2
2. 64/F	Trunk	4.5	V600E	V600E	80.9, 85.8	NE	99.0, 96.9
3. 19/F	Trunk	6.0	V600E	Wild	78.6, 74.9, 68.9	1.28	88.9, 88.7, 82.9
4. 63/M	Trunk	14.0	V600E	V600E	55.0, 54.6, 54.2	NE	80.9, 90.5, 89.3
5. 62/F	Extremities	9.0	V600E	V600E	30.8, 62.4, 60.0	NE	98.4, 95.4, 98.7
6. 61/F	Face	4.1	V600E	V600E	43.2, 55.5, 51.9	NE	95.4, 94.0, 98.2
7. 37/M	Trunk	14.5	V600E	V600E	24.2, 40.2, 48.5	NE	90.7, 96.6, 96.3
8. 63/M	Trunk	3.0	V600E	Wild	34.9, 37.3, 31.0	0.19	84.1, 75.8, 90.1
9. 79/M	Extremities	7.0	V600E	V600E	26.6, 40.3, 24.9	NE	92.9, 95.8, 94.1

ddPCR: droplet digital PCR; NE: not examined; TT: tumour thickness.

Because melanoma cells are not necessarily diploid, we cannot simply calculate the proportion of *BRAF*-mutated cells from the *BRAF*-mutant AF. At primary tumour sites in case 1, the *BRAF*^{V600E} AF was low (17.6% and 19.3% at 2 sites), despite the high proportion of melanoma cells (92.1% and 89.2%). Given that melanoma cells were homogeneous, the ratios of *BRAF*^{V600E} to *BRAF*^{wild} allele in a tumour cell were calculated to be approximately 1:4.5 and 1:3.9. Furthermore, the *BRAF*^{V600E} allele was rare (0.02%) in the metastatic lesion. Importantly, it is not uncommon for *BRAF* AF to differ between the primary and metastatic lesions. Taken together, these results indicate that melanoma cells are more likely to be heterogeneous, rather than homogeneous, in terms of *BRAF* genotype. The coexistence of *BRAF*-mutant and *BRAF*-wild-type tumour cells within the same patient has important implications for clinical decision-making. Based on the tight connection between *BRAF* genotype and clinical benefits of BRAF inhibitors, it may be necessary to assess *BRAF* mutations quantitatively. In addition, it is preferable to examine *BRAF* status in metastatic lesions (if tissue is available) because *BRAF* status may be different between lesions.

In addition, *BRAF*^{V600E} can also be detected using a *BRAF*^{V600E} mutation-specific antibody (6). However, expression of *BRAF*^{V600E} protein was not detected in a primary lesion of case 1 (data not shown), suggesting that *BRAF*^{V600E} protein may not be expressed substantially. Further studies are needed to clarify the reason for discordance between *BRAF* genotype and immunohistochemical analyses.

BRAF^{V600E} is a driver mutation that appears at an early phase of melanoma progression, implying that it is, at least initially, homogeneous (7, 8). By contrast, our results suggest that melanoma is occasionally heterogeneous in the *BRAF* genotype. Genomic instability produces not only point mutations, but also amplifications and deletions. If melanoma cells harbour multiple aberrant pathways involved in transformation, loss of the *BRAF*^{V600E} allele may occur without disrupting tumour progression. *BRAF* heterogeneity was also detected in melanomas using several methods, including single-cell PCR and pyrosequencing (5, 9). Thus, the early appearance of the *BRAF*^{V600E} allele and the ultimate heterogeneity of the *BRAF* genotype does not constitute a contradiction.

The *BRAF*^{V600E} allele was rare in the metastatic lesions of 3 cases we examined (cases 1, 3 and 8), even though the mutant allele was abundant in the corresponding primary tumours. Because metastasis is frequently caused by a tumour cell cluster, rather than a single tumour cell, in peripheral blood (10), the metastatic lesions in these cases may have derived from clusters including *BRAF*^{wild} melanoma cells along with *BRAF*^{V600E} cells, and *BRAF*^{wild} melanoma cells have predominant potential to proliferation and survival. In addition, intra-heterogeneity in the primary lesion should be related to the inter-heterogeneity between the primary and metastatic lesions. However, it is not clear whether a primary lesion with marked intra-heterogeneity results in noticeable discrepancy between lesions.

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