Intratumoral molecular heterogeneity in a BRAF-mutant, BRAF inhibitor-resistant melanoma: a case illustrating the challenges for personalized medicine

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Abstract

Targeted therapies are increasingly being used to treat a variety of cancers. Their efficacy depends upon the accurate detection and targeting of a specific mutation or aberration in the tumour. All cancers such as melanoma are molecularly heterogeneous, with drug resistant subclones present prior to treatment or emerging as a result of targeted therapies. Here we show intra-lesional molecular heterogeneity in a progressing V600E BRAF-mutant melanoma metastasis from a patient treated for 7 months with the BRAF inhibitor vemurafenib. In the single metastasis, two distinct subclones were observed, both V600E BRAF-mutant, and only one with an additional G13R NRAS-mutation. Molecular heterogeneity even at the intra-lesional level demonstrates that personalising or adjusting therapies based on genotyping of a portion of a single lesion, might not accurately depict the molecular profile or drivers of oncogenesis across the entire patient’s melanoma.
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Introduction

New tumor mutation-specific targeted therapies are revolutionizing the treatment of many solid tumors (1) including melanoma (2, 3). However, tumor molecular heterogeneity is rapidly emerging as a complicating factor in the efficacy of targeted therapies, the durability of responses and the development of drug resistance. The selective pressure induced by targeted therapies can result in the dominance or acquisition of additional driver mutations or molecular aberrations in tumor subclones.

Constitutive activating mutations of the B-raf (BRAF) gene occur in approximately half of all patients with metastatic melanoma (3). BRAF inhibitors have proven highly active (3) and improve overall survival compared with DTIC in patients with BRAF mutant metastatic melanoma (2). Eventually, most patients develop resistance to BRAF inhibition and relapse. Multiple mechanisms of resistance have been elucidated (4), including the reactivation of the MAPK pathway through the development of an NRAS mutation (5). Minority NRAS mutant subclones may pre-exist BRAF inhibitor treatment or the BRAF mutant population may acquire a secondary NRAS mutation following BRAF inhibitor exposure. The current study demonstrates tumour heterogeneity exists within a BRAF inhibitor resistant lesion, which has implications for biopsy driven personalised medicine.
Case Report

A 71 year old man presented in February 2009 with a paraumbilical subcutaneous melanoma metastasis. He had a past history of a primary cutaneous melanoma of the right lower leg diagnosed in April 2005 and a right groin recurrence in May 2007. By May 2009, he had multiple distant melanoma metastases involving the subcutis, lung, left axilla and adrenal glands and an isolated 6mm brain metastasis. The patient’s paraumbilical metastasis was tested for genomic mutations within exon 15 of the $BRAF$ gene using high resolution melt (HRM) and Sanger sequencing revealing the presence of a $BRAF^{V600E}$ point mutation. He was treated with stereotactic radiosurgery to the brain metastasis, progressed after 3 months of stable disease on temozolomide +/- ABT-888/placebo (Trial registration ID: NCT00804908) and was then enrolled on the phase 2 study of vemurafenib, BRIM2 (6). He commenced on vemurafenib (960mg twice daily) on 2 December 2009 and achieved a RECIST (Response Evaluation Criteria in Solid Tumors)-defined partial response (7), but progressed on 29 June 2010 both clinically and radiographically on computed tomography scanning (Fig 1). Excision-biopsy of subcutaneous metastases was performed one day prior to commencement of vemurafenib, on day 14 of treatment, and again on disease progression on 20 July 2010 as part of the Treat Excise Analyze for Melanoma (TEAM) Study at the Melanoma Institute Australia, Sydney, Australia, as approved by the Royal Prince Alfred Hospital Research Ethics Committee Protocol No X10-0305 & HREC/10/RPAH. His subsequent systemic therapies included single agent trametinib (previously GSK1120212, a MEK inhibitor)(8) the combination of dabrafenib (previously GSK2118436, a BRAF inhibitor) and trametinib (9). E7080 (a multi-kinase inhibitor with specific activity against VEGF), and
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finally he was re-challenged with vemurafenib (Fig 1i). For each systemic therapy, excision-biopsy of a melanoma metastasis was performed on day 4-7 days following therapy commencement and on RECIST progression, providing multiple independent tumor samples for analysis and comparison.

Results and Discussion

We sought to determine whether the patient’s initial vemurafenib resistance developed through the reactivation of the MAPK pathway due to additional mutations to the NRAS or BRAF genes. Sanger sequencing was performed on the patient’s vemurafenib resistant metastasis to detect somatic mutations in exon 1 and exon 2 of the NRAS gene and in exon 11 and exon 15 of the BRAF gene. This revealed a G13R NRAS mutation in addition to the original BRAF V600E mutation within the single vemurafenib resistant metastasis (Fig 1ii, iii and iv). We used immunocytochemistry (IHC) to analyze the expression levels of downstream signaling proteins within the progression biopsy to identify any subclones of tumor cells within the lesion. Immunohistochemistry was performed on the progressed metastasis using antibodies against Ki-67, p-ERK1/2, p-AKT, cyclin D1, MITF, p27Kip1, p16INK4a, p53, BCL-2, Mel-1, PTEN, MAP3K8 (COT), IGF1Rβ, and PDGFRβ. The staining for p-ERK1/2 expression clearly identified two areas of tumor with differential staining (Fig 2i). The percentage of immunopositive tumor cells was assessed for each subclone using a Dako ACIS III automated image analysis system. One subclone had high p-ERK1/2 expression (95% tumour cells positive, labeled subclone A, Fig 2iv), and was adjacent to melanoma cells with low expression of p-ERK1/2 (3% tumour cells positive, labeled subclone B, Fig 2v).
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proliferative rate represented by Ki-67 expression was higher in the subclone A (10% tumor cells positive) than in subclone B (3% tumor cells positive) (Fig 2vi & vii). Conversely, the percentage of cyclin D positive tumor cells was lower in subclone A than in subclone B (46% and 74%, respectively) (Fig. 2viii & viiii). The expression of the remaining proteins did not appear to differ between the subclones.

We then performed mutation testing separately on each of the subclones A and B, which were identified by their p-ERK1/2 expression. Each subclone was macrodissected and genomic DNA was extracted from each individual subclone. BRAF and NRAS mutation status of the subclones was determined by allele-specific specific real-time polymerase chain reaction (RT-PCR). In addition, subclone genomic DNA was analyzed by Mass Spectrometric SNP genotyping with OncoCarta v0.1 and the MelaCarta panel v0.1 to detect any additional somatic mutations. The genomic DNA mutational status of subclone A and B was different; subclone A was NRAS wild type and BRAFV600E mutant (Fig 2ii), while subclone B was NRAS G13R mutant and BRAFV600E mutant (Fig 2iii). The sensitivity of this allele-specific RT-PCR allowed the detection of as little as 2% mutant NRAS and BRAF allele (data not shown)(10). For this reason, it is unlikely the differential mutational status of the subclones is due to the assay sensitivity. The OncoCarta and MelaCarta did not reveal any additional mutations.

Mutational analysis using mutant-specific RT-PCR of the antecedent primary melanoma and lymph node metastases that occurred 5 and 3 years prior to the diagnosis of widely disseminated metastatic disease, revealed the presence of V600E BRAF mutation, but not
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the NRAS G13R mutation (Fig 1i). Similarly, analysis of seven metastatic tumors resected after cessation of treatment with vemurafenib and during treatment with single agent trametinib or the combination of trametinib and dabrafenib detected a BRAF V600E mutation without a NRAS G13R mutation in all biopsies (Fig 1i).

Here we show intratumoral molecular heterogeneity in a progressing V600E BRAF-mutant melanoma metastasis from a patient treated for 7 months with the BRAF inhibitor vemurafenib. Within the same individual progressing and vemurafenib-resistant metastasis, two distinct sub-populations were observed, both V600E BRAF-mutant, and only one G13R NRAS-mutant. The acquisition of an additional NRAS mutation causing MAPK pathway reactivation has been proposed as a mechanism of BRAF inhibitor resistance in BRAF mutant melanoma patients (5). In the current study, the NRAS G13R mutation may be one but not the sole driver of disease progression. This issue of tumor heterogeneity is further complicated by incomplete tumor shrinkage, at the macroscopic and radiologic levels, induced by BRAF inhibitors in the majority of tumors and patients. Thus, a biopsy of tumor progression on any BRAF inhibitor may frequently yield a mixture of tumor cells with distinct kinetic growth properties. That an NRAS mutation was not detected in the subsequent tumor biopsies raises tantalizing questions as to how the MEK inhibitor treatment alone (first sequential therapy after failure on vemurafenib alone) might have influenced the selective pressure, for or against, the double BRAF/NRAS mutant melanoma population. Our tissue biopsy series here illustrate the dynamic nature of tumor evolution on multiple distinct targeted regimens.
In this patient, we have shown that intra-lesional genetic heterogeneity is present within BRAF inhibitor-resistant metastatic melanoma and highlighted the fact that sampling error is a potential pitfall of fine needle aspiration or punch biopsy techniques. Advances in genome analysis methods such as deep sequencing (11-13) and sequencing of circulating tumor cells (14), may provide superior information regarding the heterogeneity of a patient’s tumors. These methods remain exploratory due in part to the complex analyses required. Knowledge of heterogeneity may help predict and improve a patient’s response to combination therapies based on the level and signature of mutational heterogeneity. These technologies may also help confront another likely issue arising from tumor heterogeneity: multiple mechanisms of acquired drug resistance in the same progressing tumor or multiple progressing tumors in the same patient.

However, the current method of assessing heterogeneity in a subset of sampled lesions is unlikely to adequately predict tumor heterogeneity in vivo, nor reflect the ongoing genetic changes that occur during treatment (13, 15). Thus, the strategy of biopsying metastatic disease to decide the next systemic therapy after progression on a targeted therapy will be complicated by heterogeneity. We need to anticipate multiple mechanisms of resistance, predict the critical downstream effector pathways, and treat with the most effective broad-ranging combination of therapies at first diagnosis of metastatic disease. These therapies may include the targeting of oncogenes along with immunotherapy or chemotherapy (16-18).
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**Figure Legends:**

**Figure 1.** i) Patient’s treatment timeline with biopsy points in yellow boxes and treatments in blue. ii, iii & iv) Photographs and MRI images taken of the biopsy excised on vemurafenib progression at different treatment stages (yellow arrows mark the biopsied lesion), with the Sanger sequencing result showing the detection of the NRAS G13R mutation.

**Figure 2.** i) Expression of p-ERK1/2 which is high in sub-clone A and relatively negative in sub-clone B (scale bar = 1mm), ii and iii) Allele specific RT-PCR showing the presence of NRAS wt in both subclone A and B but NRAS G13R in only Subclone B. iv) High magnification image of p-ERK1/2 expression in sub-clone A (scale bar = 100µm), v) High magnification image of p-ERK1/2 expression in sub-clone B (scale bar = 100µm), vi) Ki-67 expression in subclone A (scale bar = 100µm), vii) Ki-67 expression in sub-clone B (scale bar = 100µm), viii) CyclinD1 expression in subclone A (scale bar = 100µm), viii) CyclinD1 expression in sub-clone B (scale bar = 100µm)
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