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Emergence of *MET* hyper-amplification at progression to *MET* and *BRAF* inhibition in colorectal cancer

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Background: Combined *MET* and *BRAF* inhibition showed clinical benefit in a patient with rectal cancer carrying *BRAF*^{V600E} and *MET* amplification. However after 4 months, acquired resistance emerged and the patient deceased shortly after disease progression. The mechanism of resistance to this drug combination is unknown.

Methods: We analysed plasma circulating tumour DNA obtained at progression by exome sequencing and digital PCR. *MET* gene and mRNA *in situ* hybridisation analyses in two bioptic specimens obtained at progression were used to confirm the plasma data.

Results: We identified in plasma *MET* gene hyper-amplification as a potential mechanism underlying therapy resistance. Increased *MET* gene copy and transcript levels were detected in liver and lymph node metastatic biopsies. Finally, transduction of *MET* in *BRAF* mutant colorectal cancer cells conferred refractoriness to *BRAF* and *MET* inhibition.

Conclusions: We identified in a rectal cancer patient *MET* gene hyper-amplification as mechanism of resistance to dual *BRAF* and *MET* inhibition.

The *MET* oncogene encodes for a receptor tyrosine kinase involved in the regulation of tumour growth, survival, angiogenesis, invasion and metastases (Birchmeier *et al*, 2003; Raghav *et al*, 2012). Its amplification is associated with sensitivity to *MET* inhibition in preclinical models as well as in a subset of patients (Smolen *et al*, 2006; Camidge *et al*, 2014). *MET* gene amplification

may be present in subclones of tumour cells that are positively selected during anti-EGFR-based therapy in both NSCLC and colorectal cancer (CRC) patients (Turke *et al*, 2010; Bardelli *et al*, 2013; Pietrantonio *et al*, 2016). *MET* amplification or copy gain occurs in a fraction of *BRAF*-mutated tumours including CRC (Jardim *et al*, 2014; Pietrantonio *et al*, 2016). The combination of

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vemurafenib (BRAF inhibitor) with crizotinib (dual ALK-MET inhibitor) is being assessed in a phase I study (NCT01531361), but to date no mechanisms of primary or secondary resistance to combined BRAF and MET inhibitors have been characterised at preclinical or clinical level. Here, for the first time, we identified a molecular mechanism of clinical resistance to BRAF and MET inhibition by analysis of circulating tumour DNA (ctDNA) from plasma alongside tissue taken at progression to this combination therapy in a rectal cancer patient.

MATERIALS AND METHODS

Patient care. The study involved a 48-year-old patient with $BRAF^{V600E}$ mutant microsatellite-stable mucinous metastatic rectal cancer. Biospecimens were collected in accordance with a Fondazione IRCCS Istituto Nazionale dei Tumori Review Board-approved protocol, to which the patient provided written informed consent, and all studies were conducted in accordance with the Declaration of Helsinki. The patient's insurance company covered the cost of crizotinib + vemurafenib off-label combination therapies, to which the patient gave written informed consent. CT scans were obtained as part of routine clinical care. Additional information about specimen collection and its process (whole-exome sequencing and digital PCR on plasma samples; DNA and RNA *in situ* hybridisation (ISH)) are reported in Supplementary Material S1.

Cell lines. WiDr parental cells were obtained from Dr René Bernards (Amsterdam, The Netherlands) in July 2011. The genetic identity of parental cell lines and their resistant derivatives was confirmed by short tandem repeat profiling (Cell ID System; Promega, Madison, WI, USA) at 10 different loci. Cell lines were tested and resulted negative for mycoplasma contamination with the VenorGeM Classic Kit (Minerva Biolabs GmbH, Berlin, Germany). WiDr MET^{amp1} cell line was generated as previously described (Pietrantonio *et al*, 2016). Additional information is reported in Supplementary Material S1.

RESULTS

The first part of this molecular case study was previously published and involved the same $BRAF$ -mutated metastatic rectal cancer patient who became resistant to combined BRAF and EGFR blockade (vemurafenib + panitumumab) due to the emergence of MET amplification. The patient was shifted from EGFR to MET inhibition, and received the combination of vemurafenib and crizotinib achieving an early response (Pietrantonio *et al*, 2016). Then, partial response was confirmed after 2 months of treatment, although the computed tomography (CT) scan at 4 months showed progressive disease (PD) across all sites (Figure 1A).

To uncover the potential mechanisms of the acquired resistance, whole-exome next-generation sequencing (NGS) analysis of plasma ctDNA obtained prior to crizotinib and vemurafenib treatment and at PD was performed. DNA isolated from PBMC was used to remove germline variants from the analysis. The number of sequencing reads supporting the presence of a $BRAF^{V600E}$ mutation was comparable in the pre-treatment and post-resistance samples, indicating similar amounts of tumour-derived circulating cell-free DNA in both samples (Figure 1B). No acquired single-nucleotide variants were observed in plasma ctDNA at PD (Supplementary Table S1). However, MET copy number variation (CNV) further increased in the plasma ctDNA sample obtained at progression (Figure 1B).

To validate these findings, $BRAF^{V600E}$ mutation and MET gene copy were longitudinally monitored in plasma ctDNA by droplet digital PCR using 15 samples collected at regular intervals from

initial receipt of vemurafenib + panitumumab (Figure 1C). A rapid decrease in $BRAF^{V600E}$ mutation and MET CNV in ctDNA was observed within two weeks from the start of vemurafenib + crizotinib. The dynamics of mutant $BRAF^{V600E}$ alleles anticipated radiological progression, as mutant $BRAF$ in plasma increased again as early as 8 weeks after starting therapy. At progression, the percentage of $BRAF^{V600E}$ alleles were comparable to the pre-treatment sample, while—proportionally—a notable increase in the number of MET copies was detected (Figure 1C), thus validating the exome data.

To corroborate MET hyper-amplification, and to further exclude tumour burden-related MET CNV, we performed gene copy number analysis by $MET/CEP7$ bright-field ISH in tissue specimens obtained before and after treatment. In the liver biopsy obtained after resistance to vemurafenib + panitumumab but prior to vemurafenib + crizotinib (Figure 2, indicated as baseline 2), heterogeneous MET gene copy number (ranging from 2 to 20) had previously been reported (Pietrantonio *et al*, 2016). Upon resistance to vemurafenib + crizotinib, the patient consented to liver and inguinal lymph node tumour biopsies, both of which displayed MET hyper-amplification by ISH. Of note, the pre- and post-treatment liver biopsies were taken from the same metastatic lesion. Exome analysis performed on DNA from the liver biopsy at resistance confirmed the presence of MET amplification and ruled out other genetic mechanisms of resistance (Supplementary Table S1). Collectively, the results in liquid and tissue biopsies suggest that clones with higher levels of MET gene amplification had been selected by the treatment.

Since evaluation of MET activation has recently been proposed to better correlate with transcription rather than protein expression due to the rapid turnover of the activated protein (Bradley *et al*, 2016), we investigated MET transcript levels in tissues by RNA ISH. While MET mRNA was undetectable in the rectal primary tumour tissue, low expression level was seen in the liver biopsy taken after vemurafenib + panitumumab. Notably, upon resistance to vemurafenib + crizotinib, both available re-biopsies demonstrated MET RNA overexpression (Figure 2A).

To test whether MET overexpression is causally responsible for resistance to vemurafenib + crizotinib combination treatment, we conducted *in vitro* forward genetic experiments. We previously reported that a $BRAF$ mutant CRC cell line, WiDr, which also has increased MET gene copy number (WiDr- MET^{amp1}), was sensitive to vemurafenib + crizotinib treatment (Pietrantonio *et al*, 2016). We found that exogenous hyper-expression of MET in the same WiDr- MET^{amp1} cells (called WiDr-hyper- MET^{amp1} , Figure 2B) could confer resistance to dual BRAF and MET inhibition and prevented drug combination induced cytotoxicity (Figure 2C and D, Supplementary Figure S1).

DISCUSSION

Concomitant inhibition of BRAF and MET in CRC patients with $BRAF^{V600E}$ and MET amplification could represent a rationale therapeutic strategy (Pietrantonio *et al*, 2016). Recent work correlated MET amplification with a higher prevalence of $BRAF^{V600E}$ tumours (Jardim *et al*, 2014), which may increase the impact of this combination.

The patient reported in this work achieved 4 months of partial response under therapy with vemurafenib + crizotinib until drug resistance emerged. Our effort to uncover the gene alteration(s) driving resistance was based on ctDNA plasma sequencing. This approach has the potential to capture the inter- and intra-tumour heterogeneity present in metastatic disease (Russo *et al*, 2016). Single-nucleotide variants and CNV were comprehensively analysed by whole-exome sequencing comparing plasma ctDNA taken before

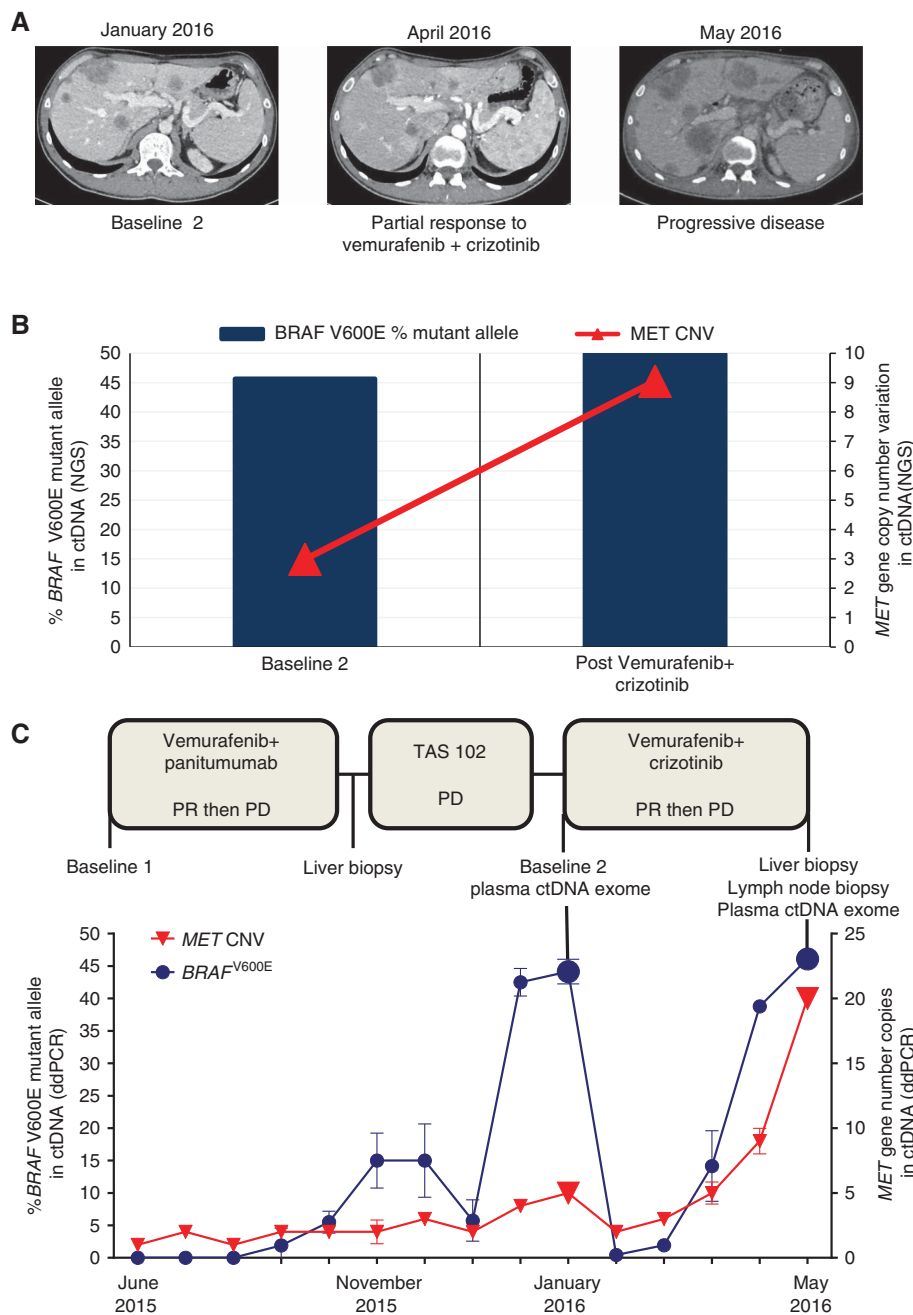
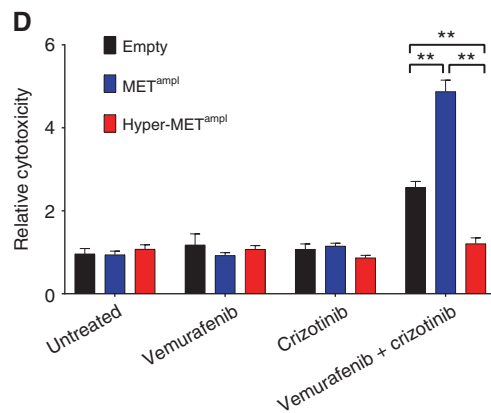
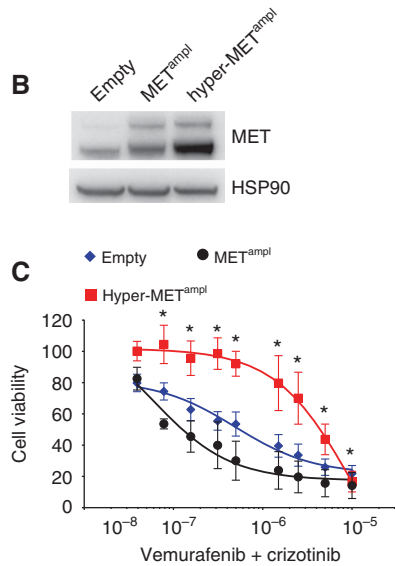
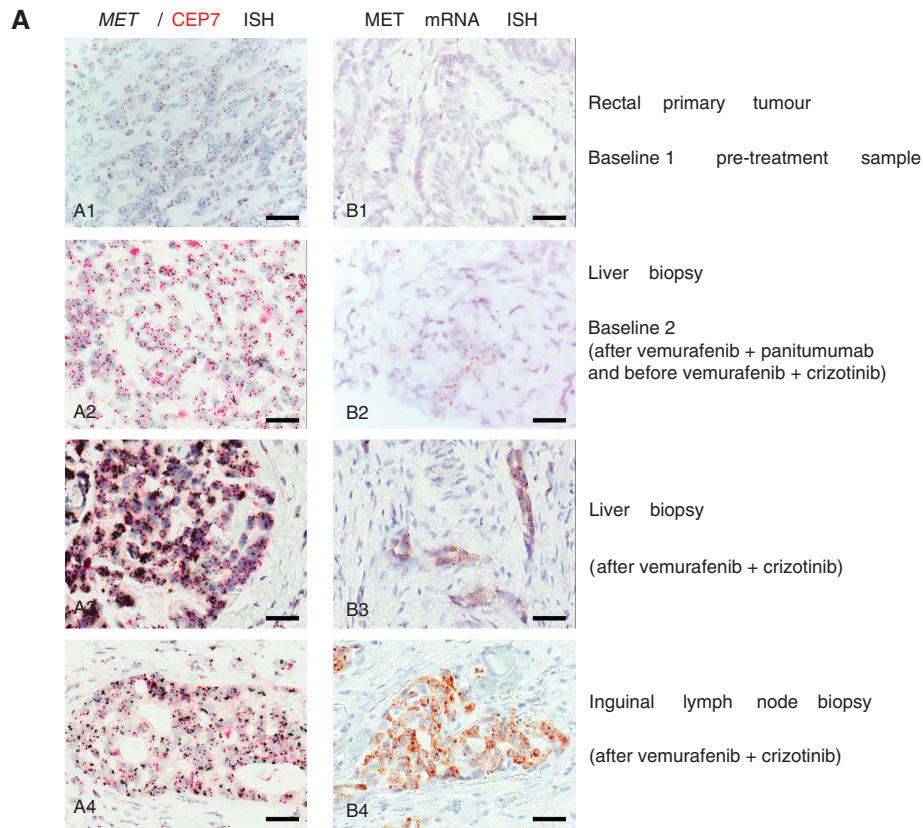


Figure 1. Emergence of *MET* gene hyper-amplification in plasma ctDNA of a *BRAF*^{V600E} metastatic colorectal cancer patient upon progression to crizotinib and vemurafenib combinatorial treatment. (A) Clinical course of the disease during treatment with vemurafenib + crizotinib. Computed tomography scans document the disease status before treatment (left), after the initial response (middle) and at disease progression (right). (B) Exome analysis of ctDNA by NGS. Mutant *BRAF*^{V600E} allele frequencies of 46 and 50% were seen in the baseline 2 plasma sample and at resistance, respectively, indicating similar amounts of ctDNA in both samples. *MET* CNV is increased when comparing ctDNA before therapy with vemurafenib + crizotinib and after disease progression. (C) Analysis of ctDNA by ddPCR. Time kinetics of the percentage of *BRAF*^{V600E} alleles (red line) or copies of *MET* gene copy number (blue line) show a decrease of both circulating markers during patient response to vemurafenib + crizotinib. At radiological disease progression, the percentage of *BRAF*^{V600E} alleles were comparable to the pre-treatment sample, while—proportionally—a notable increase in *MET* gene copy number was detected. Datapoints represent mean ± s.d. of two independent observations, each performed in duplicate. ddPCR = digital droplet PCR; PR = partial response; PD = progressive disease.

target treatment and at PD. No single-nucleotide variants previously associated with resistance to BRAF inhibition in melanoma or CRC were observed (Ahronian *et al*, 2015; Hong *et al*, 2016; Oddo *et al*, 2016). We also did not detect secondary *MET* mutations affecting the crizotinib-binding region, which had been observed either by drug-protein co-crystal structure analysis or in *MET*-amplified tumours after acquired resistance to MET inhibition (Cui *et al*, 2011; Qi *et al*, 2011; Bahcall *et al*, 2016; Heist *et al*, 2016).

In a previous study of a *MET*-amplified gastric cancer cell line treated with increasing concentrations of MET inhibitors, resistance was correlated with increased *MET* amplification accompanied by increased KRAS expression (Cepero *et al*, 2010). Our analyses identified only *MET* hyper-amplification, detected both by ISH and mRNA ISH methodologies, as the most likely genetic alteration underlying clinical acquired resistance to BRAF and MET inhibition.



Even when precision oncology is successful, the efficacy of targeted strategies is generally transient. Since the patient died about two months following disease progression, we speculate that *MET* hyper-amplification coupled with *BRAF*^{V600E} mutation not only conferred resistance to vemurafenib + crizotinib but also could be responsible for the particularly aggressive disease behaviour. Elucidating the molecular mechanisms underlying secondary resistance may help in designing further lines of therapy (Bahcall *et al*, 2016; Pietrantonio *et al*, 2016; Russo *et al*, 2016). We believe that the traits associated with the extremely high level of *MET* expression shown by the resistant tumour in this study could have been exploited as a Achilles' heel to rationally test as a further salvage line novel anti-*MET* antibody–drug conjugates, such as ABBV-399, which showed promising activity in *MET*-amplified

cancer cells and non-small cell lung cancer patients (Strickler *et al*, 2016; Wang *et al*, 2017). However, the rapid deterioration of patient conditions prevented administration of further active treatments.

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Figure 2. *MET* gene hyper-amplification and *MET* mRNA expression emerge in a patient with BRAF^{V600E} rectal cancer and confer resistance to BRAF and *MET* dual inhibition in WiDr cells. *In situ* hybridisation analyses for *MET* gene and mRNA levels were performed on the primary tumour (baseline 1, **A1** and **B1**), a liver biopsy obtained upon resistance to vemurafenib + panitumumab but before vemurafenib + crizotinib (baseline 2, **A2** and **B2**), as well as liver (**A3** and **B3**) and inguinal lymph node (**A4** and **B4**) biopsies obtained upon acquired resistance to vemurafenib + crizotinib. **(A)** Dual colour bright-field ISH for *MET* gene (black dots) and *CEP7* (red dots). The baseline sample obtained before targeted treatments does not show *MET* amplification, even if a few clones display gain of *MET* gene copy number, with 18% of neoplastic cells bearing ≥ 5 *MET* gene copy number (**A1**). The sample obtained at acquired resistance to vemurafenib + panitumumab but prior to vemurafenib + crizotinib (baseline 2) shows *MET* amplification, with a mean gene copy number of 8.8 (range 2–20) (**A2**). Upon acquired resistance to vemurafenib + crizotinib, tumour cells display *MET* ‘hyper-amplification’, with a further increase of *MET* gene copy number in both liver re-biopsy (**A3**—multiple copies and small and large clusters) and inguinal lymph node biopsy (**A4**—small and large clusters). *In situ* hybridisation for *MET* mRNA. *MET* gene transcription is below level of detection (score = 0) in baseline 1 sample (**B1**); while an increased staining of *MET* gene transcription (score = 1) is present in the liver biopsy upon acquired resistance to vemurafenib + panitumumab (**B2**). This is further increased in samples obtained upon resistance to vemurafenib + crizotinib (**B3–B4**), with evidence of strong *MET* mRNA overexpression (score = 3 in liver biopsy; score = 4 in lymph node biopsy). Scale bar, 12.5 μm . **(B)** BRAF^{V600E} mutant WiDr parental or its *MET*-amplified derivative cell line (WiDr-MET^{amp}) were transduced with either control (empty) or *MET*-expressing lentiviral vector (here called WiDr-hyper-MET^{amp}), respectively. Protein extraction and Western blotting with total *MET* antibodies revealed a gradient of *MET* protein expression among the three different cell lines. HSP90 is reported for normalisation purposes. **(C)** Cell viability by ATP assay of WiDr empty, MET^{amp} and hyper-MET^{amp} cells after treatment for 72 h with the indicated molar concentrations of vemurafenib in association with constant 0.2 μM crizotinib. **(D)** WiDr empty, MET^{amp} and hyper-MET^{amp} were treated for 72 h with 1 μM vemurafenib and/or 0.2 μM crizotinib in mono or combinatorial therapies. CellTox green cytotoxicity assay was performed to identify cells with compromised membrane integrity characteristic of cell death. Data are expressed as fold change relative to DMSO-treated control cells. Results represent mean \pm s.d. of two independent observations, each performed in duplicate or triplicate. Statistical differences in BRAF mutant cell viability or cytotoxicity between MET^{amp} and hyper-MET^{amp} cells was determined with the Mann–Whitney *U* test (**P* < 0.05, ***P* < 0.01).

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CONFLICT OF INTEREST

FP is a consultant for Bayer and SFP has received honoraria from the speakers bureaus of Roche and Amgen. FdB is a consultant for Roche, Amgen and Novartis. AB is an advisory board member for Biocartis, Horizon Discovery and Trovogene. AB and FDN have received research support from Trovogene. No potential conflicts of interest were disclosed by the other authors.

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