Identification of multiple mechanisms of resistance to vemurafenib in a patient with 
BRAF\textsuperscript{V600E}-mutated cutaneous melanoma successfully rechallenged after progression

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Running Title: Coexistence of multiple vemurafenib resistance mechanisms.

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TRANSLATIONAL RELEVANCE

Mutational activation of BRAF, a cytoplasmic serine/threonine kinase, is the most frequent genetic alteration in melanoma, with approximately 50% of tumors expressing the BRAF\textsuperscript{V600E} oncoprotein. Treatment with vemurafenib induces significant tumor regression and improves survival of patients with advanced BRAF-mutated tumors, highlighting the essential role of oncogenic BRAF in melanoma biology. Drug-resistant disease is the major cause of patient death. We evaluated by ultra-deep sequencing the mechanism(s) of acquired resistance in two subcutaneous metastases occurring in a patient with a BRAF\textsuperscript{V600E}-mutated cutaneous melanoma, successfully rechallenged after initial progression to vemurafenib, and detected two mutually exclusive mechanisms of resistance to vemurafenib. Our findings have strong clinical relevance and could serve as a proof-of-principle for future prospective studies in a larger cohort of patients.
ABSTRACT

PURPOSE. To investigate the mechanism(s) of resistance to the RAF-inhibitor vemurafenib, we performed a comprehensive analysis of the genetic alterations occurring in metastatic lesions from a patient with a BRAF<sup>V600E</sup>-mutant cutaneous melanoma who, after a first response, underwent subsequent rechallenge with this drug.

EXPERIMENTAL DESIGN. We obtained blood and tissue samples from a patient diagnosed with a BRAF<sup>V600E</sup>-mutant cutaneous melanoma that was treated with vemurafenib and achieved a near-complete response. At progression, he received additional lines of chemo/immunotherapy and was successfully re-challenged with vemurafenib. Exome and RNA sequencing were performed on a pre-treatment tumor and two subcutaneous resistant metastases, one that was present at baseline and previously responded to vemurafenib (PV1), and one that appeared de novo after reintroduction of the drug (PV2). A culture established from PV1 was also analyzed.

RESULTS. We identified two NRAS activating somatic mutations, Q61R and Q61K, affecting two main subpopulations in the metastasis PV1, and a BRAF alternative splicing, involving exons 4-10, in the metastasis PV2. These alterations, known to confer resistance to RAF inhibitors, were tumor-specific, mutually exclusive, and were not detected in pre-treatment tumor samples. In addition, the oncogenic PIK3CA<sup>H1047R</sup> mutation was detected in a subpopulation of PV1, but this mutation did not appear to play a major role in vemurafenib resistance in this metastasis.

CONCLUSIONS. This work describes the co-existence within the same patient of different molecular mechanisms of resistance to vemurafenib affecting different metastatic sites. These findings have direct implications for the clinical management of BRAF-mutant melanoma.
INTRODUCTION

Melanoma is widely known to be a molecularly heterogeneous disease; however, only recently have we been able to selectively treat patients on the basis of molecular alterations and assess response to therapy in defined subgroups (1). About half of cutaneous melanomas harbor a mutation of BRAF, primarily V600E, leading to activation of the MAPK pathway (2-4). RAF inhibitors such as vemurafenib (Zelboraf®) inhibit the MAPK pathway and cell proliferation only in BRAF-mutated tumors and have demonstrated remarkable clinical activity, with responses in approximately half of the patients in phase II and III clinical trials leading to increased overall survival (5-7). Unfortunately, the majority of responding patients eventually develop disease progression, typically within 5-7 months. Several mechanisms for acquired vemurafenib resistance have been described, mainly leading to reactivation of ERK, the downstream effector in the MAPK pathway (reviewed in (8, 9)). These mechanisms include activating NRAS mutations, BRAF gene amplification, overexpression of MAP3K8/COT, a kinase that directly activates MEK and ERK, and alternative splicing of BRAF mRNA. In the latter mechanism, variant BRAFV600E transcripts lacking exons coding for a protein region encompassing the RAS-binding domain, lead to ERK reactivation via RAS-independent BRAF dimerization (10-13). Activation of receptor tyrosine kinases (e.g. IGF1R, and PDGFRβ), may also cause resistance to vemurafenib by promoting alternative signaling pathways, notably the PI3K/AKT/mTOR pathway (11, 14). Most of these mechanisms have been identified through studies in vitro and confirmed in a few patients.

Although the standard treatment consists of a daily administration of the drug until disease progression, one study has reported a successful rechallenge with vemurafenib in two patients (15). In addition, a recent study in a mouse xenograft model suggests that interval dosing of vemurafenib may delay the emergency of genetic resistance (16). In this study, we report an in-depth analysis of resistant tumor samples from a young patient with metastatic BRAFV600E-bearing cutaneous melanoma, who responded to an initial treatment with
vemurafenib, eventually progressed, and was then successfully rechallenged with the same drug. Exome and transcriptome sequencing show for the first time the coexistence within the same patient of different genetic alterations at different metastatic sites leading to disease progression.
METHODS

Patient samples

Human tissue collection and use adhered to protocols approved by the Institutional Review and Privacy Board of the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne). The following samples were used: a formalin-fixed paraffin-embedded (FFPE) lymph node metastasis from the pre-treatment lymphadenectomy (Pre) and matched normal lymph node samples (N1) from the same surgery; two snap-frozen subcutaneous tumor samples, PV1 (surgically resected metastasis) and PV2 (needle biopsy); donor matched peripheral blood mononuclear cells (N2); and cultured cells from the subcutaneous metastasis PV1 (LAU-T1407A, named here PV1-cell line). This melanoma culture was established and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (without addition of PLX3042) and used for RNA/DNA extraction at passage 2. Patient and melanocytic tumor origin were authenticated by the genetic and expression profiles of the culture compared to the matched germline sample and original tumor tissue (see Results). Single cell cloning was performed from a stock frozen at passage 0 and clones were used for cytotoxicity experiments at passage 10-15. Cells were free of mycoplasma. An additional RNA sample from a skin metastasis carrying the BRAFV600E mutation from an advanced cutaneous melanoma patient naïve to vemurafenib was used for RNA seq.

Exome sequencing

Exome capture and library construction were performed using Agilent SureSelect Human All Exon v4 kit using DNA from the samples described above. Libraries were sequenced on Illumina HiSeq 2000, generating 100 bp paired-end reads. Data processing is described in detail in Supplementary Methods. We obtained a mean haploid exome target coverage of 356x for Pre, 321x for PV1, 268x for PV2, 94x for N1 and 76x for N2. Somatic variants were
validated using Ion Torrent sequencing technology (Life Technologies), obtaining a median target coverage of 3123x for Pre, 4902x for PV1, 2184x for PV2 and 3535x for N2.

**RNA seq**

Un-stranded paired-end RNA-seq libraries were prepared from samples PV1, PV2 and PV1-cell line with the Illumina TruSeq RNA Sample Preparation kit v2. Libraries were sequenced on Illumina HiSeq 2000, generating 83.6 million of purity filtered 100 bp paired-end fragments for PV1, 79.3 million for PV2 and 88.4 million for PV1-cell line. Sequence analysis is detailed in Supplementary Methods.

**RT/PCR detection of alternatively spliced BRAF**

To confirm the presence of alternatively spliced BRAF, cDNA prepared from total RNA using random hexamers was amplified by PCR and amplified products subjected to Sanger sequencing. Primer sequences and conditions are described in Supplementary Methods.

**In vitro cytotoxicity assays**

Sensitivity of cultured melanoma cells to BRAF, MEK, and PI3K inhibitors (PLX3042, AZD6244, CI-1040, and GDC-0941, all from Selleck) was assayed as described (17) using WST-1 reagent (Clontech) to measure cell growth. IC50 values were calculated using GraphPad Prism 5 software.
RESULTS

Patient characteristics

A 41-year-old man was diagnosed in 2008 with a non-ulcerated nodular melanoma of the trunk, Breslow thickness of 6 mm. In July 2009 he was referred to us with a recurrent metastatic disease confirmed by biopsy in the left axillary nodes. He was treated with therapeutic lymph node dissection and local radiation therapy. After ten months, he progressed systemically in the lungs, liver, bone, kidneys, and sub-cutaneous tissue. The \( \text{BRAF}^{V600E} \) mutation was detected in the biopsied lymph node, and the patient was randomized to the vemurafenib arm of the BRIM3 study (5). He received vemurafenib 960 mg/bid beginning in June 2010 and achieved a near-CR, which lasted seven months (Supplemental Figure 1), after which he progressed with the appearance of new lesions in the left kidney, retroperitoneal nodes, and an increase in the size of previously responding lesions. He then received 4 cycles of Ipilimumab followed by 2 cycles of temozolomide and 1 cycle of fotemustine with progression of disease with the appearance of new as well as increased growth of pre-existing lesions.

In the absence of effective therapeutic options in this setting, we decided to meet the expectations of the patient and in November 2011 re-introduced vemurafenib at standard dosage. At this time, the disease burden was extensive and progressive, involving the brain, lungs, right atrial wall, kidneys, adrenal glands, and several subcutaneous areas, as documented by a CT scan of the thorax, abdomen, and pelvis (Figure 1, A) and brain MRI (Figure 1, B). After only 2 weeks of treatment, the patient showed an excellent response in the brain, lungs and skin, which was confirmed at 6 weeks (Figures 1 A and B) and lasted 4 months. Some skin metastases showed responses followed by stabilization. Interestingly, one metastasis on the right arm that responded to initial vemurafenib therapy in June 2010 and eventually reappeared at progression showed some shrinkage during re-introduction of the...
drug. In addition, the patient also experienced the appearance of a new, rapidly progressing lesion involving the subcutaneous tissue of the right arm. This difference in behavior prompted us to collect snap-frozen tissue samples, referred to as PV1 and PV2, respectively (Figure 2), to investigate the molecular mechanism(s) of escape.

**Mutation profiling by exome sequencing**

To investigate the mechanisms of resistance to vemurafenib, we first performed whole-exome sequencing using the Illumina technology of a pre-treatment paraffin-embedded lymphnode metastasis (Pre) and of the two subcutaneous snap-frozen metastases of the right arm (PV1 and PV2, Figure 2). Matched blood cells (blood) and paraffin-embedded normal lymphnodes were used as control germline samples. The tumor samples were sequenced at high coverage (mean range 268x-356x). Focusing on exonic mutations, we identified 107 somatic Single Nucleotide Variants (SNVs) in Pre, 139 SNVs in PV1, and 127 SNVs in PV2, generating a set of 202 different SNVs, 82 of which were common to all 3 samples. The non-synonymous to synonymous ratios were 1.5, 2.0, and 1.6 for Pre, PV1 and PV2, respectively (1.16 for the mutations common to all 3 samples and 2.14 for non-shared mutations). The number and spectrum of base substitutions are indicated in Figure 3. C>T transitions, of which 83-86% occurred at TpC and CpC sites (suppl. Table 1), predominated in the samples, indicating UV light-induced damage. Sample PV1 contained the highest number of substitutions, suggesting a larger divergence from the primary melanoma. Furthermore, while the spectrum of mutations common to all three tumors was further enriched in C>T transitions, the latter represented only 25% of non-shared mutations (Figure 3). Private mutations (i.e. tumor-sample specific) were predominantly C>A, a type of mutation that can result from oxidative damage. Interestingly, T>C transitions were additionally enriched in the private mutations of PV1, but not PV2 (Figure 3C). T>C mutations have been found to be over-represented in mismatch repair-deficient tumors (18), thus an excess of such mutations may result from PV1-specific defects in DNA repair or be related to exposure of the progressing tumor mass to chemotherapy drugs (cfr.
Figure 2). It should be noted that we have previously reported a loss of UV-light signature among the private mutations (compared to common mutations) identified in 2 metastases removed 12 years apart from another melanoma patient, further supporting the existence of different mutagenic steps in melanoma development (17).

A set of 174 SNVs were checked using the Ion Torrent technology, and 155 SNVs (89%) were confirmed. The extremely high median coverage obtained at the mutation sites (mean range 2184x-4902x) allowed us to compute precise allele frequencies of the validated mutations in the three tumor samples (Figure 4A, suppl. Table 2). All clonal mutations in Pre were also clonal in PV1 and PV2, indicating that the two metastases arose directly from Pre or a tumor genetically very similar to Pre. More than two thirds of PV1 specific SNVs were present at low frequency (subclonal), indicating higher tumor heterogeneity. These subclonal mutations curiously included the majority of the PV1-private C>T transitions (Figure 3D), indicating a more recent acquisition of this type of mutations. Interestingly, the frequency of BRAFV600E mutation was almost double in PV2 (55.4%) compared to Pre (31.3%) and PV1 (25.1%). Copy number alteration (CNA) plots generated from the exome sequencing data showed that all three samples present a trisomy of chromosome 7q (and of the entire chromosome in PV2, suppl. Figure 2). However, the B allele frequencies of the germline heterozygous single nucleotide polymorphisms (SNPs) located on 7q are anti-correlated in PV1 and PV2 (Figure 4B). This indicates that the 7q amplifications result from independent events involving different alleles (duplication of the wild-type BRAF allele in PV1 and of the mutated allele in PV2). CNA plots also showed that these tumors present a moderate aneuploidy, with common clonal amplifications of 1q, 6p and 20p, PV1-specific 5p trisomy, and PV2-specific 7p and 15 trisomies (suppl. Figure 2, suppl. Figure 3 and suppl. Table 3). Interestingly, a chromosome 10 deletion (leading to the loss of PTEN, an event frequently associated with BRAF mutations (19)) was present in a subpopulation of Pre but was not observed in PV1 or PV2.
A resistant subcutaneous metastasis, PV1, harbours two NRAS mutations

Among the PV1-specific mutations, we found two sub-clonal NRAS mutations in codon 61, leading to Q61K and Q61R amino acid substitutions, with a frequency of 18.2% and 5.5%, respectively. The two mutations were never found on the same sequencing read, and were further enriched in a cell line derived from PV1 (29.0% and 12.1%, respectively, Figure 4C). Mutant NRAS alleles were not amplified. Taking into consideration a 30% normal tissue contamination, it is estimated that approximately 70% of the tumor cells in PV1 harbored mutant NRAS (80% in the PV1-derived culture). NRAS mutations have been shown to confer resistance to vemurafenib (11). Consistent with this, the PV1-derived cell culture was resistant to PLX-4032 but still sensitive to MEK inhibitors (suppl. Figure 4). No NRAS mutations were detected by Ion Torrent sequencing in PV2 or Pre. PV1 also harbored the oncogenic mutation H1047R in PIK3CA, the gene coding for the p110α catalytic subunit (20, 21). This mutation was subclonal in PV1 and its corresponding short-time culture (frequency 11.2% and 13%, respectively) and undetectable in Pre and PV2.

PIK3CA activating mutations have been implicated in resistance to MEK and RAF inhibitors (22, 23). We therefore explored whether the PIK3CA<sup>H1047R</sup> mutation could be responsible for vemurafenib resistance in a subpopulation of PV1. Clonal cell lines harboring this mutation (clone 5, 10, and 11, shown in Suppl. Figure 5 to have heterozygous expression) were isolated from the PV1 derived culture and tested for their sensitivity to RAF, MEK and PI3K inhibitors. All 3 PIK3CA<sup>H1047R</sup> clones, like NRAS mutated clones assayed in parallel, were resistant to PLX4032 (IC<sub>50</sub> range 9-58 µM; Figure 5A). All cultures tested were only moderately sensitive to GDC-0941 (IC<sub>50</sub> range 0.6-2.4 µM), a highly potent PI3K inhibitor that is active against both wild-type and oncogenic p110 variants, including PIK3CA<sup>H1047R</sup> (24), suggesting some activation of the PI3 kinase pathway. Combined GDC-0941 and PLX4032 treatment only slightly improved growth inhibition in 2 of the 3 PIK3CA mutated clones (Figure 5B). Similar results were obtained by co-treatment of PLX4032 with another PI3K inhibitor, LY294002 (not shown). Like the parental uncloned culture, PIK3CA and NRAS mutated clones were sensitive
to the MEK inhibitor AZD6244, with IC50 values below 1 µM, although individual PIK3CA clones showed up to 10-fold difference in sensitivity (Figure 5A), indicating that growth of these cells is still strongly dependent on MAPK pathway activation. Finally, combinations of AZD6244 with PLX4032 or GDC-0941 did not show a synergistic effect (suppl. Figure 6).

**Gene expression profiling by RNA-sequencing reveals an aberrantly spliced BRAF transcript in the resistant subcutaneous metastasis PV2**

Because some of the mechanisms responsible for resistance to vemurafenib are observed at the transcriptional or post-transcriptional level, we additionally performed poly-A+ RNA sequencing (RNA-seq) on PV1, PV2 and the PV1-derived cell line. In PV2 only, we observed an aberrantly spliced BRAF transcript that accounted for ~50% of all BRAF transcripts (Figure 6A). This splice variant contains an in-frame deletion of exons 4-10 and is one of several aberrantly spliced BRAF forms previously reported as an escape mechanism to BRAF inhibitors (12). These variants lack the RAS binding domain and can activate ERK in the presence of the inhibitor. The variant appears specific to PV2 as was not detected in PV1 or Pre, as confirmed by PCR (Figure 6B). Sequencing of the variant product showed that aberrant splicing affects the BRAF^{V600E} transcript (suppl. Figure 7). PV1 and PV2 gene expression levels were then compared to identify additional potential tumor-specific mechanisms of escape, focusing on genes belonging to the tyrosine kinase and GPCR receptor families, RAS/MAPK and AKT/mTOR signaling pathways (suppl. Figure 8, suppl. Table 4). HGF, MET and MAP3K8/COT genes, whose overexpression can drive resistance to vemurafenib (10, 25, 26), showed higher expression in either PV1 or PV2, although were not within the top 10% most differentially expressed genes. As RNAseq could not be performed on Pre due to lack of adequate RNA material, we compared PV1 and PV2 expression profiles to those obtained from a BRAF^{V600E} unrelated metastasis from a patient naïve to anti-RAF/MEK inhibitors. These comparisons showed similarly high levels of expression for the above genes. Of note, the
GPCR receptors GRM1 and EDNRB, which can activate the AKT pathway (27, 28), were overexpressed in PV2.
DISCUSSION

We have described the occurrence of different mechanisms of resistance to vemurafenib, namely activating NRAS mutations and alternative BRAF splicing, that coexist in a patient that was successfully rechallenged with vemurafenib and eventually progressed. While both mechanisms of acquired resistance have been previously described (11, 12), they have been so far reported to occur as mutually exclusive mechanisms among patients (12). A few reports have provided evidence that different mechanisms for acquired resistance to RAF inhibition can be present within the same patient. For example, Shi et al. analyzed 2 or more resistant metastases from a few patients and identified a prominent mechanism (i.e. NRAS mutation) in one but not the other tumor sample(s) (13). Similarly, Wilmott et al. clearly showed the existence of 2 phenotypically different nodules within a resistant metastasis, only one of which contained mutated NRAS (29). In either study, other mechanism(s) were not detected. Here, we clearly identify multiple resistance mechanisms to BRAF inhibition in different metastases arising in the same patient, at the same anatomical location and within the same tissue (subcutaneous metastases).

Remarkably, we identified two NRAS activating mutations affecting codon Q61 in a lesion (PV1) that was already present before the first treatment and twice underwent regression under vemurafenib. Nazarian et al. have reported the presence of 2 different NRAS mutations in resistant metastases from a patient that initially responded to vemurafenib; however, in that patient the mutations were site-specific (11). The presence of 2 subclonal NRAS mutations in the same lesion in our patient suggests that the mutations originated within this metastasis, and may reflect some type of site-specific selective pressure that confers a proliferative advantage for upstream RAS activity rather than downstream MAPK escape events. RAS-level mutations could, indeed, reactivate additional escape pathways, like PI3K/AKT/mTOR. In addition, it might also be related to the clinical history of this metastasis (see Figure 2) that allowed greater tumor evolution, as reflected in the larger number of accumulated somatic mutations and higher observed genetic heterogeneity.

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Other mechanisms, in addition to NRAS mutations and alternative BRAF splicing, may have further contributed to the acquired resistance in our patient. Indeed, NRAS mutations were estimated to account for resistance in only up to 80% of tumor cells in PV1. Among the identified somatic mutations, the PIK3CA<sup>H1047R</sup> mutation detected in an estimated 30% of cells in PV1 was a potential vemurafenib resistance candidate that we further investigated. PIK3CA mutations, of which PIK3CA<sup>H1047R</sup> is the most frequent, are common in some cancers, such as breast and colon carcinomas, but not in melanoma (30, 31). PIK3CA<sup>H1047R</sup> mutant protein has been shown to activate the AKT pathway and to contribute to MEK inhibitor resistance (22).

Results of cytotoxicity assays using PI3K, MEK and RAF inhibitors, alone or in combinations, on PIK3CA<sup>H1047R</sup> mutated cell clones isolated from PV1, did not suggest a driver role for this mutation in vemurafenib resistance. Supporting these conclusions, some clones isolated from the PV1 culture contained both PIK3CA<sup>H1047R</sup> and NRAS mutations (unpublished observation), suggesting a different role for mutant PIK3CA protein. Nevertheless, the PI3K pathway appeared to be important in both PIK3CA and NRAS mutated cells, as shown by the partial effect of GDC-0941 on cell growth, and a possible contribution of PIK3CA<sup>H1047R</sup> to vemurafenib resistance deserves a more thorough investigation. In addition, overexpression of genes such as the receptor tyrosine kinase/ligand couple MET/HGF and the G-protein coupled receptors GRM1 and ENDRB, detected by RNA sequencing, may also play a role.

The clinical complete regression of PV1 (and other metastases) before regrowth and successful vemurafenib re-challenge lends support to a state of temporary resistance in microscopic tumor deposits, possibly in conjunction with epigenetic changes or particular microenvironment conditions. In this regard, a small subpopulation of slow-proliferating, potentially drug-resistant, melanoma cells that can give rise to a highly proliferative progeny and is associated with JARID1B expression has been described (32). Moreover, a reversible drug-tolerant phenotype has been described in NSCLC cells treated with tyrosine kinase inhibitors (33).
Based on current knowledge and clinical practice, vemurafenib is stopped at tumor progression, as mechanisms of resistance may come into play limiting its efficacy. Our results clearly demonstrate that the molecular escape mechanisms can be both multiple and mutually exclusive. This has important clinical implications: firstly, local treatment of isolated progressing lesions and continuation of vemurafenib could be supported by the fact that the resistance mechanisms are not always shared. This approach is currently being tested within clinical trials (34) with preliminary results that seem to support our hypothesis. Secondly, the presence of more than one resistance mechanisms in the same patient strongly argues that single biopsy analysis at progression might not reflect the molecular complexity of tumor progression, and therefore might not be sufficient to guide selection of optimal second-line therapy. Finally, the patient in this study is the third one reported to respond to RAF-inhibitor rechallenge (15). Although this is a very small number of patients and the individual drug treatments differed (our patient was treated twice with vemurafenib, while in Neyns et al. the patients first received combined dabrafenib/trametinib and were later rechallenged either with the same combination or dabrafenib alone), the encouraging clinical results support the exploration of alternative treatment schedules with RAF inhibitors.
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FIGURE LEGENDS

Figure 1. Tumor response following reintroduction of vemurafenib. Panel A shows a thorax-abdomen CT scan; Panel B shows a brain MRI at baseline and 2 and 6 weeks after reintroduction of vemurafenib.

Figure 2. Schematic representation of the tumor samples analyzed. Pre, regional lymphnode metastasis; PV1, metastasis that was present at baseline, completely responded to 1st vemurafenib treatment, then reappeared and progressed during chemotherapy, responded again (partially) with 2nd vemurafenib treatment, then progressed; PV2, metastasis that appeared de novo during 2nd vemurafenib treatment. Ipi, ipilimumab; chem., chemotherapy; Vem., vemurafenib.

Figure 3. Somatic single-nucleotide substitutions of Pre, PV1 and PV2 samples. Panel A and B show the number and spectrum, respectively, of exonic mutations in the three metastasis Pre, PV1 and PV2 identified by whole-exome sequencing using the Illumina HiSeq platform. Panel C shows the number and type of mutations common to the three samples (COM), found in one or two samples only (not common, NOT), or private to individual samples (PREpr, PV1pr, and PV2pr). The majority of C>A in the pool of not common mutations were validated with Ion Torrent sequencing using an enzymatic DNA fragmentation and had frequencies higher than 0.2 (Suppl. Table 2), indicating that they were accumulated by the tumor and were not technical artifacts (35). D, number and type of private mutations further sub-classified as clonal (pr-clon) or subclonal (pr-sub) using frequencies obtained by the somatic variant caller MuTect (36) and taking into account normal tissue contamination.

Figure 4. Characterization of somatic mutations. Panel A shows mutant allele frequencies of validated mutations in Pre, PV1 and PV2 samples. The points on or near the axis were classified as Pre, PV1 and PV2-specific. The two clusters and outliers are clonal mutations, and were defined by fitting a three-component mixture of tri-variate Gaussian distributions on the other points using the expectation maximization algorithm implemented in the Statistics
Toolbox of Matlab. Mutations in Cluster 2 lay on the minor allele of trisomic chromosomes 1q, 6p and 20p. Most of the outliers lay in regions with copy number alteration. Panel B compares the B Allele Frequencies (BAF) of germline heterozygous SNPs located on chromosome 7q of samples PV1 and PV2. The 7q amplification involves different haplotypes. The canonical BRAF mutation V600E is indicated in red. Its location just outside the main cluster is due to 20-30% contamination with normal tissue. Panel C, sampling of 100 reads that map to NRAS Q61 codon in PV1 and PV1-derived cell line. The two NRAS mutations Q61K (C>A) and Q61R (A>G) have different frequencies and are mutually exclusive on sequencing reads. Base substitutions are indicated in red for A, blue for G, and green for T. Bases identical to hg19 are indicated in grey.

Figure 5. Sensitivity of PV1-derived single cell clones carrying the PIK3CA\textsuperscript{H1047R} mutation to RAF, PI3K and MEK inhibitors. A. Assays were performed with the indicated drugs and PIK3CA\textsuperscript{H1047R} mutant clones (PI3K Cl 5/10/11), along with the uncloned parental population and 2 NRAS mutant clones. Top panels show relative growth of cells (average and standard deviation of 3 replicates) after 4 days of incubation with drugs. Lower panels show IC50 concentrations (average and range) calculated from 2 independent experiments. B Effect of PLX4032 (PLX, 1 \(\mu\)M) and GDC-0941 (GDC, 1 \(\mu\)M) alone or in combination on cell growth. Results show average and standard deviation of 3 independent experiments.

Figure 6. Detection of alternatively spliced BRAF transcripts. Panel A shows BRAF splice junctions found by RNA-seq in samples PV1, PV2 and in the cell line derived from PV1. BRAF exons and introns are indicated at the top. Splice junctions are represented by an arc from the beginning to the end of the junction. The thickness of the arc is proportional to the number of reads covering the junction. The canonical junctions are represented below, and the alternative junctions are represented above the line. Exons 4-10 are aberrantly spliced-out in sample PV2. Panel B shows the results of RT/PCR performed with primers located in exon 3 and 11 (top panels) on the indicated samples. Alternatively spliced BRAF (97 bp product) is preferentially amplified over the longer (907 bp) canonical form in PV2, but is undetectable in PV1 and Pre
samples. Bottom panels show amplification of a cDNA fragment (exon 15) similar in size to the alternatively spliced BRAF product as control for RNA quality for the FFPE sample (Pre). PCR was performed for 35 or 40 cycles (left and right panels, respectively). Lack of amplification in reverse transcriptase (RT) samples shows specificity for cDNA.
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Figure 2. Schematic representation of the tumor samples analyzed.
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