RESEARCH BRIEF

Targeting the BRAF V600E Mutation in Multiple Myeloma

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Conflict of interest:

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Abstract

In multiple myeloma, there has been little progress in the specific therapeutic targeting of oncogenic mutations. Whole-genome sequencing data has recently revealed that a subset of patients carry an activating mutation (V600E) in BRAF kinase. To uncover the clinical relevance of this mutation in multiple myeloma, we correlated the mutation status in primary tumor samples from 379 myeloma patients with disease outcome. We found a significantly higher incidence of extramedullary disease and a shorter overall survival in mutation carriers when compared to controls. Most importantly, we report on a patient with confirmed BRAF V600E mutation and relapsed myeloma with extensive extramedullary disease, refractory to all approved therapeutic options, who has rapidly and durably responded to low doses of the mutation-specific BRAF inhibitor, vermurafenib. Collectively, we provide evidence for the development of the BRAF V600E mutation in the context of clonal evolution and describe a prognostic and therapeutic relevance of this targetable mutation.

Significance: This is the first evidence of the clinical and therapeutic relevance of BRAF V600E mutations in multiple myeloma, proving the principle of specific inhibition of driver mutations in this disease.
Introduction

Multiple myeloma is a malignancy of terminally differentiated B-lymphocytes, but its pathogenesis is yet only partially understood (1-3). While new therapeutic options have substantially increased the response rates and survival of myeloma patients over the last decade, it is still considered incurable in most cases and more effective therapies are urgently needed (4). Current treatment strategies are mechanistically based on agents without tumor cell specificity, such as proteasome inhibition (bortezomib, carfilzomib), immunomodulatory drugs with pleiotropic effects (thalidomide, lenalidomide, pomalidomide), or conventional chemotherapy. High throughput tumor genome sequencing of myeloma cells shows a diverse mutational landscape with few recurrent mutations. The most prominent set of mutations was found within the RAS pathway (2;5;6). Of immediate clinical interest, the serine-threonine kinase BRAF gene was found to be mutated in approximately 4% of all cases, with the \textit{BRAF} V600E mutation being the most common.

In malignant melanoma and hairy cell leukemia, targeting the highly prevalent \textit{BRAF} V600E mutation has recently proven to be of clinical benefit. However, whether this also applies to malignancies with a low frequency of this mutation remains unclear (7-12). In colorectal carcinoma, only 5% of patients with mutated \textit{BRAF} respond to treatment with the specific inhibitor, vemurafenib (13).

We therefore have screened tumor specimens from patients with plasma cell diseases for protein expression of mutated \textit{BRAF} V600E by mutation-specific immunohistochemistry (IHC). We here describe the clonal evolution of myeloma cells from patients with a confirmed \textit{BRAF} V600E mutation and the distinct clinical course of this cohort, thereby indicating the clinical relevance of this mutation, and provide first proof-of-principle for the therapeutic efficacy of vemurafenib in multiple myeloma.
Results

We screened for protein expression of V600E mutated BRAF in plasma cells by IHC using a mutation-specific antibody on paraffin-embedded soft tissue and bone marrow core biopsies from patients with a monoclonal gammopathy. Positive results were verified by Sanger sequencing (figure 1). A total of 421 samples (391 bone marrow biopsies, 30 soft tissue plasmacytomas) obtained from 379 patients (59 with monoclonal gammopathy of undetermined significance, 53 smoldering myelomas, 251 symptomatic myelomas, 16 AL amyloidosis) were analyzed. Patient characteristics are summarized in table 1. Two or more consecutive biopsies were available from 37 patients of the cohort.

Mutated BRAF V600E was detected in seven myeloma patients, corresponding to a prevalence of 2.8% of patients with symptomatic myeloma and 1.8% of all patients with monoclonal plasma cell disorders, respectively. One patient initially harbored the mutation in a minor subclone detectable by IHC (figure 1A,B). This could be confirmed by sequencing only after microdissection of the tissue to enrich for positive plasma cells (figure 1C). In three patients, the mutation was present at diagnosis whereas it only became detectable in an additional three patients in the context of relapsed disease, indicating clonal evolution (figure 2). As RAS is the most frequently mutated gene family in myeloma, we sequenced codons 10, 11, and 61 of exons 2 and 3 of NRAS and KRAS, respectively. While no mutations were detected in any of the 12 available samples from six patients, the BRAF-mutated subclone in patient #7 harbored a concomitant substitution of Valine for Glycine at position 60 (G60V) in KRAS, which has not been described before in multiple myeloma.

Remarkably, four of the seven BRAF mutated patients (57%) went on to develop extramedullary disease compared to 43 of 251 (17%, p=0.02) control patients with symptomatic disease (figure 2 and table 1). Progression-free survival in five of these seven
patients was very short, once the mutation was present. Details of patients’ courses of
disease are given in suppl. table 1. The overall survival of all patients with documented
follow-up of at least three months from the start of first-line treatment was then assessed.
Those patients confirmed to have the BRAF V600E mutation were found to have a
significantly shorter median overall survival of 45 months (range 6-54) as compared to 105
months (range 4 – 227 months; p=0.04) in patients without the mutation.

Patient #5, a 61-year old female Caucasian, presented with multiple soft tissue
plasmacytomas and marked B symptoms (figure 2 and suppl. table 1). She had first been
diagnosed with IgA kappa symptomatic multiple myeloma, Salmon & Durie stage IIIA, in
August 2008. Risk profiling revealed an ISS score of II. No high risk cytogenetic aberration
(i.e., t(4;14) or del17p) was detected by fluorescence in-situ hybridization (FiSH). First-line
treatment consisted of a bortezomib containing induction regimen, stem cell mobilization
and harvesting, followed by high dose melphalan and autologous blood stem cell transplant
(ASCT) in November 2008. A near complete remission (nCR) was achieved, according to
IMWG criteria (14). Fifteen months following ASCT, she developed a soft tissue
plasmacytoma of the left upper eyelid, which was treated with radiotherapy, accompanied
by systemic progressive disease. Treatment with lenalidomide/dexamethasone was initiated
in March 2010. In January 2011, the disease progressed, and she then received
bortezomib/dexamethasone, and subsequently bendamustine in October 2011, rendering
the disease refractory to lenalidomide, bortezomib, dexamethasone and bendamustine.
While still receiving bendamustine, the patient presented to our outpatient clinic with
progressive disease in June 2012.
The patient presented with profuse night sweats and opioid-dependent pain in her left arm. Clinical examination revealed multiple small cutaneous plasmacytomas (e.g., 2.1 x 1.5; 1.1 x 0.9; 1.4 x 1.2 cm) as well as a large subcutaneous plasmacytoma above the left shoulder (5.9 x 2.1 cm by whole body MRI scan), while the known osteolytic lesions remained stable. Electrophoresis of blood and urine showed a monoclonal IgA protein of 770 mg/l, 346 mg/day of kappa light chain proteinuria and free kappa light chains of 101 mg/l in serum were noted. Biopsies from the subcutaneous mass above the left shoulder as well as from one of the skin lesions showed extensive plasma cell infiltration. IHC with the BRAF V600E mutation-specific antibody demonstrated the presence of mutated BRAF V600E protein in all malignant cells. The mutation status was confirmed by sequencing of the biopsy material as well as of purified myeloma cells obtained from the large plasmacytoma, without evidence of concurrent RAS mutations. The purified myeloma cells were assessed for additional mutations, i.e. EZH2, MYD88, NOTCH1, PIK3CA, SF3B1, and TP53, that have been commonly found in other B-cell malignancies. A concomitant mutation was only detected in SF3B1 (K700E). However, the relevance of this mutation in multiple myeloma is currently unknown. In the bone marrow, less than 5% plasma cells were present at the site of the biopsy and no BRAF V600E-mutated cells could be detected.

Given the resistance of the disease to standard lines of therapy and the absence of RAS mutations, off-label treatment with the mutation-specific BRAF inhibitor, vemurafenib, was considered a rational approach. Individual informed consent was obtained and treatment was started with low-dose vemurafenib at 480 mg BID. Since she tolerated the treatment well, the dosage was increased to 720 mg BID after one week. Within two weeks, the soft tissue plasmacytomas visually reduced in size. This was accompanied by a reduced requirement for pain medication. At day 28, response parameters were assessed. Whole
body MRI scan and measurements of the three reference skin lesions showed a decrease in the sum of diameters from 14.5 x 5.7 cm to 6.4 x 1.7 cm. Serum electrophoresis showed no measurable M-spike with negative immunofixation, and urine electrophoresis revealed decreased kappa light chain proteinuria of 113.2 mg/day with positive immunofixation, resulting in a partial response of the disease by IMWG criteria (figure 3A). A re-biopsy of one of the residual skin plasmacytomas was obtained and revealed extensive tumor regression and scaring (figure 3B). While IHC still detected mutated BRAF V600E in the small area with remaining plasma cells (figure 3C), pathway activation – assessed by phosphorylation of the extracellular-signal regulated kinase (p-ERK) – and cell proliferation – represented by MIB1 – were drastically reduced compared to baseline samples (figure 3D). Furthermore, an increased number of apoptotic cells was evident and confirmed by staining for activated caspase-3. The average number of caspase-3 positive apoptotic bodies was <1 per high power field (HPF) at baseline and >10/HPF at day 28 (10 HPFs were counted per slide) (Figure 3D).

In summary, the administration of a single 4-week course of vemurafenib resulted in the achievement of a partial response, both serologically and by size. After the second course, the urine electrophoresis became negative by immunofixation. All cutaneous manifestations had disappeared. She developed manageable arthralgia and bursitic-like pain, and an intermittent tremor of both hands. During the fourth course, the patient had to be admitted to hospital due to pneumonia and treatment was temporarily discontinued. This resulted in a transient disease reactivation, which was only detectable in the urine, both quantitatively and by immunofixation (figure 3B). Once symptoms had resolved, vemurafenib was restarted with the fifth course at a lower dose of 480 mg BID. Urinary light chains rapidly returned to baseline though immunofixation of the urine remained positive (figure 3B).
Discussion

With the introduction of increasingly detailed molecular diagnostics and intervention, the clinical utility of targeted inhibition of oncogenic signal transduction cascades has been rapidly evolving. Prominent examples include imatinib targeting BCR-ABL in chronic myeloid leukemia and KIT in gastrointestinal stromal tumors (15;16), erlotinib and other agents targeting the epidermal growth factor receptor (EGFR) in non-small-cell lung cancer (17), and, most recently, vemurafenib and dabrafenib targeting BRAF in malignant melanoma or hairy cell leukaemia (7-10). The presence of activating mutations, however, does not necessarily predict for clinical and therapeutic relevance. In papillary thyroid carcinoma, BRAF mutations are associated with adverse prognosis while the therapeutic relevance remains elusive (18). In colorectal cancer, BRAF mutations seem to confer an adverse outcome but the efficacy of BRAF inhibition has so far been disappointing (13).

In multiple myeloma, two studies have reported BRAF mutations in approximately 4% of patients using genotyping approaches (5;6). The most well characterized BRAF mutation, V600E, was detected in 2.4% (four out of 199 patients) and 4% (six out of 147 patients), respectively. This is in line with our data using a proteomics based approach, which is a very cost-effective screening modality.

In our patient cohort, the activating BRAF V600E mutation appears to be associated with a clinically more aggressive form of multiple myeloma and shorter overall survival. In four of seven patients, we found that clonal dominance of BRAF-mutated plasma cells developed
over other subclones. Moreover, we observed a high incidence of extramedullary disease in more than half of the BRAF V600E positive patients, significantly higher than in patients without this mutation (table 1). This is normally a rare event in myeloma patients and associated with a dismal clinical outcome (19). However, as this mutation is rare and thus patient numbers are small, these findings await confirmation in large international trial cohorts.

In BRAF V600E mutated melanoma, activation of rescue pathways and concurrent mutations in the \textit{RAS} genes have been suggested to confer vemurafenib resistance (20;21) and drive treatment-associated cutaneous squamous-cell carcinomas and chronic myelomonocytic leukemia (22-24). The risk of secondary skin cancers, similar to other reported adverse events, also appears to be dose-dependent (9;23).

In general, \textit{KRAS}- and \textit{BRAF}-encoded proteins are mutated in many of the same types of malignancies. Concomitant mutations, however, are extremely rare, especially in the context of BRAF V600E (25). This is likely because both genes undergo gain-of-function mutations and thus represent different mechanisms of activating the same pathway. Of note in this context, mutations in the \textit{RAS} gene family are one of the most frequent recurrent genetic events in multiple myeloma (2;5;6). None of the known activating mutations were detectable in myeloma cells from our BRAF mutated patients, confirming previous reports that these mutations appear to be mutually exclusive in multiple myeloma (6). However, in the bone marrow sample from patient #7, a rare \textit{KRAS} mutation (G60V) was detected.

Moreover, this mutation was only present in the subclone of myeloma cells that concomitantly expressed BRAF V600E after tissue microdissection. This protein alteration has been described only once in colon cancer according to the COSMIC database. Although it
is predicted to be damaging by the SNAP/SIFT algorithms, no functional relevance or clinical significance has yet been identified (26).

We next hypothesized that RAS mutation negative myeloma with BRAF V600E could potentially be targetable by the mutation specific BRAF inhibitor vemurafenib. We found that pharmacologic inhibition of this single driver mutation can result in disease remission and impressive clinical benefit for a patient with refractory myeloma. It is remarkable that this response was observed at the relatively low dose of 720 mg BID and could be maintained at 480 mg BID. To avoid additional side effects we chose not to further increase the dose despite residual signal alterations within the bone marrow detectable by whole body MRI. So far, the response has been durable and no signs of progressive disease or secondary malignancies have been detected after eight months of treatment, a highly remarkable duration of response for a refractory patient with extramedullary disease. However, emerging resistance to BRAF inhibition is a therapeutic challenge in malignant melanoma. Continued follow-up of our patient will teach us whether this holds true for multiple myeloma, and provide the opportunity to apply advanced molecular means to characterize emerging rescue mechanisms in multiple myeloma as compared to other diseases.

Multiple myeloma does not harbour a unifying genomic aberration or mutation. Our data underlines the necessity for detailed molecular diagnostics early in the disease. They demonstrate the clinical feasibility of targeted inhibition of patient-specific tumor mutations, and represent a significant step towards the goal of personalizing treatment for this genetically complex malignancy. Our data provides the rationale for a validation of long-term success rate, timing and optimal dosage of BRAF inhibition in multiple myeloma by larger scale trials.
Methods

Patients and Tissue Samples

A retrospective single-centre cohort of 379 patients with a monoclonal gammopathy diagnosed between January 1992 and January 2012 was investigated by immunohistochemistry. Two or more consecutive biopsies were available from 37 patients of the cohort. In total, the series consisted of 421 formaline-fixed paraffin-embedded bone marrow (n=391) or soft tissue biopsies (n=30). The work was performed within our program for the identification of novel therapeutic targets in plasma cell dyscrasias and was approved by the Institutional Review Board (IRB). Patient characteristics are summarized in table 1.

Immunohistochemistry

Antibodies and IHC conditions have previously been described in detail (27;28). In brief, a primary BRAF-V600E mutation-specific antibody (clone VE1) was used to screen for protein expression of V600E mutated BRAF in plasma cells by immunohistochemistry. 4-6 µm sections were cut from formaline-fixed paraffin-embedded specimens and mounted on Superfrost Ultra Plus (Gerhard Menzel GmbH, Braunschweig, Germany) glass slides. The immunostaining was performed on an automated immunostainer Ventana BenchMark Ultra (Ventana Medical Systems, Tucson, Arizona, USA) using standard reagents provided by Ventana. Pretreatment with cell conditioner 1 was followed by incubation with undiluted VE1 hybridoma supernatant and consecutive chromogenic detection with optiView Universal DAB detection kit and counterstaining with hematoxilin and bluing reagent for 4 min each. The immunostained slides were evaluated by two pathologists. Microscopic figures were
taken with an Olympus BX-51 light microscope equipped with DP50-CCD camera and processed with Cell-A Software (all from Olympus, Hamburg, Germany).

**Sequencing**

Mutation analysis of *BRAF* gene was performed as previously described (24). Briefly, DNA was isolated from FFPE tissue using standard methods and mutation analysis was performed by Sanger sequencing. In case of low tumour burden the DNA was extracted from microdissected areas infiltrated by clonal plasma cells. For mutational analysis of *KRAS* and *NRAS*, exons 2 and 3 of both genes were amplified with the following primers: GTG TGA CAT GTT CTA ATA TAG TCA (*KRAS* exon 2 forward), GAA TGG TCC TGC ACC AGT AA (*KRAS* exon 2 reverse), CCA GAC TGT GTT TCT CCC TTC (*KRAS* exon 3 forward), AAC CCA CCT ATA ATG GTG AAT ATC T (*KRAS* exon 3 reverse), GAT GTG GCT CGC CAA TTA AC (*NRAS* exon 2 forward), CCG ACA AGT GAG AGA CAG GA (*NRAS* exon 2 reverse), CCC CTT ACC CTC CAC ACC (*NRAS* exon 3 forward) and CAC AAA GAT CAT CCT TTC AGA GAA (*NRAS* exon 3 reverse). Bidirectional Sanger sequencing of all PCR products were subsequently performed on a 3500 Genetic Analyzer (Life Technologies) using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies) and the mentioned PCR primers according to standard protocols.

In addition, targeted re-sequencing for mutations commonly found in B-cell malignancies was performed on the GS Junior 454 benchtop sequencer (Roche, Penzberg, Germany) using a two-step multiplex-PCR approach as previously described (29). In brief, exons with known hotspot mutations and respective splice sites of *BRAF* (exons 11 and 15), *EZH2* (exon 16), *MYD88* (exons 3 and 5), *NOTCH1* (exon 34), *PIK3CA* (exons 9 and 20), *SF3B1* (exons 14 and 15), as well as exons 4-10 of *TP53* were amplified in two multiplex-PCRs from 30 ng genomic DNA extracted from CD138-purified patient cells. Sequencing data was processed with the
GSRunProcessor (v.2.5/v.2.7) performing image and signal processing via the amplicon pipeline (Roche).

Statistical Analysis

We applied Fisher’s exact test to compare the prevalence of extramedullary disease between mutation carriers and control patients. Overall survival from the start of treatment was determined for symptomatic patients for whom a follow-up of at least three months was available and evaluated using Kaplan-Meier estimates.

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Conflict of interest

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References


Table 1: Characteristics of Samples / Patients

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<td>relapsed</td>
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<table>
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<td>57 [45 – 76]</td>
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Prevalence of extramedullary sites in symptomatic multiple myeloma was significantly higher in patients with BRAF V600E (57%) than in BRAF wildtype (18%) (p = 0.02; Fisher’s exact test).

(EMD: Incidence of extramedullary myeloma in the course of the disease)
Figure legends

Figure 1: Immunohistochemical and molecular characterization of BRAF V600E mutation status in multiple myeloma.

Samples of bone marrow core biopsies obtained from three representative myeloma patients (left, center, right) show marked plasma cell infiltration by means of IHC against CD138 (panel A). IHC analysis for BRAF V600E reveals nearly 100% BRAF mutated plasma cells in one patient (panel B, left, BRAF mut.), no BRAF mutation (center, BRAF wt) and identification of a mutated subclone (right, BRAF mut subclone). Sequence analysis of DNA extracted from the bone marrow biopsies (in case of the BRAF mutated subclone after microdissection of BRAF V600E positive tissue in order to enrich for mutated plasma cells) confirms strong presence of the $BRAF$ 1799T->A substitution that results in the BRAF V600E mutation in patient BRAF mut, BRAF wildtype in patient BRAF wt and a small proportion of BRAF V600E mutation in patient BRAF mut subclone (panel C, left to right).

Figure 2: Delineation of the clinical course and evolution of BRAF mutation status in the seven patients with BRAF V600E mutation.

The clinical courses of the seven patients with confirmed BRAF V600E mutation are depicted as bar diagrams with darker shades of grey marking further lines of therapy and white bars depicting periods without treatment. A blunt end of the bar signifies the death of the patient, an arrow-head end ongoing remission/therapy. Four of the seven patients developed extramedullary myeloma (time points indicated by arrows). At each time point a biopsy was taken (bone marrow or soft tissue), the BRAF mutation status is given by an open (wildtype) or filled (mutated) circle. This reveals three patients (#3, #4, #6) with initial BRAF
mutation, three patients (#1, #2, #5) with clonal evolution from BRAF wildtype to BRAF mutated, and one patient (#7) with a subclone harboring BRAF V600E mutation at initial diagnosis.

**Figure 3: Response to vemurafenib in patient #5.**

Comparative baseline to day 28 analyses. Whole body MRI scans reveal a marked shrinkage of a large subcutaneous plasmacytoma above the left shoulder (panel A, left). The measurable M-spike in the serum (panel A, upper right) as well as the kappa light chain proteinuria (lower right) rapidly decreased after start of treatment. Confirmed negative immunofixation in the serum and urine are depicted by respective arrows. The gray area indicate intermittent breaks in treatment. Overview histology (panel B, hematoxylin and eosin) of a skin biopsy shows regression of an infiltrative tumor. The bars depict tumor thickness (T) at baseline and day 28 as well as the extent of tumor regression (R) with scarring of subcutaneous tissue on day 28. The black boxes mark tumor areas magnified in panel C. On high magnification (panel C, left, size bar 25µm) reduction of cell size, shrinkage of nuclei and numerous apoptotic figures (arrow heads) are evident on day 28. While residual tumor remains positive for BRAF V600E on day 28 (panel C, right), p-ERK expression is completely abolished indicating decreased pathway activation after vemurafenib therapy (panel D, left). In analogy, the cell proliferation rate (IHC for MIB-1, panel C, center) is markedly reduced and the rate of apoptosis (IHC for activated caspase-3, panel C, right, arrowheads) is increased.
Figure 2

Years after diagnosis of multiple myeloma

- #1
- #2
- #3
- #4
- #5
- #6
- #7

Lines of therapy:
- 1st line
- 2nd line
- 3rd line
- 4th line
- vemurafenib

BRAF V600E status:
- ● mutated
- ○ wildtype
- ○ subclone

Extramedullary disease

Years after diagnosis of multiple myeloma
Figure 3

A

Baseline

Day 28

B

Baseline

Day 28

C

H&E

BRAF V600E

D

Baseline

Day 28
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