ABSTRACT

In multiple myeloma, there has been little progress in the specific therapeutic targeting of oncogenic mutations. Whole-genome sequencing data have recently revealed that a subset of patients carry an activating mutation (V600E) in the BRAF kinase. To uncover the clinical relevance of this mutation in multiple myeloma, we correlated the mutation status in primary tumor samples from 379 patients with myeloma with disease outcome. We found a significantly higher incidence of extramedullary disease and a shorter overall survival in mutation carriers when compared with 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 vemurafenib. Collectively, we provide evidence for the development of the BRAF V600E mutation in the context of clonal evolution and describe the 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. Cancer Discov; 3(8); 862–9. © 2013 AACR.

INTRODUCTION

Multiple myeloma is a malignancy of terminally differentiated B-lymphocytes, but its pathogenesis is yet only partially understood (1–3). Although new therapeutic options have substantially increased the response rates and survival of patients with myeloma 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 inhibitors (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 gene encoding the serine-threonine kinase \( \text{BRAF} \) was found to be mutated in approximately 4% of all cases, with the \( \text{BRAF V600E} \) mutation being the most common.

In malignant melanoma and hairy cell leukemia, targeting the highly prevalent \( \text{BRAF V600E} \) 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 \( \text{BRAF} \) 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 \( \text{BRAF V600E} \) by mutation-specific immunohistochemistry (IHC). Here, we describe the clonal evolution of myeloma cells from patients with a confirmed \( \text{BRAF V600E} \) mutation and the distinct clinical course of this cohort, thereby indicating the clinical relevance of this mutation and providing first proof-of-principle for the therapeutic efficacy of vemurafenib in multiple myeloma.

**RESULTS**

We screened for protein expression of \( \text{BRAF V600E} \) 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 (Fig. 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 with smoldering myelomas, 251 with symptomatic myelomas, 16 with Amyloid Light-chain amyloidosis) were analyzed. Patient characteristics are summarized in Table 1. Two or more consecutive biopsies were available from 37 patients of the cohort.

Mutated \( \text{BRAF V600E} \) was detected in seven patients with myeloma, 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 (Fig. 1A and B). This could be confirmed by sequencing only after microdissection of the tissue to enrich for positive plasma cells (Fig. 1C). In three patients, the mutation was present at diagnosis, whereas it became detectable in an additional three patients only in the context of...
relapsed disease, indicating clonal evolution (Fig. 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. Although 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 with 43 of 251 (17%; \( P = 0.02 \)) control patients with symptomatic disease (Fig. 2 and Table 1). Progression-free survival in five of these seven patients was very short once the mutation was present. Details of the patients’ courses of disease are given in Supplementary Table S1. The overall survival of all patients with documented follow-up of at least 3 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 to 54) as compared with 105 months (range 4 to 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 (Fig. 2 and Supplementary Table S1). She had first been diagnosed with immunoglobulin A (IgA) kappa symptomatic multiple myeloma, Salmon & Durie stage IIIa, in August 2008. Risk profiling revealed an International Staging System score of II. No high-risk cytogenetic aberration (i.e., t(4;14) or del17p) was detected by 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 was achieved, according to International Myeloma Working Group (IMWG) criteria (14). Fifteen months after 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 × 1.5; 1.1 × 0.9; 1.4 × 1.2 cm) as well as a large subcutaneous plasmacytoma above the left shoulder (5.9 × 2.1 cm by whole-body MRI scan), whereas the known osteolytic lesions remained stable. Electrophoresis of blood and urine showed a monoclonal IgA protein of 7.7 g/L and 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

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NOTE: Prevalence of extramedullary sites in symptomatic multiple myeloma was significantly higher in patients with BRAF V600E (57%) than in BRAF wild-type (18%; \( P = 0.02 \)) (Fisher Exact Test).

Abbreviations: Ig, immunoglobulin; MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, symptomatic multiple myeloma; EMD, incidence of extramedullary myeloma in the course of the disease.
BRAF in Myeloma

Lines of therapy
- First line
- Second line
- Third line
- Fourth line
- Vemurafenib
- Remission

BRAF V600E status
- Mutated
- Wild-type
- Subclone

Extramedullary disease

Years after diagnosis of multiple myeloma

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 gray 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 arrowhead 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 (wild-type) 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 wild-type to BRAF mutated, and one patient (#7) with a subclone harboring BRAF V600E mutation at initial diagnosis.

as from one of the skin lesions showed extensive plasma cell infiltration. IHC with the BRAF V600E mutation-specific antibody showed the presence of mutated 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, PIRK3CA, SF3B1, and TP53, that have been commonly found in other B-cell malignancies. A concomitant mutation was detected only 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 twice daily. Because the patient tolerated the treatment well, the dosage was increased to 720 mg twice daily after one week. Within 2 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 × 5.7 cm to 6.4 × 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 (Fig. 3A). A rebiopsy of one of the residual skin plasmacytomas was obtained and revealed extensive tumor regression and scarring (Fig. 3B). Although IHC still detected mutated BRAF V600E in the small area with remaining plasma cells (Fig. 3C), pathway activation, assessed by phosphorylation of the extracellular signal-regulated kinase (p-ERK), and cell proliferation, represented by MIB1, were drastically reduced compared with baseline samples (Fig. 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 less than one per high power field (HPF) at baseline and more than 10 per HPF at day 28 (10 HPFs were counted per slide; Fig. 3D).

In summary, the administration of a single 4-week course of vemurafenib resulted in the achievement of a partial response,
**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 (A, left). The measurable monoclonal protein in the serum (A, top right) as well as the kappa light chain proteinuria (bottom right) rapidly decreased after start of treatment. Confirmed negative immunofixation in the serum and urine are depicted by respective arrows. The gray area indicates intermittent breaks in treatment. Overview histology (B, hematoxylin and eosin, H&E) 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 C. On high magnification (left, size bar 25 μm) reduction of cell size, shrinkage of nuclei, and numerous apoptotic figures (arrowheads) are evident on day 28. Although the residual tumor remains positive for BRAF V600E on day 28 (C, right), p-ERK expression is completely abolished, indicating decreased pathway activation after vemurafenib therapy (D, left). Furthermore, the cell proliferation rate (IHC for MIB1; C, center) is markedly reduced and the rate of apoptosis (IHC for activated caspase-3; C, right, arrowheads) is increased.
both serologically and by size. After the second course, the urine electrophoresis became negative by immunofixation. All cutaneous manifestations had disappeared. The patient developed manageable arthralgia and burst-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 detectable only in the urine, both quantitatively and by immunofixation (Fig. 3B).

Once symptoms had resolved, vemurafenib was restarted with the fifth course at a lower dose of 480 mg twice daily. Urinary light chains rapidly returned to baseline, though immunofixation of the urine remained positive (Fig. 3B). The patient currently remains in stable remission after eight courses and is still undergoing continuous treatment with vemurafenib at 480 mg twice daily. Thus far, there has been no evidence of acanthomas or squamous cell carcinoma.

**DISCUSSION**

With the introduction of increasingly detailed molecular diagnostics and intervention, the clinical use 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 EGFR receptor (EGFR) in non–small cell lung cancer (17); and, most recently, vemurafenib and dabrafenib targeting BRAF in malignant melanoma or hairy cell leukemia (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, but 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% (4/199 patients) and 4% (6/147 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 seems 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). Extramedullary disease is normally a rare event in patients with myeloma and is 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 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 seems to be dose-dependent (9, 23).

In general, KRAS- and 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 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 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 Catalog of Somatic Mutations in Cancer 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 twice daily and could be maintained at 480 mg twice daily. 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 8 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 with other diseases.

Multiple myeloma does not harbor a unifying genomic aberration or mutation. Our data underlines the necessity for detailed molecular diagnostics early in the disease. They show the clinical feasibility of targeted inhibition of patient-specific somatic mutations and represent a significant step toward the goal of personalizing treatment for this genetically complex malignancy. Our data provide 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-center cohort of 379 patients with a monoclonal gammopathy diagnosed between January 1992 and January 2012 was investigated by IHC. Two or more consecutive biopsies
were available from 37 patients of the cohort. In total, the series consisted of 421 formalin-fixed paraffin-embedded (FFPE) bone marrow (n = 391) or soft-tissue biopsies (n = 30). The work was conducted within our program for the identification of novel therapeutic targets in plasma cell dyscrasias and was approved by the Institutional Review Board. 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 BRAF V600E in plasma cells by IHC. Four- to six-micrometer sections were cut from FFPE specimens and mounted on Superfrost Ultra Plus (Gerhard Menzel GmbH) glass slides. The immunostaining was conducted on a Ventana Benchmark Ultra (Ventana Medical Systems) automated immunostainer using standard reagents provided by Ventana. Pretreatment with cell conditioner 1 (Ventana Medical Systems) was followed by incubation with undiluted VE1 hydrolized supernatant and consecutive chromogenic detection with the optiView Universal DAB detection kit and counterstaining with hematoxylin and bluing reagent for 4 minutes each. The immunostained slides were evaluated by two pathologists. Microscopic figures were taken with an Olympus BX-51 light microscope equipped with a DP50-CCD camera and processed with Cell-A Software (all from Olympus).

Sequencing

Mutation analysis of the BRAF gene was conducted as previously described (24). Briefly, DNA was isolated from FFPE tissue using standard methods and mutation analysis was conducted by Sanger sequencing. In case of low tumor burden, the DNA was extracted described (24). Briefly, DNA was isolated from FFPE tissue using FFPE specimens and mounted on Superfrost Ultra Plus (Gerhard Menzel GmbH) glass slides. The immunostaining was conducted on a Ventana Benchmark Ultra (Ventana Medical Systems) automated immunostainer using standard reagents provided by Ventana. Pretreatment with cell conditioner 1 (Ventana Medical Systems) was followed by incubation with undiluted VE1 hydrolized supernatant and consecutive chromogenic detection with the optiView Universal DAB detection kit and counterstaining with hematoxylin and bluing reagent for 4 minutes each. The immunostained slides were evaluated by two pathologists. Microscopic figures were taken with an Olympus BX-51 light microscope equipped with a DP50-CCD camera and processed with Cell-A Software (all from Olympus).

Disclosure of Potential Conflicts of Interest

D. Capper has a patent pending for diagnostic use of BRAF V600E clone VE1 (EP2011/067092). T. Zenz has received honoraria from the Speakers’ Bureau of Roche. A. von Deimling has a patent pending for the diagnostic use of BRAF V600E mutant-specific antibody VE1; all terms are being managed by the German Cancer Research Center in accordance with its conflict of interest policies. H. Goldschmidt has received honoraria from the Speakers’ Bureaus of Janssen and Celgene and is a consultant/advisory board member of Janssen and Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Development of methodology: M. Andrulis, D. Capper, A. von Deimling, K. Neben

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Andrulis, N. Lehners, C. Heinig, T. Zenz, H. Goldschmidt, K. Neben, M.S. Raab

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Andrulis, N. Lehners, D. Capper, R. Penzel, J. Huellstein, A.D. Ho, K. Neben, M.S. Raab

Writing, review, and/or revision of the manuscript: M. Andrulis, N. Lehners, D. Capper, C. Heinig, T. Zenz, P. Schirmacher, A.D. Ho, H. Goldschmidt, K. Neben, M.S. Raab

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Andrulis, D. Capper, P. Schirmacher, A.D. Ho

Study supervision: P. Schirmacher, K. Neben, M.S. Raab

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REFERENCES

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