Brief Communication
Absence of *BRAF* exon 15 mutations in multiple myeloma and Waldenström's macroglobulinemia questions its validity as a therapeutic target in plasma cell neoplasias

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Abstract: Purpose: Recent whole genome and/or exome sequencing in a cohort of 32 Multiple Myeloma (MM) patients reported the incidence of *BRAF* mutations at 4%, while in another exome sequencing study, *BRAF* mutations were reported in up to 13% of cases tested. We ran a confirmatory study by using High Resolution Melting Analysis (HRMA), which is a low-cost, straightforward and sensitive screening test for detection of *BRAF* exon 15 mutations in MM and Waldenström's macroglobulinemia (WM) patients, in order to investigate their incidence in every day clinical practice. We considered this investigation to be of clinical relevance following the recent emergence of potent anti-BRAF compounds. Patients and Methods: We used genomic DNA isolated from 31 bone marrow aspirates obtained from 25 MM patients and 3 patients with WM (14 female; 14 male) who signed an informed consent. Patients' median age was 69 years (range 43-86) and median follow-up time was 45 months. Myeloma subtypes were as follows: 7 IgGκ, 6 IgGλ, 7 IgAκ, 4 IgAλ and 1 non-secretory. The bone marrow plasma cells ranged from 12 to 100% (mean/median value 45%). By International Staging System (ISS) 9/25 patients were stage I, 6/25 stage II, 7/25 stage III, while in 3 cases staging information was missing. In 3 MM cases matched paired samples at diagnosis and at relapse were also available. DNA samples were screened using HRMA. HRMA results were confirmed by subsequent ds-bi-directional sequencing (Sanger method) for somatic mutations in exon 15 of *BRAF*. Results: At a limit of detection ≥2.5% mutant allelic content by HRMA, we did not detect any *BRAF* mutations in exon 15 in any of our 31 samples. Conclusions: By using HRMA we do not confirm previously reported results. Lack of detection of *BRAF* exon 15 mutations in our MM and WM series may be related to different sensitivity of the assays used and/or the relatively small sample size. In any case, we consider that existing data should be taken into account when considering the clinical development of BRAF inhibitors in plasma cell neoplasms.

Keywords: Multiple myeloma, BRAF, mutation, WaldenstrÖm's macroglobulinemia, high resolution melting analysis

Introduction

Somatic mutations in *BRAF* are known to occur commonly in hairy-cell leukemia [1] and frequently in melanomas [2]. The most commonly reported mutation in cancer is V600E (T>A transversion) located in exon 15, which results in constitutive kinase domain activation correlating with constitutive activation of MEK and ERK1/2. [2-5]. This mutation also results in a conformational change that creates an open configuration offering improved access to the substrate and simultaneously a potentially "druggable" target for small molecule inhibitors [6]. Vemurafenib, the first BRAF inhibitor was recently approved by the FDA and the European Medicines Agency for the treatment of adult patients with *BRAF* V600 mutation positive unresectable or metastatic melanoma, following an impressively fast progress through a series of positive clinical trials [7-10]. The success story of vemurafenib in metastatic melanoma surged reasonable enthusiasm to investigate BRAF inhibitors in other cancer types

Table 1. Demographics

Patients	N	28
Age	median	68
	range	43-86
Gender	Male	14 (50%)
	Female	14 (50%)
Disease	Multiple Myeloma	25 (90%)
	Waldenstom's macro-globulinemia	3 (10%)
Multiple Myeloma subtype	IgG,	13 (52%)
	IgA	11 (44%)
	Non-secretory	1 (4%)
ISS stage	1	9
	2	6
	3	7
	unknown	3
Bone Marrow Infiltration	median	45%
	range	20-100%
Extraosseous Plasmacytoma coexistence	yes	3 (12%)
	no	22 (88%)

harboring *BRAF* V600 mutations including multiple myeloma (Clinical Trials. gov Identifier NCT01524978).

Methods

We used High Resolution Melting Analysis (HRMA), a low-cost, straightforward and sensitive screening test for detection of gene mutations. Genomic DNA was extracted using using a commercially available kit (QIAmp DNA mini kit, Qiagen) from 31 bone marrow aspirates obtained from 28 patients (14 female; 14 male); 25 multiple myeloma (MM) patients and 3 patients with Waldenstom's macroglobulinemia (WM) who signed informed consent (**Table 1**). In 3 MM cases matched paired samples at diagnosis and at relapse were available and tested. DNA samples were screened for *BRAF* mutations in Exon 15 using HRMA. All samples were subsequently bi-directionally sequenced. Primers flanking a 131 bp amplicon of *BRAF* exon 15 encompassing the V600 codon were designed. Primer sequences were as follows: ATGAAGACCTCACAGTAA and CCTC-AATTCTTACCATCC. DNA (1 ng) was amplified in a final volume of 25 ml containing 1x Platinum Taq polymerase buffer, 1 unit Platinum Taq polymerase (Invitrogen), 2.5 mmol/l MgCl$_2$, 0.125 mmol/l dNTPs, 0.5 mmol/l of each primer and 1x LC Green Plus (Idaho Technologies). PCR and HRMA were performed on a RotorGene

6000™ realtime analyser (Qiagen, Crawley, UK). PCR conditions were as follows: 95°C for 5 min followed by 45 cycles of 15 s at 95°C; a touchdown of 56°C for 15 s (1°C/cycle) and 30 s at 72°C. Following PCR amplification, products were denatured at 95°C for 1 min and cooled to 37°C for 1 min. High-resolution melt was performed from 72°C to 95°C rising at 0.2°C/s. The resulting data were analysed using Rotorgene Series software; and all PCR products were confirmed by bi-directional Sanger sequencing (ABI Prism 3130 sequencer). Serial dilutions of a cell line with single allelic *BRAF* V600E mutation (diluted in the parental cell line, both supplied by Horizon Diagnostics, Cambridge, UK) were carried out to assess HRMA sensitivity from a theoretical allelic load of 50% (**Figure 1A**).

Results

Patients' median age was 68 years (range 43-86) and median follow-up time was 45 months. Myeloma subtypes were as follows: 7 IgGκ, 6 IgGλ, 7 IgAκ, 4 IgAλ and 1 non-secretory. The bone marrow plasma cell content ranged from 20 to 100% (mean/median value 45%). By International Staging System (ISS) 9/25 patients were stage I, 6/25 stage II, 7/25 stage III, while in 3 cases staging information was missing. All patients required treatment except for three who were asymptomatic. Response to treatment varied from stable disease to complete response. Following several sensitivity analysis, HRMA was established with a limit of detection of ≥2.5% mutant allelic load (see **Figure 1A**). Sanger sequencing had an estimated sensitivity of 20% allelic content (data not shown). Neither the 28 samples obtained at diagnosis nor the 3 additional samples that were obtained at disease progression were classified as harboring any mutations in exon 15 of *BRAF*. Representative cases are shown in **Figure 1B**. As mentioned above the patient population was of all ISS stages and male and female genders were equally represented. All HRMA results were confirmed wild type by subsequent bi-directional sequencing.

Discussion

Next generation sequencing (NGS) has been used to elucidate the molecular basis of an
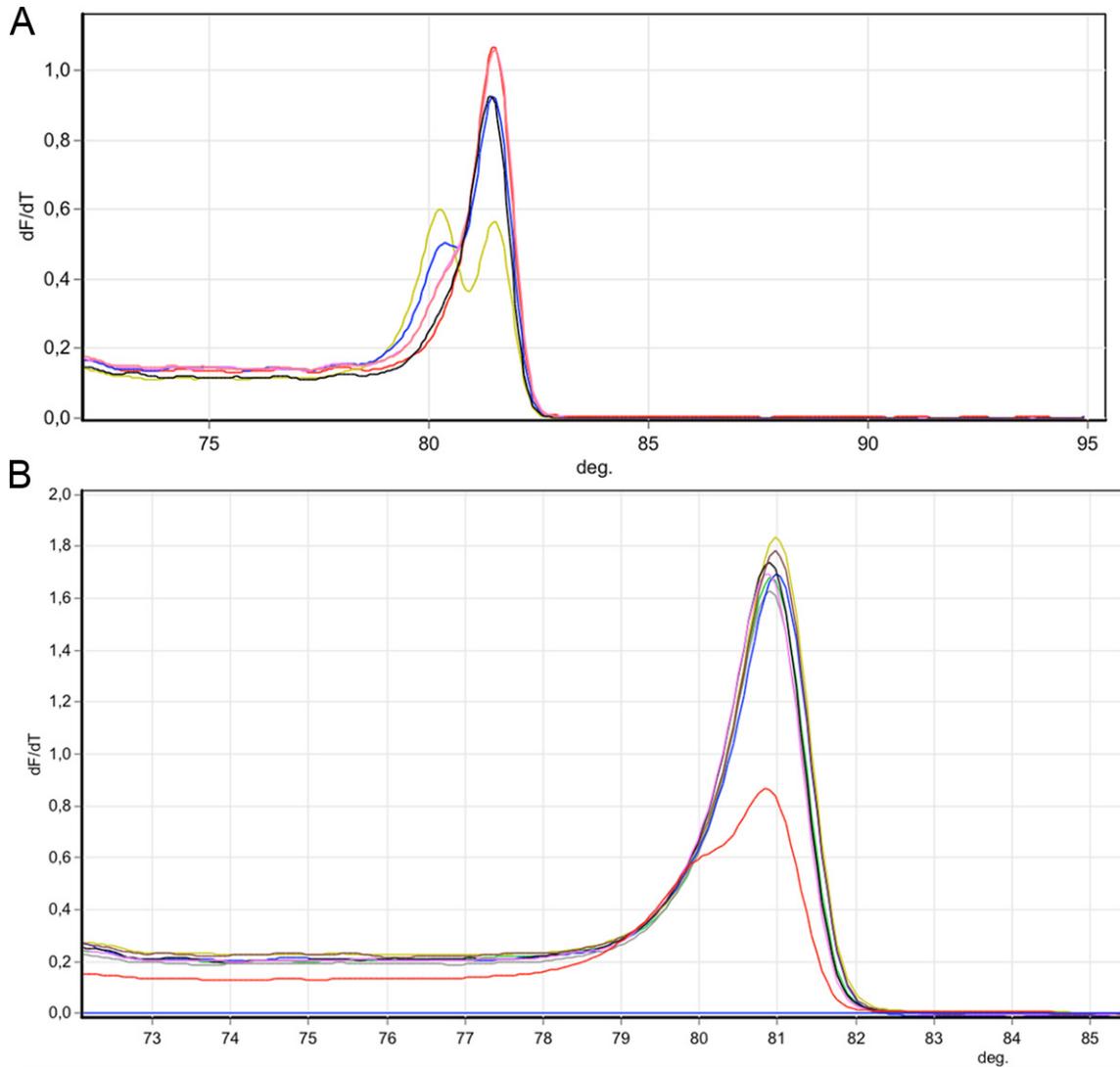
Figure 1. A. Selected HRMA results from cell line dilutions testing analytical sensitivity. Melting curves from HRMA sensitivity analysis. Not all dilutions are shown. Dilutions containing theoretical allelic content of V600E *BRAF* mutation. Yellow: 50% allelic content; Blue, 25% allelic content; Pink: 10% allelic content; Skin, 5% allelic content (note 10% and 5% essentially indistinguishable); Black: 2.5% allelic content; Red: Control 0% allelic content (Parental cell line); B. Representative HRMA results from sample set. Melting curves from HRMA of several random samples with positive and negative sample controls. Red: Positive sample containing theoretical allelic content of V600E *BRAF* mutation at 25% (Melanoma with >60% neoplastic cell content); Brown: Negative control sample a) *BRAF* wild type Melanoma sample containing >60% neoplastic cells; Green: Negative control sample b) BM aspirate from a patient with no diagnosed malignancy; Yellow: Representative Study Sample 1; Grey: Sample 5; Blue: Sample 13; Black: Sample 18; Pink: Sample 26.

increasing number of malignancies, and several "unexpected" mutations have been identified. In the first NGS study in MM, whole genome sequencing in a cohort of 32 MM patients found *BRAF* mutations in 4% of patients tested [11], and in a subsequent exome sequencing study, *BRAF* mutation frequency reached 13% [12]. In parallel, the remarkable clinical activity of anti-BRAF therapy in metastatic melanoma prompted several investigators to search for the presence of this target in other tumor types.

By using HRMA for the detection of *BRAF* exon 15 mutations in our MM and WM series we failed to confirm these previous reports and we did not identify any mutations. This might be related to the different sensitivity of the assays used (HRMA versus NGS) or the relatively small

sample size. Although HRMA was sensitive to ≥2.5% allelic content, this does not exclude the possibility that other less common mutations may have been undetected. The subsequent confirmatory analysis by Sanger sequencing supports the likelihood of a lack of mutations, however, this technique is commonly known to have a relatively poor sensitivity (≈20% allelic content as per our data). *BRAF* mutational incidence in other tumors is known to be influenced by both the analytical sensitivity of the assay, but also the neoplastic cell content of the sample [13], with recent guidelines for somatic mutational analysis (emphasis placed on solid tumors) suggesting that knowledge of the neoplastic cell content may alter decision to use one methodology over another [14]. Our data are, however, supported by other studies. Bonello L et al., failed to identify any *BRAF* mutations within exon 15 of *BRAF* in plasma cell neoplasms [15]; while Boyd E at al., who also used HRMA, detected only one MM patient out of 39 patients screened who carried two mutations within *BRAF* exon 15 (p.D594N; p. V600V) [16].

Overall we consider that currently existing data indicating a low rate of detection of mutated *BRAF* is not contradicted by our negative findings; however, it currently denotes that *BRAF* is most probably a rather poor target to undertake the development of *BRAF* inhibitors in plasma cell neoplasms without further demonstration of a baseline mutational frequency that would be clinically appropriate.

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Conflicts of interest

The authors declare no conflicts of interest.

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