Application of a *BRAF* Pyrosequencing Assay for Mutation Detection and Copy Number Analysis in Malignant Melanoma

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Mutations in the *BRAF* gene are found in the majority of cutaneous malignant melanomas and subsets of other tumors. These mutations lead to constitutive activation of BRAF with increased downstream ERK (extracellular signal-regulated kinase) signaling; therefore, the development of RAF kinase inhibitors for targeted therapy is being actively pursued. A methodology that allows sensitive, cost-effective, high-throughput analysis of *BRAF* mutations will be needed to triage patients for specific molecular-based therapies. Pyrosequencing is a high-throughput, sequencing-by-synthesis method that is particularly useful for analysis of single nucleotide polymorphisms or hotspot mutations. Mutational analysis of *BRAF* is highly amenable to pyrosequencing because the majority of mutations in this gene localize to codons 600 and 601 and consist of single or dinucleotide substitutions. In this study, DNAs from a panel of melanocyte cell lines, melanoma cell lines, and melanoma tumors were used to validate a pyrosequencing assay to detect *BRAF* mutations. The assay demonstrates high accuracy and precision for detecting common and variant exon 15 *BRAF* mutations. Further, comparison of pyrosequencing data with 100K single nucleotide polymorphism microarray data allows characterization of *BRAF* amplification events that may accompany *BRAF* mutation. Pyrosequencing serves as an excellent platform for *BRAF* genotyping of tumors from patients entering clinical trial. (*J Mol Diagn* 2007, 9:464–471; DOI: 10.2353/jmoldx.2007.060191)

Mutations in the *BRAF* gene occur in the majority of cutaneous malignant melanomas¹ and in subsets of papillary thyroid, serous ovarian, and colorectal carcinomas.^{1–4} The large majority (80 to 86%) of *BRAF* mutations in cancer are attributable to a T>A transversion in codon 600 resulting in substitution of glutamate for valine.^{1,5} This charge reversal mimics a phosphorylation event leading to constitutive activation of BRAF and increased signaling to downstream members of the MAPK (mitogen-activated protein kinase) pathway, MEK (mitogen-activated protein kinase kinase) and ERK (extracellular signal-regulated kinase).⁶ Numerous variant mutations have also been identified involving codon 600 and neighboring codons in exon 15, and less frequently in exon 11.⁵ The majority of variant mutations activate mitogen-activated protein kinase kinase leading to extracellular signal-regulated kinase activation, but rare mutations activate CRAF and extracellular signal-regulated kinase, thus bypassing mitogen-activated protein kinase kinase.^{5,7}

BRAF mutations confer a selective growth advantage and therefore provide opportunity for development of targeted molecular therapeutics, several of which are currently in trial or under development.^{8–10} As inhibitors for mutant *BRAF* enter clinical trial, *BRAF* mutation status, including knowledge of the specific mutation variant, is critical for determining drug response. Several accurate mutation detection assays have been developed to identify the common V600E mutation.^{11–14} Although such assays will detect the majority of *BRAF* mutations, variant mutations will be missed. Thus, utilization of such assays in clinical trials would fail to identify variant *BRAF* mutations, thereby failing to detect all patients with *BRAF*-mutant tumors. It will therefore be important to use a sequencing-based molecular assay that identifies the common V600E as well as variant *BRAF* mutations.

Pyrosequencing is a sequencing-based methodology that utilizes pyrophosphate molecules generated from nucleotide incorporation by *Taq* polymerase to create sequence data output. Data are displayed in the form of

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a pyrogram created by peaks representing incorporation of nucleotides in a specific order. Peak height is directly proportional to the number of individual nucleotides incorporated; pyrosequencing is thus a quantitative technology. This methodology is ideal for the genotyping of DNA in which a focal region; ie, single nucleotide polymorphism (SNP) or mutation hotspot is being interrogated. The high-throughput, low-cost features of pyrosequencing are in contrast to dideoxy cycle sequencing.

In this study, we have designed and validated a pyrosequencing-based assay for *BRAF* mutation detection. This assay shows high accuracy and precision and correctly identifies *BRAF* mutation variants as well as the common V600E mutation; therefore, it will allow determination of differential response to mitogen-activated protein kinase pathway inhibitors by mutation type in clinical trials. Further, correlation of the pyrosequencing data with SNP microarray data confirms that amplification of the *BRAF* allele is attributable to preferential increase in mutant *BRAF* copy number relative to wild-type *BRAF*.

Materials and Methods

Cell Lines and Tumors

The melanoma cell lines were cultured from primary melanomas representing all stages of melanoma progression (radial growth phase, vertical growth phase, metastatic). Melanocyte lines were generated from neonatal foreskin specimens. Melanoma cell lines were maintained in melanoma medium Tu2% and melanocyte lines were maintained in melanocyte medium.¹⁵ Genomic DNA was isolated from cell lines after proteinase K digestion and phenol/chloroform extraction. Frozen and formalin-fixed paraffin-embedded (FFPE) tumors were acquired and processed in accordance with institutional review board protocols from Fox Chase Cancer Center and the University of Pennsylvania. Genomic DNA was extracted from frozen tumors using the Puregene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN) as per the manufacturer's protocol. For DNA FFPE tumors, 1 × 5-μm sections were deparaffinized and stained using the Histogene Staining Kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's protocol. DNA was extracted using a modification of the PicoPure DNA Extraction Kit (Molecular Devices) protocol. Ten μl of Proteinase K extraction solution was pipetted over the area of interest. The pipette tip was used to scrape the area and recollect the buffer solution and cells. Collected cells were placed into a 0.5-ml microcentrifuge tube containing an additional 40 μl of Proteinase K Extraction Solution. The mixture was incubated at 65°C for 16 to 18 hours, followed by a final incubation at 95°C for 5 minutes to inactivate the proteinase K. An additional cleanup of the genomic DNA was performed using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol.

Pyrosequencing and Dideoxy Cycle Sequencing

A 228-bp region of human *BRAF* exon 15 spanning the hotspot mutation site at codon 600 was amplified by polymerase chain reaction (PCR). Primer sequences were: BraF: 5'-ATGCTTGCTCTGATAGGAA-3'; BraR: 5'-GCATCTCAGGGCCAAA-3'. The reverse primer was 5'-biotinylated to facilitate single-strand DNA template isolation for the pyrosequencing reaction. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Each PCR contained 20 to 50 ng of genomic DNA, 10 pmol of each primer, and 25 μl of Jumpstart Readymix REDTaq polymerase (Sigma, St. Louis, MO) in a total volume of 50 μl. Cycling was performed in an Eppendorf Mastercycler Gradient (Brinkman Instruments, Westbury, NY) as follows: 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 45 seconds, and a final 2-minute extension at 72°C. The protocol for DNA extracted from FFPE tissue was modified to include 45 cycles of amplification. Successful and specific amplification of the region of interest was verified by visualizing 5 μl of the PCR product on a 2% agarose gel containing ethidium bromide.

Preparation of the single-stranded DNA template for pyrosequencing was performed using the PSQ Vacuum Prep Tool (Biotage, Charlottesville, VA) according to the manufacturer's instructions. Twenty μl of biotinylated PCR product was immobilized on streptavidin-coated Sepharose high-performance beads (Amersham Biosciences, Piscataway, NJ) and processed to obtain a single-stranded DNA using the PSQ 96 Sample Preparation Kit (Biotage) according to the manufacturer's instructions. The template was incubated with 0.4 μmol/L sequencing primer (5'-GGTGATTTTGGTCTAGCTAC-3') at 80°C for 2 minutes in a PSQ96 plate. The sequencing-by-synthesis reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage) at room temperature using PyroGold reagents (Biotage). As nucleotides were dispensed, a light signal was generated proportional to the amount of each incorporated nucleotide. These light signals were detected by a charge-coupled device camera and converted to peaks in a sequencing pyrogram that was automatically generated in real time for each sample. The sequencing primer was designed to include the analysis of codons 599 to 602 to provide internal positive control peaks for nucleotide incorporation and to screen for mutations in alternate codons. Dideoxy sequencing of cell lines was performed using a previously published protocol¹⁶ with modifications. PCR products generated by hemi-nested PCR were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) before sequencing.

Cell Mixing Studies

DNA from a V600E heterozygous tumor sample was diluted with normal lymphocyte DNA to generate mixtures containing 100, 70, 50, 40, 20, and 0% tumor, respectively. Actual percent mutant allele was determined by pyrosequencing data from undiluted tumor DNA. For

each tumor dilution, a theoretical percent mutant allele was then calculated. In the cell mixing study, the linear relationship between the observed and theoretical percentages was determined by linear regression in GraphPad 4.0 software (San Diego, CA).

100K SNP Microarrays

Genomic DNA from eight melanoma cell lines was hybridized to Affymetrix 100K SNP arrays (Affymetrix, Santa Clara, CA) for determination of copy number and zygosity status across the human genome. This 100K set, comprised of two 50K microarrays (*Xba*I and *Hind*III), uses 116,204 SNPs with a mean intermolecular distance of 23.6 kb. Briefly, 250 ng of DNA from each cell line was digested with either *Xba*I or *Hind*III restriction enzymes, ligated to an adaptor that allowed linear PCR amplification of DNA, fragmented, biotin end-labeled, and hybridized to *Xba*I or *Hind*III arrays, respectively. Arrays were scanned with the GeneChip 3000 7G scanner (Affymetrix). Intensity data for each SNP were acquired with GeneChip Operating Software (GCOS; Affymetrix), and genotype calls were made using GeneChip Genotyping Analysis Software (GTYPE 4.0; Affymetrix). SNP call rates of >95% were achieved for each sample, confirming adequate data quality. The Chromosome Copy Number Analysis Tool (CNAT 3.0; Affymetrix) compares sample SNP data with a reference set of 100 normal individuals to determine copy number and likelihood of loss of heterozygosity (LOH) at an individual SNP. *BRAF* copy number data presented are given after application of a genome-smoothing algorithm that groups together SNPs over a user-defined genomic interval (0.5 Mb in present study) and represents the average of values for all intra-genic *BRAF* SNPs. Individual copy number values are associated with a *P* value that indicates the likelihood that the copy number at that SNP location deviates from 2. The LOH score is computed using a statistical algorithm based on the likelihood that a stretch of SNPs would all be homozygous.¹⁷

Results

Assay Design and Interpretation

The pyrosequencing assay was designed to begin sequence analysis with the 3' nucleotide of codon 599 and end with the 3' nucleotide of codon 602 (ie, -AGT-GAAATCT-3'). Analysis of sequence flanking the T>A hotspot within codon 600 produces internal reference peaks and allows for the detection of variant mutations (see below). A sequential nucleotide dispensation protocol was used that reflects the expected order of nucleotide incorporation and the potential T>A base change within codon 600 (Figure 1, pyrogram positions 4 and 5). In addition, negative nucleotide dispensations were inserted (C at pyrogram positions 1 and 6) to serve as internal controls for nucleotide misincorporation. Peak heights are proportional to the number of nucleotides that are incorporated with each dispensation. Therefore, in

Figure 1. Detection of V600E in melanoma cell lines. Pyrograms generated for *BRAF* wild-type (WM3208) (A), heterozygous mutant (WM 902B) (B), and homozygous mutant (WM39) (C) cell lines distinguish T versus A peaks (yellow shading). Nucleotide dispensation order is given below each pyrogram. Numerical position for each nucleotide is indicated at the top. Dispensations of C at positions 1 and 6 are included as controls for misincorporation. Dideoxy cycle sequence tracings for each cell line are shown for comparison.

the case of a wild-type sequence (Figure 1A), the generated peak heights for nucleotide dispensations at positions 2, 3, 7, 9, 10, and 11 are approximately equivalent and represent incorporation of a single nucleotide in both alleles (peak equivalent = 1.0). Note that the signal intensity of the A peak at position 8 is threefold greater and reflects the incorporation of three As in tandem for codon 601. Using the peaks at positions 2, 3, 7, 9, 10, or 11 as an internal reference, a peak equivalent ~0.5 within the variable site represents the incorporation of a nucleotide into only one of two alleles and the detection of a heterozygous mutant (Figure 1B).

Accuracy and Precision

The pyrosequencing assay was performed on DNA extracted from two melanocyte cell lines, 34 malignant melanoma cell lines, 16 frozen metastatic melanomas, and three FFPE metastatic melanomas. Pyrosequencing results were compared with dideoxy sequence tracings for exon 15 of *BRAF*. There was 100% concordance for the 34 cell lines, 15 of 16 frozen tumors (one PCR failure), and three FFPE samples (Table 1). The common V600E mutation (Figure 1) was accurately distinguished from wild type. Homozygous versus hemizygous V600E mutation with LOH was detected in 4 of 34 melanoma cell lines (Figure 1C). Several variant mutations affecting codons 600 or 601 (V600K, V600D, V600R, and K601E) were detected by aberrant pyrosequencing tracings that did not conform to the pyrogram for heterozygous mutant V600E (Figure 2 and Table 1). For these cases, the specific mutation was confirmed with dideoxy cycle sequencing.

Dichotomous diagnosis of mutation-positive or -negative samples (13 of 34 melanoma cell lines, 6 of 15 frozen

Table 1. Correlation of Pyrosequencing Results with Dideoxy Cycle Sequencing

Sample type (n)	Pyrosequencing results	Correlation with cycle sequencing*
Melanocyte cell lines (2)	Wild type = 2	100%
Melanoma cell lines (34)	Wild type = 9 V600E = 19 V600D = 4 V600R = 1 K601E = 1	100%
Frozen tumors (16)	Wild type = 9 V600E = 5 V600K = 2	100%
FFPE tumors (3)	V600E = 1 V600K = 2	100%

*For variant mutations, pyrosequencing identified the presence of variant that was defined by cycle sequencing.

tumors, two of three FFPE tumors) was always reproducible. In 2 of 34 cell lines, although the pyrogram pattern was identical, the software missed calling small T peaks. This may occur when there is instability in the pyrogram baseline in the context of lower overall amplification.

The coefficient of variation (CV) for percent mutant allele was calculated from triplicate PCR-pyrosequencing experiments for four V600E melanoma cell lines and two FFPE tumors (one each of V600E and V600K) using the mutant A peak. The average CV for the four melanoma cell lines was 1.98% (range, 0 to 6.38%). The CVs for the two FFPE tumors were 5.3% (V600E, high percentage mutant A peak) and 7.29% (V600K, low percentage mutant A peak).

Detection of Variant Mutations

In 6 of 34 melanoma cell lines, 2 of 16 frozen tumors, and two of three FFPE tumors, variant mutations involving codons 600 or 601 were identified as an aberrant pyrogram sequence pattern with altered peak heights affect-

Figure 2. Detection of *BRAF* variant mutations. Pyrograms for two variant mutations, V600R (B) and V600K (C), show distinct patterns compared with V600E (A). Changes in relative A/G peak heights at pyrogram positions 2, 3, 5, 7, and/or 8 reflect dinucleotide substitutions given at right. The specific variants are confirmed by dideoxy cycle sequencing.

Table 2. Codon 600,601 *BRAF* Mutations

Nucleotide change	Codon change	Amino acid change
c.1799T>A	GTG>GAG	p.V600E (V600E)
c.1799_1800delinsAT	GTG>GAT	p.V600D (V600D)
c.1798_1799delinsAG	GTG>AGG	p.V600R (V600R)
c.1798_1799delinsAA	GTG>AAG	p.V600K (V600K)
c.1801A>G	AAA>GAA	p.K601E (K601E)

ing peaks in addition to or different from nucleotide 1799 (c.1799T>A resulting in p.V600E). Comparison with dideoxy cycle sequence tracings confirmed the presence of variant mutations: V600D (4), V600R (1), V600K (4), or K601E (1) (Figure 2 and Table 1). All of the variant mutations involve a tandem dinucleotide substitution (Table 2). In each case, knowledge of the specific variant explains the altered pyrogram tracing created by a change in the order and/or quantity of incorporation of each nucleotide, relative to that observed with the common V600E.

Applicability to FFPE Tissue

To determine the suitability of this assay for analysis of FFPE tissue, three macrodissected FFPE tumors were tested. By increasing the PCR cycle number from 35 to 45, amplification was adequate for pyrosequencing. Matched frozen tissue was available for two FFPE tumors. Comparison of pyrograms from these two paired frozen/FFPE tumors (V600E and V600K) showed the same pattern, confirming the accuracy of this technique for FFPE material (Figure 3).

Analytic Sensitivity

To determine analytic sensitivity of the assay, mixing studies were performed. DNA dilutions prepared from mixing a V600E heterozygous tumor with normal lymphocyte DNA (100, 70, 50, 40, 20, and 0% tumor) were pyrosequenced. Based on an actual quantitated percent mutant allele in the undiluted sample as determined by pyrosequencing, a theoretical percent mutant allele was calculated for each dilution (Table 3). After pyrosequencing, an actual percentage of mutant allele was determined using the AQ mode of the PSQ96MA SNP analysis software (Biotage). Using data generated from two separate experiments, a linear relationship between the actual and theoretical percentage of mutant allele was identified, confirming that the assay is reasonably linear [Pearson correlation (r) = 0.96] (Figure 4). Pyrograms show a detectable mutant peak as low as 13 to 17% in the 20% tumor dilution, which could be reliably distinguished from the level of background noise generated in the negative control sample (Figure 5). In comparing the pyrograms with dideoxy sequence tracings from the day 1 amplifications, a small mutant peak is visible in the dideoxy sequence tracings in the 40% tumor DNA dilution, but this cannot be distinguished from background in the 20% tumor DNA dilution. These data suggest that

Figure 3. Applicability of pyrosequencing assay to FFPE tissue. Pyrograms generated from a V600E mutant tumor (A) and a V600K mutant tumor (B) are identical for paired frozen and FFPE tumors.

pyrosequencing is slightly more sensitive than dideoxy cycle sequencing for detecting the V600E mutation.

Comparison of Pyrosequencing and DNA Microarray Data

A subset of V600E mutant melanoma cell lines (4 of 19) showed a 100% mutant (A) peak. To clarify whether this mutant peak was derived from a homozygous V600E or a hemizygous V600E with loss of the wild-type allele, pyrosequencing data from selected cell lines were compared with Affymetrix 100K SNP DNA microarray data for chromosome 7 (Figure 6 and Table 4). These SNP chips provide quantitative data for gene/chromosome copy number and simultaneously determine zygosity status through calculation of a LOH score at each SNP (see Materials and Methods). *BRAF* pyrosequencing data from the eight cell lines were compared with 100K SNP chip data for chromosome 7. Data shown are for the 50K *Xba*I microarray that includes five informative (intragenic) SNPs for *BRAF*. A *BRAF* wild-type cell line, WM3211, shows two copies of chromosome 7 with retained heterozygosity as determined by the low LOH score across the whole chromosome. In contrast, WM39, a cell line that is entirely mutant as determined by pyrosequencing, shows three copies of the q arm of chromosome 7 in the region encompassing *BRAF*. A high LOH score confirms that the three copies of mutant *BRAF* arose through loss of 7q containing the wild-type *BRAF* allele, and reduplication of 7q containing mutant *BRAF*. WM793B shows retention of heterozygosity with chromosomal amplification in the region of *BRAF* (approximately four copies of *BRAF*). The pyrosequencing data show a heterozygous result, consistent with the low LOH score on 100K SNP

chip, but show a ratio of mutant/wild-type of ~2.6:1, indicating genomic amplification of the mutant *BRAF* allele. In all four of the cell lines with a heterozygous V600E, an increase in average *BRAF* copy number per cell was noted on SNP chip (range, 2.7 to 4.2). In each of these cell lines, the pyrosequencing data show an increased ratio of mutant/wild-type *BRAF*, suggesting that the mutant *BRAF* allele was preferentially amplified during melanoma genesis (Table 4).

Discussion

Pyrosequencing assays have been previously used for *BRAF* genotyping in melanoma using a nested PCR¹⁸ and in colorectal cancer.¹⁹ The pyrosequencing assay reported herein allows sensitive and accurate detection of *BRAF* exon 15 common and variant mutations using a nonnested protocol that is highly amenable to use in a clinical laboratory. The common V600E, representing >90% of *BRAF* mutations in melanoma,¹ can be accurately identified by interpretation of the pyrogram only. Because the pyrograms generated by variant mutations are more complex, we recommend performing dideoxy cycle sequencing to confirm the specific variant mutation for cases in which the pyrogram shows a pattern that is not consistent with either wild-type sequence or *BRAF* V600E. Although only a small panel of FFPE tissues was tested, increasing the PCR cycle number to 45 allowed successful amplification of the samples and accurate determination of *BRAF* mutation status (both V600E and

Table 3. Actual/Theoretical Percent Mutant Allele at Given Dilutions of a V600E Heterozygous Tumor

% Tumor	% Mutant allele (day 1)		% Mutant allele (day 2)	
	Actual	Theoretical	Actual	Theoretical
100	56		59	
70	35	39	37	41
50	22	28	23	30
40	22	22	19	24
20	17	11	13	12
0	0	0	8	0

Figure 4. Cell line DNA mixing study. Theoretical peak heights, calculated from initial percent mutant A peak in undiluted heterozygous V600E mutant tumor, are correlated with actual peak heights generated for each dilution (Pearson's correlation = 0.96), indicating a linear relationship.

Figure 5. Analytic sensitivity for V600E detection in a heterozygous tumor. Pyrograms generated from dilution series for two separate experiments (day 1 and day 2) are shown with corresponding dideoxy sequence tracings for day 1 data. The percent tumor corresponding to each data set is indicated at left.

variant V600K), indicating that this assay may be successfully applied to FFPE tissues.

The analytic sensitivity of 15 to 20% for detection of the V600E mutation is slightly higher than that achieved with standard dideoxy sequencing, suggesting higher sensitivity with pyrosequencing. This finding is in agreement with previous pyrosequencing-based analyses of *KRAS* mutations,²⁰ suggesting that pyrosequencing methods are, in general, more sensitive. Although pyrosequencing methods are methodologically quantitative in the detection of mutant peaks, the percentage of mutant peak present in an individual tumor may not reflect the exact percentage of tumor cells in the sample. This is attributable to the potential for copy number alterations (amplifications or deletions) involving both wild-type and mutant *BRAF* alleles, as well as admixed stromal tissues that could affect this determination. Because the analytic sensitivity of detecting the V600E mutation is ~15 to 20%, this assay is intended for use as a diagnostic assay but not for minimal residual disease detection. Accounting for the heterozygous state of most tumors and the presence of stromal elements, a minimum of 50% tumor tissue should be used for DNA extraction to avoid a false-negative result.

In recent years, DNA microarrays that interrogate SNPs across the human genome have been applied in oncology research to simultaneously determine chromosome copy number data and zygosity status.^{21,22} In this study, the Affymetrix 100K SNP array was hybridized with a subset of the melanoma cell lines ($n = 8$) for which *BRAF* pyrosequencing data had been generated. Included in this set was one cell line, WM39, that showed a homozygous V600E mutation by pyrosequencing. By comparing results from the two platforms, it was determined that the V600E mutation in WM39 arose through a LOH event involving the nonmutant chromosome 7 with reduplication of the *BRAF*-mutant chromosome, rather

than through biallelic V600E mutations. Similar findings have been noted in both *JAK2*- and *FLT3*-mutant hematopoietic neoplasms.^{23–25} In the case of *FLT3*, the presence of LOH has an adverse prognostic impact.²⁵ Further, comparison of data for WM793B reveals that although the nonmutant allele was retained, there is preferential amplification of the mutant *BRAF* allele in a ratio of ~2.6:1 (mutant/wild type). *BRAF* copy number gains have been identified in both follicular thyroid cancer and malignant melanoma and may occur through either gene amplification or chromosome 7 polysomy.^{26–28} Using dideoxy cycle sequence tracings, an increase in height of the mutant A peak relative to the nonmutant T peak has suggested the presence of copy number gain of mutant *BRAF* in melanoma.²⁸ Using the 100K SNP array data, we now show *BRAF* copy number gains in six of eight melanoma cell lines including five of five *BRAF* mutant cell lines. Because the pyrosequencing data are quantitative, we further show a relative increase in mutant/nonmutant *BRAF* alleles, suggesting preferential amplification of mutant *BRAF*. Thus, it seems that through mechanisms that may or may not involve LOH, mutant *BRAF* may be selectively amplified during melanoma genesis. Larger scale studies are required to determine the frequency of LOH and of *BRAF* copy number gain in melanoma and other tumors.

Functional studies of mutant *BRAF* in melanocytes confirm that it is oncogenic, leading to dysregulated cell growth.^{8,10} This has opened the door for development of *BRAF* mutant-targeting therapeutic inhibitors in melanoma. Preclinical data of the MEK inhibitor CI1040 in melanoma show selective inhibition of *BRAF* V600E mutant tumors compared with *NRAS* mutant or *BRAF*/*NRAS* wild-type tumors,²⁹ as well as in *BRAF*- or *KRAS*-mutant serous ovarian tumors.³⁰ In papillary and anaplastic thyroid cancer, RAF kinase inhibitors show similar growth inhibition in *BRAF*-mutant cell lines.^{31,32} Further, *in vitro*

Figure 6. Comparison of pyrosequencing data with SNP microarrays. **A:** SNP *Xba*I 50K microarray data for chromosome 7 displayed with CNAT 3.0 software. Melanoma cell lines analyzed and location of *BRAF* gene are indicated. For each cell line, genome smoothed chromosome copy number data are shown at the top and the LOH score is shown at the bottom. Chromosome location in megabases is given at the bottom of the data set for each cell line. Scale for copy number and LOH score indicated at right. **B:** Corresponding *BRAF* pyrosequencing data including percent T and A peaks are indicated for each cell line.

studies of *BRAF* mutations show variable levels of kinase activity,⁷ raising the possibility that response to molecular inhibitors may depend on the specific mutation present. Thus, it is highly likely that future therapeutic strategies will require knowledge of *BRAF* mutation status including the specific mutation variant.

BRAF mutation screening is also important in the molecular diagnostics of colorectal cancer. A subset of colorectal cancers (5 to 18%) has a *BRAF* mutation, the majority of which are V600E.^{1,4} Studies have shown a strong association between *BRAF* mutation and the presence of high-level microsatellite instability (MSI-H) in sporadic colorectal cancer.^{33,34} Further investigation of this association revealed that *BRAF* mutation is found in the

Table 4. Correlation of *BRAF* Copy Number with Zygosity Status and V600E Mutant/Wild-Type

Cell line	Mutation status	Average <i>BRAF</i> copy no./cell	LOH	Pyrosequencing (V600E mutant/wild-type)*
WM3211	WT	2.1	No	N/A
WM1366	<i>NRAS</i>	1.5	Yes	N/A
WM1361A	<i>NRAS</i>	2.8	No	N/A
WM39	<i>BRAF</i>	3	Yes	N/A
WM1205Lu	<i>BRAF</i>	2.7	No	1.33
WM35	<i>BRAF</i>	3.3	No	1.63
WM793B	<i>BRAF</i>	4	No	2.57
WM983C	<i>BRAF</i>	4.2	No	1.86

N/A, not available.

*Ratio given for *BRAF* V600E heterozygous cell lines only.

context of a methylation phenotype characterized by methylation of several MINT markers and gene promoters including *hMLH1*.³⁴ The association with *hMLH1* methylation explains the link between *BRAF* mutation and MSI-H. Importantly, *BRAF* is not found to be mutated in MSI-H tumors present in the setting of hereditary nonpolyposis colon cancer families, including those families with mutations in *hMLH1*.³³ Thus, *BRAF* mutation is not associated with mismatch repair deficit per se, but rather with a more generalized methylation phenotype that frequently includes methylation of *hMLH1*. Based on these findings, a strategy for determining which patients with MSI-H tumors should be subject to mismatch repair gene mutation screening has been proposed in which *BRAF*-mutant tumors (~40% of sporadic MSI-H colorectal cancers) would be exempt from such testing in the absence of other features suggestive of hereditary nonpolyposis colon cancer.³⁵ The pyrosequencing assay presented here is an excellent platform for performing *BRAF* mutation screening in this setting.

In summary, we describe a pyrosequencing assay for accurate and quantitative identification of *BRAF* mutations. The assay targets codons 600 and 601 of *BRAF* exon 15, thus capturing the large majority of *BRAF* mutations (~92%) across multiple tumor types. This assay provides a high-throughput, cost-effective approach for genotyping tumors for patients entering clinical trials, and for determining the utility of mismatch repair gene mutation screening in patients with MSI-H colorectal cancer. Finally, the quantitative nature of the pyrosequencing methodology allows interfacing of this platform with SNP array data to study the potential added role of *BRAF* gene amplification/LOH in tumorigenesis.

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