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Molecular Platforms Utilized to Detect *BRAF* V600E Mutation in Melanoma

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Metastatic melanoma (MM) is a deadly skin disease refractory to standard chemotherapy. Despite numerous clinical and pathological parameters derived to guide patient management, clinical outcomes in melanoma patients remain difficult to predict. There is a critical need to delineate the important biomarkers typical of this disease. These biomarkers will ideally illuminate those key biochemical pathways responsible for the aggressive behavior of melanoma and, in the process, unveil new opportunities for the design of rational therapeutic interventions in high-risk patients. The most common recurring mutation in cutaneous melanoma is the prooncogenic *BRAF V600E* mutation that drives melanoma cell proliferation. The development of RAF inhibitors targeted against *BRAF V600E* mutant melanoma cells has revolutionized the treatment of MM. Clinical trials with *BRAF* inhibitor vemurafenib have shown objective clinical response and improved survival in patients with MM; therefore, knowledge of the molecular signature of melanoma in patients will be important in directing management decisions. Several molecular platforms exist to analyze the mutation status of melanoma. These include Sanger sequencing, pyrosequencing, allele-specific reverse transcriptase polymerase chain reaction, mass spectrometry base sequencing (Sequenom), high-resolution melting curve analysis, and next-generation sequencing methods using microfluidics technology. The Food and Drug Administration has approved the cobas *BRAF* V600 Mutation Test developed by Roche to analyze *BRAF* mutation status in formalin-fixed paraffin-embedded tumor samples. The cobas Mutation Test has been designed specifically to detect *BRAF V600E* mutations, and the analytic performance of this assay has demonstrated >99% sensitivity in the detection of *BRAF V600E* mutation when compared with the Sanger sequencing method and confirmed with the next-generation sequencing 454-pyrosequencing technology. The lower limit of detection of the percentage of mutant alleles in a tissue sample for the cobas test is less than 4%-5%. Some cross-reactivity with other variants of mutant *BRAF* was seen with the cobas V600 platform; however, this clinical test offers highly sensitive reproducible *BRAF V600E* mutation analysis in formalin-fixed paraffin-embedded tumor samples.
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Cutaneous melanoma is a deadly skin disease well known for its aggressive clinical course and lethal metastases.[1,2] Metastatic melanoma (MM) is incurable, with a mean overall survival of 6-8 months. The mortality associated with MM stems from its refractoriness to standard chemotherapeutic agents and marginal response in a subset of patients treated with dacarbazine and high-dose interleukin-2.[3] The identification of *BRAF* mutations in the mitogen-activated protein kinase pathway in the majority of patients with cutaneous melanoma has revolutionized the treatment of advanced-stage melanoma.[4,5] This has brought selective small-molecule RAF inhibitors (eg, vemurafenib) to the clinics for treatment.

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In a phase III clinical trial (BRAF inhibitor in melanoma-3) with the RAF inhibitor vemurafenib, there was a higher response rate and a statistically significant improvement in survival in the vemurafenib group compared with those who received dacarbazine.[6]

The knowledge that melanomas harbor recurring hotspot mutations in the *BRAF* gene has rapidly brought molecular testing to the clinical stage. The cobas 4800 BRAF V600 Mutation Test developed by Roche, which is approved by the Food and Drug Administration, is an in vitro diagnostic device to detect mutant *BRAF V600E* in deoxyribonucleic acid (DNA) extracted from a formalin-fixed paraffin-embedded (FFPE) patient's sample of melanoma. The presence of the *BRAF V600E* mutation will aid in selecting patients who will be offered targeted therapy with vemurafenib.

This review will (1) discuss genetics of *BRAF* mutation in melanoma, (2) examine the various molecular platforms to detect mutant *BRAF*, and (3) highlight the clinical significance of the *BRAF* V600E mutation in melanoma.

BRAF Mutant Melanoma

BRAF is one of the 3 isoforms of RAF, a serine–threonine protein kinase and a protooncogene located on chromosome 7q32.[7] The kinase activity in all 3 isoforms requires a complex series of phosphorylation, particularly at sites near the kinase domain (eg, S338 in CRAF and S445 in *BRAF*), to relieve the inhibitory effects of the regulatory domain. However, in contrast to ARAF and CRAF, *BRAF* is constitutively phosphorylated at the S445 site, and thus demonstrates 15-20-fold higher basal kinase activity. Thus, *BRAF* requires less post-translational modifications than ARAF or CRAF to achieve maximal kinase activity.[8-10] This distinction makes *BRAF* more susceptible to mutational activation in melanoma. Activation of the RAF protein triggers mitogen-activated protein kinase signaling and cell proliferation via phosphorylation of the downstream targets mitogen-activated protein kinase and extracellular-signal regulated kinase.[11] Mutation in *BRAF* accounts for approximately 50%-60% of genetic alterations in primary cutaneous melanoma.[8,12,13] However, approximately 80% of melanocytic nevi also harbor mutant *BRAF* V600E—identical to the most common *BRAF* mutation in melanoma. Codons 595-600 from exon 15 are the most common mutation site for the *BRAF* gene, which accounts for approximately 89% of mutations in *BRAF*, followed by approximately 11% of mutations in codons 468-474.[14] More than 95% of clinically relevant *BRAF* mutations occur in exon 15 and involve a single-point mutation with a DNA base substitution from thymine to adenine (T to A) that converts valine to glutamic acid at the 600 position of the amino acid (*BRAF* V600E). Less frequently, other types of *BRAF* mutations occur, which include *V600K, V600R, K601N, L597R, L597Q, G596R*, and *D594N*.[15] Compared with wild-type (WT) *BRAF*, mutant *BRAF* V600E demonstrates an almost 500-fold increase in endogenous kinase activity.[16]

Molecular Platforms for *BRAF* Testing in Melanoma

The sequencing technology continues to evolve and offer a more thorough and complex analysis of the genetic components of a melanoma. The next-generation sequencing (NGS) or massively parallel sequencing will allow sequencing of the entire exon or whole genome.[17] Multiple sequencing molecular platforms are available to examine for *BRAF* mutations in cutaneous melanoma, and the best technological approach continues to be developed.

Sanger Sequencing

Sanger chain-termination sequencing of amplified DNA by polymerase chain reaction (PCR) was the method used to sequence the human genome.[18-20] The amplified DNA segment is randomly terminated, producing DNA segments of various lengths terminating with adenosine (A), cytosine (C), guanosine (G), or thymidine (T) bases, which are differentially labeled with fluorescent molecules. Automated instruments separate DNA segments by capillary electrophoresis and detect fluorescently labeled nucleotide sequences.[21,22] The Sanger method of sequencing led to the detection of *BRAF* mutations in cutaneous melanoma. Mutation analysis by Sanger sequencing provides a complete sequence between the sequencing primer pairs and allows for the detection of DNA base pair substitutions, deletions, and insertions.

The Applied Biosystem *BRAF* Mutation Analysis platform used this principle for the detection of *BRAF* mutations. In the shifted termination primer-extension assay, unmutated *BRAF* will allow a primer extension, and if mutated *BRAF* is present, the primer extension will prematurely terminate.[14] The sensitivity of this assay is high, with fewer than 5% of tumor cells necessary in a specimen. However, its use in the clinical setting is limited to *BRAF* testing.

The Sanger method of gene sequencing is the gold standard; however, there are some important technical and practical limitations. The length of time required to perform the test is relatively long, approximately 18-19 hours. Reading gaps of 20 base pairs from the primer site occur; however, this may not be an issue for detection of known "hot spot" mutations. This method is 10 times less sensitive than the pyrosequencing platform: the detection ratio of mutant *BRAF* V600E to WT is 1:5 by Sanger sequencing methods compared with 1:50 by pyrosequencing.[23,24] Finally, the Sanger sequencing method cannot detect changes in the chromosomal copy number and the translocations.

Pyrosequencing

Pyrosequencing is referred to as sequencing by synthesis and relies on the release of pyrophosphate (PPi) by DNA polymerase after the addition of nucleotide to the complementary DNA strand (Fig. 1A). After the initial PCR reaction, a pyrosequencing primer is annealed to the target region on the

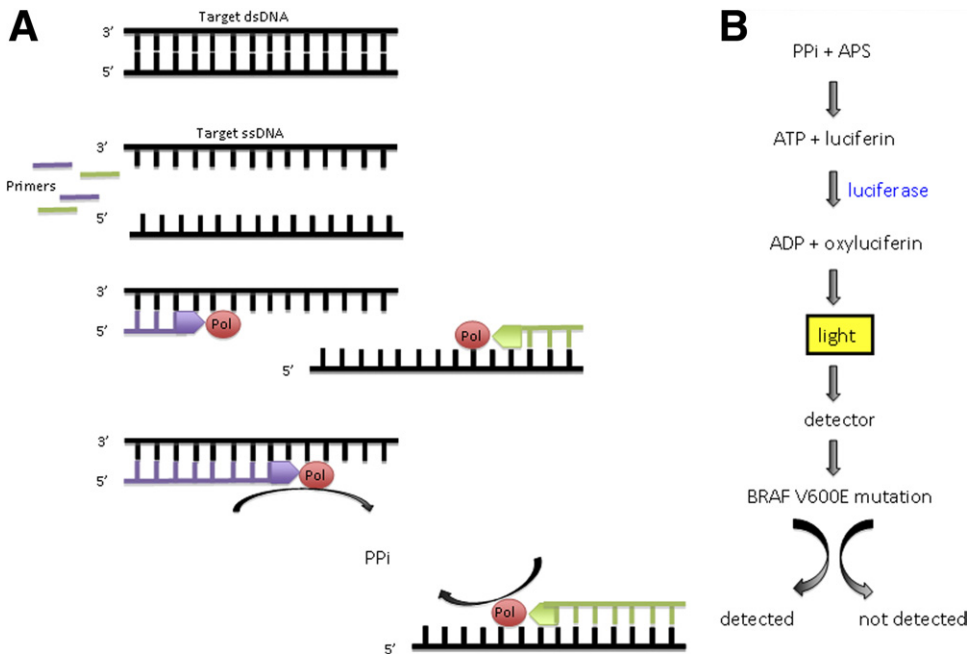

Figure 1 Pyrosequencing platform. (A) Sequencing by synthesis. Addition of nucleotides to DNA template by DNA polymerase releases pyrophosphate. (B) Chemiluminescence reaction where liberation of visible light is analyzed by detector as presence or absence of *BRAF* mutation.

amplicon in direct proximity to the mutational hot spot. Through a series of biochemical reactions, PPi combines with adenosine 5′ phosphate to give adenosine triphosphate, which is hydrolyzed by luciferase and converts luciferin to oxyluciferin, releasing a visible light (chemiluminescence reaction) (Fig. 1B).[25,26] DNA nucleotides are repeatedly dispensed in a predetermined order, and if complementary to the DNA template, the nucleotide will be added with the liberation of PPi. If the base is not incorporated in the DNA template, the nucleotide will be degraded. The instrument detects the liberated light when a base is incorporated, and the signal is plotted out on a pyrogram as a peak. The sequence can be reconstructed when analyzed by the technologist, and the presence or absence of the mutation can be determined.

The clinical application for pyrosequencing is to detect the presence or absence of known mutations within a specific segment of DNA of a single nucleotide polymorphism. Because mutations in melanoma appear to cluster in the *BRAF*, *NRAS*, and *KIT* genes, this molecular platform has been readily incorporated into the mutational analysis of melanoma. Pyrosequencing is a rapid and sensitive test for detection of the more common *BRAF V600E* mutations in addition to other variants like *V600D, V600R, V600K, and K601E*.[24] Pyrosequencing can provide the percentage of DNA that harbors *BRAF V600E* mutations, and in contrast to the Sanger sequencing methods, the detection ratio of mutant *BRAF V600E* to WT is 1:50.[23] However, pyrosequencing is limited to the length of the DNA template sequenced and is prone to errors reading through homopolymer (eg, TTTTTTTT) sequences.

Allele-specific Real-time PCR

Allele-specific (AS) real-time (RT) PCR is a molecular platform, which enriches known mutations in clinical samples to increase the sensitivity of detection. This is particularly useful in FFPE biopsies with low tumor content.[27] Primers and probes are designed to amplify and detect specific mutations (eg, *BRAF* V600E).[28] The premise of AS RT-PCR, also known as the amplification-refractory mutation system, is that nucleotide extension will not occur if the 3′ end of the primer is not complementary to the template DNA.[29] When combined with RT-PCR, the amplified mutant gene can be quantitatively measured. AS RT-PCR is a highly sensitive assay and may detect $< 1.0\%$ *BRAF* V600E admixed with WT genomic DNA.[27] AS RT-PCR is confined to known *BRAF* mutations that occur in melanoma, but demonstrates greater sensitivity in detecting *BRAF* V600E mutations in FFPE clinical samples. The AS RT-PCR platform detected *BRAF* mutations (including V600E and V600K *BRAF* alterations) in 97.2% of the melanoma tissue samples (70/72) that harbored *BRAF* mutations. Two of the samples not detected by AS RT-PCR that were detected by Sanger sequencing were *BRAF N581S* and *K601E* mutations. However, the AS RT-PCR system detected an additional 18 FFPE samples of melanoma, which failed Sanger sequencing owing to the low DNA content.[28]

Mass Spectrometry-based Sequencing (Sequenom)

The Sequenom platform uses mass spectrometry to determine the sequence of the FFPE tissue samples of melanoma. Mutational hot spots are amplified by PCR, and the amplicons are used in the iPLEX gold reaction. During this subse-

quent step, a primer is added in immediate proximity to the mutational hot spot. A base addition using mass-modified A, T, C, and G bases takes place, and the complementary base is incorporated. The mass modification of the base affects the time of flight of the resulting complex, known as the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). The MALDI-TOF is measured by the mass spectrometer, and the AS PCR products are distinguished. MALDI-TOF is a highly sensitive sequencing platform and is only limited by the design of the AS primers and the analysis of predetermined hot-spot mutations.[30] MALDI-TOF allows for the simultaneous amplification of the multiple genetic hot spots, which makes analyzing several known mutations in a single clinical sample possible. In our experience, the Sequenom platform has a slightly higher sensitivity than pyrosequencing, with dilution studies of 1:10 and 1:8, respectively.

High-Resolution Melting

High-resolution melting (HRM) relies on the PCR amplification of the DNA template and the analysis of the temperature gradient in which the double strands of the PCR products are separated or "melted." The specific temperature at which the PCR strands melt is dependent on the sequence of the constituent bases. If a sample contains WT and mutant *BRAF*, there will be differential melting curve patterns of the resultant RT-PCR products; thus, the mutant allele in the FFPE tissue sample is detected. HRM is a highly sensitive method to screen for mutations in clinical samples; however, an important limitation of this approach is the fact that the specific nucleotide alteration is not reported. Therefore, tissue samples that are positive for mutations will require additional sequencing to determine the specific nucleotide alteration by another sequencing method.[31]

The 454 Pyrosequencing (Roche)

The 454 pyrosequencing is one of the NGS technologies that allow ultradeep sequencing of entire exons. Other NGS platforms include Helicos (Helicos Bioscience Corporation, Cambridge, MA), Illumina (Illumina Inc., San Diego, CA), and SOLiD (Applied Biosystems, Carlsbad, CA) (sequential dinucleotide ligation).[17,32] We will review the NGS 454-pyrosequencing platform because this method was used to resolve the mutation discrepancies between the cobas 4800 *BRAF* V600 test and the Sanger sequencing method in clinical samples from phase II and phase III vemurafenib trials.[6,33]

The microfluidic flow chamber (micrometers in diameter) is a central component to all the NGS. The microfluidic system consists of networks of channels that allow picoliter volumes of reagents to react in a particular region of a channel.[34] The miniaturization of the NGS platform accelerates the processing and analysis of tissue samples. The 454-pyrosequencing platform uses parallel PCR amplification reactors in emulsions composed of primer, DNA template, and DNA polymerase.[32] The emulsions are then disrupted, and the DNA is sequenced. The mean error rate of the 454-pyrosequencing platform is 1.07%, with greater than half of the errors at sites of homopolymers.[35] The 454-pyrosequencing platform has the lengths of individual reads of DNA sequences of $>$ 500 base pairs and the ability to perform the test in $<$ 1 day. The 454-pyrosequencing platform is a powerful technology to sequence entire target exons.

Cobas 4800 *BRAF* V600 Mutation Test in Melanoma

The cobas 4800 *BRAF* V600 Mutation Test is based on the principles of AS RT-PCR. This platform targets a predefined 116-base pair sequence of the *BRAF* gene on exon 15. DNA-specific TaqMan probes with different fluorescent dyes are directed at WT *BRAF* 600 (GTG sequence) and mutant *BRAF* V600E (GAG sequence). Following DNA template PCR amplification and mutant enrichment (if *BRAF* V600E mutation is present in the melanoma sample), the characteristic fluorescence is measured and the detection of *BRAF* V600E mutation reported (Fig. 2). To further increase the sensitivity of the assay, amplified target PCR products are selected by introduction of AmpErase (Life Technologies, Carlsbad, CA) (uracil-*N*-glycosylase) enzyme and deoxyuridine triphosphate. Amplified products that contain deoxyuridine triphosphate will be destroyed, leaving abundant target PCR products for analysis.[36]

The lower limit of detection of *BRAF* V600E mutation on FFPE tissue with the cobas platform is 4.4% mutant alleles per 1.25 ng/μL of DNA. Testing of known *BRAF* V600E, *BRAF* WT, and *BRAF* non-V600E mutant FFPE melanoma tissue samples, determined by 454 pyrosequencing and/or Sanger sequencing, showed all *BRAF* V600E mutant tissue samples were detected by the cobas platform when the tumor content was $\geq$ 10%, with 2.3% mutant alleles per sample. Cross-reactivity with *BRAF* non-V600E mutant-melanoma samples interpreted by the cobas platform as *BRAF* V600E mutation detected was seen in 17.6% (3/17) of the samples. Two samples harbored *BRAF* V600K mutations, and one sample had a *BRAF* V600D mutant allele. The *BRAF* V600E mutation was not detected in 82.3% (14/17) of melanoma samples (tumor content range: 5%-45% and 14% median mutant alleles) with known *BRAF* non-V600E mutations (13 = V600K, 1 = V600R). The lower limit of detection before there is a loss of cross-reactivity with the cobas *BRAF* V600 system appears to be when the *BRAF* V600K mutant alleles are $<$ 31% and the V600E2 mutant alleles are $<$ 68%. There is no reported cross-reactivity with the cobas platform for the V600R mutant.[36]

The analytic performance of the cobas 4800 *BRAF* V600 test was compared with Sanger sequencing using clinical samples from phase II and phase III vemurafenib trials.[6,33,37,38] All 477 eligible patient specimens of melanoma had a valid cobas *BRAF* test result in which 0.8% (4/477) tumor samples initially had a failed cobas test result, which was validated on retesting. In contrast, Sanger sequencing had a test failure rate of 9.2% (44/477) in melanoma samples tested for *BRAF* mutation, despite being retested. Of the samples with failed test results by Sanger sequencing, 44 were subjected to the NGS 454-pyrosequencing platform. The 454 pyrosequencing identified the *BRAF* V600E mutation in ap-

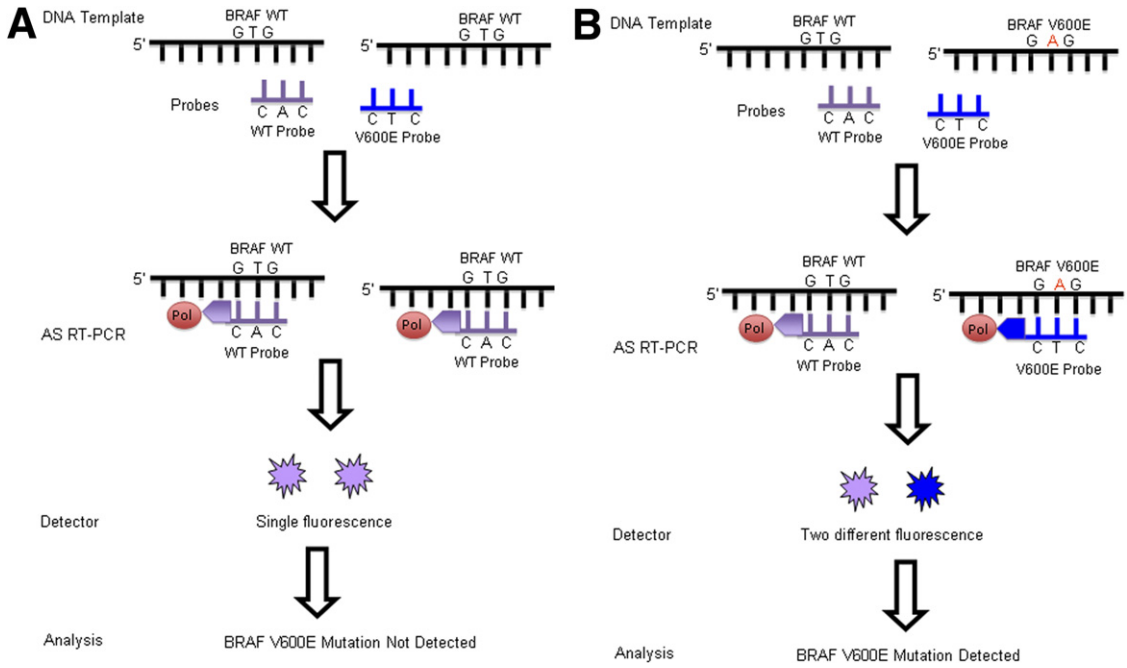

Figure 2 Simplified method of the cobas *BRAF* V600E platform. (A) Analysis of tumor DNA without *BRAF* V600E mutation. *BRAF* wild-type (WT) probes (CAC) bind to template DNA, and after allele-specific real-time polymerase chain reaction, a single fluorescence signal is emitted and reported as mutation not detected. (B) Analysis of tumor DNA with WT and *BRAF* V600E mutation. *BRAF* WT and *BRAF* V600E probes bind to their target template DNA, and after allele-specific real-time polymerase chain reaction, 2 different fluorescence signals are emitted and reported as mutation detected.

proximately 64.0% (28/44 samples) of melanoma samples with a failed test result by Sanger sequencing method. Of these cases, 18% (8/44) contained less common types of *BRAF* mutations (4 = V600K, 3 = V600E, 1 = V600M), whereas the remaining 8 cases (8/44) demonstrated absence of codon 600 *BRAF* mutations.[38]

The 433 evaluable test results from both the cobas *BRAF* and the Sanger sequencing methods demonstrated a positive agreement of 96.4% and a negative agreement of 80.0%, when the mutation detected was defined as the presence of mutant type *BRAF* V600E. There was discordance in 42 melanoma samples where the *BRAF* V600E mutation was detected by the cobas platform but not detected by the Sanger sequencing method. Sanger sequencing method did detect 64.2% (27/42) of the other types of *BRAF* mutation (26 = V600K and 1 = V600D) and 15 of 42 WT *BRAF*. However, when these "wild type" samples were subjected to 454 pyrosequencing, *BRAF* V600E mutation was detected in all 15 samples originally designated as WT by Sanger sequencing.

In the 8 discordant samples where *BRAF* V600E mutation was not detected by cobas testing and was detected by Sanger sequencing, 454 pyrosequencing identified 2 samples with WT *BRAF* and 6 samples with *BRAF* mutation (2 = V600K, 1 = V600E2, 3 = V600E). In the 3 samples where *BRAF* V600E was detected by 454 pyrosequencing, 2 of 3 specimens had a percentage of mutant alleles at or below the 5% limit of detection for cobas test.[38]

The sensitivity of detecting *BRAF* V600E by cobas 4800 on FFPE samples of melanoma is reported to be > 99%, with a specificity of 88%. The device is intended to identify patients specifically with *BRAF* V600E who may benefit from therapy with the selective *BRAF* inhibitor vemurafenib.[37-39] The advantages of the cobas 4800 V600 mutation test is its high sensitivity in detecting *BRAF* V600E mutations with a relatively small amount of input DNA that can typically be derived from one 5-μm FFPE tissue section with tumor content of at least 10% or greater, a rapid turnaround time of 8 hours, and the absence of interference from bacteria, hemoglobin, and triglycerides (Table 1). More than 89% of melanin pigment, a known inhibitor of PCR reactions, was removed with the DNA isolation procedure in the test; however, 24% of the samples had invalid tests results after 1:2 dilutions.

Because recurrent mutations in melanomas appear to be clustered at particular genomic hot spots, the cobas 4800 *BRAF* V600 mutation test is designed to detect predetermined hot-spot *BRAF* V600E mutations; however, there was some cross reactivity with non-*BRAF* V600E mutations, which is reported as "mutation detected." Those patients with non-*BRAF* V600E mutations, reported as "mutation detected," would be candidates for vemurafenib therapy. The clinical significance of this designation as it relates to response to vemurafenib therapy remains to be seen; however, it appears vemurafenib does exert inhibitory activity on non-*BRAF* V600E mutants. Preclinical studies have shown *BRAF* V600K and V600D mutant melanoma cell lines were sensitive to vemurafenib, and some patients with *BRAF* V600K had some clinical response.[6,40,41] The cobas 4800 *BRAF* V600 platform is not designed to screen for nonrecurrent genetic

Table 1 The Advantages and Disadvantages of the Cobas *BRAF* V600 Platform

Cobas *BRAF* V600 Platform	
Advantages	**Disadvantages**
High sensitivity (> 99%)	Cross-reactivity with *BRAF*-mutant variants
Rapid test turnaround time (< 1 day)	Mutation sequence not provided
Ideal for short DNA fragments isolated from formalin-fixed paraffin-embedded tissue	Not suitable for identification of new unknown mutations
Designed for recurring known hot-spot mutations	Not designed to detect *BRAF* mutant variants (eg, N581S and K601E)

mutations in melanoma; however, the *BRAF* V600E mutation appears to account for the highest percentage of mutations in cutaneous melanoma. The use of NGS platforms will bring to the clinics the ability to analyze mutations in the entire genome or target exon of cancer cells.[17]

Clinical Significance of *BRAF* Mutations

The detection of *BRAF* mutant melanoma has emerged as a central factor in the stratification of patients to deploy various targeted therapies in advanced-stage disease. In some clinical situations where patients present with MM or stage III disease without a known primary, the detection of a *BRAF* mutation may facilitate the distinction of MM and clear-cell sarcoma. The evaluation of 16 cases of clear-cell sarcoma with known Ewing sarcoma breakpoint region 1/activating transcription factor 1 fusion transcripts was all negative for *BRAF* mutations in exons 11 and 15 by HRM analysis and subsequent direct sequencing of purified amplification products.[42]

Immunohistochemical detection of *BRAF* V600E mutant melanomas is another technique that may augment the diagnostic accuracy of *BRAF* mutation analysis. *BRAF* V600E protein expressions with VE1 monoclonal antibody (Ventana, Tucson, AZ) in tumor samples with known *BRAF* V600E mutations were congruent in 97.1% (68/70) cases.[43]

Detection of the *BRAF* mutation in circulating free DNA (cfDNA) extracted from plasma and/or serum does not infer a worse prognosis in advanced-stage disease; however, a greater proportion of patients with lactate dehydrogenase LDH levels < 2 times the upper limit of normal demonstrated *BRAF* mutations in cfDNA. The concordance rate of *BRAF* mutant tumors with cfDNA was seen in 56% of the cases, and cfDNA may be an alternative source to evaluate for *BRAF* mutation status.[28]

There appears to be no association between the presence of *BRAF* V600E mutant melanoma and the site of metastasis, lactate dehydrogenase status, and Eastern Cooperative Oncology Group performance status. However, the presence of *BRAF* V600E mutation in melanoma is not associated with a favorable prognosis, which may be seen in other tumors (eg, colorectal cancer).[44] Patients with MM had a mean survival of 8.5 months with *BRAF* WT and 5.7 months in *BRAF*-mutant patients not treated with *BRAF*i.[45] The presence of mutant *BRAF* appears to impact overall survival after the diagnosis of distant metastasis or stage IV disease.[45,46] Genetic and epigenetic changes associated with the *BRAF* mutation in melanoma remain to be determined to explain for the more aggressive tumor biology and poor survival in this subset of patients with MM.

Conclusions

Molecular testing of cutaneous melanoma for targeted therapy and clinical trials have become routine practice for patient care. The Food and Drug Administration-approved cobas *BRAF* V600 Mutation Test demonstrated a high rate of sensitivity in detecting *BRAF* V600E mutations from FFPE melanoma samples. The sensitivity appears comparable with other molecular platforms like Sequenom and HRM curve analysis. There is some minor cross-reactivity to variants of *BRAF*-mutant melanomas that may be interpreted as positive *BRAF* V600E status if subsequent sequencing is not performed. The NGS platforms will offer a high degree of sensitivity and provide the mutation sequence in clinical samples tested.

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