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BRAF V600E Mutated Gene Variant as a Circulating Molecular Marker in Metastatic Melanoma Patients

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1. Introduction

Cutaneous metastatic melanoma management has recently approached the age of individualized therapy (Romano et al., 2011). The discovery that the 1799T>A point mutation in the BRAF oncogene (BRAFV600E) occurs in ~50% of melanoma lesions and that melanoma cells bearing the mutation are oncogene addicted, i.e., strictly dependent upon BRAFV600E activity for growth and survival, have pointed to BRAFV600E as a promising target for therapy. Drugs targeting BRAF have been developed, and several clinical trials are currently ongoing. Phase I-II results recently reported remarkable tumor regression in the great majority of patients bearing disseminated BRAFV600E mutated melanoma disease after treatment with BRAFV600E-specific inhibitors.

In these trials, BRAF mutational status is determined to select patients who may benefit from therapy. However, melanoma specimens are not always available to perform this analysis; moreover, a negative result in a single tumor biopsy may cover the presence of mutation-positive tumor lesions. Because blood has been proven to represent a valuable alternative source of tumor-derived cells as well as of tumor-derived DNA, several technical approaches have been studied to detect BRAFV600E in RNA/DNA extracted from blood-derived circulating tumor cells and in circulating free DNA isolated from plasma or serum. For these reasons, circulating BRAFV600E has the potential as both a specific melanoma molecular marker and a monitoring factor to be used to evaluate clinical response.

In this chapter, we summarize the clinical and biological features of BRAF mutation in melanoma. Furthermore, we report a new BRAFV600E detection assay developed in our lab that shows high sensitivity and specificity.

2. BRAFV600E mutation in melanoma

Among the genetic lesions that frequently occur in melanoma, BRAF gene mutation is the most common and is detected in about 50% of melanoma (Davies et al., 2002). The BRAF gene encodes a serine-threonine kinase belonging to the MAPK kinase pathway, also known as the RAS/RAF/MEK/ERK pathway. This signaling pathway regulates important cellular processes, including cell growth, proliferation and migration; in physiological conditions, the signaling is triggered by activated growth factor receptors, which act as binding sites for
adapter proteins that subsequently activate a cascade of kinases, including NRAS, BRAF, MEK and ERK, via phosphorylation. Activated ERK translocates to the cell nucleus, where it phosphorylates and activates many different substrates (Held et al., 2010; Poulilakos & Rosen, 2011; Young et al., 2009).

BRAF mutations identified in melanoma are in the kinase domain, which is encoded by exons 11 and 15, and are somatic. Somatic BRAF point mutations have been also detected in approximately 8% of other tumor types, including 30-70% of papillary thyroid cancers and 10% of colorectal cancers, and ovarian, breast and lung cancers (Cantwell-Dorris et al., 2011; Davies et al., 2002; Puzanov et al., 2011). No BRAF germline mutations have been found in familial or sporadic melanoma patients (Lang et al., 2003; Laud et al., 2003; Meyer et al., 2003a), although germline mutations have been shown to occur in Noonan, LEOPARD and cardio-facio-cutaneous syndromes, which are developmental disorders with overlapping features including distinctive facial dysmoria, reduced growth, cardiac defects, skeletal and ectodermal anomalies and variable cognitive deficits (Sarkozy et al., 2009; Tidyman & Raouen, 2009). The relationship between BRAF germline polymorphisms and melanoma risk has also been investigated, and data obtained suggest that BRAF can be considered a low-risk susceptibility gene for melanoma (James et al., 2005; Meyer et al., 2003b).

It has been reported that melanocortin 1 receptor (MC1R) variants increase the risk of melanoma with BRAF mutations (Fargnoli et al., 2008; Landi et al., 2006; Scherer et al., 2010). The MC1R gene, which has been identified as a low-risk melanoma susceptibility gene (Williams et al., 2010), encodes a seven-pass transmembrane G-protein receptor that binds alpha-melanocyte stimulating hormone and plays a key role in the pigmentation process (Palmer et al., 2000; Rees, 2004; Valverde et al., 1995). The MC1R gene is highly polymorphic, and gene variants determine a partial or complete loss in the ability of the receptor to transduce signals, thus impairing the switch from pheomelanin to eumelanin production in response to UV radiation exposure (Healy et al., 2000). Further studies are needed to elucidate the mechanisms causing MC1R variants to select for BRAF somatic mutations (Hacker & Hayward, 2008).

BRAF mutations result in the constitutive activation of ERK, resulting in proliferation and growth advantage of melanoma cells. In 15-30% of melanoma, the RAS/RAF/MEK/ERK pathway is constitutively activated through NRAS mutation (Sekulic et al., 2008). As BRAF and NRAS mutations are mutually exclusive, hyperactivation of the MAPK pathway is very frequent in melanoma. Although the constitutive activation of the MAPK pathway is often required to promote the growth and proliferation of neoplastic cells, BRAF mutations are prevalent in melanoma, while mutations in tyrosine kinase receptors or in RAS genes are prevalent in other tumor types. Chromosomal rearrangements leading to the formation of BRAF fusion products, characterized by the lack of the BRAF auto-inhibitory domain and the aberrant activation of the MAPK pathway, have also been reported in pilocytic astrocytoma, thyroid, prostate and gastric cancer as well as melanoma (Ciampi et al., 2005; Cin et al., 2011; Dessars et al., 2007; Palanisamy et al., 2010).

The substitution of a valine (V) for glutamic acid (E) at position 600 (V600E) accounts for >90% of BRAF mutations identified in melanoma. BRAF mutations in melanoma are an early event as they can be detected in nevi and in primary melanoma (Figure 1) (Rodolfo et al., 2004; Thomas et al., 2006). Even if 60-70% of benign and dysplastic nevi show the BRAFV600E mutation, most of them do not progress to melanoma, suggesting that further alterations are necessary to promote malignant progression. In particular, it has been proposed that BRAF mutations may induce senescence and that abrogation of the...
mechanisms regulating this cellular process are required to induce tumor progression (Michaloglou et al., 2005). In primary melanoma, BRAFV600E mutation is rarely detected in lentigo maligna lesions which arise in chronically sun-exposed skin and show a high rate of RAS mutations, and in acral and mucosal melanoma that arise in non-exposed skin, which may show KIT gene mutation (Platz et al., 2008). Melanoma occurring in childhood and adolescence, as well as those occurring in patients with a family history of melanoma, show BRAFV600E mutation (Daniotti et al., 2009). Melanoma that arise in intermittently exposed body sites, in skin lacking signs of chronic sun-induced damage, and in younger people, show a >80% rate of BRAFV600E mutation (Curtin et al., 2005). BRAF mutation frequency appears higher in advanced lesions than primary tumors, and it does not seem to be related to the site of metastases. Moreover, different studies have reported that BRAF mutation is maintained during progression from primary tumor to metastatic lesions or is acquired during the development of metastases (Houben et al., 2004; Omholt et al., 2003; Shinozaki et al., 2004). These results suggested a possible direct link between mutated BRAF and the metastatic potential of melanoma cells.

Several authors have studied the effects of BRAFV600E on global gene expression profiles of melanoma cells by microarray analysis and reported a BRAF mutation-associated gene expression signature (Pavey et al., 2004; Bloethner et al., 2005; Johansson et al., 2007). In particular, genes that encoded proteins involved in RAS/RAF/MEK/ERK signaling were
identified among the genes differentially expressed between melanoma cell lines with or without BRAF mutation (Bloethner et al., 2005). In addition, a classifier able to discriminate between BRAF mutant and BRAF wild-type melanoma with high accuracy was built, including genes encoding phosphates and other genes biologically related to melanoma progression (Pavey et al., 2004). On the contrary, Hoek et al. failed to find a BRAF signature but identified three sample cohorts that represented melanoma groups characterized by different metastatic potential (Hoek et al., 2004). This discrepancy could be explained by considering the methods used to perform the analysis of array data; in fact, when the data by Hoek were re-analyzed with another statistical approach, a BRAF signature could be identified in these data (Johansson et al., 2007). Taken together, these results support the presence of a gene expression profile associated with BRAF mutation in melanoma and point to the genes that are potentially novel therapeutic targets.

3. BRAFV600E as a therapeutic target

The frequency and specificity of BRAFV600E mutation, together with the strict dependence of melanoma cell growth and survival on BRAFV600E activity (a phenomenon called oncogene addiction), have pointed to BRAFV600E as a promising therapeutic target. Several BRAF inhibitors have been produced in the last years that have been or are currently being clinically tested (Sheperd et al., 2010). The first compound tested in clinical trials was Sorafenib (BAY43-9006), a multi-kinase inhibitor that targeted both wild-type and mutated BRAF, CRAF and other protein kinases, such as VEGFR2 and -3, PDGF, p38 MAPK, cKIT, FMS and RET (Wellbrock & Hurlstone, 2010). Sorafenib showed poor clinical activity when tested as a single agent, and in phase III trials in both front- and second-line therapies in combination with carboplatin and paclitaxol (Eisen et al., 2006; Hauschild et al., 2009). Other multi-kinase inhibitors that show a higher selectivity for BRAF than Sorafenib are currently under investigation in clinical trials (Dienstmann & Tabernero, 2011).

Several compounds that selectively inhibit BRAF have also been developed. Among them, GSK2118436 (SB-590885) has been tested in a phase I-II clinical trial and shows clinical responses in 60% of melanoma patients with BRAFV600 tumors, including patients having BRAFV600K and BRAFV600G mutations, with good tolerability (Keuffeld et al., 2010). Moreover, treatment with GSK2118436 induced a 20-100% reduction in the size of central nervous system lesions in patients with previously untreated brain metastases (Long et al., 2010).

Recently, the results of a phase I-II study that tested a specific BRAFV600E inhibitor, PLX4032 (RO5185426), were reported. Treatment with PLX4032 induced a complete or partial tumor regression in 81% of patients who had melanoma with BRAFV600E mutation, including progression-free survival for more than 7 months and manageable side effects, while patients with BRAF wild-type tumors showed no evidence of tumor regression (Flaherty et al., 2010). As a side effect, 31% of patients treated with PLX4032 developed low-grade squamous cell carcinomas, which were reported to occur also in patients treated with Sorafenib (Arnault et al., 2009). This side effect is possibly due to the selective mechanism of action of PLX4032 that shuts down only the activity of BRAFV600E while inducing the formation of BRAF-RAF1 heterodimers and RAF1-RAF1 homodimers, thus inducing hyper-activation of the MAPK pathway in both tumor cells and normal skin cells with wild-type BRAF (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010).
4. Mechanisms of resistance to BRAF inhibitors

Even if BRAFV600E tumors initially respond to PLX4032 treatment, the majority of patients relapsed within 2-18 months and developed resistance to the treatment. Furthermore, a subset of BRAFV600E tumors showed primary resistance as about 20% of patients did not respond to PLX4032 treatment (Flaherty et al., 2010). These findings indicate that the development of new therapeutic strategies using PLX4032 in combination with other targeted agents could be useful to prevent the acquisition of resistance. Several studies investigating the molecular mechanisms that promote resistance to RAF inhibitors have been recently reported. The restoration of MEK activity in BRAFV600E melanoma appears to be a crucial event in promoting the acquisition of resistance (Poulikakos & Rosen, 2011; Solit & Rosen, 2011; Solit & Sawyers, 2010; Tuma, 2011). In particular, MEK activity was restored by overexpressing other kinases such as RAF1 and COT/TPL2 (Johannessen et al., 2010) or by the de novo acquisition of a mutation in the NRAS gene (Nazarian et al., 2010). The COT gene was amplified in cell lines that showed intrinsic resistance to PLX4032 (Johannessen et al., 2010). Surprisingly, no secondary BRAF mutations were detected in tumors from patients with acquired resistance (Nazarian et al., 2010). Wagle et al. identified an activating mutation at codon 121 of MEK1 in the tumor from a patient who relapsed after developing resistance to PLX4032 treatment, thus demonstrating for the first time that resistance to PLX4032 is associated with the development of activating mutations in kinases downstream of BRAFV600E (Wagle et al., 2011). This discovery highlights the importance of establishing new combined therapies using MEK or ERK inhibitors with PLX4032 to overcome resistance. In fact, data obtained in preclinical studies demonstrated a synergism between BRAF and MEK inhibitors AZD6244 and GSK1120212 (Emery et al., 2009; Joseph et al., 2010; Paraiso et al., 2010). However, mechanisms that promote the acquisition of resistance independently of MEK activation have been described, including the increased activation of the receptor tyrosine kinases PDGFRβ (Nazarian et al., 2010) or IGF1R (Villaneuva et al., 2010), suggesting that the combination of receptor tyrosine kinase inhibitors with PLX4032 could be effective in the treatment of these patients. However in most patients, the mechanisms that promote the acquisition of resistance remain unclear (Nazarian et al., 2010).

5. BRAFV600E as a circulating disease biomarker

As blood has been proved to represent a valuable alternative source of tumor-derived cells and tumor-derived DNA/RNA, circulating BRAFV600E represents a potential circulating disease biomarker that could be useful when melanoma specimens are not available to test the BRAF mutational status for the selection of patients who will benefit from treatment with BRAF inhibitors. In addition, it could be used as a monitoring factor to evaluate clinical response.

Several studies reported that BRAFV600E is detectable in DNA/RNA extracted from circulating melanoma cells (CMC) (Kitago et al., 2009; Oldenburg et al., 2008). The assessment of CMC for monitoring the efficacy of therapeutic treatment and for predicting the disease outcome of melanoma patients has been proposed. Currently, RT-PCR and quantitative real-time RT-PCR are the methods most frequently used to detect CMC in melanoma. Both techniques are used to amplify genes expressed in melanoma cells, such as tyrosinase, MART-1, MAGE-3A and MITF (Koyanagi et al., 2010). Detection of the mutated
BRAF variant in blood samples requires the efficient isolation of CMC (Kitago et al., 2009) or the development of extremely sensitive techniques to detect the mutant sequence in a large excess of wild-type BRAF forms (Oldenburg et al., 2008).

Circulating free DNA (cfDNA) isolated from plasma or serum samples represents an alternative source of melanoma-derived DNA. Several studies reported the feasibility of detecting BRAFV600E mutation in the cfDNA from patients with melanoma (Board et al., 2009; Daniotti et al., 2007; De Giorgi et al., 2010; Pinzani et al., 2010; Yancovitz et al., 2007). Interestingly, Shinozaki and coworkers reported that the detection of circulating BRAV600E in the serum of patients treated with biochemotherapy correlates with poorer outcomes due to absence of response to the treatment (Shinozaky et al., 2007).

Some important limitations should be overcome to consider BRAFV600E as a reliable circulating disease biomarker. In fact, the studies previously mentioned demonstrated that BRAFV600E is detectable at stage IV and only in a few stage III melanoma patients, suggesting that it does not represent a marker for the detection of the disease in early-stage patients. Moreover, when matched plasma/serum and tumor samples from melanoma patients were tested for BRAFV600E, the concordance between the BRAF mutation rates of cfDNA and tumors showed some discrepancies, which could be due to a low sensitivity of the techniques used to perform the mutational analysis or to the heterogeneity of the tumor for the BRAFV600E mutation. For these reasons, different methods were developed to detect BRAFV600E mutation in high levels of BRAF wild-type DNA by increasing the specificity and sensitivity of the assays, as shown in Table 1, mainly through enriching the sample for the mutant variant or by selectively inhibiting the amplification of the BRAF wild-type form (Kitago et al., 2009; Oldenburg et al., 2008; Pinzani et al., 2011; Shinozaki et al., 2007; Yancovitz et al., 2007).

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit</th>
<th>Samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele-Specific PCR</td>
<td>1:400 mut allele in wt alleles</td>
<td>Plasma</td>
<td>Daniotti et al., 2007</td>
</tr>
<tr>
<td>Mutant-specific PCR</td>
<td>0.1 ng of mut DNA in 100 ng of wt DNA</td>
<td>Plasma</td>
<td>Yancovitz et al., 2007</td>
</tr>
<tr>
<td>PNA/LNA clamp Real Time PCR</td>
<td>1X10⁴ U mut DNA in 10 U of wt DNA</td>
<td>Serum</td>
<td>Shinozaki et al., 2007</td>
</tr>
<tr>
<td>PBAS-PCR</td>
<td>10 melanoma cells in 1ml of blood</td>
<td>CMC</td>
<td>Oldenburg et al., 2008</td>
</tr>
<tr>
<td>Real Time PCR</td>
<td>1-5 melanoma cells in 5X10⁶ PBC</td>
<td>CMC</td>
<td>Kitago et al., 2009</td>
</tr>
<tr>
<td>ARMS allele-specific Real Time PCR</td>
<td>5 copies of mut DNA in 5000 copies of wt DNA</td>
<td>Serum</td>
<td>Board et al., 2009</td>
</tr>
<tr>
<td>LNA/allele-specific Real Time PCR</td>
<td>0.3% of mut alleles in wt alleles</td>
<td>Plasma</td>
<td>Pinzani et al., 2010</td>
</tr>
<tr>
<td>COLD PCR</td>
<td>3.1% of mut alleles in wt DNA</td>
<td>FFPE tissue</td>
<td>Pinzani et al., 2011</td>
</tr>
</tbody>
</table>

PNA: Peptide Nucleic Acid; LNA: Locked Nucleic Acid; PBAS: Primer-Blocking Allele-Specific; ARMS: Amplification Refractory Mutation System; COLD: CO-amplification at Lower Denaturation temperature; mut: mutated; wt: wild-type; 1U: amount of target DNA contained in 1 µg/ml of genomic DNA; PBC: Peripheral Blood Cells; FFPE: Formalin Fixed Paraffin Embedded

Table 1. Methods developed to detect BRAFV600E.

5.1 Other melanoma circulating biomarkers
Melanoma serum markers that have significant potential both as prognostic indicators and for monitoring the treatment response include lactate dehydrogenases (LDH), S100 calcium binding protein B (S100B), and melanoma inhibitory activity (MIA) molecule.
LDH are cytochrome-c- or NAD(P)-dependent enzymes that act on either D- or L-lactate. Serum LDH is the only circulating biomarker shown to have a prognostic relevance in melanoma. Several studies have shown that high levels of circulating LDH correlate with a poor prognosis in stage IV melanoma patients and in other neoplastic diseases (Balch et al., 2009; Bedikian et al., 2008; Keilholz et al., 2002). For this reason, LDH was included in the current AJCC staging system, and its level is currently determined in melanoma patients having distant metastasis because patients with elevated LDH are assigned directly to the M1C category without considering the site of distant metastasis (Dickson & Gershenwald, 2011).

S100B is a protein that belongs to the S100 protein family and is mainly expressed by astrocytes, where it acts as a neurotrophic factor to promote neuronal survival. S100B is a well-characterized melanoma marker, and it is used as a diagnostic marker of melanocytic skin lesions in immunohistochemical staining. Several studies pointed out S100B as a prognostic marker of disease progression (Gogas et al., 2009; Jury et al., 2000) as increased serum levels in melanoma patients were predictive of disease progression. Even if not included in the AJCC staging system, Swiss and German guidelines recommend the determination of S100B serum levels in patients with Breslow thickness >1 mm every 3-6 months (Dummer et al., 2005; Garbe et al., 2007; Garbe et al., 2008).

MIA is a small protein secreted by malignant melanoma cells that exhibits an inhibitory effect on cell growth in vitro (Blesch et al., 1994). Even if a correlation between high MIA serum levels and metastatic melanoma progression has been reported (Bosserhoff et al., 1997; Stahlecker et al., 2000), MIA was shown to have lower sensitivity and specificity as a melanoma marker than S100B and LDH (Krahn et al., 2001).

Recently, microRNAs (miRNAs) have been proposed as a new class of potential circulating biomarkers that are detectable in various body fluids. miRNAs are non-coding RNAs consisting of 18-24 nucleotides that regulate mRNA and protein levels mainly by inducing mRNA degradation or by inhibiting translation (Ambros, 2004; Bartel, 2004). Recently, deregulation of a group of miRNAs was found in melanoma lesions in association with BRAF mutational status (Caramuta et al., 2010). miRNAs are also released into the extracellular space, where they can be found free or contained within vesicles such as microvesicles, exosomes, apoptotic vesicles and senescent bodies. The functional role of extracellular miRNAs as an intercellular communication system is poorly characterized (Reid et al., 2010). Extracellular miRNAs have been identified as ideal tumor circulating biomarkers because of their stability and easy quantification (Etheridge et al., 2010). For these reasons, circulating miRNAs have been investigated in many tumor types, including lung, colorectal, ovarian and pancreatic cancers, to evaluate their prognostic and diagnostic value (Reid et al., 2010). Few studies have assessed circulating miRNAs in the context of melanoma. Kanemaru et al. demonstrated that miRNA-221 serum levels are higher in melanoma patients than in healthy controls; in addition, miRNA-221 levels directly correlate with tumor thickness, staging and disease course (Kanemaru et al., 2011). In another study, 16 miRNAs were identified deregulated in blood cells of melanoma patients by comparison of miRNA expression profiles in blood cells of healthy donors; moreover, they were sufficient to distinguish melanoma patients from healthy individuals with high accuracy (Leidinger et al., 2010). Taken together, these studies suggest that miRNAs potentially could be prognostic and diagnostic circulating markers in melanoma, although larger studies and the standardization of isolation and detection techniques are needed to confirm these results.
6. Allele-specific real-time PCR-based detection of circulating BRAFV600E

It is possible to selectively eliminate the BRAF wild-type sequence and thus improve the sensitivity of the PCR performed to detect mutated circulating BRAF by taking advantage of the presence of a TspRI enzyme restriction site located at codon 600 of the BRAF wild-type sequence (Rimoldi et al., 2003). This restriction site is abrogated by BRAFV600E, and therefore it is possible to enrich for the BRAFV600E allele by selectively eliminating wild-type sequences by performing TspRI digestion. We modified the experimental conditions described by Rimoldi et al. to improve sensitivity and specificity of an allele-specific TaqMan-based real-time method to detect BRAFV600E in colorectal cancer tissues (Benlloch et al., 2006). Figure 2 summarizes the steps of the assay developed to screen for BRAFV600E mutation plasma or melanoma tissue biopsies.

![Fig. 2. Schematic representation of TaqMan-based Real-Time PCR method developed to detect few copies of BRAFV600E in a large amount of wild-type DNA.](image)

Experimental conditions of each step are reported in paragraph 6.1.

6.1 Overview of the protocol

A 224-bp fragment that includes codon 600 of the BRAF gene is amplified from 5 ng DNA in a final reaction volume of 50 µl (Figure 2A). Amplification was performed with a pre-cycling hold at 95°C for 7 min followed by 37 cycles of PCR (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) and a final extension at 72°C for 7 min using primers for exon 15 amplification reported by Davies (Davies et al., 2002). Twenty microliters of the PCR product were mixed with 1X NEB Buffer 4 supplemented with 100 µg/ml BSA (New England Biolabs) and then subjected to restriction digestion at 65°C overnight with 15 U TspRI (New England Biolabs) in a final digestion mix volume of 50 µl to enrich samples for the BRAFV600E mutant allelic variant (Figure 2B). Two microliters of the digestion product were used to perform an allele-specific TaqMan-based real-time PCR analysis (Figure 2C). The final reaction volume of 20 µl contained 10 µl 2X of TaqMan Genotyping Master Mix (Applied Biosystems), 18 pmol of each primer (BRAF-51F and BRAF-176R) and 5 pmol of each probe (BRAFmut and BRAFwt). The primer and probe sequences were reported previously by Benlloch (Benlloch et al., 2006). Amplification and detection were performed with an ABI PRISM 7900HT (Applied Biosystems) using the standard thermal profile conditions of the Absolute Quantification protocol. Data analysis was performed using the SDS (Sequence Detection System) version 2.2.2 software. Each experiment was performed in duplicate.

6.2 Results

Specificity of the technique was tested by assaying dilutions of BRAF mutated DNA (5 ng/µl) in wild-type DNA (5 ng/µl). The mutated DNA was obtained from a heterozygous melanoma cell line showing 2 copies of BRAF gene. Therefore, the allelic ratio was calculated considering one mutated allele out of 4 total alleles. Results obtained show that 1
copy of V600E allele can be detected when diluted in $8 \times 10^5$ copies of wild-type alleles. Sensitivity of the technique was assayed by testing progressive dilutions in water of the BRAF mutated DNA. Results obtained show that BRAFV600E mutation can be detected starting from $6.25 \times 10^{-5}$ ng of DNA.

This method showed an increase in both sensitivity and specificity when compared to the assays previously used in our lab to detect BRAFV600E (Daniotti et al., 2007). As shown in Figures 3 and 4, the selective elimination of the BRAF wild-type allele is a critical step required to increase both parameters. In fact, the BRAF mutated allele became detectable only when the wild-type allele was greatly reduced (Figure 3) or eliminated (Figure 4) after digestion with TspRI.

Taken together, these results indicate that the new assay has an improved sensitivity and specificity for detecting BRAFV600E when tested on genomic DNA from melanoma when diluted in an excess of wild-type DNA or when present in a few copies as in water dilutions. Preliminary results obtained by testing matched plasma and tissues samples indicate that more samples test positive for BRAFV600E compared to the previously described technique (Daniotti et al., 2007) and suggest a potential clinical application of this technique.

Fig. 3. TspRI digestion increases the specificity of BRAFV600E allele-specific real-time PCR.
TspRI digestion reduces the excess of the wild-type allele that is detected 9 cycles later compared to the undigested sample and allows the detection of the BRAFV600E allele. The red line represents the threshold line. wt: wild-type. mut: mutated.

![Amplification Plot](image)

**Fig. 4.** TspRI digestion increases the sensitivity of the BRAFV600E allele-specific real-time PCR. The amplification plot shows that the complete elimination of the BRAF wild-type template by TspRI digestion improves the sensitivity of mutated allele-specific PCR, anticipating its detection of about 14 cycles compared to undigested samples. The red line represents the threshold line. wt: wild-type. mut: mutated.

### 7. Conclusion

BRAFV600E currently represents the most specific circulating tumor marker available for cutaneous melanoma, although it will only detect about 50% of melanoma. Circulating BRAFV600E can be used to select patients to be treated with BRAF inhibitors when the tissue samples are not available for the analysis. In addition, detection methods for circulating BRAFV600E can be used to monitor the treatment response and evaluate disease relapse during follow-up. However, to use BRAFV600E as a blood marker, more sensitive technologies must be designed and validated to improve the sensitivity and specificity of the assays used to detect this mutation.
8. Acknowledgment

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9. References


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The book Research on Melanoma: A Glimpse into Current Directions and Future Trends, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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