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An inexpensive, specific and highly sensitive protocol to detect the $\text{Braf}^{\text{V600E}}$ mutation in melanoma tumor biopsies and blood

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Abstract

The $\text{Braf}^{\text{V600E}}$ mutation has been detected in patients with metastatic melanoma, colon, thyroid and other cancer. Recent studies suggested that tumors with this mutation are especially sensitive to Braf inhibitors-hence the need to reliably determine the Braf status of tumor specimens. The present technologies used to screen for this mutation fail to address the problems associated with infiltrating stromal and immune cells bearing wild type Braf alleles and thus may fail to detect the presence of mutant $\text{Braf}^{\text{V600E}}$ tumors. We have developed a rapid, inexpensive method that reduces the contamination of wild type Braf sequences from tumor biopsies. The protocol involves a series of PCR amplifications and restriction digestions that take advantage of unique features of both wild type and mutant Braf RNA at position 600. Using this protocol, mutant Braf can be detected in RNA from mixed populations with as few as 0.1% $\text{Braf}^{\text{V600E}}$ mutant cells.

Keywords

$\text{Braf}^{\text{V600E}}$; melanoma; TspR1

Introduction

The $\text{Braf}^{\text{V600E}}$ mutation has been detected in greater than 60% of patients with metastatic melanoma. It has also been observed in patients with colon [1,2,3], thyroid [4,5,6,7,8] and other cancers [9,10,11]. The present technology for determining $\text{Braf}^{\text{V600E}}$ status either utilizes mass spectrometry [12,13], real time PCR [14,15], allelic specific PCR [7,16,17], PCR using locked oligonucleotides to suppress wild type sequences [18,19,20] or direct sequencing of RNA or DNA [8,21]. Mass spectrometry, although quite reliable, requires access to a mass spectrometer and is more suited to the analysis of multiple samples carried out in large core facilities. Real time PCR and direct sequencing has the problem of contamination of wild type Braf from surrounding and infiltrating normal tissue, thus making it difficult to reliably detect the V600E mutation in mixed cell populations without extensive isolation of tumor tissue.

In this report a rapid, inexpensive method has been developed to determine the $\text{Braf}^{\text{V600E}}$ status in tumor biopsies and blood specimens that reduces the contamination of wild type sequences. This is accomplished by a series of PCR amplifications and restriction digestions that take advantage of unique features of wild type and V600E Braf RNA at position 600.

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Materials and Methods

Cell lines, tissue acquisition, peripheral blood isolation and oligonucleotides

The melanoma cell lines A375, A2058 and SK MEL 5, kidney cancer cell line 786-0, colon adenocarcinomas HT29 and DLD-1, prostate carcinoma DU145, and the breast carcinoma MCF7 were purchased from ATCC (Manassas, VA). The melanoma short term cultures WM1976, 1862, 3163 and 3727 were obtained from Dr Meenhard Herlyn of the University of Pennsylvania, human colon carcinoma cell line CloneA was obtained from Dr Ian Summerhayes of the Lahey Clinic (Burlington, MA) and the breast carcinoma MDA-MB231 was obtained from Dr Hava Avraham of the Beth Israel Deaconess Medical Center (Boston, MA). Tumor samples and peripheral blood lymphocytes were obtained from patients with advanced melanoma as part of an IRB approved tissue banking protocol (DFHCC 02-017). Peripheral blood lymphocytes (PBLs) were isolated by Ficoll density centrifugation [22]. Oligonucleotides were custom synthesized from Invitrogen (Carlsbad, CA).

The protocol

RNA from cell lines, paraffin embedded tissue or ficoll purified peripheral blood lymphocytes (PBLs) was isolated by the trizol method (Invitrogen) and (1 ug) reverse transcribed to cDNA by standard methods [23] using M-MLV reverse transcriptase (Invitrogen) and oligo (dt)₁₅ (Promega). The cDNA was PCR amplified using PCR master mix (Promega) and oligonucleotides (5'(CCATATCATTGAGACCAAATTTGAGATG)3' and 5'(GGCACTCTGCCATTAATCTCTTCATGG)3') that produced a product of 466 bp including the mutation site at position 600. The PCR conditions were 94° for 2 min followed by 40 cycles of 94° for 1 min, 60° for 2 min and 72° for 2 min with a final incubation of 72° for 7 min. After cleanup using a nucleospin extract column (Clontech), a portion of the PCR product was digested with TSPR1 (restriction site = NNCASTGNN, New England Biolabs, Beverly, MA) at 65° for 16 hours. Only wild type Braf and not V600E mutant Braf PCR product was digested by this enzyme. This digestion was added to reduce the amount of contaminating normal Braf from surrounding and infiltrating normal tissue in the biopsy sample. The TspR1 digestion is not complete resulting in some PCR product containing wild type sequence at position 600. A 1/100 dilution of the TSPR1 digested material was then PCR amplified a second time using nested oligonucleotides 5'(ACGCCAAGTCAATCATCCACAGAG)3' and 5'(CCGTACCTTACTGAGATCTGGAGACAGG)3' producing a product of 331 bp which was enriched in PCR products containing the position 600 mutation. The conditions of the PCR were the same as the first PCR except instead of 40 cycles, the amplification was 35 cycles for tissue and cell lines and 45 cycles for PBLs. This number was determined empirically based on the relative expression of Braf^{V600E} RNA in the various tissue subsets. After a second cleanup using a nucleospin extract column, the DNA was subjected to sequencing using the nested forward primer. A 1/1000 dilution of this PCR product was also reamplified using 5'(TCACAGTAAAAATAGGTGATTTTGGTCTAGCTCTAG)3' and 5'(GCTGTATGGATTTTATCTTGCATTC)3'. The PCR conditions were 94° for 2 min followed by 30 cycles of 94° for 1 min, 54° for 2 min and 72° for 2 min with a final incubation of 72° for 7 min. The forward primer has been designed with two mismatches at -4 and -3 from the 3' end. The resulting product (140 bp) from mutant but not wild type transcripts contains an Xba1 restriction site. Digestion with Xba1 for 2 hours at 37° yielded products of 108 and 32 bp which can be easily viewed on a 15% polyacrylamide gel.

Results and Discussion

Summary of the protocol

The protocol was designed to efficiently and quickly determine the V600E Braf status of any tumor biopsy without the need for expensive instrumentation and reagents. The wildtype Braf

has a valine at position 600 coded by a GTG codon. The V600E mutant has a glutamic acid at that position coded by a GAG codon. As outlined in Figure 1A, the protocol involves a series of PCR amplifications and restriction digestions that discriminate between wild type and mutant Braf at amino acid position 600. An initial RT-PCR is followed by digestion with TspR1 (restriction site = NNCASTGNN), which preferentially digests the wild type (TACAGTGAA) product but not the V600E mutated (TACAGAGAA) PCR product. In addition, none of the other less frequently reported V600 mutations (V600D, V600M, V600G, V600A, V600R, V600K, V600G) are substrates for TspR1. A second nested PCR using the digested material follows. This PCR product is subjected to sequencing using either of the nested oligonucleotides. The PCR product is also subjected to a third PCR (product 140 bp) using a unique nested forward oligonucleotide which creates an Xba 1 restriction site in the amplified product only with the mutant sequence. After digestion with Xba 1 the Braf^{V600E} products (108 bp and 32 bp) are separated on a 15% acrylamide/TAE gel.

Figure 1B illustrates how the Xba 1 site is created with V600E mutant but not wild type sequence. The Xba 1 site can be created with the V600D mutation but not any of the other known but less frequent v600 mutations. Therefore this protocol is specific for the V600E BRAF status only, although other V600 mutations can be deduced by sequencing after the second nested PCR. Since greater than 90% of BRAF mutation are V600E [24], this protocol should be sufficient for the majority of samples. The protocol was initially used to examine two cell lines: A375, a melanoma line with a Braf V600E mutation and 786-0, a kidney cancer cell line with a wild-type Braf. As shown in Figure 1C, the PCR product digested with Xba 1 from the A375 has the predicted 108 and 32 bp fragments while the Braf PCR product from the 786-0 shows no evidence of digested material. The sequence around the mutation site confirms the results from the Xba 1 digest. The Braf sequence from the A375 has the predicted GAG codon at position 600, while the Braf from 786-0 has the predicted GTG codon. The assay was further applied to cell lines representative of other cancers. As shown in Figure 1D, the colon lines DLD1 and CloneA, the breast lines MCF7 and MB231 and the prostate line DU145 do not show any evidence of Xba 1 digested material consistent with the known wild type Braf status in the majority of these cancers. However, the PCR product from the colon adenocarcinoma HT29 clearly has Xba 1 digested products consistent with its established Braf^{V600E} state [25].

The sensitivity of the protocol and the need for the TspR1 digestion

The TspR1 digestion was added after the initial PCR as a consequence of the excessive contamination by normal Braf of tumor biopsy samples from surrounding and infiltrating normal cells. To illustrate the need for the TspR1 digestion, 1 ug of total RNA from 786-0 and A375 cells was mixed in varying ratios in the presence and absence of TspR1. As shown in Figure 2, in the presence of TspR1 the digested PCR fragment, evidence of mutant Braf could be detected from as little as 1 part mutant to 999 parts of wild type Braf RNA. On the other hand, without the addition of TspR1, in order to detect mutant Braf nearly equal quantities of mutant and wild type were required, a restriction that may not be possible without refined microdissection of tissue biopsies. This protocol shows that Braf mutant RNA can be detected with as little as 100 pg of mutant tissue RNA.

An analysis of melanoma tumor biopsies

We next applied this method to melanoma tumor biopsies. To date greater than 70 biopsies have been examined from paraffin embedded archived and fresh frozen tissue. Figure 3 shows representative findings with the corroborative sequencing information. Variations are observed indicative of the degree of normal tissue in the biopsy sample. Of particular note pure wild type Braf tissue never shows any evidence of Xba 1 digested PCR fragments as seen with samples D and F and also show no evidence of an adenine (A) at the mutation site. On the other

hand there are varying degrees of Br af^{V600E} positivity. Samples such as C and E have strong mutant signals, sample B has equivalent mutant and wild-type signals, as evidenced by the overlapping A and T curves at the mutation site and finally sample A has a weak mutant signal as determined by both the Xba 1 digest and the sequence information (note: even though the sequencing software registers T at position 600 in samples A and B, there is clearly evidence of an A signal at this position in both samples). Importantly, in all cases where there is evidence of Xba 1 digested material the sequence always shows some evidence of an adenine at the mutation site.

Detection of the Br af^{V600E} mutation in peripheral blood lymphocytes

In order to detect circulating tumor cells from a metastatic melanoma patient with a known Br af^{V600E} mutation, the protocol was modified by increasing the number of cycles in the second PCR to 45. In order to determine the sensitivity of the assay in blood, varying numbers of several melanoma cell lines and short term cultures were mixed with 400,000 PBLs from a normal donor. Figure 4A shows that the assay can detect variations in expression levels of V600E BRAF in a dose dependent manner. For some lines as many as 1000 cells were necessary in order to detect an Xba 1 digested band whereas in other lines V600E BRAF transcripts can be detected with as few as 1 (A375) to 5 (WM1862) cells in 400,000 PBLs. As shown in the A375 and SK MEL 5 titrations, normal PBLs without added tumor cells show no evidence of Xba 1 digested bands. The applicability of this method as a blood test will be in its ability to discriminate changes in patient's blood samples pre and post treatment and thus the units of quantitation are not as important as the relative intensity of the V600E Xba 1 digested bands.

The assay was next applied to the blood of a patient with metastatic melanoma with a known Br af^{V600E} mutation. As shown in Figure 4B, Xba 1 digested PCR fragments were easily detected with either 1 or 10 ug of starting RNA from the patient's PBLs but as in Figure 4A undetected with either 1 or 10 ug of RNA from a normal donor.

A comparison to other methods

We have presented an inexpensive, specific and highly sensitive method to detect the Br af^{V600E} mutation. Alternative methods make use of real time PCR [14,15], mass spectrometry [12,13], allelic specific PCR [7,16,17], PCR using locked oligonucleotides to suppress wild type sequences [18,19,20] or direct sequencing [8,21] of RNA or DNA to preferentially distinguish the mutant V600E from wild type Br af . Methods that incorporate real time PCR tend to be expensive due to the use of fluorescent probes and the need for expensive real time PCR instrumentation or access to core facilities. These methods cost in the range of \$50–\$80/sample. However once the PCR conditions are optimized with respect to annealing temperature, real time PCR has the advantage of specificity albeit to the detriment of sensitivity. The protocol presented here can generate Br af status for any sample for under \$10. In addition, none of these other methods offer the sensitivity and specificity of this protocol. Methods involving real time PCR reported sensitivity in which as little as 10% of the total tissue is composed of V600E positive cells. Even the sequenom method which relies on mass spectrometry to distinguish between mutant and wild type differences requires at least 20% mutant sequences. The methods which has reported to be nearly as sensitive and specific as well as inexpensive and simple as the method reported here are allelic specific PCR [7,16, 17] and locked oligonucleotides in conjunction with PCR. They report the ability to detect 1–2% Br af mutant sequence in a pool of wild type sequence. This is still several orders of magnitude less sensitive than our protocol. As was shown in figure 2, mutant product can be detected in the presence of a thousand fold excess of wild type RNA. Since many tumor biopsies contain a large amount of normal skin tissue, it is likely that these other methods have missed the presence of mutant Br af and thus underestimate the frequency of the Br af^{V600E} mutation in patients with metastatic melanoma. Our protocol reduces the background from wild type

Braf with the use of TspR1, a restriction enzyme that preferentially digests only the wild type sequence from the first PCR product. Although quite effective at digesting the wild type PCR fragment the digestion is not complete, leaving enough wild type products for the rest of the assay to allow for detection of pure wild type tumors. In fact this enzyme has previously been used to access Braf mutations [8,26]. In those reports the presence of undigested PCR product was claimed to be indicative of Braf V600E mutant transcripts. This findings needs to be questioned in light of our observation about the incomplete digestion by TspR1. What may be interpreted as mutant PCR fragment may in fact be undigested wild type fragments.

The power of the protocol is in its superior sensitivity and therefore can be used for the detection of circulating tumor cells from patients with metastatic melanoma and possibly other cancers where the Braf^{V600E} mutation has been documented such as thyroid and colon cancer. In fact, with a simple modification of the cycle number in the second PCR, one melanoma cell was detected in a mixture with 400,000 PBLs. Potentially using this protocol, disease progression, disease regression and disease recurrence can be documented. In addition this protocol can be used to detect microscopic disease in sentinel lymph nodes which would otherwise be undetected by standard immunohistochemistry.

Finally, many protocols use DNA as a template for analysis due to concerns about RNA degradation in paraffin embedded tumor biopsies. As a consequence of the extreme PCR amplification of the cDNA that arose from the RNA in the tissue, this protocol is able to tolerate some RNA degradation and still detect a Braf PCR signal. In fact using this protocol, we have yet to observe a tissue sample where the final Braf PCR product was not amplified. This concern is not an issue with fresh blood or tissue samples where RNA isolation is performed immediately. As a consequence using RNA as a template, this protocol allows for a greater degree of sensitivity than DNA based assays, especially those melanoma cells that have high expression levels of V600E BRAF RNA.

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Figure 1.

(A) Schematic outline of the protocol determining the Bra^f V600 status, as described in results and discussion. (B) The rationale behind the final Xba I digestion. The final PCR uses a unique forward oligonucleotide with mismatches at positions -3 and -4 (bold letters). This creates an Xba I restriction site if the mutant Adenine (A) and not the wild type Thymidine (T) is present in the secondary PCR template. Digestion with Xba I produces products of 108 and 32 bp. Arrows indicate the potential Xba I cleavage site. (C) The sequence at Bra^f position 600 and the accompanying Xba I digestion for wild type (786-0) and mutant (A375) cell lines. The arrows in the sequence indicate the mutation site. The undigested (140 bp) and Xba I digested

(108 and 32 bp) fragments are indicated. **(D)** Xba 1 digestion of Braf PCR from non-melanoma cell lines. U = PCR product prior to the addition of Xba 1; X = Xba 1 digested PCR product.

Figure 2. Accessing the sensitivity of the protocol in the presence (top) and absence (bottom) of TspR1. Varying ratios of wild type (786-0) to mutant (A375) RNA was subjected to the protocol. TspR1 was added according to the protocol or not added. The undigested and Xba 1 digested fragments are indicated.

Figure 3. Testing the protocol on melanoma biopsies. The sequence at Braf position 600 and the accompanying Xba 1 digestion for melanoma tumor biopsies. The arrows in the sequence indicate the mutation site. The undigested and Xba 1 digested fragment are indicated. The intensity of the 108 bp band correlates with the relative peak height of adenine (green) to thymidine (red) at position 600.

Figure 4.

Testing the protocol on peripheral blood lymphocytes. **(A)** Xba I digestion of the Braf PCR from a titration of mutant A375, A2058, SK MEL 5, WM 1976, WM 1862, WM 3163, and WM 3727 cells in 400,000 PBLs. Data is presented as the number of melanoma cells in 400,000 PBLs. **(B)** Xba I digestion of the Braf PCR from PBLs from a normal donor (N) and a patient (B39) with a known V600E mutation. Either 1 or 10 ug of RNA was used in the assay.