REVIEW ARTICLE

Importance of BRAF Testing in Unresectable or Metastatic Melanoma

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ABSTRACT

BRAF is a protein kinase in the mitogen-activated protein kinase pathway that regulates cell proliferation, differentiation, and survival. Mutation in the BRAF gene can lead to aberrant activation of the pathway, which occurs in a variety of human cancers such as melanoma, papillary thyroid cancer, colorectal cancer, and hairy cell leukaemia. BRAF mutations have been reported to occur in 40 to 60% of melanoma tumours, most often affecting codon 600. The most common mutation is a prooncogenic valine-to-glutamic acid (V600E) conversion that drives melanoma cell proliferation. BRAF mutation represents an attractive target for molecular therapy and, in recent years, BRAF inhibitors have created a paradigm shift in melanoma treatment as they significantly improve tumour response and prolong survival of patients with BRAF-mutant unresectable, metastatic melanoma. Accurate and rapid detection of BRAF mutations thus plays a central role in patient selection for optimal, personalised therapy. Several molecular methods are available to analyse BRAF mutation status and these include Sanger DNA sequencing, real-time polymerase chain reaction, pyrosequencing, and high-resolution melting curve analysis. Each of these methods has differing sensitivities, specificities, and costs. This article highlights the key methods for BRAF mutation analysis and summarises the clinical significance of BRAF V600E mutation in melanoma.

Key Words: Immunohistochemistry; Melanoma; Proto-oncogene proteins B-raf

中文摘要

對不可切除或有轉移性黑色素瘤中BRAF基因測試的重要性

馬紹鈞

BRAF是絲裂原活化蛋白激酶通路中的一種蛋白激酶，負責調節細胞增生、分裂和存活。BRAF基因突變可引致通路異常激活，出現於多種人類癌症中，例如黑色素瘤、乳頭狀甲狀腺癌、結直腸癌及毛細胞白血病。在黑色素瘤中，有40%至60%出現BRAF基因突變，通常影響codon 600，最常見的突變是一種驅動黑色素瘤細胞增生的促致癌性纈氨酸-谷氨酸(V600E)轉換。BRAF基因突變是分子治療中一個具吸引力的標靶：在有BRAF基因突變、不可切除轉移性黑色素瘤的病人中，由於BRAF抑制劑能顯著改善腫瘤反應及延長存活，在近年間已經造成黑色素瘤治療的思維典範轉移。因此，BRAF基因突變的準確快速檢測，在選擇病人接受最適切的個人化治療中擔當了核心的角色。現已有幾種分子方法分析BRAF基因突變狀態，包括桑格DNA測序(Sanger DNA sequencing)、實時聚合酶鍵反應(real-time polymerase chain reaction)、焦磷酸測序技術(pyrosequencing)及高分辨率熔解曲
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INTRODUCTION

BRAF is a member of the RAF family of serine/threonine protein kinases. It is a protein component downstream of RAS in the mitogen-activated protein kinase (MAPK) pathway, which is activated by growth factor binding to transmembrane receptor kinases or G-protein-coupled receptors, ultimately regulating cell proliferation, differentiation, and survival. The BRAF gene is located at chromosome 7q34 and spans 190 kb. Mutation of the BRAF gene is one of the mechanisms that leads to constitutive activation of the MAPK signalling pathway and occurs in many human cancers, including melanoma (40-60%), papillary thyroid cancer (45%), colorectal cancer (5-15%), low-grade ovarian tumours (35%), pilocytic astrocytoma (60–80%), non–small-cell lung cancer (1-3%), hairy cell leukaemia (100%), and myeloma (4%).

BRAF mutation in melanoma occurs in about 40 to 60% of melanomas, of which approximately 80% are V600E mutations. Other less frequent BRAF mutations include V600K (16%), V600D/V600R (3%) and, rarely, outside of codon 600 (1%; e.g. D594N, G469E). BRAF mutation in melanoma is characterised by presentation at a young age and low cumulative ultraviolet exposure. In metastatic melanoma, BRAF mutation has been shown to be associated with poor survival, although this is not consistently demonstrated across studies. It is less common in mucosal melanoma and absent in uveal melanoma, which suggests that different melanoma subtypes may have different pathogenic pathways of development.

BRAF MUTATION TESTING IN MELANOMA

With the availability of BRAF inhibitors such as vemurafenib for the treatment of unresectable, metastatic melanoma harbouring the BRAF V600E mutation, testing for BRAF mutations has become increasingly important in clinical practice. Several methods are available for detection of BRAF mutations in tumours. These include real-time quantitative polymerase chain reaction (RQ-PCR), Sanger DNA sequencing, pyrosequencing and high-resolution melting curve analysis. Recently, immunohistochemical detection of BRAF V600E mutation has also been validated as a reliable test in tumours that frequently carry the BRAF V600E mutation. An automated immunohistochemistry assay will likely provide a simple, rapid, and potentially more economical method for BRAF V600E mutation testing in routine clinical practice.

Real-time Quantitative Polymerase Chain Reaction: the BRAF Inhibitor Companion Diagnostic Test

RQ-PCR by the Cobas 4800 BRAF V600 mutation test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) is the US Food and Drug Administration (FDA)–approved and CE-marked companion diagnostic for selecting patients with BRAF V600E mutation–positive melanoma for treatment with vemurafenib. Because this method requires formalin-fixed, paraffin-embedded (FFPE) sections containing at least 50% melanoma cells, haematoxylin-eosin staining and examination by a pathologist are first performed to estimate the tumour content. If the sample contains less than 50% tumour cells, the tissue is macrodissected where possible. The DNA from an FFPE tissue section can then be extracted and quantified, and a fixed concentration of 125 ng of DNA at 5 ng/μl is subjected to amplification and mutation detection using an automated RQ-PCR system.
The main advantages of the Cobas RQ-PCR test include its rapid and simple methodology; the assay can be completed in less than 8 hours after receiving the tumour sample. The test has been validated in pivotal clinical trials to select patients with BRAF V600E mutation for vemurafenib treatment. Analytical sensitivity analysis shows that the lower limit of detection of BRAF V600E mutation using this RQ-PCR assay in FFPE tissue sample is 5% mutation level in a 125 ng/25 μl DNA concentration. This is considerably higher than the limit of detection of 15 to 20% for Sanger sequencing.

Although the RQ-PCR test is designed for detecting BRAF V600E mutation, it has some cross-reactivity with non-V600E mutations. In clinical trials, it can detect about 70% of V600K mutations as well as V600D.14 It is not known to have cross-reactivity with V600R or other BRAF mutations outside of the V600 codon. Limitations of the test include its inability to identify new unknown mutations or provide the mutation sequence.

Sanger DNA Sequencing: Experience from a Local Hospital

Sanger DNA sequencing is the standard method for detecting BRAF mutations in the Hong Kong Sanatorium and Hospital. Unlike RQ-PCR, mutation analysis by Sanger sequencing provides a complete sequence between the selected sequencing primers and allows for detection of BRAF V600E and non-V600E mutations. The time required to perform the test is longer than that for RQ-PCR (approximately 18-19 hours) and the method is less sensitive, with a detection limit of 15 to 20%.

From January 2011 to May 2013, a total of 17 malignant melanoma FFPE samples were tested for BRAF mutations by Sanger sequencing at the Hong Kong Sanatorium and Hospital. These samples were predominantly from metastatic tumours (n = 11); few samples came from primary sites (n = 4), and paired metastatic and primary sites (n = 2). BRAF mutations were identified in seven (41%) samples and these included the V600E (n = 4), V600K (n = 1), V600R (n = 1), and D594G (n = 1) mutations (Table). Patients with BRAF mutation–positive melanoma included three Caucasian and four Chinese patients; six patients had metastatic disease, while one had mucosal melanoma involving the anal canal; the age of all patients ranged from 29 to 62 years.

**Immunohistochemistry: a Promising Approach to Detect BRAF V600E Mutation**

A recent advance in BRAF mutation detection is the development of a BRAF V600E mutation-specific monoclonal antibody (VE1) that allows identification of BRAF V600E mutation by immunohistochemistry. It has been validated in primary and metastatic melanoma, as well as in a range of other tumours frequently carrying the BRAF V600E mutation.15 An automated immunohistochemical test using the VE1 antibody (Ventana Medical Systems, Tucson, AZ, USA) is being made available, making BRAF V600E mutation detection more rapid, and potentially cheaper and more sensitive than existing methods.

The sensitivity and specificity of BRAF V600E mutation testing by immunohistochemistry using the VE1 antibody was investigated in a study of 100 patients with stage IIIIC unresectable or stage IV melanoma. This study analysed BRAF mutation status in FFPE melanoma samples independently by high-resolution melting curve analysis and immunohistochemistry using VE1 antibody.16 Results showed that the antibody had 97% (n = 37/38) sensitivity and 98% (n = 58/59) specificity for detecting BRAF V600E mutations. It was

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**Table.** Melanoma patients tested positive for BRAF mutations by Sanger sequencing in the Hong Kong Sanatorium and Hospital from January 2011 to May 2013.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>Site</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>M*</td>
<td>44</td>
<td>Metastasis to the lung</td>
<td>c.1798_99delinsAA</td>
<td>p.Val600Lys (V600K)</td>
</tr>
<tr>
<td>F</td>
<td>38</td>
<td>Right axillary lymph nodes</td>
<td>c.1799T&gt;A</td>
<td>p.Val600Glu (V600E)</td>
</tr>
<tr>
<td>M*</td>
<td>62</td>
<td>Intestine and mesentery</td>
<td>c.1799T&gt;A</td>
<td>p.Val600Glu (V600E)</td>
</tr>
<tr>
<td>M*</td>
<td>53</td>
<td>Right axillary lymph nodes</td>
<td>c.1798_99GT&gt;AG</td>
<td>p.Val600Arg (V600F)</td>
</tr>
<tr>
<td>F</td>
<td>59</td>
<td>Anal canal</td>
<td>c.1781A&gt;G</td>
<td>Asp594Gly (D594G)</td>
</tr>
<tr>
<td>M</td>
<td>29</td>
<td>Inguinal lymph nodes</td>
<td>c.1799T&gt;A</td>
<td>p.Val600Glu (V600E)</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>Left inguinal lymph nodes</td>
<td>c.1799T&gt;A</td>
<td>p.Val600Glu (V600E)</td>
</tr>
</tbody>
</table>

Abbreviations: M = male; F = female.
* Caucasian patient.
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specifically for V600E mutation and none of the non-V600E samples stained positive. Five cases showed discordant BRAF mutation results, of which three mutation-positive cases by immunohistochemistry were confirmed by additional molecular testing, suggesting that they were missed by the initial molecular tests. Two cases remained discordant, one of which was false-negative and the other false-positive by immunohistochemistry.16

BRAF MUTATIONS: IMPORTANCE IN CLINICAL PRACTICE

The presence of BRAF mutation in unresectable or metastatic melanoma has emerged as an important factor in selecting patients for targeted therapy. In the phase III BRAF Inhibitor in Melanoma 3 (BRIM-3) study comparing vemurafenib with dacarbazine in 675 patients with previously untreated, metastatic melanoma that tested positive for the BRAF V600E mutation by the Cobas RQ-PCR assay, vemurafenib was associated with a 63% relative risk reduction for death and 74% relative risk reduction for either death or disease progression compared with dacarbazine (p < 0.001 for both comparisons).5 This study included 20 patients with non-V600E mutations (n = 19 with V600K and n = 1 with V600D); among these, 10 patients with V600K mutation were randomised to the vemurafenib arm and four (40%) patients reported a partial response. This suggests that melanomas harbouring V600K mutations are also sensitive to vemurafenib. Further studies are needed to investigate the efficacy of BRAF inhibitors in melanomas with other less frequent, non-V600E mutations.

FUTURE CONSIDERATIONS

In addition to BRAF, other mutations have been observed in melanomas as potential targets for molecular therapy. KIT mutations are observed in about 10% of melanomas and are most common in mucosal and acral melanomas (10-40%).15 These mutations do not overlap with BRAF mutations and do not necessarily correlate with KIT copy number or CD117 expression. KIT is an established therapeutic target for several other cancers; in melanoma, some patients harbouring specific KIT mutations have been shown to respond to treatment with the KIT inhibitor imatinib. Identifying patients with metastatic melanoma harbouring functionally relevant KIT mutations may be helpful for selecting patients most likely to respond to KIT-targeted therapy. Finally, BRAF V600E mutation is known to play a role in other cancers, such as colorectal cancer, papillary thyroid cancer, and hairy cell leukaemia. The effects of BRAF inhibitors, alone and in combination therapies, in various BRAF-mutant tumour types are currently being investigated for tumour response and patient benefits.

CONCLUSION

Molecular testing for BRAF mutations in melanoma plays a vital role in selecting patients for appropriate, personalised therapy. The potent BRAF inhibitor vemurafenib has been shown in clinical trials to provide substantial benefits in overall and progression-free survival, in patients with BRAF V600E-positive, unresectable or metastatic melanoma. There are various molecular methods for detecting BRAF V600E mutations, including Sanger DNA sequencing, RQ-PCR, pyrosequencing, and high-resolution melting curve analysis. Each of these methods has differing sensitivities, specificities, costs, and equipment requirements. The RQ-PCR mutation assay is an FDA-approved, CE-marked companion diagnostic test to identify melanoma patients with V600E mutations for treatment with vemurafenib. In studies, this method was more sensitive than Sanger sequencing for detecting V600E mutations; furthermore, it can also detect V600K and V600D mutations. In the near future, BRAF mutation testing by immunohistochemical analysis will likely further improve the care for melanoma patients by providing a rapid, sensitive, and potentially more cost-effective method for identifying patients most likely to benefit from BRAF-targeted therapy. For a personalised approach in cancer therapy, reliable molecular diagnostic testing is essential to ensure that effective targeted therapies are given to appropriate patients.

REFERENCES