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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）、實時聚合酶鏈反應（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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線分析（high-resolution melting curve analysis）。這些方法的敏感度、特異性及成本各有不同。本文重點介紹*BRAF*基因突變分析的主要方法，並總結了*BRAF* V600E突變在黑色素瘤中的臨床顯著性。

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.[1] 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%).[2,3] The most common *BRAF* mutation in tumours is a single point mutation, a valine-to-glutamic acid substitution at codon 600 (V600E; initially designated V599E) in the exon 15 activating domain.[2] The mutation mimics the effect of phosphorylation of either T599 or S602, resulting in constitutively elevated kinase activity and activation of the MAPK pathway.[4] *BRAF* V600E mutation represents an attractive target for molecular therapy and, in recent years, *BRAF* kinase inhibitors have created a paradigm shift in melanoma treatment as they significantly improve tumour response and survival in patients with *BRAF*-mutant unresectable, metastatic melanoma.[5,6] For an optimal, personalised and therapeutic approach, accurate and a robust molecular assay to detect *BRAF* mutations is now an important part of clinical practice. Here, we summarise the key methods for *BRAF* mutation detection and highlight the clinical significance of *BRAF* V600E mutation in melanoma.

BRAF MUTATION IN MELANOMA

BRAF mutation 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).[7] *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.[8-11] 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.[12]

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* V600 mutation–positive melanoma for treatment with vemurafenib.[13] 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.[14]

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-V600 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 IIIC 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

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

Sex	Age (years)	Site	Nucleotide change	Amino acid change
M*	44	Metastasis to the lung	c.1798_99delinsAA	p.Val600Lys (V600K)
F	38	Right axillary lymph nodes	c.1799T>A	p.Val600Glu (V600E)
M*	62	Intestine and mesentery	c.1799T>A	p.Val600Glu (V600E)
M*	53	Right axillary lymph nodes	c.1798_99GT>AG	p.Val600Arg (V600R)
F	59	Anal canal	c.1781A>G	p.Asp594Gly (D594G)
M	29	Inguinal lymph nodes	c.1799T>A	p.Val600Glu (V600E)
F	60	Left inguinal lymph nodes	c.1799T>A	p.Val600Glu (V600E)

Abbreviations: M = male; F = female.
* Caucasian patient.

specific only 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%).[17] 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.

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