

***BRAF* mutations in non-small cell lung cancer**

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Background: *BRAF* is a proto-oncogene encoding a serine/threonine protein kinase which promotes cell proliferation and survival. *BRAF* mutations are commonly seen in melanoma and papillary thyroid carcinoma. We aimed to investigate the prevalence and clinicopathological features of *BRAF* mutations in non-small cell lung cancer (NSCLC) cases submitted for routine mutation testing at our institution.

Methods: Mutation analysis for *BRAF*, *EGFR* and *KRAS* was performed using Sequenom MassARRAY platform with OncoCarta panel v1.0. Pathological features were reviewed and immunohistochemistry for *BRAF* V600E was also performed.

Results: Seven out of 273 cases (2.6%) had *BRAF* mutations (three males and four females, median age 70 years, all smokers), with six adenocarcinomas and one NSCLC, not otherwise specified (NOS). All had wild-type *EGFR* and *KRAS*. The identified *BRAF* mutations were V600E (4/7, 58%), K601N, L597Q and G469V. *BRAF* V600E immunohistochemistry was positive in two cases with V600E and negative in one case with K601N (tissue available in three cases only). No significant difference in age or gender was found (*BRAF* mutant *vs.* wild-type).

Conclusions: *BRAF* mutations occur in a small proportion of NSCLC that lack other driver mutations. The clinicopathological profile differs from that of *EGFR* mutant tumours. The potential benefits of *BRAF*-inhibitors should be investigated.

Keywords: Lung cancer; *BRAF* mutation; genetic testing

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Introduction

The discovery of epidermal growth factor receptor (*EGFR*) mutations in non-small cell lung cancer (NSCLC) has allowed effective targeted therapy with EGFR tyrosine kinase inhibitors in patients that harbour these mutations (1). However, the majority of NSCLC cases have wild-type *EGFR* and it is now known that many other mutations can drive oncogenic pathways, including *KRAS* and less commonly, *BRAF* (2). *BRAF* is a proto-oncogene encoding a serine/threonine protein kinase which is a downstream effector protein of RAS and transduces signalling through the mitogen-activated protein kinase pathway to promote cell proliferation and survival. This pathway functions

downstream of various receptor tyrosine kinases such as *EGFR* and is a key mediator of oncogenesis (3).

BRAF mutations are commonly seen in a range of malignancies, including hairy-cell leukemia (100%) (4), melanoma (~40%) (5), papillary thyroid carcinoma (30-50%) and colorectal carcinoma (~10%) (6). The V600E mutation has been shown to constitutively activate *BRAF* which phosphorylates the downstream effectors MEK and subsequently ERK (7). ERK, in turn, activates transcription factors such as c-fos and Elk-1, driving cell cycle progression and survival (8). The importance of the *BRAF* pathway is well established in melanoma, as *BRAF* inhibitors have been shown to significantly increase progression free survival

of patients with advanced stage melanoma harbouring the *BRAF* V600E mutation (9). This raises the possibility that *BRAF* mutations may also be a feasible target in NSCLC. *BRAF* mutations in NSCLC are not well characterised in the literature due to their low prevalence. In this study we aimed to investigate the prevalence and clinicopathological features of *BRAF* mutations in NSCLC.

Methods

Patients

We retrospectively reviewed 273 NSCLC cases that underwent mutation testing upon request of the treating oncologist at Royal Prince Alfred Hospital between March 2012 and March 2014. The patients underwent either a resection or a diagnostic procedure (biopsy or cytological specimen) and the tissue was formalin fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The H&E sections were reviewed by a pathologist (SOT or WC) to ensure adequate tumour cells were present and to mark representative areas for deoxyribonucleic acid (DNA) extraction. Histological subtypes were classified according to the IASLC/ATS/ERS classification (10). This study was approved by the Human Research Ethics Committee of Royal Prince Alfred Hospital.

Mutation detection

DNA was extracted from the formalin fixed paraffin embedded tissue using NucleoSpin FFPE DNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instruction with 2 hr proteinase digestion. The quantity of the extracted DNA was assessed using Qubit® Fluorometer (Life Technologies, Mulgrave, Australia). A minimum of 300 ng of DNA was required for optimal mutational analysis. Samples were amplified for 238 variant targets in a 24-multiplex PCR using the OncoCarta Panel v1.0 Kit (*ABL1*, *AKT1*, *AKT2*, *BRAF*, *CDK*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *JAK2*, *KIT*, *MET*, *HRAS*, *KRAS*, *NRAS*, *PDGFR*, *PIK3CA*, and *RET*) and analyzed based on the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) technology on the Sequenom MassArray platform (11,12). The targeted mutations in the 19 oncogenes comprising the OncoCarta v1.0 Panel are reported to be biologically significant in carcinogenesis or progression in a range of malignancies. These mutational analyses and immunohistochemistry described below were performed at

an Australian National Association of Testing Authorities (NATA) accredited medical laboratory.

Immunohistochemistry

BRAF V600E immunohistochemistry was performed on sections cut at four microns. Tissue was pre-treated on a Ventana Benchmark Ultra (Roche) with CC1 (Roche) for 64 minutes. The anti-*BRAF* (VE1) mouse monoclonal antibody (Spring Bioscience) was used at 1:100 dilution with 16 minutes incubation. Staining was performed using the OptiView DAB Immunohistochemistry Detection kit (Roche) for 8 minutes. Cases with 1+, 2+ and 3+ staining were regarded as positive and cases with no staining were regarded as negative.

Statistical analysis

Fisher's exact test was used to evaluate the difference in gender distribution between *BRAF* wild-type and mutant patients while Welch's *t*-test was used to evaluate the age difference. Data were analysed using the R environment for statistical computing (13).

Results

Patient characteristics

The patient characteristics are summarised in *Table 1*. A total of 273 cases of NSCLC were tested and 7 (2.6%) were found to have *BRAF* mutations. Of patients with *BRAF* mutations, there were three males and four females, median age 70 years, range from 51 to 76 years. All seven patients were former smokers with smoking history ranged from 3 to 90 pack years. *BRAF* wild-type was found in 266 cases with 141 males and 125 females, median age 66.5 years. There was no significant difference in gender distribution ($P=0.71$) or age ($P=0.65$) between *BRAF* wild-type and mutant patients. Due to incomplete data on smoking history and tumour type in the *BRAF* wild-type group, statistical analysis could not be performed.

Six cases were adenocarcinomas and one case was a non-small cell carcinoma, not otherwise specified (NOS). The tumour diagnosed as non-small cell carcinoma, NOS, was from a fine needle aspiration specimen. Two cases of adenocarcinoma were diagnosed on bronchial biopsy or washing. One case was diagnosed on core biopsy showing a mixture of acinar, papillary and lepidic patterns. In patients who underwent a resection, the histological subtypes

Table 1 Patient clinical characteristics and *BRAF* genotype

Patient no.	Age (years)	Gender	Smoking status	Procedure	Predominant histological subtype	Other components	<i>BRAF</i> mutation
1	51	F	Ex-smoker 15 pk yrs	Lung FNA	Non-small cell carcinoma, NOS		V600E
2	57	F	Ex-smoker pk yrs not known	Resection	Adenocarcinoma—lepidic	Papillary	G469V
3	70	F	Ex-smoker 10 pk yrs	Resection	Adenocarcinoma—micropapillary	Lepidic	V600E
4	70	M	Ex-smoker 90 pk yrs	Lung core biopsy	Adenocarcinoma—acinar	Papillary and lepidic	K601N
5	73	F	Ex-smoker 40 pk yrs	Bronchial washing	Adenocarcinoma		V600E
6	74	M	Ex-smoker 40 pk yrs	Bronchial biopsy	Adenocarcinoma		L597Q
7	76	M	Ex-smoker 3 pk yrs	Resection	Adenocarcinoma—micropapillary	Acinar	V600E

F, female; M, male; NOS, not otherwise specified.

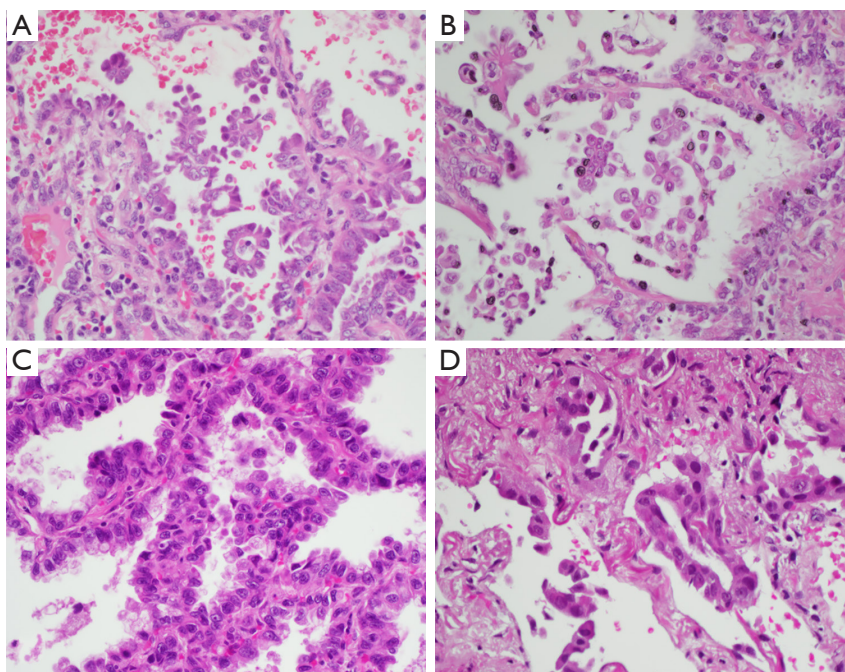


Figure 1 (A,B) Micropapillary pattern in patients 3 and 7 respectively; (C) lepidic pattern in patient 2; (D) acinar pattern in patient 4. (H&E 400× magnification).

were lepidic predominant with papillary component, micropapillary predominant with lepidic component and micropapillary predominant with acinar component. Representative H&E sections are shown in *Figure 1*.

***BRAF* mutation genotypes**

Four *BRAF* mutation genotypes were identified. Three mutations were located in exon 15 which included V600E (c.1799T>A, 58%, n=4), K601N (c.1803A>T, 14%, n=1) and L597Q (c.1790T>A, 14%, n=1). One mutation was

found in exon 11 which was G469V (c.1406G>T, 14%, n=1). Representative spectra are shown in *Figure 2*. A female predominance of V600E mutations was noted (3 out of 4 V600E mutations). Furthermore, both patients with a micropapillary component harboured V600E mutation. No patient with a *BRAF* mutation had a concomitant *EGFR* or *KRAS* mutation.

Immunohistochemistry

Due to limited availability of tissue, *BRAF* V600E

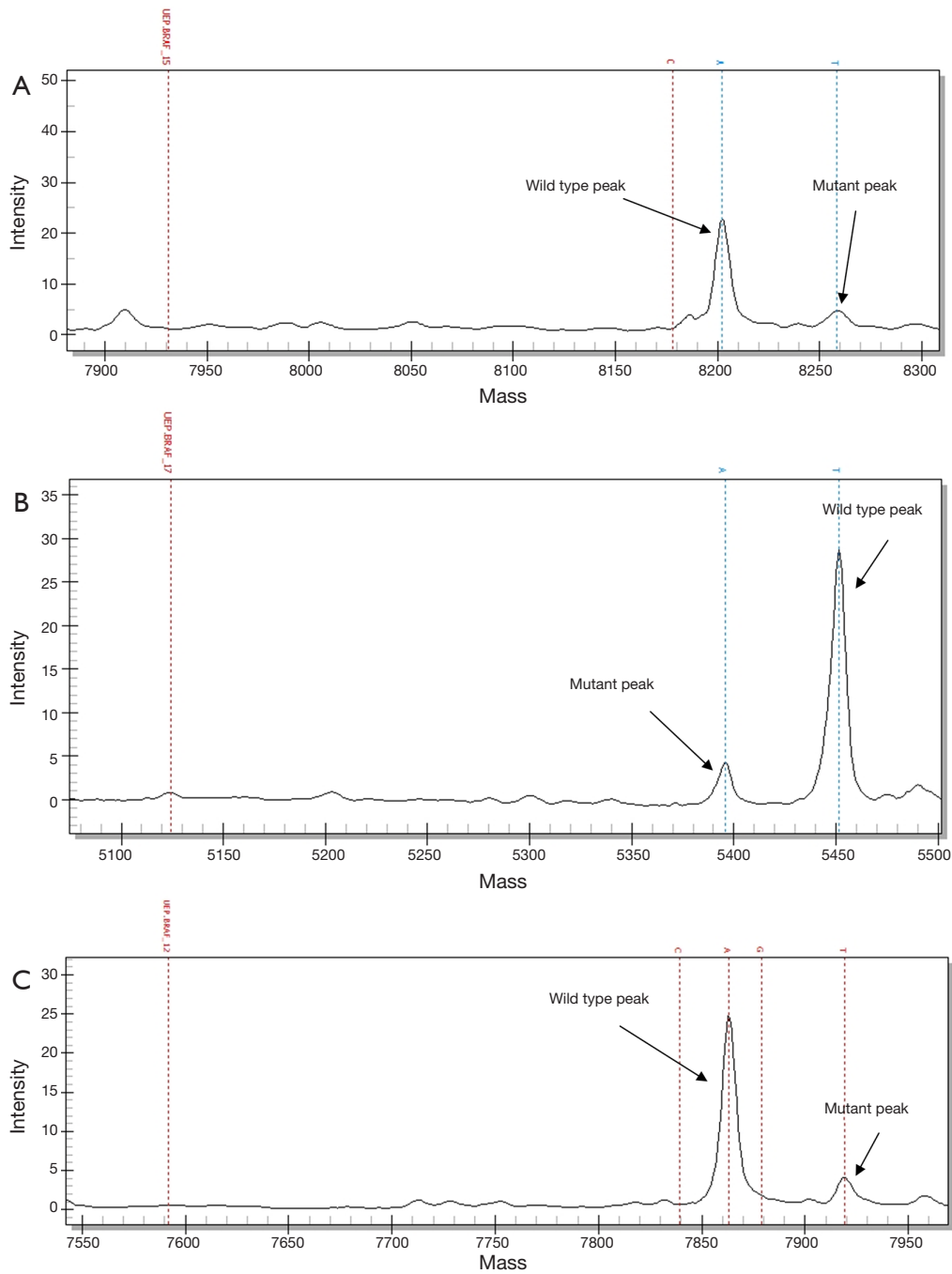


Figure 2 Mass spectrometry of PCR products showing *BRAF* mutations (all three profiles showing the changes at the antisense strand of DNA). (A) *BRAF* V600E mutation (patient 1) with a mutant adenine (A) peak substituting for thymine (T) at position 1799 (c.1799T>A); (B) *BRAF* K601N mutation (patient 4) with a mutant T peak substituting for A (c.1803A>T); (C) *BRAF* L597Q mutation (patient 6) with a mutant A peak substituting for T (c.1790T>A).

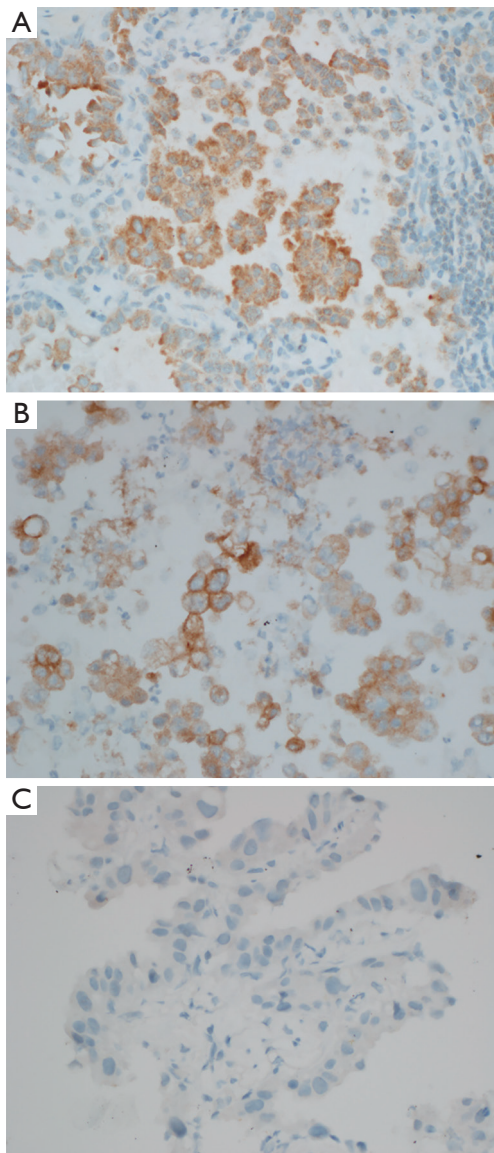


Figure 3 *BRAF* V600E immunohistochemistry for (A) patient 3 (V600E mutation), (B) patient 5 (V600E mutation) and (C) patient 4 (K601N mutation). (400× magnification).

immunohistochemistry was only performed in three cases. *BRAF* V600E immunohistochemistry was positive in two cases with V600E mutation and negative in one case with K601N mutation (Figure 3). Thus the immunohistochemistry results were consistent with the Sequenom MassArray platform results.

Discussion

In our population of Australian patients with NSCLC that underwent mutation testing, we found *BRAF* mutations occurred in 2.6% of patients who were all former smokers. This is consistent with other studies reporting *BRAF* mutation prevalence between 2-5% in NSCLC (14-16). While this is much less common than *EGFR* mutations that occur in approximately 15% of lung adenocarcinomas in Western populations (17), there were approximately 6,000 new cases of NSCLC diagnosed in Australia in 2007 (18), giving a predicted number of 156 patients with *BRAF* mutant lung cancer. These patients could potentially benefit from targeted therapy as *BRAF* V600E NSCLC has shown some response to dabrafenib (19). The prevalence rate is only slightly lower than that of *ALK* gene rearrangements that are found in ~3-5% of lung adenocarcinomas (20).

We found all patients with *BRAF* mutation had a smoking history, in contrast with *EGFR* mutations which commonly occur in non-smokers (21). Although others have also reported an association between *BRAF* mutation and smoking (16), one study reported V600E mutation to be associated with non-smokers while non-V600E mutations were associated smokers (14). Discrepancies between studies may be due to low numbers of *BRAF* mutant cases in each study, relating to the low prevalence of *BRAF* mutations in NSCLC.

A potential limitation of the targeted approach to mutation detection employed in the current study is that very rare mutations not on the OncoCarta panel may not be detected, such as *BRAF* mutations involving amino acids 421, 436, 439 and 471. However, these mutations represent less than 2% of all reported *BRAF* mutations in NSCLC (22), making it highly unlikely for the overall *BRAF* mutation prevalence to be under-represented in the current study. Furthermore our testing is more comprehensive than that performed by many centres who currently focus only of the *BRAF* V600 codon.

Although the current study did not find a significant difference in gender distribution or age between *BRAF* wild-type and mutant patients, the small number of patients in the *BRAF* mutant group makes it difficult to draw definitive conclusions. Interestingly, a predominance of *BRAF* V600E mutation in females has been reported by others (14) while we found a non-statistically significant trend towards female predominance. *BRAF* mutation is also more commonly found in females in colorectal cancer (23,24). This finding is akin to the female predominance of *EGFR* mutations and may represent a similar underlying mechanism. An association between *EGFR* mutation and oestrogen receptor

has been found, possibly indicating a hormonally driven phenomenon (25). However, a clear mechanism has yet to be substantiated.

The *BRAF* V600E mutation has been previously reported to be associated with the aggressive micropapillary subtype of lung adenocarcinoma (14,26). This finding is supported by the current study as both patients with a micropapillary component showed *BRAF* V600E mutation. However, it is difficult to be conclusive due to the small number of patients in the current study precluding statistical analysis.

The most common *BRAF* mutation in melanoma is the V600E mutation, which accounts for more than 90% of mutations (6). However, the current study shows that *BRAF* V600E mutation only accounts for 58% of mutations in NSCLC. This finding is supported by others who found the non-V600E mutation rate to be between 50-89% (16,27). Although the biological significance of this is unknown, this raises the possibility that *BRAF*-related oncogenesis in NSCLC arises from a different mechanism compared to melanomas with V600E mutations. It has been shown that the V600E mutation confers a much higher kinase activity compared to other mutations within the kinase domain (7). The G469V mutation found in the current study occurs in the P-loop which is the ATP binding site. Mutations within the P-loop have been shown to have a lower activity compared to wild-type *BRAF* (7), therefore whether these mutations drive oncogenesis is uncertain. Similarly, rare mutations at codons 439 and 440 (AKT phosphorylation motif) have been reported in NSCLC and they do not increase the oncogenic properties of *BRAF* (28). This indicates that the genotype of the *BRAF* mutation may be an important therapeutic consideration. Unfortunately, there is currently only limited phase I clinical trial data for RAF inhibitors in NSCLC (19) and the significance of the different mutation spectrum remains uncertain.

In conclusion, the current study confirmed that a small proportion of NSCLC patients harbour *BRAF* mutations. Their clinicopathological characteristics appear to differ from patients with *EGFR* mutations and their genotype differs from that found in melanoma. Further work needs to be done to determine whether this small subset of patients will benefit from *BRAF* inhibitors.

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