Identification of BRAF\textsuperscript{V600E} in thyroid neoplasia may be useful because it is specific for malignancy, connotes a worse prognosis, and is the target of novel therapies currently under investigation. Sanger sequencing is the ‘gold standard’ for mutation detection but is subject to sampling error and requires resources beyond many diagnostic pathology laboratories. In this study, we compared immunohistochemistry (IHC) using a BRAF\textsuperscript{V600E} mutation-specific MAB to Sanger sequencing on DNA from formalin-fixed paraffin-embedded tissue, in a well-characterized cohort of 101 papillary thyroid carcinoma (PTC) patients. For all cases, an IHC result was available; however, five cases failed Sanger sequencing. Of the 96 cases with molecular data, 68 (71\%) were BRAF\textsuperscript{V600E} positive by IHC and 59 (61\%) were BRAF\textsuperscript{V600E} positive by sequencing. Eleven cases were discordant. One case was negative by IHC and initially positive by sequencing. Repeat sequencing of that sample and sequencing of a macrodissected sample were negative for BRAF\textsuperscript{V600E}. Of ten cases positive by IHC but negative by sequencing on whole sections, repeat sequencing on macrodissected tissue confirmed the IHC result in seven cases (suggesting that these were false negatives of sequencing on whole sections). In three cases, repeat sequencing on recut tissue remained negative (including using massive parallel sequencing), but these cases demonstrated relatively low neoplastic cellularity. We conclude that IHC for BRAF\textsuperscript{V600E} is more sensitive and specific than Sanger sequencing in the routine diagnostic setting and may represent the new gold standard for detection of BRAF\textsuperscript{V600E} mutation in PTC.
*BRAF*<sup>V600E</sup> and aggressive behavior of PTC (Xing *et al.* 2005, Kebebew *et al.* 2007, Lupi *et al.* 2007, Basolo *et al.* 2010, O’Neill *et al.* 2010), suggesting that *BRAF*<sup>V600E</sup> status could be used for risk stratification and individualized clinical management of patients with PTC, and therapeutic agents specifically targeting BRAF are under active investigation in clinical trials (http://clinicaltrials.gov/ct2/show/NCT01286753).

Although traditional Sanger sequencing is still considered the gold standard for mutation identification, many novel techniques have been developed to identify *BRAF* mutation including single-strand conformation polymorphism, restriction fragment length polymorphism (RFLP), mass-array spectrometry, pyrosequencing, and mutation-specific PCR. These methods are PCR based. They therefore require significant infrastructure; are technically demanding; and subject to tissue heterogeneity, sampling error, and suboptimal preservation of DNA in formalin-fixed paraffin-embedded (FFPE) tissue. For these reasons, *BRAF*<sup>V600E</sup> testing has been difficult to deploy clinically particularly in resource poor settings.

Recently, Capper *et al.* (2011) developed a novel mouse monoclonal mutation-specific anti-*BRAF*<sup>V600E</sup> antibody (clone VE1 Spring Bioscience, Pleasanton, CA, USA), which is suitable for immunohistochemistry (IHC) on routinely processed FFPE tissue. This antibody has shown initial promise in the detection of *BRAF*<sup>V600E</sup> in PTC (Capper *et al.* 2011, Koperek *et al.* 2012). In this study, we evaluate this *BRAF*<sup>V600E</sup>-specific antibody and compare it with Sanger sequencing in a large cohort of Australian PTC patients, most of whom we had previously determined *BRAF*<sup>V600E</sup> status determined by RFLP against long-term clinical data (O’Neill *et al.* 2010).

**Materials and methods**

**Tissue samples**

Patients were identified from a prospectively maintained endocrine surgical database after approval from the local institutional human research ethics committee. FFPE tumor blocks were available for 101 PTC patients in the archives of the Department of Anatomical Pathology at the Royal North Shore Hospital, and their acquisition was regulated by the New South Wales Human Tissue Act 1983. Fresh sections were cut from archived blocks and independently reviewed by a single pathologist to confirm the diagnosis (A Chou). The single paraffin block with the largest dimension of tumor regardless of the ratio of neoplastic to non-neoplastic tissue was chosen for both IHC and sequencing.

**Immunohistochemistry**

IHC was performed on FFPE tissue using a novel mouse MAB (clone VE1). This previously described antibody was raised to react with *BRAF*<sup>V600E</sup> but not with wild-type *BRAF* or non-V600E mutations and was developed by two of us (D Capper and A von Deimling) (11). All slides were examined independently by three observers (A J Gill, A Clarkson and T Dodds) who were blinded to each other’s results and all molecular data. The immunohistochemical staining was recorded as being either positive or negative. (Supplementary Materials and methods, see section on supplementary data given at the end of this article).

**Genomic DNA extraction and PCR**

From each paraffin block confirmed to contain tumor, a ribbon of 10×10 μm sections was placed in an eppendorf container with care being taken to prevent tissue cross-contamination. Genomic DNA was extracted from the FFPE sections using a QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer’s instructions. A 224 bp region of the *BRAF* coding sequence encompassing codon 600 was amplified by PCR using HotStar Taq DNA polymerase (Qiagen) using forward 5′-TCATAATGCTTGCTCTGATAGGA and reverse 5′-GGCCAAAAATTTACAGTGGA primers.

**Sanger sequencing**

PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega) according to manufacturer’s instructions. Each sample was sequenced using forward and reverse primers on an ABI PRISM 3700 platform (Applied Biosystems, Foster City, CA, USA) (service provided by Australian Genome Research Facility, Sydney, Australia).

**Discordant cases**

In cases where IHC and sequencing were discordant, both tests were initially repeated on the same sample and massive parallel sequencing was performed. Repeat Sanger sequencing and massive parallel sequencing were then also performed on a second sample of PTC which was macro-dissected from tumor blocks so as to avoid adjacent non-neoplastic thyroid tissue. Micro-dissection (separation of individual tumor cells from non-neoplastic cells within the main tumor volume) was not performed.
Massive parallel sequencing

Massive parallel sequencing was performed using an Illumina MiSeq platform and custom amplicon array including BRAF (TruSeq Amplicon – Cancer Panel, Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. BRAF<sup>V600E</sup> was considered positive if the variant was detected at Qscore ≥ 30 and coverage > 60.

Results

Five cases failed to amplify or yield a result by Sanger sequencing, all of which yielded a result by IHC. Of the remaining 96 cases with molecular data, 68 (71%) were positive for BRAF<sup>V600E</sup> by IHC and 59 (61%) were BRAF<sup>V600E</sup> positive by sequencing (Table 1). Other BRAF mutations were not identified by sequencing. Eleven cases were discordant, comprising ten cases that were positive by IHC but negative by sequencing and one case that was negative by IHC but positive by sequencing.

All the three observers who interpreted the IHC showed complete agreement (kappa score 1). All but five BRAF<sup>V600E</sup>-positive cases demonstrated diffuse homogenous staining in all malignant cells (Fig. 1). The remainder showed definite positive staining in more than 60% of malignant cells with a gradual transition to weaker and then negative staining in areas (particularly in the central portion of large tumors). Some faint non-specific staining was identified in colloid and some macrophages, but this was easily differentiated from true positive staining (Supplementary Figure 1, see section on supplementary data given at the end of this article). Non-neoplastic thyroid tissue, benign inflammatory cells and stroma were consistently negative.

The 11 discordant cases were extensively investigated. Repeat immunostaining on all cases confirmed the original IHC result. In the one case that was negative for BRAF<sup>V600E</sup> by IHC but initially positive by sequencing, both repeat sequencing of that sample and sequencing of a repeat macrodissected sample were negative for BRAF<sup>V600E</sup>. Of the ten cases positive for BRAF<sup>V600E</sup> by IHC but negative by Sanger sequencing, repeat Sanger sequencing on the same samples (shavings from whole blocks containing cancer) remained negative. Using massive parallel sequencing, BRAF<sup>V600E</sup> was detected in only three of the shavings from these cases at variant frequencies 0.096, 0.308, and 0.318. When Sanger and massive parallel sequencing were performed on macrodissected tumor, BRAF<sup>V600E</sup> was identified in 7/10 cases by both approaches. For the remaining three cases where repeat Sanger and massive parallel sequencing remained negative, it was noted that there was a relatively low ratio of neoplastic to non-neoplastic (benign thyroid, stromal, and inflammatory) cells, with an estimated neoplastic percentage of <35% (Fig. 1E and F).

This study included 87 samples from a previously reported cohort of patients where we noted that BRAF<sup>V600E</sup> was associated with increased risk of clinically evident disease recurrence and/or death (6). We re-analyzed our clinical data in these cases according to IHC results from this study and found that BRAF<sup>V600E</sup> remained significantly associated with the composite end point of clinically evident disease recurrence and/or death (Supplementary Tables 1 and 2, and Supplementary Figure 2, see section on supplementary data given at the end of this article).

Discussion

Accurate pathological diagnosis is central to the treatment of malignancy and underpins translational research. The development of personalized medicine strategies has created an urgent need for rapid and accurate molecular characterization of cancers. In this study, we have compared detection of BRAF<sup>V600E</sup> by IHC to Sanger sequencing in 101 PTC samples. We found that 68/96 (71%) PTCs were positive for BRAF<sup>V600E</sup> by IHC, whereas 59/96 (61%) PTCs were positive for BRAF<sup>V600E</sup> by sequencing. In 11 discordant cases, further sampling and re-testing favored the original IHC result in eight cases, suggesting that IHC is more sensitive for detecting BRAF<sup>V600E</sup> than Sanger sequencing from FFPE tissue performed on whole sections.

Sanger sequencing is still considered the gold standard for identifying oncogenes in solid tumors. However, it is laborious and time-consuming and may fail to accurately diagnose an oncogene if the tumor is poorly sampled or heterogeneous (Giannini et al. 2007). A particular limitation of Sanger sequencing (and other PCR-based molecular methods) in the routine diagnostic setting is the need to ensure that amplified DNA is mainly from neoplastic cells, rather than from non-neoplastic cells.

Table 1 BRAF<sup>V600E</sup> status by IHC and Sanger sequencing on whole sections

<table>
<thead>
<tr>
<th>BRAF&lt;sup&gt;V600E&lt;/sup&gt; status</th>
<th>IHC</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>68 (71)</td>
<td>59 (61)</td>
</tr>
<tr>
<td>-</td>
<td>28 (29)</td>
<td>37 (39)</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>96</td>
</tr>
</tbody>
</table>
than being diluted by excess non-neoplastic tissue. False negatives by Sanger sequencing can therefore occur (as in our study) when whole sample sections are used. This may only partly be overcome by macrodissection. For example, in one report, \textit{BRAF}^{V600E} was detected in 36.9\% of macrodissected PTCs by Sanger sequencing but in 53.6\% by pyrosequencing (Guerra et al. 2012a, b).

Our study demonstrates that massive parallel sequencing only partly addresses the issue of sample heterogeneity given that it was negative when performed on whole sections (confirmed by an experienced pathologist to definitely contain PTC) from four cases subsequently proven to be mutated on macrodissected tissue. In this context, it is important to note that the three ‘false positives’ of IHC compared to sequencing on macrodissected tissue were associated with large numbers of non-neoplastic cells intimately admixed with tumor. In these cases, the estimated tumor cellularity was <35\%. Therefore, it is possible that these may still represent false negatives of sequencing due to low neoplastic cellularity.

\textbf{Figure 1} Serial H&E and \textit{BRAF}^{V600E}-stained sections of a case that was positive by both Sanger sequencing and IHC (A, B, C and D) and a case that was positive by IHC but consistently negative by sequencing (E and F). The homogenous positive staining of all malignant cells is emphasized as is the negative staining in non-neoplastic cells. In E and F, it is noted that the malignant cells are outnumbered by benign stromal and inflammatory cells, and in this instance, it was estimated that neoplastic cells constituted <35\% of all cells in the macrodissected area (original magnifications 40× (A and B), 400× (C and D), and 100× (E and F)).
It is interesting to note that we found diffuse and homogenous positive staining for BRAF V600E in all but five positive cases, a finding that has also been reported by Koperek et al. (2012). It has previously been reported that the VE1 antibody may show false-negative staining when IHC is performed on older sections or on tissue that has previously been frozen, undergone decalcification or is pre-necrotic, indicating that the antibody may be sensitive to false-negative staining under suboptimal conditions (Capper et al. 2011). The gradual transition from positive to negative staining that we observed in these cases with focal negative staining being more prominent in the center of large tumors rather than demonstrating distinct clonal nodules of positive and negative cells is a pattern that we normally associate with technical issues related to processing, fixation, and antibody performance rather than true clonal heterogeneity. At face value, our data therefore suggest that BRAF V600E expression is homogenous in tumor cells within a particular PTC and therefore an early clonal event, even if those cancer cells are admixed with non-neoplastic epithelium, stromal cells, and/or infiltrating macrophages. However, other reports have suggested that BRAF V600E may be clonally heterogeneous within single tumors based on pyrosequencing of macro-dissected cases corrected for lymphoreticular infiltration, subcloning of PTC cells in tissue culture, and laser capture microdissection of four cases (Guerra et al. 2012a,b). Further studies will therefore be required to resolve this apparent paradox.

In summary, our findings demonstrate that IHC using a BRAF V600E mutation-specific antibody is a rapid, robust, and reliable method for detecting this oncogene, which can readily be deployed in most diagnostic surgical pathology laboratories with a high degree of sensitivity and specificity.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0239.

**Declaration of interest**

Under a licensing agreement between Ventana Medical Systems, Inc., Tucson, Arizona, and the German Cancer Research Center, Dr Capper and Dr von Deimling are entitled to a share of royalties received by the German Cancer Research Center on the sales of VE1 antibody. The terms of this arrangement are being managed by the German Cancer Research Center in accordance with its conflict of interest policies.

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**References**


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