ORIGINAL ARTICLE

Detection of BRAF V600E activating mutation in papillary thyroid carcinoma using PCR with allele-specific fluorescent probe melting curve analysis

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Background: A single hotspot mutation at nucleotide 1799 of the *BRAF* gene has been identified as the most common genetic event in papillary thyroid carcinoma (PTC), with a prevalence of 29–83%.

Aims: To use a PCR assay to molecularly characterise the *BRAF* activating point mutation in a series of PTC and benign thyroid cases and correlate the mutation results with histological findings.

Methods: Formalin-fixed paraffin-embedded (FFPE) sections were evaluated for the *BRAF* V600E mutation using LightCycler PCR with allele-specific fluorescent probe melting curve analysis (LCPCR).

Results: 42 (37 PTC; 5 benign) surgical tissue samples were analysed for the *BRAF* V600E activating point mutation. Using LCPCR and direct DNA sequencing, the *BRAF* mutation was identified in 23/37 (62.2%) PTC FFPE samples. DNA sequencing results demonstrated confirmation of the mutation.

Conclusions: Detection of *BRAF*-activating mutations in PTC suggests new approaches to management and treatment of this disease that may prove worthwhile. Identification of the *BRAF* V600E activating mutation in routine FFPE pathology samples by a rapid laboratory method such as LCPCR could have significant value.

Although thyroid cancer represents only 1% of all human malignancies, it accounts for more than 90% of all endocrine cancers.¹ The incidence of thyroid cancer has risen in the United States, from a rate of 3.6 per 100 000 in 1973 to 8.7 per 100 000 in 2002.² The increase in thyroid cancer incidence can be attributed primarily to an increase in the incidence of papillary thyroid carcinoma (PTC). For the time period 1973 to 2002, the incidence of PTC increased from 2.7 to 7.7 per 100 000, a 2.9-fold increase.² In a report of 15 700 patients in the United States, overall survival rates, corrected for age and sex, were 98% for PTC, 92% for follicular carcinoma, 80% for medullary carcinoma, and 13% for anaplastic carcinoma.³ Among the most curable of cancers, PTC tends to remain localised in the thyroid gland, but in time it may metastasise to regional lymph nodes and, less commonly, to the lungs. Peak incidence of PTC is in the fifth decade of life and it occurs nearly three times more frequently in women than in men.⁴

A single hotspot mutation at nucleotide 1799 of the *BRAF* gene has been identified as the most common genetic event in PTC, with a prevalence of 29–83%.^{5–24} Activating *BRAF* mutations may be an important event in the development of PTC. This mutation had been formerly termed T1796A, based on the NCBI GenBank nucleotide sequence NM 004333, which missed a codon (three nucleotides) in exon 1 of the *BRAF* gene. With the correct version of the NCBI GenBank nucleotide sequence NT 007914 available, this *BRAF* mutation is now designated T1799A.²⁵ This thymine (T) to adenine (A) transversion mutation (T→A) results in the substitution of valine with glutamate in codon 600 (V600E, formerly V599E) and converts *BRAF* into a dominant transforming protein that causes constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, independent of RAS activation.²⁶ Amino acid 600 lies in the kinase domain of *BRAF*, and the V600E mutation makes the enzyme more active than wild-type *BRAF*. The resulting protein shows increased kinase activity that can transform NIH3T3 cells.²⁶ This suggests that therapy with RAF kinase inhibitors may be of use in this disease. If the response

to RAF kinase inhibition is dependent on the presence of an activated *BRAF* protein, it will be necessary to evaluate cases of PTC for the presence or absence of mutations.

The *BRAF* V600E activating point mutation appears to be highly specific for PTC, with no benign or other well-differentiated thyroid neoplasm having been found to harbour this mutation. Moreover, some studies have suggested that *BRAF* mutation may serve as a novel prognostic biomarker that predicts poor clinicopathological outcomes, helping to identify patients who should undergo more aggressive clinical follow-up.^{7 9 27} While these preliminary reports are somewhat controversial^{17 18 21 28–30} and additional studies are required to establish the efficacy of this marker, these findings suggest that *BRAF* mutation detection may serve as a useful tool for diagnosis, management and treatment of PTC.

LightCycler PCR with allele specific fluorescent probe melting curve analysis (LCPCR) has been used successfully to detect *BRAF* activating point mutations in PTC.⁷ Recently, we reported on the clinical utility of this method for detecting *BRAF* mutations in a series of indeterminate thyroid fine needle aspirate (FNA) cytology samples.³¹ Based on the detection of either a fluorescent reporter probe or double-stranded DNA-binding dyes, “real-time” PCR instruments, such as the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) can provide quantitative information regarding target nucleic acid sequences. The LightCycler is a microvolume fluorimeter integrated with a thermal cycler. Using post-amplification melting curve analysis, the LightCycler instrument can also be used to differentiate alleles for the purpose of determining sequence variations or point mutations. In this report, we used LCPCR to molecularly characterise the *BRAF* activating point mutation in a series of PTC and benign thyroid cases and correlate the mutation results with histological findings.

Abbreviations: FFPE, formalin-fixed paraffin-embedded; FNA, fine needle aspirate; FVPTC, follicular variant of PTC; LCPCR, LightCycler PCR with allele-specific fluorescent probe melting curve analysis; MAPK, mitogen-activated protein kinase; PTC, papillary thyroid carcinoma; WT, wild type

Figure 1 Melting curve analysis of *BRAF* mutations in thyroid surgical tissue samples. Fluorescently labelled oligonucleotide probes were used with extracted DNA to detect mutations in exon 15 of *BRAF*. Multiple probes complementary to the wild-type (WT) sequences were placed within the same reaction, and the different sites were identified by their specific probe/target melting temperatures. The position of each probe/target melting temperature and the relative ratio of the melting peak areas determined WT profiles. After amplification in a LightCycler, the instrument begins a melting programme where the reactions are cooled to anneal the probes and then slowly heated (0.1°C/s) while fluorescence is continuously monitored. Somatic mutations are identified by changes from a characteristic WT melting curve profile. When melting curves from non-mutated and mutated samples are compared, additional melting peaks or changes in peak-area ratios indicate a sequence alteration (nucleotide mismatch) under the probe. Melting curve analysis revealed that the WT *BRAF* sequence T_m was $64.92^{\circ}\text{C} \pm 0.35^{\circ}\text{C}$, the mutation at nucleotide 1799, resulted in a shift to $60.11^{\circ}\text{C} \pm 0.46^{\circ}\text{C}$.

METHODS

This retrospective study was approved by the University of Utah Institutional Review Board (#13005). It was conducted in accordance with all federal regulations governing human research. Study samples were identified from a database, established by the University of Utah Department of Surgery (University of Utah Institutional Review Board #11565), of all patients having undergone treatment for cancer of the thyroid gland at the University of Utah Hospitals and Clinics from 1994 to 2004.

From the thyroid cancer database, we identified those individuals with a formalin-fixed, paraffin-embedded (FFPE) tissue confirming PTC. An experienced surgical pathologist performed additional review of each case in order to confirm the diagnosis. In order to be classified as the follicular variant of PTC (FVPTC), 90% or more of the tumour showed a follicular pattern. Conversely, if less than 90% of the tumour demonstrated a follicular pattern it was classified as classic PTC. The surgical pathology tissue blocks were retrieved from the surgical pathology laboratory tissue block archives. Additionally, we identified FFPE thyroid tissue samples demonstrating benign findings. These samples were selected to serve as negative controls.

Genomic DNA was isolated from the FFPE surgical tissue resections using a proteinase K digestion method described previously.³¹ Briefly, from the FFPE tissue block, 5 µm sections were cut. One tissue section was H&E stained and coverslipped for review; the area of the tissue section containing the tumour was identified. The remaining unstained sections were deparaffinised and the slides were allowed to air-dry completely. Following deparaffinisation, the unstained tissue section slide was then inverted over the H&E stained slide and the area of tumour was marked on the underside of the unstained tissue section slide. A scalpel blade was used to manually scrape the areas of the tissue containing the tumour cells of interest. Following manual microdissection, proteinase K (3 mg/ml) digestion solution (50 mM Tris, 1 mM EDTA, pH 8.0, 1% Tween 20) was pipetted onto the scraped area of the slide to pick up any remaining cells.

The samples were incubated at 55°C for 12–16 hours. Following centrifugation, the supernatant was transferred into a newly labelled microcentrifuge tube. The samples were then placed into a 95°C heat block for 10 minutes to inactivate the proteinase K. The FFPE tissue sample DNA was diluted to a working concentration of 50 ng/µl prior to amplification. Following DNA extraction, all samples were stored at -20°C prior to analysis.

Control material for the LCPCR detection of the *BRAF* V600E mutation was developed from three human cell lines. One human PTC-derived cell line (NPA), which contains a homozygous T→A transversion mutation in codon 600 of the *BRAF* gene, was used as a positive control. One follicular thyroid carcinoma (ROW-1), and one colorectal carcinoma (HCT116) cell line, both of which contain the wild-type *BRAF* sequence, were used as negative controls.

A pair of oligonucleotide primers were designed to amplify a 250 base-pair region of exon 15 in the *BRAF* gene: forward: 5'-CTC TTC ATA ATG CTT GCT CTG ATA GG-3'; reverse: 5'-TAG TAA CTC AGC AGC ATC TCA GG-3' (Integrated DNA Technologies, Coralville, IA, USA). Amplicon size was confirmed using a 2% agarose DNA gel.

Two fluorescent hybridisation probes were designed to detect the *BRAF* V600E wild-type sequence⁷: sensor: 5'-AGC TAC AGT GAA ATC TCG ATG GAG-fluorescein-3'; anchor: 5'-LCRed640-GGT CCC ATC AGT TTG AAC AGT TGT CTG GA-phosphate-3' with the sensor probe spanning nucleotide position 1799 (Idaho Technologies, Salt Lake City, UT, USA).

Amplification was performed in glass capillaries using 50 ng of FFPE sample DNA in a 10 µl total volume. The reaction mixture underwent 45 cycles of rapid PCR consisting of denaturation at 94°C for 1 second, annealing at 55°C for 20 seconds, and extension at 72°C for 10 seconds. Transition rates were 20°C/s from denaturation to annealing, 20°C/s annealing to extension, and 20°C/s extension to denaturation.

Following PCR, the reaction mixture is cooled in order to allow amplicon/probe heteroduplexes to form. When the probes hybridise next to each other on their target DNA sequences, the fluorescent molecules are brought close together and fluorescence

Figure 2 Representative sequence chromatographs of a region of exon 15 of the *BRAF* gene. Arrow denotes heterozygous T→A mutation (A) and wild-type (B) in papillary thyroid carcinoma.

resonance energy transfer occurs. Slowly heating the amplicon/probe heteroduplex causes the probe to denature away from the amplicon, resulting in an increase in the distance between the two dyes. Using LCPCR, the decrease in fluorescence that results when the probe denatures or “melts” away from the amplicon is measured to generate a melting curve. The difference in melting profiles between mismatched probe/target and perfectly matched probe/target can be used to characterise amplification products and indicate the presence of a mutation.

Post-amplification fluorescent melting curve analysis was performed by gradual heating of the samples at a rate of 0.1°C per second from 45°C to 95°C. Fluorescent melting peaks were determined by plotting of the negative derivative of fluorescence (F) with respect to temperature (T), or $-dF/dT$.

A limit of detection experiment was conducted to determine the percent of tumour with normal cell contamination in which abnormal melts were detectable by LCPCR. The NPA cell line was diluted with human genomic wild-type (WT) DNA to 99% tumour, 95% tumour, 90% tumour, 75% tumour, 50% tumour, 25% tumour, 10% tumour, and 5% tumour. In addition, 100% NPA and 100% WT samples were tested. Each dilution was run in duplicate using LCPCR.

All PCR products that showed deviation from the WT genomic DNA melting peak and all benign tissue control samples underwent direct sequencing of *BRAF* gene exon 15.

RESULTS

A total of 42 archival FFPE (PTC 37; benign 5) surgical tissue samples were evaluated for the *BRAF* V600E point mutation using LCPCR. On pathologist review, 18/37 (48.6%) cases were classified histologically as classic PTC, 15/37 (41.0%) were classified as FVPTC, 2/37 (5.4%) was classified as a mixed classic and follicular tumour and 2/37 (5.4%) were classified as tall cell variant.

Results of the detection limit experiments confirmed that the 1 base pair change in the *BRAF* mutation was detectable down to the level of 25% tumour when a homozygous mutant cell line (NPA) was used as a control. Consequently, it was determined that the results of LCPCR for detection of the heterozygous *BRAF* V600E mutation in FFPE samples containing less than 50% tumour cells may not be accurate.

Using LCPCR and direct DNA sequencing, the *BRAF* activating point mutation was identified in 23/37 (62.2%) PTC FFPE

samples. Melting curve analysis revealed that the WT sequence (GTG) T_m was $64.92^\circ\text{C} \pm 0.35^\circ\text{C}$, while the mutant T_m was $60.11^\circ\text{C} \pm 0.46^\circ\text{C}$ (fig 1).

Bidirectional DNA sequencing revealed that all *BRAF* mutations were heterozygous and involved a T→A substitution at nucleotide 1799 (fig 2). All five benign thyroid FFPE samples were found to be negative for the *BRAF* mutation. The LCPCR assay demonstrated 100% concordance between melting curve and DNA sequence results (table 1).

The *BRAF* V600E activating point mutation was identified in 14/18 (77.8%) cases of classic PTC, 5/15 (33.3%) cases of follicular variant of PTC, 2/2 (100%) cases classified as mixed classic/follicular and 2/2 (100%) cases classified as tall cell variant.

CONCLUSIONS

A number of molecular events have been identified in thyroid cancer. However, the predictive value of these markers for clinical use has been limited to date due to a lack of specificity, sensitivity, or both.³² Consequently, investigators have focused on the discovery of additional targets associated with the molecular genetics of thyroid cancer. The RAS/RAF/MEK/ERK MAPK cascade mediates cellular responses to growth signals. The pathway consists of a cell surface receptor that, on ligand binding, activates a small binding protein of the RAS family, leading to activation of a series of protein phosphorylations and changes in transcription and cell physiology. Following activation of RAS, phosphorylation of the RAF family of proteins occurs, which include RAF1 (cRAF), ARAF, and BRAF, all of which act as intermediaries to transducer signals to the cell nucleus through phosphorylation of various downstream proteins. The mutation at V600E in the *BRAF* kinase gene appears to be an attractive molecular marker for thyroid cancer diagnosis as it has been found to be the most common genetic event in PTC, while being highly specific for this tumour.^{5–24} Studies have shown that the *BRAF* V600E activating point mutation is present in approximately 44% of all classic PTCs (range 29–83%) (table 2).

The results of this study appear similar to these previous reports of *BRAF* mutation in PTC. The LCPCR method identified the *BRAF* V600E activating point mutation in 23/37 (62.2%) PTC cases.

Although the LCPCR method may be a useful molecular tool for identification of *BRAF* mutations, overall tumour cell content of the sample is critical. Limit of detection experiments in the present study confirmed that LCPCR in samples containing less than 50% tumour cells might not be accurate. While this constraint may not affect FFPE tissue samples in which careful manual microdissection can help limit the amount of WT DNA present in the PCR reaction, it could limit the application of this method in FNA samples in which sampling often requires the needle to traverse through adjacent normal parenchyma. In a previous report on the utility of *BRAF* V600E mutation detection in cytologically indeterminate thyroid nodules, we identified six FNA samples that were negative for the *BRAF* V600E mutation in which the corresponding FFPE tissue samples were positive.³¹ Of these, 5/6 FNA samples contained less than 50% tumour cells. Nikiforova *et al* reported that using a technique such as laser capture microdissection can significantly enhance the sensitivity for identifying mutant DNA in the presence of WT DNA, as the captured sample contains almost exclusively tumour cells.⁷ It is possible that this method could be used to increase the potential for use of LCPCR for *BRAF* mutation detection in FNA samples.

Because the hybridisation probes used in the present study were designed as a perfect match to the *BRAF* WT genotype,

Table 1 Detection of *BRAF* V600E mutation in benign thyroid lesions and papillary thyroid carcinoma using DNA isolated from formalin-fixed paraffin-embedded samples

Sample	Diagnosis	<i>BRAF</i> V600E mutation	
		LCPCR result	Sequencing result
1	PTC; follicular variant	MUT	MUT
2	PTC; follicular variant	MUT	MUT
3	PTC	MUT	MUT
4	PTC	WT	WT
5	PTC	MUT	MUT
6	PTC	MUT	MUT
7	PTC	WT	WT
8	PTC	MUT	MUT
9	PTC	MUT	MUT
10	PTC; tall cell variant	MUT	MUT
11	PTC	WT	WT
12	PTC; tall cell variant	MUT	MUT
13	PTC; follicular variant	MUT	MUT
14	PTC	MUT	MUT
15	PTC	MUT	MUT
16	PTC; follicular variant	WT	WT
17	PTC; follicular variant	WT	WT
18	PTC	MUT	MUT
19	PTC; follicular variant	WT	WT
20	PTC	MUT	MUT
21	PTC	MUT	MUT
22	PTC; follicular variant	MUT	MUT
23	PTC	MUT	MUT
24	PTC; mixed	MUT	MUT
25	PTC; follicular variant	WT	WT
26	PTC; follicular variant	WT	WT
27	PTC; follicular variant	WT	WT
28	PTC; follicular variant	WT	WT
29	PTC; follicular variant	WT	WT
30	PTC	MUT	MUT
31	PTC; follicular variant	WT	WT
32	PTC; follicular variant	WT	WT
33	PTC; mixed	MUT	MUT
34	PTC	MUT	MUT
35	PTC; follicular variant	MUT	MUT
36	PTC	MUT	MUT
37	PTC	WT	WT
38	Colloid nodule	WT	WT
39	Follicular neoplasm	WT	WT
40	Hurthle cell neoplasm	WT	WT
41	Hyperplastic nodule	WT	WT
42	Adenomatous nodule	WT	WT

LCPCR, LightCycler PCR with allele-specific fluorescent probe melting curve analysis; PTC, papillary thyroid carcinoma; WT, wild-type *BRAF* sequence; MUT, *BRAF* V600E heterozygous mutation.

other mutations covered by the probe would likely lead to a different melting temperature profile and probable mutation detection. Recent studies have shown that up to 9% of all cases of FVPTC demonstrate a mutation in codon 601 of the *BRAF* gene, resulting in the substitution of lysine with glutamate (K601E).^{5, 28} Of the 15 FVPTC cases in the present study, none were positive for the K601E mutation. However, because the number of FVPTC cases in our series was small, a much larger number of cases is needed to validate the previously published reports of K601E mutation in FVPTC cases.

While papillary thyroid tumours may have a pure papillary histopathology, they may also contain an admixture of follicular elements. Although the *BRAF* V600E mutation is primarily associated with PTCs with papillary architecture, it is less commonly associated with FVPTC and the reported prevalence of this mutation in these cases varies widely, ranging from 0% to 42%.^{5, 17, 18, 33} As a result, some investigators have suggested that the usefulness of *BRAF* V600E as a diagnostic marker of FVPTC is limited. The histopathological diagnosis of FVPTC can be difficult, however and significant interobserver variation exists. Lloyd *et al* evaluated the

Table 2 Prevalence of *BRAF* V600E mutation in papillary thyroid carcinoma

Series	<i>BRAF</i> mutation frequency	
	PTC (%)	Benign (%)
Kimura <i>et al</i> , 2003	28/78 (36)	0/26 (0)
Cohen <i>et al</i> , 2003	24/35 (69)	0/20 (0)
Xu <i>et al</i> , 2003	21/56 (38)	0/24 (0)
Soares <i>et al</i> , 2003	23/50 (46)	0/72 (0)
Fukushima <i>et al</i> , 2003	40/76 (53)	
Namba <i>et al</i> , 2003	49/170 (29)	0/20 (0)
Nikiforova <i>et al</i> , 2003	45/119 (38)	0/111 (0)
Xing <i>et al</i> , 2004	18/30 (60)	0/9 (0)
Xing <i>et al</i> , 2004	14/28 (50)	0/54 (0)
Xing <i>et al</i> , 2004	8/16 (50)	0/21 (0)
Trovisco <i>et al</i> , 2004	45/124 (36)	
Kim <i>et al</i> , 2004	58/70 (83)	
Nikiforova <i>et al</i> , 2004	30/82 (37)	
Cohen <i>et al</i> , 2004	36/95 (38)	0/32 (0)
Fratini <i>et al</i> , 2004	19/60 (32)	
Fugazzola <i>et al</i> , 2004	18/56 (32)	0/1 (0)
Puxeddu <i>et al</i> , 2004	24/60 (40)	0/6 (0)
Penko <i>et al</i> , 2004	97/232 (42)	
Salvatore <i>et al</i> , 2004	26/69 (38)	
Sedliarou <i>et al</i> , 2004	13/56 (28)	
Vasil'ev <i>et al</i> , 2004	55/91 (60)	0/24 (0)
Perren <i>et al</i> , 2004	7/15 (47)	
Hayashida <i>et al</i> , 2004	37/72 (51)	0/45 (0)
Nakamura <i>et al</i> , 2005	16/42 (38)	0/42 (0)
Present study	23/37 (62)	0/5 (0)
Total	774/1819 (43%)	0/512

correlation between 10 experienced thyroid pathologists in the diagnosis of FVPTC in 87 tumours and determined that the diagnostic criteria used to establish a diagnosis of FVPTC, including pseudo-inclusions, nuclear grooves, and powdery nuclei, are not uniformly recognised by experts.³⁴ A concordant diagnosis of FVPTC was made by all 10 reviewers with a cumulative frequency of only 39%. In the present study, the *BRAF* V600E mutation was identified in 5/15 (33.3%) cases of follicular variant. Additional studies may confirm that *BRAF* mutation analysis in this subset of difficult PTC cases could prove to be a useful adjunct to conventional diagnostic methods.

Because of its high prevalence rate, and its association with tumours presenting at advanced stages, *BRAF* has become an attractive target for small molecule kinase inhibitors, which are currently in development. BAY43-9006 (sorafenib) is a novel signal transduction inhibitor that is intended to prevent tumour growth by blocking the RAS signalling pathway, and stop tumour angiogenesis by inhibiting the vascular endothelial growth factor receptor-2/platelet derived growth factor receptor- β signalling cascade. Sorafenib is in clinical trial investigation as an antineoplastic agent in a variety of cancers,³⁵⁻³⁷ including well-differentiated thyroid cancer.³⁸

Identification of the *BRAF* V600E activating point mutation by a rapid laboratory method could have significant value. Detection of single DNA nucleotide gene mutations, such as *BRAF*, using a rapid real-time PCR method, appears feasible using routine FFPE tissue samples. The presence of *BRAF*-activating mutations in PTC suggests that new approaches to treating this disease involving RAF kinase inhibitors may prove worthwhile, and that mutation analysis by LCPCR might help guide therapy and management of these patients.

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Take-home messages

- BRAF V600E gene mutation is the most common genetic event in papillary thyroid carcinoma (PTC) (29–83%), and is highly specific for PTC.
- This mutation converts BRAF into a dominant transforming protein that causes constitutive activation of the mitogen-activated protein kinase pathway.
- Evaluation of cases of PTC for the presence or absence of BRAF mutation may be clinically useful to determine PTC in indeterminate thyroid nodules, predicting prognosis, and response to RAF kinase inhibition.
- PCR with allele specific fluorescent probe melting curve analysis (LCPCR) can be successfully used to detect the presence of BRAF activating point mutations using formalin-fixed paraffin-embedded tissue.
- The V600E mutation was identified using LCPCR in 62% of a series of PTC; 78% in classical PTC and 33% follicular variant.

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