

Detection of *BRAF* V600E mutation in thyroid fine-needle aspiration specimens by High Resolution Melting (HRM) analysis

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Abstract

Aim: The aim of our study was to test the feasibility of High Resolution Melting (HRM) analysis for detection of *BRAF* V600E mutation in various types of fine-needle aspiration (FNA) specimens from patients with papillary thyroid carcinoma (PTC). **Materials and Methods:** We analyzed fresh thyroid aspirates and smears from eight cases of PTC: three classic PTCs (CPTC), three follicular variant of PTCs (FVPTC), one tall cell, and one oncocyctic variant of PTC. DNA extraction was performed using a MasterPure purification kit. The isolated DNA quantity was assessed using a NanoDrop spectrophotometer and the DNA quality was tested by PCR amplification of β -globin gene and by native DNA electrophoresis. HRM was performed on a LightCycler 480 (Roche). We amplified the 15th exon of *BRAF* gene, using selected primers to flank the *BRAF* V600E mutation point. **Results:** For all types of cytological specimens, the quantity of isolated DNA was adequate and allowed amplification. Similarly, the DNA quality control did not show signs of DNA degradation and the DNA was amplifiable for β -globin gene. Four cases revealed the *BRAF* V600E mutation: two CPTCs, one oncocyctic PTC, one tall cell PTC. None of the three cases of FVPTC had this mutation. **Conclusions:** HRM analysis represents a feasible and reproducible molecular technique, offering new perspectives for detecting *BRAF* mutation in various FNA specimens. In our study, *BRAF* V600E mutation revealed a strong association with specific histological variants of PTC: highly specific for CPTC, tall cell or oncocyctic PTC, but negative in all cases of FVPTC.

Keywords: papillary thyroid carcinoma, fine-needle aspiration, *BRAF* V600E mutation, High Resolution Melting analysis.

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, accounting for more than 80% of the cases [1]. According to the literature, 30 to 70% of PTC cases harbor the *BRAF* gene mutation [2, 3], also referred to as the “genetic signature of PTC” [4].

BRAF mutation occurs on the *p* arm of the 7th chromosome, where the gene is situated. The vast majority (>95%) of *BRAF* mutations found in thyroid cancer are a thymine to adenine transversion at nucleotide 1799 (T1799A) [5], leading to a substitution of valine by glutamic acid at the residue 600 of the protein (V600E). The result of the genetic alteration is activation of the mitogen-activated protein kinase (MARK) signaling pathway [6], which plays a major role in the regulation of cell growth, division and proliferation [7] and is responsible for the malignant transformation in PTC [8].

Several studies have demonstrated that *BRAF* V600E mutation can be detected in fine-needle aspiration (FNA) specimens using different modern molecular techniques [4, 9–11]. It has been shown that *BRAF* V600E mutation testing not only may improve the cytological FNA

diagnosis, but also provides a novel tool for pre-operative risk stratification of thyroid cancer [12].

Direct DNA sequencing has been the most widely used method for *BRAF* V600E mutation detection [13–15], although it has limitations in terms of sensitivity, especially if only few mutated follicular cells are present in the FNA specimen [10, 11].

The aim of our study was to test the feasibility of a new, innovative PCR (Polymerase Chain Reaction) method, High Resolution Melting (HRM) analysis for detection of *BRAF* V600E mutation in our laboratory, using various types of FNA specimens from patients with PTC.

As a second objective, we have compared the *BRAF* V600E mutation status to several pathological parameters, including histopathological subtype, multifocality, extra-thyroidal extension and lymph node metastasis.

Materials and Methods

Case selection

Fresh FNA specimens (drop and washout solution), as well as Giemsa stained smears from eight cases of PTC registered at the Department of Pathology, Centre

Hospitalier Lyon Sud, Hospices Civils de Lyon, from January to May 2011 were tested for detection of *BRAF* V600E mutation. Our study group included three cases of classic or conventional PTC (CPTC), three cases of follicular variant of PTC (FVPTC), one case of tall cell variant and one case of oncocytic variant of PTC.

The histological subtypes of PTC were classified according to *WHO* criteria [1]. The microscopic diagnosis of PTC was exclusively based on nuclear features: enlargement, overlapping, irregularity of the nuclear contours, grooves, clearing or a ground glass appearance and nuclear pseudoinclusions. CPTCs had a characteristic papillary architecture that was pure or admixed with a variable proportion of follicles. The tumors defined as FVPTC revealed an exclusively follicular architecture, characteristic PTC nuclear changes in most of the cells lining these follicles and virtually no papillary structures. Tall cell variant of PTC was characterized by predominance (more the 50%) of tall columnar cells, whose heights were at least three times their widths. The diagnosis of oncocytic variant of PTC was set based on the presence of a majority of oncocytic tumor cells, with abundant, dense eosinophilic, granular cytoplasm and nuclear features identical to those seen in CPTC.

The ethics committee of the medical faculty and the state medical board agreed to these investigations, and informed consent was obtained from all of the patients included in this study.

DNA extraction

DNA extraction was performed using the Blood and Cell Culture Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, after a prior incubation of one hour at 56°C with 20-μL proteinase K and 200-μL DNA extraction buffer. Regarding the smeared specimens (two specimens for each case), the material on the surface of each slide was scraped using a sterile knife and gathered in the center of the slide. A drop of tissue lysing solution was added to the scraped material and then the material was easily pipetted into the collection tube. For assessment of DNA extraction quality, we used a negative extraction control, represented by a DNA-free phosphate buffer saline (PBS) solution that underwent all the extraction steps, similarly to the rest of the study material.

Isolated DNA was quantified by spectrophotometric absorption at 260 nm and evaluation of A 260/A 280 ratio (NanoDrop1000 Spectrophotometer, Pharmacia Biotech GeneQuant II).

The DNA quality was further tested by PCR amplification of β-globin gene and by native DNA electrophoresis on an agarose gel.

HRM technique

HRM was performed on a LightCycler 480 (Roche Diagnostics, Vienna, Austria). We amplified the 15th exon of *BRAF* gene, using a pair of selected primers (forward and reverse) to flank the *BRAF* V600E mutation point (Primers purify HPLC, Sigma). The 147 bp amplicon was evaluated for the ability to detect *BRAF* V600E mutation. Amplification was performed using a reaction mixture comprising 2 μL of DNA solution, 0.2 μL of each primer, 10 μL of LightCycler LC480 Probe Master (Roche), 1 μL Resolight Dye solution (Roche) and water to a final volume of 20 μL. For each set of reactions, two positive, three negative and one 1/2 positive controls were used and all reactions were performed in duplicate.

The reaction mixture was then subject to PCR amplification, denaturation and reannealing, allowing for heteroduplex formation. For detection of sequence variation and presence of *BRAF* V600E mutation, differences in melting curves of the amplicons were analyzed. Due to a sequence mismatch, heteroduplex DNA melts at a different temperature than homoduplex DNA. Resolight Dye solution (Roche), a fluorescent dye that binds only to double stranded DNA, allowed detection of this temperature variation, translated by different shapes of melting curves obtained with homoduplex and heteroduplex samples, respectively.

Samples were considered mutated when significant fluorescence level for all duplicated fell out-side the range of variation for wild-type control and in the corresponding range for positive controls.

Results

Table 1 describes the clinico-pathological characteristics of the eight cases of PTC included in our study.

Table 1 – Clinico-pathological data and molecular findings (*BRAF* status) in FNA specimens from eight cases of PTC

No.	Age [years] / Sex	Tumor size [mm]	Cytological preoperative diagnosis (according to Bethesda System)	Histopathological diagnosis	Multifocality	Extra-thyroidal extension	Lymph node metastasis	<i>BRAF</i> status
1.	38 / F	22	FLUS/AUS (class III)	FVPTC	+	-	-	-
2.	54 / F	15	Suspicious for malignancy (class V)	CPTC	+	-	-	inconclusive
3.	70 / M	20	FN/SFN (class IV)	FVPTC	+	-	-	-
4.	55 / F	8	Malignant (class VI)	PTC, tall cell variant	+	-	-	+
5.	72 / F	21	Malignant (class VI)	FVPTC	-	-	-	-
6.	64 / F	25	Malignant (class VI)	PTC, oncocytic variant	+	+	-	+
7.	63 / F	22	Malignant (class VI)	CPTC	+	+	-	+
8.	63 / F	13	Malignant (class VI)	CPTC	+	-	-	+

FLUS/AUS: follicular lesion of undetermined significance/atypia of undetermined significance; FN/SFN: follicular neoplasm/suspicious for follicular neoplasm; PTC: Papillary Thyroid Carcinoma; CPTC: Classic Papillary Thyroid Carcinoma; FVPTC: Follicular Variant of Papillary Thyroid Carcinoma.

All but one of the patients were females and the patients had a mean age of 60 years. All cases of CPTC, oncocyctic PTC and tall cell variant of PTC corresponded to Bethesda V or VI diagnostic categories on preoperative cytology. By contrast, only one case of FVPTC was addressed as malignant (Bethesda VI) on cytology, while the remaining two cases had a prior diagnosis on cytology consistent with Bethesda III or IV categories.

For all types of cytological specimens (drop and washout solution, as well as Giemsa stained smears) of all eight cases of PTC, the quantity of isolated DNA was adequate and allowed amplification (DNA concentration values ranged from 9.4 to 110.1 ng/ μ L and the A 260/A 280 ratio was in between the 1.8–2.2 interval). The DNA quality control did not show signs of DNA degradation and the DNA was amplifiable for β -globin gene in all types of cytological specimens (Figures 1 and 2).

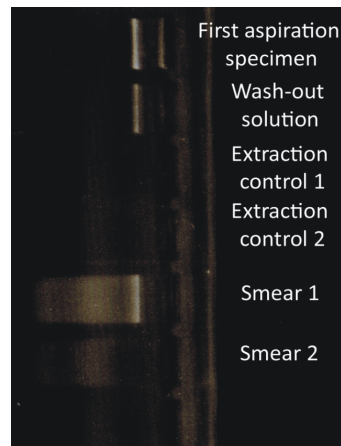
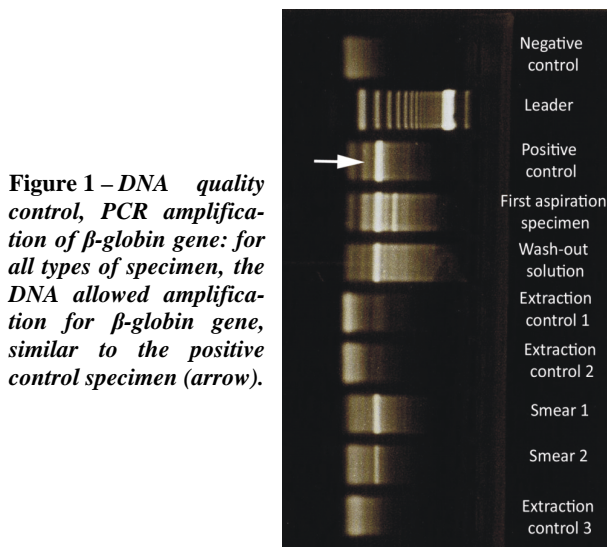


Figure 2 – DNA quality control, native DNA electrophoresis: example of one case in which DNA was adequate for all types of specimens, except for Smear 2 where DNA fragmentation could be observed.

Regarding the HRM technique, the amplification curves for all types of specimens reached a similar plateau height (Figure 3) and the resulting melting curves revealed a characteristic shape, different for heterozygous or mutated samples and different for homozygous or non-mutated samples (Figure 4), thus allowing the detection of *BRAF* V600E mutation. Furthermore, we were able to detect the mutation using all types of cytological specimens (drop and washout aspirates, as well as Giemsa stained smears).

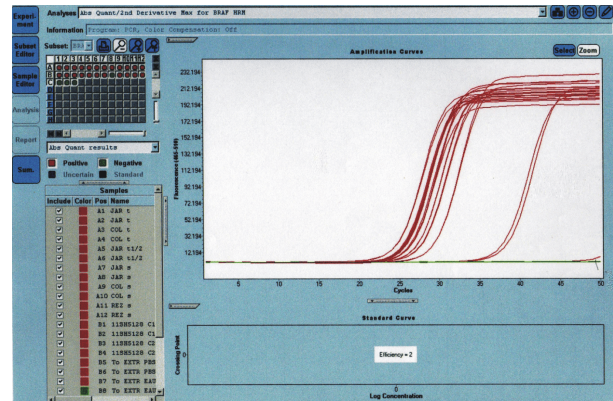


Figure 3 – HRM analysis, amplification curves: the curves for all specimens reached a similar plateau height.

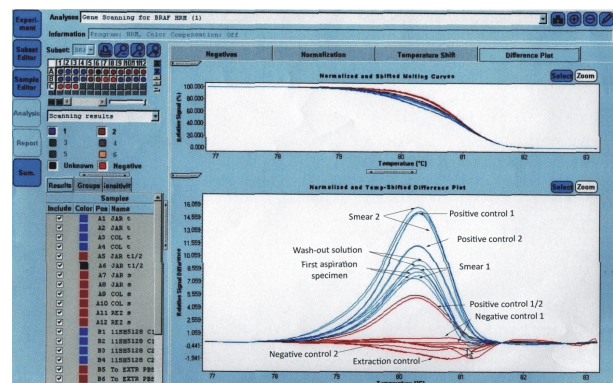


Figure 4 – HRM analysis: the resulting melting curves revealed a characteristic shape, different for heterozygous or mutated samples (blue) and different for homozygous or non-mutated samples (red). We used two positive, three negative and one-half positive controls (all controls being priori tested by DNA sequencing). *BRAF* V600E mutation was positive in this case of classic PTC.

The results on the *BRAF* V600E mutation status for all the cases included in our study are presented in Table 1. Four cases revealed this mutation, including two cases of CPTC, one case of oncocyctic PTC and on case of tall cell variant of PTC. None of the three cases of FVPTC had the *BRAF* V600E mutation. Interestingly, one case of CPTC showed an inconclusive result regarding its mutated or non-mutated status. The two cases of PTC with extra-thyroidal extension were both *BRAF* V600E mutated.

Discussion

In this study, we have evaluated the feasibility of a new, innovative PCR technique (HRM technique) for detecting of *BRAF* V600E mutation in FNA samples from patients with PTC. Our results revealed that *BRAF* V600E can perfectly be detected by HRM technique using various types of cytological specimens (drop and washout aspirates, as well as Giemsa stained smears).

BRAF has been one of the most common studied genes in thyroid cancers in the recent years and search of *BRAF* mutation alone or in combinations with other oncogenes (*RAS*, *BRAF*, *RET/PTC*) has been reported with encouraging results, both as a potential diagnostic

and prognostic marker for thyroid cancer [2, 3, 16]. Thus, this reliable diagnostic test may influence clinical diagnosis and therapeutic decisions.

HRM analysis is a recently developed molecular technique proved to be applicable for detection of various clinically relevant human mutations, including *BRAF* V600E mutation [17]. It allows high-throughput analysis, without any post-PCR processing, features that are required characteristics for research and clinical application.

BRAF V600E mutation has a strong association with specific histological variants of PTC. It is highly prevalent in the tall cell variant of PTC, where it occurs in 70–80% of cases [2, 18], in CPTC (about 55% of cases) [2, 18], in oncocytic variant of PTC (about 50% of cases) [19] and is absent or very rare (about 5% of cases) in FVPTC [18]. Although we have evaluated only eight cases of PTC, our results mirror the close correlation between the *BRAF* V600E gene mutation and the histological subtype of PTC. All but one of the CPTC cases and both tall cell and oncocytic variant of PTC cases were positive for the *BRAF* V600E mutation, while none of the three cases of FVPTC were *BRAF* V600E mutated.

Although very specific for CPTC, tall cell or oncocytic variant of PTC, detection of *BRAF* V600E mutation was not a reliable marker for FVPTC in our study. The three cases of FVPTC, two consistent with Bethesda III or IV and one with Bethesda VI diagnostic category in cytology, revealed a negative *BRAF* V600E status.

BRAF V600E is generally accepted as a reliable prognostic marker and its association with more aggressive tumor characteristics is well documented [3, 14]. In our study, only two cases of PTC were associated with extra-thyroidal extension and they were both *BRAF* V600E mutated.

✉ Conclusions

We have demonstrated that HRM represents a feasible and reproducible molecular technique, offering new perspectives for detecting *BRAF* V600E mutation in various FNA specimens.

In our study *BRAF* V600E mutation revealed a strong association with specific histological variants of PTC and with some pathological indicators of aggressive behavior such as extra-thyroidal extension. Although very specific for CPTC, tall cell or oncocytic variant of PTC, *BRAF* V600E mutation did not proved to be a reliable diagnostic marker for FVPTC.

Abbreviations

CPTC: Classic / Conventional Papillary Thyroid Carcinoma.

FNA: Fine-Needle Aspiration.

FVPTC: Follicular Variant of Papillary Thyroid Carcinoma.

HRM analysis: High Resolution Melting analysis.

PCR: Polymerase Chain Reaction.

PTC: Papillary Thyroid Carcinoma.

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References

- [1] DeLellis RA, Williams ED, Pathology of the thyroid and parathyroid. In: DeLellis RA, Lloyd RV, Heitz PU (eds), *Pathology and genetics of tumors of endocrine organs*, IARC Press, Lyon, 2004, 57–66.
- [2] Xing M, *BRAF* mutation in thyroid cancer, *Endocr Relat Cancer*, 2005, 12(2):245–262.
- [3] Nikiforov YE, *Molecular analysis of thyroid tumors*, *Mod Pathol*, 2011, 24(Suppl 2):S34–S43.
- [4] Kucukodaci Z, Akar E, Haholu A, Baloglu H, *A valuable adjunct to FNA diagnosis of papillary thyroid carcinoma: in-house PCR assay for BRAF T1799A (V600E)*, *Diagn Cytopathol*, 2011, 39(6):424–427.
- [5] Garnett MJ, Marais R, *Guilty as charged: B-Raf is a human oncogene*, *Cancer Cell*, 2004, 6(4):313–319.
- [6] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA, *Mutations of the BRAF gene in human cancer*, *Nature*, 2002, 417(6892):949–954.
- [7] Hilger RA, Scheulen ME, Strumberg D, *The Ras-Raf-MEK-ERK pathway in the treatment of cancer*, *Onkologie*, 2002, 25(6):511–518.
- [8] Mercer KE, Pritchard CA, *Raf proteins and cancer: B-Raf is identified as a mutational target*, *Biochim Biophys Acta*, 2003, 1653(1):25–40.
- [9] Dujardin F, Pagès JC, Collin C, de Calan L, Lecomte P, Guyétant S, *BRAF V600E mutation in papillary thyroid carcinoma: prevalence and detection in fine needle aspiration specimens*, *Ann Pathol*, 2010, 30(4):252–262.
- [10] Kim SK, Kim DL, Han HS, Kim WS, Kim SJ, Moon WJ, Oh SY, Hwang TS, *Pyrosequencing analysis for detection of a BRAFV600E mutation in an FNAB specimen of thyroid nodules*, *Diagn Mol Pathol*, 2008, 17(2):118–125.
- [11] Kwak JY, Kim EK, Kim JK, Han JH, Hong SW, Park TS, Choi JR, *Dual priming oligonucleotide-based multiplex PCR analysis for detection of BRAFV600E mutation in FNAB samples of thyroid nodules in BRAFV600E mutation-prevalent area*, *Head Neck*, 2010, 32(4):490–498.
- [12] Xing M, Clark D, Guan H, Ji M, Dackiw A, Carson KA, Kim M, Tufaro A, Ladenson P, Zeiger M, Tufano R, *BRAF mutation testing of thyroid fine-needle aspiration biopsy specimens for preoperative risk stratification in papillary thyroid cancer*, *J Clin Oncol*, 2009, 27(18):2977–2982.
- [13] Chung KW, Yang SK, Lee GK, Kim EY, Kwon S, Lee SH, Park do J, Lee HS, Cho BY, Lee ES, Kim SW, *Detection of BRAFV600E mutation on fine needle aspiration specimens of thyroid nodule refines cyto-pathology diagnosis, especially in BRAFV600E mutation-prevalent area*, *Clin Endocrinol (Oxf)*, 2006, 65(5):660–666.
- [14] Xing M, Westra WH, Tufano RP, Cohen Y, Rosenbaum E, Rhoden KJ, Carson KA, Vasko V, Larin A, Tallini G, Tolaney S, Holt EH, Hui P, Umbricht CB, Basaria S, Ewertz M, Tufaro AP, Califano JA, Ringel MD, Zeiger MA, Sidransky D, Ladenson PW, *BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer*, *J Clin Endocrinol Metab*, 2005, 90(12):6373–6379.
- [15] Kim SJ, Lee KE, Myong JP, Park JH, Jeon YK, Min HS, Park SY, Jung KC, Koo do H, Youn YK, *BRAF V600E mutation is associated with tumor aggressiveness in papillary thyroid cancer*, *World J Surg*, 2012, 36(2):310–317.

- [16] Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, Fagin JA, Falciglia M, Weber K, Nikiforova MN, *Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules*, J Clin Endocrinol Metab, 2009, 94(6):2092–2098.
- [17] Pichler M, Balic M, Stadelmeyer E, Ausch C, Wild M, Guelly C, Bauernhofer T, Samonigg H, Hoefler G, Dandachi N, *Evaluation of high-resolution melting analysis as a diagnostic tool to detect the BRAF V600E mutation in colorectal tumors*, J Mol Diagn, 2009, 11(2):140–147.
- [18] Adeniran AJ, Zhu Z, Gandhi M, Steward DL, Fidler JP, Giordano TJ, Biddinger PW, Nikiforov YE, *Correlation between genetic alterations and microscopic features, clinical manifestations, and prognostic characteristics of thyroid papillary carcinomas*, Am J Surg Pathol, 2006, 30(2):216–222.
- [19] Trovisco V, Soares P, Preto A, de Castro IV, Lima J, Castro P, Máximo V, Botelho T, Moreira S, Meireles AM, Magalhães J, Abrosimov A, Cameselle-Teijeiro J, Sobrinho-Simões M, *Type and prevalence of BRAF mutations are closely associated with papillary thyroid carcinoma histotype and patients' age but not with tumour aggressiveness*, Virchows Arch, 2005, 446(6):589–595.

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