การตรวจการกลายของยีน *BRAF* ในโรคมะเร็งต่อมไทรอยด์

Molecular Testing of *BRAF* Mutation in Thyroid Cancer

อัญชลี ไหลพิริยกุล[1], **รังสิมา อรุณโรจน์**[2], **ยิ่งลักษม์ วิเศษศิริ**[2] **และ บุษบา ฤกษ์อำนวยโชค**[2]*

Aunchalee Laipiriyakul[1], Rangsima Aroonroch[2], Yingluck Visessiri[2] and Budsaba Rerkamnuaychoke[2]

[1]*หลักสูตรวิทยาศาสตร์มหาบัณฑิต สาขาวิชาพยาธิวิทยาคลินิก;* [2]*ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพ 10400*

[1]*Clinical Pathology program;* [2]*Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400*

**Corresponding author: budsaba.rer@mahidol.ac.th*

บทคัดย่อ

โรคมะเร็งต่อมไทรอยด์เป็นโรคที่พบบ่อยที่สุดในกลุ่มของมะเร็งต่อมไร้ท่อ และชนิดที่พบมากที่สุดคือมะเร็งต่อมไทรอยด์ชนิด papillary ซึ่งมีหลายรายงานแสดงความเกี่ยวข้องและความจำเพาะของมะเร็งต่อมไทรอยด์ชนิดนี้กับการกลายของยีน *BRAF* ชนิด V600E ซึ่งการตรวจการกลายทางห้องปฏิบัติการจะมีส่วนช่วยในการวินิจฉัยแยกโรคให้ชัดเจนยิ่งขึ้น ซึ่งจะมีประโยชน์ต่อการวางแผนการรักษาและพยากรณ์โรค ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธี denaturing high pressure liquid chromatography (DHPLC) สำหรับตรวจหาการกลายของยีน *BRAF* ชนิด V600E ในมะเร็งต่อมไทรอยด์ จากตัวอย่าง formalin-fixed, paraffin-embedded tissue (FFPE) จำนวน 50 ตัวอย่าง และตัวอย่าง fine needle aspiration biopsy (FNA) จำนวน 18 ตัวอย่าง จากคนไข้โรคมะเร็งต่อมไทรอยด์ มาสกัด DNA และตรวจการกลายของยีน *BRAF* ชนิด V600E โดยวิธี DHPLC และยืนยันผลโดยใช้วิธี direct sequencing จากผลการทดลองพบว่าวิธี DHPLC สามารถตรวจหาการกลายของยีน *BRAF* V600E ได้ และให้ผลตรงกับวิธี direct sequencing ในทุกตัวอย่าง ดังนั้นวีธี DHPLC จึงเป็นอีกทางเลือกหนึ่งที่สามารถใช้ในการตรวจการกลายของยีน *BRAF* ชนิด V600E ได้อย่างมีประสิทธิภาพ

ABSTRACT

Thyroid carcinoma is the most prevalent form of malignant endocrine tumor. The most common type of which is papillary thyroid carcinoma (PTC). Several studies suggested the correlation and specificity of papillary thyroid carcinoma with *BRAF* V600E mutation. The laboratory detection of *BRAF* V600E mutation in PTC samples can assist clinicians for treatment planning, prognostication and diagnosis of thyroid cancer. The objective of this study was to develope DHPLC technique to detect *BRAF* V600E mutation in PTC. We performed the assay in 50 FFPE samples and 18 FNA samples from thyroid patients. Then, the extracted DNA was detected for *BRAF* V600E mutation by DHPLC and confirmed by direct sequencing. The result showed that DHPLC gave the same results of *BRAF* V600E. DHPLC is an alternative promising tool for detecting *BRAF* V600E mutation.

คำสำคัญ: การกลายของยีน *BRAF*, มะเร็งต่อมไทรอยด์, ดีเอ็ชพีแอลซี

Keywords: *BRAF* mutation, thyroid cancer, DHPLC

INTRODUCTION

Thyroid cancer is the most common benign and malignant endocrine neoplasms (Xing et al., 2004). Among all types of thyroid cancer, PTC has the most frequent incidence (80%) (Kucukodaci et al., 2011). About 10-30% of the FNA results are diagnosed as 'indeterminated' and this determination has significant worry and uncertainty on the patients and also a diagnostic problem to the clinicians (Chung et al., 2006). In *RAF* kinases, *BRAF* appears to be the strongest activator of mitogen activated protein kinase (MAPK) signaling pathway among other *RAF* members (Trovisco et al., 2006). *BRAF* gene is situated on human chromosome 7 (Kucukodaci et al., 2011). The most frequent mutation is characterized by a missense substitution of thymine at 15th *BRAF* exon to adenine (T1799A) and results in coding changes in codon 600 and causes production of glutamate replacing of valine (V600E) (Trovisco et al., 2006; Kucukodaci et al., 2011). The most common *BRAF* mutations in thyroid cancer are V600E and specific for PTC type of thyroid cancer (Kucukodaci et al., 2011; Nikiforov et al., 2011). Detection of the *BRAF* mutation is a valuable tool for cytological and pathological diagnosis of thyroid cancer for better tumor prognostication and better therapy (Kucukodaci et al., 2011; Nikiforov et al., 2011). There are many laboratory techniques to detect *BRAF* V600E. Direct DNA sequencing assay has been the most widely used for mutation detection but this method requires many steps that cause tedious work, time consuming and high cost (Kucukodaci et al., 2011). In this study, alternative detection method was applied to used DHPLC method. This method provided a practical, inexpensive, potential sensitivity, relative ease of use, and rapid assay to detect *BRAF* V600E mutation on paraffin block tissues and FNA samples.

MATERIALS AND METHODS

1. Biological samples

Fifty samples of block tissues of PTC (during 2001-2002) and 18 samples of current FNA from thyroid patients. Patient samples were the remaining samples from routine service of Endocrine Pathology, Department of Pathology, Ramathibodi Hospital, Mahidol University. This work was reviewed and approved by Committee on Human Rights related to Researches Involving Human subjects based on Helsinki Declaration (ID 07-54-54).

2. DNA preparation

DNAs were extracted from FNAs using the QIAamp DNA Mini kit according to the manufacturer's instructions and applied to DNA Mini kit. For block tissue, samples were deparaffinized with incubation buffer and DNA was extracted using the QIAamp DNA Mini kit.

3. Polymerase chain reaction

PCR reactions were performed specific primers: 5'-TCATAATGCTTGCTCTGATAGGA-3' and 5'-GGGCCAAAAATTTAATCAGTGGA-3' as forward and reverse primers respectively. Cycling conditions were as following: initial denaturation at 94ºC for 7 minutes, 40 cycles of denaturation at 94ºC for 30 seconds, annealing step at 56ºC for 30 seconds, extension step at 72ºC for 30 seconds and final step at 72ºC for 10 minutes. A single major PCR products of 225 bp were separated on 2 % agarose gel.

4. DHPLC

The PCR products were analyzed on a DHPLC WAVE® system 4500 (Transgenomic®, USA). The optimum temperature of 55.6°C was chosen for *BRAF* V600E mutation. For analysis of somatic mutations in tumors, one potential drawback is that DHPLC requires that both wild-type and mutant DNA are present so that heteroduplexes can form (Keller et al., 2001). The sample chromatogram was compared with a reference pattern and mutant positive samples and subsequently confirmed by DNA sequencing using ABI Prism 3130 Genetic Analyzer. DNA sequences were compared with those of the normal *BRAF* gene exon 15 in the GenBank database (EU600171.1) using sequence assembly software.

RESULTS AND DISCUSSION

The result showed that 10 FFPE and 16 FNA samples had detectable amplified products of *BRAF* V600E mutation but 40 FFPE and 2 FNA samples were undetectable (Table 1). Then, the amplified products were subjected to DHPLC to detect point mutation of the *BRAF* V600E. In FFPE, *BRAF* V600E mutation was detected in 4 out of 10 samples while, in FNA was not.

The characteristics of difference between wild type and *BRAF* V600E mutation gene was detected using DHPLC at optimum temperature. In wild type, chromatogram pattern showed a clear single peak but the mutant pattern was double peak with the variable degree of mutation in each sample. All positive samples were confirmed by a direct sequencing assay (Figure 1).

Table 1 The result of DNA amplification and *BRAF* V600E mutation detection from FFP and FNA samples

Amplification	FFPE	FNA	*BRAF* mutation positive		*BRAF* mutation negative	
			FFPE	FNA	FFPE	FNA
PCR product	10	16	4	0	6	16
No PCR product	40	2	-	-	-	-
Total	50	18	4	0	6	16

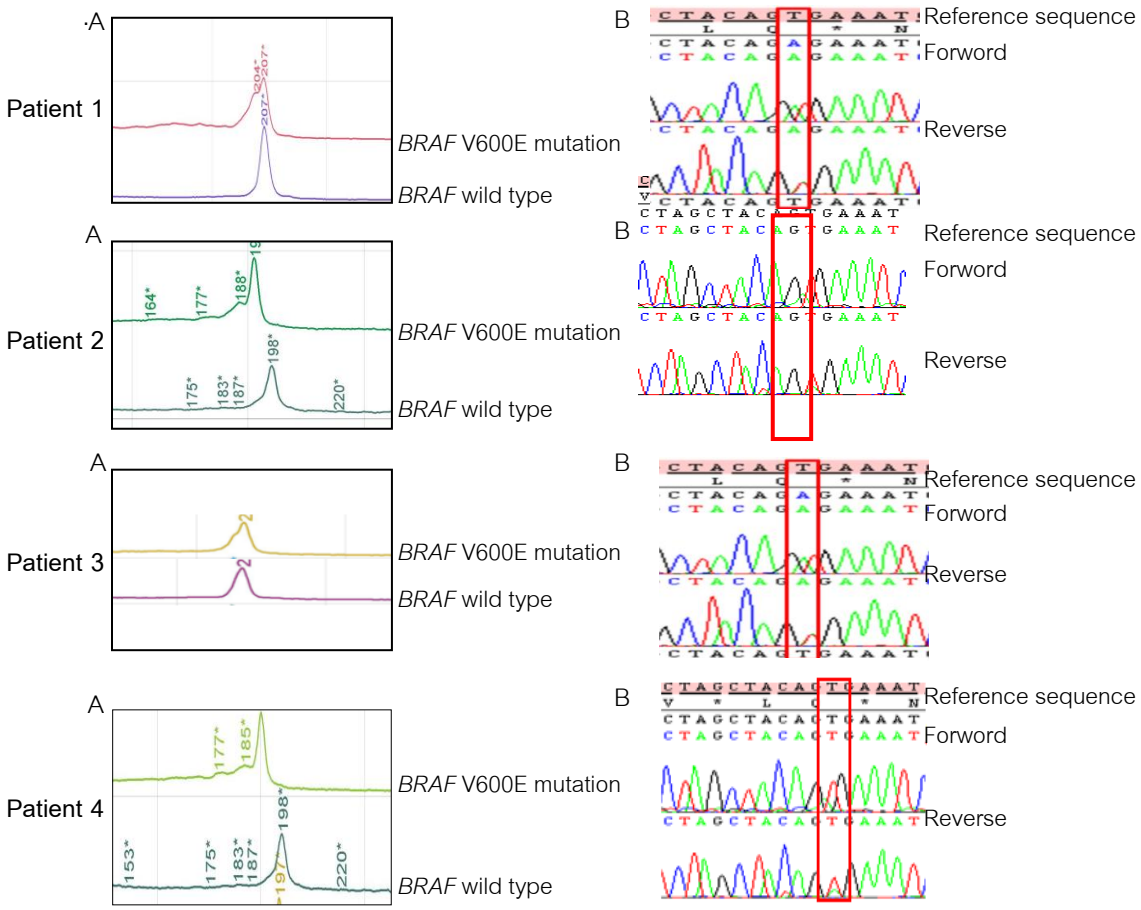

Figure 1 *BRAF* V600E mutation detected in 4 patients PTC. (A) Chromatogram pattern of wild type and *BRAF* V600E mutation by detected DHPLC. (B) Results of direct sequencing between wild type and *BRAF* V600E mutation.

DNA sequencing of the amplified DNA from positive samples by DHPLC showed the presence of *BRAF* V600E mutation. All positive samples had T to A transversion at nucleotide 1799 as previously reported about that activating in *BRAF* V600E mutations is expressed in human cancer cell lines and more common in certain clinical and pathologic subsets of melanoma, colorectal cancer, and thyroid cancer (Nucera et al., 2011; Flaherty et al., 2010). In PTC, *BRAF* V600E mutation is the most common presenting 29% to 83% of all (Kim et al., 2013). Therefore, the result of *BRAF* V600E mutation by DHPLC and direct sequencing are consistent. Moreover, numerous reports have documented the high accuracy and excellent sensitivity of DHPLC (96%-100%) (Lilleberg et al., 2003) while, in direct sequencing, the sensitivity of detection was 15% or more was interpreted as having a heterozygous mutation genotype (Kim et al., 2013). In this study, FFPE samples were stored during 2001 to 2002 which was considered to be stored for a long time. Therefore, the quality of DNA was affected and caused unamplifiable result. There are multiple factors that contribute to quality of DNA from the tissues, the nature of clinical samples, tissue processing, fixation conditions, formaldehyde concentration, pH, temperature on tissue fixation and length of sample storage time (Wang et al., 2007). For our FNA samples, *BRAF* V600E mutation negative result can be explained by several reasons. Normally, standard FNA procedure is not performed in up to 20% of cases because of low cell numbers in the sample. FNA contains a mixture of a large quantity of blood, vascular endothelial, interstitial cells, stromal cells and normal thyroid follicular cells (Hayashida et al., 2004; Musholt et al., 2010). Therefore, the results of molecular testing from these samples can be indeterminate or inadequate. There are risks of false-negative results (Chuang et al., 2010). Moreover, DNA extraction step is another reason affecting to yield of DNA.

CONCLUSION

DHPLC is a convenient molecular testing for detecting *BRAF* V600E mutation in PTC samples. DHPLC detection of *BRAF* V600E mutation demonstrated equivalent result to direct DNA sequencing. Moreover, this method is rapid, simple and inexpensive comparing to direct DNA sequencing, Therefore, the development of DHPLC method to detect other mutations is useful and can be applied in many genetic testing.

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