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BRAF^{V600E} Mutation Analysis in Fine-Needle Aspiration Cytology of Fixed Slide Specimens in Patients with Papillary Thyroid Carcinoma

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Abstract

Background: Papillary thyroid carcinoma is the most frequent type of thyroid cancer. The BRAF^{V600E} mutation is associated with tumor progression. We explored the utility of the BRAF molecular testing on fine needle aspiration fixed specimens of patients with confirmed diagnoses of papillary thyroid carcinoma.

Methods: Fixed thyroid cytology slide specimens of 19 patients with Bethesda II to VI reports were used to detect BRAF^{V600E} mutation by pyrosequencing of extracted DNA.

Results: BRAF^{V600E} mutation was detected in 25% of the specimens with Bethesda category III and IV nodules and in 73% of the nodules with Bethesda category V and VI.

Conclusion: BRAF mutation analysis can be performed on fixed fine needle aspiration cytology specimens. Although the frequency of the mutation is higher in specimens with higher Bethesda category scores, it could support clinical decision-making in thyroid nodules with intermediate Bethesda category scores.

Keywords: Papillary Thyroid Cancer, Fine Needle Aspiration, BRAFV600E

Conflicts of Interest: None declared
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Introduction

Thyroid cancers are the most prevalent neoplasms found in the endocrine system. Generally, differentiated thyroid lesions can be divided into two categories: papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). PTC accounts for approximately 73.9%, while the Follicular Variant of PTC carcinoma accounts for approximately 26.1% (1). Surgery is the primary treatment, and radioiodine therapy has also been used to treat certain types of thyroid cancer. Multi-kinase and tyrosine kinase inhibitors are used for iodine-resistant patients (2, 3). The inhibitory drugs target genes such as BRAF^{V600E}, RET, and TRK (3).

The BRAF gene, located on chromosome 7 (7q34), encodes a cytoplasmic serine/threonine kinase enzyme. This enzyme is critical in regulating the induction of the mitogen-activated protein kinase (MAPK) signaling pathway, which mediates cell proliferation, differentiation, survival and growth (4).

Pathogenic mutations in the BRAF gene occur in more than 19% of cancers. More than 90% of BRAF mutations are V600E mutations (5, 6).

The BRAF gene mutation has been identified in 70% of melanoma, 45% of thyroid cancer, 40% of pancreatic cancer, and 20% of colon cancer patients (7). In thyroid cells, the BRAF gene mutation has been associated with hypermethylation. The prevalence rate of the BRAF gene mutation has been reported 45% in thyroid cancer (8).

Detection of somatic variants in tumor tissue is a major challenge due to the high heterogeneity of cancer cells. Tumor tissue cells often have a limited number of mutated cells, which are surrounded by a large number of normal

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↑What is "already known" in this topic:
A molecular marker combined with cytology analysis can improve the diagnosis accuracy in thyroid nodules.

→What this article adds:
Based on the results, the detection of BRAFV600E gene mutation in the cytology specimens could improve clinical decisions in thyroid nodules with undetermined statue, but further investigation should be done.

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cells. Somatic mutations occur at low frequencies and require high-sensitivity methods for detection. Molecular detection methods such as PCR-based methods, including allele-specific PCR (AS-PCR) (9), real-time PCR, HRM (10), COLD-PCR, ddPCR (11), immunohistochemistry (IHC), sequencing methods such as Sanger (12), pyrosequencing (13), and next-generation sequencing (NGS) are used to detect variants. The sensitivity and specificity of detection methods are varying, except ddPCR, most of them have 1-5% sensitivity. The sensitivity of qPCR and NGS methods is approximately 5%. Although Sanger sequencing has high specificity, its sensitivity is relatively low at 15-20% (12). Pyrosequencing has higher sensitivity than Sanger and is able to detect about 5% of mutant alleles (14). A pyrosequencing assay was used in this study to increase the detection sensitivity of the low-frequency mutant alleles.

Methods

Study population: This case series study included 19 patients with Bethesda II-VI thyroid nodules admitted to the thyroid clinic of the Endocrinology and Metabolism Institute, Iran University of Medical Sciences. Cytology specimens were collected from all patients suspected of PTC. The samples were obtained via a Fine Needle Aspiration (FNA) procedure under guided ultrasound. A written consent was obtained from the included patients, and they were informed about the objective of this study. The cytological evaluation was performed by a single expert pathologist. The presence of malignant cells was confirmed and the Bethesda groups were also identified.

Sample preparation: Alcohol-fixed smears that contained lesion cells were utilized for BRAF^{V600E} molecular analysis. For DNA extraction from alcohol-fixed FNA specimens, the FNA slides were first de-coverslipped by soaking in xylene at room temperature for up to 72 hours. The decoverslip slides were resoaked in xylene for five minutes to remove the remaining mounting media. During this process, cellular contents adhere well to the slide. Cells on glass slides were collected by scraping the slides with a sterile scalpel blade. The processes were carried out by wetting the slide with approximately 30 µl of lysis buffer to help collect the cells as clump form into 2ml tubes. DNA extraction was conducted using a commercially available kit of Qiagen (Valencia, California, USA). DNA was evaluated for quantity and quality through a Nano-drop spectrophotometer (MAESTRO-Gene, Taiwan).

Molecular detection: Pyrosequencing technology was employed to identify BRAF^{V600E}. In order to evaluate the target gene, the 205 bp region of exon 15 containing codon 600 of the BRAF gene was amplified using the PCR method. The sequence of primers includes BRAF_F:5′-ATGCTTGCTCTGATAGGAA-3′ and BRAF_R: 5′-TCAGTGGAAAAATAGCCTCAATTC-3’. The reverse primer was biotinylated at its 5' end to facilitate the pyrosequencing process. Each PCR reaction contained 20 to 50 ng of genomic DNA, 10 picomoles of each primer and 25 microliters of Taq DNA Polymerase Master Mix RED enzyme buffer (Amplicon, Denmark) in a total volume of 50 microliters. The PCR process (Applied Biosystems, USA) was carried out using temperature cycling, which includes initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds. The final amplification was performed at 72°C for 2 minutes. To verify the accuracy and specificity of the amplification, we observed 5 microliters of PCR product on a 2% agarose gel. Then, 20 microliters of the biotin PCR product were added to 6 microliters of streptavidin sepharose beads. The temperature was increased to make single-stranded DNA (ssDNA), biotinylated ssDNA attached to streptavidin sepharose beads. PyroMark Q48 (QIAGEN) used dispense sequence to identify sequence variation. The primer was designed to analyze codons 599 to 602.

Statistical analysis

In this study, the descriptive analysis of quantitative variables was carried out by reporting the mean and standard deviation. The descriptive analysis of qualitative parameters was performed using frequencies and regarded percentages. The chi-squared test and the Fisher exact test were used to compare the BRAFV600E mutation between different subgroups. Mann-Whitney test was also implemented to compare the means of age and tumor size based on the status of BRAF V600E mutation. All statistical analyses were performed using SPSS version 20. A *P* value of <0.05 was considered statistically significant.

Results

The average age of the patients at the time of diagnosis was 45.2 (±9.3) years. Seventy-four percent (N=14) of the participants were female. Table 1 provides detailed information about the clinical characteristics of the participants. Cytology examination of the specimens classified 15 patients as Bethesda category V & VI. In addition, 4 patients were classified as Bethesda category III & IV. The average size of the nodules was 2.4 cm (±1.5). Of all cases with available relevant data (N=11), 54.5% of the patients had suspicious cervical lymph nodes detected by ultrasound imaging.

The pyrosequencing began with the analysis of codon 599 and ended with codon 602 analysis. Therefore, a T>A sequence analysis of codon 600 was performed to identify

Table 1. Characteristics of the study participants

	Bethesda III-IV	Bethesda V-VI
Age_ Median(Min_Max yrs.)	52.5 (34-59)	42 (33-59)
Nodule size (cm, Min_Max)	2.5 (0.3-4)	2.2 (0.3-6)
Lymphatic node involvement (%) **	1(50%)	5(55.5%)
BRAF V600E (%)	1 (25)	11 (73.33)

*Min: Minimum. Max: Maximum, ** based on available data

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the mutation. The results showed that 73.33% of the patients with Bethesda V & VI categories and 25% with Bethesda III & IV categories had the mutation. There was no significant statistical relationship between age, gender, and tumor size with the occurrence of the $BRAF^{V600E}$ mutation ($P > 0.05$).

Discussion

BRAF mutation has been reported in 60-74% of people with PTC, 4-45% in ATC, and 1.7% in FTC (15). The prevalence of $BRAFV^{600E}$ mutation in PTC (51%) is higher compared to FVPTC (24.1%) and FTC (14%). In PTC patients, the presence of the $BRAF^{V600E}$ mutation is associated with some clinical features, including age at diagnosis, distant and lymph node metastases, progression of TNM stage, and risk of recurrence (16, 17).

$BRAF^{V600E}$ mutation has been identified in 43.6% of papillary microadenomas and 42.4% of small PTC (18). 80-83% of this mutation occurs in tall cells and 12-18% in follicular cells of PTC patients (6). BRAF p.V600E mutation results in thymine to adenine change at position 1799 and as a result of substituting valine (V) to glutamate (E) at amino acid codon 600. Less frequent mutations at position 1799 include V600K (valine to lysine), V600R (valine to arginine), and V600M (valine to methionine) (5, 6). Studies have shown that the $BRAF^{V600E}$ mutation usually occurs in younger individuals, whereas the V600K mutation is more common in older patients and tends to affect the head and neck region (19). In the study of Al-Masri et al. (2021), the frequency of $BRAF^{V600E}$ in patients with PTC was reported to be 71%. In this study, genetic testing was performed on FFPE tissue and an inverse relationship was observed between tumor size and the presence of mutation ($P = 0.009$) (20). In a study on the Iranian population, the frequency of $BRAF^{V600E}$ mutation was reported at 77.8% in FFPE tissue samples with PTC (21). In the study of Dadgarnia et al. the prevalence of the $BRAF^{V600E}$ mutation in Iranian patients with suspected PTC (76 FNA samples) has been reported at 21.1%. The results showed a significant relationship between the mutation and pathological findings ($P = 0.001$) (22). $BRAF^{V600E}$ mutation is associated with an increased risk of recurrence as well as reduce the effectiveness of treatment and radioiodine therapy (23). To improve PTC detection in uncertain cytology, analyzing $BRAF^{V600E}$ in aspiration samples may increase the accuracy rates of diagnostic (24). In addition, detecting the mutation in low-risk patients before surgery may provide a valuable prognosis for disease management (25). It has been observed that mutations in the BRAF gene occur with a frequency of 90.2% in Asiana PTC patients. The prevalence of $BRAF^{V600E}$ in East Asia is reported to be 76.4%. However, there is weak evidence about the frequency of the variant in the population of Iran, Thailand, Singapore, and other countries (26).

In this study, we investigated the frequency of the mutation in cytology samples in the two groups of patients with suspected PTC (Bethesda III-VI). The results indicated the presence of eleven samples with $BRAF^{V600E}$ mutation in the Bethesda V-VI (positive PTC) and one $BRAF^{V600E}$ mutation in the Bethesda III-IV group. In our study, the rate of $BRAF^{V600E}$ detection was 63.1% among the patients included. Analysis of molecular markers and cytology of FNA samples can improve the accuracy of thyroid nodule diagnosis before surgery (27). Only 5-15% of thyroid nodules are reported as malignant and require surgery based on the FNA cytology test. However, a histological study will be necessary in 30% of patients for cytological features alone can't provide an accurate diagnosis (28). BRAF variant assay on FNA samples showed higher sensitivity in diagnosing thyroid nodules compared to cytology analysis alone (29-31).

Conclusion

Our research found that the $BRAF^{V600E}$ mutation was present in over 73.3% of patients with Bethesda V & VI, while it was found in only 25% of patients classified as Bethesda III & IV. Therefore, identifying the BRAF gene mutation in cytology samples can be a valuable diagnostic tool for diagnosing PTC from suspected PTC.

Authors' Contributions

Conception and design: MH and MEK; Development of methodology: MH and FA; Acquisition, analysis, and interpretation of data: SC; Writing, review, and/or revision of the manuscript: SC and MEK; Administrative, technical, or material support: MEK and MH; Study supervision: MEK; All authors read and approved the final manuscript.

Ethical Considerations

All phases of the study were approved by the Ethics Committee of the Iran University of Medical Sciences No. IR.IUMS.REC.1396.30870.

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Conflict of Interests

The authors declare that they have no competing interests.

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