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BRAF^{V600E} Mutation Analysis from May-Grünwald Giemsa-Stained Cytological Samples as an Adjunct in Identification of High-Risk Papillary Thyroid Carcinoma

Aneeta Patel,

Department of Pediatrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4712, USA

Joanna Klubo-Gwiezdzinska,

Department of Medicine, Division of Endocrinology, Washington Hospital Center, 110 Irving Street Northwest, Washington, DC, USA

Victoria Hoperia,

Center for Endocrine Surgery, Kharkivske Shose 121, Kyiv 02091, Ukraine

Alexander Larin,

Center for Endocrine Surgery, Kharkivske Shose 121, Kyiv 02091, Ukraine

Kirk Jensen,

Department of Pediatrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4712, USA

Andrew Bauer, and

Department of Pediatrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4712, USA

Vasyl Vasko

Department of Pediatrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4712, USA

Abstract

The *BRAF*^{V600E} mutation is specific for thyroid papillary cancer (PTC) and correlates with PTCs invasiveness. This study investigated whether detection of *BRAF*^{V600E} mutation can be performed on routinely stained FNABs. We also examined if establishment of the *BRAF*^{V600E} mutation could help in identification of patients at higher risk for metastatic disease. DNA was isolated from 134 FNABs samples (20 follicular neoplasm, ten suspicious for malignancy, and 104 malignant) using Pinpoint Slide DNA Isolation System. *BRAF*^{V600E} mutation was detected by PCR followed by sequencing. DNA was successfully extracted from all examined FNABs samples. In follicular neoplasm, suspicious for malignancy and malignant FNABs, *BRAF*^{V600E} mutation was found in 0/20 (0%), 2/10 (20%), and 47/104 (45.2%) of cases, respectively. Extra-thyroidal extension was detected in 35/47 (74.4%) *BRAF*^{V600E} positive and in 24/57 (42.1%) wild-type BRAF cases (p=0.001). Metastases were detected in 37/47 (78.7%) *BRAF*^{V600E} positive and in 28/57 (49.1%)

vasyl.vasko@gmail.com.

wild-type BRAF cases (p=0.002). Our results showed that stained FNAB specimens can be used for DNA extraction and assessment of $BRAF^{V600E}$ mutation. Detection of $BRAF^{V600E}$ mutation had limited value in diagnoses of malignancy in follicular neoplasms but can ascertain malignancy in subset of suspicious for malignancy FNABs. In malignant FNABs, $BRAF^{V600E}$ mutation was significantly associated with presence of extra-thyroidal extension and metastases after surgery.

Keywords

Thyroid cancer; FNAB; BRAF mutation

Introduction

Papillary thyroid cancer (PTC) is the most common thyroid cancer, accounting for 80% or more of all thyroid cancers [1]. The majority of patients with PTC are effectively treated with thyroidectomy and postoperative I^{131} therapy, but up to 20% of thyroid cancer patients develop persistent/recurrent disease which is associated with increased morbidity and mortality.

The extent of initial surgery for PTC has a significant impact on disease recurrence [2, 3]. It is generally agreed that therapeutic neck dissection should be performed to remove macroscopic lymph node metastases, in order to reduce the likelihood of persistent/recurrent PTC. Whether routine prophylactic central neck dissection warrants the greater risk of complications remains debatable. These controversies are due to the imprecision in the risk estimation of thyroid cancer aggressiveness based on clinical information and the available preoperative testing.

Fine-needle aspiration biopsy (FNAB) is currently considered the most effective technique for morphological diagnosis of thyroid nodules [4, 5]. The Bethesda System for Reporting Thyroid Cytopathology has provided a diagnostic terminology including benign (B), atypia of undetermined significance (AUS), follicular neoplasm (FN), suspicious for malignancy (SM), malignancy (M), and non-diagnostic (ND) for the interpretation of FNAB. The meta review analysis showed that risk of malignancy in diagnostic categories B, AUS, FN, SM, M and ND was 6%, 16%, 25%, 62%, 97%, and 12%, respectively [6]. Patients with follicular neoplasm, suspicious for malignancy, and malignant FNABs are commonly referred for surgery. However, preoperative FNAB cannot always accurately guide the extent of optimal surgical intervention.

With malignant cytologic findings on FNAB, a preoperative neck ultrasound is recommended to examine the cervical lymph nodes in all patients undergoing thyroidectomy [7]. Preoperative ultrasound can identify suspicious cervical adenopathy in 20–31% of cases, potentially altering the surgical approach in this group of patients [8, 9]. Preoperative ultrasound, however, will only identify 50% of the lymph nodes found at surgery, due to the presence of the overlying thyroid gland [10]. Clearly, there is a necessity for additional preoperative risk stratification tools, such as the detection of molecular characteristics associated with more aggressive tumors.

BRAF mutations are common in human cancers, with a prevalence of up to 66–83% in melanoma and thyroid cancer [11]. The oncogenic $BRAF^{V600E}$ mutation has been widely found in PTC, as well as in some anaplastic thyroid cancers, with prevalence in PTC of approximately 45%. Previous studies have established an association between BRAF mutations and aggressive clinicopathologic characteristics of primary PTC, including extra-thyroidal extension, lymph node metastasis, histologic subtypes with a poorer prognosis, and advanced disease stages [12, 13]. Recent data have demonstrated that BRAF mutation testing of FNAB samples provides a novel tool to the preoperative identification of PTC patients at higher risk for extensive disease [14].

The aim of this study was to determine whether routinely stained FNAB specimens could be used for DNA extraction and assessment of the $BRAF^{V600E}$ mutation. We sought to establish whether detection of $BRAF^{V600E}$ mutation could improve the cytologic diagnosis in cases of follicular neoplasm and suspicious for malignancy FNABs. We also investigated whether establishment of $BRAF^{V600E}$ mutation in malignant FNABs could help in the preoperative identification of patients at higher risk for metastatic disease.

Material and Methods

Thyroid Tissue Samples

Thyroid tissue samples were obtained in accordance with protocols approved by the Human Use Committee at The Center for Endocrine Surgery, Kiev, Ukraine, and maintained in an archival bank approved by the Uniformed Services University of the Health Sciences, Bethesda, MD. FNAB specimens from 134 patients were examined. All FNAB specimens were stained by the May-Grünwald Giemsa (MGG) technique. The cytological diagnoses consisted of three categories that indicate surgery: follicular neoplasm (20 cases), suspicious for malignancy (ten cases), and malignant (104 cases). All patients were treated at the Center for Endocrine Surgery and underwent thyroidectomy in the Department of Surgery. The initial treatment was total thyroidectomy and, when necessary, central neck and/or laterocervical lymph node dissection. Histological diagnoses were made independently, in a blinded fashion, by two pathologists (V.H. and V.V.) and tumors were classified according to the histopathological typing of the World Health Organization.

Comparison of FNAB diagnoses to the final histology showed that among follicular neoplasms there were four follicular adenomas (FA), five Hürthle cell adenomas, four follicular carcinomas (FC), and seven follicular variant of papillary carcinomas (FVPC). In suspicious for malignancy FNABs there were one FA, one FC, three FVPC, and five PTCs. All malignant FNABs were classified as malignant tumors on histology and included 11 FVPC and 93 classical PTCs.

DNA Extraction and Detection of BRAF Mutation

DNA was extracted from routinely prepared FNAB samples with a proprietary DNA extraction kit (Zymo Research, Irvine, CA) according to the manufacturer's procedure. In brief, the samples were covered with the "pinpoint solution," air-dried and scraped with a blade into a tube. Extraction buffer and proteinase K were added, and samples were

incubated at 55°C for 4 h and at 98°C for 10 min. DNA was eluted using the DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions.

Amplification of the *BRAF* gene, exon 15, was performed using 20 ng of DNA, 12.5 μL of GoTaq Hot Start Mastermix (Promega, Madison, WI), 10 pM of forward primer 5′-TCATAATGCTTGCTCTGATAGGA-3′, 100 nM of reverse primer 5′-GGCCAAAAATTTAATCAGTGGA-3′, and DNase/RNase-free water up to a final volume of 22 μL. Conventional PCR amplification protocol was composed of an initial activating step at 94°C for 15 min, a cycling step (40 cycles) performed as follows: 94°C for 30 s, 58°C for 1 min, 72°C for 30 s, and of a final extension at 72°C for 10 min. The PCR product was subjected to the restriction endonuclease TspR1 (New England BioLabs, Ipswich, MA) treatment. In brief, 7 μL of the PCR product was incubated with 1 μL of TspRl, 1 μL of NEbuffer 4 and BSA (New England Biolabs, Ipswich, MA) at 65°C for 2 h.

The denatured PCR products were electrophoresed in a 2% agarose gel (Invitrogen, Carlsbad, CA). Digestion of the 237-base pair (bp) PCR fragment with restriction endonuclease TspRl yielded two major bands of 117 and 87 bp for the wild-type allele. The $BRAF^{V600E}$ mutation abolished the restriction sites, resulting in a prominent band of 237 bp from the mutant allele and residual bands from the normal allele.

To confirm the results, direct sequencing was performed for selected samples. The amplified products were purified with a QIAGEN PCR purification kit and sequenced using the forward primer described previously with Big Dye Terminator (ABI Systems, Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Avant Genetic Analyzer (Perkin-Elmer, Waltham, MA).

Statistical Analysis

Data were analyzed using SPSS software and $p<0.05$ denoted the presence of a significant difference.

Results

Evaluation of Sample Adequacy for Molecular Testing

To determine whether detection of $BRAF^{V600E}$ mutation can be an adjunct technique to standard cytological examination, we extracted DNA from FNAB samples that already had been stained by the MGG technique and were subsequently used for routine cytological analysis. This approach was helpful for the identification of highly cellular slides and localization of areas of interest for DNA extraction. The quantity of DNA was assessed before molecular testing and ranged from 1 μg to 10 μg. The quality of DNA was assessed by PCR amplification of the *BRAF* gene. All examined FNAB samples demonstrated satisfactory DNA quality with a PCR amplification cycle threshold less than 35 cycles.

Detection of *BRAF*^{V600E} Mutation in Follicular Neoplasms and Suspicious for Malignancy FNABs Samples

To determine whether detection of *BRAF*^{V600E} mutation could improve diagnostic accuracy of FNABs, we examined the *BRAF*^{V600E} mutation status on preoperative FNAB specimens from follicular neoplasm (20 cases) and suspicious for malignancy lesions (ten cases). The results of *BRAF* mutation status as a function of histology are summarized in Table 1.

In the group of follicular neoplasm, *BRAF*^{V600E} mutation was not found in any of examined cases. In suspicious for malignancy FNABs, *BRAF*^{V600E} mutation was found in two of ten cases (one FVPC and in one PTC).

Detection of *BRAF*^{V600E} Mutation in Malignant FNABs

We examined the *BRAF*^{V600E} mutation status on preoperative FNAB specimens from 104 PTC patients and assessed the relationship to clinicopathologic characteristics of these patients. *BRAF*^{V600E} mutation was found in 43 of 93 (46.2%) conventional PTC patients and in four of 11 (36.3%) follicular variant PTC patients, with an overall prevalence of 45.2%.

By univariate analysis of our data set, no significant statistical correlation was found between *BRAF*^{V600E} and patient's age or gender (see Table 2). The frequency of *BRAF*^{V600E} mutation was not significantly different between patients who were younger than 45 years and patients whose age was 45 years and older at the time of surgery.

The incidence of *BRAF*^{V600E} mutation has also been examined in function of pathological parameters such as tumor size, multifocality, extra-thyroidal invasion, and presence of nodal metastases. In this series of samples, no significant association was found between tumor size and BRAF mutation status. Results demonstrating association between *BRAF*^{V600E} mutation and pathological characteristic of thyroid tumors are summarized in Table 3. Multifocal growth was more frequently detected in *BRAF*^{V600E}-positive than in mutation-negative PTCs, but the difference was not statistically significant. There was a significant association of *BRAF*^{V600E} mutation in preoperative FNAB specimens with the presence of extra-thyroidal extension and lymph node metastases after histological examination of postoperative tissue samples.

Discussion

Fine-needle aspiration biopsy is an effective method for preoperative evaluation of thyroid nodules. Molecular methods such as mutation detection or expression profile analysis are promising tools to improve the diagnostic accuracy of FNABs. The efficacy of molecular testing largely depends on quality and quantity of isolated nucleic acids from FNAB samples. The collection of cytological material for molecular testing requires supplementary steps during FNAB procedure to include additional aspiration of nodules, needle washout with nucleic acids preserving solution and storage of FNABs samples at low temperature. In this current study, we initially sought to determine whether routinely stained FNAB specimens can be used for DNA extraction. Our results demonstrated that initial cytological examination of stained samples is useful in determining areas of high cellular density for

further DNA extraction. After identification of the extraction site, DNA was successfully isolated from routinely stained FNAB samples, and the quality of the DNA isolated from stained samples was sufficient for genetic analysis. In our study, the amount of isolated DNA was higher compared to previously reported data, when nucleic acids were isolated from material obtained from FNAB needle washout [15, 16].

Molecular evidence has demonstrated associations between BRAF mutations and thyroid cancer. Experimental studies, including transgenic mouse models, have shown ability of the $BRAF^{V600E}$ mutation to promote aggressiveness and progression of PTC [11, 17]. Previous studies suggested that identification of a $BRAF^{V600E}$ mutation in thyroid aspirates could help distinguish between benign and malignant tumors when the cytologic examination is inconclusive [15]. Several multivariate analyses have demonstrated that the presence of $BRAF^{V600E}$ mutation in PTC is an independent risk factor for disease persistence/recurrence [11, 18, 19].

We examined $BRAF^{V600E}$ mutation status in a series of FNABs that were classified as follicular neoplasm or suspicious for malignancy. For patients with these cytological diagnoses, the ATA guidelines recommend either thyroid lobectomy or total thyroidectomy. We sought to establish whether detection of $BRAF^{V600E}$ mutation could ascertain the diagnosis and guide the extent of surgery in these cases. In patients with follicular neoplasm, $BRAF^{V600E}$ mutation was not detected in any samples, including 11 histologically confirmed thyroid cancers. These results suggest that detection of $BRAF^{V600E}$ mutation has a limited value in patients with FNAB diagnoses follicular neoplasm. In suspicious for malignancy FNABs, we detected $BRAF^{V600E}$ mutation in two of ten examined cases. Two of seven histologically confirmed cancers in this category were BRAF positive. These results suggest that detection of $BRAF^{V600E}$ mutation may be helpful to ascertain a diagnoses of cancer in subset of patients with suspicious for malignancy FNABs.

In the current study, the $BRAF^{V600E}$ mutation was detected in 45% of malignant FNABs. These results are comparable with previously published data on prevalence of the $BRAF^{V600E}$ mutation in papillary thyroid cancers. Numerous studies from patients of various ethnic and geographic backgrounds have demonstrated an association between the $BRAF^{V600E}$ mutation with aggressive clinicopathologic characteristics of PTCs such as extathyroidal extension and lymph nodes metastases [12, 13, 19]. Our study also showed that $BRAF^{V600E}$ mutation in malignant FNAB was significantly associated with the presence of extra-thyroidal invasion and lymph node metastases. It has to be noted that lymph node metastases were detected in 49.1% of $BRAF^{V600E}$-negative PTCs. These results are consistent with previously reported data showing the presence of metastases in 44% of $BRAF^{V600E}$-negative PTCs [20].

Together, these data indicate that detection of a BRAF mutation in malignant FNAB samples is a promising tool for evaluation of PTC aggressiveness, however, absence of BRAF mutation in malignant FNABs does not rule out the possibility of lymph nodes metastases. Additional studies are needed to determine whether detection of a BRAF mutation will have greater predictive value for the predicting the presence of lymph node metastases compared

to preoperative neck ultrasound. This could be important for selection of appropriate surgery in patients with malignant FNABs results but with negative preoperative neck ultrasound.

We detected $BRAF^{V600E}$ mutation in five of 21 (23.8%) histologically confirmed FVPC. A $BRAF^{V600E}$ mutation was present in 0% of follicular lesions, 33.3% of lesions suspicious for malignancy and 36.3% of lesions with malignant cytology. Our data are similar to results from a study examining BRAF mutation status in 187 FVPC in function of cytological diagnoses [21]. In this study, a $BRAF^{V600E}$ mutation was detected in 11% of FVPC diagnosed as follicular neoplasm, in 27.6% of FVPC diagnosed as suspicious for carcinoma and in 54.5% of FVPC diagnosed as malignant by FNABs. These results show that FVPC diagnosed as malignant on standard FNAB more frequently harbor BRAF mutation compared with the FVPC falling into the follicular neoplasm cytological group.

In conclusion, we have demonstrated that stained FNAB specimens can be used for DNA extraction and assessment of the $BRAF^{V600E}$ mutation. Detection of the $BRAF^{V600B}$ mutation has limited value in establishing a malignant diagnosis in follicular neoplasm but can ascertain diagnoses in subset of patients with FNAB suspicious for malignancy. In malignant FNABs, $BRAF^{V600E}$ mutation is associated with risk of extra-thyroidal extension and metastases; however, further studies are needed to determine the cohort of thyroid cancer patients that would benefit from preoperative testing.

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Table 1

Detection of *BRAF* mutation in indeterminate FNABs

Histology	Follicular neoplasm (20)		Suspicious for carcinoma (10)	
	***BRAF* (+)**	***BRAF* (−)**	***BRAF* (+)**	***BRAF* (−)**
FA (10)	0	9	0	1
FC (5)	0	4	0	1
FVPC (10)	0	7	1	2
PTC (5)	0	0	1	4

Table 2

Association of *BRAF* mutation with demographic characteristics of patients

	***BRAF* positive (47 cases)**	***BRAF* negative (57 cases)**	***p* value**
Age at diagnosis (year)			
Mean/standard deviation	42.4/13.3	40.3/17.3	0.49
Gender			
Male/female	8/39	13/44	0.62

Table 3

Association of *BRAF* mutation with pathologic characteristics of tumors

	***BRAF* positive (47 cases)**	***BRAF* negative (57 cases)**	***p* value**
Multifocality (yes)	21 (44.6%)	16 (28.1%)	0.1
Extra-thyroidal extension (yes)	35 (74.4%)	24 (42.1%)	0.001
Lymph node metastases (yes)	37 (78.7%)	28 (49.1%)	0.002