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Detection of *BRAF* Mutations on Direct Smears of Thyroid Fine-Needle Aspirates Through Cell Transfer Technique

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ABSTRACT

Objectives: *To determine the utility of the cell transfer technique (CTT) for* BRAF *molecular testing on thyroid fine-needle aspiration (FNA) specimens.*

Methods: *Polymerase chain reaction (PCR)–based* BRAF *molecular testing was performed on tissues obtained through CTT from both air-dried and ethanol-fixed direct smears of thyroid FNA specimens and then compared with the corresponding thyroidectomy formalin-fixed, paraffin-embedded (FFPE) tissues on 30 cases.*

Results: BRAF *testing was successfully performed on 29 of 30 air-dried CTT, 27 of 30 ethanol-fixed CTT, and 27 of 30 FFPE tissues. The results exhibited 11, 13, and 13* BRAF *mutations and 18, 14, and 14 wild types for the air-dried CTT, the ethanol-fixed CTT, and the FFPE tissues, respectively. The concordance rate was 96% between air-dried and ethanol-fixed CTT tissues, 88% between air-dried CTT and FFPE tissues, and 92% between ethanol-fixed CTT and FFPE tissues.*

Conclusions: *PCR-based* BRAF *mutational testing can be reliably performed on the direct smears of the thyroid FNA specimens through the application of CTT.*

Papillary thyroid carcinoma (PTC) accounts for 80% of all thyroid cancers in the United States, making it the most common thyroid malignancy.[1] Fine-needle aspiration (FNA) biopsy has emerged as an important diagnostic tool in the workup of suspicious thyroid nodules. Papillary carcinoma, in particular, displays characteristic nuclear features that often allow for a cytologic diagnosis based on morphology alone. Some cases, however, have only a few of the diagnostic features or are of limited cellularity. These cases are often placed into the category of follicular lesions of undetermined significance (FLUS) or suspicious for malignancy.

In recent years, it has been shown that a mutation of the *BRAF* gene is the most common genetic alteration in PTC and is detected in 29% to 69% of PTCs.[2-4] Most important, when dealing with primary thyroid neoplasms, *BRAF* mutations are found in PTC and in poorly differentiated or anaplastic thyroid carcinoma derived from PTC.[5] Therefore, molecular techniques that can identify this *BRAF* mutation can be an important ancillary diagnostic tool in the diagnosis of PTC, especially in cases that would otherwise be classified as atypia of undetermined significance or suspicious for malignancy. In addition, numerous studies have shown that *BRAF* mutations in PTC are associated with characteristics that are predictive of tumor progression and recurrence such as extracapsular invasion, lymph node metastasis, and advanced tumor stage.[6] Finally, with the advent of novel pharmaceutical agents that target the mitogen-activated protein kinase pathway that is abnormally activated by *BRAF* mutations, detection of *BRAF* mutations will likely have an impact on the treatment of PTC. Thus, analysis of specimens for the *BRAF* mutation can have diagnostic, prognostic, and therapeutic implications.

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The use of formalin-fixed, paraffin-embedded (FFPE) cell blocks prepared from FNA specimens is generally considered a reliable source of tumor cells for molecular assessment as well as immunohistochemical studies that can detect *BRAF* mutations.[7] In particular, thyroid FNA is commonly used for preoperational diagnosis because high vascularity of the thyroid tissue can cause complications with more invasive procedures such as core biopsy. Cell blocks, however, are not routinely made or sometimes lack adequate cellularity to perform these ancillary studies. At our institution, the cell transfer technique (CTT), using direct cytologic smears as a source of tumor cells, has proven to be a reliable method for performing immunohistochemical and molecular studies when cell blocks lack adequate cellularity.[8] Our goal, therefore, was to establish CTT as a viable option for isolating tumor cells for ancillary *BRAF* mutation analysis via the polymerase chain reaction (PCR)–based platforms. To validate this technique, we concurrently tested corresponding FFPE tissue from follow-up surgical resection alongside tissue obtained from direct smears.

Materials and Methods

This study was approved by the Indiana University Institutional Review Board. A computerized search of our anatomic pathology information system was performed for a 26-month period (January 2011 through February 2013). To have an adequate number of positive *BRAF* mutation cases, we selected thyroidectomy specimens with final diagnoses of PTC and two cases of follicular neoplasm that had corresponding FNA performed. Cases of benign thyroid lesions were also included.

In total, there were 30 FNA specimens from 30 patients, including six men and 24 women in our study. The patients' ages ranged from 26 to 84 years, with a median age of 45 years and a mean age of 45.7 years. Nodules were present in the left lobe in 15 cases, in the right lobe in 14 cases, and in the isthmus in one case. The final histologic diagnoses of 30 cases included 18 classic PTCs, four follicular variant PTCs, one follicular carcinoma, one follicular adenoma, and six benign nonneoplastic lesions.

The slides from direct smears of thyroid aspirates and histologic slides of the corresponding thyroid nodules were reexamined. Both cytologic and surgical pathology reports as well as pertinent clinical history and radiographic results were reviewed. One ethanol-fixed smear, one air-dried direct smear, and one corresponding FFPE block that contained lesional cells were selected for *BRAF* molecular testing. The areas on the direct smears containing abnormal cells were marked by a pathologist (H.H.W.), and photographs were taken from these areas, in which the tumor cells of interest were then collected by CTT and sent for molecular testing.

Cell Transfer Technique

CTT was performed using clean technique as follows: (1) the coverslip was removed using fresh histologic-grade xylene (Fisher Scientific, Pittsburgh, PA), (2) a thin layer of Mount Quick media (Daido Sangyo, Tokyo, Japan) was spread uniformly over the top of the cellular material, (3) the slide was then placed in a 60°C oven for approximately 2 to 3 hours (or until hardened to the touch), (4) a Sharpie marker was used on the surface of the dried media to divide the slide into multiple areas of interest, (5) the slide was then placed into a clean Coplin jar of deionized water and submerged into a warm water bath at 45 ± 3°C for 30 minutes to 2 hours or until the media were soft enough to easily peel away from the slide, and (6) the media were cut along the marked areas, and each cut section was placed in an Eppendorf 2.0-mL safe-lock centrifuge tube and sent for molecular testing.

DNA Extraction

DNA extraction from FFPE tissue and cytologic specimens was performed using the Qiagen QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA). A microdissection was performed for the extraction of DNA from FFPE tissue. For the cytologic specimens, a modification from the manufacturer's recommendations was made. Samples were incubated at room temperature for 5 minutes with 1 mL xylene and then centrifuged at 15,000 rpm for 5 minutes. Xylene was removed from the pellet and followed by the ethanol wash recommended by the manufacturer. DNA concentration was determined using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

BRAF Mutations

Samples were analyzed by a Qiagen *BRAF* RGQ PCR run on the Rotor-Gene Q MDx (Qiagen) following the manufacturer's recommendations. Genomic DNA was used to detect five mutations in codon 600 of the *BRAF* gene. The real-time PCR detection system employed both Scorpions and Amplification Refractory Mutation System technologies (Qiagen) on the Rotor-Gene Q instrument. This assay can detect somatic mutations, including V600E, V600E complex, V600D, V600K, and V600R, although it is unable to differentiate the V600E and V600E complex mutations. Overall, this is a two-step procedure, with the first step being a control assay to assess the total *BRAF* DNA content in a sample and the second step combining both the mutation and control assays to determine the presence or absence of mutated DNA. The threshold at which the signal is detected above background is called the cycle threshold. Sample delta cycle threshold values are calculated as the difference between the mutation assay cycle threshold and the wild-type assay cycle threshold from the same sample. Samples are subsequently classified as "mutation positive"

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Table 1
Comparison of *BRAF* Mutation Results Between Cytologic and Formalin-Fixed, Paraffin-Embedded Tissue

	FNA Dx	DNA-AD, ng	*BRAF*-AD	DNA-EF, ng	*BRAF*-EF	SP Dx	DNA-FFPE, ng	*BRAF*-FFPE
1	B	8.6	WT	31.5	Failed	B	161.5	WT
2	B	5	WT	0.6	WT	B	72.4	WT
3	B	5.3	WT	0.2	WT	B	107.2	WT
4	FLUS	2.4	WT	4.3	Failed	B	147.6	Failed
5	FLUS	1.7	WT	7.9	WT	PTC	32	V600E
6	FLUS	1.4	WT	1.3	Failed	PTC	176.5	V600E
7	FLUS	6.4	WT	6.7	WT	FVPTC	223.9	WT
8	FLUS	6.3	WT	30.7	WT	B	207.9	WT
9	FLUS	5.2	WT	20	WT	B	44.7	WT
10	FN	13.4	WT	25.1	WT	FA	100.8	WT
11	PTC	9.4	WT	1.7	V600E	PTC	258.8	V600E
12	PTC	5.1	V600E	1.4	V600E	PTC	266.1	V600E
13	PTC	3.5	V600E	5.5	V600E	PTC	90.4	V600E
14	PTC	1.1	V600E	18.6	V600E	PTC	68.9	V600E
15	PTC	1.5	V600E	24	V600E	PTC	153.6	V600E
16	PTC	7.2	V600E	3.7	V600E	PTC	217.2	V600E
17	PTC	3.8	V600E	4.1	V600E	PTC	100.1	V600E
18	PTC	4.3	V600E	14.8	V600E	PTC	155.2	V600E
19	PTC	3.4	V600E	27.1	V600E	PTC	265.3	V600E
20	PTC	1.2	V600E	2.9	V600E	PTC	109.5	V600E
21	PTC	3.6	V600E	15.8	V600E	PTC	410.2	V600E
22	PTC	16.9	WT	7.8	WT	PTC	425.1	WT
23	PTC	0.2	Failed	1.2	V600E	PTC	75.2	WT
24	PTC	3.7	WT	0.1	WT	PTC	86.8	WT
25	S/PTC	2.1	WT	2.2	WT	PTC	263.3	Failed
26	S/PTC	1.3	V600E	13.2	V600E	FVPTC	123.6	Failed
27	S/PTC	3.8	WT	3	WT	PTC	317.8	WT
28	S/PTC	1.6	WT	9	WT	FC	96.2	WT
29	S/PTC	3.5	WT	0.2	WT	FVPTC	56.3	WT
30	S/PTC	3.2	WT	8.6	WT	FVPTC	28.1	WT

AD, air-dried cell transfer cytology; B, benign nonneoplastic lesion; Dx, diagnosis; EF, ethanol-fixed cell transfer cytology; FA, follicular adenoma; FC, follicular carcinoma; FFPE, formalin fixed, paraffin embedded; FLUS, follicular lesion of undetermined significance; FN, follicular neoplasm; FNA, fine-needle aspiration; FVPTC, follicular variant of papillary thyroid carcinoma; PTC, papillary thyroid carcinoma; SP, surgical pathology; S/PTC, suspicious for papillary thyroid carcinoma; WT, wild type.

if they give a delta cycle threshold less than the stated cutoff value for the assay. If the delta cycle threshold is above the cutoff, the sample is classified as "mutation not detected." Appropriate positive and negative controls were run with each sample.

Results

All CTT tissue submitted for *BRAF* testing contained more than 100 cells. The DNA yield ranged from 0.1 to 31.5 mg (median, 3.7 mg; mean, 9.6 mg) for ethanol-fixed and 0.2 to 16.9 mg (median, 5.5 mg; mean, 4.7 mg) for air-dried cytologic samples and 28.1 to 425.1 mg (median, 123.6 mg; mean, 158.2 mg) for FFPE tissues.

BRAF testing was successfully performed on 29 of 30 air-dried CTT (97%), 27 of 30 ethanol-fixed CTT (90%), and 27 of 30 FFPE (90%) specimens. The results included 11 *BRAF* mutations and 18 wild types (WTs) for the air-dried CTT specimens, 13 *BRAF* mutations and 14 WTs for the ethanol-fixed CTT specimens, and 13 *BRAF* mutations and 14 WTs for the FFPE specimens **Table 1**. The concordance rate was 96% between air-dried and ethanol-fixed CTT specimens, 88% between air-dried CTT and FFPE specimens, and 92% between ethanol-fixed CTT and FFPE specimens. There was one case in which mutation was detected on CTT specimens but not on the corresponding FFPE tissues (case 23). Conversely, two other cases had mutations identified on the FFPE tissue but not on the corresponding CTT specimen (cases 5 and 6). Using the FFPE tissue as the gold standard, the sensitivity and specificity for detection of *BRAF* mutation on CTT direct smears were 85% and 93%, respectively.

The original FNA diagnoses of these 30 cases included three benign nonneoplastic lesions, six FLUS, one follicular neoplasm, six cases suspicious for PTC, and 14 PTCs (Table 1). All the benign and malignant FNA diagnoses were confirmed by the follow-up thyroidectomies. The follow-up of six cases suspicious for PTC revealed two PTCs, three follicular variant PTCs, and one follicular carcinoma. The follow-up of the follicular neoplasm case revealed a follicular adenoma on the resection specimen. Of the six

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FLUS cases, the follow-up showed three benign nonneoplastic lesions, two PTCs, and one follicular variant of PTC. *BRAF* V600E mutation was noted in 15 cases, of which the thyroidectomy specimens showed classic PTC in 14 cases and follicular variant PTC in one case. Ethanol-fixed CTT specimens had the same *BRAF* mutation detection rate as FFPE specimens (13/15), while the air-dried specimens had a lower rate (11/15).

Discussion

BRAF mutation is the single most common mutation associated with thyroid carcinoma, and it is a potential stratification tool for planning the extent of thyroid surgery and as a potential therapeutic target in PTC.[9] The addition of molecular analysis of *BRAF* mutation on cytologic specimens has increased 20% to 25% yield of sensitivity in the detection of thyroid cancer compared with cytology alone.[10-12] CTT is a feasible method for obtaining cellular material from FNA direct smears for immunocytochemical stains.[13] It has also proven useful for obtaining adequate DNA from tumor cells for the molecular testing to identify *EGFR* and *KRAS* mutations from FNA smears of adenocarcinoma of the lung. Wu et al[8] have found a high agreement rate (97%) between the specimens obtained from the FNA smears by CTT and FFPE tissue for *EGFR* and *KRAS* mutations in cases of pulmonary adenocarcinoma. In our institution, cell blocks of the thyroid FNA usually contain scant cellularity. The ability to visualize and select the abnormal cells through CTT provides an advantage over the recut of the cell block tissue, especially when the tumor cells are fewer and the surrounding normal tissues are abundant in the block, factors that may cause a false-negative result for the cell block tissue. In the current study, we confirm the usefulness of CTT to obtain adequate materials for *BRAF* molecular analysis from thyroid FNA smears. We required at least 100 cells to be transferred for each case, and *BRAF* testing was successfully performed on 29 of 30 air-dried (97%) and 27 of 30 ethanol-fixed cell-transferred smears (90%) that compared with a test failure/invalid rate of 9.2% for Sanger sequencing.[14] There was a 96% agreement of the final molecular results between the two methods. The agreement between cytologic and FFPE specimens was 88% for air-dried and 92% for ethanol-fixed CTT. Both air-dried and ethanol-fixed smears are feasible for providing DNA materials for molecular testing for *BRAF* mutations. However, in our small series of study, ethanol-fixed CTT showed a slightly higher successful rate in detecting *BRAF* mutation compared with the air-dried CTT (13 vs 11 cases), but the number of cases is too small to draw a conclusion.

There were three discordant cases in our study. Two false-negative cases with cytologic diagnosis of FLUS revealed WT on CTT smears, while the corresponding FFPE tissues, which were diagnostic of papillary carcinoma, demonstrated *BRAF* V600E mutations (cases 5 and 6). Retrospective review of these two cases showed abundant colloid with scattered macrophages and few groups of atypical follicular epithelium on the smears. The false-negative molecular testing results on these two FLUS cases are most likely due to low volume of tumor cells with contaminant benign follicular cells and colloid. The third discrepant case in our study was a case of papillary carcinoma (case 23) that was diagnosed by cytology and confirmed on thyroidectomy. In this case, *BRAF* mutation was found on the ethanol-fixed CTT cytologic samples, but the corresponding FFPE tissue demonstrated only WT. False-positive *BRAF* mutation has been shown to be rare by Kim et al,[11] who used dual-priming oligonucleotide–based multiplex PCR analysis to identify five cases of false-positive *BRAF* V600E mutation in a study of 1,074 patients. A false-positive result can be due to sampling error; a small focus of micropapillary carcinoma might have not been sampled when the thyroidectomy specimen was examined. The discrepancy of this last case (case 23) is most likely due to *BRAF* genetic heterogeneity even though intratumoral *BRAF* genetic heterogeneity rarely occurs in PTC. Walts et al[15] used the PCR method for *BRAF* V600E detection and discovered discordant *BRAF* results in 4.8% of the pairs studied for PTC. The other possibility is that the decalcification process of the targeted tumor nodule on case 23 might have caused degeneration of the DNA materials, which may also contribute to the false-negative *BRAF* mutational result on the FFPE tissue.[7]

CTT has been proven useful to provide adequate DNA materials for the detection of *BRAF* mutations in cases with cytologic diagnoses of papillary carcinoma or suspicious for papillary carcinoma. In our study, there was 100% sensitivity using ethanol-fixed CTT cytologic materials for *BRAF* detection in this group of patients. One possible potential for the application of CTT on direct smears of the thyroid aspirates is to triage the cases with indeterminate FNA diagnoses such as FLUS or "atypia of undetermined significance." *BRAF* has a very high cancer predictive rate; in the study by Kim et al,[11] 221 (98%) of 226 cases of *BRAF* V600E–positive FNA cytology correlated with thyroid carcinoma on the follow-up thyroidectomies, so positive *BRAF* will warrant a total thyroidectomy with possible neck lymph node dissection, even with indeterminate FNA diagnoses for patients.

CTT provides a useful option for cytology specimens in which cell blocks lack adequate cellularity for ancillary studies.

One of the advantages of CTT over a common microdissection technique (using a scalpel to scrape the cells from the slides) is the capability of CTT to perform

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immunocytochemistry at the same time. Immunocytochemistry on CTT smears has been validated and routinely performed in our laboratory.[13] Recently immunocytochemistry in the detection of *BRAF* V600E in the FNA of PTC was validated on cell block slides.[7] Potentially, we can perform immunocytochemistry for *BRAF* mutation on CTT first as a cheaper screening test and then conduct molecular testing as the confirmation method either on the same CTT pieces that immunocytochemistry was performed on or on different pieces of CTT that are present on the same smears.

Although all six FLUS cases in our study showed no evidence of *BRAF* mutations on CTT samples, it may potentially provide additional sensitivity for the diagnosis of PTC in cases of indeterminate cytologic diagnoses. A large-scale correlation study is needed to verify the utility of CTT for thyroid FNA cases with indeterminate diagnoses.

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