Molecular Diagnosis Using Residual Liquid-Based Cytology Materials for Patients with Nondiagnostic or Indeterminate Thyroid Nodules

Hyemi Kwon¹, Won Gu Kim¹, Markus Eszlinger², Ralf Pascke², Dong Eun Song³, Mijin Kim¹, Suyeon Park¹, Min Ji Jeon¹, Tae Yong Kim¹, Young Kee Shong¹, Won Bae Kim¹

¹Division of Endocrinology and Metabolism, Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; ²Division of Endocrinology and Metabolism, Department of Oncology and Arnie Charbonneau Cancer Institute, Cummings School of Medicine, University of Calgary, Calgary, AB, Canada; ³Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

Background: Molecular analysis for common somatic mutations in thyroid cancer can improve diagnostic accuracy of fine-needle aspiration cytology (FNAC) in the nondiagnostic or indeterminate category of thyroid nodules. In this study, we evaluated the feasibility of molecular diagnosis from residual liquid-based cytology (LBC) material after cytological diagnosis.

Methods: This prospective study enrolled 53 patients with thyroid nodules diagnosed as nondiagnostic, atypia of undetermined significance (AUS), or follicular lesion of undetermined significance (FLUS) after FNAC. DNAs and RNAs were isolated from residual LBC materials. \(BRAF^{V600E}\) and \(RAS\) point mutations, \(PAX8/\text{peroxisome proliferator-activated receptor }\gamma (PPAR\gamma)\), \(RET/PTC1\), and \(RET/PTC3\) rearrangements were evaluated by real-time polymerase chain reaction and pyrosequencing.

Results: All DNAs from 53 residual LBC samples could be analysed and point mutations were detected in 10 samples (19%). In 17 AUS nodules, seven samples (41%) had point mutations including \(BRAF (n=4)\), \(NRAS (n=2)\), and \(KRAS (n=1)\). In 20 FLUS nodules, three samples (15%) had \(NRAS\) point mutations. RNA from only one FLUS nodule could be analysed for rearrangements and there was no abnormality.

Conclusion: Molecular analysis for \(BRAF\) and \(RAS\) mutations was feasible in residual LBC materials and might be useful for diagnosis of indeterminate thyroid nodules.

Keywords: Molecular diagnostic techniques; Biopsy, fine-needle; Thyroid aspiration; Thyroid nodule; Thyroid neoplasms

INTRODUCTION

Fine-needle aspiration cytology (FNAC) is a reliable, cost-effective, and safe diagnostic method for the evaluation of thyroid nodules [1]. The prevalence of thyroid nodules has been estimated to be about 4% to 10% in adults on physical palpation of the thyroid gland [2,3]. It is much higher on thyroid ultrasonography (US), as 20% to 70% [2,4]. However, the over-all malignancy risk of thyroid nodules is only 5% to 10% [5,6]. By means of FNAC, 15% to 30% samples were classified as inde-
terminate category, including atypia of undetermined significance (AUS) or follicular lesion of undetermined significance (FLUS), follicular neoplasm (FN) or suspicious for follicular neoplasm (SFN) [1,6]. The risk of malignancy for AUS/FLUS thyroid nodules was 5% to 15% and repeated fine-needle aspiration (FNA) was recommended [7]. Molecular testing for common somatic mutations of thyroid cancer provides the opportunity to improve the diagnostic accuracy of FNAC in the indeterminate category of thyroid nodules [5-9].

However, routine use of molecular analysis for all FNAC samples remains controversial and is not cost-effective [10]. Usually, molecular diagnosis can be applicable for thyroid nodules which are diagnosed as indeterminate in the first FNAC results. Since currently available molecular tests need fresh FNAC material obtained by standard FNA procedures, an additional FNA is required for molecular analysis. A recent study suggested the feasibility of air-dried FNAC samples for molecular analysis including common point mutations and rearrangements in thyroid cancer [11]. This study suggested that additional molecular analysis using air-dried FNAC samples could improve the sensitivity of FNAC from 67% to 75% [11].

Recently, liquid-based cytology (LBC) is widely used for the preparation of cytological specimens instead of routine air-dried slides. One of the most common U.S. Food and Drug Administration-approved methods for the LBC samples is to use an ethanol-based fixative solution (CytoRich, TriPath Imaging, Burlington, NC, USA). The FNAC samples are collected in this solution, centrifuged twice and slowly transferred on to a single slide by a computer-assisted device and finally stained with a hematoxylin-eosin stain (SurePath, TriPath Imaging) [12]. After the centrifugation, the pellet is used for diagnosis but the supernatant is discarded. If the residual supernatant could be used for molecular diagnosis, repeated FNAs could be avoided for indeterminate thyroid nodules. A recent study demonstrated that residual ThinPrep (Hologic Inc., Bedford, MA, USA) material from thyroid FNAC was adequate for molecular analysis in most of the cases [13]. The majority of $BRAF$ mutations were found in cases classified cytologically as malignant or suspicious for malignancy [13]. However, the usefulness of $BRAF$ testing was limited by the low rate of $BRAF$-positive cases in other categories such as AUS/FLUS or FN/SFN [13].

The aim of this study was to evaluate the feasibility of molecular diagnosis using residual supernatant of SurePath samples after cytological diagnosis. We evaluated $BRAF$, $NRAS$, $HRAS$, and $KRAS$ point mutations and rearrangements of $PAX8$/peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$), $RET/PTC1$, and $RET/PTC3$ using residual SurePath samples of cytological non-diagnostic or indeterminate thyroid nodules such as AUS/FLUS or FN/SFN categories.

**METHODS**

**Patients and samples**

This prospective study enrolled 53 thyroid nodules from 53 patients, which were diagnosed nondiagnostic or indeterminate thyroid nodules after thyroid FNAC from July 2014 to September 2014 in Asan Medical Center. The thyroid nodules were classified as non-diagnostic ($n=16$), AUS ($n=17$), and FLUS ($n=20$). The supernatant of LBC material after the centrifugation for collecting pellets to make slides for cytological diagnosis was collected. The samples were blinded to the medical record number, age, gender, and other personal information with the exception of FNAC results. The study was approved by the Institutional Review Board of the Asan Medical Center, Seoul, Korea. Informed consents were obtained from all patients.

**US guided FNAC**

As we previously described, all US examinations were performed using one of two US systems; an iU22 unit or an HDI-5000 (Philips Healthcare, Bothell, WA, USA) equipped with a linear, high-frequency probe (4 to 15 MHz) [14]. The scanning protocol included both transverse and longitudinal real-time imaging of the thyroid. FNA was performed under US guidance using a 23-gauge needle connected to a 10-mL syringe. All US examinations and FNAs were performed by experienced radiologists or endocrinologists.

**Cytological diagnosis**

Cytological diagnoses were made by experienced endocrine cytopathologist (D.E.S). FNAC diagnoses were categorized into six categories using the Bethesda System as follows: nondiagnostic or unsatisfactory, benign, AUS or FLUS, FN or SFN, suspicious for malignancy, and malignant [1].

**DNA and RNA extraction from residual SurePath material**

Centrifugation of residual SurePath material was performed at 20,000 rpm for 2 minutes and the pellet was used. DNAs and RNAs were extracted from the samples using the AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany). RNA was extracted from the upper phase of the initial extraction step, and DNA was extracted from the lower phase as previously reported [11,13].
cDNA synthesis
According to the manufacturer’s protocol, cDNA was synthesized from RNA using the miScript Reverse Transcription Kit (Qiagen) [11]. A 7.5 μL template RNA was added to a master mix consisting of 2 μL miScript RT buffer and 0.5 μL miScript Reverse Transcriptase Mix. The mixture was incubated for 60 minutes at 37°C and 5 minutes at 95°C.

Detection of point mutations and rearrangements by high-resolution melting peak analysis
BRAF (codon 600), NRAS (codon 61), KRAS (codon 12/13), and HRAS (codon 61) point mutations were detected by real-time polymerase chain reaction (RT-PCR) and high-resolution melting (HRM), using the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche) as previously reported [11,13,15]. PAX8/PPARγ, RET/PTC1, and RET/PTC3 rearrangements were also evaluated after cDNA synthesis from RNAs. PCRs were performed as previously reported [11].

Detection of point mutations and rearrangements by pyrosequencing
BRAF (codon 600), NRAS (codon 61), KRAS (codon 12/13), and HRAS (codon 61) point mutations were detected by pyrosequencing on a PyroMark Q24 (Qiagen) according to the manufacturer’s instructions. Pyrosequencing was applied if the samples were considered as mutation positive or showed ambiguous results by HRM peak analysis. When the samples were determined wild-type by HRM peak analysis, some samples of them were analyzed by pyrosequencing as previously reported [11].

Statistical analysis
Statistical analysis was conducted by using SPSS version 21.0 (IBM Co., Armonk, NY, USA). Graphs were produced using Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). Continuous variables such as age and primary nodule size were presented as mean±standard deviation.

RESULTS
Evaluation for BRAF or RAS point mutations
All DNAs from 53 residual LBC samples could be analysed for point mutations by RT-PCR or pyrosequencing. Point mutations were detected in 10 of 53 samples (19%) (Table 1, Fig. 1). There

Table 1. Polymerase Chain Reaction and Pyrosequencing Results for Point Mutations of Atypia of Undetermined Significance and Follicular Lesion of Undetermined Significance Samples

| Cytology | BRAF       | NRAS | KRAS | HRAS |
|----------|------------|------|------|------|
| A2       | V600E (10%)| Wild type | Wild type | Wild type |
| A5       | Wild type  | Q61R (27%) | Wild type | Wild type |
| A6       | V600E (12%)| Wild type | Wild type | Wild type |
| A9       | V600E (19%)| Wild type | Wild type | Wild type |
| A14      | Wild type  | Q61R (35%) | Wild type | Wild type |
| A15      | Wild type  | Wild type | G12V (16%) | Wild type |
| A16      | V600E (14%)| Wild type | Wild type | Wild type |
| F2       | Wild type  | Q61R (32%) | Wild type | Wild type |
| F8       | Wild type  | Q61K (35%) | Wild type | Wild type |
| F18      | Wild type  | Q61R (20%) | Wild type | Wild type |

In pyrosequencing, samples showing a mutation level greater than 10% were considered as positive mutation.
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Fig. 2. Only one RNA sample from a follicular lesion of undetermined significance (FLUS) thyroid nodule could be analysed for rearrangements. AUS, atypia of undetermined significance.

were no BRAF or RAS point mutations in 16 nondiagnostic thyroid nodules. In 17 AUS thyroid nodules, seven samples (41%) had point mutations including BRAF (n=4), NRAS (n=2), and KRAS (n=1). In 20 FLUS thyroid nodules, three samples (15%) had NRAS (n=3) point mutations.

In AUS thyroid nodules, the BRAF V600E point mutation was detected in sample A2, A6, A9, and A16 by pyrosequencing (mutation percentage 10%, 12%, 19%, and 14%, respectively). NRAS point mutations were detected in sample A5 (Q61R, mutation percentage 27%) and A14 (Q61R, mutation percentage 35%). There was only one KRAS G12V point mutation in sample A15 (mutation percentage 16%). There were no HRAS mutations in AUS thyroid nodules.

In FLUS thyroid nodules, NRAS point mutations were detected in sample F2 (Q61R, mutation percentage 32%), F8 (Q61K, mutation percentage 35%), and F18 (Q61R, mutation percentage 20%). There was no BRAF, KRAS, and HRAS mutations in FLUS thyroid nodules.

Evaluation for rearrangements

Only one RNA sample from a FLUS thyroid nodule could be analysed for rearrangements (Fig. 2). There were no PAX8/PPARY, RET/PTC1, or RET/PTC3 rearrangements in this sample. The other RNA samples were not amplified by PCR.

DISCUSSION

In this study, molecular analysis for BRAF and RAS point mutations was feasible using residual supernatant of LBC SurePath samples after FNA. We found BRAF or RAS point mutations in 10 of 37 AUS/FLUS nodules (27%). However, we did not find point mutations in nondiagnostic thyroid nodules, and RNAs from residual SurePath material were not applicable for molecular analysis of gene rearrangements.

FNA is the procedure of choice in the evaluation of thyroid nodules [6]. Once the needle is withdrawn from the lesion, the material is extruded onto glass slides for a conventional smear which is fixed with 95% ethyl alcohol for the Papanicolaou stain. Otherwise, the smear may be air-dried and then stained with May-Grunwald Giemsa. The thin-layer or LBC technique has been used popularly as an alternative technique for conventional smear due to cost-effectiveness, time-sparing, and simple application with a semi-automated device [10,12]. It is originally developed for application in gynecologic cervical smears. In gynecologic area, the diagnostic utility of cell blocks prepared from residual LBC material was applied for detection of human papilloma virus by immunohistochemistry or in situ hybridization method [16].

A single mutational marker or the molecular testing panel were useful and provided a significant increase in the diagnostic accuracy of FNAC of up to 95% [17-21]. Especially, PTC may carry BRAF, NRAS, or RET/PTC mutations in a total of 70% of cases [9,17,18,20-24]. The revised guidelines of the American Thyroid Association suggested that molecular analysis, such as BRAF, RAS, PAX8/PPARY, and RET/PTC, may be considered in patients with indeterminate FNAC to help guide clinical management and improve the final diagnostic accuracy [6,24].

A repeated FNAC is required for molecular testing, because it is usually applied with fresh FNAC material before fixation. Molecular analysis using residual LBC material takes advantage to avoid repeated FNA or needle biopsy. In this study, we used the supernatant of LBC samples after isolation of the cell pellet which was used for cytological diagnosis. We could apply molecular testing only in nondiagnostic or AUS/FLUS nodules which were diagnosed by cytopathological examination. This approach could enhance cost-effectiveness. The nucleic acids are stable in the preservative solution for up to 6 months after sampling [12,25]. Isolated DNAs from residual LBC SurePath samples were stable and useful for mutational analysis in the present study. The previous study using residual ThinPrep material reported that adequate results were obtained in 402 of 597 cases (67%) [13]. In this study, all DNAs from 53 residual LBC SurePath samples could be analysed for point mutations by RT-PCR or pyrosequencing.

In this study, we found the BRAF V600E mutation in four AUS nodules. There was no BRAF V600E mutation in a FLUS nodule. The AUS nodules had several atypical nuclear features including nuclear grooves, prominent nucleoli, elongated nuclei and cytoplasm, and/or intranuclear cytoplasmic inclusions. The FLUS nodules had a prominent population of microfollicles or...
Hurthle cells but did not otherwise fulfill the criteria for FN/SFN. The \textit{BRAF} V600E mutation is known to be found in 29\% to 69\% of classical PTC and in 80\% of tall cell variant of PTC, but in only 10\% to 25\% of follicular variant of PTC and very rarely in follicular thyroid carcinoma \cite{17,20,21,26,27}. When a \textit{BRAF} V600E mutation was detected in FNAC specimens, the possibility of PTC was approximately 99\% \cite{23,26,28-30}. In this study, RNAs isolated from the supernatant of LBC samples were not useful for molecular diagnosis. RNA from only one sample could be analyzed for rearrangements. The other samples were not amplified in RT-PCR. Since we used Surepath which includes a small amount of formalin for preservation \cite{31}. Limited availability of RNAs from residual LBC samples is most likely due to a chemical linkage between proteins and RNAs generated by formalin \cite{32,33}.

This prospective study has several limitations. First, we could not evaluate sensitivity and specificity of molecular analysis in residual LBC materials because our prospective study protocol was approved by the Institutional Review Board in condition of blind for other medical records including follow-up information. We could be accessible only for the cytological results and residual LBC samples of the study patients. Therefore, we could not obtain final pathological results. Second, our study included only a limited number of patients to indicate general application of molecular testing from residual LBC material. However, this study provides a basis for future progression in the development of a larger prospective study using residual LBC samples for a DNA based molecular analysis of point mutations.

In conclusions, molecular analysis for \textit{BRAF} and \textit{RAS} mutations was feasible in residual LBC materials and might be useful for the diagnosis of indeterminate thyroid nodules. This approach might be expected to reduce repeated FNA, needle biopsy, or diagnostic surgery for indeterminate thyroid nodules.

**CONFLICTS OF INTEREST**

No potential conflict of interest relevant to this article was reported.

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