ORIGINAL

Measurement of *TFF3* mRNA in aspirates from thyroid nodules using mesh filtration: The first clinical trial in 130 cases

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Abstract. Measurement of gene expression levels in thyroid tumor cells in aspirates was difficult because it is interfered with peripheral blood cells or infiltrating lymphocytes. In this study, we established a novel method to separate thyroid tumor cells from blood cells efficiently with mesh filtration. The expression level of trefoil factor 3 (*TFF3*) mRNA was estimated using *LGALS3* mRNA as an internal control (T/G ratio) in 148 preoperative thyroid aspirates. Intra-assay coefficients of variation (CV) of T/G ratio for high, moderate, and low samples were 6.5%, 2.5%, and 9.7%, respectively, and inter-assay CV for high, moderate, and low samples were 27.7%, 21.9%, and 38.2%, respectively. Nondiagnostic samples in terms of T/G ratio and cytology were 12.2% and 16.9%, respectively. We observed no interference with the data by contaminating blood cells. Among these patients, 12 patients received more than two repeated aspirations. We did not observe a marked day-to-day variation except in two cases. All 13 preoperative aspirates diagnosed as malignant by cytology showed an extremely low T/G ratio, whereas 93 aspirates diagnosed as benign by cytology showed extremely varied T/G ratios and 21.5% of them showed a T/G ratio below the cut-off value. Eleven cases underwent surgery. All nodules showing a low T/G ratio were diagnosed as papillary carcinoma by pathological diagnosis. However, one nodule diagnosed as follicular adenoma after surgery showed a high T/G ratio. Our present method may be a promising preoperative test for measuring mRNAs in thyroid aspirates.

Key words: Follicular thyroid carcinoma, Trefoil factor 3 (*TFF3*), Aspiration biopsy cytology, Molecular-based diagnosis, Mesh filtration

THYROID NODULE is a common clinical problem. Although the majority of thyroid nodules are benign and asymptomatic, thyroid carcinoma accounts for about 5% of thyroid nodules [1]. There are three major types of thyroid cancer believed to be derived from thyroid follicular cells: anaplastic carcinomas, papillary carcinomas, and follicular carcinomas. Fine needle aspiration biopsy (FNAB) is the recommended initial test for the diagnosis of thyroid nodule to decide whether patients should undergo surgical resection. The results of FNAB cytology can be classified as benign (70% of

cases), malignant (5 to 10% of cases), intermediate or suspicious (10 to 20% of cases), or nondiagnostic (10 to 20% of cases) [2, 3]. Although FNAB is the most accurate tool for preoperative diagnosis of thyroid malignancy, differential diagnosis between thyroid follicular adenomas and follicular carcinomas is quite difficult since they have similar cytological appearances [4]. Therefore, most patients who have been diagnosed with thyroid follicular tumors require follow-up examinations for the rest of their life or are referred for surgical resection without definite therapeutic necessity. Thus, a preoperative diagnostic method for follicular tumor has been anticipated for a long time.

Recent molecular analysis of follicular thyroid lesions suggests that follicular carcinoma and follicular adenoma have characteristic gene expression profiles [5-7]. However, most molecular markers described in

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these studies failed to show reproducible results in follow-up studies.

In 2004, we found that the decreased expression of trefoil factor 3 (*TFF3*) mRNA is a marker of follicular carcinomas, especially those with a high risk of invasion or metastasis, and follicular adenomas and carcinomas can be efficiently distinguished by measuring *TFF3*/galectin-3 (*LGALS3*) mRNA ratio (T/G ratio) [8, 9]. In the follow-up studies, many researchers at other institutes confirmed the utility of *TFF3* mRNA in diagnosing follicular carcinoma [10-17]. A more intriguing property of *TFF3* mRNA is that papillary and anaplastic thyroid carcinomas also show low T/G ratios [9, 18]. Thus, T/G ratio is a universal indicator to diagnose all follicular cell-derived thyroid tumors.

We have developed a method to diagnose thyroid malignancy named aspiration biopsy nucleic acid diagnosis (ABND), which utilizes nucleic acids from tumor cells obtained by FNAB. ABND has been applied to the diagnosis of thyroid papillary, anaplastic, medullary carcinomas, and B-cell lymphoma [19-23]. However, in the case of *TFF3* mRNA, the precise measurement of its expression levels in thyroid tumor cells in aspirates was difficult because it is interfered with peripheral blood cells or infiltrating lymphocytes that are aspirated simultaneously by FNAB. In fact, there has been only a few reports measuring *TFF3* mRNA using thyroid aspirates [13-16]. Thus, selection of thyroid tumor cells in aspirated samples is crucial for the precise measurement of the T/G ratio in aspirates.

In our previous study, we established a novel method to separate thyroid tumor cells from blood cells using mesh filtration [24]. After mesh filtration, T/G ratios in aspirates corresponded well to those of corresponding tumor tissues. Furthermore, in the present study, we found that addition of hemolytic process before mesh filtration prevents the clotted blood cells from blocking the mesh filter, which enabled the selection of thyroid tumor cells in aspirates with a large volume of aspirated peripheral blood. Using this method, we started a clinical trial and measured the T/G ratios in 148 aspirates obtained from 130 patients.

Materials and Methods

Extraction of RNA from thyroid tissue samples

The study protocol was approved by the institutional ethics committee. The tumor samples were obtained by surgery after obtaining the patients' informed con-

sent and tumors were classified according to the WHO histological classification of thyroid tumors by experienced pathologists. Thirty-one follicular adenomas, 22 follicular carcinomas, and 15 papillary carcinomas were collected. All tissues were frozen in liquid nitrogen immediately after resection. Total RNA was extracted according to the method described previously [24].

Extraction of RNA from aspirates

Extraction of total RNA from thyroid aspirates was performed as described previously with some modifications (Fig. 1) [24]. Size of the thyroid nodules was measured by ultrasonography. We obtained 148 aspirates from thyroid nodules or neck lymph node metastases in 130 patients after obtaining the patients' informed consent. The study protocol was approved by the institutional ethics committee. A syringe with a 22-gauge needle was used for FNAB to obtain an aspirate. Aspirated samples were first used for preparing glass slides for cytological examinations, and then left-over cells inside the needle were flushed into a 1.5-mL tube containing 180 μ L of 0.2% NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) to hemolyze red cells. Then, 900 μ L of RNeasy lysis buffer (Takara Bio, Shiga, Japan) was added, and the sample was stored at 4°C. The sample was placed in a Cell Strainer with a 35- μ m nylon mesh (BD Falcon, CT, U.S.A) and then centrifuged at 150 g for 1 minute. Cells left on the mesh were lysed with 400 μ L of a denaturing solution (D solution) containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol solution, and then total RNA was extracted.

Reverse transcription

Reverse transcription was performed using either 1 μ g of total RNA from tissues or all recovered total RNA from an aspirate in an RT mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 0.5 mM deoxynucleotide triphosphates (dNTPs) (Takara Bio), 200 U M-MLV reverse transcriptase (Invitrogen, Tokyo, Japan), 2 U/ μ L RNase inhibitor (Takara Bio), and 2.5 μ M random hexamer (Takara Bio) in a total volume of 10 μ L. The conditions for reverse transcription were 25°C for 10 minutes, 37°C for 50 minutes, and 70°C for 15 minutes.

Real-time quantitative polymerase chain reaction

We measured *TFF3* (GenBank NM003226) and *LGALS3* (GenBank NM002306) mRNAs.

Fig. 1 Recovery of thyroid tumor cells in the aspirates

An aspirated sample was first used for preparing a glass slide for cytological examinations (A), and then leftover cells inside the needle were flushed into a 1.5-mL tube containing 0.2% NaCl and 1 mM EDTA to hemolyze red cells (B). RNA later was added and then the sample was stored at 4°C (C). The sample was placed in a Cell Strainer and then centrifuged (D).

Thyroglobulin (*TG*, GenBank X05615), CD45 (*PTPRC*, GenBank Y00062), and TSP-1 (*THBS-1*, GenBank X14787) mRNAs were also measured to estimate the number of thyroid tumor cells, white blood cells, and fibroblasts in the aspirates, respectively [24, 25]. Real-time quantitative polymerase chain reaction (TaqMan PCR) using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster, CA, U.S.A.) and Platinum qPCR SuperMix-UDG (Invitrogen) was performed according to the manufacturer's protocol. One microliter of the first strand cDNA was used in the following assay. Two primers and one TaqMan probe used for quantification of *TFF3*, *LGALS3*, *TG*, *PTPRC*, and *THBS-1* mRNAs were as follows:

TFF3F (0.5 μ M): 5'-AATGCACCTTCTGAGGCACCT-3' (base 452-472), TFF3R (0.5 μ M): 5'-CGTTAAGACATCAGGCTCCAGAT-3' (base 623-601), and TFF3-TM (10 pmol): 5'-FAM-CATCTCAGCTTTCTGTCCCTTTGCTCCC-TAMRA-3' (base 547-574); LGALS3F (0.5 μ M): 5'-CTTATAACCTGCCTTTGCCTGG-3' (base 368-389), LGALS3R (0.5 μ M): 5'-GC AACATCATTCCTCTTTGGA-3' (base 464-485), and LGALS3-TM (10 pmol): 5'-FAM-AGTGGTGCCTCGCATGCTGATAACAA-TAMRA-3' (base 393-418);

TGF (0.5 μ M): 5'-CTGCTGGCTCCACCTTGTTT-3' (base 2071-2090), TGR (0.5 μ M): 5'-CAGGGCGTGGGGCATTTCTT-3' (base 2230-2211), and TG-TM (10 pmol): 5'-FAM-CACTTCG AGTCCAGGAA TGGCCTGACCCT-TAMRA-3' (2196-2167); PTPRCF (0.5 μ M): 5'-ATCATAAACTGTGTGCAGAC TCAA-3' (base 3997-4020), PTPRCR (0.5 μ M): 5'-CACACATACCACCTTTATGTACTATCTC-3' (base 4139-4112), and PTPRC-TM (10 pmol): 5'-FAM-TG ACCTCAAGATGTCCTCCTTGTCTACTCA-TA MRA-3' (4044-4074); and THBS1F (0.5 μ M): 5'-CCTGATCCCCACCCTTACTCA -3' (base 4291-4311), THBS1R (0.5 μ M): 5'-CACTGCCTTACACTG GTTTGA-3' (base 4366-4343), and THBS1-TM (10 pmol): 5'-FAM-CACCTTGCAGTGGCCAGAATTA GGAAT-TAMRA-3' (base 4313-4340); respectively.

All primers and probes were purchased from Operon Biotechnologies (Tokyo, Japan). The conditions for TaqMan PCR were as follows: 95°C for 2 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Recombinant pGEM Easy T-Vector (Promega, Tokyo, Japan) containing *TFF3*, *LGALS3*, *TG*, *PTPRC*, or *THBS-1* cDNA was constructed by PCR-cloning with the same set of primers used in TaqMan PCR, and

these products were then used as standard samples. The amplification plots of the PCR reaction were used to determine the threshold cycle (C_T). The C_T value represented the PCR cycle at which an increase in reporter fluorescence (ΔR_n) above the line of the optimal value (optimal ΔR_n) was first detected. The initial copy number of the target mRNA was calculated by a plot of the C_T against the input target quantity. The results are shown as the mean from duplicate determinations.

Statistical analysis

Data were analyzed with JMP7 software (SAS Institute, SAS Campus Drive Cary, NC, U.S.A.). A receiver operator characteristic (ROC) curve was constructed to find the optimum values of the T/G ratio for distinguishing follicular carcinoma from follicular adenomas. Statistical analysis of differences between the groups was carried out using the Mann-Whitney *U* test. A *P* value less than 0.05 was considered significant. The correlation between the tumor size and the T/G ratio was analyzed by the Pearson product-moment correlation coefficient method.

Results

Estimation of the cut-off value

In this study, we estimated the cut-off value to differentiate malignant thyroid tumors from benign tumors using tissue samples according to the assumption that a T/G ratio in a tumor corresponds well to that in its aspirate, since we do not have any data on T/G ratios in thyroid aspirates and, in our previous study, we confirmed that, using the mesh filtration method, the T/G ratios in tumor tissues corresponded well to those in their aspirates [24]. We measured the T/G ratios in 31 follicular adenomas, 22 follicular carcinomas, and 15 papillary carcinomas. Among the 22 follicular carcinomas, 7 cases were widely invasive carcinoma and minimally invasive carcinoma with distant metastasis (Fig. 2).

The ROC curve analysis for distinguishing follicular carcinoma from adenoma determined the cut-off value as 1.89. Using this cut-off value, the sensitivity and specificity were 72.3 and 87.1%, respectively. Since the distinction of follicular adenomas from minimally invasive follicular carcinomas without distant metastasis is quite difficult even by pathological examination, another aspect to decide the cut-off value was to set it to cover all clinically problematic tumors including papillary carcinomas, widely invasive follicular carci-

Fig. 2 T/G ratios in thyroid tumor tissues to set the cut-off value 1, Follicular adenomas; 2, minimally invasive carcinomas without distant metastasis; 3, widely invasive follicular carcinomas and minimally invasive follicular carcinomas with distant metastasis; and 4, papillary carcinomas. The broken line represents the cut-off value determined by the T/G ratios in clinically problematic malignant tumors.

nomas, and minimally invasive follicular carcinomas with distant metastasis. This distinction has a significant impact on prognosis since the prognosis is more severe in these tumors [26]. In this regard, the cut-off value was set at 1.50. Since there was only a small difference between the two cut-off values, we used 1.50 in this study.

T/G ratio in the preoperative aspirates obtained from thyroid nodules

We measured the T/G ratios in the preoperative aspirates, and compared them with their cytology. Intra-assay coefficients of variation (CV) for high, moderate, and low samples were 6.5%, 2.5%, and 9.7%, respectively, and inter-assay CV for high, moderate, and low samples were 27.7%, 21.9%, and 38.2%, respectively. For samples in which both *TFF3* and *LGALS3* mRNAs were less than 30 copies, T/G ratios tended to show extremely low values, probably because these samples did not contain thyroid epithelial cells (data not shown). Thus, we regarded these samples as nondiagnostic in terms of T/G ratio. Nondiagnostic samples in terms of T/G ratio and cytology were 12.2% and 16.9%, respectively. There was a considerable discrep-

Fig. 3 T/G ratios in the preoperative aspirates obtained from thyroid nodules. Open circles indicate operated nodules.

ancy in nondiagnostic samples between T/G ratio and cytological examinations (Table 1). This was probably due to the fact that T/G ratio was measured using the leftover samples in the needles, which did not always contain the same number of tumor cells as prepared for cytological examinations.

All 13 aspirates from 11 cases diagnosed as malignant by cytology showed low T/G ratio (Fig. 3). 93 aspirates diagnosed as benign by cytology showed extremely varied T/G ratios from 0 to 239 and 20 (21.5%) aspirates showed a T/G ratio below the cut-off value. Among these patients, 11 cases underwent surgery and 16 corresponding tumors or lymph node metastases were resected. The T/G ratios in the cases that underwent surgery are summarized in Table 2. Among the tumors diagnosed as papillary carcinoma, one tumor was nondiagnostic in terms of the preoperative T/G ratio but all the rest showed low T/G ratios in the preoperative aspirates. One tumor was diagnosed as follicular adenoma after surgery and its preoperative aspirate showed high T/G ratio (8.04).

Estimation of contaminating blood cells and fibroblasts in the aspirates

We measured the copy number of *PTPRC* mRNA, a marker of white blood cells, in the aspirates to estimate the blood cell removal efficiency with the combination of hemolysis and mesh filtration. Aspirates contaminated with more than 10 μ L of blood were classified as

Table 1 Comparison of the percentage of nondiagnostic samples between T/G ratio and cytology

	T/G ratio	Diagnostic 130 (87.8%)	Nondiagnostic 18 (12.2%)
Cytology			
Diagnostic 123 (83.1%)		111 (75.0%)	12 (8.1%)
Nondiagnostic 25 (16.9%)		19 (12.8%)	6 (4.1%)

bloody aspirates. We compared the expression level of *PTPRC* mRNA between 86 normal and 24 bloody aspirates and there was no significant difference between the two (Fig. 4A). These results suggested that blood cells in aspirates were efficiently removed by the present method and showed little effect on measurement of T/G ratio.

A considerable number of samples that were diagnosed as benign in cytology showed a T/G ratio below the cut-off values. Since contamination of a large number of fibroblasts can decrease T/G ratio since they express a high copy number of *LGALS3* mRNA but not *TFF3* mRNA, we measured *THBS-1* mRNA, which is expressed in thyroid-derived fibroblasts in a restricted manner [25]. To assess the proportion of fibroblasts to thyroid tumor cells, we calculated *THBS-1/TG* mRNA ratio in 64 aspirates diagnosed as benign by cytology. There was a significant difference between the aspirates with T/G ratios above and below the cut-off value ($P < 0.01$) (Fig. 4B). These results suggested that contaminating fibroblasts might decrease the T/G ratio in

Table 2 T/G ratios in the preoperative aspirates and pathological diagnoses in the corresponding tumor tissues in the cases that underwent surgery

Case	Thyroid tumor	Intrathyroidal metastasis	Lymph node metastasis 1	Lymph node metastasis 2	Pathological diagnosis
1	0.15	0.15			Papillary carcinoma
2	0.21 ^a		0.53		Papillary carcinoma
3	0	0.001			Papillary carcinoma
4	0.002				Papillary carcinoma
5			0.01		Papillary carcinoma
6	0.44				Papillary carcinoma
7	0.006				Papillary carcinoma
8	0.19				Papillary carcinoma
9	0.71 ^b		0.59 ^b	0.24	Papillary carcinoma
10	0.05				Papillary carcinoma
11	8.04				Follicular adenoma

a: T/G ratio was nondiagnostic due to low copy numbers (*TFF3* mRNA, 4.4; *LGALS3* mRNA, 21.0) and the tumor was diagnosed as an adenomatous nodule by cytology.

b: Cytology was nondiagnostic due to the lack of epithelial cells.

Fig. 4 The expression of *PTPRC* (A) and *THBS-1* (B) mRNAs in the aspirates

Fig. 5 T/G ratios in the aspirates from nodules for which repeated aspiration was performed.

some samples, which could be a cause of a false positive result.

T/G ratios in repeated aspirations in the same nodules

Among these patients, 12 patients received more than two repeated aspirations, since a definite cytological diagnosis could not be made with a single FNAB. We did not observe a marked day-to-day variation except in two cases. The first case showed extremely

low T/G ratio in the first aspirate (0.00) compared with those in the second (5.59) and third aspirates (6.99). The second case showed extremely low T/G ratio in the second aspirate (0.02) compared with that in the first aspirate (2.33) (Fig. 5). We assumed that these low T/G ratios were caused by contaminating fibroblasts, since in these samples, we could hardly detect *TG* mRNA (1.2 and 1.0 copies), while *LGALS3* mRNA was expressed abundantly (1316 and 1673 copies). Furthermore, there were no thyroid epithelial cells in the glass slides for cytology. Unfortunately, owing to the limited volume of these samples, we could not confirm this assumption by measuring *THBS-1* mRNA.

Comparison between tumor size and the T/G ratio

We measured the size of the aspirated thyroid nodules and analyzed if it has any relation with the T/G ratio. There was no correlation between tumor size and the T/G ratio (Fig. 6).

Discussion

In this study, we carried out the first clinical trial of ABND measuring *TFF3* mRNA. It is noteworthy that, even using the leftover cells in the needle, we could measure T/G ratio successfully in nearly 90% of the aspirates.

Considering the following findings, the present study

Fig. 6 Comparison between the tumor size and the T/G ratio

demonstrated that the T/G ratios measured in thyroid aspirates using the present methods are likely to represent those in the corresponding thyroid nodules. First, the T/G ratios in aspirates showed a distribution similar to that of thyroid tumors reported previously [9]. For example, all aspirates diagnosed as malignant by cytological examinations showed a low T/G ratio and all samples aspirated from tumors diagnosed as papillary carcinoma after surgery showed a low T/G ratio. Furthermore, aspirates diagnosed as benign by cytological examinations showed extremely varied T/G ratios. Second, repeated aspirates from the same nodule usually showed similar T/G ratios, which suggested the T/G ratio in each aspirate corresponds well to that of the corresponding nodule. Inter-assay CVs of the present T/G ratio measurement were higher than many other clinical laboratory tests. This was because the T/G ratios were determined by real-time quantitative RT-PCR measuring a small quantity of mRNA, which usually shows a high CV [27]. However, we consider this level of high CVs is not a big problem in our test, since *TFF3* mRNA expression was reported to be more than 30 times greater in follicular adenomas than in follicular carcinomas [12].

In the present method, the contamination of a large volume of aspirated peripheral blood showed no effect on T/G ratio. This is a great advantage for ABND, especially when this method is applied for follicular tumors, since they are usually rich in blood vessels [4]. Furthermore, the present method of RNA recovery takes only a few minutes for sample preparation and does not require laborious technical procedures. The samples can be stored at 4°C for at least one week [24]. Total cost of the analysis is only 10 US dollars. These features are also advantageous when we consider its use in a daily clinical context.

Thyroid-derived fibroblasts in connective tissues are difficult to be removed by mesh filtration. Thus, contaminating fibroblasts can be a cause of a false positive result. About 20% of aspirates diagnosed as benign by cytology showed a T/G ratio below the cut-off value. The actual T/G ratio in some of these aspirates might be higher, since we observed a large copy number of *THBS-1* mRNA in them. However, this might not be a serious problem. Since ABND will be used as a screening test, a small percentage of false positive cases are allowable. Furthermore, contaminating fibroblasts can be detected by measuring *THBS-1* mRNA. False positive results might be reduced by recommend-

ing repeated aspiration when we find a sample with a high copy number of *THBS-1* mRNA. Otherwise, when we can accumulate the data on how *THBS-1* expression alters the T/G ratio in the aspirates, we may be able to calculate and adjust a real T/G ratio based on the amount of *THBS-1* expression.

In this study, the T/G ratios in thyroid nodules showed no correlation with their size. This finding supports the previous observation that long standing thyroid nodules do not usually change their cytological appearances, and also probably biological characteristics [28].

It would be a great advance in the diagnosis of thyroid tumor if the expression level of *TFF3* mRNA can be measured in thyroid aspirates, since it is regarded as one of the promising markers to distinguish thyroid carcinoma including follicular carcinoma. Although *TFF3* mRNA was reported to be the best marker in many studies, the discrepancy between pathological and *TFF3* mRNA-based diagnosis was often problematic since the accuracy in diagnosing follicular adenomas and carcinomas is only around 80% [8, 9, 12]. This is partly due to the difficulty in pathological distinction between follicular adenomas and minimally invasive follicular carcinoma without distant metastasis. In fact, local recurrence or distant metastasis sometimes occurs in tumors pathologically diagnosed as definite benign tumors, suggesting that the present WHO criteria in diagnosing follicular carcinoma, in which only a capsule or vascular invasion is regarded as a sign of malignancy, do not always reflect the biological characteristics of the tumors [29, 30]. Taniguchi *et al.* measured the expression levels of more than 2500 genes expressed in follicular tumors and they examined whether the expression level of any genes or any set of genes could distinguish follicular carcinomas from adenomas. However, the accuracy never exceeded 90% even when they used a combination of the top 60 genes [10]. This study clearly indicated that at least 10% of the pathological diagnoses of follicular tumors do not correctly reflect their biological characteristics.

Interestingly, *TFF3* mRNA-based molecular diagnosis of “clinically evident” follicular carcinomas, including widely invasive follicular carcinomas and minimally invasive carcinomas with distant metastasis, showed a perfect match with the pathological diagnosis and the discrepancy between molecular-based diagnosis and pathological diagnosis was restricted to minimally invasive follicular carcinomas without distant metastasis [8, 11]. From a clinical point of view, it is

clear that we should focus our efforts on *TFF3* mRNA underexpressing tumors, which contain anaplastic and papillary carcinomas, widely invasive follicular carcinomas, and minimally invasive follicular carcinomas with distant metastasis. The present method would be a great help to screen such tumors preoperatively. The discussion about the discrepancy between pathological and molecular-based diagnoses in minimally invasive follicular carcinomas without distant metastasis might be of academic but not clinical interest. Probably, we should regard molecular-based diagnoses including the *TFF3* mRNA-based diagnosis as independent criteria from the classical pathological diagnosis to decide surgical resection.

The above considerations lead to the recognition that our test has two advantages over the conventional cytological examinations. First, using this test, the majority of follicular carcinomas, especially widely invasive follicular carcinomas and minimally invasive follicular carcinomas with a distant metastasis, are expected to be distinguished from follicular adenoma. Second, cytologically intermediate cases can be classified objectively into benign or malignant by measuring the T/G ratio.

Unfortunately, in the present study, only one patient with benign cytology underwent surgery. Thus, we

could not obtain data to estimate the sensitivity and specificity of ABND in diagnosing thyroid follicular tumors including follicular carcinoma. We expected these situations since immediate surgical resection is not usually recommended to a patient with a tumor diagnosed as benign by cytology. Follicular carcinoma is a relatively rare tumor and it is rare that a follicular carcinoma is diagnosed as malignant by cytological examinations [4]. Further clinical study on a large scale should be carried out for several years to confirm the usefulness of this method in diagnosing follicular carcinoma.

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Appendix

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