ABSTRACT

Recently, the restricted expression of oncofetal fibronectin mRNA was reported in thyroid papillary and anaplastic carcinomas. In this study, by extracting RNA from the leftover cells inside the needles used for fine-needle aspiration biopsy, we establish a new method for gene diagnosis of these carcinomas without further invasiveness to the patient (aspiration biopsy-reverse transcription-PCR, ABRP). RNA was extracted from 177 fine-needle aspiration biopsies of thyroid nodules that were suspicious for malignancy, and then the gene diagnoses made by reverse transcription-PCR detection of oncofetal fibronectin mRNA were compared with cytological diagnoses. Thirty-five (94.6%) of 37 samples that were diagnosed as papillary or anaplastic carcinomas by cytological examination showed a positive result by gene diagnosis, whereas only 4 (3.7%) of 109 samples that were cytologically diagnosed negative for both carcinomas showed a positive result. Among all of the cases, 50 patients underwent surgery, and a histological diagnosis was consequently made. The sensitivity and specificity of this method were 96.9 and 100%, respectively. A combined examination using both genetic and cytological approaches may contribute to a more precise preoperative diagnosis of papillary and anaplastic carcinomas.

INTRODUCTION

Thyroid tumors are often diagnosed by FNAB3 as well as by ultrasonography. Cytological examination of FNAB by a skilful pathologist who is an expert in thyroid tumors provides the most reliable information for the diagnosis of thyroid neoplasms (1–3). In some clinical situations, however, slide samples are not adequate for cytological examination because of poor fixation, and a well-trained expert pathologist is not always available for diagnosis. In such cases, a more objective method is required for exact diagnosis. Gene diagnosis by analyzing nucleic acids from aspirates may be used for this purpose. Most previous trials, however, have failed to provide much information on the clinical features of thyroid tumors (4, 5). Thyroid cancers are known to overexpress a number of genes, such as EGF receptor, c-erbB, IGF-I, p53, dipeptidyl aminopeptidase IV, c-Met, and CD44E (6–12). However, mRNAs of these genes are not adequate for gene diagnosis by RT-PCR because their expression is not restricted in cancer cells and because not all of the cancer tissues overexpress their mRNAs. As for genes expressed in blood cells, they cannot be used for this purpose because substantial contamination of blood cells often occurs in FNAB.

In our previous study (13), we used a PCR-based method, Sequence Specific Differential Display, to screen for specific mRNAs in thyroid carcinomas, and succeeded in finding several mRNAs the expression of which is restricted in cancer tissues. One such gene was oncofetal fibronectin. Fibronectins are high-molecular-mass adhesive glycoproteins present in the extracellular matrix and in body fluids (14). Oncofetal fibronectin is characterized by the presence of the oncofetal domain (IIICS domain), which is absent in normal fibronectin (15). Many researchers have used this antibody in immunohistochemical analysis to indicate the existence of oncofetal fibronectin in such malignant tissues as oral, breast, colon, and gastric cancers (16–19); in addition, a recent study (20) showed the expression of intracellular fibronectin in thyroid carcinomas. In our previous study of tissues from 98 thyroid tissue samples (13), we found that papillary and anaplastic carcinomas could be distinguished from normal thyroid tissues, follicular adenomas, and follicular carcinomas by RT-PCR amplifying oncofetal fibronectin cDNA. Subsequently, the restricted expression of oncofetal fibronectin mRNA in papillary and anaplastic carcinomas was further confirmed by in situ hybridization (21). Thus, oncofetal fibronectin is considered to be an ideal target for gene diagnosis and therapy of these two carcinomas, which together comprise about 90% of all malignant thyroid tumors in iodide-sufficient counties (22). Accurate preoperative diagnosis of papillary carcinomas is clinically important because they usually indicate the need for surgical dissection.

In a previous study (23), we introduced a new method of preoperative gene diagnosis of thyroid carcinomas. This technique, ABRP, allows us to perform cytological and gene diagnoses simultaneously. After preparing a slide glass for cytological examination, we extract RNA from leftover cells within the needle used for FNAB for RT-PCR analysis. ABRP thus provides both RNA information and a cytological diagnosis without further invasiveness to the patient. The RNA extracted from a single FNAB provides us with sufficient cDNA for many as 20 PCR examinations. Furthermore, the results of cytological and gene diagnoses using the same RNA can be compared. Most importantly, only a few minutes are required to produce both a slide glass and a RNA sample. Thus, it is possible to collect samples for gene diagnosis in the midst of clinical work.

In this study, we first performed ABRP using the 72 surgically dissected benign and malignant thyroid tissues to confirm the usefulness of this method. Next, to establish the method of preoperative gene diagnosis of thyroid papillary and anaplastic carcinomas, ABRP was performed in 155 patients, and the results of the cytological and gene diagnoses were compared. Finally, the sensitivity and specificity of ABRP were determined postoperatively.

MATERIALS AND METHODS

ABRP Using Surgical Tissues. Seventy-two thyroid tissues (23 normal thyroid tissues from the opposite lobe of the thyroid carcinomas, 14 adeno-
mous goiters, 13 follicular adenomas, 3 follicular carcinomas, 18 papillary carcinomas, and 1 anaplastic carcinoma) were surgically dissected and used immediately. ABRP was performed as previously described (Ref. 23; Fig. 1). A syringe with a 22-gauge needle was used for FNAB from each tissue sample. Samples were prepared on a slide glass for cytological examinations, and then leftover cells inside the needle were lysed rapidly and flushed into a 1.5-ml tube using a denaturing solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The tube was stored at 4°C. Total cellular RNA was extracted by the method of Chomczynski and Sacchi (24). After Papinicolaou staining, slide glasses were used to make sure the tumor cells were aspirated from the tissues (25). Reverse transcription was performed using the whole RNA extracted in a reverse transcription mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 0.5 mM dNTPs, 200 units M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD), 2 units/μl RNase inhibitor (Takara, Shiga, Japan), and 2.5 μM oligo dT (Life Technologies) in a total volume of 20 μl at 37°C for 60 min. One μl of first strand cDNA was used as a template for the PCR reaction with specific primers for either oncofetal fibronectin (26) or thyroglobulin (27). For the amplification of oncofetal fibronectin cDNA, a poly(A)-anchor primer (13) was used instead of a 3’ specific primer to prevent false positive results. The oligonucleotides used as primers were: (a) thyroglobulin 5’ (5’-GTGCGACACCTCATCGT) (base 7011–7027); (b) thyroglobulin 3’ (5’-AATTCTGCAGTGCCTGGT) (base 7657–7674); (c) oncofetal fibronectin 5’ (5’-AAAGCATAGCCAAGACCATAAC) (base 6127–6148); and (d) oncofetal fibronectin 3’ (5’-ATGCGAATTCGTITnTnTTrnT). All of the primers were purchased from Life Technologies. Each reaction mixture consisted of 1 μl of cDNA, 0.5 μM of each primer, 1 μl of 10 X Ex Taq buffer, 0.8 μl of dNTP mix, 0.5 units of Ex Taq polymerase, and nuclease-free water to a final volume of 10 μl. The 10X Ex Taq buffer, dNTP mix, and Ex Taq polymerase were obtained from Perkin-Elmer Cetus (Emeryville, CA). The reaction mixture was subjected to 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). After PCR amplification, the reaction mixture was run on 1% SeaKem GTG agarose gel (Takara) in Tris-HCl/acetate/EDTA (TAE) buffer. The gel was then stained with ethidium bromide.

**Gene Diagnosis by ABRP.** A total of 177 aspirates from 155 patients with nodular goiter (21 males and 134 females; age range, 18–85 years) who were suspected of having a malignant thyroid tumor were obtained by UG-FNAB using a syringe with a 22-gauge needle as described previously (2). After a preparation of the slide-glass sample for cytological examination, total RNA was extracted from leftover cells inside the needle, and RT-PCR analysis was performed as described previously. Thyroglobulin mRNA, which exists only in the thyroid follicular cells (28), was used as the positive internal control; the PCR reaction with specific primers for either oncofetal fibronectin (26) or thyroglobulin (27). For the amplification of oncofetal fibronectin cDNA, a poly(A)-anchor primer (13) was used instead of a 3’ specific primer to prevent false positive results. The oligonucleotides used as primers were: (a) thyroglobulin 5’ (5’-GTGCGACACCTCATCGT) (base 7011–7027); (b) thyroglobulin 3’ (5’-AATTCTGCAGTGCCTGGT) (base 7657–7674); (c) oncofetal fibronectin 5’ (5’-AAAGCATAGCCAAGACCATAAC) (base 6127–6148); and (d) oncofetal fibronectin 3’ (5’-ATGCGAATTCGTITnTnTTrnT).

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**Cytological Examinations.** After Papinicolaou staining, slide-glass samples were classified into the following categories: (a) negative cytology, benign nodules or follicular tumors; (b) suspicious cytology, presence of atypical cells that are suspected for papillary or anaplastic carcinoma; (c) positive cytology, papillary or anaplastic carcinoma; and (d) inadequate materials, insufficient materials because of poor fixation or scanty follicular cells.

**Histological Examinations.** After the operation, thyroid tumors were classified histopathologically according to the WHO histological classification of thyroid tumors (29). The sensitivity of ABRP was calculated as:

\[
\text{Sensitivity} = \frac{\text{Number of samples in which RT-PCR detected oncofetal fibronectin mRNA}}{\text{Total number of tumors other than papillary and anaplastic carcinomas}}
\]

and the specificity of ABRP as:

\[
\text{Specificity} = \frac{\text{Number of samples in which RT-PCR did not detect oncofetal fibronectin mRNA}}{\text{Total number of tumors other than papillary and anaplastic carcinomas}}
\]

The sensitivity of cytology was calculated as:

\[
\text{Sensitivity} = \frac{\text{Number of samples of positive and suspicious cytology from papillary and anaplastic carcinomas}}{\text{Number of papillary and anaplastic carcinomas}}
\]

and the specificity of cytology was calculated as:

\[
\text{Specificity} = \frac{\text{Number of samples of negative cytology from other tumors than papillary and anaplastic carcinomas}}{\text{Number of other tumors than papillary and anaplastic carcinomas}}
\]

**RESULTS**

To confirm the reliability of ABRP, we aspirated 72 thyroid tissues and examined the expression of oncofetal fibronectin and thyroglobulin.
onfFN mRNA by RT-PCR. A representative gel image is shown in Fig. 2. Thyroglobulin mRNA was detected in all of the samples, including one anaplastic carcinoma sample. Oncofetal fibronectin mRNA was detected in all of the papillary and anaplastic carcinomas but not in normal thyroid tissues, follicular adenomas, adenomatous goiters, or follicular carcinomas (Table 1). Next, 177 aspirates from 155 patients were examined. A representative gel image is shown in Fig. 3. Oncofetal fibronectin mRNA was detected in 94.6, 66.7, and 3.7% of the samples that were diagnosed as positive, suspicious, and negative cytology, respectively (Table 2). The total numbers of the inadequate materials were 28 (15.8%) for the cytological diagnosis and 9 (5.1%) for the gene diagnosis. Among the 155 patients, 50 underwent surgery, and, thus, histological diagnoses were made in these cases (Table 3). Both the gene and cytological diagnoses agreed with the histological diagnosis in 47 of 48 cases. The sensitivity and specificity were 96.9 and 100% for the gene diagnosis, and 100 and 93.8% for the cytological diagnosis, respectively (Table 4).

**DISCUSSION**

Nodular thyroid disease is a frequently observed condition, especially in women. The prevalence of palpable thyroid nodules is reported to be 4 to 7% in adults (30). FNAB has been used as a common method of preoperative diagnosis of thyroid nodules, and its safety and reliability have been established by many studies. For a successful diagnosis, FNAB requires the following three conditions: (a) accurate aspiration by a well-trained thyroidologist; (b) quick and accurate preparation of slide samples; and (c) intensive cytological examination by a skilled pathologist. Accurate aspiration may be performed by the use of UG-FNAB (2). The training for the preparation of slide samples is relatively easy. However, the final step, that of the cytological examination, is often problematic. Diagnosis by a pathologist specializing in thyroid tumors may not always be possible. Thus, in many cases, screeners who are not familiar with thyroid diseases are engaged in the diagnosis. In fact, the requirement for an expert cytopathologist has discouraged the wide use of FNAB in a number of countries, and the wide variance in the previously reported sensitivity and specificity of FNAB may be the result, at least in part, of varying skills among the cytopathologists (31). Objective diagnosis by FNAB may be established by genetic analysis. However, many previous trials failed to yield a sensitivity and specificity comparable to that of cytological examinations because no target genes capable of clearly distinguishing malignant and benign thyroid tumors had yet been found. Our recent finding of the restricted expression of oncofetal fibronectin mRNA in thyroid papillary and anaplastic carcinomas enabled us to establish an efficient method of gene diagnosis by ABRP.

In the present study, the sensitivity and specificity of ABRP in the diagnosis of papillary and anaplastic carcinomas were 96.9 and 100%, respectively. These results are almost the same as those derived from cytological examination by a skillful pathologist. Previous studies reported the sensitivity and the specificity of FNAB for thyroid malignancy as 65–98% and 72–100%, respectively (31). However, it is difficult to directly compare these previous results and our present results for two reasons:

(a) in most of the reports, 10–30% of the samples are cytologically determined as "intermediate" or "suspicious" (32). Therefore, the results differ considerably depending on whether these samples were seen as negative or positive (for example, including intermediate samples in the positive group increases the sensitivity but decreases the specificity); and

(b) especially in the reports from iodide-deficient countries, a considerable number of cases of follicular carcinomas, which show a negative result by ABRP, are included in the category of "malignant"

**Table 1 ABRP from tissue samples**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total number</th>
<th>onfFN (+)</th>
<th>onfFN (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid</td>
<td>23</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Adenomatous goiter</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>13</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>18</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Anaplastic carcinoma</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* onfFN, oncofetal fibronectin; +, detected; -, not detected.

**Table 2 Comparison between the cytology and ABRP**

<table>
<thead>
<tr>
<th>Cytology (n = 177)</th>
<th>Positive (n = 37)</th>
<th>Suspicious (n = 3)</th>
<th>Negative (n = 109)</th>
<th>Inadequate (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology (%)</td>
<td>(20.9)</td>
<td>(1.7)</td>
<td>(61.6)</td>
<td>(15.8)</td>
</tr>
<tr>
<td>Tg (+)</td>
<td>35 (94.6%)</td>
<td>2 (66.7%)</td>
<td>4 (3.7%)</td>
<td>4 (14.3%)</td>
</tr>
<tr>
<td>Tg (-)</td>
<td>1 (2.7%)</td>
<td>1 (33.3%)</td>
<td>101 (92.7%)</td>
<td>20 (71.4%)</td>
</tr>
</tbody>
</table>

* Tg, thyroglobulin; onfFN, oncofetal; +, detected; -, undetected.
tumors without classification by the histological typing of papillary, follicular, and anaplastic carcinomas.

Differentiation of follicular adenomas and carcinomas by ABRP is not possible at present. However, we do not consider this to be a serious limitation of this method, because the preoperative diagnosis of follicular carcinomas is also quite difficult by cytological examination (33). This method may be more useful in iodide-sufficient countries like Japan because the prevalence of follicular carcinomas is very low.

In the present study, 1 of the 50 cases operated on was misdiagnosed by ABRP. We have not been able to determine the reason for the differences between the cytology and the gene diagnosis. Possibly, the majority of cancer cells aspirated by FNAB were washed out with the blood onto the slide glass, as sometimes occurred when we aspirated a large volume of blood or cystic fluid by FNAB. This is one of the major causes of the unsuccessful detection of thyroglobulin mRNA in the ABRP samples and renders these samples inadequate for gene diagnosis. One case could not be diagnosed by cytological examination because of poor fixation but was correctly diagnosed by ABRP. We, therefore, consider ABRP to be as useful and accurate as cytological examination.

Four of 109 samples that diagnosed as negative cytology showed a positive result in the gene diagnosis. We could not clarify the reason for this discrepancy because these patients had not undergone an operation. Repeated FNAB may be needed in these cases for a more accurate diagnosis to decide whether an operation is warranted. The carcinoma cells may have remained inside the needle, failing to transfer onto the slide glass, which sometimes happens when only a small number of cells is aspirated or when a microcarcinoma (the high prevalence of which was reported previously; Ref. 34) is aspirated.

In this study, thyroglobulin mRNA was used as an internal control for the gene diagnosis to confirm the existence of thyroid follicular tumors without classification by the histological typing of papillary, follicular, and anaplastic carcinomas.

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In this study, thyroglobulin mRNA was used as an internal control for the gene diagnosis to confirm the existence of thyroid follicular tumors. As described by Arturi et al. (35), ABRP may be used for the diagnosis of lymph node or bone metastases of differentiated thyroid carcinomas. This is because ABRP rapidly provides samples for gene diagnosis without further invasion to the patient; the existence of thyroglobulin mRNA confirms the diagnosis of carcinoma of thyroid origin.

The total RNA recovered from the leftover cells is usually less than 50 ng (23). When the clinical usefulness of gene diagnosis is confirmed, the use of the whole samples aspirated may improve the sensitivity and specificity of this method. Smaller percentages of samples (5.1%) have been determined to be inadequate compared with cytological examination (15.8%). This result indicates that gene diagnosis requires a smaller number of cells than does cytological examination. The method of ultra fine-needle biopsy using 24- to 26-gauge needles ought to be established because it reduces invasion into the patients. Furthermore, up to six multiple aspirations from a single nodule to prevent false negative results, as recommended by Hamburger et al. (36), may not be necessary when a combined diagnosis using genetic and cytological examinations is made possible.

ABRP requires only a few minutes to produce both a slide glass and RNA samples, far less than the time required in previous studies in which aspirated materials were divided into aliquots (35). The total cost of RT-PCR analysis of both thyroglobulin and oncofetal fibronectin mRNAs is only $4.00 (500 yen), much cheaper than that of most other tests of thyroid carcinomas. Therefore, RT-PCR detection of oncofetal fibronectin mRNA may be used for the first screening of papillary and anaplastic carcinomas.

ACKNOWLEDGMENTS

We thank Mayumi Hisago for technical assistance.

REFERENCES

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Table 3 Comparison between histology and ABRP

<table>
<thead>
<tr>
<th>Histology n = 50 [no. of cases]</th>
<th>Cytology (no. of cases)</th>
<th>Tg (+) a</th>
<th>Tg (+) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary carcinoma [33]</td>
<td>Positive (29)</td>
<td>27 (81.8%)</td>
<td>1 (3.0%)</td>
</tr>
<tr>
<td></td>
<td>Suspicious (2)</td>
<td>2 (6.1%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td></td>
<td>Inadequate (2)</td>
<td>1 (3.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Anaplastic carcinoma [1]</td>
<td>Positive (1)</td>
<td>1 (100%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Follicular carcinoma [4]</td>
<td>Positive (1)</td>
<td>0 (0.0%)</td>
<td>1 (25.0%)</td>
</tr>
<tr>
<td></td>
<td>Negative (3)</td>
<td>0 (0.0%)</td>
<td>3 (75.0%)</td>
</tr>
<tr>
<td>Follicular adenoma [6]</td>
<td>Negative (6)</td>
<td>0 (0.0%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Adenomatous goiter [5]</td>
<td>Negative (5)</td>
<td>0 (0.0%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Graves' disease [7]</td>
<td>Negative (1)</td>
<td>0 (0.0%)</td>
<td>1 (100%)</td>
</tr>
</tbody>
</table>

Table 4 Sensitivity, specificity, and accuracy of ABRP

<table>
<thead>
<tr>
<th>Cytology, %</th>
<th>ABRP, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100 (32/32)</td>
</tr>
<tr>
<td>Specificity</td>
<td>93.8 (15/16)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>97.9 (47/48)</td>
</tr>
</tbody>
</table>


Accurate and Objective Preoperative Diagnosis of Thyroid Papillary Carcinomas by Reverse Transcription-PCR Detection of Oncofetal Fibronectin Messenger RNA in Fine-Needle Aspiration Biopsies

Toru Takano, Akira Miyauchi, Tamotsu Yokozawa, et al.


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