Preoperative Diagnosis of Thyroid Papillary and Anaplastic Carcinomas by Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction of Oncofetal Fibronectin Messenger RNA

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Abstract

The restricted expression of oncofetal fibronectin (onfFN) mRNA in thyroid papillary and anaplastic carcinomas was recently reported. In this study, we measured the copy number of onfFN mRNA in RNAs extracted from fine needle aspiration biopsies by real-time quantitative reverse transcription-PCR using thyroglobulin mRNA as an internal control. By measuring the onfFN:thyroglobulin mRNA ratio, preoperative aspirates from 31 papillary carcinomas and an anaplastic carcinoma can be distinguished from those from 5 adenomatous goiters, 5 follicular adenomas, and 4 follicular carcinomas. Thus, quantification of onfFN by real-time quantitative reverse transcription-PCR may be useful for the preoperative diagnosis of papillary and anaplastic carcinomas.

Introduction

Thyroid tumors are often diagnosed by FNAB as well as by ultrasonography. Cytological examination of FNAB by a skillful pathologist who is an expert in thyroid tumors provides the most reliable information for the diagnosis of thyroid neoplasms. In some clinical situations, however, slide samples are not adequate for cytological examinations due to 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. In a previous study, we introduced a new method of preoperative molecular-based diagnosis of thyroid carcinomas. This technique, ABRP, allows us to perform cytological and molecular-based diagnoses simultaneously by extracting RNA from leftover cells within the needle used for FNAB. Thus, ABRP provides both RNA information and a cytological diagnosis without further invasion to the patient. onfFN mRNA is abundantly expressed only in papillary and anaplastic carcinomas (3–5). Thus, by using ABRP to measure the real-time quantitative level of onfFN mRNA in FNABs, these carcinomas may be accurately diagnosed preoperatively. Recently, an automated system that measures the quantity of a small amount of mRNA by monitoring the amplification rate in each PCR cycle (real-time quantitative RT-PCR) was developed (6, 7). By using this system in measuring onfFN mRNA, a fully automated system for use in clinical laboratories may be established. Because thyroglobulin mRNA is expressed in most of the differentiated thyroid tumors of epithelial ancestry and its expression was reportedly decreased in some of the thyroid carcinomas, this gene is considered to be one of the most important for the internal control of thyroid cells. Thus, by measuring the ratio of onfFN to thyroglobulin mRNA, we may be able to distinguish thyroid carcinomas from benign tumors using only a small number of tumor cells. In light of the above, we calculated the relative expression level of onfFN to thyroglobulin mRNA by real-time quantitative RT-PCR in RNAs extracted from tumor tissues and FNABs. We then examined its clinical usefulness.

Materials and Methods

Extraction of RNA from Thyroid Tissues. Tissue samples from 14 follicular adenomas, 6 follicular carcinomas, 20 papillary carcinomas, 2 anaplastic carcinomas, 12 adenomatous goiters, and 34 normal thyroid tissues in the opposite lobe of the thyroid carcinomas, 13 adenomatous goiters, 15 follicular adenomas, 2 follicular carcinomas, 18 papillary carcinomas, and 1 anaplastic carcinoma) were surgically dissected and immediately used. A syringe with a 22-gauge needle was used for FNAB from each tissue sample. Samples were prepared on a glass slide 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 described above. After Papanicolaou staining, slide glasses were used to make sure the tumor cells were aspirated from the tissues.

RNA Extraction from FNABs. Aspirates from patients with a nodular goiter who were suspected of having a malignant thyroid tumor were obtained by ultrasound-guided FNAB using a syringe with a 22-gauge needle (11). RNA recovery was performed as described above. After the operation, the thyroid tumors were classified histopathologically according to the WHO histological classification of thyroid tumors.

Reverse Transcription. Reverse transcription was performed using either 1 μg of total RNA (tissues samples) or the whole RNA extracted (aspirates) in a RT mixture containing 50 m Tris-HCl (pH 8.3), 75 m KCl, 10 m DTT, 3 m MgCl2, 0.5 m dNTPs, 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 2 units/μl RNase inhibitor (Takara, Shiga, Japan), and 2.5 μl oligo(dT) (Life Technologies, Inc.) in a total volume of 20 μl at 37°C for 60 min.

Real-Time Quantitative PCR. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and a TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp., Foster City, CA) was performed according to the manufacturer’s protocol. One μl of the first strand cDNA was used in the following assay. The two primers and one TaqMan probe used for the quantification of onfFN, thyroglobulin, and GAPDH mRNA were (13–15):

onfFN5 (0.5 μM; 5′-TCTTTCATGGACCAGAGATCT-3′; bases 5932–5951),

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3 The abbreviations used are: FNAB, fine needle aspiration biopsy; ABRP, aspiration biopsy-reverse transcription-PCR; onfFN, oncofetal fibronectin; RT-PCR, reverse transcription-PCR; FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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measuring onfFN mRNA, a fully automated system for used in monitoring the amplification rate in each PCR cycle (real-time quanti-
onfFN3 (0.5 μM; 5'-TATGGTCTGGGCTATGCT-3'; bases 6128–6147), and onfFN-TM (10 pmol; 5'-FAM-AGCAACCATCTCCTCTCTCTCACT-TAMRA-3'; bases 671–693), respectively; Tg5 (0.5 μM; 5'-GAGAAGAGCCCTGCTG-3'; bases 7980–7999), Tg3 (0.5 μM; 5'-CAGCTCACTGCACAT-TAMRA-3'; bases 8128–8147), and Tg-TM (10 pmol; 5'-FAM-TCCTAGGCATCCTGC-3'; bases 8054–8076), respectively; and GAPDH5 (0.5 μM; 5'-TCCATGACAACTTTGATC-3'; bases 551–570), GAPDH3 (0.5 μM; 5'-AAGGTCTCCCTGAGCTAGA-3'; bases 715–734), and GAPDH-TM (10 pmol; 5'-FAM-AGCAACCATCTCCTCTCTCTCACT-TAMRA-3'; bases 671–693), respectively. The conditions for the TaqMan PCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A recombinant pGEM T-vector (Promega, Tokyo, Japan) containing onfFN, thyroglobulin, or GAPDH cDNA were constructed by PCR cloning with the same set of primers used in TaqMan PCR and were used as standard samples. The representative amplification plots of onfFN cDNA are shown in Fig. 1A. These plots were used to determine the threshold cycle (Ct) represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value (optimal ΔRn) was first detected. A plot of the Ct against the input target quantity (common logarithmic scale) is shown in Fig. 1B. The initial copy number of the target mRNA was calculated from this plot.

Statistical Analysis. Statistical analysis of differences between the groups was carried out using the Mann-Whitney U test. Ps of <0.05 were considered significant.

Results

The expression levels of thyroglobulin and onfFN mRNAs in thyroid tissues were measured by real-time quantitative RT-PCR. The majority of the samples showed the copy number of GAPDH mRNA to be ~10^7 (data not shown). Compared with normal thyroid tissues, follicular adenomas, papillary carcinomas, and follicular carcinomas showed a decreased thyroglobulin:GAPDH mRNA ratio (Fig. 2A). In two anaplastic carcinomas, the thyroglobulin mRNA was barely detectable by RT-PCR. The majority of papillary carcinomas, one follicular carcinoma, and two anaplastic carcinomas showed increased values of the onfFN:GAPDH mRNA ratio (Fig. 2B). Due to the decreased thyroglobulin:GAPDH mRNA ratio and the increased onfFN:GAPDH mRNA ratio, the onfFN:thyroglobulin mRNA ratio was >0.1 in all of the papillary and anaplastic carcinomas (Fig. 2C). On the other hand, the onfFN:thyroglobulin mRNA ratio in all of the other tissues was <0.1. Two anaplastic carcinomas showed extremely high values of the onfFN:thyroglobulin mRNA ratio.

Next, mRNAs in the aspirates from fresh tissues were examined (Fig. 3). The copy number of GAPDH mRNA ranged from 10^5 to 10^7 (data not shown). Aspirates from papillary and anaplastic carcinomas showed greatly increased values of the onfFN:thyroglobulin mRNA ratio, and all of the aspirates from other tissues showed an onfFN:thyroglobulin mRNA ratio of <0.1, which was consistent with the results obtained using the tissue samples.

RNAs were extracted from leftover cells inside the needles used for
FNAB from patients with a nodular goiter. Those samples that showed an extremely low copy number of both onfFN ($<10^5$) and thyroglobulin ($<10^5$) mRNA were not used for further analysis because they were likely to contain many contaminated blood cells rather than thyroid tumor cells. Among the patients, 46 underwent surgery (5 adenomatous goiters, 5 follicular adenomas, 31 papillary carcinomas, 4 follicular carcinomas, and 1 anaplastic carcinoma). Preoperative aspirates from the papillary and anaplastic carcinomas showed greatly increased values of the onfFN: thyroglobulin mRNA ratio (Fig. 4). On the other hand, aspirates from other tissues showed an onfFN:thyroglobulin mRNA ratio of $<0.06$. Thus, samples from the papillary and anaplastic carcinomas were clearly distinguished from those from the adenomatous goiters, follicular adenomas, and follicular carcinomas. A preoperative aspirate from an anaplastic carcinoma showed an extremely increased value of onfFN:thyroglobulin mRNA due to the decreased expression of thyroglobulin mRNA.

**Discussion**

Nodular thyroid disease is a frequently observed condition, especially in women. The prevalence of palpable thyroid nodules is reported to be 4–7% in adults (16), and most patients are diagnosed by preoperative cytological examinations. Because cytological examinations require a skillful cytopathologist, which is not always available, a more objective criterion is needed. Our recent trial of detection of onfFN mRNA in FNAB by semiquantitative RT-PCR showed the sensitivity and specificity in the diagnosis of papillary and anaplastic carcinomas to be 96.9 and 100%, respectively. This is compatible with diagnosis by a skillful cytopathologist (17). However, considering the future clinical use, an automated system rather than a procedure using gel electrophoresis is preferable. By real-time quantitative RT-PCR, in which data are automatically analyzed by a computer, we may establish a system that can be used in the routine clinical work.

Previous studies have shown a restricted expression of onfFN mRNA in papillary and anaplastic carcinomas that was not detectable by Northern blot or in situ hybridization in normal thyroid tissues or follicular tumors (3, 4). onfFN mRNA is detectable in the latter two tissues only by RT-PCR using primers that allow for highly sensitive PCR amplification, such as the onfFN5 and onfFN3 described in this study. In this study, the copy number of onfFN mRNA in papillary carcinomas estimated by real-time quantitative RT-PCR is ~10–100 times greater than that in follicular tumors, which makes it possible to distinguish papillary carcinomas from follicular tumors by using a small number of tumor cells. Furthermore, by using thyroglobulin mRNA as an internal control in measuring the copy number of onfFN mRNA in FNABs, papillary and anaplastic carcinomas could be more clearly distinguished from benign nodules such as follicular adenomas and adenomatous goiters. This suggests that the increased value of onfFN: thyroglobulin mRNA ratio in FNABs is an objective diagnostic criterion of papillary and anaplastic carcinomas, and real-time quantitative RT-PCR may be a useful molecular adjunct of aspiration biopsy-cytology of thyroid tumors.

Anaplastic thyroid carcinoma is a rare but highly aggressive neoplasm in humans (18). It is highly resistant to any form of therapy, and because of its rapid growth, it is rarely cured. Thus, an accurate and rapid diagnosis is vital for early determination of a treatment course. In some cases, however, accurate diagnosis of an anaplastic carcinoma is difficult, such as when the cytological examinations are hindered by necrosis, hemorrhage, or contamination of the differentiated carcinoma cells that often accompany anaplastic carcinomas. Our results indicated aspirates from anaplastic carcinomas show extremely high values of the onfFN:thyroglobulin mRNA ratio due to the decreased expression of thyroglobulin mRNA. Because entire real-time quantitative RT-PCR analysis takes only 6 h, a rapid diagnosis of anaplastic carcinomas is possible using materials from patients who have suffered from the rapid growth of a thyroid tumor.

In this study, preoperative samples from patients which showed an extremely low copy number of both onfFN and thyroglobulin mRNA, ~10% of all of the samples, were not used for further analysis. This is because, by using these, we could not obtain reproducible results of quantification of onfFN and thyroglobulin mRNA by real-time quantitative RT-PCR. This was probably due to the interference of cDNAs
derived from the contaminated blood cells. Development of a quantifying system that measures a small copy number of mRNA more accurately is needed because otherwise repeated FNABs may be required for these patents to confirm the diagnosis.

Differentiation of follicular adenomas and carcinomas by real-time quantitative RT-PCR 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. Furthermore, when a new target gene the expression of which differs in follicular adenomas and carcinomas is found, this method can be also applied to distinguish these two.

By real-time quantitative RT-PCR, we can quantify the copy number of mRNAs in a small volume of clinical materials. Thus, by measuring the expression of multiple mRNAs, including onfFN and thyroglobulin in FNABs, we may be able to obtain more information on the clinical features of thyroid tumors.

References

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