Rapid Detection of Specific Messenger RNAs in Thyroid Carcinomas by Reverse Transcription-PCR with Degenerate Primers: Specific Expression of Oncofetal Fibronectin Messenger RNA in Papillary Carcinoma

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ABSTRACT

The search for mRNAs that are specifically expressed in cancer tissues is important for gene diagnosis and therapy. However, finding such mRNAs in human cancers is usually very difficult, both because of the limited volume of RNA obtainable from the tissues and the many technical difficulties of RNA analysis. To address these problems, the present study compared mRNA from thyroid cancer tissues with those from normal and benign tissues by reverse transcription-PCR using two degenerate primers. Amplified cDNAs were separated by electrophoresis with nondenaturing acrylamide gel, then three bands that are increased in cancer tissues were selected, reamplified by PCR, and cloned into T-vector. One of the bands was determined by sequencing analysis to be oncofetal fibronectin. The expression of oncofetal fibronectin mRNA in benign and malignant tissues was examined by Northern blot and reverse transcription-PCR using specific primers of its cDNA sequence, and its increased expression was observed only in papillary and anaplastic carcinomas. Thus, the present method rapidly detected specific mRNAs in cancer tissues, and one of these, oncofetal fibronectin mRNA, is a good target for gene diagnosis of papillary carcinoma.

INTRODUCTION

The search for mRNAs that are exclusively expressed in cancer tissues is vital to gene diagnosis and other technologies in the treatment of cancer. If such mRNAs exist, their promoters could potentially be used for targeted gene therapy (1), whereas the proteins encoded by their sequences could be used in targeted immunotherapy (2). Furthermore, cancers could be diagnosed with only a limited volume of biopsies or other samples by the use of RT-PCR to screen for specific mRNA sequences in cancer cells (3, 4).

Thyroid tumors of epithelial descent are classified histopathologically into three types: follicular adenoma, differentiated carcinoma (papillary carcinoma and follicular carcinoma), and undifferentiated anaplastic carcinoma (5). Both ultrasound sonography and FNAB (6) are commonly used to diagnose thyroid tumors. However, in some clinical situations, an accurate diagnosis requires a more objective method than mere cytological examination. Gene diagnosis by analyzing nucleic acids in aspirates may be used for this purpose, although in most trials gene diagnosis has failed to give as much information on clinical features of thyroid tumors as cytological diagnosis (7, 8).

When using only a small number of cells, gene analysis of mRNA is much easier than that of DNA because the copy number of each mRNA is usually larger than that of the corresponding DNA. Previous studies have reported the overexpression of some genes in thyroid cancers, such as EGF receptor (9), c-erbB (10), IGF-I (11), p53 (12), dipeptidyl aminopeptidase IV (13), and CD44E (14). However, mRNA of these genes cannot be used for gene diagnosis by RT-PCR because their expression is not strictly restricted in cancer cells and not all cancer tissues overexpress their mRNA.

In consideration of the above facts, we intended to screen for specific mRNAs in thyroid cancers by a PCR-based method. Differential display is the most commonly used method for identifying specific mRNAs in two different groups of RNA (15) because specific RNAs can be more rapidly screened by this than by any other method. However, this cannot be used directly to screen human tissues because of the following: first, most of the PCR products in differential display are derived from the 3' untranslated region of mRNA sequences in which individual variations, which can be an obstacle for identifying specific changes in cancer tissues, are often observed; and second, because most of the bands of difference in the first screening of differential display are reported as false positive (16), the second screening by Northern or slot blots is hard to perform using only a limited volume of cancer tissues.

Therefore, we developed a new screening system, the SS-DD, by modifying the system by Liang and Pardee (15). First, instead of an arbitrary decamer for the 5' primer, we used a degenerate primer with a length of 16 bases. This effectively prevented false-positive results by allowing us to perform PCR cycles with a high annealing temperature. Second, to avoid the screening in the 3' untranslated regions, Ex-Taq, which has proofreading activity, was used to ensure the PCR reaction in order to produce longer PCR products (17); then, PCR products that were longer than about 500 bp were subjected to screening. Lastly, up to six sets of normal and tumor samples were compared simultaneously on the same gel image.

In the present study, we subjected six normal thyroid tissues, six papillary carcinomas, four follicular adenomas, and two follicular carcinomas to the screening and detected three mRNAs that were specifically expressed in thyroid cancers. The specificity of each mRNA was further examined by Northern blotting and RT-PCR.

MATERIALS AND METHODS

Extraction of RNA from Thyroid Tissues. Tumors in the present study were obtained by surgery (Table 1). Whenever possible, the normal tissues from the opposite lobe were also obtained. All tissues were frozen in liquid nitrogen immediately after resection. RNA was extracted according to the method of Chomczynski and Sacchi (18). SS-DD Method. RNA from six normal thyroid tissues, six papillary carcinomas, four follicular adenomas, and two follicular carcinomas was used for the screening by the SS-DD. Ten μg of extracted total RNA were digested with 12 units DNase I (Takara, Shiga, Japan) at 37°C for 30 min. After extraction with phenol-chloroform, RNA was precipitated with ethanol then reconstituted with distilled water. One μg of total RNA was used as a template of RT. RNA was denatured for 4 min at 60°C then chilled rapidly on ice. RT was performed in RT mixture containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 0.5 mM dNTPs, 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 2 units/μl Ex-Taq, which has proofreading activity, was used to ensure the PCR reaction in order to produce longer PCR products (17); then, PCR products that were longer than about 500 bp were subjected to screening. Lastly, up to six sets of normal and tumor samples were compared simultaneously on the same gel image.

In the present study, we subjected six normal thyroid tissues, six papillary carcinomas, four follicular adenomas, and two follicular carcinomas to the screening and detected three mRNAs that were specifically expressed in thyroid cancers. The specificity of each mRNA was further examined by Northern blotting and RT-PCR.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: RT, reverse transcription; FNAB, fine-needle aspiration biopsy; SS-DD, sequence-specific differential display; dNTP, deoxynucleotide triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC2, prohormone convertase-2.
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Table 1 Thyroid tissues used in this study

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Pathological diagnosis</th>
<th>Sample No.</th>
<th>Pathological diagnosis</th>
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<tbody>
<tr>
<td>1</td>
<td>Normal thyroid (the opposite lobe of No. 2)</td>
<td>11</td>
<td>Normal thyroid (the opposite lobe of No. 12)</td>
</tr>
<tr>
<td>2</td>
<td>Papillary carcinoma (poorly differentiated)</td>
<td>12</td>
<td>Papillary carcinoma (poorly differentiated)</td>
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<tr>
<td>3</td>
<td>Normal thyroid (the opposite lobe of No. 4)</td>
<td>13</td>
<td>Papillary carcinoma (poorly differentiated)</td>
</tr>
<tr>
<td>4</td>
<td>Papillary carcinoma (poorly differentiated)</td>
<td>14</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>5</td>
<td>Normal thyroid (the opposite lobe of No. 6)</td>
<td>15</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>6</td>
<td>Papillary carcinoma (well differentiated)</td>
<td>16</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>7</td>
<td>Normal thyroid (the opposite lobe of No. 8)</td>
<td>17</td>
<td>Follicular adenoma (widely invasive)</td>
</tr>
<tr>
<td>8</td>
<td>Papillary carcinoma (well differentiated)</td>
<td>18</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>9</td>
<td>Normal thyroid (the opposite lobe of No. 10)</td>
<td>19</td>
<td>Follicular adenoma (widely invasive)</td>
</tr>
<tr>
<td>10</td>
<td>Papillary carcinoma (well differentiated)</td>
<td>20</td>
<td>Follicular adenoma (widely invasive)</td>
</tr>
<tr>
<td>11</td>
<td>Normal thyroid (the opposite lobe of No. 12)</td>
<td>21</td>
<td>Follicular adenoma</td>
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<tr>
<td>12</td>
<td>Papillary carcinoma (well differentiated)</td>
<td>22</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>13</td>
<td>Follicular adenoma</td>
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<td>15</td>
<td>Follicular adenoma</td>
<td>25</td>
<td>Anaplastic carcinoma</td>
</tr>
</tbody>
</table>

RNase inhibitor (RNasin; Takara), and 2.5 μM oligodeoxynucleotidic acid (Funakoshi, Tokyo, Japan) in a total volume of 20 μl at 37°C for 60 min. All primers were purchased from Funakoshi. Two degenerate primers were used in the following PCR reaction. One, [TK: 5' < GA(C/T)GTCCTGA(A/T) (CG)(CT)T(A/T)(CT)(G <3')] was originally designed to correspond to the sequence of the 3' region of the catalytic domain of tyrosine kinases (19), and the other, [DD: 5' < GTTTTTTTTTTTTTTTTTTT(T/A)(G/C) <3'], was designed to correspond to the poly(A) tail. Each reaction mixture consisted of 1 μl of cDNA, 4 μM each primer, 4 μl of 10 X Ex Taq Buffer, 4 μl of dNTP mix, 2 units of Ex Taq polymerase, and nuclease-free water to a final volume of 40 μl. Ex Taq Buffer (10X), dNTP mix, and Ex Taq polymerase were obtained from Perkin-Elmer Corp. (Emeryville, CA). The reaction mixture was subjected to 2 min of denaturation at 94°C, then two cycles of denaturation (94°C; 1 min), annealing (40°C; 2 min), and extension (72°C; 5 min), followed by 35 cycles of denaturation (94°C; 1 min), annealing (55°C; 1 min), and extension (72°C; 3 min with 5 s of auto-segment extension for each cycle) using a DNA thermal cycler (Perkin-Elmer Corp.). To prevent false-positive results, each set of samples to be compared was set side-by-side in the DNA thermal cycler. After PCR amplification, 3.5 μl of reaction mixture was separated on 3.5% acrylamide gel in Tris-HCl/acetate/EDA (IAE) buffer. Amplified PCR products were extracted from the gel with Suprec-Ol (lakara), then cloned into pMOSBlue vector by using pMOSBlue I-vector kit (Amersham Corp., Buckinghamshire, United Kingdom). Digoxigenin-labeled cRNA probes were synthesized with recombinant pMOSBlue vectors using a DIG RNA labeling kit (Boehringer-Mannheim). Chemilumigrams of the bands were obtained by exposing the membrane to Hyperfilm-ECL (Amersham).

RT-PCR Method. For 5' primers, PCRs were performed using a specific primers for each gene (20–22), and for 3' primer, DDR was used to avoid the false-negative results due to the individual variations of 3' untranslated regions except GAPDH cDNA (23). The sequences of the primers are as follows: TP1, 5'-ATGCTGTAATATACACACATC; TP2 (oncofetal fibronectin), 5'-AAGGCATAAGCAGACATAC; TF1, 5'-TGCCCTGAGAGATC-

Fig. 1. Representative image of SS-DD of papillary carcinomas. After PCR, PCR products derived from normal thyroid tissues (N) and papillary carcinomas (P) were separated on an acrylamide gel. The gel was stained with Sybr Green I, and the fluorescent image was detected with a Fluor Imager.

Fig. 2. Representative image of SS-DD of follicular tumors. After PCR, PCR products derived from normal thyroid tissues (N), follicular adenomas (FA), and follicular carcinomas (FC) were separated on an acrylamide gel.

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RESULTS

Screening of Specific mRNAs in Cancer Tissues by SS-DD. Tissue samples used in Figs. 1 through 5 are shown in Table 1. RNAs from papillary carcinomas and normal thyroid tissues from six patients were compared by SS-DD with primers TK and DD (Fig. 1). Both the presence and absence of each band were unstable when the length of the PCR products was less than approximately 500 bp. Also, in this region, differences in the lengths of the PCR products were observed between the individuals, probably due to individual variations in the 3' untranslated regions. Many weak bands that were about 1000–1500 bp and that appeared only in normal thyroid tissues were detected. Two bands were found, TP1 and TP2, which had intensities that increased in papillary carcinomas. The TP1 band increased relative to normal thyroid tissue in five of six papillary carcinomas. The TP2 band was not detected in normal tissues, but an intense band was found in all of the papillary carcinomas. The same screening was performed using RNAs of thyroid follicular tumors (Fig. 2). In this experiment, RNAs from follicular tumors were compared with those from normal tissues from papillary carcinoma patients due to a lack of normal thyroid tissues from follicular tumor patients. Neither an increase of TP1 nor of TP2 was observed in this screening. Some bands decreased in follicular tumors, but no band was found that increased in all of the follicular tumors. However, one band, TF1, appeared in only one of the two follicular carcinomas (sample 18). These three bands, TP1, TP2, and TF1, were cut out from the gel, then extracted PCR products were reamplified by PCR with primers TKR and DDR. Reamplified PCR products were cloned into pMOSBlue vectors and sequenced. Sequencing analysis showed that all of the three clones were amplified by both of the two primers, TK and DD. TP2 coded oncofetal fibronectin. The sequence of TF1 probe was observed in some papillary carcinomas. The TPI band increased relative to normal tissue and a papillary carcinoma in the Northern blot. The TP1 probe hybridized two transcripts (8.5 and 12 kb) in the papillary carcinoma. Although the expression of TP1 in normal tissues could be detected in the screening by SS-DD, no band could be observed in sample 3 by Northern blotting. Using the TF1 probe, we observed a weak band at 5 kb in sample 18, but no hybridization was detected in the normal thyroid tissue.

Northern Blotting. Total RNAs from one normal tissue (sample 1), five papillary carcinomas (samples 2, 6, 8, 10, and 12), four follicular adenomas (samples 13, 14, 15, and 16), and two follicular carcinomas (samples 17 and 18) were used for Northern blotting (Fig. 4). Expression of oncofetal fibronectin mRNA was restricted in papillary carcinomas. The expression of TP1 in normal tissues could be detected in the screening by SS-DD, no band could be observed in sample 3 by Northern blotting. Using the TF1 probe, we observed a weak band at 5 kb in sample 18, but no hybridization was detected in the normal thyroid tissue.

The Second Screening by Northern Blotting. The specificity of the three cDNA clones was examined by Northern blotting using two RNA samples that showed a clear difference in the first screening by SS-DD (Fig. 3). For TP1 and TP2, samples 3 and 4 were used and for TF1, samples 11 and 18. The TP1 (oncofetal fibronectin) probe hybridized 8.6 kb transcript, and its expression showed a clear difference between a normal tissue and a papillary carcinoma in the Northern blot. The TP1 probe hybridized two transcripts (8.5 and 12 kb) in the papillary carcinoma. Although the expression of TP1 in normal tissues could be detected in the screening by SS-DD, no band could be observed in sample 3 by Northern blotting. Using the TF1 probe, we observed a weak band at 5 kb in sample 18, but no hybridization was detected in the normal thyroid tissue.

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Fig. 3. Second screening by Northern blotting. Northern blots with TP1, TP2, and TF1 probes were performed, and then the results were compared with the gel images of SS-DD (top). Twenty µg of total RNA was loaded in each lane. The gels stained with ethidium bromide (bottom) show that an almost equal amount of RNA was loaded in each lane. Arrows, the positions of the bands of difference in the SS-DD images.
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Fig. 4. Expression of TP1, oncofetal fibronectin (oncFN), and TF1 mRNAs. Twenty μg of total RNA from one normal thyroid tissue (NT), five papillary carcinomas, four follicular adenomas, and two follicular carcinomas (FC) were subjected to Northern blot analysis using TP1, oncofetal fibronectin, and TF1 cRNA probes. Ethidium bromide staining of the gel (bottom) shows that an almost equal amount of RNA was loaded in each lane.

Fig. 5. RT-PCR analysis of the expression of TP1, oncofetal fibronectin (oncFN), and TF1 mRNAs. cDNAs from 6 normal thyroid tissues, 5 follicular adenomas, 2 follicular carcinomas, and 1 anaplastic carcinoma were amplified by RT-PCR with specific primers for each gene. Thyroglobulin and GAPDH cDNA were also amplified as controls. GAPDH mRNA was detectable in all of the samples. Thyroglobulin mRNA was observed in all samples except an anaplastic carcinoma. Oncofetal fibronectin mRNA was detected neither in normal thyroid tissues nor in thyroid follicular tumors, although it was clearly detected in all papillary carcinomas, which indicates that its expression is highly restricted in papillary carcinomas. Furthermore, an anaplastic carcinoma also expressed oncofetal fibronectin mRNA (Fig. 5). The TP1 band was clearly detected in all papillary carcinomas. However, because this band can also be detected in some normal thyroid tissues and follicular tumors, its expression is probably increased, but not restricted, in papillary carcinomas. A strong band of TF1 was observed in one of two follicular carcinomas. However, a weak band was also detected in some papillary carcinomas and other follicular tumors. To confirm the restricted expression of oncofetal fibronectin mRNA in papillary and anaplastic carcinomas, we examined its expression in a total of 98 samples (29 normal thyroid tissues, 13 adenomatous goiters, 19 follicular adenomas, 30 papillary carcinomas, 5 follicular carcinomas, and 2 anaplastic carcinomas), including the samples in Table 1, by the use of RT-PCR with the specific primer for TP2 (oncofetal fibronectin) and the anchor primer DDR. Oncofetal fibronectin mRNA was detected only in 30 papillary carcinomas and 2 anaplastic carcinomas (data not shown).

DISCUSSION

In this study, we developed a new screening system to detect mRNAs specifically expressed in cancer tissues and succeeded in
detecting three mRNAs that show increased expression in thyroid cancer. Although differential display was first reported in 1992 and has been studied frequently since, few of these studies have used normal and cancer tissues from actual patients. This suggests the difficulty of the direct application of differential display to human tissues. We intended to overcome this difficulty by using degenerate primers and Ex Taq to perform PCR cycles with a higher annealing temperature and to screen PCR products with a large molecular weight. In this way, we hoped to avoid false-positive results and interference by individual variations in the 3' untranslated region of mRNAs. This seems to have been successful; as shown in Fig. 1 and Fig. 2, we obtained the differential gel image between normal and cancer tissues with high reproducibility in the region of high molecular weight. We confirmed the reproducibility of the positive results with RT-PCR using the specific primers. Furthermore, the increased expression of two of the three genes screened in the SS-DD image was clearly shown by Northern blotting. One of the most important advantages of SS-DD is that one can estimate the importance of the differentially expressed genes by examining the display, because up to six sets of screening are performed in one image. For instance, TP2 was estimated to be more important than TP1 because the TP2 band was present in all papillary carcinomas and absent in all normal tissues. This allows us more rapid and efficient screening of the specifically expressed genes in cancer tissues.

We have cloned more than 10 genes, the bands of which appear only in normal tissues. Although TK was originally designed to correspond to the sequence of the 3' region of the catalytic domain of tyrosine kinases, no known tyrosine kinases were included, although most of the sequences had no homology to known genes with well-defined biological characters (data not shown). SS-DD seems to have low selectivity to the target gene family because only the 5' primer used in PCR is specific.

One of the most important results in this study is the much-increased expression of oncofetal fibronectin mRNA in papillary and anaplastic carcinomas, which can be easily detected by RT-PCR. It has been reported that small papillary carcinomas are easily distinguished from thyroid follicular cells and that the incidence of occult papillary carcinoma is surprisingly high (24). Thus, our data suggest distinct differences between the biological features of papillary carcinoma cells and those of thyroid follicular cells, differences that probably stem from the very beginning of the cancer development. Fibronectins are high molecular mass adhesive glycoproteins present in the extracellular matrix and in body fluids (25). Oncofetal fibronectin is characterized by the presence of the oncofetal domain, which is absent in normal fibronectin. One of the oncofetal domains, IIICS, is recognized by a monoclonal antibody called FDC-6 (26), and many researchers have used this antibody to report the existence of oncofetal fibronectin in malignant tissues such as oral, breast, colon, and gastric cancers by immunohistochemistry (27-30). Thus, oncofetal fibronectin mRNA may be expressed not only in thyroid carcinomas but also in many types of malignant tumor. Oncofetal fibronectin can be used as more specific tumor maker of papillary carcinoma than the widely used maker, thyroglobulin (31), if a sufficient number of molecules is released into the blood stream from the cancer tissue. Also, our preliminary study by RT-PCR showed the expression of mRNA sequence of another oncofetal domain besides the IIICS domain, ED-B, in papillary and anaplastic carcinomas (data not shown). These data are consistent with the study by Kaczmarek et al. (32) in breast cancer by immunohistochemistry, which showed the existence of both IIICS and ED-B domain in cancer tissues.

Furthermore, we may be able to establish a new classification of thyroid tumors of epithelial descendancy. Thyroid tumors are classified into two groups: one expresses oncofetal fibronectin mRNA and the other does not. The former is papillary carcinoma and the latter is follicular adenoma and carcinoma. Furthermore, expression of oncofetal fibronectin mRNA can be used as one of the criteria of the diagnosis of papillary carcinoma, and papillary carcinomas may be more correctly diagnosed by in situ hybridization of oncofetal fibronectin mRNA.

The TP1 probe hybridized two large transcripts (8.5 and 12 kb). Although we have not analyzed their complete sequences, the determined sequence of the 5' end of TP1 showed homology to the genes already known. PC2 is a member of a family of cellular endoproteolytic processing enzyme and its mRNA was reported to be expressed in neuroendocrine tumors, e.g., medullary carcinomas (33). The TP1 band was clearly observed in sample 18 by both SS-DD and RT-PCR with the specific primer. The sequence of TP1 was the same as exon 11-12 of PC2. However, primer DD did not anneal to the expected position of exon 12. Moreover, in sample 18, Northern blot did not detect the transcript of PC2 (2.8 kb; Ref. 21) reported previously, but rather a weak hybridization at 5 kb. Therefore, we assume that the amplified gene in one of the two follicular carcinomas may not be PC2, but a homologue of the PC2 gene.

Oncofetal fibronectin mRNA is a good target of gene diagnosis of papillary carcinomas by FNAB because papillary carcinomas and other tumors or normal thyroid follicular cells are easily distinguished by RT-PCR. Gene diagnoses of papillary carcinomas by FNAB that are more objective and accurate than cytological examinations will soon be established. Furthermore, in the near future, gene therapy for papillary carcinoma that selects oncofetal fibronectin as a target molecule will be considered.

REFERENCES
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