Expression of Oncofetal Fibronectin mRNA in Thyroid Anaplastic Carcinoma

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Received March 7, 2007; accepted April 28, 2007; published online August 18, 2007

Background: Oncofetal fibronectin (onfFN) is a fetal protein, the expression of which is observed in papillary thyroid carcinomas but not in follicular tumors or in normal thyroid. Its expression in anaplastic thyroid carcinoma (ATC), however, has not been clarified, since only a few cases had been examined in previous studies.

Methods: We examined the expression levels of onfFN mRNA in ATC tissues and cell lines derived from ATC by real-time quantitative reverse transcription-polymerase chain reaction (PCR) and in situ hybridization.

Results: Increased expression of onfFN mRNA was observed in all cases of ATC regardless of the type of accompanying differentiated carcinoma and five of six ATC cell lines. Furthermore, expression of onfFN mRNA was observed in the majority of ATC cells in all six tissues examined by in situ hybridization.

Conclusion: These results confirm that expression of onfFN mRNA characterizes not only papillary thyroid carcinoma but also ATC. onfFN mRNA or protein may be a useful marker to identify anaplastic carcinoma cells and may be considered as an optimistic target in molecular-based therapy of ATC.

Key words: molecular Dx – tumor markers – immunotherapy

INTRODUCTION

Thyroid tumors are relatively common, especially in women of middle age. In differentiated thyroid carcinomas, including papillary and follicular carcinomas, distant metastases and local recurrence occur frequently, but fatal cases are rare, since these carcinomas are usually slow in growth. In contrast, poorly differentiated anaplastic thyroid carcinoma (ATC) is a rare tumor and has a relentless and deadly clinical course with rapid progression and dissemination (1). Recently, it has been hypothesized that thyroid cancer cells are derived from remnants of the fetal thyroid instead of normal follicular cells and ATCs are derived from thyroid cancer stem cells (TSCs) (2-4). Thus, genes which are expressed restrictedly in ATCs are of great interest since they may present a clue to study the nature of TSCs.

Because ATCs are very rare tumors, there are only a few reports on specific gene expression in ATCs. In our previous studies, we reported overexpression of oncofetal fibronectin (onfFN), a fetal protein, in papillary thyroid carcinomas and in some ATCs (5-8). In these studies, however, it was not clear to what extent overexpression of onfFN is observed in ATCs, owing to the limitation in the sample number of ATCs examined in the studies. In this study, 12 ATCs and six cell lines derived from ATC were used for analysis of the expression levels on onfFN mRNA. Furthermore, localization of onfFN mRNA in the tissue sections were examined by in situ hybridization study.

MATERIALS AND METHODS

Extraction of RNA from Thyroid Tissues

Tissue samples from 20 follicular adenomas, 17 follicular carcinomas, 19 papillary carcinomas, 12 ATCs and 20 normal thyroid tissues at the opposite lobe of a carcinoma were
Table 1. Characteristics of anaplastic thyroid carcinomas used in this study

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age</th>
<th>Gender</th>
<th>Histological subtype</th>
<th>Accompanying differentiated carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>77</td>
<td>F</td>
<td>Giant cell</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>F</td>
<td>Spindle cell</td>
<td>Papillary carcinoma</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>F</td>
<td>Pleomorphic</td>
<td>Papillary carcinoma</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>M</td>
<td>Pleomorphic</td>
<td>Papillary carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>82</td>
<td>F</td>
<td>Spindle cell</td>
<td>—</td>
</tr>
<tr>
<td>6*</td>
<td>80</td>
<td>F</td>
<td>Squamoid cell</td>
<td>Papillary carcinoma</td>
</tr>
<tr>
<td>7*</td>
<td>35</td>
<td>F</td>
<td>Giant cell</td>
<td>Follicular carcinoma</td>
</tr>
<tr>
<td>8*</td>
<td>61</td>
<td>F</td>
<td>Giant cell</td>
<td>Follicular carcinoma</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>F</td>
<td>Giant cell</td>
<td>Follicular carcinoma</td>
</tr>
<tr>
<td>10*</td>
<td>73</td>
<td>F</td>
<td>Giant cell</td>
<td>—</td>
</tr>
<tr>
<td>11*</td>
<td>80</td>
<td>F</td>
<td>Pleomorphic</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>54</td>
<td>F</td>
<td>Spindle cell</td>
<td>—</td>
</tr>
</tbody>
</table>

*Cases used in the in situ hybridization study.

collected by surgery after obtaining the patients’ informed consent. Clinical and pathological characteristics of ATCs are shown in Table 1. Among 12 cases of ATC, four and three accompanied papillary and follicular carcinoma, respectively. All tissues were frozen in liquid nitrogen immediately after resection. The study protocol was approved by the institutional ethical committee. Six cell lines, derived from an ATC, SW579, KMH2, 8305C, 8505C, ASH-3 and TCO-1, were cultured in RPMI1640 with 10% fetal bovine serum (Invitrogen, Shiga, Japan), 200 U/mL reverse transcriptase (Invitrogen), 2 U/μl RNase inhibitor (Takara) and 2.5 μM random hexamer (Invitrogen) in a total volume of 20 μl at 42°C for 60 min.

**Reverse Transcription**

Reverse transcription was performed using 1 μg of total RNA in an RT mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl2, 0.5 mM deoxyribonucleotide triphosphates (dNTPs) (Takara, Shiga, Japan), 200 U M-MLV reverse transcriptase (Invitrogen), 2 U/μl RNase inhibitor (Takara) and 2.5 μM random hexamer (Invitrogen) in a total volume of 20 μl at 42°C for 60 min.

**Real-time Quantitative Polymerase Chain Reaction**

Real-time quantitative polymerase chain reaction (TaqMan PCR) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was performed as described previously (8). One microliter of the first-strand cDNA was used in the following assay. The two primers and one TaqMan probe used for the quantification of onfFN IIICS sequence (HSFIB1, GenBank X02761) and beta-actin (ACTB, GenBank X00351) mRNAs were, respectively:

- [onfFN (0.5 μM): 5’-TCTTCATGGACCAGAGATCT-3’ (base 5932–5951)]
- [onfFNR (0.5 μM): 5’-TATGGTTCTGGCCTATGCCT-3’ (base 6147–6128)]
- [onfFNF (0.5 μM): 5’-AGCAACCCAGTGTTGGCACA-3’ (base 6045–6066)]; and
- [ACF (0.5 μM): 5’-TGGACATCCCGAAAGACCTG-3’ (base 901–920)]
- [ACR (0.5 μM): 5’-CCGGATCGCCAGGATCTT-3’ (base 1066–1047)]
- [AC-TM (140 nM): 5’-CACCACCATGTCGCC-GCCATTGCC-3’ (base 947–971)].

All the primers and probes were purchased from Operon Biotechnologies (Tokyo, Japan). The conditions for the TaqMan PCR were as follows: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Recombinant pGEM Easy T-Vector (Promega, Madison, WI, USA) containing either onfFN or beta-actin cDNA was constructed by PCR-cloning with the same set of primers used in TaqMan PCR and used as standard samples. The amplification plots of the PCR reaction were used to determine the threshold cycle (Ct). The Ct value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value (optimal ΔRn) was first detected. The initial copy number of the target mRNA was calculated by a plot of the Ct against the input target quantity.

**In situ Hybridization Study**

An in situ hybridization was performed essentially as described previously with some modifications (6). A digoxigenin-labeled single-strand RNA probe was prepared using a DIG RNA labeling kit (Roche, Tokyo, Japan) according to the manufacturer’s instructions. For the generation of the antisense probe of the IIICS sequence, a sequence of human fibronectin cDNA (base 5889–6148) obtained from a papillary carcinoma was subcloned into pGEM Easy plasmid (Promega). Non-labeled single-strand RNA probe was prepared using 1 mM ATP, CTP, GTP and UTP (Roche) instead of an NTP labeling mix.

Ten-micrometer-thick frozen sections were cut out from the tissues. They were air-dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; Wako, Osaka, Japan) at 4°C for overnight. They were washed with PBS,
then dehydrated with ethanol series and air-dried, then stored at \(-80^\circ\text{C}\) until use. Before hybridization, they were digested with 2\,\mu\text{g/ml} \text{proteinase K} (\text{Roche}), fixed again with 4\% paraformaldehyde in PBS for 10\,\text{min}, and then treated with 0.3\% hydrogen peroxide in methanol for 20\,\text{min}. They were washed once with distilled water and twice with PBS. A hybridization solution with 0.5\,\text{ng/\mu l antisense probe} or a negative control mixture of 0.5\,\text{ng/\mu l antisense probe} and 50\,\text{ng/\mu l non-labeled probe} was prepared with mRNA \textit{in situ} Hybridization Solution (Dako Japan, Tokyo, Japan). A 50\,\mu l aliquot of hybridization solution was placed on each section, and sections were covered with siliconized coverglass and incubated at 50\,\text{C} for 16\,\text{h} in a moisture chamber. After hybridization, the slides were washed in 5\times\text{SSC} (1\times\text{SSC} = 0.15\,\text{M sodium chloride, 0.015\,M sodium citrate}) briefly and in 50\% formamide, 2\times\text{SSC} at 50\,\text{C} for 30\,\text{min} and RNase A treatment (10\,\mu\text{g/ml}, Wako) was carried out at 37\,\text{C} for 30\,\text{min}. The slide was treated with 2\times\text{SSC}, 0.2\times\text{SSC}, then 0.1\times\text{SSC} at 50\,\text{C} for 20\,\text{min}. Hybridized digoxigenin-labeled probes were detected with a horseradish peroxidase (HRP)-labeled anti-DIG antibody and a GenPoint System (Dako Japan) according to the manufacturer’s protocol.

**Statistical Analysis**

Statistical analysis of differences between the groups was carried out using the Mann–Whitney \textit{U}-test. A \textit{P}-value of <0.05 was considered significant.

**RESULTS**

High expression levels of onfFN mRNA were observed in papillary carcinomas and fibroblast cultures, which was concordant with the previous studies (5,9). The expression levels of onfFN mRNA were high in all 12 ATC tissues. All ATC cell lines but KMH-2 showed almost the same levels of onfFN mRNA expression as those of ATC tissues (Table 2, Fig. 1). \textit{In situ} hybridization study using a digoxigenin labeled-antisense probe of the IIICS sequence was performed in six tissues of ATC, listed in Table 1. All tissue sections of ATCs were positive for staining of onfFN mRNA. onfFN mRNA was expressed in the cytoplasm of the majority of the ATC cells (Fig. 2).

**DISCUSSION**

With rare exceptions, nearly all patients with ATC die from their tumor within several months (1). Unfortunately, at present no chemotherapeutic agent or combination of agents exists with sufficient antineoplastic activity against ATC to alter its fatal outcome. Cancer immunotherapy would be considered in such situations. However, because of the rarity of ATC, only a small number of articles have been published on genes expressed in ATC in a restricted manner.

![Figure 1. Expression levels of oncofetal fibronectin mRNA in anaplastic thyroid carcinomas. The results are shown as means from duplicate determinations. N, normal thyroid tissues; FA, follicular adenomas; FC, follicular carcinomas; PC, papillary carcinomas; anaplastic carcinoma; CL, cell lines; and FB, thyroid-derived fibroblasts.](image)

Since ATCs are quite undifferentiated tumors, they do not express various kinds of thyroid specific genes such as thyroglobulin, thyroid peroxidase and sodium/iodide symporter (NIS) (10,11); thus they are easily distinguished from differentiated thyroid tumors such as papillary carcinoma (8). In some of the previous studies, some genes such as \textit{krt1} tubulin (12), UbcH10 (13) and thymosin B10 (14) were reported to be overexpressed in ATC. However, the reason for overexpression of these genes in ATC seems to be closely related to the cell’s high proliferation rate. The molecular-based therapy of ATC targeting these genes might be also

**Table 2. Expression levels of oncofetal fibronectin mRNA in thyroid tumors, cell lines and fibroblast cultures**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number</th>
<th>onfFN mRNA/\beta-actin mRNA (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid</td>
<td>20</td>
<td>0.16 ± 0.10</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>20</td>
<td>0.25 ± 0.16</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>17</td>
<td>0.54 ± 0.35</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>19</td>
<td>12.09 ± 10.08</td>
</tr>
<tr>
<td>Anaplastic carcinoma</td>
<td>12</td>
<td>12.68 ± 6.70</td>
</tr>
<tr>
<td>Cell line</td>
<td>6</td>
<td>10.54 ± 11.92</td>
</tr>
<tr>
<td>Fibroblast culture</td>
<td>7</td>
<td>18.01 ± 6.84</td>
</tr>
</tbody>
</table>
showed that onfFN mRNA was expressed in the majority of cancer cells. Case 6 was a squamoid cell anaplastic thyroid carcinoma which showed an intense cytoplasmic staining in cancer cells. Case 11 was a pleomorphic anaplastic thyroid carcinoma which was rich in connective tissue. It showed a staining in the cytoplasm of cancer cells and a slight staining in the connective tissue; possibly a staining in fibroblasts. Case 11 was a pleomorphic anaplastic thyroid carcinoma showing an intense cytoplasmic staining in cancer cells.

Figure 2. In situ hybridization study on the expression of oncofetal fibronectin mRNA. (A), (C) and (D) show the staining with anti-sense probe in cases 1, 6 and 11, respectively (×200). The bar represents a scale of 100 μm. In (C), a high magnification photo (×630) is shown on the right-hand side. (B) The staining of case 1 with anti-sense probe added with excess non-labeled probe as a negative control. Case 1 was a giant cell anaplastic thyroid carcinoma which showed an intense cytoplasmic staining in cancer cells. Case 6 was a squamoid cell anaplastic thyroid carcinoma which was rich in connective tissue. It showed a staining in the cytoplasm of cancer cells and a slight staining in the connective tissue; possibly a staining in fibroblasts. Case 11 was a pleomorphic anaplastic thyroid carcinoma showing an intense cytoplasmic staining in cancer cells.

Fibronectins are high-molecular-mass adhesive glycoproteins present in the extracellular matrix and body fluids (15), and onfFN is characterized by the presence of the oncofetal domain, which is absent in normal fibronectin. The oncofetal domain is recognized by a monoclonal antibody called FDC-6, which many researchers have used to report the existence of onfFN in a wide variety of fetal tissues and malignant tissues (16).

The role of onfFN in thyroid cancer cells is not clear, although some studies demonstrated modulation of the adhesive behavior of tumors by onfFN and its receptors (17). Some researchers assume that epithelial—mesenchymal transformation (EMT) is one of the characteristics of cancer cells, since mesenchymal cells have the ability, unlike true epithelia, to invade and migrate through the extracellular matrix (ECM) to create dramatic cell transpositions (18). This might explain why onfFN mRNAs are overexpressed in ATCs, since fibroblasts are abundantly expressed in some mesenchymal cells such as fibroblasts. Alternatively, as we assumed in a previous paper, the expression of onfFN mRNA is not caused by malignant transformation, but by thyroid cancer cells expressing onfFN mRNA because they derive from fetal cells which ubiquitously express onfFN mRNA (3).

Overexpression of onfFN mRNA was observed in all ATCs studied, regardless of their histological subtypes. It should be noted that, even though follicular carcinomas did not express onfFN mRNA in previous studies (5,6), all three ATCs accompanied follicular carcinoma expressed onfFN mRNA abundantly. Furthermore, an in situ hybridization study showed that onfFN mRNA was expressed in the majority of the anaplastic carcinoma cells in the tissue sections. These findings indicate that onfFN mRNA is constantly expressed in ATC cells regardless of the conditions. These phenomena were not observed with the gene expression, which was reported to be specific in ATC previously. In ATC cell lines, five of six cell lines showed high expression levels of onfFN mRNA. The low expression level of onfFN mRNA in KMH-2 may be explained by altered gene expression profiles from the originating ATC caused by repeated passages (19).

Our present study indicates that onfFN mRNA is a molecular marker not only for papillary thyroid carcinoma but also for ATC. From the therapeutic point of view, onfFN mRNA or protein may be considered to be a potential target of molecular-based therapy such as cancer immunotherapy of ATC, since onfFN mRNA is expressed in fetal or cancer cells in a restricted manner.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for Scientific Research C, 2005–6, no. 18590531, Research Grant of the Princess Takamatsu Cancer Research Fund and Foundation for Promotion of Cancer Research in Japan.

Conflict of interest statement

None declared.

References


