High prevalence of cyclooxygenase 2 expression in papillary thyroid carcinoma

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Abstract

Background: Cyclooxygenase-2 (COX-2) seems to play a role in the development and carcinogenesis of papillary thyroid carcinoma. Its incidence of expression and potential application as a tumor marker remain to be elucidated.

Materials and methods: Immunohistochemical staining for COX-2 expression was performed for 30 papillary thyroid carcinoma (PTC) and 40 benign thyroid specimens. COX-2 mRNA expression was analyzed using a reverse transcriptase-polymerase chain reaction (RT-PCR) for paired fresh frozen tissues removed from surgically resected PTC specimens.

Results: COX-2 expression was detected by immunohistochemistry in 27 of 30 (90%) PTC but was absent in 40 benign thyroid specimens, including 27 nodular hyperplasia, 7 follicular adenoma and 6 lymphocytic thyroiditis. Two of the three COX-2 negative carcinomas were follicular variant of PTC. RT-PCR analysis confirmed COX-2 mRNA over-expression in 14 of 20 (70%) paired specimens of PTC. Real-time quantitative RT-PCR showed that the level of COX-2 mRNA expression was significantly higher in PTC than in both the adjacent non-cancerous tissues and the benign thyroid specimens.

Conclusion: COX-2 is frequently expressed in PTC but not in benign thyroid specimens. COX-2 expression may serve as a useful molecular marker for PTC in cases of diagnostic difficulty.

Introduction

Cyclooxygenases catalyze the formation of prostaglandins from arachidonic acid and two cyclooxygenase isoforms, COX-1 and COX-2, have been identified. COX-1 is known as a housekeeping gene and is ubiquitously expressed in most tissues. On the other hand, COX-2 is an early expressed gene and is induced by various stimulating factors such as growth factors, oncogenes and cytokines. Recent studies have demonstrated that COX-2 plays a role in carcinogenesis and carcinoma progression in many epithelial tumors including thyroid carcinomas (1–6).

The expression of COX-2 in human thyroid cancer cell lines as well as in benign and malignant thyroid tissues has been studied (6). The finding that COX-2 is upregulated in human thyroid cancer but not in benign thyroid nodules suggests that COX-2 expression may serve as a marker for thyroid malignancy and may have an important role during the development of thyroid cancer (6). However, the frequencies of COX-2 expression vary widely in different types of thyroid cancers (6–11) and COX-2 expression is also detected in a small proportion of benign thyroid tissues, especially follicular adenoma and Hashimoto thyroiditis (8–11). The usefulness of COX-2 as a marker of thyroid malignancy has been challenged (8) but its potential role in carcinogenesis continues to arouse significant interest (6–11). Despite its clinical heterogeneity, papillary thyroid carcinoma (PTC) is characterized by a consistent set of molecular changes different from other types of thyroid cancer. COX-2 seems to be upregulated in PTC but not in other thyroid carcinomas. In this study, we have examined a relatively large number of PTC and benign thyroid specimens for COX-2 expression using immunohistochemistry in order to assess the feasibility of using COX-2 as a marker for malignancy. The level of COX-2 mRNA expression was analyzed and compared between malignant, adjacent normal and benign thyroid tissues.

Materials and methods

Immunohistochemistry

Paraffin blocks of thyroid specimens surgically excised from 70 patients were selected for the present study. These included 30 PTC and 40 benign thyroid specimens. There were 17 men and 53 women.
Patients ranged in age from 15 to 82 years (median, 50 years). Patients with PTC were staged according to the UICC pTNM classification (12). There were 7 men and 23 women with ages ranging from 15 to 81 years (median, 46 years). The size of the primary tumor ranged from 0.5 to 8.5 cm (median, 2.1 cm). According to the UICC pTNM classification, there were 16 patients with stage I, 4 with stage II, 9 with stage III and 1 with stage IV disease. To examine the histopathology of thyroid tissues, 4–μm thick sections were cut from the paraffin blocks, mounted on polylysine-coated slides and stained with hematoxylin-eosin for light microscopy. Sections were also dewaxed in xylene, rehydrated in descending alcohols, and blocked for endogenous peroxidase and avidin-biotin activities. After blocking with 3% BSA in PBS, the sections were incubated with mouse monoclonal antibody against human COX-2 (Cayman Chemical, Ann Arbor, MI, USA) or an isotype-matched control antibody (Zymed Laboratories Inc., South San Francisco, CA, USA) at a dilution of 1:500 overnight at 4°C. After washing with PBS, the slides were incubated with biotinylated secondary antibody (DakoCytomation A/S, Glostrup, Denmark) for 45 min at room temperature, washed and visualized with diaminobenzidine substrate. Tissue sections were counter-stained in aqueous hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount. Histological review was performed by a single pathologist (K Y L) blinded to the COX-2 assay of the specimens. Histological subtyping of PTC was reported accordingly and 8 of the tumors were positive. Twenty PTC specimens that originated from the same patients as those used for the immunohistochemical analysis were retrieved for analysis. There were 5 men and 15 women with a median age of 47.5 years (range, 15 to 81 years). The size of the primary tumor ranged from 1.1 to 8.5 cm (median, 2.5 cm). According to the UICC pTNM classification, there were 10 patients with stage I, 1 with stage II and 9 with stage III disease. To determine the relative amount of mRNA transcripts in tissue samples by RT-PCR, total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Ambion, Austin, TX, USA). As previously described (13), cDNA was synthesized with 1 μg DNase-treated total RNA by oligo(dT)\textsubscript{12–18} primer using Superscript II RNase\textsuperscript{−} reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Gene-specific primers for COX-2 and β-microglobulin were designed using the Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA). The primers and probe sequence used are summarized in Table 1. PCR was performed in 1 × PCR buffer (25 μl) containing 200 μmol/l dNTPs, 1.5 mmol/l MgCl\textsubscript{2} and 0.8 μmol/l of each paired primer, using the ABI 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR was carried out at 94°C for 10 min, followed by

### RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Tissue samples were obtained from our frozen tissue bank collected prospectively during thyroidectomy from 1999 to 2000. Macroscopically, the site and size (maximum dimension) of thyroid tumors were recorded in fresh tissue. One representative section of the tumor was snap frozen in liquid nitrogen and stored at −80°C until processed. Adjacent apparently normal thyroid tissue, usually located in the contralateral lobe, was also obtained. Other standard sections from the tumor were taken, fixed in 10% buffered formalin and embedded in paraffin wax. To examine the histopathology of thyroid tissue, 10-μm thick sections were cut from frozen blocks, mounted on glass slides and stained with hematoxylin-eosin for light microscopic analysis.

Twelve PTC specimens that originated from the same patients as those used for the immunohistochemical analysis were retrieved for analysis. There were 5 men and 15 women with a median age of 47.5 years (range, 15 to 81 years). The size of the primary tumor ranged from 1.1 to 8.5 cm (median, 2.5 cm). According to the UICC pTNM classification, there were 10 patients with stage I, 1 with stage II and 9 with stage III disease. To determine the relative amount of mRNA transcripts in tissue samples by RT-PCR, total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Ambion, Austin, TX, USA). As previously described (13), cDNA was synthesized with 1 μg DNase-treated total RNA by oligo(dT)\textsubscript{12–18} primer using Superscript II RNase\textsuperscript{−} reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Gene-specific primers for COX-2 and β-microglobulin were designed using the Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA). The primers and probe sequence used are summarized in Table 1. PCR was performed in 1 × PCR buffer (25 μl) containing 200 μmol/l dNTPs, 1.5 mmol/l MgCl\textsubscript{2} and 0.8 μmol/l of each paired primer, using the ABI 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR was carried out at 94°C for 10 min, followed by

### Table 1 Sequence of primers and probes used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer set</th>
<th>Oligonucleotide sequence</th>
<th>Temp. (°C)</th>
<th>Expected PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward primer</td>
<td>5′-GGTCTGGTGCGTGCTGATGATG-3′</td>
<td>69.4</td>
<td>724</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5′-GTCCTTTCAAGGAGAATGGTG-3′</td>
<td>62.1</td>
<td></td>
</tr>
<tr>
<td>β-microglobulin</td>
<td>Forward primer</td>
<td>5′-AGCAGAGAATTGGAAGTCAAA-3′</td>
<td>56.4</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5′-ATGCTGCTTACATGTCTCGA-3′</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td>Real-time Q-PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward primer</td>
<td>5′-GAATCATTCAACCAGGCAATATTG-3′</td>
<td>69.4</td>
<td>724</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5′-TTTCGTAAGCTGCGGGTGGAAC-3′</td>
<td>62.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5′-FAM-TCCTCTACCCAGCAACGCTGCA-TAMRA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal 18 s</td>
<td>Forward primer</td>
<td>5′-CGGTACACATCCAAAGGAA-3′</td>
<td>69.4</td>
<td>724</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5′-GCTGAAATTACCCGAGCT-3′</td>
<td>62.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5′-VIC-TGCTGGACACACTTGCCCTC-TAMRA-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Temp., temperature.
30 cycles at 94°C for 40s, 60°C for 40s and 72°C for 1 min and a final elongation step at 72°C for 10 min. The β-microglobulin housekeeping gene was included in all experiments as an internal control. PCR products were resolved by 2% agarose gel electrophoresis and visualized after staining with ethidium bromide under UV illumination. The gel images were captured and analyzed by the Syngene CCD BIO acquisition system (Hitachi Genetics, Alameda, CA, USA).

**Real-time quantitative RT-PCR for COX-2 mRNA expression**

Eight paired samples used in the previous experiment and 6 additional benign thyroid specimens were subjected to a real-time quantitative RT-PCR (Q-PCR) assay for COX-2 mRNA expression. Oligonucleotide primers and TaqMan probes for COX-2 and 18s ribosomal genes in Q-PCR were designed using the ABI PRISM Primer Express Software (Applied Biosystems) (Table 1). The fluorescence dyes EAM and VIC were used respectively as reporter signals. Ribosomal 18s was used as an internal control. Real-time Q-PCR was performed according to the previously described procedures (14). To determine COX-2 expression levels in different patients, 2 µl of each cDNA were subjected to AmpliTaq Gold DNA polymerase in 1 x PCR buffer (25 µl) containing 200 µmol/l dNTPs, 1.5 mmol/l MgCl₂, 0.5 µmol/l of each of the paired primers, and 200 nmol/l TaqMan probe. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min. All reactions were performed at least in duplicate and the analysis was conducted using the ABI Prism 7700 detection system (Applied Biosystems).

The relative gene expression data were analyzed according to the description by Livak and Schmittgen (15). The relative amount of COX-2 mRNA, normalized to an internal control ribosomal 18s and relative to a calibrator, was defined as $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = (CT_{\text{r18s}} - CT_{\text{cox-2 sample}}) - (CT_{\text{r18s calibrator}} - CT_{\text{cox-2 calibrator}})$. The amplification plots of COX-2 and ribosomal 18s are shown in Fig. 1. Differentiation in normalized reporter signal (ΔRn, Y axis) was plotted against cycle number. Ct (X axis). Ct was defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. The Ct value corrected with the input target mRNA levels, and a lower Ct value indicated a higher starting copy number. A sample of benign normal thyroid tumor was designated as the calibrator to compare the relative amount of target in different samples and to adjust for the plate-to-plate variation in amplification efficiency (Fig. 1).

**Results**

Using immunohistochemical evaluation, COX-2 expression was not observed in normal follicular cells or stromal cells in either normal/benign thyroid tissues or tissues adjacent to the tumors. COX-2 expression was detected in 27 of 30 (90%) PTC tumour specimens (Fig. 2). The expression was diffusely positive in all 27 but was negative in 3 cases of PTC. However, none of the 40 benign thyroid specimens showed COX-2 expression ($P < 0.001$ by chi-square test). COX-2 expression was not detected in 27 nodular hyperplasia, 7 follicular adenoma and 6 lymphocytic thyroiditis specimens. Twenty-seven patients had a pure PTC and three were diagnosed as having follicular variant of PTC. In PTC, the incidence of COX-2 expression was significantly lower for patients with follicular variant compared with that of pure PTC ($P = 0.001$; Fischer’s exact test). Twenty-six of twenty-seven (96%) patients with a normal variant of PTC expressed COX-2 compared with 1 of 3 patients (33%) with a follicular variant. COX-2 was expressed in 14 of 17 patients with stage I-II disease compared with all 13 patients with stage III-IV disease but the difference was not statistically significant. No other relationship between COX-2 expression and clinicopathological features of PTC, including age and stage of disease, could be found because of the high incidence of COX-2 expression in PTC.

RT-PCR assay of COX-2 mRNA confirmed overexpression in 14 of 20 paired specimens (70%) (Fig. 3). Real-time Q-PCR for COX-2 mRNA expression was performed on 8 paired PTC tissues and 6 benign thyroid tissues. The level of COX-2 mRNA expression was significantly higher in tumors compared with that in the adjacent or contralateral paired non-cancerous thyroid tissues. COX-2 mRNA expression in tumor was $16.6 \pm 3.9$ (mean $\pm$ S.E.) compared with $5.8 \pm 1.6$ in noncancerous tissues (Student’s t-test, $P = 0.023$). In addition, the level of COX-2 mRNA expression of either the tumors ($P = 0.006$) or non-tumorous thyroid tissues ($P = 0.031$) was much higher compared with that of benign or normal thyroid tissues ($1.2 \pm 1.0$) (Fig. 4).

**Discussion**

COX-2 expression has been reported in various human epithelial malignancies, including colorectal (1), gastric (2), breast (3), esophageal (4) and lung (5) cancers. Depending on the methods of detection and the type of malignancy, COX-2 expression was detected in up to 100% of the tumors and its prognostic relevance remained controversial (16). Previous studies showed that COX-2 was expressed in human thyocytes in vitro, and that COX-2 expression was induced by proinflammatory cytokines in human thyroid epithelial cells (17, 18). COX-2 expression was shown to be present in various types of thyroid carcinomas but the expression was low in non-neoplastic thyroid tissue of tumor samples (6). In addition, COX-2 was not expressed in normal or benign thyroid tissue (6) but was subsequently found to be occasionally expressed in benign follicular cells, including follicular adenoma.
and thyroiditis (8–11). COX-2 was most frequently upregulated in PTC compared with other types of thyroid malignancy (6–10). COX-2 expression was detected in 84 of 108 (78%) (7), 4 of 5 (80%) (10), 40 of 49 (82%) (9), and 19 of 19 (100%) (8) PTC specimens using immunohistochemistry. Our study showed that COX-2 overexpression was frequently detected in the majority of patients with PTC using immunohistochem-

Figure 1 (A) Amplification plots of ribosomal 18s as internal control for different thyroid tissues – emission intensity of the reporter (VIC) dye (ΔRn, Y axis) versus cycle number (X axis). The relative amount of mRNA in each sample is normalized to an internal control ribosomal 18s, \( \Delta C_T = C_T \) of COX-2 – \( C_T \) of 18s. (B) Amplification plots of COX-2 expression in different thyroid tissues – emission intensity of the reporter (FAM) dye (ΔRn, Y axis) versus cycle number (X axis). Benign/normal, non-tumorous contralateral thyroid, and tumor tissues are represented by grey, dark grey and black color plots respectively.
COX-2 expression in papillary thyroid carcinoma

mRNA was demonstrated in PTC compared with both adjacent non-tumorous and benign thyroid tissues. On the other hand, the lower incidence of COX-2 mRNA expression using RT-PCR for paired specimens when compared with immunohistochemistry could be attributed to a lower level of COX-2 expression in the adjacent tissue because the determination of COX-2 expression in tumor was calculated with reference to that of adjacent non-tumorous tissue.

It was shown that COX-2 expression using immunohistochemistry was significantly lower in anaplastic and follicular carcinomas than in PTC (9). In addition, COX-2 expression in PTC was significantly reduced in cases with old age, large size, advanced stage, satellite tumors, and the presence of a solid, scirrhous or trabecular growth pattern (9). Therefore, it was suggested that COX-2 activation seemed to be involved in the early phase of pathogenesis of thyroid carcinoma (8, 9). However, COX-2 expression was also shown to increase with age and age-related increase in COX-2 expression could explain the more aggressive behavior of PTC in the older age group (7). In our study, COX-2 expression was higher in PTC at an advanced stage but the difference was not statistically significant. The potential relationship between COX-2 expression and the clinical characteristics of PTC could not be identified because of the high prevalence of COX-2 expression in PTC. The role of COX-2 activation in carcinogenesis of PTC remains unclear.

The expression of COX-2 in follicular variant of PTC has not been analyzed in previous studies. Follicular variant of PTC is a common variant of PTC and constitutes 9 to 22.5% of cases (19). Although the clinical behavior of follicular variant of PTC did not differ significantly from that of the usual type of PTC, others suggested a difference in lymph node involvement, distant metastases...
and prognosis (19). In this study, cases of follicular variant of PTC were often negative for COX-2. Lam et al. have also reported that RET oncogene activation (a genetic activation specific for PTC) was often negative in follicular variant of PTC (20). Thus, follicular variant of PTC may have a different genetic mutation pathway in carcinogenesis from the usual type of PTC.

Efforts are being made to find a molecular marker to improve diagnostic accuracy for thyroid carcinomas. In order for the marker to be clinically useful, it should be able to reliably distinguish benign from malignant nodules during cytological diagnosis of thyroid nodules, it should be confirmed by several different investigators as a useful tool in the diagnosis of thyroid cancer, it should have prognostic significance, and it should provide insights into the pathogenesis and treatment options in these patients (21). It was suggested that COX-2 expression might be employed as a molecular marker for diagnosing thyroid malignancy during fine needle aspiration cytology for thyroid nodules (6). However, COX-2 expression has been shown to be significantly lower in follicular carcinoma and its expression has been documented in a small proportion of patients with benign pathologies including follicular adenoma and thyroiditis (8–11). Therefore, its application as a marker in diagnosing thyroid malignancy for thyroid nodule would be limited (8). On the other hand, based on our present study, COX-2 expression has consistently been documented in a relatively large proportion of patients with PTC but not in benign thyroid specimens. COX-2 may be applied as a molecular marker for the identification of patients with PTC in cases of diagnostic difficulty. In addition, the finding of COX-2 expression in PTC but not in benign thyroid specimens suggests that it might have a role in carcinogenesis of PTC. The use of real-time quantitative PCR may be a more sensitive tool in assessing COX-2 expression.

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References


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