Abstract: Background. Recent studies have demonstrated that cyclooxygenase-2 (COX-2) expression is associated with the carcinogenesis of numerous neoplasms. The aim of this study was to evaluate the role of COX-2 in medullary thyroid carcinoma (MTC).

Methods. Tissue specimens of thyroid neoplasms were obtained from 22 patients with MTC and 15 control subjects with nonmalignant thyroid specimens.

Results. This immunohistochemical study confirms the presence of COX-2 in a significant number of MTCs. A large area of staining was noted in only 2 patients in the control group (13%) compared with 18 (82%) in the medullary carcinoma group. On a scale of 0 to 3, the average area of positive staining measured 2.35 in the study group and 0.9 in the control group ($p < .0001$). The average intensity of staining on a scale of 0 to 5 (deep brown) was 2.15 and 0.8 mm, respectively ($p < .001$).

Conclusion. COX-2 is expressed significantly in MTC including a larger area of staining and greater intensity than in nonmalignant thyroid tissue. These findings may have important treatment implications for the use of COX-2 inhibitors in patients with MTC.

Keywords: COX-2; colloid goiter; medullary thyroid carcinoma

Medullary thyroid carcinoma (MTC) is an endocrine neoplasm that originates in the C cells of the thyroid gland. It accounts for 5% to 10% of all thyroid carcinomas. Seventy-five percent of cases are sporadic and 25% are familial. The clinical course ranges from indolent to extremely aggressive. The effectiveness of treatment is largely dependent on the stage of disease at diagnosis; patients with the sporadic type are commonly diagnosed at a late stage. Tumors confined to the thyroid are treated with total thyroidectomy and microdissection of cervicomediastinal lymph nodes, with a 95% success rate. However, the risk of recurrence is about 50%, and the factors predicting recurrence remain unclear. In patients with unresectable or distant metastases, the prognosis is poor; there is no effective treatment, and less than 30% survive more than 10 years after diagnosis. MTC is hardly radiosensitive, and according to most reports, radiotherapy has palliative value only. Radioactive iodine is not a useful treatment modality in persistent or metastatic MTC because the C cells, which do not originate from thyroid follicles, fail to trap iodine. Chemotherapy is generally used in pa-
patients with rapidly progressive and metastatic disease, but none of the regimens has proven particularly effective, and the response rate is generally low.12–14 Thus, new therapeutic modalities are urgently needed to control the disease. Recently, investigators suggested the use of biological agents, such as somatostatin analogs, which have an antiproliferative effect and inhibit angiogenesis.15 Others evaluated the effectiveness of interferon.16 However, both studies included only very small groups of patients.

Cyclooxygenase-2 (COX-2), or prostaglandin-endoperoxide H synthase-2, catalyzes the formation of prostaglandins from arachidonic acid. Recent studies have demonstrated that elevated COX-2 expression is associated with the carcinogenesis of numerous neoplasms, including head and neck squamous cell carcinoma,17 and colorectal,18 breast,19 lung,20 pancreatic,21 gastric,22 hepatocellular,23 and skin.24 Cancer COX-2 may promote carcinogenesis by inhibiting apoptosis25 or promoting angiogenesis,26,27 or cell proliferation, or by inducing an immune suppression state via inhibition of the synthesis of immune regulatory products, such as natural killer cells or tumor necrosis factor.28,29 The enzyme was found to have therapeutic importance in premalignant colorectal adenoma.30

In 1968, Williams et al31 reported high levels of prostaglandin in plasma and tumor tissues of patients with MTC. However, the presence of COX-2 enzyme has not been specifically evaluated. The aim of the present study was to determine the role of the COX-2 enzyme in medullary carcinoma.

MATERIALS AND METHODS
Tissue specimens of thyroid neoplasms were obtained from 22 patients with a diagnosis of MTC being treated in our center (study group). For comparison, 15 specimens were obtained from 15 subjects with nonmalignant follicular adenoma or colloid goiter, or normal thyroid tissue obtained in postmortem (n = 5 each; control group). Patients and controls had a similar demographic background (age, sex).

Immunohistochemical Study. Tissues were fixed with 10% formalin, embedded in paraffin, and cut into 4 mm thickness and stained immunohistochemically with rabbit polyclonal anti-human COX-2 antibody (IBL, Gunma, Japan). The sections were dewaxed and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 25 minutes. For antigen activation, sections were immersed in 0.04 mol/L citrate buffer, boiled, and incubated in a microwave oven at 95°C for 10 minutes. After the sections were rinsed with 0.005 mm triethanolamine-buffered saline (TBS) at pH 7.6, 20% swine serum (Wako, Osaka, Japan) was applied for 20 minutes to block nonspecific reactions. The sections were then incubated with the primary antibody at a dilution of 1:150 at room temperature overnight, rinsed with TBS, and treated with peroxidase-labeled anti-mouse and anti-rabbit immunoglobulins (Dako, Tokyo, Japan) for 30 minutes. The peroxide reaction was visualized by incubating the sections with 0.02% 3,3-diaminobenzidinetetrahydrochloride (DAB) in 0.05 mol Tris buffer with 0.01% hydrogen peroxide (Nichirei). The sections were counterstained with mouse hematoxylin. Sections for negative controls were prepared using mouse and rabbit immunoglobulin instead of the primary antibody.

Immunohistochemical Evaluation. Immunostaining was verified with 2 specimens from colon carcinomas as controls, which are known to stain positively for COX-2. Cells were considered positive when immunoreactivity was clearly observed in the cytoplasm. At least 1000 cells in 5 randomly selected fields were carefully monitored and classified into 4 categories: strong positivity (++++), >66% positive cells; diffuse 2(++), >33% positive cells; heterogeneous 1(+), 10% to 33% positive cells; and negative 0 (−), <10% positive cells. Intensity was defined according to the color of the staining: deep brown 3(+++); brown 2(++) or yellowish-brown 1(+); 0 when only faint color appeared. Specimens from the 2 groups were compared for the percentage of the area that stained positive and for the degree of intensity of the positive staining (Tables 1 and 2).

Statistical Analysis. Statistical analyses were performed using chi-square and parametric Mann-Whitney tests. A p value of <.05 was considered statistically significant.

RESULTS
The study group consisted of 12 men (55%) and 10 women (45%) of average age 47 years at surgery (range, 15–77 years). In 3 patients, the medullary tumor measured <1.5 cm, and the remaining thyroid tissue was normal. In all the other patients, the medullary carcinoma was larger than 2.5 cm. In 1 patient, there was a concomitant papillary
carcinoma. The women-to-men ratio was 2:1 (10 women, 5 men) in the control group, and average age at surgery was 50 years.

Figure 1 demonstrates the immunostaining in the control group, Figure 2 shows the immunostaining in the MTC group, and Figure 3 demonstrates the immunostaining of the invasive carcinoma into the surrounding vessels. On a scale of 0 to 3, comparison of the percentage of area stained positive by immunohistochemistry between the groups revealed an average area of positive staining of 2.35 in the study group and 0.9 in the control group. Large area involvement (++ or ++++) was noted in 18 patients (82%) in the study group and only 2 patients (13%) in the control group. This difference was statistically significant ($p < .0001$) (Table 1, Figure 4). The subgroups among the control group were too small for statistical analysis. There were 9 patients in the control group with a grade zero of staining compared with only 1 patient with MTC.

Comparison of intensity of immunostaining (Table 2, Figure 5) revealed an average intensity of 2.15 (dark brown) in the study group and 0.8 in the control group ($p < .0001$). In only 1 control specimen was there significant (++ or ++++) intensity (7%) compared with 16 study group specimens (72%).

Spearman test demonstrated a direct correlation between area of involvement and staining intensity ($p = .044$). Two patients in the control group had either significant intensity or large area of staining. The first was a patient with follicular adenoma who had both high intensity and large area of staining, and the second was a patient with a normal thyroid. In the 3 patients with small cancerous thyroid nodules, there was significant staining in the tumor area, while the background was pale.

**DISCUSSION**

The relationship between COX-2 expression and thyroid tumors has been investigated in several studies. COX-2 expression was found to be significantly elevated in papillary carcinoma compared
with follicular adenomas, with only occasional staining of normal cells, and compared with multinodular goiter, anaplastic carcinoma, and normal thyroid tissue, all of which were devoid of immunostaining.\textsuperscript{32,33} Smith et al,\textsuperscript{34} however, demonstrated immunostaining in cell lines of normal thyroid tissue. In our study, positive immunostaining was noted in only a small percentage of nonmalignant specimens, compared with 82\% of the MTC specimens. The positive control specimens had no evidence of malignant transformation or any special characteristics.

Williams et al,\textsuperscript{31} in a search of an explanation for the diarrhea associated with medullary carcinoma, found that the levels of prostaglandin in the tumor tissue were high. It was, however, 36 years later that Quidville et al\textsuperscript{35} evaluated the expression of COX-2 in cells from a mouse model of human MTC. Although they found low levels of COX-2 in the tumor tissue itself, levels were high in the macrophages surrounding the tumors. Treatment with indomethacin, a prostaglandin inhibitor, reduced tumor growth and lowered the level of calcitonin in the peripheral blood.

To the best of our knowledge, the expression of COX-2 in human MTC has not been investigated before. On immunohistochemical study, clearly positive staining was noted in 82\% of MTC specimens. The staining was widely distributed in the medullary thyroid carcinoma tissue as well as in the lymph node metastases, and the area involved was significantly greater than in the control group. We intentionally chose groups with a similar background as far as sex to rule out factors that could influence the tissue itself. Although the small size of our group precludes specific conclusions regarding pathogenesis, the presence of intense staining of endothelial cells in blood vessels surrounding the tumors and the tumor cells in the blood vessels which did not exist in the control group may suggest that COX-2 induces angiogenesis in MTC. This may account for the relatively high rate of distant metastases in these tumors. This finding may have important implications for the therapeutic use of COX-2 inhibitors. In parallel to our finding, the finding of COX-2, which was found to be overexpressed in colon cancer is virtually undetectable in normal intestinal mucosa. Recent clinical studies using specific COX-2 inhibitors have shown a 3-fold target of action: (1) successful use in precancerous colon treatment regimen\textsuperscript{36}; (2) reduction in intestinal polyp burden in familial polyposis; and (3) prevention of adenomas and regulation of angiogenesis, resulting in the reduction of liver metastases. We hope that patients with MTC expressing COX-2 will benefit from the anti–COX-2 inhibitors.
In conclusion, the present preliminary study demonstrates a significant elevation of COX-2 levels in MTC. Animal and clinical studies are needed to evaluate the promising role of COX-2 inhibitors in the treatment of affected patients.

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REFERENCES


