Immunoexpression of Multidrug-Resistance Protein 2 and Cyclooxygenase 2 in Medullary Thyroid Carcinomas

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Context.—Chemoresistance is due to the expression of multidrug-resistance proteins (MRPs). Cyclooxygenase 2 (COX2), a key enzyme in prostaglandins synthesis, upregulates MRPI. MRPI is overexpressed in medullary thyroid carcinomas (MTCs), but it is not involved in resistance to doxorubicin and cisplatin, which are commonly used in MTC treatment. MRPI is specifically involved in resistance to both chemotherapeutic agents, but no data exist on the expression of MRPII and COX2 in MTC.

Objective.—To evaluate MRPI and COX2 expressions in MTC.

Design.—We analyzed immunohistochemical expression of MRPI and COX2 in 12 MTCs and in 6 lymph node metastases. Results were correlated with pTNM and clinical stage.

Results.—MRPI and COX2 expressions were observed only in tumor samples and metastases. Nine MTCs, all pTNM stage T4, were positive for MRPI, whereas 3 MTCs, pTNM stages T2 and T3, were unreactive for MRPI. Six metastatic MTCs at stage T4 showed higher proportion of MRPII cells, compared with primary tumors. All 12 MTCs were positive for COX2. Three MTCs, pTNM stage T2 and T3, showed COX2 positivity in all cells. The proportion of COX2I cells decreased with increased pTNM stage. Four out of 6 metastatic MTCs, stage T4, showed a lower proportion of COX2I cells, compared with primary tumors. The proportion of MRPII cells was inversely related to the proportion of COX2I cells.

Conclusions.—MRPI and COX2 expression correlated with pTNM stage. High MRPI and low COX2 expression may explain resistance to doxorubicin and cisplatin, which is observed in advanced stage MTC. Evaluation of the expression pattern of these 2 proteins may be useful to predict chemosensitivity of these types of tumors.

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The cellular capacity to resist cytotoxic agents is mediated by multidrug transporters. Multidrug transporters reduce the effects of chemotherapeutics by actively pumping them out of the cells. Multidrug transporters are adenosine triphosphate-binding cassette transporters containing families of membranous proteins and include multidrug resistance proteins (MRPs) 1 to 4. Specific differences distinguish MRPI protein from MRPII. The former is localized on the lateral plasma membrane, whereas the latter is detected on the apical plasma membrane of the polarized cells. MRPI overexpression is more strongly associated with vincristine resistance, whereas MRPII overexpression is more frequently involved with cisplatin and doxorubicin resistance. In addition, even when the 2 proteins transport the same type of drugs, they show different affinity. Therefore, these observations suggest that MRPI and MRPII play a different role in chemoresistance. Chemoresistance specificity of multidrug transporters may depend on cellular context.

One of the major regulators of MRPI expression is cyclooxygenase 2 (COX2), a key enzyme in the prostaglandin biosynthetic pathway that has recently received considerable attention because of its role in human cancers. Evidence exists that COX2 plays an important role in carcinogenesis through the promotion of angiogenesis, the stimulation of cell growth, and the inhibition of apoptosis. COX2 is also involved in increasing expression and activity of MRPI, but its involvement in MRPII expression remains to be clarified.

Medullary thyroid carcinomas (MTCs) are endocrine neoplasms arising from the parafollicular C cells. A portion of these cancers that have not been cured by surgery are usually treated by chemotherapy and frequently show chemoresistance. MTC cell lines and tissues express high levels of MRPI and it has been suggested that this finding may be used to predict treatment responsiveness. However, current chemotherapeutic protocols of MTC include doxorubicin and cisplatin, resistance to which is modulated by MRPII. No data are available regarding MRPII expression in MTC.

COX2 expression has been previously evaluated in benign and malignant thyroid tumors arising from follicular epithelium. To date, few data exist on COX2 expression in MTC.

MRPII and COX2 in Medullary Thyroid Carcinoma —Ruggeri et al
Multidrug-Resistance Protein 1 (MRP1) and Cyclooxygenase 2 (COX2) Expression Patterns and Clinicopathologic Features of 12 Medullary Thyroid Carcinomas (MTCs)

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age, y/sex</th>
<th>MTC Form</th>
<th>Histologic Variants*</th>
<th>pTNM†</th>
<th>Clinical Stage‡</th>
<th>Reactive MRP2 Cells‡</th>
<th>Intensity MRP2 Reaction§</th>
<th>Reactive COX2 Cells§</th>
<th>Intensity COX2 Reaction§</th>
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</table>

* MTCs were diagnosed according to Armed Forces Institute of Pathology criteria.
† Pathologic (pTNM) and clinical stages have been determined, according to the revised criteria of the American Joint Committee of Cancer. 38
‡ MTCs were diagnosed according to Armed Forces Institute of Pathology criteria.
§ Staining was scored on a 5-point scale from 0 (absent) through 4+ (very intense) as specified in “Materials and Methods.”
¶ MEN indicates multiple endocrine neoplasia.

In this study we evaluated the immunopexpression of MRP2 and COX2 in MTC histologic samples and correlated expression of these 2 proteins with pTNM and clinical stage.

**MATERIALS AND METHODS**

**Tissue Collection**

Specimens of 12 MTCs plus 6 corresponding lymph node metastases were retrieved from the archives of the Department of Human Pathology, University of Messina, Italy. Appropriate clinical data were available for each MTC sample. There were 11 female patients and 1 male patient with a mean age ± SD of 57.6 ± 15.1 years at the time of thyroidectomy. None of the patients received chemotherapeutic drugs before surgery. Among the MTCs examined, 11 MTC were sporadic and 1 MTC was associated with the multiple endocrine neoplasia IIB syndrome. At the time of diagnosis, 6 of the MTC patients had metastases to regional neck lymph nodes and 2 of them showed distant metastases (liver). According to the criteria proposed by the Armed Forces Institute of Pathology, the lesions were histologically classified as 11 MTC usual type and 1 MTC small cell variant. 37 MTCs were divided according to their staging (TNM classification). 38 Based on pTNM staging, 1 MTC was classified as pT2, 2 as pT3, and 9 as pT4. Based on clinical staging, 1 MTC case was considered at stage II, 2 at stage III, and 9 at stage IV (6 at stage IVA, 1 at stage IVB, and 2 at stage IVc) (see Table). Specimens of 6 normal thyroids, harvested at autopsy, and 10 colloid nodules, obtained at surgery, were used as controls. In addition, all MTC samples were studied in comparison with the adjacent non-tumor thyroid tissue and C cell hyperplasia.

Specimens were fixed in 4% formalin and embedded in blocks of paraffin. Each block was cut into 5-μm serial sections to perform hematoxylin-eosin stain and immunohistochemistry. The experimental protocol was approved by the local ethics committee in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

**Immunohistochemistry**

Immunohistochemistry was performed separately, using mouse monoclonal antibodies raised against human MRP2/ cMOAT (immunoglobulin IgG2a, clone M, III-6, amino acids sequences 1339–1541, 1:100, Alexis Biochemicals, Lausen, Switzerland), COX2 (IgG1, amino acids sequences 580–599, 1:200, Cayman Chemical Company, Ann Arbor, Mich), and chromogranin A (IgG1, clone Lk2H10, 1:100, Immunotech, Marseille, France), respectively, and a rabbit polyclonal antibody raised against rabbit anti-human calcitonin (Lot 083, 1:400, Dako-Cytomation, Glostrup, Denmark).

Antigen retrieval technique was carried out as described by Gown et al. 39 In brief, the sections were deparaffinized in xylene and rehydrated through graded alcohol and deionized water. En-
MRP2 and COX2 Immunohistochemistry

MRP2 and COX2 positive immunoreactions were observed only in MTC samples and lymph node metastases. The controls included in this study, such as adjacent non-tumor thyroid tissue, C cell hyperplasias, colloid nodules, and normal thyroids did not show any MRP2 or COX2 immunostaining.

MRP2 was expressed in 9 (75%) of 12 MTCs and in all 6 lymph node metastases (100%) (Table). In all 6 metastatic MTCs, both the primary tumor and the corresponding lymph node metastases showed MRP2 immunoreaction, but the rate of positive cells was different. In fact, in primary MTCs, a positive MRP2 immunoreaction was detected in 20% to 40% of cells (mean 29%), whereas in the corresponding metastatic lymph nodes, the reactivity for MRP2 was observed in a higher proportion of cells (25%–60%, mean 38%, P = .01) (Table). The immunostaining of MRP2 was located on the membrane and the cytoplasm in both primary and metastatic MTC cells (Figure 1, A and B). In primary MTCs, MRP2 immunostaining was frequently observed even on the cellular membrane opposite to the eccentric nucleus (Figure 1, arrow in A). Moreover, in primary MTCs, the MRP2 staining grade was moderate (cases 4–6), intense (cases 7–11), or very intense (case 12), respectively, whereas it was almost always intense in metastatic MTCs (Table).

COX2 immunoreaction was observed in all MTCs analyzed (100%), as well as in all 6 lymph node metastases (100%) (Table). Primary MTCs showed a higher expression of COX2 compared with the corresponding metastatic lesions. In fact, the cellular proportion reacting to COX2 ranged between 10% and 50% (mean 22%) in the former, whereas it ranged between 10% and 40% (mean 17%) in the latter (P = .04) (Table). In both primary and metastatic MTCs, the immunostaining for COX2 was located in the cytoplasm of reactive cells (Figure 1, C and D). The staining grade was intense (cases 7–12) or very intense (cases

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**Figure 1.** Multidrug-resistance protein 2 (MRP2) and cyclooxygenase 2 (COX2) immunohistochemistry in medullary thyroid carcinomas (MTCs). Cytoplasmic and membranous MRP2 immunostaining in primary (A) and matching metastatic (B) MTC (no. 7). Cytoplasmic COX2 immunostaining in primary (C) and matching metastatic (D) MTC (no. 9) (immunoperoxidase, original magnification ×150 [A], ×200 [B], ×130 [C], and ×250 [D]).
Figure 2. Percentage of multidrug-resistance protein 2 (MRP2) and cyclooxygenase 2 (COX2) positive cells in medullary thyroid carcinomas. Correlation between MRP2 and COX2 expression in various pathologic and clinical stages of primary (A) and metastatic (B) medullary thyroid carcinomas. Data are from the Table.

1–6) in primary MTCs and always intense in MTC metastases (Table).

Comparing MRP2 expression with COX2 immunoreactivity, the proportion of MRP2+ cells was inversely related to the proportion of COX2+ cells in both primary and metastatic MTCs (r = 0.96 and 0.92, respectively) (Figure 2, A and B).

Moderate to strong cytoplasmic staining for both calcitonin and chromogranin A was found in all cases of MTC, lymph node metastases, and C cell hyperplasia (data not shown).

**Correlation of MRP2 and COX2 Expression With pTNM and Clinical Stage**

When MRP2 reactivity was correlated with pTNM and clinical stage, we found that only MTCs extending outside the thyroid capsule, and invading larynx or trachea (ie, pT4 stage), showed MRP2 immunostaining. In 3 MTCs at stage pT2 and pT3, MRP2 expression was absent. Moreover, a clear correlation was seen considering the clinical stages. In fact, all MTCs at pT4 stage, and classified as clinical stages IVA, IVB, or IVC, showed high level of expression of MRP2 (Table).

The results of the correlation between COX2 expression and pTNM and clinical stage were opposite compared with that obtained with MRP2 expression. Very intense and diffuse COX2 immunoreaction (100% of positive cells) was observed in MTCs at stage pT2 and pT3. COX2 immunoreaction was still observed in stage pT4, but the percentage of positive cells, ranging from 10% to 80%, was reduced compared with the previous stages (pT2 and pT3) combined (P = .005). Even for COX2 immunoreaction, a correlation with clinical stage was observed. In fact, the lowest percentage of COX2+ cells was observed in lesions either at stage IVB or at stage IVC.

**COMMENT**

MTC accounts for 5% to 10% of thyroid cancers and occurs either in sporadic (75%) or inherited form (25%).41–43 Hereditary MTC arises through defects of the RET proto-oncogene and is diagnosed in the context of multiple endocrine neoplasia 2A and 2B as well as in family groups.
Both sporadic and familial MTC forms produce calcitonin, whose serum levels are used as a diagnostic marker. Sporadic MTC usually presents as an isolated and unilateral mass, whereas the heritable forms are typically multifocal and tend to be bilateral. These latter types also are more frequently accompanied by C-cell hyperplasia compared with the sporadic types.

Prognosis and treatment effectiveness are largely related to the tumor stage, and an early diagnosis is essential to obtain the best cure rate, because these tumors are often diagnosed at an advanced stage and metastases frequently occur as early events. The treatment of choice for MTC is surgery. However, in patients with progressive metastatic tumor and without surgical options, the unique alternative therapeutic approach is represented by chemotherapy. A small cohort of studies has evaluated the efficacy of pharmacologic treatments in MTC. Doxorubicin alone or in combination with cisplatin appears to be the drug of choice for this type of tumor. Although these studies showed that chemotherapy is able to reduce tumor mass, no improvement in survival rate was observed. To explain the low efficacy of chemotherapeutic agents, a potential role of multidrug transporters was suggested. Particularly, resistance to both doxorubicin and cisplatin has been associated with MRP2 expression.

The present study was conducted to evaluate the role of MRP2 expression as a potential candidate to select lesions more likely to be treated with chemotherapeutic agents.

We demonstrated that MRP2 is constitutively expressed in MTC samples, before any drug treatment, at higher pTNM stages (T4), whereas no expression was observed in lower pTNM stages (T2 and T3). This could explain the scarce response rate of MTC patients to chemotherapeutic strategies, based on doxorubicin and cisplatin, in advanced and metastatic tumors. MRP2 expression may be considered a useful predictor of chemosensitivity in clinical practice to plan chemotherapy with doxorubicin and cisplatin. In addition, our results suggest that resistance to chemotherapy may be circumvented by modulating MRP2 expression, focusing the attention of researchers on MRP2 as a novel therapeutic target.

The clinical course of MTC patients depends on many clinical (age, stage, gender, hereditary type of MTC), biochemical (serum preoperative and postoperative calcitonin), pathologic (tumor size, lymph node and distant metastases, extrathyroidal invasion, and multifocality), and molecular (specific RET mutations) prognostic factors. The list of MTC prognostic factors is progressively increasing and efforts have been made to assign each single factor to a specific MTC stage. Recently, the staging criteria for MTC have been revised. According to these criteria, tumors previously classified as pT4 have been further subdivided into 2 additional subgroups, pT4a and pT4b. As a consequence, clinical stages have been modified accordingly. Therefore, MTCs, previously classified as clinical stage III or IV, now are included into the new stages of IVA, IVB, or IVC. No changes have been made with respect to the age at diagnosis. The new criteria continue to not consider age younger than 45 years as a major good prognostic factor. In routine practice, clinicians attempting to predict MTC outcome should take into account the disease stage at presentation, as assessed by the pTNM system and clinical stage.

Our results indicate that a good correlation is observed for both MRP2 and COX2 expression with both the pathologic and clinical staging criteria. The correlation between the expression pattern of these 2 proteins and the pathologic and clinical characteristic of the tumor suggests that MTCs expressing MRP2 are potentially resistant to chemotherapy, but more cases are needed to confirm these results.

We observed that MRP2 expression increased significantly in advanced stages of disease and that higher expression levels were observed in T4 stages. Normal C cells, on the contrary, do not express MRP2, suggesting that MTC may develop resistance to doxorubicin and cisplatin by increasing MRP2 expression. Thus, MRP2 could have a role in MTC progression and metastatic migration of malignant C cells. The observation concerning a higher MRP2 expression in MTC cells from lymph node metastases, compared with MTC cells from matching primary thyroid tumors, reinforces this hypothesis. We believe that these results may be useful in clinical and pathologic practice. MRP2 could play a role as unfavorable prognostic factor of MTC and, because it can distinguish hyperplastic from malignant C cells, it may be used as a diagnostic marker too.

Our study indicates that a negative correlation exists in MTC between COX2 and MRP2 expression, and this pattern is quite different from that observed with MRPs. COX2 expression is associated with MRPs upregulation, whereas our data, obtained in 12 MTCs, indicate that COX2 is associated with MRP2 downregulation. The COX2 expression that we observed in all our specimens is in agreement with previous reports from other authors. However, in such studies no correlation with pTNM or clinical stage was performed. We studied COX2 expression in the various stages of MTC and in lymph node metastases thereof, using the recently revised criteria for MTC staging. We observed a decreasing COX2 immunoreaction along with increasing pTNM and clinical stages. According to these data, COX2 immunoexpression may also be considered as a possible favorable prognostic marker, specific for early stages of MTC progression. Moreover, these results let us suppose that COX2 may potentially act through MRP2 to limit malignant C cells growth, but further experiments are needed to confirm this hypothesis.

In conclusion, the evaluation of MRP2 and COX2 expression in MTC specimens may improve diagnostic evaluation and contribute to a better characterization and treatment of this type of tumor. A low MRP2 expression, together with a high COX2 expression, is associated with lower pTNM and clinical stage and indicates that the lesion would be less resistant to doxorubicin and cisplatin treatment. On the contrary, in more advanced stages of MTC, the high MRP2 expression and the low COX2 expression may predict resistance to drug treatment and, therefore, chemotherapy would not be indicated in these patients. Because of the relatively small number of cases examined, additional studies with larger numbers of cases are required to confirm our preliminary results.

References


