Commentary

Mixed Medullary and Follicular Carcinoma of the Thyroid

On the Search for Its Histogenesis

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Medullary Thyroid Carcinoma, the Great Simulator

The recognition of the pathological features of medullary thyroid carcinoma (MTC) by Horn¹ and Hazzard et al² in the 1950s and the demonstration that it derived from the calcitonin-producing parafollicular cells^{3,4} allowed the distinction of such a tumor type from the more common follicular cell tumors.

Five typical pathological features are usually present in MTCs: 1) amyloid-rich stroma; 2) trabecular and nesting arrangement; 3) scarce atypia and nuclear uniformity; 4) neurosecretory granules; and 5) calcitonin and CEA immunostaining. However, MTC has a great ability to show unusual features that may mimic other tumors⁵: acini; tubules (follicles)⁶; papillae⁷; small,⁸ giant,⁹ clear,¹⁰ or oxyphilic^{11,12} cells; squamous differentiation¹²; and mucin secretion.¹³ MTC may also resemble vascular tumors.¹⁴

The occurrence of pseudoacinar, follicular, or papillary growth patterns in MTC was particularly controversial. Although in some cases they have an artifactual origin,⁵ it is now well accepted that MTC, like carcinoids and many other neuroendocrine carcinomas, may display glandular features. In fact, elegant electron-microscopic studies showed the presence of microvilli on the surface of MTC cells lining glands or papillae.^{15,16} These structures should not be considered of follicular origin unless thyroglobulin expression is convincingly demonstrated.

Mixed Medullary and Follicular Carcinoma, a Controversial Entity

In the early 1980s, several authors started describing tumors that combined features of MTC and follicular cell

carcinomas. Since then, individual cases and short series of tumors have appeared in the literature.^{17–24} During this period, it has become clear that mixed medullary and follicular carcinoma is a rather controversial neoplasm. Some authors have voiced reservations about its consideration as a real entity,²⁵ its histogenesis, and its diagnostic criteria.²⁶

In 1988, in the second edition of the WHO booklet *Histological Typing of Thyroid Tumours*, mixed medullary-follicular carcinomas were defined as tumors showing the morphological features of both a MTC with immunoreactivity for calcitonin and a follicular carcinoma with immunoreactivity for thyroglobulin.²⁷ By following these strict criteria, true mixed medullary and follicular carcinomas are rare. They should be distinguished from MTCs with follicles⁶ or papillae⁷ as well as from MTCs with entrapped normal follicles at their infiltrating edges. They should also be separated from follicular cell tumors with trabecular, solid, or insular patterns, such as hyalinizing trabecular adenomas or carcinomas,^{28–30} and poorly differentiated follicular carcinomas³¹; they may resemble MTC and may even contain a minor population of neuroendocrine cells.

For many years, immunohistochemistry was almost the exclusive tool for the study of mixed medullary and follicular carcinomas. As mentioned above, staining for both calcitonin and thyroglobulin was required to establish the diagnosis. However, this finding was considered to be necessary but not exclusive for this type of tumor. Occasional immunostaining for thyroglobulin in otherwise typical MTCs was initially explained by osmosis, passive absorption, or transfer of thyroglobulin from entrapped normal follicular cells to neoplastic cells.³² A similar phenomenon is frequently seen in thyroid metastases of carcinomas from other organs.³³

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Soon immunostaining for both thyroglobulin and calcitonin was demonstrated in lymph node metastases of some MTCs.³⁴ Still, in these cases, draining of thyroglobulin from damaged follicular thyroid tissue infiltrated by the tumor into regional lymph nodes was an alternative explanation.³⁵ The later demonstration of colocalization of both hormones in distant metastases made osmosis or passive absorption very unlikely. It is worth mentioning that several authors recommended caution in the interpretation of thyroglobulin immunostaining, because of the lack of absolute specificity of some polyclonal and monoclonal antibodies that could recognize epitopes on several other molecules, such as mucins.³⁶

In 1987, Holm et al reported a series of classical MTCs that showed thyroglobulin immunoreactivity.³⁷ These authors claimed that thyroglobulin-positive MTC was an unusual variant of MTC, which carried a better prognosis than its thyroglobulin-negative counterpart. The existence of thyroglobulin immunoreactive MTC explained the reports of incorporation of radioactive iodine in rare cases of MTC. In fact, MTC is a tumor known to be capable of producing a great variety of hormonal and nonhormonal substances, such as histaminase, somatostatin,³⁸ catecholamines, or ACTH. The existence of these tumors raised three important questions: Why could a typical MTC not produce thyroglobulin ectopically? Should an otherwise typical, thyroglobulin immunoreactive MTC be considered as a true mixed medullary and follicular carcinoma? Should the patients be treated with ¹³¹I?

It soon became obvious that in daily practice, there were tumors that combined some features of medullary and follicular cell carcinomas, without fulfilling the strict criteria of the WHO. The spectrum of these tumors included MTC with thyroglobulin immunoreactive cells at one extreme³⁹ and carcinomas of follicular cell origin with neuroendocrine cells at the other. In fact, it was shown that coexpression of follicular and parafollicular markers was particularly common in hyalinizing trabecular tumors as well as in papillary, mucoepidermoid, mucinous, and poorly differentiated carcinomas.^{19,39,40} Furthermore, even tumors fulfilling the WHO criteria showed a great variation in microscopic appearance, particularly in the follicular component, which was reported to exhibit areas of oxyphilic,²³ poorly differentiated,²¹ and even anaplastic carcinoma.⁴¹

The pathological scenario of mixed medullary and follicular thyroid carcinoma was further complicated by the description of cases of MTC that contained intimately admixed populations of cells with the features of papillary carcinoma as well as thyroglobulin immunoreactivity.⁴²⁻⁴⁴ The areas of MTC, which predominated in the vast majority of the reported cases, were thyroglobulin negative but immunoreacted for calcitonin and CEA. Admixtures of both components were also identified in lymph node metastases.

Moreover, distinction between a true mixed medullary and follicular carcinoma and a tumor resulting from the collision of two different medullary and follicular cell carcinomas was not always easy. In some cases, the distribution of the two neoplastic components in the thyroid

gland allowed their classification as obvious collision tumors.⁴⁵ In other cases, the extension of the neoplastic growth of the two components complicated the pathological interpretation. In fact, examples of concurrent (independent), medullary and papillary or follicular carcinomas in the same thyroid gland or even localized in the same thyroid lobe have been reported.⁴⁶⁻⁴⁸

After a careful review of the literature available, I have formed the impression that the term "mixed medullary and follicular thyroid carcinoma" has been used to designate a heterogeneous group of neoplasms. I believe that some of the reported cases represent MTCs with follicles, MTCs with thyroglobulin expression, collision tumors, and even poorly differentiated carcinomas containing neuroendocrine cells. Obviously, many other reported tumors are convincing examples of true mixed medullary and follicular carcinomas, but exhibiting a great variability in the morphological appearance of the follicular component. In this regard, I think that the hypothesis proposed by Volante et al in this issue of *The American Journal of Pathology*⁴⁹ agrees with such a point of view. The "hostage hypothesis" would explain perfectly the histological variability of the follicular cell component of true mixed medullary and follicular thyroid carcinomas; MTC would contain a hyperplastic (polyclonal) follicular proliferation in some cases, but a fully developed neoplastic (monoclonal) component in others. The neoplastic proliferation would be able to acquire either a follicular or a papillary phenotype in different cases.

Molecular Pathology Techniques and Assessment of Cell Clonality

Once it was clear that immunohistochemistry was not going to answer all of the questions raised by the existence of mixed medullary and follicular carcinomas, several authors began to apply molecular pathology techniques. Noel et al first demonstrated by Northern blot and *in situ* hybridization the presence of calcitonin and thyroglobulin mRNAs in tumor cells of two cases.⁵⁰ Papotti et al studied 11 cases by combined immunohistochemistry and *in situ* hybridization.²³ They detected separated thyroglobulin and calcitonin gene expression in the vast majority of the tumors, although concurrent expression of the two genes was seen occasionally in cells of two neoplasms. Although these molecular studies clearly rendered interesting results, they did not provide conclusive evidence of the histogenesis of this tumor type.

Several methods can be used to assess the independent or common origin of two different components of a neoplasm. They have been applied to a great variety of tumors showing divergent differentiation (carcinosarcomas of different organs, malignant mixed müllerian tumors),^{51,52} as well as to establish the independent or metastatic origin of simultaneously occurring tumors (synchronous mucinous tumors of the appendix and the ovaries, simultaneous endometrioid adenocarcinomas of the uterus and the ovaries).⁵³⁻⁵⁵ They include loss of heterozygosity (LOH), gene mutation, and clonal X-inactivation analyses. The most reliable of them are those

addressing the molecular alterations that occur in the early stages of tumor development.

Although LOH may indicate inactivation of tumor suppressor genes involved in the early steps of tumorigenesis, there is evidence suggesting that LOH may also reflect the existence of the genetic instability that occurs at more advanced steps.⁵⁶ Several studies have shown different patterns of LOH at different areas of the same tumor as a result of tumor heterogeneity.^{57,58} Although these data suggest that LOH analysis is not the best way to assess monoclonality in neoplasias, it can provide interesting information. In other words, different LOH patterns do not necessarily indicate a different origin for two tumor components; but the concordance in LOH pattern in two different cell populations is highly suggestive of a common clonal origin.^{59,60}

Mutation analysis of genes involved in early steps of tumorigenesis is a good method for assessing the common origin of two different tumor components. In fact, a few years ago we used such an approach to study a series of synchronous mucinous tumors of the appendix and the ovaries.⁵³ By using a very sensitive RFLP-polymerase chain reaction method, we found a concordant k-RAS mutation pattern in both the appendiceal and the ovarian tumors in each case, suggesting that they had a common clonal origin, giving support to the currently well-accepted idea that the ovarian neoplasms were metastases from the primary appendiceal tumors. Similar approaches have been used in different tumor settings.⁶⁰ The only objection against this method is that a common carcinogenic agent theoretically could be capable of inducing the same genetic alteration in two different tumors developed as a result of a field effect. However, comparative analysis of simultaneous colonic adenomas and carcinomas (which are supposed to be the result of common carcinogenic agents) has shown different k-RAS mutation patterns in these synchronous lesions.⁶¹

Although Volante et al⁴⁹ could have chosen k-RAS for the assessment of clonality in mixed medullary and follicular carcinomas (k-RAS mutations are early events in follicular cell tumorigenesis,⁶² while very infrequent in MTC), they decided to study RET and gsp gene mutations. The RET proto-oncogene (rearranged during transfection) is located on chromosome 10q11.2, contains 21 exons spread over a genomic region of approximately 60 kb, and encodes a cell-surface receptor with tyrosine kinase activity.⁶³ RET mRNA is expressed in some tissues and tumors presumably developed from migratory cells of the embryonal neural crest, such as parathyroid cells, thyroid C-cells, and adrenal medullary cells.⁶⁴ Germline missense point mutations of RET are responsible for familial MTC and MEN type II.^{65–67} However, somatic point mutations in exons 15 and 16, and less frequently in exons 11 and 13, are common genetic alterations of sporadic MTCs.^{68–70} Interestingly, RET proto-oncogene abnormalities also occur in papillary thyroid carcinomas, but in the form of somatic rearrangements and not of point mutations.⁷¹ The detection of any somatic RET point mutation in the follicular component of a mixed medullary and follicular thyroid carcinoma would necessarily indicate that such follicular elements had

resulted from differentiation of a preexisting MTC component. However, the presence of a somatic RET point mutation in the MTC component and its absence in the follicular carcinoma areas would be indicative of two separated origins. Again, some caution has to be exercised in interpreting RET somatic mutations in MTC, since they can occur in specific subclones as a result of tumor progression.⁷²

X-linked clonality assays can be used in informative females for determination of clonal derivation of cell populations.⁷³ During the process of embryogenesis, either the maternally derived or the paternally derived X chromosome in each cell is randomly and permanently inactivated. The choice, once made, is stable through subsequent cell cycles. X chromosome inactivation is demonstrated by differential methylation patterns between the active and inactive alleles. The distinction is based on polymorphisms that exist in the general population at different loci on chromosome X. Several assays have been used: glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate kinase (PGK), hypoxanthine phosphoribosyl transferase (HPRT),⁷⁴ the hypervariable M27 β locus (DXS255),⁷⁵ and the human androgen receptor gene (HUMARA).⁷⁶ The HUMARA assay (the one used by Volante et al) takes advantage of a highly polymorphic (>90%) trinucleotide that is in the coding region of the first exon of the gene and is closely linked to four methylation sites. The HUMARA assay can be applied to archival paraffin-embedded tissues; X-chromosome inactivation patterns are determined by polymerase chain reaction amplification after predigestion with one of the methylation-sensitive restriction endonucleases *HpaII* and *HhaI*.⁷⁶ In polyclonal cell populations, both X alleles are amplified and visualized as two bands on denaturing gels, whereas only a single band is obtained from monoclonal cell populations. Demonstration of different patterns of X-chromosome inactivation in two components of the same tumor is a clear indication that each of them has a different clonal origin. However, when cells from two tumor components show identical patterns of X-chromosome inactivation, there remains a 50% probability that they could be of different clonal cell origins.

Application of X-linked clonality analyses to thyroid lesions has yielded interesting results. Several studies have shown that monoclonality is not exclusive of follicular cell tumors,^{77–80} because a high proportion of follicular nodules of multinodular goiters exhibit a monoclonal pattern. In fact, polyclonal and monoclonal nodules may coexist in a goiter; the monoclonal ones probably develop from primarily polyclonal nodules.⁸⁰ Contrariwise, a polyclonal pattern of X-inactivation was surprisingly obtained in some examples of MTC.⁸¹

X-linked clonality analyses have two main limitations. First, despite their high sensitivity, these polymerase chain reaction-based clonality assays might not be capable of detecting a minor monoclonal cell population in a large background of normal⁷⁶ polyclonal cells. The second limitation refers to the problem of Lyonization.⁷⁴ According to Lyon's hypothesis, X chromosomes are inactivated on a random basis during early embryogenesis. Given the small number of cells at that time, it is

unreasonable to expect that every polyclonal somatic tissue in every adult will contain equal numbers of inactivated paternally and maternally derived X chromosomes. The balance of maternal and paternal X chromosome inactivation follows a binomial distribution. Although the phenomenon of skewed lyonization is estimated to be infrequent, it can mimic clonal derivation of cells in a nonneoplastic polyclonal cell population.

Histogenetic Hypotheses

Since their description, several histogenetic hypotheses have been offered to explain the existence of the full spectrum of tumors that combine the features of medullary and follicular carcinomas:

1. Recapitulation or origin from uncommitted stem cells capable of differentiating toward both follicular and C-cell lineage.
2. Development from C-cells and thyroid follicles derived from remnants of the ultimobranchial body and solid cell nests.^{82,83}
3. Differentiation of MTC cells toward a follicular phenotype by the acquisition of additional molecular alterations.
4. Ectopic production of thyroglobulin in classical MTCs.
5. Simultaneous transformation of both follicular and C-cells, either as collision tumors, or as a result of a field effect.

None of these hypotheses have successfully answered all of the different questions raised by embryologists, pathologists, or molecular biologists. Now the article of Volante et al⁴⁹ provides a provocative sixth hypothesis, the so-called hostage hypothesis. The collection of cases from three well-known institutions with great experience in endocrine pathology and the application of diverse molecular techniques (RET and gsp mutations, LOH on six chromosomes, and clonal X-inactivation analysis with the HUMARA assay) have allowed the authors to gather a sufficient number of cases of this rare entity to obtain significant results. In brief, the "hostage hypothesis" proposes that mixed medullary and follicular carcinomas derive from two different cells. First, the neoplastic transformation of parafollicular C cells would lead to the development of a MTC with entrapment of normal follicles at its periphery. The microenvironment provided by MTC cells would subsequently stimulate the proliferation of the trapped follicular cells, resulting in hyperplastic and adenomatous follicles, which ultimately would acquire a fully developed neoplastic phenotype (either follicular or papillary), capable of producing lymph node or distant metastases. The definitive confirmation of this "hostage hypothesis" will require the identification of the unknown trophic factors that would be necessary for the stimulation of the follicular cells, as well as the detection of these substances in the MTC cells of the mixed medullary and follicular carcinomas and their absence in classical MTCs.

In my opinion, there is a rationale for such a new hypothesis. For many years follicular and parafollicular C-cells were regarded as two separate cell types, with

little biochemical relationship between them. However, in recent years, it has been shown that there are some interconnections between them.⁸⁴ After Hubert Wolfe's description of C-cell hyperplasia in the vicinity of familial MTC and its recognition as a precursor lesion,⁸⁵ C-cell hyperplasia was also found to be associated with several other conditions. In fact, C-cell hyperplasia was recognized in some patients with Hashimoto thyroiditis⁸⁶ as well as in thyroid tissue adjacent to follicular and papillary neoplasms.⁸⁷ If we accept that follicular cell lesions may be associated with C-cell hyperplasia, why could we not accept the possibility that MTC provides the appropriate microenvironment to stimulate follicular cell proliferation?

The relationship between tumor cells and their surrounding tissues is far from being well understood in some endocrine neoplasias. It might well be possible that some MTCs secrete endocrine/paracrine growth factors that could induce proliferation or differentiation of non-neoplastic associated cells. The demonstration that sustentacular cells are more frequently present in familial than in sporadic MTCs⁸⁸ gives support to this hypothesis. These phenomena may be similar to the proliferation of Schwann cells in maturing neuroblastomas, which probably respond to the production of growth factors by tumor cells.⁸⁹

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