

# Mixed Medullary-Follicular Thyroid Carcinoma

## *Molecular Evidence for a Dual Origin of Tumor Components*

Marco Volante,\* Mauro Papotti,\* Jürgen Roth,†  
Parvin Saremaslani,‡ Ernst J. M. Speel,‡  
Ricardo V. Lloyd,§ J. Aidan Carney,§  
Philipp U. Heitz,‡ Gianni Bussolati,\* and  
Paul Komminoth†‡

*From the Department of Biomedical Sciences and Oncology,\*  
University of Turin, Turin, Italy; the Division of Cell and  
Molecular Pathology† and the Department of Pathology,‡  
University of Zürich, Zürich, Switzerland; and the Department of  
Pathology and Laboratory Medicine,§ Mayo Clinic and  
Foundation, Rochester, Minnesota*

**Mixed medullary-follicular carcinomas (MMFCs) are tumors of the thyroid that display morphological and immunohistochemical features of both medullary and follicular neoplasms. The histogenetic origin and possible molecular mechanisms leading to MMFCs are still unclear. To address these questions, we have isolated the two histological components of 12 MMFCs by (laser-based) microdissection, analyzed them for mutations in the *RET* proto-oncogene and allelic losses of nine loci on six chromosomes, and studied the clonal composition of MMFCs in female patients. Our results provide strong evidence that the follicular and medullary components in MMFCs are not derived from a single progenitor cell, because the seven tumors amenable for analysis consistently exhibited a different pattern of mutations, allelic losses, and clonal composition. We also demonstrate that follicular structures in MMFCs are often oligo/polyclonal and more frequently exhibit hyperplastic than neoplastic histological features, indicating that at least a subset of MMFCs are composed of a medullary thyroid carcinoma containing hyperplastic follicles. (*Am J Pathol* 1999, 155:1499–1509)**

Medullary thyroid carcinomas (MTCs) comprise 5–10% of all thyroid carcinomas. Although a majority of these tumors occur sporadically, about 20% have a familial background.<sup>1</sup> MTCs are assumed to evolve from the neural crest or ultimobranchial body-derived C-cells<sup>2,3</sup> and are regarded as being closely related to tumors of the disseminated neuroendocrine system. In the late 1970s it was noted that the histological appearance of MTC may

be “atypical” and that follicular structures can be encountered in these tumors in addition to typical medullary features.<sup>4</sup> Subsequently it was shown that in addition to the characteristic calcitonin immunoreactivity in such atypical MTCs, thyroglobulin (Tg) was detectable in the foci having a follicular appearance, and that the same histological and immunohistochemical pattern was also present in their metastatic lesions. Hence it was proposed that these tumors might represent a new entity, which was termed “mixed medullary-follicular carcinoma” (MMFC).<sup>5,6</sup> In the second edition of *Histological Typing of Thyroid Tumors*,<sup>7</sup> Hedinger and associates defined MMFC as “tumors which show the morphological features of both a medullary carcinoma with immunoreactivity for calcitonin and a follicular carcinoma with immunoreactivity for thyroglobulin,” both in the primary tumor and metastatic lesions. Since these early reports, several authors have published results for mixed tumors fulfilling these WHO criteria. More recently there have also been reports on mixed tumors with papillary patterns<sup>8–11</sup> and MMFCs with a familial setting.<sup>12–14</sup> However, MMFCs exhibiting a dual endocrine and neuroendocrine differentiation appear to be rather rare, with little more than 30 well-documented tumors on record to date<sup>5,6,8–13,15–22</sup>. The existence of the entity of MMFC was further consolidated by demonstrating 1) Tg mRNA expression in follicular components<sup>11,13</sup> and 2) mixed patterns of follicles and MTCs in organ metastases.<sup>16</sup> Therefore, these data excluded absorption of Tg or cross-reaction of Tg antibodies to high-molecular-weight 27S Tg (so called C-Tg) in C-cells as causes for Tg positivity in MMFCs and the presence of nonneoplastic follicles, which rarely occur in regional lymph nodes,<sup>23</sup> as a cause for mixed patterns in lymph node metastases of MMFCs.

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Dedicated to the memory of the late Prof. Ch. Hedinger (December 5, 1917–January 12, 1999), former head of the Institute of Pathology at the University of Zürich, Zürich, Switzerland.

Supported in part by grants from the Italian Ministry of University and Research (M.V., M.P.).

Accepted for publication July 12, 1999.

Address reprint requests to Dr. Paul Komminoth, Division of Cell and Molecular Pathology, Department of Pathology, Schmelzbergstrasse 12, University of Zürich, CH-8091 Zürich, Switzerland. E-mail: paul.komminoth@pty.usz.ch.

**Table 1.** Clinicopathological Data

No.	Sex/age	Location/size (cm)	Pathology	Metastasis/recurrences	Follow-up, years
T1	M/68	L/4	MTC with Tg-positive follicles (5%)* + solid follicular carcinoma (40%)	Lymph nodes (mixed) (10%)	NED 1
T2	F/53	L/5	Papillary carcinoma and MTC	Lymph node (mixed) (5%)	DOD 8
T3	M/55	n.n.	MTC with Tg-positive follicles (15%) + focus of follicular carcinoma	None	NED 5
T4	M/74	R/2	MTC with Tg-positive follicles (25%)	None	DOD 1
T5	M/68	Isthmus/3	MTC with Tg-positive follicles (15%)	Lymph nodes (mixed) (10%) Local recurr. (2y)/bone (3y)	DOD 8
T6	M/68	n.n.	Oxyphilic carcinoma (80%) + MTC	Lung	DOD 1
M7	F/26	L/2.5	MTC with Tg-positive follicles (10%)	None	NED 0.4
M8	F/49	R/1 + L/0.2 MEN2A	MTC with Tg-positive follicles (15%)	None	NED 3.2
M9	F/42	L/5.5	MTC with Tg-positive follicles (15%)	Mediastinum	n.n.
M10	M/58	L/n.n.	MTC with Tg-positive follicles (25%)	None	NED 1
M11	M/69	R/1.7 + L/1.1 MEN2A	MTC with Tg-positive follicles (10%)	Lymph nodes (mixed) (5%)	AWD 3
Z12	M/35	R/5	MTC with Tg-positive follicles (15%)	Lymph nodes (mixed) (5%) Local recurr. (1y)/liver (2y)	n.n.

L, left lobe; R, right lobe; n.n., not known; MTC, medullary thyroid carcinoma; Tg, thyroglobulin; NED, no evidence of disease; AWD, alive with disease; DOD, died of disease; y, years.

\*Approximate percentage of the follicular component.

Because of the variety of patterns and admixed components in MMFCs, several terms have been proposed to designate these neoplasms, including "mixed follicular-parafollicular carcinoma,"<sup>20</sup> "compound medullary-papillary carcinoma,"<sup>8</sup> "composite carcinomas of the thyroid,"<sup>24</sup> "differentiated thyroid carcinoma, intermediate type,"<sup>25</sup> and "stem cell carcinoma."<sup>9</sup> The last term was proposed because the majority of authors believe that MMFCs are derived from neoplastically transformed uncommitted stem cells with the capacity to differentiate into tumor components with morphological and histochemical characteristics of both follicular and medullary neoplasms of the thyroid.<sup>9,10,12,13,17,18,20-22</sup> This hypothesis was further supported by reports demonstrating Tg and CT coexpression in tumor cells of MMFCs<sup>13,26</sup> and the development of occasional mixed tumors with Tg-positive cells in a transgenic murine model for MTC.<sup>27</sup> However, the stem cell theory has never been proved by direct evidence. Thus the embryological background and possible molecular mechanisms leading to MMFC are still unclear.

The aim of the present study was to determine whether the two tumor components in MMFC are derived from the same cell clone or whether they arise independently. For this purpose, the two tumor components of each MMFC were separated by (laser-based) microdissection and individually analyzed for allelic losses at nine different chromosomal loci, somatic mutations of the *RET* and *gsα* gene, as well as clonal composition in female patients.<sup>28,29</sup>

## Materials and Methods

### Materials

A total of 12 MMFCs of the thyroid were collected from the files of the Mayo Clinic (Rochester, MN) (five cases), the Department of Pathology, University of Turin (Turin, Italy) (six cases), and the Department of Pathology, University

of Zürich (Zürich, Switzerland) (one case). Tumor specimens had been fixed in 10% buffered formaldehyde solution and embedded in paraffin according to standard procedures.

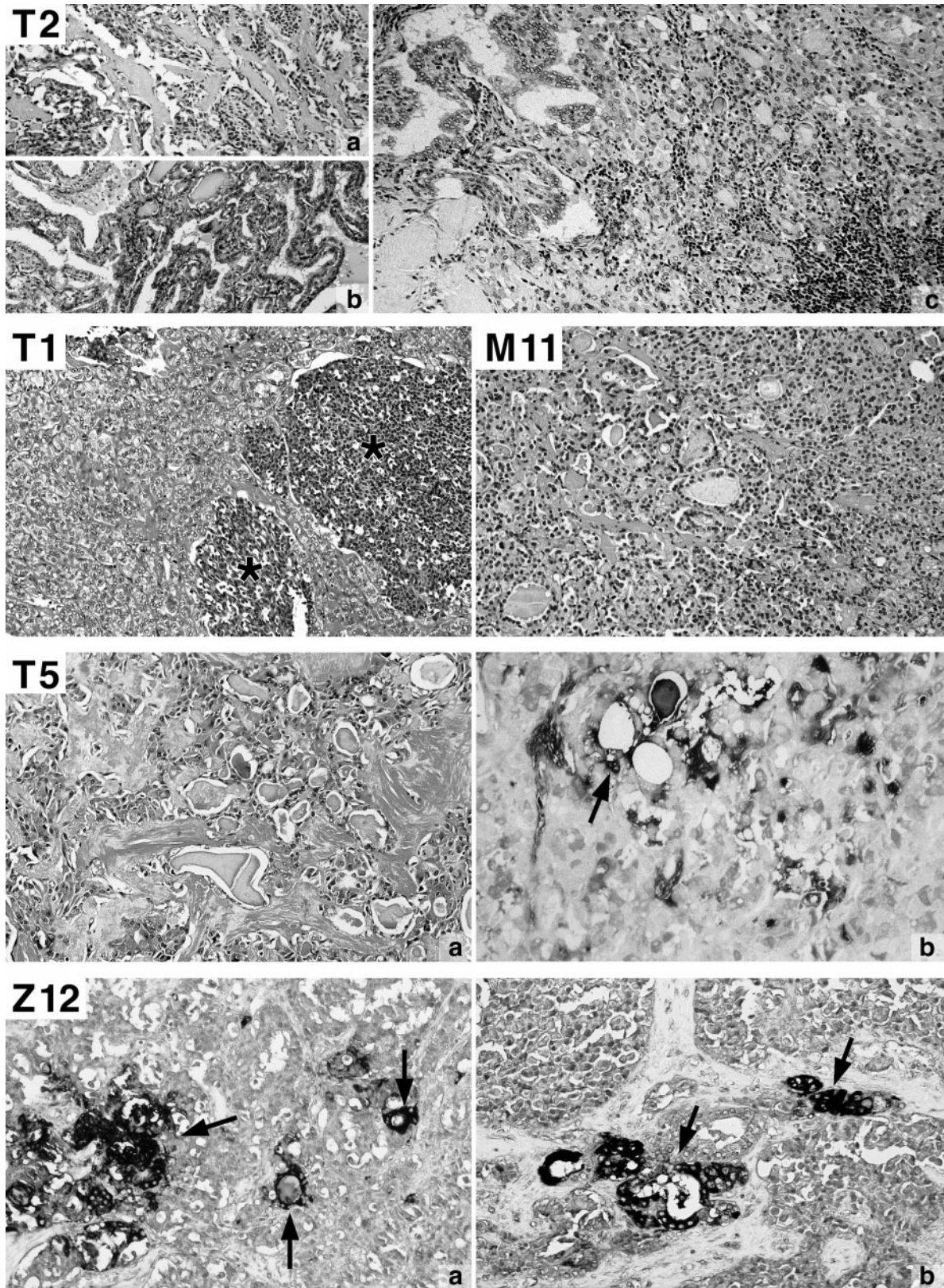
All tumors had previously been studied by conventional histology, immunohistochemistry, or *in situ* hybridization.<sup>5,11</sup> Ten tumors met all histopathological criteria for the diagnosis of MMFC as defined by the WHO classification.<sup>7</sup> One tumor (file number T5; Table 1, Figure 1), which presented as a primary tumor with components of a MTC and follicular variant of a papillary carcinoma, exhibited papillary features in the lymph node metastases together with a medullary component. Another tumor (file number T2) was included in the study because of the mixed medullary-papillary features encountered in the lymph node metastases despite the fact that two separate primary papillary and medullary carcinomas were identified simultaneously in the same thyroid lobe (Figure 1).

Clinical data were collected and follow-up information was obtained for all but two patients (Table 1).

### Immunohistochemistry

Freshly cut sections from archival paraffin blocks were stained with hematoxylin and eosin (H&E) and used for light microscopic examination. Serial sections (4 μm) of all 12 tumors were cut and mounted on Superfrost Plus glass slides (Menzel Gläser, Germany). Immunoreactive sites for calcitonin (CT) (polyclonal, 1:200; Dako, Glostrup, Denmark) and thyroglobulin (Tg) (monoclonal, 1:50; Dako) were detected using the avidin-biotin-complex (ABC) systems.<sup>30</sup>

Double immunostaining of CT and Tg was performed using the APAAP system<sup>31</sup> and naphthol phosphate/fast red TR (Dako) as a chromogen for CT, and subsequently the ABC system and diaminobenzidine (Walter, Kiel, Germany) for Tg immunohistochemistry.



**Figure 1.** Histological appearance of some studied mixed medullary-follicular thyroid carcinomas. **T2:** Two separate primary tumors (**a**: medullary thyroid carcinoma; **b**: papillary carcinoma) and collision tumor in the lymph node metastasis (**c**). **T1:** Mixed tumor with a solid follicular tumor component (\*). **M11:** Mixed tumor with approximately 10% thyroglobulin-positive follicles. **T5:** Mixed tumor with thyroglobulin-positive (**arrow**; **b**) follicles and amyloid stroma in the medullary part (**a**). **Z12:** Thyroglobulin-positive follicles (**arrows**) in a mixed tumor (**a**: primary; **b**: lymph node metastases). **T2, T1, M11, T5a:** Hematoxylin and eosin (H&E)  $\times 200$ . **T5b, Z12:** Double immunostaining with black reaction product for thyroglobulin and red for calcitonin,  $\times 200$ .

**Table 2.** PCR Primers

Primer	Orientation	Sequence	PCR product (bp)	Ann. T <sup>†</sup>
RET primers (exons 10, 11, 15, 16)				
10	Sense	5'-GCGCCCCAGGAGGCTGAGTG-3'		
	Antisense	5'-CGTGGTGGTCCCGGCCG-3'	182	63°C
10N*	Sense	5'-CTCAGGGGGCAGCATTGTT-3'		
	Antisense	5'-CACTCACCTGGATGCTT-3'	132	56°C
11	Sense	5'-CCTCTGCGGTGCCAAGCCTC-3'		
	Antisense	5'-TGTGGGCAAACCTTGTGGTAGCA-3'	155	58°C
15	Sense	5'-GACTGCTGCTATTTTCCTC-3'		
	Antisense	5'-ATGGTGCACCTGGGATCCCT-3'	199	53°C
15N*	Sense	5'-TGCTATTTTCCTCAC-3'		
	Antisense	5'-CCGGGACTGGGCAC-3'	155	48°C
16	Sense	5'-AGGGATAGGGCCTGGGCTT-3'		
	Antisense	5'-TAACCTCCACCCCAAGAG-3'	192	56°C
16N*	Sense	5'-AGAGTTAGAGTAACTTCAATGTC-3'		
	Antisense	5'-TAACCTCCACCCCAAGAGA-3'	151	45°C
Gs $\alpha$ gene primers (exons 8 and 9)				
GsE8	Sense	5'-TTACTGTTTCGGTTGGCT-3'		
	Antisense	5'-AGAGGGACTGGGGTGAA-3'	192	52°C
GsE8N*	Sense	5'-CGGTTGGCTTTGGTGAGA-3'		
	Antisense	5'-CAGAAACCATGATCTCTGT-3'	163	52°C
GsE9	Sense	5'-TTTCTTGACATTCACCCAG-3'		
	Antisense	5'-AGCGACCCTGATCCCTAA-3'	186	56°C
GsE9N*	Sense	5'-CACCCAGTCCCTCTGGAA-3'		
	Antisense	5'-CGTTCATTACGAACAGCCA-3'	140	62°C
Androgen receptor primers (exon 1)				
AR	Sense	5'-GAGGAGCTTCCAGAATCTG-3'		
	Antisense	5'-GATGGGCTTGGGGAGA-3'	233	59°C
ARN*	Sense	5'-TCCAGAACTGTTCAGAGC-3'		
	Antisense	5'-TGGGGAGAACCATCTCACC-3'	216	59°C

\*Nested primers.  
<sup>†</sup>Specific annealing temperature.

## Molecular Analyses

### DNA Extraction

Tumor DNA was isolated from five 10- $\mu$ m paraffin sections of each tumor as described.<sup>29</sup> In 11 of the 12 cases, nontumorous tissue of the same patient was also available, and DNA was extracted as well.

Selected cases were submitted to laser-based microdissection (PALM Laser-Microbeam Systems GmbH, Germany)<sup>32,33</sup> to obtain isolated single neoplastic follicles and single nests of medullary carcinoma. The dewaxed and hematoxylin-stained tissue was placed in 0.5-ml Eppendorf tubes (Eppendorf GmbH, Hamburg, Germany) and boiled for 10 minutes at 94°C in 18  $\mu$ l 1 $\times$  polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl; GeneAmp, Perkin Elmer, Roche, NJ). Then 0.5  $\mu$ g of proteinase K diluted in 1  $\mu$ l of double-distilled water (ddH<sub>2</sub>O) was added to each sample and incubated at 55°C for 24 hours. Then samples were incubated for 10 minutes at 94°C to inactivate the proteinase K. Next 7.5  $\mu$ l of the solution was added to the appropriate PCR mixture for further PCR amplification, under the conditions described below.

For clonality analysis,<sup>29</sup> microdissected tissue samples were placed in 0.5-ml Eppendorf tubes and boiled for 10 minutes at 94°C in 18  $\mu$ l 1 $\times$  restriction enzyme Buffer L (Boehringer Mannheim GmbH, Mannheim, Germany). After proteinase K treatment, as described above,

10 U of *Hpa*II (in 1  $\mu$ l of ddH<sub>2</sub>O) was added, and the samples were incubated at 37°C overnight. Then inactivation of the enzyme was performed at 94°C for 10 minutes, and 7.5  $\mu$ l of the final solution was used as a template for further PCR amplification.

### Nonisotopic PCR-SSCP and HDE RET Mutation Analysis

All 12 cases were screened for somatic or germline mutations of the *RET* proto-oncogene in exons 10, 11, 15, and 16 by the PCR-based single-strand conformation polymorphism (SSCP) and heteroduplex gel electrophoresis (HDE) analysis as recently described,<sup>34</sup> using primers and PCR conditions as detailed in Table 2. Two tumors (file numbers M8 and M11) were from known MEN 2A patients.

Blood-derived DNA of healthy persons and from patients carrying *RET* point mutations were used as negative and positive controls, respectively.

### Restriction Analysis

Twenty microliters of PCR products from *RET* exons 15 and 16 was digested overnight at 37°C in 50  $\mu$ l of medium and low-salt buffer containing 10 U of the restriction enzyme *Alu*I (exon 15) and *Fok*I (exon 16), respectively

**Table 3.** Microsatellite Primers

Primer	Chromosomal location	PCR product (bp)	Ann. T <sup>†</sup>
D1S188	1p32	149–173	55°C
D3S1110	3p25.3-p25.1	~66	50°C
D3S1100	3p22-p21.3	154–170	52°C
D7S480	7q31-q35	189–206	55°C
D7S490	7q31-q35	92–106	55°C
D10S564	10q23	252–262	55°C
(Sense: 5'-TGGGAATGTGTCTTTATCCA-3' Antisense: 5'-AGCTCTAACATAGAGGCCAGAT-3')*			
D11S4936	11q13	~142	55°C
(Sense: 5'-GCTTGCCAGTGAGCCGAGATTG-3' Antisense: 5'-CAAAACAACGACACAAAAAGCC-3')*			
D22S257	22q11.2	108–130	55°C
D22S1043	22q13	108–130	55°C

\*Sequences of the related primer.

†Specific annealing temperature.

(Boehringer Mannheim). Restriction fragments were analyzed by 6% polyacrylamide gel electrophoresis and silver staining.<sup>35</sup>

#### Nonisotopic PCR-SSCP *Gsα* Gene Mutation Analysis

Four MMFCs (file numbers T1–3 and T6) with a clearly distinct neoplastic follicular or papillary component were analyzed for mutations in exons 8 and 9 of the *Gsα* gene. Oligonucleotide primers flanking exons 8 and 9 (Table 2) were added in a concentration of 1 mmol/L in a 50- $\mu$ l mixture containing 100–300 ng of template DNA, 0.2 mmol/L dNTPs, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim). Thirty-five cycles of amplification were performed with a programmable thermal cycler (GeneAmp PCR System 9600), and the SSCP method was performed as described for the *RET* analysis. Nested primers were used for an additional amplification in cases with low DNA yield.

#### Loss of Heterozygosity Analysis

Eleven MMFC (T2–Z12), from which additional nontumorous tissue from the same patient was available, were examined for loss of heterozygosity (LOH) in microsatellite regions of chromosomes 1, 3, 7, 10, 11, and 22, using a recently described PCR-based approach.<sup>36,37</sup> The primer pairs D1S188, D3S1110, D3S1100, D7S480, D7S490, D22S257, and D22S1043 were purchased from Research Genetics (Huntsville, AL). The chromosomal location, the annealing temperatures, and the sequence of the other two primers, D10S564 and D11S4936, are detailed in Table 3. For LOH analysis, only patients heterozygous for a given DNA sequence were considered informative, whereas the presence of either homozygosity or an unclear distinction between paternal and maternal alleles was considered uninformative. A twofold difference in relative allele intensity ratios between tumor DNA and normal DNA was considered as allelic loss.

#### Clonality Analysis

The clonal composition of the tumors from the four female patients (T2, M7, M8, M9) was studied by analyzing the inactivation patterns of a polymorphic X-linked region encoding the androgen receptor (AR) gene as recently described.<sup>29</sup> Patients were considered heterozygous if PCR amplification of digested and undigested DNA from nonneoplastic tissue showed two major PCR products. Genomic DNA from paraffin-embedded tissue of a monoclonal non-Hodgkin lymphoma (female) patient and of a healthy male individual were used as controls for *Hpa*II digestion.

#### Results

The clinicopathological findings and the morphological patterns of the tumors, which have been discussed in detail in previous reports,<sup>5,11</sup> are shown in Figure 1 and are summarized in Table 1.

The ratio of the two tumor components varied from 10% to 80% for the medullary portion either separate from (two cases) or intermingled with the follicular component (eight cases) or both (two cases). When clearly distinct from the medullary portion, the follicular component presented various morphological features, ranging from well-differentiated papillary and follicular carcinoma to oxyphilic and poorly differentiated (solid) carcinoma. In five cases, mixed features were found in the lymph node metastases (T1, T2, T5, M11, Z12). In patient T2, a mixed papillary/medullary lymph node metastasis was present, although the primary tumors were topographically separated (Figure 1). In patient T5, a lymph node metastasis with features of a cystic papillary carcinoma associated with a MTC in the cyst wall developed from a MTC with prominent follicular features (follicular variant of papillary carcinoma) in the primary tumor (not shown). The other three tumors (T1, M11, and Z12) with lymph node metastases exhibited similar histological features in the primary tumors and their metastases (eg, Z12, Figure 1).

**Table 4.** Results

No.	Sample	RET		G $\alpha$ gene	LOH								Clonality (HUMARA)	
		Exon	Type		D1S188	D3S1110	D3S1100	D7S480	D7S490	D10S564	D11S4936	D22S257		D22S1043
T1	MTC	—	—	—	nm	nm	nm	nm	nm	nm	nm	nm	nm	
	FOL	—	—	—										
T2	MTC	16 (M918T)	S	—	□	□	□	□	□	ni	ni	□	□	ni
	FOL	—	—	—	□	■	□	□	□	ni	ni	□	□	ni
T3	MTC	—	—	—	□	□	□	□	□	□	□	□	□	
	FOL	—	—	—	□	□	□	□	□	□	□	□	□	
T4	MTC	—	—	—	np	np	□	□	■	np	□	np	np	
	FOL( $\mu$ dissect.)	—	—	—				□	□					
T5	MTC	15 (A883F)*	S	—	np	□	np	■	■	np	□	□	□	
	FOL( $\mu$ dissect.)	—	—	—				□	□					
M6	MTC	—	—	—	□	□	□	□	□	□	□	□	□	
	FOL	—	—	—	□	□	□	□	□	□	□	□	□	
M7	MTC	11 (C634W)	G	—	□	□	□	□	□	ni	□	□	□	M
	FOL( $\mu$ dissect.)	—	—	—										P
	MIXED	—	—	—										P
M8	MTC	10 (C618A)	G	—	np	□	□	np	np	□	□	□	□	M
	FOL( $\mu$ dissect.)	—	—	—										M
	MIXED	—	—	—										P
M9	MTC	—	—	—	■	□	ni	□	□	□	□	□	□	M
	FOL( $\mu$ dissect.)	—	—	—	□									P
	MIXED	—	—	—										P
M10	MTC	—	—	—	□	□	□	□	□	□	□	□	□	
M11	MTC	10 (C618S)	G	—	□	□	□	□	□	□	ni	□	□	
Z12	MTC	16 (M918T)	S	—	□	□	□	□	□	□	np	np	np	
	FOL( $\mu$ dissect.)	—	—	—										

MTC, medullary component; FOL, follicular component; MIXED, mixed component; S, somatic; G, germline; \*mutation present in subclones of tumor cells; np, no successful PCR; ni, not informative; nm, no normal tissue available; □, retention of heterozygosity; ■, loss of heterozygosity; M, monoclonal; P, polyclonal.

All tumors stained positively for calcitonin in the medullary but not in the follicular component, whereas thyroglobulin immunostaining was observed both in follicular (all tumors) and in solid tumor areas (7/12 cases).

### Mutation Analysis

The results are summarized in Table 4, and representative examples are shown in Figures 2 and 3. The sequence of *RET* exons 10, 11, 15, and 16 was studied in tumor DNA of all tumors and in the related germline DNA from normal tissue of 11 patients. Two of our patients (M8 and M11) were known MEN2A disease gene carriers.

PCR-SSCP analysis of *RET* exon 10 revealed single-strand conformation variants (SSCVs) in both tumor and germline DNA of two different patients, and the sequence analysis of PCR products revealed two different germline missense mutations at codon 618, C618A (M8) and C618S (M11). The analysis of *RET* exon 11 revealed a SSCV in one patient (M7), and the sequencing analysis revealed a mutation in normal and tumor DNA at codon 634 (C634W), defining this patient as a thus far unknown MEN2A disease gene carrier. The analysis of *RET* exon 15 revealed abnormal SSCP patterns in tumor DNA of one patient (T5). Because no SSCP variation could be detected in the DNA from a metastasis of the same patient, we dissected six different regions of the primary tumor and were able to demonstrate the presence of the abnormal band only in three subpopulations of tumor cells (Figure 2, T5). Sequence analysis revealed the presence of a mutation at codon 883 (A883F), which was confirmed by *AluI* restriction enzyme digestion (not shown). Microdissected follicles from the positive MMFC regions, however, showed no SSCP alterations.

The heteroduplex assay for exon 16 showed abnormal patterns in tumor DNA of two patients (T2 and T12). In

one tumor (T2) aberrant band patterns (heteroduplexes) were present in PCR products from DNA of the MTC but not of the papillary component. The same result was obtained for the other tumor (Z12), in which follicles were microdissected from the lymph node metastasis (Figure 3). The presence of a M918T mutation at codon 918 in the positive samples was confirmed by *FokI* restriction enzyme analysis.

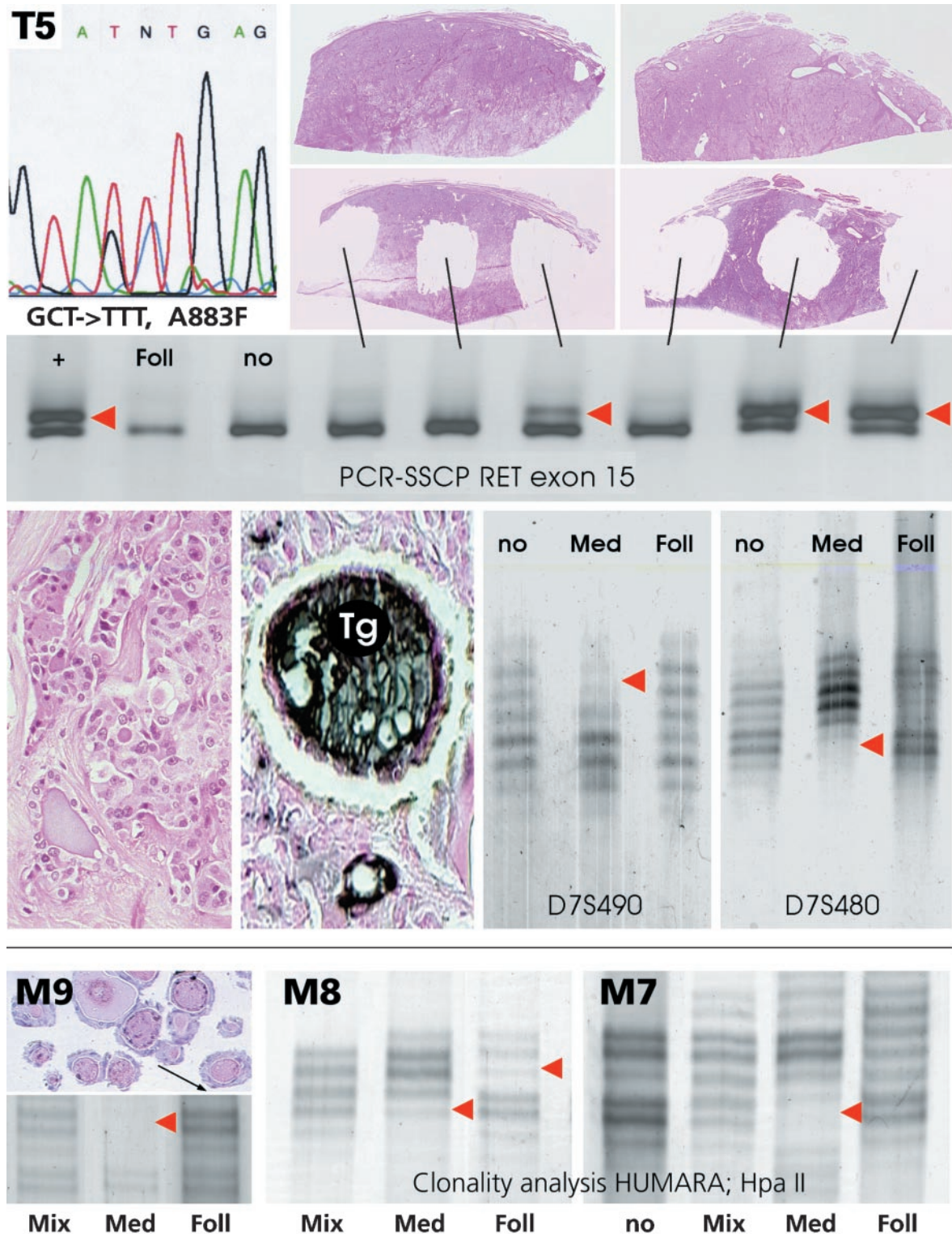
No mutations were found in the G $\alpha$  gene in any MMFC (data not shown).

### LOH Analysis

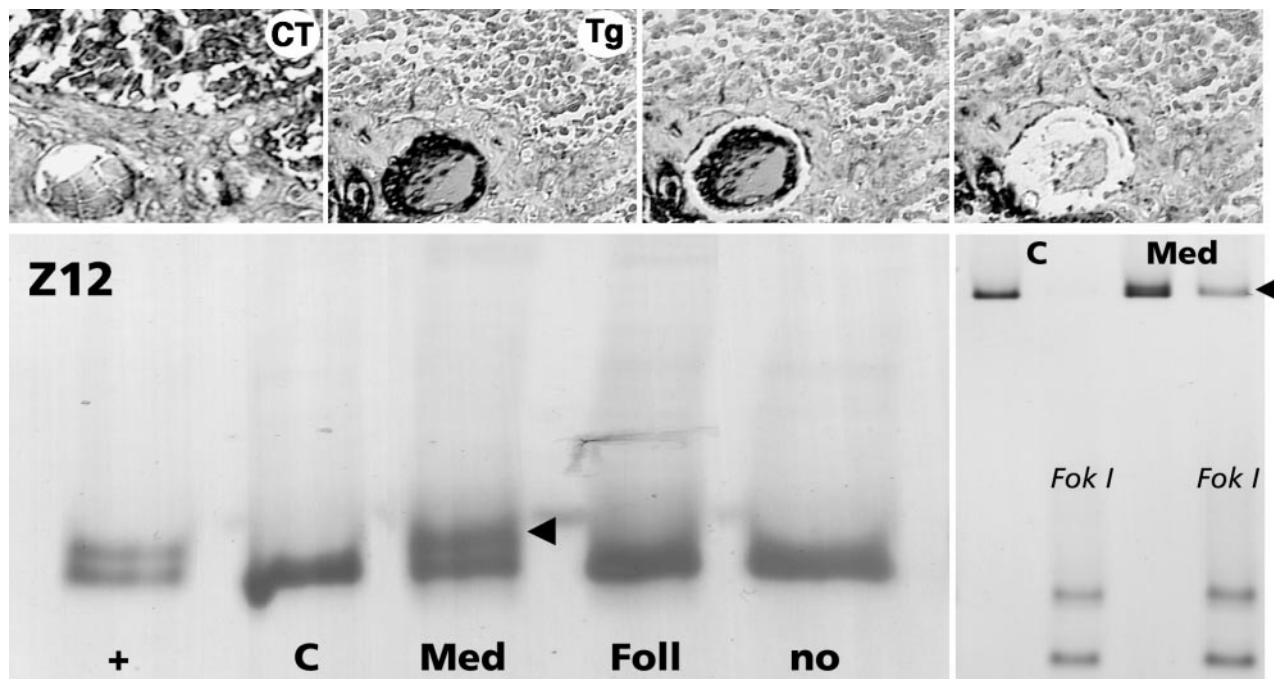
We analyzed allelic losses of nine loci on six chromosomes in the MTC and follicular components of all MMFCs. Three tumors exhibited LOH in the MTC portion (one each at D1S188 and D7S490; and one tumor at both D7S480 and D7S490), which was not present in the respective microdissected follicular component of the MMFC (Table 4, Figure 2). One additional MMFC had a LOH at D3S1110 in the follicular tumor, part which was not detectable in the MTC component (data not shown).

### Clonality Analysis

Four MMFCs were from female patients, and three of these were informative for PCR-based clonality analysis by identification of X-chromosomal inactivation. In all three MMFCs, a monoclonal X-chromosomal pattern was identified in the medullary portion, but the respective follicular components exhibited a polyclonal pattern in two patients (Figure 2; M9, M7) and a monoclonal pattern with inactivation of the allele opposite that of the medullary component in the remaining MMFC (Figure 2; M8).



**Figure 2.** Mutation analysis (**top**) and loss of heterozygosity analysis (**middle**) of microdissected medullary and follicular components from a mixed medullary-follicular carcinoma (**T5**). Note that in the medullary carcinoma portion the single-strand conformation analysis of *RET* exon 15 exhibits aberrant band patterns (**red arrowheads**) in a subset of the tumor (which is indicative of the presence of a somatic A883F missense mutation; **upper left**) but not in the follicular component (Foll) or nontumorous tissue (no). Note also that a loss of heterozygosity at the microsatellite loci D7S490 and D7S480 is only detectable in the medullary portion (Med) of the tumor (**red arrowheads**) and not in the microdissected thyroglobulin-positive (Tg) follicular part (Foll). **Bottom, M9, M8, M7:** Comparison of the X-chromosomal inactivation (clonality) pattern in the microdissected medullary carcinoma and follicular components of three mixed medullary-follicular thyroid carcinomas from informative female patients (for details see Materials and Methods). Note that all three tumors exhibit a monoclonal pattern in the medullary portion (Med), which is defined by the loss of one allele after *Hpa*II digestion of DNA (**red arrowheads**), whereas a polyclonal pattern similar to that in normal tissue (no) or whole tumor extract (Mix) is encountered in the follicular component (Foll) of two tumors (**M9** and **M7**), and a monoclonal pattern with inactivation of the opposite allele is present in the third tumor (**M8**).



**Figure 3.** Mutation analysis of *RET* exon 16 in the microdissected calcitonin-positive (CT) medullary and thyroglobulin-positive (Tg) follicular portions of a mixed medullary-follicular thyroid carcinoma (lymph node metastasis; Z12). Note that a heteroduplex formation (lower left, red arrowhead) is only detectable in the medullary (Med) but not in the follicular (Foll) tumor component. The presence of a somatic M918T point mutation was confirmed by *Fok I* restriction analysis of PCR products (lower right, red arrowhead).

### Discussion

The most interesting and equally controversial aspect of MMFC is its histogenetic and pathogenetic origin. The various hypotheses proposed to explain the occurrence of MMFCs and the molecular changes to be expected are listed in Table 5. We report here the first molecular analysis of the two components in MMFC of the thyroid. Our data strongly suggest that the follicular and medullary components in MMFCs are not derived from a single stem cell, because they consistently exhibited different patterns of *RET* proto-oncogene mutation, LOH, and X-chromosomal inactivation (clonality) in seven tumors that were suitable for clonal analysis or had detectable molecular aberrations at the genes and loci investigated (Table 4). Furthermore, we demonstrate that the follicular structures in MMFCs are often polyclonal and more frequently exhibit hyperplastic than neoplastic histological characteristics.

The currently favored "stem cell" theory of MMFC is based on studies of chicken and dog thyroid in which

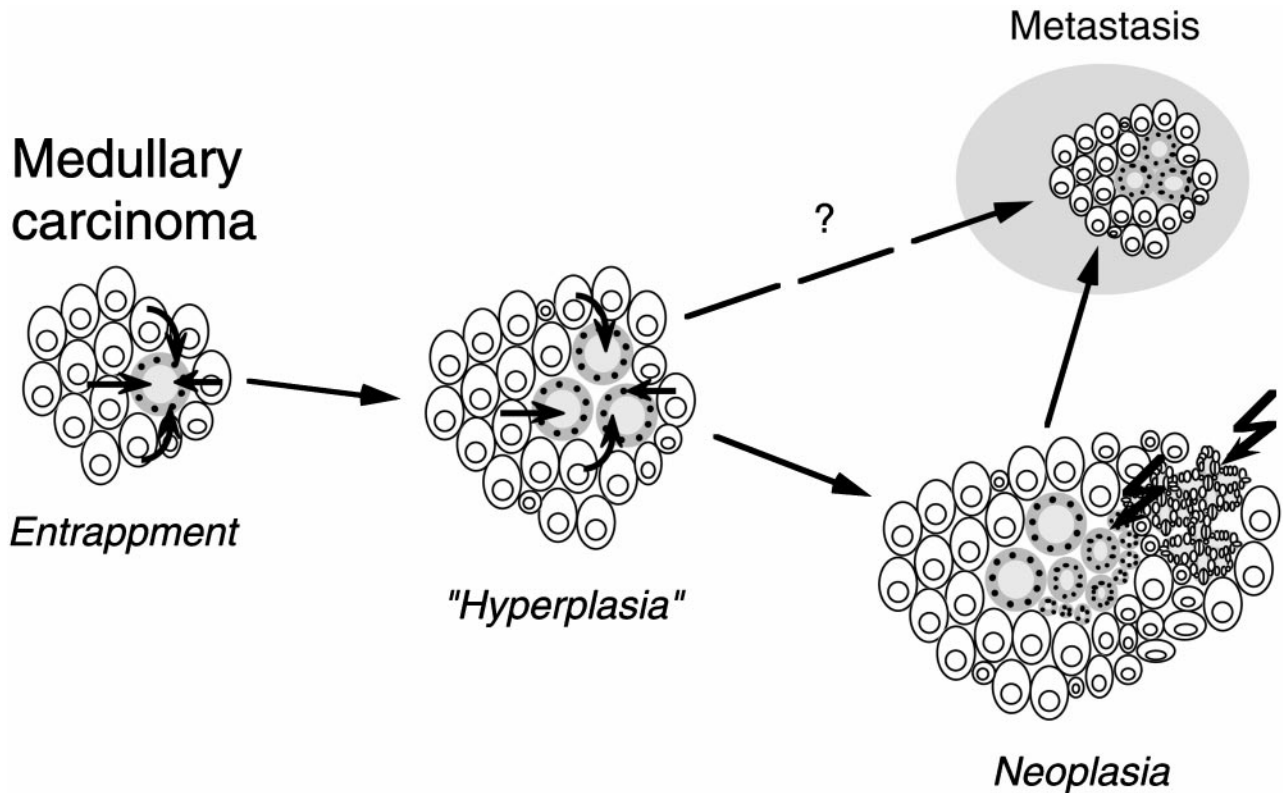
ultimobranchial remnants can give rise to both thyroid follicles and C-cells,<sup>38,39</sup> a finding that could be confirmed for humans as well.<sup>3</sup> Thus it was proposed that the neoplastic transformation of an uncommitted stem cell from the ultimobranchial body might give rise to MTC with dual (endocrine and neuroendocrine) differentiation. According to the stem cell hypothesis, the two tumor components of MMFCs should exhibit the same X-inactivation pattern (as a sign for clonal growth), and molecular alterations such as LOH and mutational status should be very similar (Table 5). However, our findings clearly demonstrate differences for both the clonal and the LOH/mutation pattern of the two tumor components. Specifically, in three MMFCs of female patients the MTC component exhibited a monoclonal pattern, whereas the follicle part displayed a polyclonal pattern in two tumors and a monoclonal pattern with inactivation of the opposite allele in one tumor. Furthermore, whereas three MMFCs demonstrated a LOH at different loci in the medullary part, a normal composition was detectable in the follicular part of the same patients. In an additional tumor we found a LOH at the locus D3S1110 in the follicular component but not in the MTC part. Our mutation analysis revealed additional differences: three sporadic MMFCs contained somatic *RET* mutations that could not be detected in their follicular components or in one examined lymph node metastasis. In summary, different molecular and clonal patterns were found in the two components of all seven MMFCs amenable for molecular analysis by three independent molecular approaches.

Therefore, the present results do not support the "stem cell" theory of development of MMFC. Likewise, our find-

**Table 5.** Hypotheses about the Pathogenesis of Mixed Thyroid Tumors

Hypothesis	Expected results from the two components	
	Clonality assay	LOH/mutation assay
"Stem cell" theory	Same clonality	Same (similar) pattern
"Divergent differentiation"	Same clonality	Different (similar) pattern
"Field effect"	Different clonality	Different pattern
"Collision effect"	Different clonality	Different pattern
"Hostage" theory	Different clonality	Different pattern





**Figure 4.** Diagram illustrating the "hostage theory" of the evolution of mixed medullary-follicular thyroid carcinomas. Entrapped nonneoplastic follicles are stimulated by trophic factors leading to hyperplastic follicular foci ("hyperplasia"). Acquired genetic defects in follicular cells lead to neoplastic transformation and development of follicular or papillary carcinoma components that can give rise to mixed metastases.

ings seem to exclude the "divergent differentiation" theory, assuming that some MTC cells differentiate toward a follicular phenotype by the acquisition of additional molecular defects (Table 5). Such tumors should exhibit a common clonality pattern in the two tumor components, which was not detectable in the MMFCs studied.

Another pathogenetic mechanism that has been put forward for MMFC is the "collision theory" (Table 5). It assumes that two independently arising tumors, either a MTC and a follicular carcinoma, or a MTC and a papillary carcinoma, collide in the thyroid. In fact, several authors have reported such neoplasms,<sup>40-43</sup> and others have pointed out that such tumors may imitate a primary MMFC.<sup>8,24</sup> Our series included one patient who had two topographically separated primaries consisting of a MTC and a papillary carcinoma and mixed metastases in regional lymph nodes. This indicates that a MTC can entrap and disseminate foci of another thyroid neoplasms. The same holds true for residual thyroid follicles, which can become entrapped by MTCs. Thus MTC exhibits a tumor growth different from that of most other human cancers, which usually destroy the tissue in which they arise.

Our molecular analysis does not entirely rule out the "field effect" hypothesis (Table 5), although we did not find a common LOH or mutation in the components investigated. The field effect hypothesis assumes a common oncogenic stimulus triggering neoplastic transformation of both follicular and C-cells,<sup>24</sup> which leads to the

simultaneous development of two different tumor components.<sup>44</sup>

Yet another pathogenetic mechanism may exist in at least one subset of MMFC. As already pointed out, MTCs may exhibit entrapped, well-preserved residual follicles. Although it is common to see such residual follicles at the periphery of classical MTCs, it is typical for MMFCs that the follicular structures are found deep within the tumor and that they exhibit cytological features different from that of the surrounding nonneoplastic follicles. It is conceivable that some unknown trophic factors keep the entrapped follicles alive and might even stimulate them to proliferate, and conversely that some follicles might secrete substances preventing them from being destroyed by the MTC.<sup>45</sup> Such a "symbiosis" of neoplastic C-cells and follicular cells might lead to a combined growth of follicular and C-cell structures and result in the mixed tumor pattern of the majority of MMFCs. The microenvironment provided by the MTC might result in hyperplastic and adenomatous follicular foci, which eventually may become neoplastic through the acquisition of molecular defects (Figure 4). Such a scenario could explain the spectrum of follicular patterns and the occasional neoplastic foci in MMFCs as well as the clonal and molecular changes found in this study. The question that arises from this "hostage theory" is whether such follicular structures can metastasize together with the MTC component (Fig-

ure 4). This aspect, however, cannot be answered at present and will require additional detailed analysis.

An important question remains: namely whether follicles with Tg immunoreactivity in MMFC are really neoplastic or merely hyperplastic or adenomatous, as suggested by Sobrinho-Simões.<sup>46</sup> Thus, in the group of reported "true" MMFCs, the majority of tumors exhibited follicular components in both primary stage and metastases that have to be classified as hyperplastic or adenomatous by conventional histology<sup>4,6,13-18,20,22,26</sup> (similar to our three cases amenable for clonal analysis), and only the minority of tumors contained morphologically undoubtably neoplastic non-MTC components.<sup>19,21</sup> Follicular structures fulfilling the conventional criteria of neoplastic follicular lesions in our series were identified in four MMFCs. Unfortunately, none of the four tumors were amenable for clonality analysis (three MMFCs from male patients and one not informative). Thus no definite answer about the clonal composition of the latter tumors can be given. One of those tumors, however, exhibited a *RET* mutation and a LOH at D7S480 and D7S490 in the medullary component but not in the follicular carcinoma portion.

One might argue that our molecular results are more easily explained by the fact that we have only accidentally analyzed entrapped residual follicles. However, we have only microdissected follicular structures deep within the MMFC, which exhibited a histology different from that of the peritumoral nonneoplastic follicles (eg, enlarged or clear nuclei), or which occurred in clusters. Thus we believe that our results are obtained from follicular components typical for MMFCs. Other technical reasons, eg, resulting in false clonality patterns, can be excluded because appropriate controls have been included in each assay and methods have been evaluated and tested in previous studies.<sup>29,37</sup>

From all of the data of the literature and the results of our study, it appears that the group of so-called MMFCs is in fact a rather heterogeneous group with various morphological features and different pathogenetic mechanisms. It includes 1) neoplasms that have to be classified as collision tumors, ie, that are composed of two separately arising tumors with different morphologies and an intermingled growth; 2) "true" mixed carcinomas with features as defined by the WHO classification<sup>7</sup>; and 3) MTCs with Tg-expressing cells that might be derived from a common stem cell.<sup>26</sup> However, the latter tumor type should be separated from MMFCs because they do not present histological evidence of follicles or papillae.

In summary, our present investigation provides molecular evidence that Tg-positive follicles or clusters of follicles and the medullary portion in MMFCs are not derived from a common stem cell. We therefore postulate that the pathogenetic mechanisms of MMFC might be different from those ones previously proposed. These findings provide new insights into the development of tumors with mixed morphology and raise the possibility that trophic factors secreted by neoplastic and host cells might have a significant role in the tumorigenesis of MMFC.

## Acknowledgments

We thank Seraina Muletta-Feurer and Katrin Rütlimann for technical support, André Barghorn for helpful discussions and technical advice, Madeleine Pfaltz for providing tissue samples, the group of Dieter Zimmermann for performing the sequence analyses, and Norbert Wey and Ida Schmieder for photographic and computer-assisted reproductions.

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